EXPRESSION OF MAMMALIAN MYOGLOBIN GENES <u>IN</u> <u>VIVO</u>

POLLY ANNE WELLER

Thesis submitted for the degree of

Doctor of Philosophy

in the University of Leicester

1986

ProQuest Number: U367056

All rights reserved

INFORMATION TO ALL USERS
The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript
and there are missing pages, these will be noted. Also, if material had to be removed,
a note will indicate the deletion.
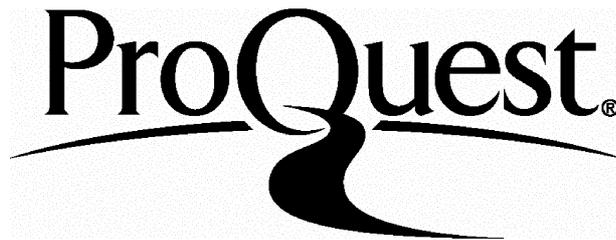
ProQuest U367056

PUBLICATIONS

Some of this work has been published:

Jeffreys,A.J., Blanchetot,A. and Weller,P. 1983. Myoglobin genes. pp487#508. In: "Brussels Hemoglobin Symposium" eds: Schnek,A.G. and Paul,C. Pub: Presses Universitaires de Bruxelles, A.S.B.L.

Weller,P., Jeffreys,A.J., Wilson,V. and Blanchetot,A. 1984. Organisation of the human myoglobin gene. EMBO J. 3:439-446.

ACKNOWLEDGEMENTS

DEDICATION

to Philippa and Peter

ABBREVIATIONS

A_n	absorbance at wavelength n nm
BCIG	5-bromo-4-chloro-3-indolyl-β-D-galacto-pyranoside
BSA	bovine serum albumin
cpm	counts per minute
DEPC	diethylpyrocarbonate
DMSO	dimethylsulphoxide
DNase	deoxyribonuclease
DS	dextran sulphate
DTT	dithiothreitol
EDTA	diaminoethanetetra-acetic acid
IMS	industrial methylated spirit
IPTG	isopropyl-β-D-galacto-pyranoside
MHC	myosin heavy chain
MLC	myosin light chain
MOPS	3-(N-morpholino)propanesulphonic acid
mya	million years ago
dNTP	2'-deoxynucleoside triphosphate. N = adenosine, guanosine, thymidine, cytidine
ddNTP	2'3'-dideoxynucleoside triphosphate
PEG	polyethylene glycol 6000
PIPES	piperazine-N,N' bis(2-ethane-sulphonic acid)
POPOP	1,4-di-2(4-methyl-5-phenyl)oxazolyl
PPO	2,5-diphenyl oxazole
PVP	polyvinypyrrolidine
RNase	ribonuclease
SDS	sodium dodecyl sulphate
SR	sarcoplasmic reticulum
SSC	saline sodium citrate
TCA	trichloroacetic acid
TEMED	N,N,N'N'-tetramethyl-ethylinediamine
TnT	troponin T
Tris	tris(hydroxymethyl)aminomethane

CONTENTS

Chapter 1

INTRODUCTION

1. Myoglobin

i) Structure of myoglobin

Myoglobin is a small globular protein of molecular weight approximately 17,800 daltons which is composed of a single polypeptide and the prosthetic group haem. Haem, a coordination compound of protoporphyrin IX with an atom of iron, is also the prosthetic group in a number of other proteins including haemoglobin, cytochromes b, c_1 and c and some enzymes such as catalase and peroxidase. It is haem which gives myoglobin, haemoglobin and the cytochromes their distinctive colours and enables myoglobin and haemoglobin to bind oxygen reversably. The iron atom of the haem may be in the ferrous (+2) or ferric (+3) oxidation state: ferromyoglobin and ferrimyoglobin (or metmyoglobin). Only ferromyoglobin has the capacity to bind oxygen (see Antonini and Brunori, 1971).

Myoglobin was the first protein for which the three dimensional structure was determined by X-ray crystallography by Kendrew and his collegues, at increasingly high resolutions in the late 1950s and early 1960s (Kendrew et al., 1958; 1960; 1961; Kendrew 1963; Nobbs et al., 1966). The results of these studies of sperm whale myoglobin and a further study by Takano (1977) revealed that myoglobin was a very small compact protein with approximately 75% of the amino acid chain folded into exclusively right handed α-helix.

The hydrophobic haem group is located in a pocket or crevice in the myoglobin molecule, surrounded by non-polar residues, but with the polar propionate side groups at the surface. It is the non-polar environment of the haem group which enables myoglobin to bind oxygen stably, as although isolated haem can bind oxygen, in the surrounding water the ferrous haem

1

is rapidly oxidised to the non-oxygen binding ferric form. This oxidation is prevented in the myoglobin molecule by the exclusion of water.

The iron atom of the haem has four of its six coordination sites occupied with bonds to the porphyrin ring structure. Of the remaining two, one is linked to the histidine residue, F8 (amino acid 93). The iron atom lies out of the plane of the porphyrin ring on the side of the proximal histidine F8. The sixth coordination position is available for the binding of oxygen (see Antonini and Brunori, 1971). Ferrimyoglobin has a water molecule at this position.

The amino acid sequence of myoglobin from a large number of species is known (Romerra-Herrera et al., 1978). All mammalian myoglobins so far sequenced are 153 amino acids long. Amino acid sequences of human myoglobin from adult skeletal and cardiac muscles are identical, and the same as that from foetal muscle (Schneiderman, 1962).

ii) Function of myoglobin

Myoglobin occurs in the muscles of both vertebrates and invertebrates. In vertebrates it is the major haemoprotein of both skeletal and cardiac muscle. The amount of myoglobin present in muscle is very variable and depends both on the type of muscle and on the species in consideration. (see sections 1.6 and 1.7).

The function of both myoglobin and haemoglobin in vertebrates is the binding and transport of the oxygen required for aerobic metabolism. The three dimensional structures of myoglobin and individual chains of the haemoglobin tetramers are strikingly similar, much more so than comparison of their respective amino acid sequences would suggest (Perutz, 1964; Perutz et al., 1965).

The oxygen dissociation curve of myoglobin is hyperbolic, whereas the haemoglobin curve is sigmoidal, a result of the cooperativity in the binding of oxygen between the four haem groups of the haemoglobin

2

tetramer. Binding of oxygen to myoglobin, unlike to haemoglobin, is not affected by change in the pH or CO_2 concentration (the Bohr effect), nor does myoglobin bind either H^+ or CO_2. The affinity of myoglobin for oxygen at low oxygen partial pressures is greater than that of haemoglobin. Hence oxygen released by haemoglobin in the tissue capillaries can be bound by myoglobin in the tissues.

The idea that muscle myoglobin actually facilitated diffusion of oxygen within the tissue was first suggested by Wittenberg (1959), who demonstrated accelerated diffusion of oxygen through agar membranes which contained haemoglobin. Model systems were developed to gain an understanding of how myoglobin could contribute to diffusion of oxygen within muscle. Such systems demonstrated that myoglobin (and haemoglobin) facilitated flux of oxygen brought about by the translational diffusion of oxygenated myoglobin molecules (see Wittenberg, 1970).

Wittenberg (1970) suggested that the rate of oxygen supply to the muscle mitochondria is influenced by three factors: i) the diffusion path length from capillary to mitochondrion, ii) the difference between capillary and mitochondrial oxygen tensions and iii) an oxygen diffusion term made up from myoglobin-facilitated diffusion and the natural diffusivity of dissolved oxygen within the muscle fibre. A number of workers have suggested that myoglobin acted as a short term oxygen store (e.g. Anstrand et al., 1960), but Wittenberg (1970) has shown that both (red) skeletal and cardiac muscles in sustained contraction operate under steady state conditions. Under these steady state conditions, the parameters of oxygen flux mentioned above remain constant.

The effect of increasing the work performed by muscle is an increase in oxygen demand and a subsequent decrease in capillary oxygen tension. The steady state is restored in both skeletal and cardiac muscle by increasing the number of open capillaries within the muscle, thus reducing the effective oxygen diffusion path length. Blood flow into capillaries

3

is controlled by the precapillary sphincters in response to local oxygen concentration.

Two requirements for myoglobin-facilitated oxygen diffusion in such a steady state system are steep gradients of free and myoglobin-bound oxygen within the muscle fibre. The difference in oxygen tension between capillary and mitochondrion is estimated by Wittenberg (1970) to be more than ten-fold. He also estimates that at a pO_2 of 17 torr, the pO_2 of venous blood, myoglobin in the tissue is approximately 80% saturated with oxygen, indicating that conditions are appropriate for myoglobin-facilitated diffusion.

Conclusive evidence for the role of myoglobin in facilitation of oxygen diffusion was provided by Wittenberg et al. (1975). They demonstrated that abolition of myoglobin function in bundles of pigeon breast muscle in low oxygen tension reduced the steady state oxygen consumption by approximately half. At higher oxygen tensions, where oxygen availability does not limit respiration, oxygen uptake was not affected by abolition of myoglobin function, showing that utilisation of oxygen by mitochondria was not impaired.

1.2 Muscle structure and function

i) Structure of skeletal muscle

In order to understand the role of myoglobin, it is necessary to consider the structure and metabolism of the different types of muscle tissue in which it occurs. Muscle is a highly specialised tissue, the function of which is the conversion of chemical energy into mechanical work. Skeletal muscle comprises a large number of cross-striated muscle fibres which are long and cylindrical, usually 10-100μm in diameter. Each muscle fibre is actually a syncytium which contains many hundreds or thousands of nuclei. The bulk of the muscle fibre is occupied by the contractile apparatus, cross-striated myofibrils which run almost the

4

entire length of the fibre and in most mammalian muscles occupy 75-85% of the fibre volume (Eisenberg and Kuda, 1976). The structure of the sarcomere, the unit of the contractile apparatus is shown schematically in Figure 1.1.

The muscle fibre contains two distinct membranous systems which ramify between the myofibrils. These are the transverse tubular system (T-system) and the sarcoplasmic reticulum (SR). Depolarisation of the sarcolemma is transmitted into the interior of the fibre by the T-tubules and release of calcium from the SR is triggered by depolarisation across the triadic junction between T-tubules and SR (Endo, 1977). These ions bind to the thin fibres, specifically to troponin C, causing conformational changes which are transmitted to tropomyosin and then actin, enabling the contraction reaction to start (see Ebashi, 1974). In the resting state, calcium ions are sequestered within the SR by an active transport system (see Tada et al. 1978, for review).

Muscle is an intensely respiring tissue and the energy for contractile activity comes mainly from the oxidative metabolism of mitochondria. The number of mitochondria reflects the metabolic pattern of the muscle fibre (Gauthier, 1969). In white fibres, mitochondria encircle each myofibril at the level of the Z line and occupy 8 to 10% of the fibre volume. Red fibres have two additional populations of mitochondria, one arranged in longitudinal columns regularly arrayed in the spaces between the myofibrils and the other as a dense aggregation of large mitochondria under the sarcolemma, often in the region of the nucleus (Wittenberg, 1970).

A single motoneuron innervates a number of muscle fibres. The number varies with the muscle, but may be as many as thousands. Fibres innervated by a single neuron constitute a motor unit and may be grouped within the muscle or scattered. All fibres in the same motor unit have the same structural and physiological properties and contract together in

Figure 1.1 Fine structure of the contractile apparatus

The Figure shows the structure of one sarcomere in schematic form, taken from Squire (1981). The A and I bands consist of separate arrays of interdigitating filaments. The A band comprises thick myosin filaments together with interdigitating thin actin filaments which also contain tropomyosin and troponin. The I band is the thin actin filaments alone and the H zone is the part of the A band not overlapped by actin filaments. The M line crosslinks the myosin filaments and the Z line, in which the actin filaments are anchored, divides one sarcomere from the next along the length of the myofibril. On contraction the length of the A bands remains constant, while that of the I band varies. Cross-sections through a region of overlap of thick and thin filaments show that individual filaments are arranged in a regular hexagonal array with one thick filament surrounded by six thin ones.

Other proteins have been localised to the myofibrillar structure and these include C-protein, which is a component of the thick filaments (Offer et al., 1973) and creatine kinase and myomesin, which are M line proteins (Turner et al., 1973; Eppenberger et al., 1981). The Z line has been shown to contain α-actinin (Lane et al., 1977).
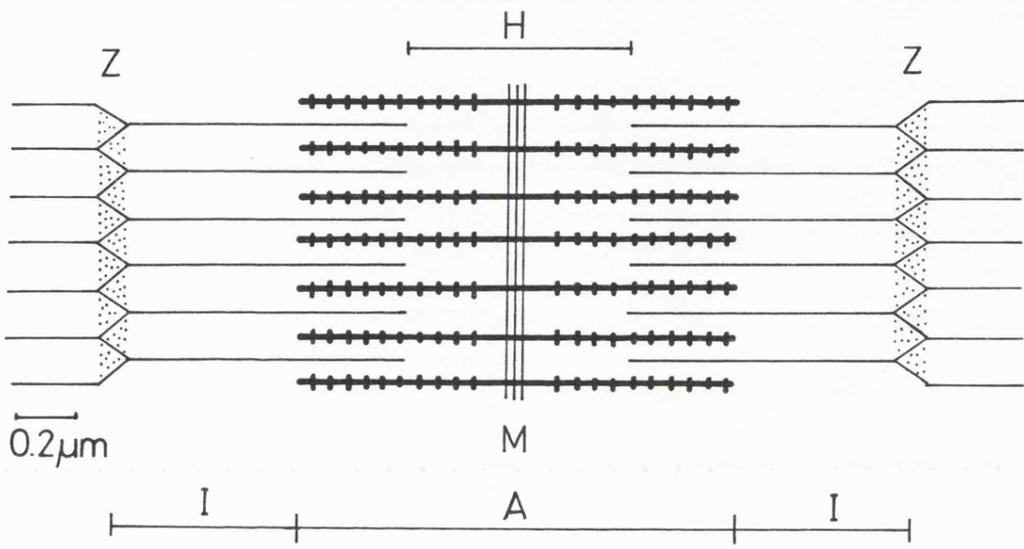
an all or nothing response (Burke et al. 1971). The force generated by a muscle is therefore proportional to the number and size of the active motor units, which are recruited in a set hierarchical order; slow motor units activated before fast (Henneman et al., 1974; Monster and Chan, 1977).

ii) Metabolism of skeletal muscle and the diversity of muscle fibre type

The level of expression of the myoglobin gene is closely related to muscle metabolism, both within an individual fibre and in the whole muscle. Muscle fibres can be divided into categories using a number of different criteria. Very few muscles contain exclusively one fibre type (see Ishikawa, 1983). Levels of myoglobin are closely related to muscle metabolism and vary in different types of muscle fibre. A discussion of the fibre types of skeletal muscle in relation to their function and metabolism is therefore relevant.

Fresh muscles can be distinguished macroscopically on the basis of colour into red, white and a range of intermediates. The colour of muscle is due to the variable content of myoglobin and mitochondrial cytochromes, as first recognised by Lawrie (1952; 1953a,b). The degree of redness can be attributed to the ratio of red to white muscle fibres. Red fibres can be distinguished from white histochemically by high levels of mitochondrial enzymes (Beatty and Bocek, 1970) and also ultrastructurally by more numerous mitochondria, lipid droplets and glycogen granules (see Ishikawa, 1983).

Red fibres are associated with a dense network of capillaries. Capillary density has been correlated with the oxidative capacity of muscle fibres (Folkow and Halicka, 1968). Individual white fibres are surrounded by on average far fewer capillaries than red fibres (Wittenberg, 1970). Myoglobin concentration has also been correlated with blood flow (Wooten and Reis, 1972). These characteristics are indicative

6

of the high level of oxidative metabolism in red muscle (Henneman and Olson, 1965).

In white muscle the main source of energy is glycolysis. Because glycolysis is relatively inefficient, activity can only be maintained for short periods before rest is neccessary for the removal of lactate and import of more glucose from the liver. In contrast, the main source of energy in red muscle is derived from the oxidation of fatty acids via the citric acid cycle and oxidative phosphorylation in the mitochondria, hence the increased requirement for oxygen and elevated myoglobin concentration (see Close, 1972).

Skeletal muscle fibres can also be distinguished physiologically, on the basis of speed of contraction, into slow (tonic) and fast (twitch) (Close, 1967; Burke et al. 1971). This classification is not identical to the red/white one: the bat cricothyroid muscle, used in emission of high frequency sound, is a fast muscle but has small fibres, rich in mitochondria (Revel, 1962). In mammalian muscle, twitch fibres are generally divided into slow-twitch (type I) and fast-twitch (type II). This division is closely related to myosin ATPase activity. Fast-twitch muscles have a higher myosin ATPase activity than slow-twitch (Barany and Close, 1971). Further subdivision of type II fibres can be made, into type IIA, B and C on the basis of the pH at which the myosin ATPase is inactivated (Brooke and Kaiser, 1974). Contractile proteins including myosins and troponins of fast- and slow-twitch fibres are different (Dhoot et al., 1978; Gauthier and Lowey, 1979).

In mammals, typical slow (tonic) fibres, which show a number of differences from fast (twitch) fibres, are confined to eye and ear muscles and muscle spindles (Ishikawa, 1983). Most mammalian muscles are composed of mixed populations of distinct types of twitch muscle fibres. Ratios of fibre type vary in different muscles and contribute to their variable properties. Histochemical analysis of individual fibres shows that they

7

may contain many different combinations of enzyme activities (see Khan, 1976). Myoglobin has been shown by histochemical staining to occur in type I fibres, although some intermediately staining fibres have also been found (Morita et al., 1970). Many fibre characteristics show a continuous distribution between two extremes (Pette, 1980) and many characteristics including mitochondrial oxygen uptake, respiratory enzyme activity and myoglobin concentration can be altered by exercise and also by electrical stimulation (see section 1.7). Hence no simple classification scheme can be applied to mammalian muscle fibres, though a number has been suggested (See Ishikawa, 1983). Skeletal muscle is therefore a highly heterogeneous tissue containing a large number of phenotypically distinct fibre populations, which in addition show a high degree of plasticity in adaptive responses to functional demands.

iii) Cardiac muscle

In many repects, including the arrangement of the contractile apparatus, cardiac muscle is very similar to skeletal muscle and has similar levels of myoglobin (see section 1.6).

There is no arrangement of the contractile apparatus into long syncytial fibres; cardiac muscle is cellular, although the structure and mechanism of contraction of the contractile apparatus is essentially identical to that of skeletal muscle. Low electrical resistance is maintained between the cells by gap junctions (Barr et al., 1965). The organisation of the T-system and SR is generally very similar to that in skeletal muscle. Mitochondria are very numerous and are arranged in longitudinal rows along the myofibrils and in aggregates near the nucleus (see Simpson et al. 1973). The capillary density is much higher in cardiac than in skeletal muscle (Wittenberg, 1970).

The heart represents a functional syncytium, as impulses entering the ventricular myocardium are eventually conducted to all other regions

of the ventricles via the low electrical resistance of the cell-cell

junctions. In the heart there is therefore no analogy to the tension

modulation in skeletal muscle achieved by activation of a variable number

of motor units.

The metabolism of the heart is very closely linked to its energy

needs. Normally energy is derived from fatty acid oxidation, though

glycolysis is stimulated if the tissue becomes hypoxic (see Neely and

Morgan, 1974). Utilisation of fatty acid oxidation as the primary energy

source accounts for the large numbers of mitochondria in cardiac muscle

and also the relatively high myoglobin concentrations (see section 1.6).

iv) Smooth muscle

The smooth muscle of vertebrates occurs chiefly in the walls of both

large and small hollow organ systems, including the circulatory system,

digestive system, urinary tract and uterus. Smooth muscles are not

adapted for speed, but for slow rhythmic contraction and are different

from striated muscle in many ways, including myoglobin concentration (see

section 1.6).

Smooth muscle, like cardiac, is cellular rather than syncytial

although the cells are much smaller (Hamoir, 1977). As much as a third to

half the protein content of smooth muscle may be contained in

extracellular collagen and elastin networks (Hamoir, 1977). Smooth muscle

cells do contain thin (actin) and thick (myosin) filaments, although the

thin filaments contain no troponin. Unlike striated muscle, the length of

both thick and thin filaments is variable and there is very little

evidence of the ordered structural array seen in skeletal muscle.

In addition, smooth muscle contains large numbers of intermediate,

or 10 nm filaments, which also occur to a lesser extent in skeletal and

cardiac muscle. These are made up of desmin (Lazarides and Hubbard, 1976)

and occasionally vimentin (Gabbiani et al., 1981) and are integrated into

a network extending throughout the muscle fibre. Smooth muscle
contraction is thought to operate by a sliding filament mechanism but like
invertebrate (molluscan) muscle regulation, the calcium regulation of
contraction is via myosin light chain phosphorylation, and not via the
troponin-tropomyosin system of vertebrate skeletal muscle (see Hartshome
and Siemankowski, 1981).

The SR is not as extensive in smooth muscle as in striated, nor is
it distributed regularly within the cell, although there is sufficient to
function as a calcium store in excitation/contraction coupling (Devine et
al., 1972). The peripheral SR forms couplings with invaginations in the
plasma membrane as there is no T-system (see Shoenberg, 1977).
Mitochondria are not as abundant in smooth muscle as in striated and most
are located near the nucleus. Many mitochondria are also located close to
the cell membrane or surface invaginations and have been suggested to act
as sites of calcium storage (Somlyo and Somlyo, 1971; Batra, 1973). The
metabolism of smooth muscle (dog carotid) shows the same characteristics
(in terms of activity ratios of specific enzymes) as those of red skeletal
muscle, but the levels of these enzymes are approximately 10-fold lower
(Bass et al., 1969).

1.3 Muscle development in vivo

Muscle tissue is formed following fusion of undifferentiated
myoblasts to form multinucleate syncytia called myotubes. This cell
fusion, which takes place early in embryonic development, is closely
associated with a burst of mRNA and protein synthesis and an accumulation
of muscle-specific proteins and structures (see Buckingham, 1977). The
general pattern of myogenesis is similar in skeletal and cardiac muscles.
In cardiac muscle, however, the process is less synchronous and mitotic
activity does not cease before the initiation of the synthesis of the
contractile proteins in the differentiating myocytes. This process of

myoblast fusion occurs at different developmental stages in skeletal and cardiac muscles. In the mouse, cardiac myoblasts appear at 7 days gestation and cardiac contraction begins as early as 8 days. In contrast, skeletal muscle myoblasts do not appear until 11 days gestation and myotubes cannot be detected until 14 to 15 days, when the muscles first become contractile (Rugh, 1968). Muscle development and maturation, however, continues for a considerable time after birth; the characteristically adult arrangement of couplings between the SR and the T-system does not develop in mice until 10 to 14 days after birth (Edge, 1970).

There is a marked increase in the number of mitochondria in myotubes, compared with myoblasts. Myotubes have been shown to have an increased reliance on oxidative phosphorylation as an energy source (Konigsberg, 1964). Progressive structural differentiation of mitochondria, seen as an increased number of cristae, occurs in parallel with musle development, presumably reflecting the increased oxidative metabolism of the developing tissue (see Fischman, 1972). The thick and thin filament components of the contractile apparatus appear oriented axially in the cytoplasm of myotubes. Such aggregates increase both in girth and length and are eventually organised into the sarcomeric pattern (see Goldspink, 1980).

The maturity of skeletal muscle at birth depends not only on the particular muscle, but also on the species in consideration. Animals of some species are born more developed than others. Direct comparison of different species is therefore not always possible. For example, differentiation into twitch fibres type I and type II, as judged by histochemical staining, is complete in the human foetus by 20 weeks gestation. In contrast, differences between fibres are not very apparent in neonate mice, rats or cats (Briskey et al., 1970). The diameter of fibres at birth is small, but increases with age (Rowe and Goldspink,

11

1969). Fibres also lengthen during growth of the muscle (Griffin et al., 1971). The number of fibres, however remains unchanged, as muscle fibres do not divide (Rowe and Goldspink, 1969; Strickland and Goldspink, 1973). Satellite cells fuse to muscle fibres during development, thus increasing the number of nuclei (Moss and Leblond, 1970), but no DNA replication or nuclear division occurs (Stockdale and Holtzer, 1961).

At birth skeletal muscle is already innervated but each muscle fibre at this stage is innervated by more than one neuron. This polyneural innervation disappears however, so that 15 day old rats have muscle fibres innervated by a single axon (O'Brien et al., 1978). At birth, muscle fibres of most animals are still uniformly functionally immature and combine characteristics not seen together in adult muscle fibre types. The speed of contraction and relaxation is uniformly slow, but fast muscle isoforms of the contractile proteins (eg. myosin light chain) may be present (Gauthier et al., 1978).

The development of immature muscle fibres into fully differentiated slow- and fast-twitch adult fibres has been shown to depend on innervation. Buller et al. (1960a,b) showed that the speed of contraction of fast-twitch muscles of new-born kittens increased considerably in normal development, while that of slow-twitch did not much change. Severing the efferent nerve supply had the effect of increasing the contraction speed of the slow muscle. This and other work (see Jolesz and Streter, 1981) suggests that both muscle fibres and motorneurons acquire properties of fast-twitch muscle in the absence of the low-frequency activation pattern characteristic of slow-twitch muscle.

Two types of fibres can be identified as early as 18 days gestation in rat leg muscle by their ability to stain for fast and slow isoforms of troponins I, C and T. Since impulse activities of nerves in foetal muscle do not differ and muscle is polyinnervated at this stage, Perry and Dhoot (1980) have suggested that innervation of foetal muscle may not be the

12

primary factor for the stimulation of differential gene expression. However, Gordon and Van Essen (1985) have recently suggested that muscle fibres do indeed begin to differentiate physiologically and histochemically while still polyinnervated and that the motorneurons themselves are also effectively differentiated into specific types. There is no doubt, however, that the final differentiation into type I and type II fibres requires the concomittant differentiation of slow and fast motor units, although the relative contributions of 'myogenic' and 'neurogenic' influences on fibre type differentiation still remains unclear (see Gordon and van Essen, 1985).

The developmental appearance of individual muscle proteins, as judged by histochemical staining techniques varies both in different species and from one muscle to another in the same animal, some enzymes showing differentiation before others (see Dubowitz, 1970). In addition, there are changes in the isoforms of individual proteins (both enzymes of metabolism and the contractile proteins) expressed at different stages during muscle development, before the characteristic adult patterns of muscle gene expression are attained (see section 1.4).

1.4 <u>Multiple isoforms of muscle proteins</u>

It is clear that muscle proteins, and particularly the contractile proteins, characteristically occur as multiple isoforms, encoded by multigene families. Different patterns of isoforms are expressed in different adult muscle tissues. Different isoforms occur in the distinct fibre types of adult skeletal muscle and yet others are specific to foetal or newborn muscle tissue. The occurrence of different isoforms of the contractile proteins and the likely minimum number of genes in each family has been reviewed by Buckingham (1983), and will be mentioned only briefly here.

Myosin heavy chain proteins (MHC) provide a good example with which

to illustrate the complexity of the types of tissue- and developmental-
specific isoforms which occur both in muscle and non-muscle tissues.
There are two distinct non-muscle MHCs which are tissue-specific.
Distinct MHCs occur in adult smooth muscle and in the heart where there is
an atrial and two ventricular isoforms. Fast-twitch (type II) and
slow-twitch (type I) skeletal muscles also have distinct MHC isoforms and
MHCs may even be distinct in type IIA and IIB fibres (Billeter et
al.,1981). In addition these workers also found that a fast myosin light
chain isoform (LC_{1f}) occurs in slow twitch (type I) fibres, as well as the
two slow isoforms, and that hybrid myosin molecules are present.

In skeletal muscle three developmental stage-specific isoforms of
MHC have been described (Whalen et al., 1981). The foetal form persists
in rat until a few days after birth when the neonatal form, MHC_{neo}, first
appears. The adult fast and slow isoforms, MHC_f and MHC_s, then appear and
have completely replaced MHC_{neo} after about two weeks, when fibre
differentiation is complete. The timing of these changes is thought to be
related to the development of innervation of the muscle fibres. In some
species a different ventricular isoform, MHC_{v1}, appears around the time of
birth. The adult ventricular form may be MHC_{v3} (the embryonic form) or
MHC_{v1}, depending on the species. There is therefore a considerable number
of MHC isoforms, which it has been estimated are thought to be transcribed
from a MHC multigene family of 11 to 13 functional genes (see Buckingham,
1983).

Multiple tissue- and developmental-specific isoforms of the
contractile proteins myosin light chain, troponins C, I and T, tropomyosin
and actin also occur, but the number of isoforms and their pattern of
expression are different for each.

Estimates of minimum gene number for each of the contractile protein
gene families can be made from the number of protein isoforms detected
(see Buckingham, 1983). However, this takes no account of isoforms not

yet detected, or of duplicated identical genes or pseudogenes. For example, several experimental techniques have indicated that there are approximately twenty actin genes in the human genome (Humphries et al., 1981), though only six isoforms are known. By using isoform-specific cDNA hybridisation probes, skeletal and cardiac α-actins have been found to be single copy, whereas the cytoplasmic β and γ-actins are present in the human genome in multiple copies, most of which are almost certainly pseudogenes (Ponte et al, 1983).

An alternative way of generating diversity among proteins is by deriving multiple isoforms from a single gene, by way of alternative RNA splicing. This has been found to occur with several cellular genes, including some of the contractile protein genes. Myosin light chain mRNAs MLC$_1$ and MLC$_3$ from fast skeletal muscle are identical in nucleotide sequence over the 3' non-translated regions and codons specifying the majority of the amino acid sequence, but differ markedly at the 5' end. They have been shown to derive from the same gene by a combination of transcription from two separate promoters and differential splicing of the transcripts (Nabeshima et al., 1984; Periasamy et al., 1984). Likewise, multiple mRNAs are transcribed from the single MHC and tropomyosin I genes in Drosophila (Rozek and Davidson, 1983; Basi et al.,1984). Ruis-Opazo et al. (1985) have shown that three distinct α-tropomyosin mRNAs occur in rat, which are tissue-specific, developmentally regulated and most likely transcribed from the same gene. They include two striated muscle isoforms and a smooth muscle isoform.

The most complex example of generation of multiple isoforms from a single gene described so far is that is that of troponin T (TnT). Breitbart et al. (1985) isolated four distinct TnT cDNAs from rat fast skeletal muscle which shared discontinuous subsegments of sequence. Sequencing of the rat TnT gene showed that it consisted of 18 small exons with a characteristic split codon structure. A total of ten different

mRNAs containing different exon combinations, as well as invariant exons, were detected by S1 mapping, giving a theoretical maximum of as many as 64 distinct TnT mRNAs. They also demonstrated that at least two of the TnT mRNAs are generated from identical primary transcripts.

Muscle protein diversity is generated both by multiple genes and by differential splicing of transcripts from a single gene. The relative importance of each mechanism depends on the gene family. The potential for diversity among the myofibrillar proteins is therefore very considerable. In this way the muscle cell can retain the essential function of a particular protein, while simultaneously modifying other characteristics in a reversable manner important both in the development of the different adult muscle phenotypes and in the adaptability and plasticity of muscle in response to numerous internal and external stimuli.

1.5 The mechanism of myogenesis

i) Myoblast fusion

The process of myoblast fusion and the development of myotubes and muscle fibres can be observed in embryonic muscle. However, the fusion process is asynchronous, making the analysis of specific stages all but impossible. This has been overcome by the use of tissue culture techniques, with which a more synchronous fusion of both embryonic myoblast cultures and myoblast cell lines can be achieved.

Myoblast fusion in culture has been shown to be dependent on both culture medium and cell density. Fusion is a very specific process and will not occur between myoblasts and non-muscle cells. Both during proliferation of myoblasts and after their withdrawal from the cell cycle in G_1, the cells align along their longitudinal axes. This is thought to be in some way mediated by fibronectin (Chiquet et al., 1981). Following alignment, fusion is thought to proceed by a series of distinct stages,

16

defined by the resistance of myoblast aggregates in suspension to first

EDTA and then trypsin, referred to as recognition, followed by adhesion

and then membrane union (see Wakelam, 1985). Structures with the

appearance of gap junctions have been observed between apposed myoblast

membranes in the adhesion stage and myoblasts have also been identified as

being electrically coupled, but not fused. Gap junctions are also seen in

19 day foetal rat muscle in vivo (Rash and Staehelin, 1974).

Fusion of myoblasts has been suggested to be initiated at a single

membrane site with a single cytoplasmic link (Lipton and Konigsberg,

1972). Particle-free regions have been observed by freeze fracture

electron microscopy in myoblast plasma membranes at the fusion site

(Kalderon and Gilula, 1979). The fusion rate is very sensitive to

temperature, indicating that the fluidity of myoblast membranes is

important for fusion. In fact, an increased membrane fluidity has been

demonstrated on the onset of fusion by a number of techniques (see

Wakelam, 1985).

It has been suggested (see Wakelam, 1985) that onset of fusion in

myoblasts is stimulated by a diffusible signal, most probably receptor

activation. Embryo extract is essential for the fusion of primary

myoblasts and it is likely that the active component has a neuronal origin

(Wakelam and Pette, 1983).

Myoblast fusion is calcium-dependent, but low calcium concentrations

do not inhibit myoblast proliferation and alignment. Other divalent

cations cannot substitute for calcium in myoblast fusion (Schudt et al.,

1973). The kinetics of fusion have shown that multiple processes

involving calcium are likely to be involved.

There is good evidence for the involvement of surface proteins in

myoblast fusion. Glycoproteins particularly are implicated, as both

binding of surface glycoproteins by concanavalin A and inhibition of

protein glycosylation inhibit fusion (Burnstein and Shainberg, 1979;

17

Gilfix and Sanwal, 1980). The synthesis of a number of proteins has been found to increase with the onset of fusion and to decrease thereafter. These include fibronectin and a number of other unidentified cell-surface proteins (Walsh and Phillips, 1981). There are also developmentally regulated changes in phosphorylation of unidentified plasma membrane proteins. Studies with monoclonal antibodies have shown that quantitative changes in cell surface antigens occur, as well as changes in their distribution. Developmental changes in antigenic expression have been observed and used to define stages in muscle differentiation in vitro (see Walsh et al. 1984; Kaufman and Foster, 1984). However, the identity of these antigens is still unclear.

It is thought that myoblast fusion may be mediated by a specific proteinase: limited proteolysis can stimulate fusion in other systems and inhibition of metalloendoproteinase activity has been found to inhibit fusion (Couch and Strittmatter, 1983). Decreased amounts of high and increased amounts of low molecular weight surface proteins have been found during fusion (Couch and Strittmatter, 1983). A calcium-activated proteinase has been reported to appear in myoblasts around the time of fusion (see Wakelam, 1985).

The lipid components of the myoblast membrane have also been studied in relation to myoblast fusion. There is an increase in the lipids known to have fusogenic properties on the external leaflet of the myoblast membrane when compared with fibroblast membranes (Sessions and Horwitz, 1981; 1983). Membrane inositol phospholipids are broken down on fusion and there is an apparent increase in levels of 1,2-diacylglycerol and phosphatidic acid, both known to have fusogenic properties (Wakelam, 1983).

Membrane fusion has been suggested to proceed by removal of the hydration barriers which exist between apposed membranes. This could be achieved by breakdown of specific proteins and lipids, including inositol

18

phospholipids which have large, polar, heavily hydrated head groups, and which may also cross-link to other membrane components causing membrane rigidity. This breakdown has been shown to have some dependence on calcium. Protein and/or phospholipid breakdown could then result in the removal of the inhibitors of fusion which could then proceed (see Wakelam, 1985).

The fusion of myoblasts, which is a key developmental stage in the embryogenesis of skeletal muscle, and with which the induction of muscle gene expression is closely associated, is therefore a closely regulated and controlled process involving numerous modifications in membrane lipids and proteins, the exact roles of which, however, still remain unclear.

ii) Expression of muscle-specific genes in myogenesis

Work on the induction of expression of muscle-specific genes, like that on myoblast fusion mechanisms, has been carried out using tissue culture techniques to study the differentiation of either primary myoblast cultures or cultures of established myoblast cell lines (derived predominantly from rat and mouse). The use of cloned cDNA probes to measure mRNA levels for muscle-specific genes has shown that levels of mRNA parallel protein levels and therefore that control of expression of these genes is likely to be at the level of transcription. The accumulation of mRNA as a direct result of withdrawal of myoblasts from the cell cycle has also been shown to be a significant factor in the 500-fold accumulation of MHC mRNA during myogenesis of L6E9 cells (Nadal-Ginard et al., 1982).

The stoichiometry of the synthesis of several of the contractile proteins in myogenesis has been examined using primary myoblast cultures from quail muscle. The proteins studied (α-actin, tropomyosins α and β, MHC, MLC_1, MLC_2 and troponin C) were synthesised on differentiation at approximately equimolar rates, with the exception of a 2.5-fold elevated

rate of α-actin synthesis, suggesting a possible coordinate regulation mechanism (Devlin and Emerson, 1978; 1979). Studies using specific cDNA probes, however, have shown that muscle-specific mRNAs do not all appear at the same stage in myoblast differentiation. In a study of differentiation of mouse T984 myoblasts, MHC and skeletal muscle α-actin mRNAs were detectable from 96 hours in culture and reached a maximum at 144 hours (myotubes first appear at 120 hours in this system). In contrast, MLC_1 and MLC_3 did not appear until later (from 144 hours) and were preceded by the embryonic isoform MLC_{1emb}, both mRNA and protein levels of which then decrease (Buckingham et al., 1982). It is important to note that because of the differing homology between probes and mRNAs and also differences in labelling, it is difficult to determine the exact time of the onset of mRNA synthesis and the relative levels of different muscle-specific mRNAs.

There is a tendency for differentiating myoblasts to synthesise the appropriate embryonic muscle protein isoforms, where these occur, e.g. differentiating L6 rat myoblasts synthesise the MHC_{emb} isoform (Whalen et al., 1982). In some cases both adult fast and slow muscle isoforms are expressed. For example, myotubes derived from chick presumptive slow muscle express both slow and fast MLC_1 and MLC_2 isoforms, suggesting that the adult pattern of MLC expression, characteristic of fast or slow muscle is established later in muscle development (Stockdale et al., 1981).

Synthesis of proteins other than those of the contractile apparatus is also induced on myoblast differentiation (see Pearson, 1980). These include enzymes of muscle metabolism (e.g. creatine kinase, adenylate kinase, glucose-6-phosphate dehydrogenase, glycogen synthetase, glycogen phosphorylase, etc.) and also mitochondrial enzymes including succinate cytochrome c reductase and membrane proteins including acetylcholine receptor and acetylcholinesterase.

Some enzymes show changes in the isozymes expressed following

myogenesis. These include fructose diphosphate aldolase, phosphofructokinase and creatine kinase (see Pearson, 1980). Creatine kinase (CK) undergoes a large increase in total enzymatic activity (~600-fold) and also an isozymic transition from the brain form, BB-CK, which is predominant in myoblasts, to the muscle form, MM-CK, which is located in the myofibrillar M line in differentiated muscle. M-CK mRNA is found to begin to accumulate very early in mouse MM14 myoblast differentiation; only two or three hours after the first cells become committed and as much as six hours before fusion begins (Chamberlain et al., 1985). These authors point out, however, that the timing and magnitude of CK induction varies considerably between myogenic cells derived from different species.

The synthesis of many proteins is induced in myogenesis, but that of a large number must also be repressed; the overall complexity of the myotube transcript population in only about half that of the myoblast, as assayed by non-repetitive DNA hybridisation (Ordahl and Caplan, 1976). However, little attention has been focused on characterisation of these proteins. One interesting example is the cellular thymidine kinase gene. Expression of this gene is shut down on terminal differentiation of myoblasts. Transformation experiments have shown that information sufficient to specify this muscle-specific regulation is entirely intragenic (Merrill et al., 1984).

iii) Control mechanisms in myogenesis

During myogenesis myoblasts cease DNA synthesis, fuse and start to express a wide variety of muscle-specific genes. The order and interdependence of these events is not clear, although it has been known for some time that inhibition of myoblast fusion, for example by reducing the calcium concentration in the growth medium, does not necessarily inhibit muscle protein synthesis (e.g. Emerson and Beckner, 1975).

Nadal-Ginard et al. (1982) have shown that muscle gene expression can be reversibly induced in G_1 myoblasts, indicating that myoblast commitment ie. irreversible withdrawal from the cell cycle, and biochemical differentiation are separate events in myogenesis, although both are required to produce a stable phenotype. An alternative mechanism for the generation of post-mitotic differentiated muscle cells, whereby committed stem cells each undergo four divisions to produce 16 terminally differentiated muscle cells has been proposed (Smith Quinn et al., 1985). This model accounts for the otherwise strange observation that chick myogenic clones contain a multiple of 16, or just under such a multiple, differentiated cells.

Recent work involving cell hybrids and heterokaryons between muscle and non-muscle cells has thrown some light on the problem of myogeneic control mechanisms. Human muscle genes (MLC_1, MLC_2 and muscle creatine kinase) were found to be induced in heterokaryons formed between human amniocytes and differentiated mouse myocytes, thus indicating the presence of cytoplasmic activators of muscle-gene expression within the mouse muscle cells. These activators are capable of inducing muscle gene expression in nuclei of a different species and of a cell type which would never normally express these genes. Moreover, human muscle gene products were detectable only 24 hours after cell fusion and at a level per nucleus comparable to that of the endogenous mouse proteins (Blau et al., 1983). Further work using the same heterokaryon system (Chiu and Blau, 1984) showed that induction of human amniocyte muscle genes occurred in the absence of DNA synthesis. The conformation of the chromatin in these cells is therefore accessible to the cytoplasmic muscle regulators in the absence of any modifications which require DNA synthesis. Furthermore, in heterokaryons where DNA synthesis was not inhibited, the human amniotic fibroblast nuclei were shown to be in cell cycle phases G_1, S or G_2, and muscle gene expression was induced in nuclei in each phase. This

demonstrates that muscle gene expression is not restricted to the G_1 phase of the cell cycle, although in normal differentiation myoblasts become committed in G_1 and in the absence of DNA synthesis (Pinset and Whalen, 1985).

In similar experiments, Wright (1984) made heterokaryons between differentiation-defective L6 rat myoblasts and differentiated chick myocytes. Rat embryonic and adult MLC_1 were induced, indicating that these rat genes were responsive to chick inducing factors and that the defective myoblasts were not producing large amounts of suppressors of muscle gene expression. Wright then went on to make a series of cell hybrids with fusion-competent (fus^+) and fusion defective (fus^-) L6 myoblasts. From the observed frequency of differentiation of hybrids, Wright hypothesised that a mechanism involving a threshold occupancy of multiple receptor sites is required to trigger the terminal differentiation of myoblasts. To test this hypothesis, Wright (1985) carried out repeated selection of myoblasts resistant to increasing concentrations of 5-bromodeoxyuridine (BUdR), which is known to inhibit differentiation in a number of systems. When hybrids were made between BUdR-resistant myoblasts and fus^- myoblasts, the frequency of differentiation was significantly higher than in hybrids between normal and fus^- myoblasts. This is consistent with the hypothesis that the selected cells were overproducing the factor or factors involved in the decision to differentiate, although, as Wright points out, the mechanism for this is unknown and it is likely that BUdR affects multiple processes in the sequence of steps between commitment and the expression of structural proteins. The availability of a system where expression of such factors may be amplified might, however, allow such regulatory genes to be identified.

Gene expression in general has been shown to be correlated with the methylation pattern of sequences surrounding the gene (Doefler, 1983).

Hypomethylation is often associated with the induction of gene expression and the change in methylation has been suggested to precede expression. For example, the rat phosphoenolpyruvate carboxykinase gene is hypermethylated in tissues in which it is not expressed and hypomethylated where it is expressed (adult liver). In the foetal liver, which does not yet express the gene, there is a partial hypomethylation, suggesting the gene is competent to be expressed. Treatment with 5-azacytidine causes hypomethylation and subsequent activation of this gene (Benvenisty et al., 1985). With regard to muscle-specific genes, treatment of HeLa cells with 5-azacytidine makes them capable of expressing a muscle-specific surface antigen and muscle creatine kinase in heterokaryons formed with mouse muscle cells. 5-azacytidine is presumed to act by reducing methylation of these genes in HeLa cell nuclei, rendering them capable of responding to putative trans-acting muscle regulatory factors (Chiu and Blau, 1985). Hypomethylation on a genomic scale has been shown to be associated with terminal differentiation of other cell lines (Bestor et al., 1984). Curiously, the methylation of individual muscle-specific genes is not correlated with their expression. Shani et al. (1984) found that the patterns of methylation of the rat skeletal muscle α-actin and MLC_2 genes were similar in DNA from myoblasts and myotubes and that in these cells the genes were unexpectedly more methylated than in non-muscle tissues.

Transcriptionally active genes have also been shown to be associated with an altered chromatin conformation which confers sensitivity to DNase I digestion. Muscle-specific genes, including the rat MLC_2 (Shani et al., 1982) and skeletal muscle α-actin genes (Carmon et al., 1982) have been found to be sensitive to DNase I in differentiated cultures, but not in proliferating myoblasts.

Coordinate expression of muscle proteins raises the possisility that their genes may be linked. However, no linkage of muscle genes has been found in mammals, except for a loose linkage between MHC_f and MLC_{1emb}

in the mouse (Robert et al., 1985).

Muscle gene promoters seem to be typical of those of genes transcribed by RNA polymerase II (see Chambon et al., 1984). As yet there is no evidence for muscle-specific gene promoter or enhancer elements which could be involved in muscle differentiation and gene induction. However, significant sequence conservation has been noted in the 5' region between homologous genes in different species. These include homology between human and rat fast MLC_2 genes (Nudel et al., 1984), and between chicken and rat α-actin genes, where there is significant sequence conservation between 230 and 80bp upstream from the cap site (Nudel et al., 1985). Conserved regions also occur in the 3' non-translated sequences of actin genes (Ordahl and Cooper, 1983; Mayer et al., 1984). The best demonstration that control elements for a particular gene are present in a particular sequence and are conserved between species comes from the observation that genes can be introduced into the cells of another species and are expressed normally in differentiation. For example, the chicken skeletal muscle α-actin gene is regulated in differentiation of stably transfected rat myogeneic cells (Nudel et al., 1985). Similarly, the rat MLC_2 gene is correctly expressed in the skeletal muscle of transgenic mice (Shani et al., 1985). All the information required for regulated espression is contained in the 5' flanking sequences of the rat actin gene. This sequence, fused to the chloramphenicol acetyl transferase gene, specifies correct gene induction in transfected L6 myoblasts (Melloul et al., 1984).

There has been some interesting recent work on the activation of muscle actin genes in developing Xenopus embryos. α-skeletal and α-cardiac actins are coordinately induced at the end of gastrulation. Both are located in mesoderm destined to form embryonic muscle. Cytoskeletal actin transcripts, in contrast, are present throughout the embryo (Mohun et al., 1984). Gurdon et al. (1985) have shown that cardiac α-actin mRNA

is synthesised in a sub-population of animal cells in response to an inducing stimulus produced by the vegetal cells. The minimum cell contact time required for induction is very short; 1.5 to 2.5 hours, about the same time as taken for the diffusion of small molecules between inducing and responding cells. mRNA can be detected within 5 to 7 hours of induction. However, nothing is yet known of the nature or mechanism of action of this putative induction regulator.

1.6 Myoglobin in human muscle

Chapters 4 and 5 deal with the expression of the myoglobin gene in different adult human muscle tissues and in developing muscle. This section summarises what is already known about myoglobin levels in human muscle.

Myoglobin concentration is related to muscle type and therefore to muscle fibre type (James, 1968). Many authors have reported myoglobin concentrations in muscles of undefined or mixed fibre type, or have omitted to mention the source of the muscle used. The variation in myoglobin concentration accountable to differing skeletal muscle fibre type in one species, however, is almost undoubtedly smaller than the interspecific variations described in this thesis. Variation between different skeletal muscle types within a single species will therefore be disregarded.

A number of estimates of myoglobin concentrations in human skeletal muscle have been published. Concentrations vary from 2.1mg myoglobin /g wet weight (Perkoff and Tyler, 1958) to 7.0mg/g (Biorck, 1949), with the majority of values at approximately 5mg/g (e.g. Moller and Sylven, 1981, for femoral limb muscle). Similarly there have been a number of estimates of myoglobin concentration in human cardiac muscle. These too are somewhat variable, but approximate to 3mg/g, slightly lower than concentrations in skeletal muscle (Biorck, 1949). Wittenberg (1970)

speculates that the higher myoglobin concentration in red skeletal muscle compared with heart may be due to a longer oxygen diffusion radius in skeletal than in cardiac muscle (see section 1.1ii).

Evidence for the presence of myoglobin in smooth muscle is contradictory. Early work using spectroscopic methods reported a low concentration of 0.5mg/g in uterine muscle (Biork, 1949). More recently a report was published by Fasold et al. (1970) stating that myoglobin could not be detected in either human uterus or taenia coli. This conclusion was based on an immunological assay for myoglobin, which the authors claim was sensitive to a myoglobin concentration as low as 0.001mg/g (see chapter 4).

Some evidence is also available on the levels of myoglobin in immature muscle. Using myoglobin antiserum in an immunological assay for myoglobin, Kagen and Christian (1966) claimed to detect myoglobin in human foetal skeletal muscle at 20 weeks gestation at a level as low as 0.5% of that in adult muscle and found that levels of skeletal muscle myoglobin increased with development such that at birth the level was approximately 10% of adult. Using a spectrophotometric method for determining concentrations of myoglobin, Longo et al. (1973) found barely detectable levels of myoglobin (less than 1% of adult levels) in skeletal muscle of lamb foetuses described as 'near term'. Tipler et al. (1978) could not detect myoglobin in human skeletal muscle extracts from foetuses of 19 to 24 weeks gestation by SDS-polyacrylamide gel electrophoresis and staining with Coomassie brilliant blue. These reports suggest that foetal skeletal muscle does contain low levels of myoglobin, but it is not clear how much, nor at which stage myoglobin first appears.

In contrast to the extremely low levels of myoglobin in foetal skeletal muscle, the myoglobin concentration has been found to be significantly higher in foetal cardiac muscle. Myoglobin can be detected in extracts from 21 week human cardiac muscle by SDS-polyacrylamide gel

electrophoresis and staining with Coomassie brilliant blue (Tipler et al.,
1978). In near term lambs, Longo et al. (1973) found that although there
were barely detectable levels of myoglobin in skeletal muscle, the level
in cardiac muscle was as high as 50% of the adult cardiac myoglobin
concentration, possibly reflecting their different work loads in utero.

1.7 Adaptations in levels of myoglobin

One of the characteristic features of muscle as a tissue is its high
degree of plasticity and its many adaptations in response to both internal
and external stimuli. This section describes adaptations specifically in
myoglobin concentrations, both those seen in different species and also
those seen in individuals in response to stimuli which also affect many
other aspects of muscle physiology.

The concentration of myoglobin in human skeletal muscle is
approximately 5mg/g wet weightof muscle. This is a fairly typical figure
for larger terrestrial mammals. Smaller mammals, in contrast, tend to
have lower skeletal muscle myoglobin levels. For example, the myoglobin
concentration in rabbit skeletal muscle is 0.2mg/g (Lawrie, 1953a) and
that in mouse is estimated to be as low as 0.3-0.5mg/g (Weller et al., in
preparation). Histochemical staining shows high numbers of fast-twitch
(type II) fibres in rodent muscle, which show no staining for myoglobin
(James, 1968) and large numbers of fibres with high ATPase activity,
indicative of innervation by fast motor neurons. The proportion of fibres
with high ATPase activity was found to decrease dramatically with body
size, particularly in the diaphragm (Davies and Gunn, 1971).

Some species have significantly elevated myoglobin levels. These
include those living in an environment with reduced oxygen tension; for
example diving and fossorial species and those living at high altitude.
Reynafarje and Morrison (1962) found that wild Peruvian rodents of the
genus Phyllotis (pericotes) from a height of over 4,000m had twice as much

myoglobin in diaphragm and leg muscles as a sub-species from sea level. The mole rat Spalax and pocket gophers Geomyidae, fossorial rodents, also have elevated skeletal muscle myoglobin (see Nevo, 1979).

Perhaps the most dramatic increase in myoglobin levels occurs in diving species, both mammals and birds. Levels in skeletal muscles of these species can be as high as 90mg/g of muscle (wet weight) in sperm whale, (Scholander, 1940). Aquatic species which do not dive for such prolonged periods show less elevated myoglobin levels, for example, 38mg/g in porpoise (Blessing, 1972) and 26mg/g in sea otter (Lenfant et al., 1973). Diving birds (penguins) also show elevated myoglobin levels (approximately 40mg/g) in several species (Weber et al., 1974).

That skeletal muscle myoglobin concentration is directly related to diving ability is most likely an oversimpification, as the physiological responses to diving, especially of the cardiovascular system, are complex. Moreover, myoglobin levels in some of the deep diving species do not appear to be remarkably high. The blue whale and the harp seal have only approximately 10mg/g and 7mg/g myoglobin, respectively (Lawrie, 1953a; George et al., 1971). However, there remains no doubt that most diving mammals have greatly elevated myoglobin levels and this has long been seen as an adaptation to a diving physiology, to provide an increased oxygen store (see Butler and Jones, 1982).

The provision of additional oxygen stored as oxygenated myoglobin is only one adaptation in the specialised physiology of diving mammals. Most natural dives of these species are under aerobic metabolism. This is achieved by a number of adaptations. Oxygen stores in both blood and tissues are higher (per kg body mass) than in terrestrial mammals. On diving, the heart rate slows considerably and blood supply to the gut, kidneys, liver and inactive skeletal muscle is reduced while blood pressure and flow rate to heart and brain are maintained. Only during abnormally long or strenuous dives does tissue metabolism become anaerobic

(see Butler and Jones, 1982).

So far in this section only the adaptations in myoglobin concentration which occur in individual species of a particular habitat or physiology have been mentioned. However, myoglobin concentrations can also be altered within a single individual in response to a number of internal and external stimuli. I have already mentioned that species living at high altitude can have elevated skeletal muscle myoglobin levels when compared with related lowland species. This adaptive effect of altitude has also been demonstrated by Vaughan and Pace (1956) and Tappan and Reynafarje (1957), who found that rats and guinea pigs, respectively, reared at an altitude of about 4,000m showed significantly higher levels of both cardiac and skeletal muscle myoglobin. People living at high altitude also have elevated skeletal muscle myoglobin (Reynafarje, 1962). Interestingly, Rosenmann and Morrison (1965) and Morrison et al. (1966) have found a seasonal fluctuation of as much as 2.5-fold in skeletal muscle myoglobin levels of the snowshoe hare and northern red-backed vole.

Another way in which levels of myoglobin can be increased is by muscular exercise, particularly endurance exercise. Exercised rats were found to have significantly increased hind limb muscle myoglobin concentrations (Lawrie, 1953c; Pattengale and Holloszy, 1967). Similarly, immobilised rats showed a decrease in myoglobin level (Lawrie, 1953c). Change in myoglobin concentration is only one of a whole pattern of responses to increased muscular exercise (see Salmons and Henriksson, 1981). There is also a marked increase in the capacity for oxidative metabolism which results from an increase in levels of enzymes of the citric acid cycle, respiratory chain and fatty acid metabolism (Holloszy and Booth, 1976). Mitochondria increase in size and number in parallel with increased enzyme activities. The capillary density has also been observed to increase as a response to endurance exercise (Mai et al., 1970). However, there is no transformation of fibre type seen as a result

of electrical stimulation (see below), nor is there any change in fibre number (Salmons and Henriksson, 1981).

Chronic low frequency stimulation of fast-twitch skeletal muscle leads to an orderly sequence of changes which ultimately bring about transformation of fibres from fast-twitch (type II) to slow-twitch (type I), over a period of several weeks. This can also be achieved in part by innervating a fast muscle e.g. flexor digitorum longus with the nerve which supplies a slow muscle e.g. soleus. These changes include a 2-fold increase in myoglobin concentration in the flexor digitorum longus cross-innervated in this way (McPherson and Tokunaga, 1967). Other changes as a result of stimulation of a fast muscle include an increase in the oxidative metabolic capacity (Pette et al., 1973) and capillary density (Brown et al., 1976), as described for responses to increased exercise. There are changes in the MHC and MLCs, resulting in accumulation of isoforms characteristic of slow muscle (e.g. Streter et al., 1973). There is also a reduction in the extent of the T-system and SR to a distribution typical of slow muscle and an increase in the numbers of mitochondria (Salmons and Henriksson, 1981). These changes parallel the simultaneous changes in isometric contractile properties which increasingly resemble those of a slow-twitch muscle (Salmons and Vrbova, 1969). On removal of stimulation, the fast muscle slowly regains its former characteristics (see Salmons and Henriksson, 1981).

The extensive response of skeletal muscle to a number of both internal and external stimuli demonstrates the high degree of plasticity of this tissue in its ability to modulate function while maintaining specific tissue characteristics. Such changes in phenotype must reflect alterations in the pattern of gene expression, including that of the myoglobin gene, the expression of which is altered in response to a number of these stimuli. As yet, however, we have no knowledge of the mechanisms by which these patterns of gene expression are controlled.

1.8 The myoglobin gene

i) Structure and expression of the globin genes

Myoglobin is a member of the globin gene family, perhaps the most extensively studied gene family. Myoglobin and the other globin genes are thought to have arisen as a result of successive duplications of an ancestral globin-like gene and the subsequent divergence of the resulting genes. The time of divergence of myoglobin from the haemoglobin ancestor has been separately estimated as 800mya, which is before the emergence of the vertebrates, (Hunt et al., 1978) and 500mya (Czelusniak et al., 1982), very early in vertebrate evolution. The initial myoglobin/haemoglobin duplication was then followed by sequential duplication events leading to the modern functional haemoglobin genes (see Efstratiadis et al., 1980).

In man these genes are the embryonic ζ and adult α2 and α1 genes in the α-like gene cluster, closely linked in this order on chromosome 16 and the embryonic ε, foetal $^G\gamma$ and $^A\gamma$ and adult δ and β of the β-like cluster, also closely linked in this order on chromosome 11. The genes in each cluster are orientated in the same direction.

All functional vertebrate globin genes characterised have a similar three exon and two intron structure. The positions at which the introns interrupt the coding sequence are precisely homologous in all genes; between codons 30 and 31 and codons 104 and 105 in the human β-globin genes (Efstratiadis et al., 1980). The central exon encodes the haem-binding domain of globin and the first and third exons encode domains required for stability of the haemoglobin tetrameric complex (Go, 1981).

A large number of globin genes has now been cloned and sequenced. The genes show similarity in length of introns and 3' and 5' non-translated regions (Blanchetot et al., 1983). There are also similarities in the promoter elements of the 5' flanking sequences. Globin gene promoters, like those of many other genes transcribed by RNA polymerase II, have a TATA box approximately 30bp upstream from the cap

site. There is another conserved sequence CCAAT at approximately ≈75.

These sequences have been shown to be involved in determination of correct

initiation and rate of transcription, respectively (Grosveld et al.,

1982). In addition, a dimerised CACCC element is present in the ≈100

region of β-globin genes (Dierks et al., 1983).

During development, the globin genes first start to be expressed

during the differentiation of erythroid precursor cells. Globin mRNA is

first detected in proerythroblasts and becomes the most abundant mRNA in

reticulocytes. Erythropoiesis in very early mammalian embryos occurs in

the yolk sac and then transfers gradually to the foetal liver and then the

bone marrow, which is the site of erythropoiesis in the adult (see Collins

and Weissman, 1984; Karlsson and Nienhuis, 1985). At all times α and β

like chains are expressed in a 1:1 ratio. Embryonic globin genes are

expressed early in development. In man the embryonic α-like chain is ζ

and the corresponding β-chain is ε. In man the ε chain is replaced by γ,

product of the $^G\gamma$ and $^A\gamma$ genes, during later prenatal development. There

is no foetal α-like chain in man. In the third trimester, the switch

from γ to β begins. Production of γ decreases and synthesis of β

increases coordinately, keeping the total β+γ relatively constant. By six

months after birth the level of foetal haemoglobin is less than 1%.

Synthesis of the minor β-like component δ also begins just prior to birth.

Although our knowledge of the structure of the globin gene clusters

is very extensive, our understanding of the control of tissue-specific and

developmentally regulated globin gene expression remains poor. A major

problem in approaching these questions has been the lack of an

experimental system in which gene switching can be generated with cloned

globin genes. It has been shown that globin genes introduced into

erythroid cell lines (including MEL cells) with limited amounts of

flanking DNA, can specifically be activated on differentiation of the

cells in vitro (eg. Charnay et al., 1984). Recently, Magram et al.

(1985), in the first demonstration of stage-specific regulation of a cloned globin gene, have shown that a hybrid mouse/human adult β-globin gene is correctly expressed in the foetal liver and adult bone marrow of transgenic mice. Significantly, this gene is not expressed in embryonic blood cells where normally only the mouse embryonic β-like genes y and βh1 are expressed. Enough information for correct developmental regulation is therefore contained within the 5kb DNA fragment on which the gene was injected. Magram et al. hypothesise that the switch of β-globins from embryonic to adult in the mouse is mediated by specific trans-acting factors. Trans-acting factors have also been implicated more directly in the control of globin gene expression. HPFH (hereditary persistance of foetal haemoglobin) is a condition in which $^G\gamma$ and $^A\gamma$ genes are not turned off and the switch from foetal to adult haemoglobin does not occur. Foetal sheep serum was found to contain a factor which turned off the $^G\gamma$ and $^A\gamma$ genes in HPFH cells (Papayannopoulou et al., 1984).

ii) The number of myoglobin genes

Unlike the globins described above and unlike many of the muscle-specific proteins described earlier, adult and foetal myoglobins are identical (Schneiderman, 1962). Also unlike the globins, there are surprisingly few known human myoglobin variants. Only four have been characterised to date, all of which are single amino acid substitutions (Boulton et al., 1969; 1971a,b,c). In none of these cases was the variant myoglobin associated with any muscle disorder. About 50% of the myoglobin of these individuals was found to be variant. In addition, where myoglobin of the parents was analysed (in uncharacterised variants, Boyer et al, 1963), the variant was found to account for approximately 50% of the myoglobin of one parent only, as expected for a heterozygote. This is therefore evidence that myoglobin is specified by a single functional gene in man.

iii) Structure of the grey seal (<u>Halichoerus grypus</u>) myoglobin gene

The globin gene family represents one of the best characterised gene families. However, until recently, nothing was known about the myoglobin gene. Knowledge of myoglobin gene structure was potentially interesting in that the gene diverged from the globin gene ancestor very early, before the α/β globin divergence, and so could provide information about events which occurred early in globin evolution. A study of a mammalian myoglobin genes also permits analysis of expression of the myoglobin gene, which, alone of the globin genes has been incorporated into the myogenic developmental system, itself extensively studied.

Initial attempts at cDNA cloning of human myoglobin mRNA failed (Wood, PhD thesis, 1984). Grey seal skeletal muscle, rich in myoglobin and also therefore likely to be a rich source of myoglobin mRNA, was therefore used for cDNA cloning (Wood <u>et al</u>., 1982). A seal myoglobin cDNA was identified, and hybridisation to Southern blots of restriction endonuclease-digested seal DNA indicated that the grey seal genome contained a single functional myoglobin gene (Wood <u>et al</u>., 1982).

The grey seal myoglobin cDNA was used as a probe to isolate the myoglobin gene from a grey seal genomic library. Like the other globin genes, the gene was found to consist of three exons and two introns (Blanchetot <u>et al</u>., 1983). The coding seqence is interrupted by the introns at positions exactly homologous to those in α- and β-globin genes. The three intron and two exon structure of haemoglobin genes is now known to extend to myoglobin genes, showing that this gene organisation is ancient and must pre-date the divergence of haemoglobin and myoglobin. Other globin gene structures, however, are known. Plant leghaemoglobin genes have three introns, the first and third in homologous positions to the vertebrate globin gene introns (Jensen <u>et al</u>.,1981), suggesting that at least these two introns are ancestral to animal and plant globin genes. The third intron, which splits the second exon of vertebrate globin

35

genes, was either lost from the animal globin gene ancestor, or gained by the leghaemoglobin gene, after the plant/animal divergence. In addition, all introns have been lost from insect (Chironomus) larval globin genes (Antoine and Nessing, 1984).

The most striking feature of the seal myoglobin gene, compared with α- and β-globin genes, is its length; 9.2kb. The 5' and 3' non-translated regions are 70 and 548nt, respectively, both considerably longer than those of α- and β-globin genes (Blanchetot et al., 1983). Thus a myoglobin mRNA length of 1083nt, excluding the poly A tail, is predicted from sequence and S1 nuclease protection mapping data. This is consistent with a mRNA of approximately 1400nt detected in Northern blot hybridisations of grey seal poly(A)[+] RNA using the myoglobin cDNA as a probe (Wood et al., 1982).

The seal myoglobin gene 5' flanking region contains features different from those of α- and β-globin genes. There is a conventional TATA box 32bp upstream from the cap site, but there is no CCAAT box, normally occurring at around position -75 in α- and β-globin genes. Instead the region from approximately -60 to +100 is occupied by an unusually purine-rich sequence. The 3' non-translated region contains the conventional AATAAA polyadenylation signal, 22bp from the polyadenylation site.

It has been pointed out that the globin genes (α, β and leghaemoglobin genes, and particularly the first introns of these genes) show remarkable constancy in length. This led to the speculation that intron length might in some way be important in gene expression (van den Berg et al., 1978). The extremely elongated seal myoglobin gene introns indicate, however, that short non-coding regions are not a pre-requisite for globin gene expression. However, as pointed out by Blanchetot et al. (1983), it is not clear whether the unusually elongated gene structure and unusual promoter features are peculiar to the grey seal, perhaps as a

36

recent adaptation to a diving physiology, or whether they represent a general feature of mammalian myoglobin genes.

1.9 Objectives of the research

The work in this thesis is concerned with the structure of the human myoglobin gene and with myoglobin gene expression. The main questions considered are: 1), is the structure of the seal myoglobin gene typical of mammalian myoglobin genes, and how do seal and human myoglobin genes compare? 2), is myoglobin expression restricted to muscle, and at what levels is it expressed? 3), when, and at what levels is the myoglobin gene expressed during muscle development? 4), what is the molecular basis of the adaptation leading to high myoglobin levels in the grey seal? and 5), can an in vitro model system be developed for studying myoglobin expression?

Chapter 2

MATERIALS AND METHODS

2.1 DNA, RNA and Tissue

DNA had previously been prepared from human placenta. Human adult

skeletal calf muscle (amputation, 67 year old male) was supplied by Dr

Simon Walker (Pathology Department, University of Leicester). Grey seal

skeletal muscle (Halichoerus grypus), from which DNA had already been

prepared, was supplied by Dr John Prime (British Antarctic Survey,

Cambridge). Embryonic, juvenile and adult mice (outbred) were obtained from

the animal unit (Royal Infirmary, Leicester). Various human muscle RNA

samples were kindly provided by Dr Yvonne Edwards (University College,

London). Cell line RNAs from L6 and G8 myoblasts and myotubes and 1R

fibroblasts were from Dr Mike Webb (Institute of Neurology, University of

London). Mouse liver DNA (DBA/2) was provided by Dr Susan Adams, and G8

myoblast and myotube DNAs by Melanie Price.

2.2 Plasmids, bacteriophage and bacterial strains

pAM91 was a gift from Dr Steve Humphries (St Mary's Hospital Medical

School, London) and the myosin light chain cDNA plasmid was a gift from Dr

Yvonne Edwards (University College, London).

The lambda replacement vector λL47.1 (Loenen and Brammar, 1980) was

used to make the human genomic DNA library. Plasmid pAT153 (Twigg and

Sherratt, 1980) was used for subcloning DNA fragments from recombinant

bacteriophage. The M13 vector M13mp8 (Messing and Vieira, 1982) was used in

shotgun cloning of DNA fragments for sequencing, and M13mp18 (Yanish-Perron

et al., 1985) was used for cloning of DNA fragments to make single-stranded

cDNAs for probes and for the message abundance assay.

Escherichia coli strains used are listed in Table 2.1.

Table 2.1   Genotypes of E. coli strains

Strain	Genotype	Reference
HB101	F⁻ recA13, hsdS20, lacY1, rpsL20, ara-14, galK2, xyl-5, supE44, mtl-1, proA2	1
ED8910	supE44, supF58, recB21, recC22, hsdS, metB, lacY1, galK2, galT22	2
JM101	Δ(lac-pro), supE44, thi-1, F'traD36, proAB, lacI^q, ZΔM15	3
JM103	Δ(lac-pro), thi-1, rpsL20, supE44, endA, sbcB15, hsdR4, proAB, lacI^q, F'traD36, ZΔM15	3
BHB2688	N205 recA (λimm434, cIts, b2, red3, Eam4, Sam7/λ	4
BHB2690	N205 recA (λimm434, cIts, b2, red3, Dam15, Sam7/λ	4
WL87	803, supE, supF, hsdR$_k$, hsdM$_k$, tonA, trpR, metB,)	5
WL95	P2 lysogen of WL87	5

References:

1   Maniatis et al. (1982)

2   Loenen and Brammar (1980)

3   Messing (1981)

4   Hohn (1979)

5   from W.J.Brammar

2.3. Enzymes, antibiotics, chemicals and reagents

The sources of the most important of these are listed below. All other chemicals were analytical grade.

	source
deoxyribonucleotide triphosphates	Bethesda Research Labs
S1 nuclease	"
urea	"
M13mp8 and M13mp18 RF DNAs	"
restriction enzymes, unless stated otherwise	"
lysozyme	Sigma
dextran sulphate (sodium salt)	"
dithiothreitol	"
spermidine trichloride	"
ficoll 400	"
salmon sperm DNA (sodium salt)	"
bovine serum albumin	"
dimethyldichlorosilane	"
piperidine	"
isopropyl-β-D-galacto-pyranoside (IPTG)	"
ampicillin	"
N,N,N',N'-tetramethyl-ethylenediamine (TEMED)	"
polyethylene glycol (PEG)	"
diethylpyrocarbonate (DEPC)	"
thymine	"
3-(N-morpholino)propanesulphonic acid (MOPS)	"
piperazine-N,N' bis(2-ethane-sulphonic acid) (PIPES)	"
DNA polymerase I (Klenow enzyme)	Boehringer
calf intestinal phosphatase	"
calf liver tRNA	"
dideoxyribonucleoside triphosphates	"
DNase I	Worthington
T4 polynucleotide kinase	New England Biolabs
T4 DNA ligase	"
avian myeloblastosis virus reverse transcriptase	Life Sciences Inc.
polyvinylpyrrolidine (PVP)	Fisons
2,5-diphenyloxazole (PPO)	"
1,4-di-2(4-methyl-5-phenyl oxazolyl (POPOP)	"
Acrylamide	Unisciences
bisacrylamide	Bio-Rad Laboratories
ficoll 400	Pharmacia
sephadex G50	"
oligo(dT)cellulose	Collaborative Research
Agarose (standard and low melting point)	FMC Corporation
5-bromo-4-chloro-3-indolyl-β-D-galacto-pyranoside (BCIG)	Bachem Inc.
E.coli DNA polymerase I	Amersham
rabbit reticulocyte lysate	"
[14C] methylated protein mixture	"
[α-32P]dCTP, [α-32P]dATP, [γ-32P]ATP, and [35S]methionine	"

2.4 Media

Liquid and solid media used were as follows:

Luria broth (10g Difco Bacto Tryptone; 5g Difco Bacto yeast extract; 5g NaCl per litre). Luria agar plates were prepared by solidifying liquid medium with 15g Difco Bacto-agar per litre. For soft agar overlays 6g agar per litre were used.

BLA contained 10g BBL trypticase, 5g NaCl and 15g sterilin agar per litre. BTL soft agar overlay was as BLA, but contained only 6g of agar per litre. BBL agar, used for M13 work, contained 10g BBL trypticase, 5g NaCl and 5g $MgSO_4$ per litre, and was solidified with either 15g or 6g of agar as above. Other soft agar overlays were supplemented with 10mM $MgCl_2$

Glucose supplemented minimal medium, used to maintain strains JM101 and JM103 contained 42mM Na_2HPO_4, 22mM KH_2PO_4, 18mM NH_4Cl, 8mM NaCl, 22mM glucose, 0.1mM $CaCl_2$, 1mM $MgSO_4$, 3μM thiamine HCl and 0.17mM proline.

2.5 General techniques

2.5.1 General techniques for handling DNA and RNA

i) Phenol extraction

DNA and RNA solutions were mixed with 0.5 to 1 volume of phenol; chloroform; isoamyl alcohol; 8 hydroxyquinoline (100:100:4:0.4, w:v:v:w) saturated with 10mM Tris-HCl, pH 7.5. The upper aqueous phase, containing the DNA or RNA was removed and the phenol layer was rextracted with an equal volume of 10mM Tris-HCl, pH 7.5. The phenol was AR grade and was not redistilled.

ii) Ethanol precipitation

DNA and RNA were precipitated from solution by the addition of 0.1 volume of 2M Sodium acetate, pH 6.5, and 2.5 volumes of ethanol. The mixture was chilled for 5 minutes in an IMS/dry ice bath. Precipitated DNAs or RNAs were pelleted either at 13,000g for 30 minutes at 0°C (Sorvall HB4 rotor), or in an Eppendorf centrifuge for 5 minutes at maximum speed. The supernatant was discarded and the pellet rinsed with 80% ethanol, centrifuged briefly, and the 80% ethanol removed. DNA and RNA pellets were

dried under vacuum and redissolved as appropriate for further manipulation.

iii) <u>Note on handling RNA</u>

All solutions, glassware and centrifuge tubes etc. used in preparation or manipulation of RNA were made up with, or pre-treated with, a 0.1% solution of diethylpyrocarbonate (DEPC) in distilled water. Solutions and equipment were left overnight at 37°C before inactivation of DEPC by autoclaving.

2.5.2 <u>Restriction endonuclease digestion of DNA</u>

DNAs were digested in the manufacturer's recommended buffer, at a final concentration of ≤ 0.5mg/ml, at 37°C for 1 hour, unless otherwise stated. The efficiency of restriction endonuclease activity was enhanced by the routine addition of spermidine trichloride to a final concentration of 4mM. This was particularly important if the DNA had previously been recovered from an agarose gel (Bouche, 1981). Complete digestion was checked by electrophoresing an aliquot containing ~0.2µg of DNA in a suitable agarose gel against marker DNAs of known molecular weight. Incompletely digested DNA was incubated for longer with additional restriction endonuclease. After complete digestion, EDTA was added to a final concentration of 20mM, the DNA was recovered by phenol extraction and ethanol precipitation, and redissolved as appropriate for further manipulation.

2.5.3 <u>Preparative agarose gel electrophoresis and recovery of DNA from gels</u>

Preparation of samples, gel loading and electrophoresis were as described by Jeffreys <u>et al.</u> (1980). The quantity of DNA and slot size were such that no more than 0.5µg DNA per mm^2 of gel slot surface was loaded. This prevented overloading of the system. DNA in preparative gels was visualised by the fluorescence of bound ethidium bromide under long

41

wave ultraviolet light.

Two different procedures were used to recover DNA from gels:

i) <u>Vertical dialysis membrane</u>. DNA was electrophoresed onto a vertical

dialysis membrane inserted into a slot cut into the gel, as described by

Yang <u>et al</u>., (1979). The DNA was rinsed off the membrane with sterile

water.

ii) <u>DE81 paper</u>. DNA was electrophoresed onto Whatman DE81 DEAE-cellulose

paper, and recovered by a modification of the method of Dretzen <u>et al</u>.

(1981). DE81 paper was prepared by soaking in 2.5M NaCl and rinsing five

times in distilled water, and stored in 1mM EDTA at 4°C. The desired DNA

fragments were first separated by agarose gel electrophoresis, then cut out

of the gel in small pieces of agarose. These agarose blocks were then

wrapped in DE81 paper and replaced in the gel in slots cut away from any

other DNA in the gel. The DNA was electrophoresed onto the paper at high

voltage (200V). The gel slices were then removed from the paper and

unwrapped. The DNA on the DE81 paper was visualised by ethidium bromide

fluorescence under long wave ultraviolet light and the rest of the paper

cut away and discarded. DNA was recovered by vortexing the paper until it

disintigrated in high salt buffer (1M NaCl, 50mM Tris-HCl, pH7.5, 1mM

EDTA), and then incubating at 37°C for 15 minutes followed by 3 minutes at

65°C, to release the DNA from the paper. DNA was separated from fragmented

DE81 by centrifugation through a small polyallomer wool column in a

bench-top MSE centrifuge at maximum speed for a minute. The DNA was

recovered by phenol extraction and ethanol precipitation before further

manipulation.

2.5.4 <u>Photography and autoradiography</u>

DNA and RNA in agarose gels was visualised by fluorescence of bound

ethidium bromide using a short wave ultraviolet transilluminator

(Ultra-violet Products Inc., California, USA). Gels were photographed

using a Polariod MP-3 land camera and Polariod 4x5 type-52 or type-57 film.
Autoradiographs and Coomassie brilliant blue-stained gels were photographed
on a white light transilluminator, using a Nikon F camera and Kodak AHU
35mm film which was processed according to the manufacturer's instructions
using Kodak D19 developer and May and Bakers' Amfix fixer.

All autoradiography used Kodak X-ray film (35x40 or 13x18cm X-Omat RP
or 13x18cm X-Omat AR. Exposure times were judged by the cpm (counts per
minute) detected using a hand-held mini-monitor (Mini-Instruments Ltd, g-m
monitor, type 5.10). Agarose gels to be autoradiographed were first dried
to a glass plate using a commercial hair drier. Exposure to X-ray film in
the presence of an intensifying screen (Ilford Tungstate) was at -80°C,
otherwise at room temperature.

X-ray film was developed by immersion for 3 minutes in developer
(Kodak DX-80), followed by a rinse in dilute acetic acid and fixation for 3
minutes in Kodak FX-40 fixer plus HX-40 hardener.

2.5.5 Preparation of RNA from tissue

i) Total RNA

This method is an adaptation of that used by Minty et al. (1981). Up
to 10g of tissue, previously stored at -80°C, was immersed in liquid
nitrogen, then pulverised in a mortar and pestle, before being homogenised
in a Waring Blendor in approximately 10ml of lysis buffer per gramme of
tissue (lysis buffer = 6M urea, 3M LiCl, 20µg/ml heparin, 10mM sodium
acetate, pH 5.6, 0.1% SDS. The SDS was added from a concentrated stock
solution just before use). Homogenisation was for a total of 4 minutes, in
bursts of about 30 seconds. The homogenate was left at 4°C for 24 hours
then spun at 13,000g for 30 minutes at 4°C. The supernatant was discarded
and the pellet resuspended in 10ml 6M urea, 3M LiCl by vortexing.
Insoluble material was pelleted again by spinning at 13,000g for 30 minutes
at 4°C. The pellet was resuspended in 10ml 0.2M sodium acetate, pH 5.6,

43

0.1% SDS by vortexing. The RNA, now in solution was then extracted twice

with phenol and once with chloroform, centrifuging at 3,000g each time,

before ethanol precipitation, with a $-80°C$ chill. RNA was recovered by

spinning at 13,000g for 30 minutes at 0°C, was washed with 80% ethanol,

dried under vacuum and redissolved in a small volume of water.

For samples containing any bone tissue, the RNA solution was dialysed

overnight, after chloroform extraction, against 2mM Tris-HCl, pH 7.5, 0.1mM

EDTA to remove phosphate ions before ethanol precipitation. RNA

concentrations were determined by measurement of the optical density at

260nm using a Cecil Instruments CE 202 ultraviolet spectrophotometer with

CE 235 Micro-sipette control attachment. An A_{260} of 24 is equivalent to a

concentration of 1mg/ml of RNA.

Quality of RNA preparations were tested by gel electrophoresis in a

1.5% agarose gel.

ii) Polyadenylated RNA

Polyadenylated RNA (poly(A)$^+$ RNA) was purified by oligo(dT) cellulose

chromotography, as described by Aviv and Leder (1972). Columns of 50mg

(bed volume 0.2-0.3 ml) were prepared in siliconised Pasteur pipettes

plugged with polyallomer wool and equilibrated with several bed volumes of

1x binding buffer (0.5M LiCl, 0.5% SDS, 1mM EDTA, 10mM Tris-HCl, pH 7.5).

Total RNA was heat-shocked for 3 minutes at 65°C and cooled on ice before

the addition of an equal volume of 2x binding buffer. RNA was loaded onto

the column and the eluate collected, heat-shocked and re-loaded onto the

column twice more. Unbound RNA was washed through the column with 15-20

bed volumes of 1x binding buffer, and the poly(A)$^+$ RNA eluted with 10 bed

volumes of elution buffer (0.05% SDS, 1mM EDTA, 10mM Tris-HCl, pH 7.5).

Poly(A)$^+$ RNA was recovered by ethanol precipitation with a $-80°C$

chill and centrifugation at 13,000g for 30 minutes at 4°C. RNA pellets

were washed with 80% ethanol, dried under vacuum and redissolved in a small volume of water. Concentrations and quality of poly(A)$^+$ RNA were determined in the same way as for total RNA (see section 8).

2.5.6 Agarose gel electrophoresis and transfer to nitrocellulose filters

i) DNA (Southern blotting)

Horizontal slab gels with loading slots from 3-7mm long were electrophoresed in buffer (40mM Tris-acetate, 2mM EDTA, pH7.7) containing 0.5μg/ml ethidium bromide (Aaij and Borst, 1972). Gel size varied with the number of samples, from 5x7cm (minigel) to 20x20cm (mapping gel). The agarose concentration was varied between 0.4 and 3% (w/v), according to the anticipated DNA fragment sizes. Molecular weight markers were either λ x HindIII or pBR322 x Sau3A, or both. DNA samples were mixed with 0.5 volume of agarose beads (0.2% agarose gel in 20mM EDTA, 10% glycerol and a small amount of Bromophenol blue as an electrophoretic marker; this was prepared as described by Schaffner et al., (1976)). Gels were electrophoresed at 120V for a short time or at10-15V overnight, as appropriate, until the bromophenol blue had migrated approximately two thirds of the length of the gel.

For mapping, DNA was electrophoresed double-stranded on 0.5% agarose gels with 5-7mm slots. When DNA was to be transferred to nitrocellulose (Sartorius, 0.45μm pore size) the gel was denatured by acid/alkali treatment. After being photographed, gels were soaked twice in 0.25M HCl for 7 minutes each to reduce the size of the DNA fragments in situ by partial depurination. The DNA was denatured by soaking twice in 0.5M NaOH, 1M NaCl for 15 minutes each. This was followed by two 15 minute washes in 0.5M Tris-HCl, 3M NaCl, pH 7.5. Transfer was by a modification of the method of Southern (1975), as described by Barrie (PhD thesis, 1982).

ii) RNA (Northern blotting)

To test the quality and approximate concentration of RNA, samples

were electrophoresed in horizontal agarose gels as for DNA, the only difference being that the samples were heat-shocked at 65°C for 3 minutes prior to loading, and the DNA size markers were single-stranded (denatured by adding 0.1 volume of 1.5M NaOH, 0.1M EDTA 5 minutes before loading). Good separation was generally obtained with 1.5% agarose gels.

RNA was transferred to nitrocellulose by an adaptation of the method of Thomas (1980). Horizontal slab gels were used, as for DNA, with the same sized slots and were about 7mm thick. Agarose was dissolved in 0.5 gel volume of water, to which was then added 0.5 volume of 2x gel buffer (4.4M formaldehyde, 50mM sodium phosphate buffer, pH 7.6). RNA samples to be loaded were vacuum dried, then redissolved in 10µl 50% formamide, 2.2M formaldehyde, 20mM sodium phosphate buffer, pH 7.6, and denatured at 60°C for 10 minutes. 1 vol agarose beads was added before loading (agarose beads = 0.2% suspension of agarose in 2.2M formaldehyde, 20mM sodium phosphate buffer, pH 7.6, 10% glycerol and a very small amount of Bromophenol blue). DNA markers (λ x HindIII and/or pBR322 x Sau3A) were run single stranded, as described above. Gels were electrophoresed in 2.2M formaldehyde, 10mM sodium phosphate buffer, pH 7.6 at 100V, until the Bromophenol blue had migrated ~8cm from the slots (generally about 3 hours).

After electrophoresis, some formaldehyde was driven out of the gel by shaking gently in water at 60°C for 10 minutes, then in water at room temperature for 10 minutes. Gels were soaked in 20xSSC (saline sodium citrate; 1xSSC = 0.15M NaCl, 15mM trisodium citrate, pH 7.0). RNA was transferred to nitrocellulose as described for DNA (Barrie, PhD thesis, 1982), except that the nitrocellulose and first sheet of Whatman 3MM paper were soaked in 20xSSC, rather than 3xSSC, and after transfer, the filter was baked at 80°C for only 2 hours.

2.6 Filter hybridisations

2.6.1 Preparation of RNA and DNA spot hybridisation filters

i) <u>RNA</u>. The total amount of RNA required for making the desired dilution series was vacuum dried and redissolved in an appropriate volume of TE + 1mg/ml calf liver tRNA. (TE = 10mM Tris-HCl, pH 7.5, 1mM EDTA). The RNA was heat-shocked at 65°C for 3 minutes and then a 1 in 2 dilution series was made using TE + 1mg/ml tRNA. 2µl of each dilution was spotted onto a nitrocellulose filter. The largest amount of RNA spotted was generally 5-10µg total RNA or 0.5-1µg poly(A)$^+$ RNA. Filters were allowed to dry at room temperature, and then baked at 80°C for 2 hours before hybridisation (see section 2.6.3).

ii) <u>DNA</u>. The amount of DNA required for making the dilution series was vacuum dried and redissolved in 1M NaCl, 0.1M NaOH, 10mM EDTA. The DNA was denatured by heating to 100°C for 3 minutes. A 1 in 2 dilution series was made in the same solution and 2µl of each dilution was spotted onto a nitrocellulose filter which was allowed to dry and baked at 80°C for 4 hours. The maximum amount of DNA spotted was generally 5ng (this was purified DNA fragments used as hybridisation probes). Hybridisation was as for RNA filters.

2.6.2 Labelling of DNA probes with ^{32}P by "nick translation"

The method used to label DNA probes was that of Weller et al. (1984). 50-100ng of DNA in 5µl of water was denatured by heating in a boiling water bath for 3 minutes, then cooled on ice and the following were added:

2.5µl	10x nick mix (0.5M Tris-HCl, pH7.5, 50mM MgCl$_2$, 0.1M 2,mercaptoethanol
2µl	each of 50µM dATP, dGTP and dTTP
1µl	0.1M spermidine
1µl	8ng/ml DNase I ≈ freshly diluted from 1mg/ml stock in H$_2$O
1.5µl	[α-^{32}P]dCTP (0.37MBq/µl, 111TBq/mMol)
5 units	E.coli polymerase I
7µl	water

47

The reaction mix was incubated at 15°C for 90 minutes. A small

sample (1µl) was sometimes removed to check incorporation by

electrophoresing in a small agarose gel, drying the gel and

autoradiographing it. 50% incorporation, or better, was achieved, even

with substrates which labelled poorly by conventional nick translation of

double-stranded DNA. The reasons for this increased incorporation are not

known, but in no way affect behaviour in filter hybridisations. Reaction

was stopped by adding 25µl of 0.5% SDS, 12.5mM EDTA, 10mM Tris-HCl, pH 7.5,

and then was extracted with phenol. 100µg of high molecular weight salmon

sperm DNA (prepared as described by Harris, (PhD thesis 1985)) was added as

a carrier and the DNAs were ethanol precipitated without chilling or

centrifugation. The supernatant was removed and the DNA redissolved in

0.2ml 10mM Tris-HCl, pH 7.5, before being reprecipitated in the same way.

The DNA was rinsed with 80% ethanol and redissolved in 0.5ml 10mM Tris-HCl,

pH 7.5. Specific activities of 10^7 to 10^8 dpm/µg were routinely achieved.

2.6.3 Filter hybridisation

i) DNA filters

Filter hybridisations were carried out as described in detail by

Barrie, (PhD thesis, 1982). Filters were cut into strips and prehybridised

at 65°C in a gently rocking water bath in the following succession of

solutions (each was degassed under vacuum).

10 minutes	3 x SSC	(This was not degassed)
45 "	1 x Denhardts	0.2% Ficoll 400, 0.2% polyvinylpyrrolidine, 0.2% BSA in 3 x SSC
30 "	1 x CFHM	CFHM = complete filter hybridisation mix (1 x Denhardts plus 0.1% SDS and 50µg/ml denatured salmon sperm DNA in 1 x SSC
30 "	1 x CFHM + DS	CFHM plus 9% dextran sulphate (DS)

The [^{32}P]labelled probe DNA (<10ng/ml) was added to the hybridisation

solution (1 x CFHM + DS), after being denatured by heating to 100°C for 5

minutes (for nick translated probes) or for long enough to melt the agarose (single-stranded M13-derived probes). The presence of dextran sulphate greatly increases the hybridisation kinetics (Wahl et al., 1979). Genomic DNA filters and λHM recombinant filters were hybridised at 65°C overnight (dextran sulphate was omitted from lambda recombinant filter hybridisations), while colony hybridisation filters and M13 recombinant phage spot filters were generally hybridised at 65°C for 3-5 hours, in the presence of dextran sulphate.

Unbound labelled DNA was washed off the filters in several changes of 1 x CFHM, changing the wash solution more frequently at first, until no more radioactivity could be detected in the washings. Filters were rinsed in 3xSSC, blotted dry on Whatman 3MM paper, allowed to dry at room temperature, then reconstructed onto a glass plate for autoradiography. Filters to be re-hybridised with other probes were washed extensively in water at 65°C to remove previously hybridised [^{32}P]labelled DNA.

ii) RNA filters

RNA filter hybridisations (Northern blot analysis) were carried out using the method of Thomas (1980). Filters were cut into strips and prehybridised at 42°C in a gently rocking water bath for 4-20 hours in prehybridisation buffer [50% (v:v) formamide, 5xSSC, 250μg/ml denatured salmon sperm DNA, 1 x Denhardts, 50mM sodium phosphate buffer, pH 6.5. (Formamide was deionised by stirring with a small amount of 'Amberlite' monobed resin MB-1 (BDH) for 1-2 hours)]. Filters were transfered to hybridisation buffer to which the probe had been added, exactly as for DNA filter hybridisations (hybridisation buffer = 4 parts prehybridisation buffer plus 1 part 50% dextran sulphate). RNA filters were always hybridised at 42°C overnight

Filters were washed at room temperature in soap boxes in 4 changes of 2xSSC, 0.1% SDS, then 4 changes of 1xSSC, 0.1% SDS and finally in 2 or 3

changes of either 0.1xSSC, 0.1%SDS (when the probe sequence was 100%

homologous to the RNA on the filters) or otherwise in 0.25xSSC, 0.1% SDS.

These final washes were carried out at 50°C in a shaking water bath.

Filters were blotted on Whatman 3MM, allowed to dry and reconstructed for

autoradiography as for DNA filters. Filters to be rehybridised with other

probes were washed extensively in water at 42°C to remove previously

hybridised [^{32}P]labelled DNA and, if necessary, left for any remaining

radioactivity to decay.

DNA mobility marker filter strips from Northern blots were hybridised

using RNA filter hybridisation conditions.

2.7 Purification and quantification of myoglobin

2.7.1 Partial purification of myoglobin by isoelectric focussing and measurement of absorption spectra

At all stages below marked '*', a sample was reserved for measurement

of absorption spectra.

A known mass of muscle tissue (2-3g) was homogenised thoroughly in

20ml of water in a Waring Blendor. The homogenate was centrifuged at

110,000g for an hour at 4°C. The supernatant was decanted carefully and

kept on ice*. 10ml of this supernatant was dialysed overnight against

water at 4°C and the new volume measured*. 8ml of this was concentrated by

dialysis against solid PEG 6,000 to a volume of 350μl*. 100μl of this

concentrate (10μl of the seal muscle extract) was loaded onto a LKB

Ampholine PAG plate, pH 3.5→9.0. Samples were focussed at 20W, 1.5kV for

about 90 minutes, until all the pigmented proteins were resolved. Human

haemoglobin was also focussed as a marker.

The major non-haemoglobin red pigmented band (myoglobin) and the

prominent brown pigmented band (metmyoglobin), which focussed nearer to the

cathode, were excised from the gel and the protein extracted by elution

from syringed gel slices in 1ml of water. A blank piece of gel was also

extracted. Gel fragments were removed by centrifugation and the total

volume of the recovered protein solution was made up to 1.55ml before the

final samples were taken for measurement of absorption spectra[*].

Absorption spectra were measured using a scanning spectrophotometer

(Unicam SP1800 plus Unicam SP1805 programme controller and AR 25 linear

recorder), using 1ml diluted samples in disposable plastic cuvettes over a

wavelength range μ = 450–700nm.

2.7.2 in vitro translation of RNA

Both total and poly(A)$^+$ RNAs were translated in the rabbit

reticulocyte lysate system . 0.5–1µg of RNA was translated in a final

volume of 6µl containing 4µl reticulocyte lysate plus [^{35}S]methionine at a

final concentration of 37MBq/ml (Amersham, 50TBq/mmol). This reaction was

scaled up six–fold when myoglobin was to be purified from the remainder of

the translation products. Reactions were carried out at 30°C for 1 hour,

then terminated by the addition of a chase of unlabelled L–methionine (to a

concentration of 10mM) and heat–treated pancreatic RNase (to a

concentration of 0.1mg/ml) and incubation at 37°C for 15 minutes.

2.7.3 Analysis of in vitro translation products by SDS–polyacrylamide gel electrophoresis

Labelled products from in vitro translations were analysed by SDS–

polyacrylamide gel electrophoresis (Laemmli, 1970). Vertical slab gels

were used in a Raven slab gel apparatus (IN/96). Buffer composition and

recipes for the gels used are given in Table 2.2. The gel apparatus was

used with an acrylamide plug of the same composition as the separating gel.

Using combs with 7mm teeth 12 samples could be electrophoresed on each gel.

Samples were heated to 100°C for 2 minutes in 2 volumes of sample buffer

before loading. Unlabelled protein samples were electrophoresed in the

outside lanes to prevent "smiling" of the samples.

Table 2.2 Solutions and gel compositions for SDS-polyacrylamide gel
electrophoresis

Solution	Composition
buffer A	75mM Tris-HCl, 0.2% (w:v) SDS, pH 8.0.
buffer B	25mM Tris-HCl, 0.2% (w:v) SDS, pH 6.8.
acrylamide	44% (w:v) acrylamide, 0.8% (w:v) bisacrylamide.
electrophoresis buffer	25mM Tris-HCl, 192mM glycine, 0.19% (w:v) SDS, pH 8.3-8.6.
sample buffer	625mM Tris-HCl pH 6.8, 2% (w:v) SDS, 10% (v:v) glycerol, 5% (v:v) 2-mercaptoethanol, pinch bromophenol blue.

Gel recipes (in ml)

	stacking gel	separating gels	
	7%	18%	20%
buffer A	-	13.5	13.5
buffer B	10	-	-
acrylamide solution	3.3	11.2	12.0
water	6.7	1.35	0.55
ammonium persulphate (10mg/ml, fresh)	0.5	0.95	0.95
TEMED	0.04	0.075	0.075

Gels were run at 25mA for about 4 hours or overnight at 6mA, as appropriate, until the bromophenol blue had migrated to within 5mm of the bottom of the gel. Gels were then stained for about 3 hours, or overnight, shaking gently in 25% (v:v) isopropanol, 10% (v:v) glacial acetic acid, 0.05% (w:v) Coomassie brilliant blue. Diffusion destaining was carried out by shaking gently in several changes of the same solution, Coomassie blue omitted. Gels were photographed on a white light transilluminator, then fluorographed. Fluorography was carried out by dehydration of the gel by shaking for an hour in 2 changes of dimethylsulphoxide (DMSO), followed by an hour in DMSO plus 22% 2,5-diphenyloxazole (PPO). PPO was precipitated in situ by shaking the gel in water for 2 hours (Bonner and Laskey, 1974). Gels were then dried onto Whatman 3MM paper using a Bio-rad gel drier, before exposure to X-ray film, with an intensifier screen, at -80°C.

2.7.4   Measurement of total incorporation of [^{35}S]methionine in in vitro translations

2µl of each in vitro translation sample was spotted onto a 3mm strip of Whatman 3MM filter paper and allowed to dry. Proteins were precipitated with trichloroacetic acid (TCA) as follows: in 5% TCA on ice for 30 minutes, heated to 90°C for 10 minutes then hot TCA poured off and replaced with cold. Samples were left on ice for 10 minutes, then washed five times with 5% TCA on ice and then three times with acetone before being allowed to dry. Filter paper strips were put in plastic scintillation vials with 2 to 3 ml of non-aqueous scintillation fluid [toluene with 0.5% (w:v) PPO and 0.03% (w:v) POPOP] and counted on the ^{14}C channel of a Packard 3255 liquid scintillation spectrometer.

2.7.5   Quantitation of myoglobin from in vitro translations by isoelectric focussing and polyacrylamide gel electrophoresis

3µg of total or poly(A)$^+$ RNA was translated using the rabbit

reticulocyte lysate system in a total volume of 35μl (see section 2.7.1). A 9μl aliquot of each reaction was removed to be electrophoresed in an SDS-polyacrylamide gel and for measuring the total incorporation of [^{35}S]methionine (see section 2.7.4). The remaining lysate reaction was loaded onto an LKB Ampholine PAG plate and focussed as described above (section 2.7.3). Purified oxygenated myoglobin and metmyoglobin from the appropriate species were focussed on each side of the in vitro translation samples as markers. Gel slices from the appropriate positions indicated by the purified myoglobins were cut out of the gel and transferred to Eppendorf tubes. 20μl of polyacrylamide sample buffer was added and allowed to diffuse into the gel slices for 90 minutes. The gel slices were then incubated at 100°C for 5 minutes and loaded directly into the slots of an 18% SDS-polyacrylamide gel. Samples were electrophoresed and the gel fluorographed as described in section 2.7.2

The amount of [^{35}S]methionine incorporated in individual bands was measured as described by Ames (1974). The dried gel was removed from the filter paper backing with a small spatula and the required bands were cut out and placed in plastic scintillation vials. 50μl distilled water was added to each and they were allowed to stand for 10 minutes. 5ml of NCS scintillation fluid [(92% (v:v) toluene, 8% (v:v) NCS solubiliser, 0.37% (w:v) PPO, 0.0056% (w:v) POPOP] was added. The vials were stoppered, vortexed and incubated overnight at 37°C before vortexing again and scintillation counting on the [^{14}C] channel of a Packard 3235 liquid scintillation spectrometer.

2.8   Isolation of the myoglobin gene from a λL47.1 genomic library

2.8.1   Preparation of partial digests of human DNA using restriction endonuclease Sau3A

Twenty aliquots of 50μg of human DNA were digested at 250μg/ml in the appropriate buffer for 20 minutes at 37°C with increasing amounts of Sau3A,

ranging from 0.5 to 25 units. 0.5µg samples were removed from each digest and tested for degree of digestion by agarose gel electrophoresis. The digests were pooled and the DNA was recovered by phenol extraction and ethanol precipitation.

The partially digested DNA was electrophoresed in a preparative 0.4% agarose gel, flanked by λ x HindIII molecular weight markers. Sau3A fragments between 10 and 20kb were recovered by electrophoresis onto a vertical dialysis membrane as described in section 2.5.3.

2.8.2   Preparation of λL47.1 vector arms

Vector arms were prepared by Dr A.J.Jeffreys. 200µg of λL47.1 DNA was digested to completion with restriction endonuclease BamHI. DNA was recovered by phenol extraction and ethanol precipitation. The left and right arms were recovered by electrophoresis onto a dialysis membrane in a preparative 0.4% agarose gel and the DNA was purified as described in section 2.5.3.

2.8.3   Preparation of λL47.1 human recombinants

The cohesive termini of the vector arms were first annealed by incubating equimolar amounts of left and right arms in a small volume of 0.1M Tris-HCl, pH7.5, 10mM MgCl$_2$ at 42°C for 1 hour. Annealed vector arms were stored at -20°C.

For ligation, 8µg annealed arms were mixed with 5µg size-selected human DNA fragments and the mixture was incubated at 4°C overnight in ligase buffer (50mM Tris-HCl, 10mM MgCl$_2$, 20mMDTT, 1mM ATP, 50µg/ml BSA, pH 7.8) in a total volume of 75µl and in the presence of 1600 units of T4 DNA ligase. 4µl was removed for testing, the remainder being stored at -20°C. To this 4µl was added 20µl 66mM Tris-HCl, 10mM MgCl$_2$, pH 7.5 and the tube was heated to 65°C to melt annealed cohesive termini. The sample was then split in two. One half was alkali denatured and both were electrophoresed

in a 0.4% agarose gel to test the effectiveness of ligation. Ligated DNA

was recovered by ethanol precipitation and dissolved in 230µl of buffer A

(20mM Tris-HCl, pH 8.0, 3mM $MgCl_2$, 7mM 2-mercaptoethanol, 1mM EDTA).

2.8.4 in vitro packaging

The method used was a modification of the procedure of Enquist and

Sternberg (1979), using E.coli λ lysogens BHB2688 and BHB2690 (Hohn, 1979).

The method used to make the packaging extracts was exactly that described

by Barrie (PhD thesis, 1982)

For in vitro packaging the following components were mixed, in order,

on ice:

    20µl DNA in buffer A
    2.5µl M1 buffer (6mM Tris-HCl, pH 7.5, 30mM spermidine,
        60mM putrescene, 18mM $MgCl_2$, 15mM ATP, 28mM 2-mercaptoethanol)
    15µl   sonicated extract of BHB2690
    25µl   freeze-thaw lysate of BHB2688

Mixtures were incubated at 25°C for one hour and were then diluted by

the addition of 550µl λ buffer (6mM Tris-HCl, 10mM $MgSO_4$, 0.005% gelatin,

pH 7.5) and kept on ice. A half-volume reaction using 100ng intact λL47.1

was also set up to test the efficiency of the packaging reaction. After

incubation, this sample was made up to 1ml with λ buffer and a 1 in 10

dilution series, to 10^{-5} was made, also in λ buffer.

Overnight cultures of E.coli strains WL87 and WL95 (which is a P2

lysogen of WL87 for recombinant selection) were grown in Luria broth plus

10mM $MgSO_4$ and 0.2% maltose. Cultures were diluted to a third in the same

medium and grown shaking at 37°C for two hours.

To assay recombinants, 1µl from each packaging reaction was mixed

with 100µl of λ buffer and two 1 in 10 dilutions of this were made (in λ

buffer). 100µl of L95 were added to each tube. To make four 'library'

plates, each packaging reaction was aliquoted to give 150µl in each of 4

tubes, to each of which 150µl L95 was added. 100µl of WL87was added to

each tube in the dilution series of the λL47.1 control packaging reaction.

Finally, the WL95 phenotype was checked by mixing 1μl of the λL47.1

packaging reaction with 100μl of WL95. Other controls included plating of

WL87 and WL95 cells only.

After dilution of packaging reactions and addition of the appropriate

volume of cells, tubes were left to stand for 15 miutes for phage

adsorption, and then 3ml of BTL soft agar, supplemented with 10mM $MgCl_2$ and

0.2% maltose was added and the mixture was plated onto BLA plates.

Plates were incubated at 37°C overnight, with the lids ajar for the

first hour to allow drying. λL47.1 was packaged with an efficiency of 10^7

pfu/μg DNA. The recombinant yield was 4×10^5 pfu/μg ligated DNA. A total

of 3×10^6 recombinants, generated from a number of packaging reactions was

screened.

2.8.5 Selection of recombinant phage containing myoglobin gene sequences

The method used was a modification of that of Benton and Davis

(1977). After phage growth the agar was hardened slightly by cooling at

4°C for 15 minutes. 82mm diameter nitrocellulose filters (Schleicher and

Schull) were placed directly onto the agar, avoiding air bubbles. Phage

were allowed to transfer onto the filters for 5 minutes, while the plates

were marked for future reꟼorientation of filters. Filters were removed

from the plates and floated on 0.1M NaOH, 1.5M NaCl for 1 minute, followed

by neutralisation for a minute on 2xSSC, 0.2M Tris-HCl, pH 7.5. They were

then blotted dry and baked for 4 hours at 80°C.

Filters were hybridised overnight in the presence of dextran

sulphate, with an appropriate [^{32}P]labelled probe, and then washed (see

section 2.6.3). Autoradiography of the washed filters was generally for 1

to 4 days, with an intensifying screen.

2.8.6. Purification of positive recombinant clones and phage amplification

Positively hybridising plaques or regions were picked and resuspended in 0.5ml λ buffer to which a drop of chloroform was added to lyse cells. 100μl of this was replated in serial dilution on E.coli ED8910. Plates were incubated overnight at 37°C and those plates with dense, but not confluent, plaques were rescreened in the same way. This purification procedure was repeated until each plate contained only positively hybridising plaques. Single plaques were then picked for phage amplification and storage, as described by Barrie (PhD thesis, 1982).

2.8.7 Large scale preparation of recombinant λ phage

Phage lysates were prepared by a modification of the method of Blattner et al., (1977). Once recombinant phage had been purified, 5 well-separated plaques were picked (as 3mm diameter plugs, including some of the surrounding bacterial lawn), and used to inoculate 200ml of Luria broth plus 10mM MgSO₄ and 20μg/ml thymine, in a 2l unbaffled flask. After shaking gently overnight at 37°C, successful phage growth was indicated by the presence of cell debris in an otherwise nonᴬturbid culture. Chloroform was added to 0.5% (v:v) and the culture was left to stand to lyse all remaining cells. Lysates were cleared by centrifugation at 13,000g for 10 minutes at 4°C. Phage were harvested and phage DNA prepared as described by Harris (PhD thesis, 1985).

2.8.8 Restriction endonuclease mapping of recombinant phage

0.5μg of each recombinant phage DNA was digested singly and in all possible pairwise combinations with a series of restriction endonucleases (BamHI, BglII, EcoRI and HindIII). Digests were electrophoresed, without being recovered, in 0.5% agarose gels until the bromophenol blue had migrated 8cm from the slots. The gels were photographed and the DNA denatured in situ before transfer to nitrocellulose, see section 2.5.6.

DNA fragments containing sequences homologous to myoglobin probe sequences were identified by hybridisation and autoradiography.

2.9 Subcloning and characterisation of myoglobin gene sequences

2.9.1 Preparation of plasmid vector pAT153 DNA for subcloning

20μg of plasmid pAT153 was linearised by digestion with either restriction endonuclease BamHI or EcoRI. 0.15 units of calf intestinal phosphatase were added to the digest reaction for the last 30 minutes of digestion. DNA was recovered by two phenol extractions and ethanol precipitation.

2.9.2 Preparation of λ recombinant DNA for subcloning

5μg of recombinant λ DNA was digested with the appropriate restriction endonuclease (BamHI or EcoRI), and the desired fragment(s) recovered by agarose gel electrophoresis onto DE81 paper (see section 2.5.3).

2.9.3 Ligation of DNA fragments into pAT153

Fragments isolated from restriction endonuclease digestions of recombinant λHM DNAs were ligated with linearised, phosphatased pAT153 in equimolar proportions. Ligation reactions containing a total of 1μg of DNA, or less, were set up in 10μl ligase buffer (see section 2.8.2), plus 4mM spermidine and 80 units of T4 DNA ligase. Ligation was tested by agarose gel electrophoresis.

2.9.4 Transformation

Transformations were carried out using a modification of the method of Cohen et al., (1972).

E.coli HB101 was grown shaking overnight in Luria broth plus 20μg/ml thymine, and then diluted 1 in 100 into fresh medium and grown to $A_{600}=0.2$.

This dilution and growth to A_{600} of 0.2 was repeated once. 40ml of cells

were pelleted by centrifugation at 4,000g at 4°C for 5 minutes and then

were resuspended in 20ml of ice cold 0.1M $MgCl_2$. Cells were repelleted and

resuspended in 20ml ice cold 0.1M $CaCl_2$ and left on ice for 20 minutes.

Finally, cells were pelleted and resuspended in 2ml ice cold 0.1M $CaCl_2$ and

kept on ice until required.

200µl competent cells, 100µl 1xSSC and approximately 200ng ligated

DNA in ~10µl were mixed and kept on ice for 30 minutes. Tubes were heat

shocked at 42°C for 2 minutes, before being replaced on ice for a further

20 minutes. 1ml Luria broth plus 20µl/ml thymine was added and the tubes

were shaken gently at 37°C for 60-90 minutes. Cells were then pelleted and

resuspended in 0.1ml of Luria broth before plating on selective medium

(Luria agar plus 20µg/ml thymine, 25µg/ml ampicillin). Plates were

incubated overnight at 37°C.

2.9.5   Screening of transformants

The method used was a modification of the filter hybridisation method

of Grunstein and Hogness (1975), which has been described in detail by

Barrie, (PhD thesis, 1982). Probes used for detection of the desired

clones were appropriate to the fragment cloned.

2.9.6   Preparation of plasmid DNA

All strains were grown at 37°C in Luria broth plus 20µg/ml thymine

and 25µg/ml ampicillin.

i) Small-scale plasmid preparations

This method is a modification of the small-scale alkaline extraction

method of Birnboim and Doly (1979). 1.5ml of an overnight culture of the

plasmid-containing strain were pelleted by a 20 second spin in an Eppendorf

centrifuge. Cells were resuspended in 100µl of ice-cold lysis solution

(25mM Tris-HCl, 10mM EDTA, 50mM sucrose, pH 8.0 containing freshly added

lysozyme to 1mg/ml). After 10 minutes on ice, 200µl ice-cold alkaline-SDS

solution (0.2M NaOH, 1% SDS) was added and the tubes were left for a

further 5 minutes on ice for cells to lyse. Protein and chromosomal DNA

were precipitated by the addition of 150µl of ice-cold 3M potassium

acetate, pH 5.2. The tubes were left for 10 minutes on ice. Tubes were

centrifuged for five minutes in an Eppendorf centrifuge and plasmid DNA was

recovered from 1.5ml of supernatant by ethanol precipitation. DNA was

purified by phenol extraction and ethanol precipitation. Plasmid DNA could

then be characterised by restriction endonuclease digestion in the presence

of RNase to digest the large amount of RNA produced by this method.

ii) Large scale plasmid preparations

This is essentially a scaled up version of the method above, using

the same solutions, but with an additional CsCl gradient centrifugation

step.

1ml of an overnight culture of the plasmid-carrying strain was used

to innoculate each of two 400ml volumes of medium which were grown

overnight. Cells were pelleted by centrifugation at 4,200g for 5 minutes

at 4°C and resuspended in 40ml of ice-cold lysis solution (as above, but

containing 2mg/ml freshly added lysozyme). The cell suspensions were kept

on ice for five minutes, then 80ml ice-cold alkaline-SDS solution were

added and the mixture kept on ice for a further five minutes. To this

solution 60ml of ice-cold 3M potassium acetate, pH 5.2, were added.

Precipitate was removed by centrifugation at 6,000g for 10 minutes at 4°C

and the supernatant recovered by pouring through a polyallomer wool plug in

a glass funnel. Nucleic acids were precipitated with 0.5 volume of

propan-2-ol and recovered by centrifugation at 4,200g, 4°C for 10 minutes.

The pellet was gently rinsed with 80% ethanol and then with diethyl ether,

which was blown off. The nucleic acids were dissolved in 10ml of TE buffer

(TE = 10mM Tris-HCl, 1mM EDTA, pH 7.5).

The volume was adjusted gravimetrically to 20ml with TE and then 4ml

of 5mg/ml ethidium bromide and 23.76g CsCl were added, to give a final

refractive index of 1.392 to 1.394. This solution was split between two

Beckman polyallomer (16x76mm) "Quickseal" tubes and centrifuged at

110,000g, 15°C for 40 hours. The plasmid band was removed from the CsCl

gradients using a 5ml syringe and fine gauge hypodermic needle. Ethidium

bromide was removed by repeated extraction with propan-2-ol, saturated with

CsCl/H$_2$O. Two volumes of water were added and the DNA was recovered by

ethanol precipitation at 13,000g, 0°C for 30 minutes. DNA was dissolved in

10mM Tris-HCl, pH 7.5 and the yield and quality assessed by measurement of

the optical density at 260nm and agarose gel electrophoresis, respectively.

2.9.7 Restriction mapping of recombinant plasmids

Detailed restriction maps were produced by the method of Smith and

Birnstiel (1976). The method used is described in detail by Harris (PhD

thesis, 1985). Fragments to be mapped were [^{32}P]labelled at the 5' ends

with polynucleotide kinase and [γ-^{32}P]ATP. A restriction endonuclease was

used to cut DNA fragments labelled at both ends and the required uniquely

end-labelled fragment recovered from a gel (see section 2.5.3). Partial

digestion of this end-labelled fragment with a range of restriction

endonucleases was then carried out and fragments were electrophoresed in an

agarose gel which was dried onto a glass plate and autoradiographed.

2.10 M13 sequencing

2.10.1 Preparation of M13 recombinants

i) Sonication of Plasmid DNA

Sonication of approximately 15µg of DNA in a total volume of 30µl

was carried out in an Eppendorf tube in a sonicating waterbath (Kerry

Ultrasonics Ltd) containing 1-2cm of water. The tube was placed on the

bottom of the waterbath for 4 x 30 second bursts of sonication. Between

each burst the solution was placed on ice and given a quick spin to bring

the solution back to the bottom of the tube. The appearance of a "mist" on the sides of the tube was an indication of successful sonication. The desired size range of fragments was 600-1,200bp. A 1μl aliquot was electrophoresed against pBR332 x Sau3A markers on an agarose gel to check this. Further 30 second bursts of sonication were carried out, if incomplete first time. DNA was recovered after phenol extraction by two ethanol precipitations, vacuum dried then redissolved into 10μl of water.

ii) End-repair of sonicated DNA and size-selection

The sonicated DNA was end-repaired by incubation overnight at 15°C in the following;

DNA	(in water)	20μl
10x ligase mix	(500mM Tris-HCl,100mM MgCl$_2$ 100mM DTT,pH 7.5)	3μl
TM buffer	(100mM Tris-HCl,100mM MgCl$_2$,pH 7.5)	3μl
0.1M spermidine		1.2μl
sequence chase mix	(0.25mM solution of each dNTP in TE buffer)	2μl
DNA polymerase I	(10 units of large fragment Klenow enzyme)	2μl

DNA was then electrophoresed in a 1.5% preparative agarose gel with pBR322 x Sau3A markers. DNA between 400 and 900bp was recovered onto DE81 paper (section 2.5.3) and then redissolved in 20μl of water.

iii) Preparation of stock M13 vector DNA

2μg of M13mp8 RF DNA was cleaved with SmaI, and simultaneously phosphatased as previously described (2.9.1). DNA was then recovered, after phenol extraction, by two ethanol precipitations, vacuum dried and redissolved in 10mM Tris-HCl, pH 7.5 and adjusted to 20μg/ml.

iv) Ligation of sonicated DNA into M13 vector DNA

The following ligation reaction mixes were prepared;

Size selected DNA fragments (ii)	1µl	2µl	4µl
Phosphatased M13mp8 RF DNA (iii)	1µl	1µl	1µl
10mM ATP	1µl	1µl	1µl
20mM Spermidine	2µl	2µl	2µl
10x Ligase buffer	1µl	1µl	1µl
water	4µl	3µl	1µl

400 units of T4 DNA ligase were added to each and tubes were incubated overnight at 15°C. A further 0.5µl of 10mM ATP and 200 units of T4 DNA ligase were then added and incubated at 4°C for an additional 1-4 days after which they could be stored at -80°C indefinitely.

v) Transfection of E.coli JM101 or JM103

Competent cells were prepared by a modification of the method of Kushner (1978). JM103 (or JM101) was grown, with shaking, overnight at 37°C in Luria broth containing thiamine (0.0002%). 0.5ml of cells were diluted 1 in 100 in the same medium and grown to A_6A_{00} = 0.3. The culture was kept at room temperature while 1.4 ml aliquots were pelleted by a 30 second spin in an Eppendorf centrifuge (the number of tubes prepared is the same as the number of ligation reactions). The supernatant was removed and the cells were gently resuspended in 0.5ml of MR (MR= 10mM MOPS, 10mM RbCl, pH 7.0,). The cells were pelleted again and the supernatant removed as before. The cells were resuspended in 0.5ml of MRC (MRC= 100mM MOPS, 10mM RbCl, 50mM $CaCl_2$, pH 6.5) and left on ice for 30 minutes. Cells were pelleted and resuspended in 0.15ml of MRC and kept on ice until required. To each tube of "competent" cells was added 3µl DMSO and 5µl of the ligation mix. The mixture was left on ice for an hour, shocked at 55°C for 35 seconds, cooled on ice for 1 minute, then held at room temperature. Before plating, the cells were transferred to a glass tube containing 200µl of JM103 or JM101 log phase cells (those held at room temperature), 25µl of 25mg/ml BCIG (in dimethylformamide) and 25µl of IPTG (in water). 3ml of BBL soft agar was added and the mixture poured on BBL agar plates and incubated overnight at 37°C.

Purified recombinant plasmid DNA fragments to be cloned in M13 were

ligated, in approximately equimolar vector:fragment ratios, into M13mp18 RF

DNA which had been cleaved with the appropriate restriction endonuclease(s)

and treated with calf intestinal phosphatase, as previously described

(section 2.9.1). Ligated DNA was used to transfect JM101 in exactly the

same way as for JM103.

White plaques were toothpicked into 1ml λ buffer and 100μl of this

was used to innoculate 1.5ml of a 1 in 100 dilution of an overnight culture

of JM103 in Luria broth. Cultures were grown with fast shaking at 37°C for

$5^1/_2$ hours. Phage were collected by PEG precipitation as described by

Weller et al. (1984). Cells were pelleted by two five minute spins in an

Eppendorf centrifuge with a 180° twist between spins. 1ml of supernatant

was transferred to fresh tubes and phage were precipitated by the addition

of 200μl of 10% PEG 6000, 2.5M NaCl and incubation at 15°C for 15 minutes.

Phage were pelleted by two five minute spins and all the supernatant was

very carefully removed with a drawn out pasteur pipette. Finally, the

phage pellets were resuspended in 100μl 1.1M sodium acetate, pH 7.0.

Clones were screened for the desired inserts by spotting 1μl phage plus 1μl

of (3M NaCl, 0.2M NaOH, 0.1% SDS, 0.1% bromophenol blue) onto a gridded

nitrocellulose filter (Schleicher and Schull). After 15 minutes drying at

room temperature, the filter was washed in 3xSSC, baked at 80°C for 4 hours

and hybridised with an appropriate [^{32}P]labelled probe. Phage DNAs were

purified by phenol extraction and ethanol precipitation. DNAs were

redissolved in 30μl of water at 60°C for 15 minutes.

2.10.2 Sequencing of M13 recombinants

i) Sequencing reactions

Sequencing of M13 recombinant clones was based on the methods of

Sanger et al., (1978) and Biggin et al., (1984) for M13 dideoxyribo-

nucleotide chain-termination using α-^{32}P-dATP and α-^{32}S-dATP respectively.

Quantities given are for 15 sequencing templates which is a comfortable

number to process at any one time. Reactions were carried out in 1.5ml

Eppendorf tubes and all centrifugations were done in an Eppendorf

centrifuge.

To ensure the cloned template DNAs were fully redissolved after

preparation, they were incubated at 60°C for 10 minutes prior to annealing.

Each clone was annealed with the 17-mer universal primer by mixing 5μl of

clone DNA and 5μl of primer mix (0.18μg/ml 17-mer primer, 10mM Tris-HCl,

pH 8.0, 10mM MgCl$_2$) and incubating at 60°C for an hour with a quick

centrifugation after 30 minutes. The annealed mix was held at room

temperature. For each clone, 4 reaction tubes were prepared containing

2μl of annealed clone plus 2μl of either a 'T','C','G', or 'A' dNTP mix.

dNTP mixes for sequencing

	'T'	'C'	'G'	'A'
0.5mM dTTP	12.5	250	250	250
0.5mM dGTP	250	250	12.5	250
0.5mM dCTP	250	12.5	250	250
10mM ddTTP	12.5(6.2)			
10mM ddCTP		2(4)		
10mM ddGTP			4(8)	
10mM ddATP				0.75(1.2)
TE buffer	500	500	500	250

TE buffer= 10mM Tris-HCl, pH 8.0, 0.1mM EDTA). 0.5mM dNTP and 10mM

ddNTPs were prepared in TE buffer. The figure in brackets is the amount

of ddNTP added to the mix when [³⁵S]dATP was the radiolabelled substrate.

To each reaction tube in turn was added 2μl of freshly prepared

'Klenow' mix {[³²P]mix = 117μl TE buffer, 3.3μl Klenow polymerase (5

units/μl), 10μl [α-³²P]dATP, 0.7μl 50μM dATP; [³⁵S]mix = 114μl H$_2$O, 7μl

Klenow polymerase, 10μl [α-³⁵S]dATP)}. The tube was given a gentle mix

then incubated at 37°C for 20 minutes. 2μl of sequence chase mix (0.25mM

each of dATP, dGTP, dTTP, dCTP made up in TE buffer) was then added to

each tube in turn (in the same order as before), mixed and incubated a

further 20 minutes at 37°C. Tubes were prepared for loading onto a sequencing gel by adding 4μl of formamide dye (stock solution containing 10ml deionised formamide, 10mg xylencyanol FF, 10mg bromophenol blue, 0.2ml 0.5M EDTA, pH 8.0). [³²P]labelled substrates were loaded as soon as possible while [³⁵S]labelled substrates could be stored for several days at ∼20°C, prior to the addition of the formamide dye.

ii) Sequencing gels

Substrates were electrophoresed in 40cm, buffer gradient gels (Biggin et al., 1983). A 'sharks-tooth' comb was used which enabled 15∼18 clones to be electrophoresed in a single gel. The standard mixes used to prepare two buffer gradient gels are below:

	0.5x	2.5x
acrylamide	17.1g	2.28g
bisacrylamide	0.9g	0.12g
urea	150g	20g
sucrose	∼	2g
10 x TBE (1M Tris∼borate, 20mM EDTA, pH 8.3	15ml	10ml
H₂O to final volume	300ml	40ml

The solutions were filtered through two 9cm Whatman no.1 filters, using a Buchner funnel and vacuum line then 150ml (0.5x) and 20ml (2.5x) were degassed before addition of:

	0.5x	2.5x
10% ammonium persulphate	1.05ml	0.14ml
TEMED (prior to pouring)	72μl	9.6μl

1.5μl [³²P] or 2.5μl [³⁵S] samples of each sequencing reaction were boiled for 3 minutes before loading onto the gel. After electrophoresis for 3∼8 hours at 1.4-1.7kV, the gels were fixed in 10% (v:v) methanol, 10% (v:v) acetic acid for 15 minutes, then dried onto Whatman 3MM paper using a Bio-rad gel drier. Gels were autoradiographed at room temperature for

16 hours to 4 days.

2.10.3 Computing

M13 clone sequences were aligned against reference sequences and each

other using a version of the Staden (1980) programme modified to run on a

Digital PDP 11/44 minicomputer. This minicomputer was also used in

conjunction with the word-processing package Word 11 to format the DNA

sequences as presented.

2.11 Preparation of [³²P]labelled single-stranded cDNAs from M13

recombinants

2.11.1 For hybridisation probes

The method used was that described by Jeffreys et al. (1985).

Approximately 0.5-1μg of single-stranded M13 recombinant DNA was annealed

with 4ng of 17-mer sequencing primer (Duckworth et al., 1981) in 10μl of

[10mM $MgCl_2$, 10mM Tris-HCl, pH 8.0] at 60°C for 30 minutes. Primer

extension was carried out by adding 16μl of [80μM dATP, 80μM dTTP, 80μM

dGTP, 10mM Tris-HCl, 0.1mM EDTA (pH 8.0),] plus 3μl (1.11MBq) [α-³²P]dCTP

(111TBq/mmol) in the presence of 5 units of Klenow polymerase by incubating

at 37°C for 15 minutes. Extension was completed by adding 2.5μl of 0.5mM

dCTP and chasing at 37°C for a further 15 minutes. The DNA was cleaved at

a suitable restriction endonuclease site, either in the insert, or in the

M13 polylinker distal to the insert, and then was denatured by adding a

tenth volume of 1.5M NaOH, 0.1M EDTA. The [³²P]labelled single-stranded

DNA fragment extending from the primer was recovered by electrophoresis in

a 2% low melting point agarose gel. The excised fragment was melted at

100°C for 2 minutes with 1mg of alkali-denatured salmon sperm carrier DNA

and 500μl of water and was then added directly to a pre-warmed

hybridisation chamber containing hybridisation solution (for either

Northern or Southern hybridisations). Filters were hybridised and washed

67

as described previously (section 2.6.3). Specific activities achieved by this labelling method were in excess of 10^9 cpm/μg DNA.

2.11.2 For the DNA/RNA hybridisation assay for measuring myoglobin mRNA abundance

The same general method as above was used, in a scaled up version, except that [α-^{32}P]dCTP was mixed with unlabelled dCTP and there was no chase. Approximately 5μg of single-stranded M13 recombinant DNA (M13.HEX2) was annealed with 50ng of primer in 20μl 10mM MgCl$_2$, 10mM Tris-HCl, pH 8.0. Primer extension was carried out by adding, in order:

2 μl	each of 20mM dATP, 20mM dGTP and 20mM dTTP
7.2μl*	1mM dCTP (unlabelled)
16 μl	10mM Tris-HCl, 1mM EDTA, pH 8.0
10 μl	[α-^{32}P]dCTP (3,000Ci/mMol)
20 units	Klenow polymerase

* Amount varied depending on the specific activity required, but generally as here.

After the addition of the [α-^{32}P]dCTP, 1μl was removed for the exact input to be calculated by serial dilution and Cerenkov counting using a Packard 3255 spectrophotometer. The mixture was incubated at 37°C for 30 minutes, then the DNA was cleaved in the polylinker, distal to the insert, by adding 6μl of 10x restriction endonuclease core buffer (BRL), 2.5μl 0.1M spermidine and 20 units of Sst I and incubating at 37°C for 20 minutes. The DNA was alkali-denatured as above and the 316bp fragment recovered from a 2% agarose gel (not low melting point) by elution from the gel slice, which was mashed in 1ml of water treated with DEPC. DNA was collected by ethanol precpitation and redissolved in 100μl of water, then purified by phenol extraction and ethanol precipitation. The incorporation of ^{32}P was measured by Cerenkov counting and the yield of DNA calculated from the input of [α-^{32}P]dCTP and the ratio of labelled to unlabelled dCTP in the primer extension reaction.

68

2.12   Assay of myoglobin mRNA abundance in polyadenylated RNA

This method involves hybridisation of a constant known amount of

[^{32}p]labelled single-stranded DNA, complementary to myoglobin mRNA (made

from the recombinant M13.HEX2 as described in the section above, and

referred to hereafter as antisense DNA), with increasing amounts of

poly(A)$^+$ RNA. The degree of hybridisation was assessed by agarose gel

electrophoresis and autoradiography.

The labelled antisense DNA (usually 1ng) was hybridised with between

4ng and 3μg of poly(A)$^+$ RNA in 10μl of 1xSSC plus 100μg/ml calf liver tRNA.

Hybridisations were carried out, under liquid paraffin, at 65°C overnight

(or for varying lengths of time). Liquid paraffin was removed by

extracting twice with diethyl ether and blowing off the remaining ether.

10μl agarose beads were added to each tube and the samples were loaded

directly onto a 2% agarose gel, with alkali-denatured DNA size markers.

After electrophoresis the gel was photographed and then dried onto a glass

plate for autoradiography, without an intensifier screen. After

autoradiography, the amount of radioactivity incorporated in particular

bands or regions of the gel was determined by cutting out those regions and

Cerenkov counting. Myoglobin mRNA abundance can be calculated from the

amount of mRNA which just hybridises with a certain amount of antisense

DNA.

2.13   S1 nuclease protection mapping of human myoglobin RNA

This was carried out using the method of Berk and Sharp (1977), as

modified by Weaver and Weissman (1979). Plasmid pHM.27.B2.9 was digested

with restriction endonucleases NcoI and SstI to give a number of fragments.

The digested DNA was treated with alkaline phosphatase and

[^{32}P]end-labelled with polynucleotide kinase before recovery of the desired

fragment, which extended 1kb 5' from the NcoI site at the initiation codon

of the myoglobin gene.

i) Phosphatasing of the SstI/NcoI digest of pHM27.B2.9

Approximately 30μg of pHM.27.B2.9 was digested with SstI and NcoI and simultaneously treated with 2 units of alkaline phosphatase to remove the terminal 5' phosphate groups. Great care was taken to eliminate all phosphatase activity prior to labelling. Phosphatase was first inactivated by adding 2μl of 0.5M EDTA; 2.5μl of 20xTNE (TNE = 10mM Tris-HCl, 100mM NaCl, 1mM EDTA, pH 8.0) and 5μl 10% SDS and heating to 65°C for 15 minutes. DNA was then extracted twice with phenol, ethanol precipitated and redissolved in 100μl of 10mM Tris-HCl, pH 7.5. DNA was spun through a G50 sephadex column (1ml bed volume, pre-prepared by spinning twice with 100μl 10mM Tris-HCl, pH 7.5). The column was washed with 100μl of 10mM Tris-HCl, pH 7.5, and the DNA was recovered by ethanol prepcipitation. The efficiency of the phosphatase treatment was tested by self-ligation of the DNA.

ii) Labelling the DNA with T4 polynucleotide kinase and [γ-^{32}P]ATP

30μg of restriction endonuclease-digested, phosphatased DNA was 5' end-labelled in 15μl of kinase buffer [kinase buffer = 50mM Tris-HCl, 10mM MgCl$_2$, 5mM DTT, 4mM spermidine, 0.1mM EDTA (pH 7.6)] in the presence of 1.11MBq [γ-^{32}P]ATP (111TBq/mmol) and 5 units of T4 polynucleotide kinase, for 30 minutes at 37°C. The reaction was terminated by the addition of 1μl of 0.5M EDTA, pH 8.0, and the labelled DNA was phenol extracted. The required DNA fragment was then purified by agarose gel electrophoresis and recovered using DE81 paper (section 2.5.3).

Because of the choice of enzymes, label was incorporated almost exclusively at the protruding 5' (NcoI) terminus, the 5' terminus (SstI) of the other strand being recessed and labelled much less efficiently. There was therefore no necessity to cleave the fragment with another restriction endonuclease to obtain a fragment labelled at one end only. The SstI-NcoI fragment was labelled to a specific activity of ~1-2x10^5 cpm/μg DNA.

iii) DNA/RNA hybridisations and S1 nuclease digestion

Between 50 and 100μg of human muscle total RNA was vacuum dried with 100μg of tRNA and 30,000cpm of the kinase-labelled DNA fragment (containing approximately 100ng of DNA). The dried DNA and RNA was redissolved thoroughly in 40μl hybridisation buffer [40mM PIPES, 400mM NaCl, 1mM EDTA (pH 6.4) in 80% (v:v) deionised formamide]. Tubes were incubated, sealed with conformable tape, completely submerged to eliminate evaporation, at 85°C for 12 minutes and were then transferred directly to 52°C for either 3 hours or overnight incubation (15 hours). 10μl of each hybridisation mix was aliquoted into each of four tubes containing 300μl of ice-cold S1 nuclease reaction buffer [50mM NaCl, 30mM sodium acetate (pH 4.6), 1mM ZnSO₄, 5% (v:v) glycerol]. Differing amounts of S1 nuclease were added to three out of the four tubes for each hybridisation (ranging between 20 and 400 units). The fourth tube was left untreated. Tubes were incubated at 37°C for 30 minutes and the reaction was then terminated by the addition of 2μl of 0.5M EDTA, pH 8.0. 20μg of carrier tRNA was added to each tube and the nucleic acids were recovered by ethanol precipitation and redissolved in 5μl of water. 3.5μl sequencing formamide dye mix [1mg/ml xylene cyanol, 1mg/ml bromophenol blue, 10mM EDTA (pH 8.0), in deionised formamide] was added to each tube. Tubes were incubated at 100°C for 2 minutes before loading the samples onto sequencing-type polyacrylamide gels.

iv) Preparation of size-markers

Size-markers for S1 nuclease-protected fragments were prepared by subjecting approximately 10⁵cpm of the end-labelled probe fragment to the chemical cleavage sequencing method of Maxam and Gilbert (1980). The labelled fragment was split equally between two of the chemical modification reactions; G+A and T+C, which were carried out exactly as described by Maxam and Gilbert (1980). Before loading on the gel, 11μl of formamide dye mix was added and sequencing reactions were denatured by

incubation at 90°C for 1 minute and then cooled on ice. 5µl was loaded per track.

v) Preparation of polyacrylamide gels

S1 nuclease-protected fragments and Maxam and Gilbert sequencing substrates were electrophoresed in 40cm, 8% polyacrylamide gels, 0.35mm thick. Acrylamide for two gels was made up from:

acrylamide	15.2g
bisacrylamide	0.8g
urea	100g
10xTBE buffer	10ml
water	up to 200ml

The solution was filtered through two 9cm diameter Whatman No1. filters. For one gel, 1.4ml freshly made 10% (w:v) ammonium persulphate was added to 100ml of solution, followed by 25µl TEMED, just before the gel was poured. A conventional square-toothed gel comb was used. Gels were electrophoresed at approximately 1,200V until the bromophenol blue had migrated to within 5cm of the bottom of the gel. Gels were fixed and dried as described in section 2.10.2, before autoradiography, with an intensifying screen, for 7 to 14 days.

2.14   Mapping of possible S1 nuclease-sensitive sites in the myoglobin gene

2.14.1   S1 nuclease-treatment of plasmid DNAs

Test digests of 0.5µg plasmid DNA were carried out with varying concentrations of S1 nuclease (between 0.01 and 50 units per µl) in order to determine the S1 concentration at which most of the plasmid was linearised, but not degraded. Reactions were carried out in 10µl of S1 reaction buffer (see section 2.13), for 10 minutes at 37°C. The reaction was terminated by the addition of 0.5µl of 0.5M EDTA, pH 8.0, and the DNA was electrophoresed in a 0.5% agarose gel. The most appropriate

concentration of S1 nuclease was found to be 0.05 units/µl. Approximately

10µg of plasmid DNA was then digested in a scaled up version of the test

digest, using this concentration of S1 nuclease and the same reaction

conditions. Reaction was terminated by the addition of 10µl of 0.5M EDTA,

pH 8.0. DNA was extracted with phenol and recovered by ethanol

precipitation

2.14.2   Restriction mapping of S1 nuclease-sensitive sites

Restriction endonuclease digests of 0.5µg of plasmid DNA were carried

out in pairs, ie S1-treated and untreated. Digests were carried out in

10µl of the appropriate restriction endonuclease buffer, then

electrophoresed directly in 0.5% agarose gels. Restriction endonucleases

chosen were those that cleaved the plasmid DNAs only 2-4 times, giving a

small number of bands, against which new S1-generated bands would be

clearly visible. Gels were photographed and the approximate positions of

S1 nuclease-sensitive sites were determined from the mobilities of new

bands in restriction endonuclease digests of S1-treated DNAs.

2.14.3   Fine-scale mapping of S1 nuclease-sensitive sites

From the approximate position of S1 nuclease-sensitive sites

determined as above, and fine-scale restriction maps of the recombinant

plasmid inserts (section 2.9.7), enzymes were chosen which had a cutting

site within about 500bp of the approximate position of the S1-sensitive

site. Restriction endonuclease digestion of S1-treated and untreated

plasmid DNA was carried out with these enzymes, as described above.

Complete digestion was checked by agarose gel electrophoresis of an aliquot

of the reaction, then digested DNA was phenol extracted and recovered by

ethanol precipitation. DNA fragments (approx 1µg) were then labelled with

either $[\alpha-^{32}P]$dCTP or $[\alpha-^{32}P]$dATP using reverse transcriptase. Reactions

were carried out in 10µl of 'fill-in' mix [0.5M Tris-HCl, 10mM

73

2-mercaptoethanol, 50mM MgCl$_2$, 50μM appropriate dNTPs] and 2.8MBq of either [α-^{32}P]dCTP or [α-^{32}P]dATP, as appropriate to the enzyme used. Tubes were incubated at 37°C for 15 minutes and the reactions terminated by adding 0.5μl of 0.5M EDTA, pH 8.0. λ x HindIII and pBR322 x Sau3A DNA size markers were labelled in the same way. 5μl of each sample was electrophoresed in a 2% agarose gel which was dried onto a glass plate and autoradiographed.

2.15   Containment

All experiments described in this thesis were undertaken with reference to the Genetic Manipulation Advisory Group's guidelines on safety and containment conditions for such work.

Chapter 3

CLONING AND CHARACTERISATION OF THE HUMAN MYOGLOBIN GENE

3.1 Introduction

Characterisation of the human myoglobin gene would yield information

relating directly to myoglobin gene structure and evolution, but such

characterisation is also a pre-requisite for the study of myoglobin gene

expression.

Observations of the few myoglobin variants which have been

described, together with the presence of a single myoglobin gene in the

grey seal, combine to suggest that the human genome also contains a single

functional myoglobin gene. The first myoglobin gene to be characterised,

that of the grey seal (Halichoerus grypus), was isolated via cDNA cloning

and has since been sequenced (Wood et al., 1982; Blanchetot et al., 1983).

The availability of a cloned mammalian myoglobin gene enabled the human

gene to be isolated from a genomic library by cross-hybridisation using

probes from the seal gene.

3.2 Human DNA contains a single functional myoglobin gene

The grey seal myoglobin cDNA clone pSM178 (Wood et al., 1982),

containing only part of the 3' non-translated sequence of the seal

myoglobin gene, does not cross-hybridise to human DNA (data not shown).

More suitable probes containing myoglobin coding sequences were prepared

from clones of the seal myoglobin gene (Blanchetot et al., 1983), (Figure

3.2). Hybridisation of probes from seal exons 1 and 3 to restriction

endonuclease digests of human DNA, carried out by Dr A.J.Jeffreys,

revealed a single major hybridising fragment (Weller et al., 1984). In

contrast, exon 2 detected a complex set of fragments in all restriction

endonuclease digests tested, the pattern of which did not vary between six

individuals. This pattern could be simplified to a major hybridising component by increasing the post-hybridisation washing stringency from 1xSSC at 65°C to 0.2xSSC at 65°C. Human myoglobin is therefore specified by a single gene, but the human genome also appears to contain an additional sequence family related to the central exon of the seal myoglobin gene.

3.3 Cloning strategy

Human DNA fragments generated by partial digestion with Sau3A were ligated into the BamHI sites of the replacement vector λL47.1 (Loenen and Brammar, 1980). The use of partially digested human DNA of fragment size 10-20kb generated by Sau3A ensures that a pseudo-random collection of large DNA fragments is generated, and therefore that overlapping clones can be isolated.

3.4 Preparation of human DNA fragments and ligation into λL47.1

Aliquots of human DNA were digested with increasing amounts of Sau3A, pooled, and fragments from 10-20kb isolated by preparative gel electrophoresis onto a vertical dialysis membrane as described in section 2.5.3.

The vector was prepared by digesting λL47.1 DNA with BamHI and separating the left and right arm fragments from the central inessential fragment by preparative gel electrophoresis (section 2.9.2). The cohesive termini of the left and right arms were annealed and vector arms and human DNA partials were ligated in a 2:1 mass ratio.

3.5 In vitro packaging and screening of recombinant λ-phage

To reduce the possibility of recombinational rearrangement of phage DNA, in vitro packaged recombinant phage should be used to infect a Rec⁻ E. coli strain (Fritsch et al., 1980). Previous work using the recBC host

ED8910, however, yielded only low plating frequencies (Barrie, PhD thesis 1982). Initial infections were therefore carried out using a Rec$^+$ host, WL95, which is a P2 lysogen of WL87, and should allow growth of recombinants only. The efficiency of the packaging reaction was tested using λL47.1 DNA plated onto WL87. Packaging efficiencies of 1-2x10^7 pfu/μg λL47.1 DNA and ~3x10^5 pfu/μg ligated DNA were achieved.

A total of approximately 3x10^6 recombinants (corresponding to 15 genome equivalents), from a number of different packaging reactions was screened. The screening method was that of Benton and Davis (1977). Nitrocellulose filter 'lifts' were hybridised with exon sequence probes from the seal myoglobin gene, labelled with ^{32}P by nick translation (see Figure 3.1). The exon 2 probe was not used for screening because of its apparent cross-hybridisation with sequences not related to the single functional myoglobin gene (Weller et al., 1984). In some cases duplicate 'lifts' were taken and filters hybridised one with the seal exon 1 probe and the other with the exon 3 probe, in an albeit unsuccessful attempt to obtain full length myoglobin gene clones. Approximately 35 positively hybridising regions were detected and picked for plaque purification. After three rounds of rescreening a total of ten positively hybridising recombinants termed λHM2-32 was isolated. Figure 3.3 shows that only the tenth recombinant to be characterised contained the extreme 3' end of the gene. The number of positively hybridising recombinants is consistent with the human myoglobin gene being present in a single copy. DNA from these recombinants was prepared by the method of Blattner et al. (1977), (section 2.8.6)

3.6 Characterisation of recombinant phage

A restriction map of each recombinant was constructed by digestion of DNA with the four restriction endonucleases HindIII, EcoRI, BamHI and BglII, in both single and double digests. Fragments containing sequences

77

homologous to regions of the seal myoglobin gene were identified by Southern blot hybridisation using seal myoglobin gene exon probes [^{32}P]labelled by nick translation.

Once the restriction map for each recombinant had been constructed, it was possible to overlap the individual clones on the basis of shared restriction fragment pattern. The general approach to the mapping of λ recombinants is shown in Figure 3.1, using λHM17, which contains human myoglobin exons 2 and 3, as an example. A composite restriction map compiled fom the ten individual clones isolated, extending over approximately 23kb and containing the entire sequence of the human myoglobin gene is shown in Figure 3.2, together with the region represented in each of the human myoglobin λHM recombinants.

3.7 Subcloning and fine scale mapping of the human myoglobin gene

BamHI restriction fragments from λHM27 and an EcoRI fragment from λHM17 were subcloned into pAT153 (Twigg and Sherratt, 1980) to give pHM.27.B1.1, pHM.27.B2.9 and pHM.17.E1 (Figure 3.2). Detailed restriction endonuclease site cleavage maps covering the majority of the human myoglobin gene were constructed using the partial digestion method of Smith and Birnstiel, (1976), (section 2.9.8)

Figure 3.3 shows the method of construction of such a map for the region containing the third exon and 3' non-translated sequence. A uniquely [^{32}P]end-labelled EcoRI-SstI fragment, recovered from pHM.17.E1, was subjected to partial digestion by a range of restriction endonucleases. The partially digested DNA fragments were separated by agarose gel electrophoresis and the gel was dried and autoradiographed before map construction. Figure 3.4 shows the resulting composite restriction endonuclease cleavage map constructed from three individual mapping experiments.

Figure 3.1   Characterisation of λHM17.

A.   0.5µg aliquots of λHM17 DNA were digested with combinations of the restriction endonucleases BamHI (B), EcoRI (E), BglII (Bg) and HindIII (H), as indicated. Fragments were separated in a 0.5% agarose gel and the gel was then photographed. Molecular weight markers (M) are λ x HindIII DNA fragments. Marker sizes in this and all other Figures are given in kb.

B.   DNA fragments in the gel shown in A were acid/alkali denatured in situ and transferred to nitrocellulose by Southern blotting. Fragments containing sequences homologous to seal myoglobin exon 3 were identified by filter hybridisation with the seal exon 3 probe, [^{32}P]labelled by nick translation. Hybridisation was in 1xSSC at 65°C overnight, without dextran sulphate.

C.   Restriction endonuclease cleavage map of the recombinant λHM17 which has an insert of 10.5kb. Alignment of this restriction map and those of the other human myoglobin gene λ-recombinants (see Figure 3.2) with the genomic map of the human myoglobin gene (Jeffreys et al. 1983) indicates that this clone contains exons 2 and 3 of the functional human myoglobin gene. The positions of the exons within the 5.6kb BglII-EcoRI fragment could not be determined from this data, but their positions, determined by fine-scale mapping are indicated; filled boxes, coding sequences; and open box, 3' non-translated region.

Figure 3.2   Organisation of the human myoglobin gene

A. The organisation of the seal myoglobin gene with coding sequences
indicated by filled boxes and non-translated mRNA sequences by open boxes.
Exon sequence probes were purified after cleavage of seal myoglobin
subclones pSM19.5 and pSM1.17 (Blanchetot et al. 1983) with the indicated
restriction endonucleases;

>   Exon 1 probe   46bp 5' flanking sequence, 70bp 5' untranslated
>   sequence and 84bp coding sequence
>   Exon 2 probe   25bp first intron sequence, 233bp coding sequence
>   Exon 3 probe   66bp second intron sequence, 147 coding sequence and
>   67bp 3' untranslated sequence

B. Restriction map of the region of human DNA containing the myoglobin
gene constructed from maps of the individual λHM recombinants isolated
from the genomic library by screening with [^{32}P]labelled seal myoglobin
probes. The individual recombinants are shown aligned according to the
human sequence that they contain. Coding sequences were located by
Southern blot hybridisation of restriction endonuclease-digested λHM DNAs
with the appropriate seal myoglobin gene exon probe. BamHI restriction
fragments from λHM27 and an EcoRI fragment from λHM17 were subcloned into
pAT153 to give pHM.27.B2.9, pHM.27.B1.1 and pHM.17.E1. Regions of the
myoglobin gene which have been sequenced are represented by hatched boxes.

(A)

seal Mb

exon 3
exon 2
exon 1

probes

Bgl II
Hinf I

Hinf I
Rsa I

Hind III
Ava II

1 Kb

1 Kb

(B)

human Mb

Hind III
Eco RI
Bgl II

Eco RI

Bgl II
Bam HI
Eco RI

Eco RI

Bgl II

Hind III

Bgl II

Bam HI
Hind III

Hind III

λ HM

17
27
32
24
2
3
18
20
19
23

pHM.27.B2.9
pHM.27.B1.1
pHM.17.E1

1 Kb
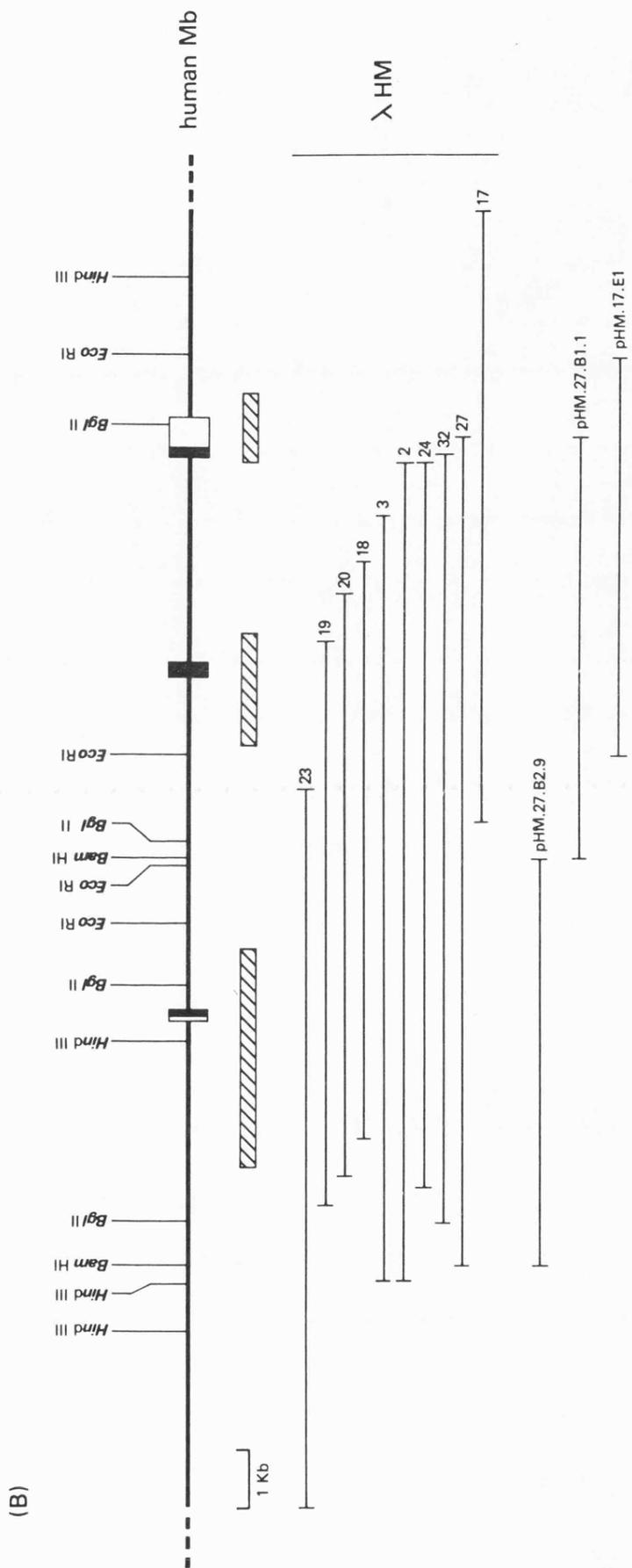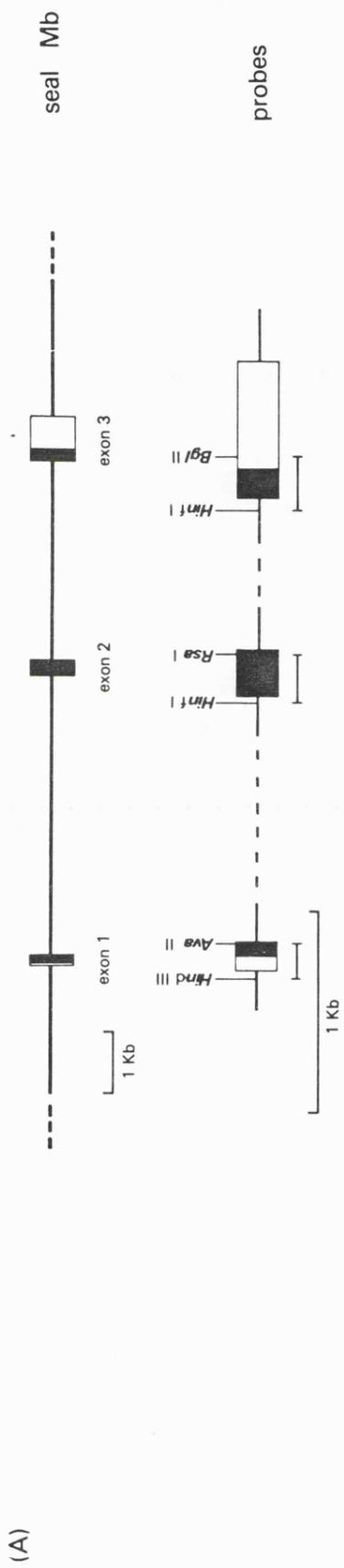
Figure 3.3 Fine-scale restriction mapping of the third exon of the human
myoglobin gene

The method used was that of Smith and Birnstiel (1976), see section 2.9.8

A. Preparation of ^{32}P end-labelled fragment for restriction mapping.
A map of plasmid pHM.17.E1 with EcoRI (E) and SstI (S) restriction sites
marked is shown. pAT153 sequences are represented as a narrow open box.
Myoglobin gene exons 2 and 3 are indicated with boxes; filled for coding
sequences, open for non-translated mRNA sequences. The plasmid was
digested with EcoRI. Only the fragment relevant to isolation of the
required labelled fragment is shown (1). The EcoRI fragments were
labelled with ^{32}P at the 5' terminal phosphates (2) and then cleaved with
SstI to give six fragments from which the uniquely end-labelled 2.1kb
SstI-EcoRI fragment shown was purified (3).

B. Partial digestion of end-labelled fragment. The end-labelled
SstI-EcoRI fragment, isolated as described in A, was subjected to partial
digestion by the range of restriction endonucleases shown. DNA fragments
were separated on a 1.5% agarose gel which was dried onto a glass plate
and autoradiographed. Molecular weight markers were a mixture of
end-labelled λ x HindIII and pBR322 x Sau3A

C. The detailed restriction endonuclease cleavage site map for the third
exon and 3' flanking sequence of the human myoglobin gene was derived from
the autoradiograph shown in B. The approximate positions of the coding
and 3' non-translated sequences are shown. Coding and non-coding
sequences are represented as in A.

Figure 3.4   <u>Detailed restriction map of the human myoglobin gene</u>

The map is derived from three separate experiments, including that described in Figure 3.3 and extends over ~4kb and ~1kb of 5' and 3' flanking sequence respectively, but there is a gap of 3.1kb in the first intron. The approximate positions of the exons of the myoglobin gene are shown above the map.

exon 1

exon 2

exon 3

B

E E

E

1 kb

Sst I
BstEII
NcoI
HpaII
Taq I
Hinf I
Dde I
Ava II
Sau 96I
Sau 3A
Alu I
Hae III
Rsa I
EcoRV
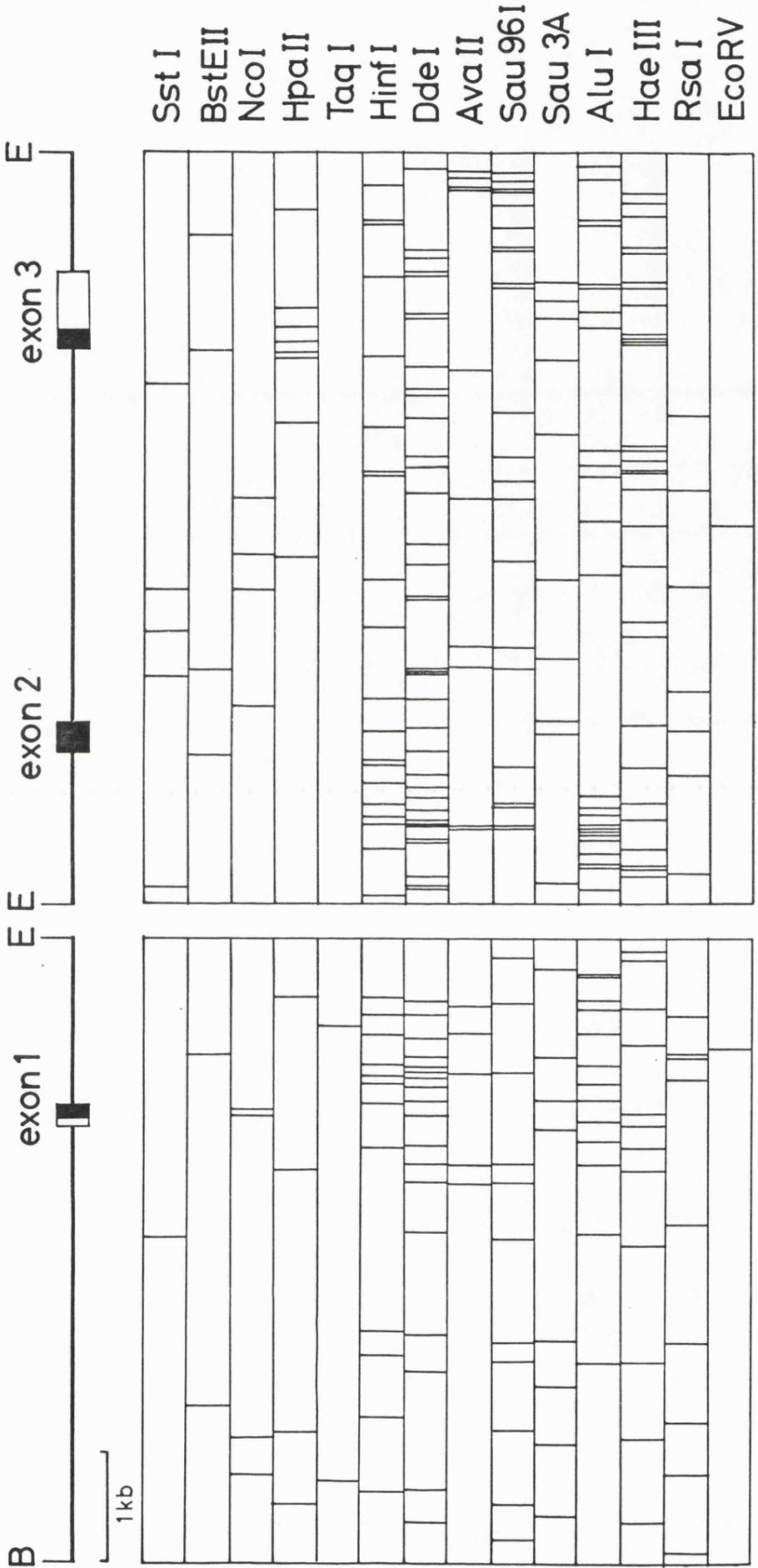
3.8 Sequencing of the human myoglobin gene

The sequencing of the human myoglobin gene was caried out by Dr A.J.Jeffreys and Mrs V.Wilson. M13 recombinant phage were generated by shotgun cloning of myoglobin gene recombinant plasmids. Phage were screened for required inserts by filter hybridisation. DNA was purified and DNA sequences were determined using the dideoxynucleotide chain termination method of Sanger et al. (1980).

The entire sequence of the human myoglobin gene is presented in Figure 3.5. The amino acid sequence derived from the nucleotide sequence corresponds exactly with the sequence determined by Romera⁼Herrera and Lehmann (1974), thus establishing that the cloned gene is the functional gene in man. The human myoglobin gene has the same three exon and two intron structural organisation as the seal myoglobin gene, with long intron sequences interrupting the coding sequence at precisely the same positions, and a similarly long 3' non⁼translated region.

The principal difference between the two genes is an elongation of the first intron from 4.8kb in seal to 5.8kb in man. The position of the cap site has been deduced from the homologous sequence in the seal myoglobin gene, (see also chapter 7). The 5' flanking sequence contains a conventional TATA box 33bp upstream from the putative cap site, and a highly purine⁼rich sequence which is located 68⁼114bp upstream from the cap site, also seen in the seal gene. This sequence spans the region which normally contains the CCAAT box, approximately 80bp upstream from the cap site. This element is absent from the human myoglobin gene. The polyadenylation signal, AATAAA, and 3' terminus of the mRNA were located by homology with sites in the seal gene. The 5' and 3' non⁼translated regions are 70 and 531nt, respectively, which predict a human myoglobin mRNA of 1066nt, similar to the 1083nt seal myoglobin mRNA (Blanchetot et al., 1983).

Figure 3.5   DNA sequence of the human myoglobin gene

     Sequences present in mature myoglobin mRNA are shown in upper case and the amino acid sequence is shown above the coding sequences. Positions of the cap site (see chapter 7) and the poly(A) addition site were deduced from the homologous sequence locations in the grey seal myoglobin gene (Blanchetot et al., 1983). The 5' polypurine sequence, located 68 to 114bp upstream from the capsite, the TATA box and AATAAA polyadenylation signal are underlined. The numbering of the sequence relates to the human myoglobin sequence as published (Weller et al., 1984).

exon 1

```
tcctccactctctttctttgcaaaaccatccacccatttactcaataaacatttattcagttcaaacttggcacaaagcaccatgtgaggcccaagagat  1600
acgtgggttaataaaacagagctcctgccctcctgaaaactgcaaagaaaggggcgtggcttcctgagttcaaatcccaactctgccagcgactagctgt  1700
acatcagtgatgtttccctactttctctcaattaaataggga taatgtcagtacctatcacattgggaggtcttgcgggggattaaatgagttaccaaatg  1800
ccaagtgtttgggacagggcctggcacccagcaaagtctcttgtgagtgctggctgctattatcctaatggagaagatggcatgaaaaccaggaaatagg  1900
atgccctttgggaagcaatgcaacaggaacttacacaaagaaaggaaaggaggaagcaattagtggtgtctcaaagggaqtatgtcaagaaaaacttttca  2000
gagggaaacctttgagcagggccatgaaaacaggagttctctaagagagattgtggacttgcctgggaccacctggctataagcacaaaaccatccggttcc  2100
tttctgtcacttctggcgggtgaggggtctctggcaaagggcagaaggtgcgtgagaggttgcgaatggcaggactgtcctggccagccggggcacctg  2200
gtggccaagcttagaaacatgacaggtcctcttgggagggctgaccgcagggagcgttgggtttcaggctgctggcgtcggcttctgtggtgccctttct  2300
gtcggctatgagagtccagacagtgcccaacctcctcccccttctttccacacgcacaaccaccccaccccctgtggcctgagctgtcctgcctcgccaca  2400

atggcacctgccctaaaatagcttcccatgtgagggctagagaaaaggaaaagattagaccctccctggatgagagagagaaagtgaaggagggcaggggga  2500
                                                                                          CAP
gggggacagcgagccattgagcgatctttgtcaagcatcccagaaggtataaaaacgccctgggaccaggcagcctcaAACCCCAGCTGTTGGGGCCAG  2600
                                   iniGlyLeuSerAspGlyGluTrpGlnLeuValLeuAsnValTrpGlyLy
GACACCCAGTGAGCCCATACTTGCTCTTTTTGTCTTCTTCAGACTGCGCCATGGGGCTCAGCGACGGGGAATGGCAGTTGGTGCTGAACGTCTGGGGGAA  2700
sValGluAlaAspIleProGlyHisGlyGlnGluValLeuIleAr(g)
GGTGGAGGCTGACATCCCAGGCCATGGGCAGGAAGTCCTCATCAGgtaaaagtgaagagattccattgcccctgccacccacacctaagatcaagggtgt  2800

tcagctgcaggtggaaagtttgcacgtgggg taggtcagttggctgcattagttaagggtgttagaacggtcacttgcttttttcttttgcttttaagtgt  2900
cagggattggactcaggagagggaaaggagccatttcaggctgatatcagcagctggaggaagcatgagaatcaaacctaggatgctcagagtccaccag  3000
gaagaattttagaattatagacagtcagagttaacaagggtcctgagagattttgtacagccacctctcttacaggatgaggacaaaaagcgactgacaa  3100
ggggaggacatttccagagtcacagctcattaaatgctcttaaagtgtcaaggttaagacatgctcttcaagggagacagatctggttctagacttggc  3200
tctgccactgagccactgggtgacctttgggaaggtactcaacctctcggagcctcaatttcctctcctgtacagtgaggggatatcctaatatctatat  3300
cctagaggagatgtgagaattaaatataaataatgcatgcaagaggcctggcatggttcctggcatatactgagtcctagaaatgttagtagctattactg  3400
atgaagcccaggctagggacctttcaaagcattgcaattagagaacagaagatagaggctcattagtgaccttcgatgttgagtatgtctctagtttgag  3500
aggtctgaatgatgtggtctgcaagtatatcctgccttctaccacaagggattccagaatacaccaaagaaaacaaaattcctgaggtttgtaaatagagg  3600
gtggctgtggtttgtacatagaagctcatctcctcgttgccttctatcccaaaggtgatacactcttctcttggcccctttccctcaccattctgagctgg  3700
ttccctcagaagtctaataggttaagaatcaacgtttctgccaacgggaggaaggaagtgggcgccgg
```

exon 2

```
gtgaggggactgggaggttcagagagtcacagcttgggctcaaaacaagcaagaggtttctgagtgtgaggattgctctggagtggaatggccctcacag  800
gtaggagtgagcctcctgtagctagaggtatttaagcagctgaaggacaatccctgggcaggaagctgcagagatggtcgcagcgtggactagaactgct  900
gttttggtcactcagacctcattccagcctggcttctctggacagcaccctgcaatagtgagctggtgactttacgcctcagaacctcggtttccacat  1000
ctgtaaaatgggaattatatgacactcactatgtgccagacaccctgttggtacatagcacactatctcacttaatccttcaatgagggacaagttat  1100
ccccatccctatatgaggaagctgaggcacagagaggtgaagtgaatggcccaaggtcacacagctgggaagacagggagctaaacttgaactctagtc  1200
                                                 (Ar)gLeuPheLysGlyHisProGluThrLeuGluLysPhe
tggctgcccccagacctcacaccgcacctccatgccgactccagccttccctgtgcccacagGCTCTTTAAGGGTCACCCAGAGACTCTGGAGAAGTTT  1300
AspLysPheLysHisLeuLysSerGluAspGluMetLysAlaSerGluAspLeuLysLysHisGlyAlaThrValLeuThrAlaLeuGlyGlyIleLeuL
GACAAGTTCAAGCACCTGAAGTCAGAGGACGAGATGAAGGCATCTGAGGACTTAAAGAAGCATGGTGCCACTGTGCTCACCGCCCTGGGTGGCATCCTTA  1400
ysLysLysGlyHisHisGluAlaGluIleLysProLeuAlaGlnSerHisAlaThrLysHisLysIleProValLysTyrLeuGlu
AGAAGAAGGGGCATCATGAGGCAGAGATTAAGCCCCTGGCACAGTCGCATGCCACCAAGCACAAGATCCCCGTGAAGTACCTGGAGgtaggaggcagagc  1500

ctgggcaggtgggaggatgcggggaaggcctcgggtggggcaatgggatctgggttcgagtccaagctcagccactaacttgtgggatgacctatgccac  1600
tcttctctgtgccccaggtttctcatttgtaaaggggactgccacccactttgccttcctcctgggattgttgagaatgaacacatttagcatttttaat  1700
ttagtatgccaaattcacatcttattaccaaagaggaaagggagagggggatattgggtgcaaaatttgcatcctctccatgggtaggtaggtaccattatcata  1800
tccacttgatagatggggaaactgaggctcacagaggttaagcagcttgtccacggtcacaggaggtggataatggcagagccaagattcaaacgcaggt  1900
ctctattactacagaaccccagcccctaactgctgtgccactgggagtctggtacatgcaggacttatgtggcaggagctc
```

exon 3

```
ggtcctggaataaagagaaggtaggaggacaactgactcccatctggcccctggcttgtcccaccctggtgaccattttctctcctcaccctccctgcag  100
PheIleSerGluCysIleIleGlnValLeuGlnSerLysHisProGlyAspPheGlyAlaAspAlaGlnGlyAlaMetAsnLysAlaLeuGluLeuPheA
TTCATCTCGGAATGCATCATCCAGGTTCTGCAGAGCAAGCATCCCGGGGACTTTGGTGCTGATGCCCAGGGGGCCATGAACAAGGCCCTGGAGCTGTTCC  200
rgLysAspMetAlaSerAsnTyrLysGluLeuGlyPheGluGlyTer
GGAAGGACATGGCCTCCAACTACAAGGAGCTGGGCTTCCAGGGCTAGGCCCCTGCCGCTCCCACCCCCACCCATCTGGGCCCCGGGTTCAAGAGAGAGCG  300
GGGTCTGATCTCGTGTAGCCATATAGAGTTTGCTTCTGAGTGTCTGCTTTGTTTAGTAGAGGTGGGCAGGAGGAGCTGAGGGGCTGGGGCTGGGGTGTTG  400
AAGTTGGCTTTGCATGCCCAGCGATGCGCCTCCCTGTGGGATGTCATCACCCTGGGAACCGGGAGTGCCCTTGGCTCACTGTGTTCTGCATGGTTTGGAT  500
CTGAATTAATTGTCCTTTCTTCTAAATCCCAACCGAACTTCTTCCAACCTCCAAACTGGCTGTAACCCCCAAATCCAAGCCATTAACTACACCTGACAGTA  600
GCAATTGTCTGATTAATCACTGGCCCCTTGAAGACAGCAGAATGTCCCTTTGCAATGAGGAGGAGATCTGGGCTGGGCGGGCCAGCTGGGGAAGCATTTG  700
                                                                           poly(A)
ACTATCTGGAACTTGTGTGTGCCTCCTCAGGTATGGCAGTGACTCACCTGGTTTTAATAAAACAACCTGCAACATCTCagtttctgcctggcattttttca  800
tctcctagagtaaatgatgcccccaccagcaccagcatcaaggaagaaatgggaggaaggcagacctgggcttgtgtgtgcagagagagctcaggaaaga  900
gggagaaggggaggaggaaaggcaggagggtgagagggacaggagcccaccctccctgggccaccgctcagaggcaggcccagtgcagggcatgggaaat  1000
ggaaaggggacaggctggccccagccttgggagcacctctctcttcgggggaggtgggaggcagcgaacagacctctgcaatacgaggagagagtgacaggt  1100
gcgccaggctgtgggaacccagagagaggggaagccatcatcatcatggctgcaataccttcagtaacgtgggaaggtcaccctgctagtaagtggcag  1200
agctgggactcaaactatggcctgga
```

3.9 Summary

The single functional human myoglobin gene has been isolated in a series of overlapping genomic λ-recombinant clones and characterised by detailed restriction endonuclease mapping and sequencing. The human myoglobin gene has an overall organisation very similar to that of the grey seal myoglobin gene with the typical α^A and β-globin three exon and two intron structure, but with greatly elongated introns and 3' non-translated sequence.

TISSUE-SPECIFIC EXPRESSION OF MYOGLOBIN mRNA

4.1   Introduction

A great deal of work has been published over a considerable period

of time concerning estimation of the concentration of myoglobin in

different muscle types and in different animal species (see Chapter 1).

As myoglobin is the product of a single gene, it would be interesting to

compare myoglobin levels in the different tissues in which the gene is

expressed.   Myoglobin is known to be present at similar concentrations in

the skeletal and cardiac muscle of man (see Chapter 1).   Early work using

spectrophotometric techniques indicated that myoglobin was also present in

smooth muscle (Biorck, 1949), but later work using an immunoprecipitation

technique failed to detect any myoglobin (Fasold et al., 1970).   In

contrast, smooth muscle of the chicken (gizzard) has been shown to contain

fairly high levels of myoglobin (Grœschel-Stewart et al. 1971).

The concentration of myoglobin in the skeletal muscle of small

mammals tends to be low.   The level in mouse skeletal muscle has been

estimated at 0.3-0.5mg/g wet weight (Weller et al., in preparation), which

is approximately 20-fold lower than the level in human skeletal muscle.

This chapter is concerned with measurement of relative myoglobin

mRNA levels in different tissues, in an attempt to gain a better

understanding of the tissue-specific pattern of expression of the

myoglobin gene.   The method used is Northern blot analysis of poly(A)$^+$

RNAs made from various tissue samples, using a number of human and seal

myoglobin gene probes to estimate the relative levels of myoglobin mRNA

present.   Myoglobin protein concentrations in these tissues have been

measured by Western blotting and the results are presented elsewhere

(Weller et al., in preparation, and see Chapter 9).

4.2 Preparation of RNA from a range of tissues

Initial preparation of RNA involved gentle SDS lysis of muscle samples homogenised in ice-cold 75mM NaCl, 50mM EDTA, pH 8.0 (Wood et al., 1982). Lysates were extracted with phenol and the nucleic acids were precipitated with ethanol. RNA was separated from DNA by sedimentation through caesium chloride gradients. Agarose gel electrophoresis showed that the RNA was degraded (Figure 4.1A,B) and the yield was low, so another method was used.

RNA was routinely prepared by homogenising frozen tissue in a lysis buffer containing high concentrations of urea and lithium chloride, in which DNA is soluble, but RNA is not (Minty et al., 1981). RNA was recovered by centrifugation, redissolved and then purified by phenol extraction and ethanol precipitation (2.5.5). Yield and quality of RNA were much better using this method (Figure 4.1C). Yields of RNA per gram of the various tissues from which RNA was prepared are given in Table 4.1. Yields from muscle tissue were consistently lower than from non-muscle tissues.

Polyadenylated (poly(A)$^+$) RNA was purified from total RNA preparations by oligo(dT)cellulose chromatography (Aviv and Leder, 1972), see section 2.5.5. Quality and yield of poly(A)$^+$ RNA were determined as for total RNA (Figure 4.1D). The yield of poly(A)$^+$ RNA after one passage over an oligo(dT)cellulose column was generally 2-3%, but occasionally up to 5% (Table 4.1). About 50% of this was recovered after passage over a second column.

4.3 Relative myoglobin mRNA levels in human skeletal, smooth and cardiac muscles

In order to see whether myogolobin mRNA could be detected in human muscle RNAs by Northern blot analysis and hybridisation with human myoglobin gene probes, three different amounts of each of two adult

Figure 4.1   Agarose gel electrophoresis of total and polyadenylated RNAs

Initial preparations of RNA from muscle samples used the SDS lysis method, described by Wood et al., (1982). Total nucleic acids were prepared by phenol extraction and ethanol precipitation of the cell lysate and RNA was purified by CsCl gradient centrifugation. Molecular weight markers (M) were pBR322 x Sau3A and/or λ x HindIII (single-stranded).

A.   Total nucleic acids prepared by the SDS lysis method from seal (S) and human (H) skeletal muscle were electrophoresed in a 1.5% agarose gel. The RNA appears as a smear below the high molecular weight DNA band, with the 18S and 28S rRNAs superimposed. A lower molecular weight tRNA band is also visible.

B.   RNA from the same preparation shown in A was electrophoresed in a 1.5% agarose gel after CsCl gradient centrifugation. The RNA is partially degraded; the mean size of RNA molecules is smaller than in A and the rRNA bands are no longer visible. Lanes marked 'OH' show further degradation of RNA on treatment with alkali.

C.   Total RNA from three separate preparations of seal (S) and human (H) skeletal muscle, using the urea/LiCl method (section 2.5.5). 2μg from each preparation was heat-shocked (65°C for 3 minutes) to remove secondary structure before being electrophoresed in a 1% agarose gel. The tracks appear almost identical; the quality of RNA obtained using this method was better than with the SDS lysis method. 18S, 28S rRNAs and tRNA are indicated

D.   Polyadenylated RNA was prepared by passage over oligo(dT)cellulose columns. Aliquots of three preparations of total and polyadenylated RNAs (urea/LiCl method) from mouse liver (L), kidney (K) and spleen (S) were heat shocked before electrophoresis in a 1% agarose gel. After oligo(dT)chromatography the relative intensity of the rRNAs is reduced and there is no tRNA present. The migration positions of 18S, 28S rRNAs and tRNA are indicated.
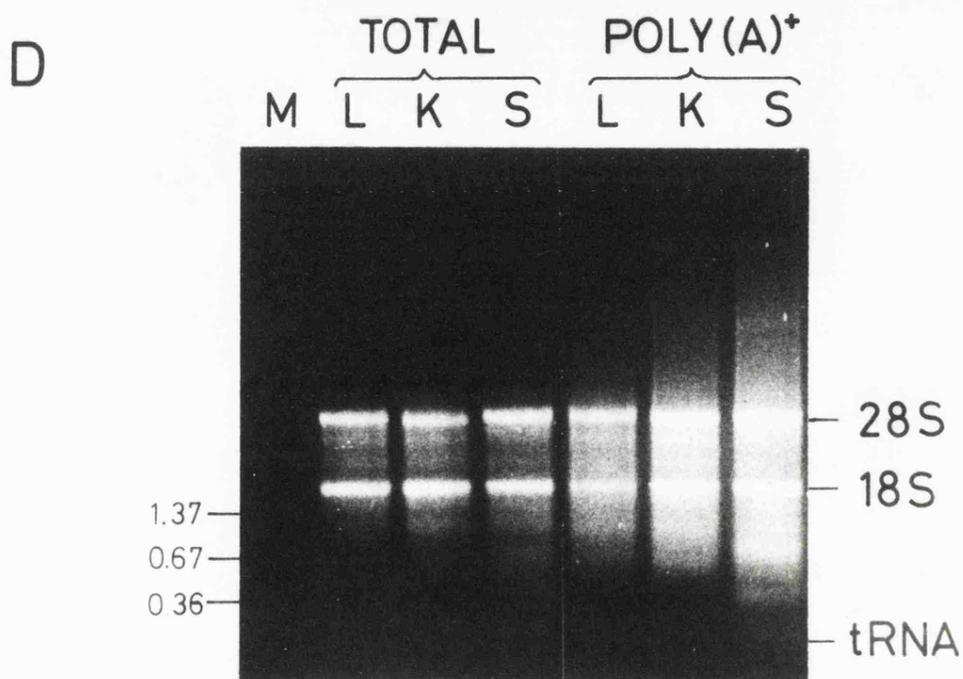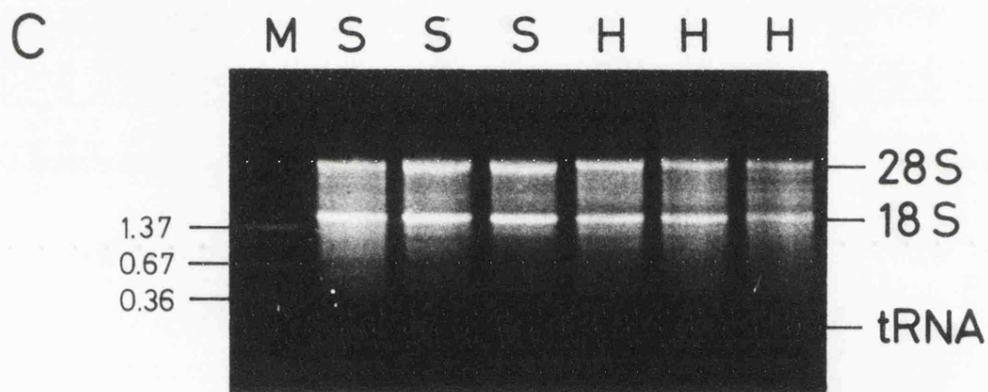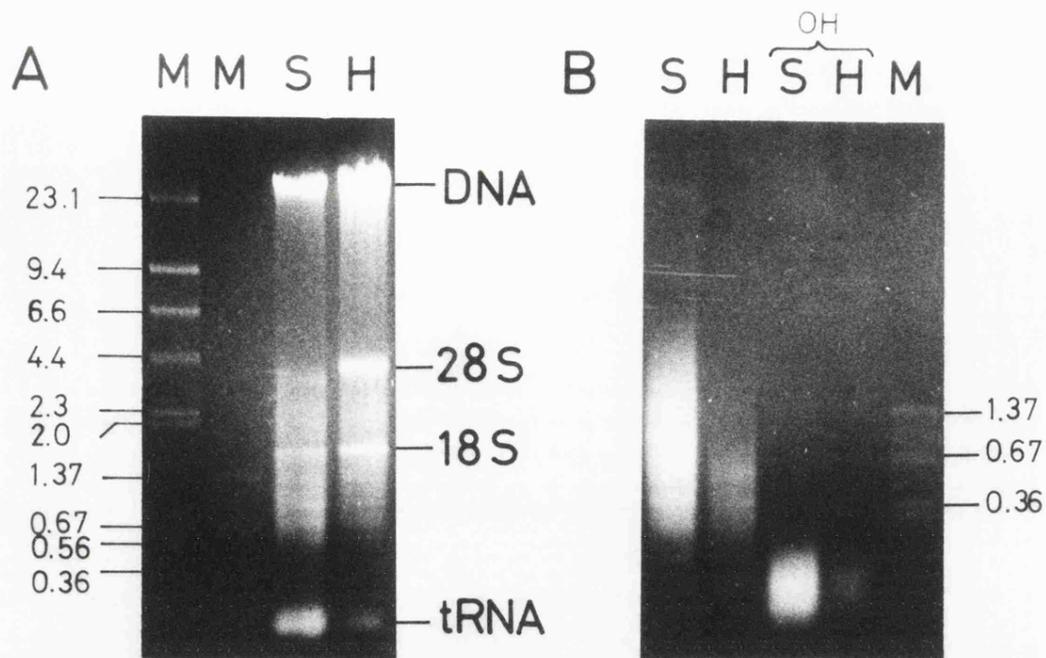
Table 4.1   Yields of total and polyadenylated RNA from various tissues

     The yield of total RNA from RNA preparations by both the SDS lysis and urea/LiCl methods are given.   Yields were determined by measuring the A_{260} of diluted aliquots.   Figures for human and seal skeletal muscle are means from several preparations.   The SDS lysis method gave low yields of relatively poor quality RNA and so was abandoned in favour of the urea/LiCl method, which gave consistently better yields of higher quality RNA.

Table 4.1 Yields of total and polyadenylated RNA from various tissues

Tissue	Yield total RNA mg/g tissue		Yield poly(A)+ RNA
	SDS lysis	urea/LiCl	(% total RNA)
grey seal skeletal muscle	0.051	0.270	approx. 2%
human skeletal muscle (gastrocnemius)	0.070	0.190	approx. 2%
mouse skeletal muscle		0.280,0.335	2.9%
human cardiac muscle		0.35	2.25%
mouse cardiac muscle		0.22	4
mouse skeletal muscle			
15 days gestation		2.70	5.0%
16		2.60	3.9%
17		2.50	3.4%
18		0.55	2.0%
19		1.90	3.7%
birth		0.62	2.0%
1 day		0.58	2.0%
6 days		0.46	5.0%
mouse non-muscle - liver		approx. 5	approx. 2%
kidney		approx. 2	approx. 2%
spleen		approx. 2	approx. 2%

skeletal muscle poly(A)$^+$ RNA samples were electrophoresed and transferred to nitrocellulose (2.5.6). The filter was hybridised with a 316nt human myoglobin exon 2 single-stranded antisense probe. Details of this probe and others from the human and seal myoglobin genes are given in Figure 4.2. The resulting autoradiograph is shown in Figure 4.3A.

A single myoglobin mRNA is detected in human skeletal muscle RNA. It has a mobility of approximately 1400nt, which is consistent with the mRNA length of 1066nt, predicted from the sequence of the human myoglobin gene, plus the poly(A) tail. There is no evidence for any mRNA precursors.

Having established that myoglobin mRNA can be detected in RNA from skeletal muscle, levels of myoglobin mRNA were also investigated in cardiac and smooth muscle RNAs, in comparison with skeletal muscle (Figure 4.3B and C). Myogobin mRNA with the same mobility as in skeletal muscle is present in both cardiac and smooth muscles. There is no evidence for precursors of mature mRNA in either tissue. The level of myoglobin mRNA in cardiac muscle is similar to that in skeletal muscle, but there is quite a large difference in concentrations between different RNA samples from the same tissue type (compare Figure 4.3A and B, which are from the same experiment). In contrast, myoglobin mRNA is present at very low levels in smooth muscle (Figure 4.3C), probably less than 1% of the level in skeletal muscle (not shown). Myoglobin protein has also been detected at low levels in smooth muscle by Western blotting (Weller et al., in preparation). This establishes that the myoglobin gene is expressed in human smooth muscle.

4.4 Cross-hybridisation between human and mouse myoglobin sequences

Study of myoglobin gene expression would be considerably easier using a mouse rather than a human system, particularly for looking at developmental expression. However, in order to estimate mouse myoglobin

Figure 4.2   Hybridisation probes from the seal and human myoglobin genes

Sequences transcribed are shown as boxes: open boxes, non-translated sequences; filled boxes, coding sequence. Only restriction endonuclease sites relevant to probe fragments are shown.

A.   Probes from the seal and human myoglobin genes. Two equivalent 3' non-translated sequence probes, from the seal and human genes, S3' and H3', were isolated from pSM.1.17 and pHM.17.E1, respectively.

The constructs M13.SEX3 and M13.HEX3 were made by 'filling in' the ends of the fragments shown, using the Klenow fragment of DNA polymerase I, and cloning the blunt-ended fragments into the SmaI site of M13mp18 RF DNA. The BstEII-HaeIII fragment, H2, was used as a probe labelled with ^{32}P by nick translation and was also cloned into M13mp18 as above, to generate M13.HEX2. Single-stranded probes were generated from M13 constructs by the primer extension method of Jeffreys et al., (1985), see section 2.11.1. Two probes were generated from M13.HEX2; one the full length of the insert, and the other, by cleavage with RsaI, equivalent to the pure coding-sequence fragment BstEII-RsaI.

B.   Probe fragment lengths and composition in terms of coding, non-coding and M13 linker and primer sequences are given. A mouse myoglobin exon 1 probe was generated from M13.MEX1, which was isolated during the recent cloning and sequencing of the mouse myoglobin gene (Blanchetot et al., in preparation).

A.

seal — exons 1, 2, 3. Restriction sites: Dde I, Dde I, Sau 96I, Hinf I. Probes: M13.SEX3, S3'

human — exons 1, 2, 3. Restriction sites: BstEII, Rsa I, HaeIII, BstEII, Alu I, HpaII, Hinf I. Probes: H2, M13.HEX3, H3', 1) 2) M13.HEX2
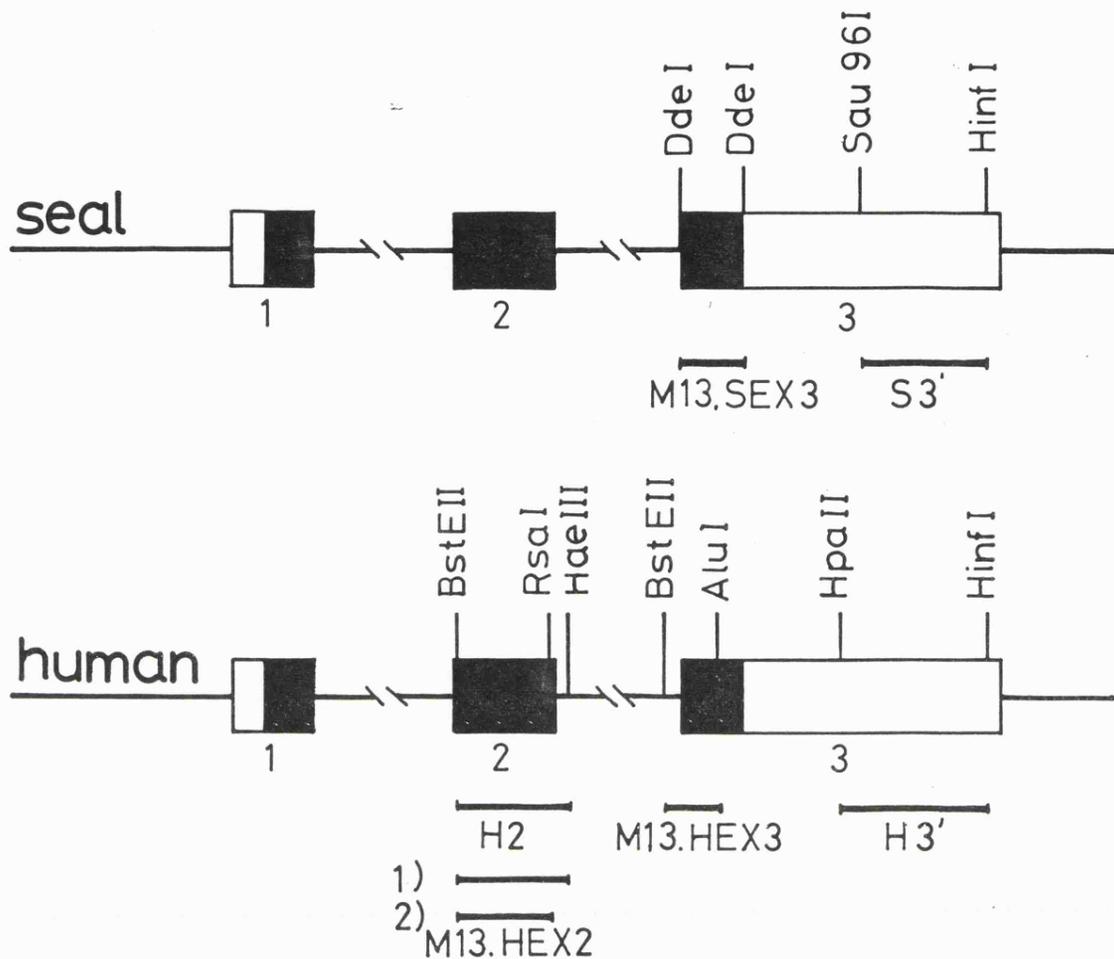
B.

Probe		Length / bp	Probe sequence comprises
seal:	S3'	269	269bp 3' non-translated sequence
	M13.SEX3	204	138bp exon 3, remainder primer and polylinker
human:	H2	254	212bp exon 2, 42bp second intron
	M13.HEX2 1)	316	212bp exon 2, 42bp second intron, remainder primer and polylinker
	M13.HEX2 2)	261	204bp exon 2, remainder primer and polylinker
	H3'	285	285bp 3' non-translated sequence
	M13.HEX3	194	93bp exon 3, 32bp second intron, remainder primer and polylinker
mouse:	M13.MEX1	190	119bp exon 1, 13bp 5'flanking sequence, remainder primer and polylinker

Figure 4.3   Relative myoglobin mRNA levels in human skeletal, smooth and cardiac muscles

Myoglobin mRNA levels were estimated by Northern blot analysis and hybridisation with human myoglobin exon 2 probes.

A. Skeletal muscle. 0.5, 0.25 and 0.125µg (a,b and c respectively) of each of two skeletal muscle poly(A)$^+$ RNA samples were electrophoresed and transferred to nitrocellulose as described (2.5.6). The filter was hybridised with the 316nt single-stranded human exon 2 probe generated from M13.HEX2 (see Figure 4.2). Exposure was for 5 days, without an intensifier screen.

B. Cardiac muscle. 0.5, 0.25 and 0.125µg (a,b and c respectively) of each of two cardiac muscle poly(A)$^+$ RNAs (car1 and car2), isolated from different tissue samples were electrophoresed, blotted and hybridised in the same experiment as in A.

C. Smooth muscle. 1µg of each of two skeletal muscle poly(A)$^+$ RNAs (S1 and S2) and one smooth muscle (Uterine, U) were electrophoresed and transferred to nitrocellulose. The filter was hybridised with the 268bp BstEII-HaeIII human exon 2 probe (Figure 4.2). Exposure was for 2 weeks with an intensifier screen. Skeletal muscle RNA tracks are therefore overexposed and appear smeared.
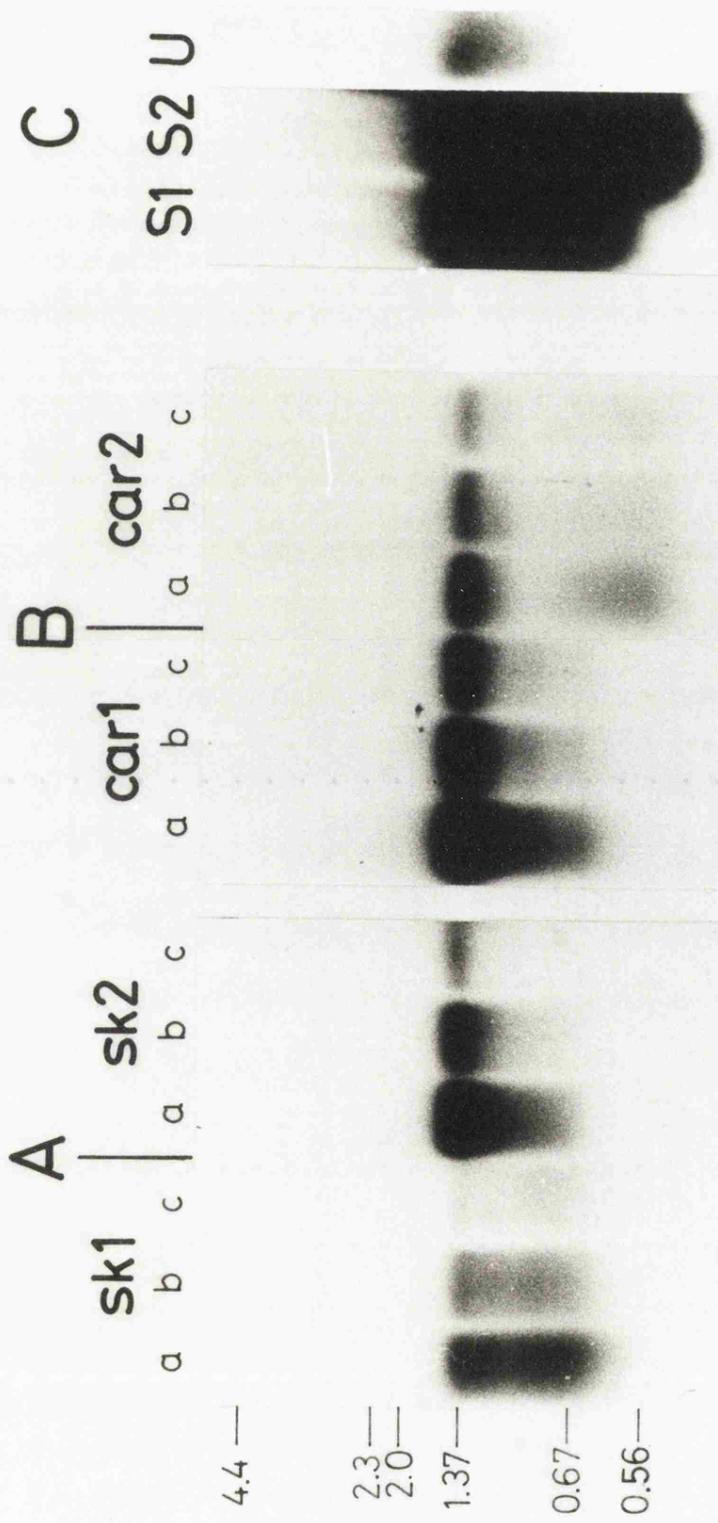
mRNA levels, it is first neccessary to show that human myoglobin gene probes hybridise approximately equally well with human and mouse myoglobin sequences. Cross-hybridisation between a human myoglobin probe and human, seal, rat and mouse (DBA/2) myoglobin genes was tested by Southern blot hybridisation (2.5.6). The filter was hybridised with the 316nt human myoglobin exon 2 probe generated from M13.HEX2 (see Figure 4.2) under Northern blot hybridisation conditions (2.6.3), and washed at a stringency of 0.25xSSC at 50°C, as these were the conditions to be used in subsequent hybridisations between human and mouse sequences. The resulting autoradiograph is shown in Figure 4.4.

The presence of a single hybridising fragment in each track indicates a single myoglobin gene in each species. The appearance of two fragments in mouse DNA digested with BclI could be due to heterozygosity or to partial restriction endonuclease digestion, as there is no additional hybridising fragment in the HindIII digest and there is no BclI site in the region of the mouse gene homologous to the probe used (Blanchetot et al., in preparation). It is interesting that unlike the seal myoglobin exon 2 probe (Weller et al., 1984), this human exon 2 probe does not detect multiple hybridising components in human DNA.

Strong cross-hybridisation between human and mouse myoglobin sequences therefore enables mouse myoglobin mRNA levels to be determined using human myoglobin gene probes.

4.5 Relative levels of myoglobin mRNA in mouse skeletal and cardiac muscle

Estimates of myoglobin mRNA levels in mouse skeletal muscle and heart were made by Northern blotting. Total RNA was used rather than poly(A)$^+$ RNA as the yield of total cardiac muscle RNA was too low to prepare enough poly(A)$^+$ RNA. The filter was hybridised with the 316nt single-stranded human antisense exon 2 probe (Figure 4.5A). The mouse

Figure 4.4   Cross-hybridisation between human and seal, rat and mouse
myoglobin sequences

5µg aliquots of genomic DNA from the species man (H), seal (S), rat
(R) and mouse (M) were digested with BamHI or BclI. DNA fragments were
electrophoresed in a 0.5% agarose gel, then acid/alkali denatured in situ
and transferred to nitrocellulose by Southern blotting. The filter was
hybridised with the single-stranded 316nt human exon 2 probe (Figure 4.2).
Hybridisation was under standard conditions for Northern blot filters
(section 2.6.3) and filters were washed at 50°C in 0.25xSSC.
Autoradiography was for 4 days with an intensifier screen. The appearance
of two bands in mouse DNA cleaved with BclI could be due to heterozygosity
or to incomplete restriction endonuclease digestion [based on sequence
data, there are no BclI sites in exon 2 of the mouse gene (Blanchetot et
al., in preparation)].
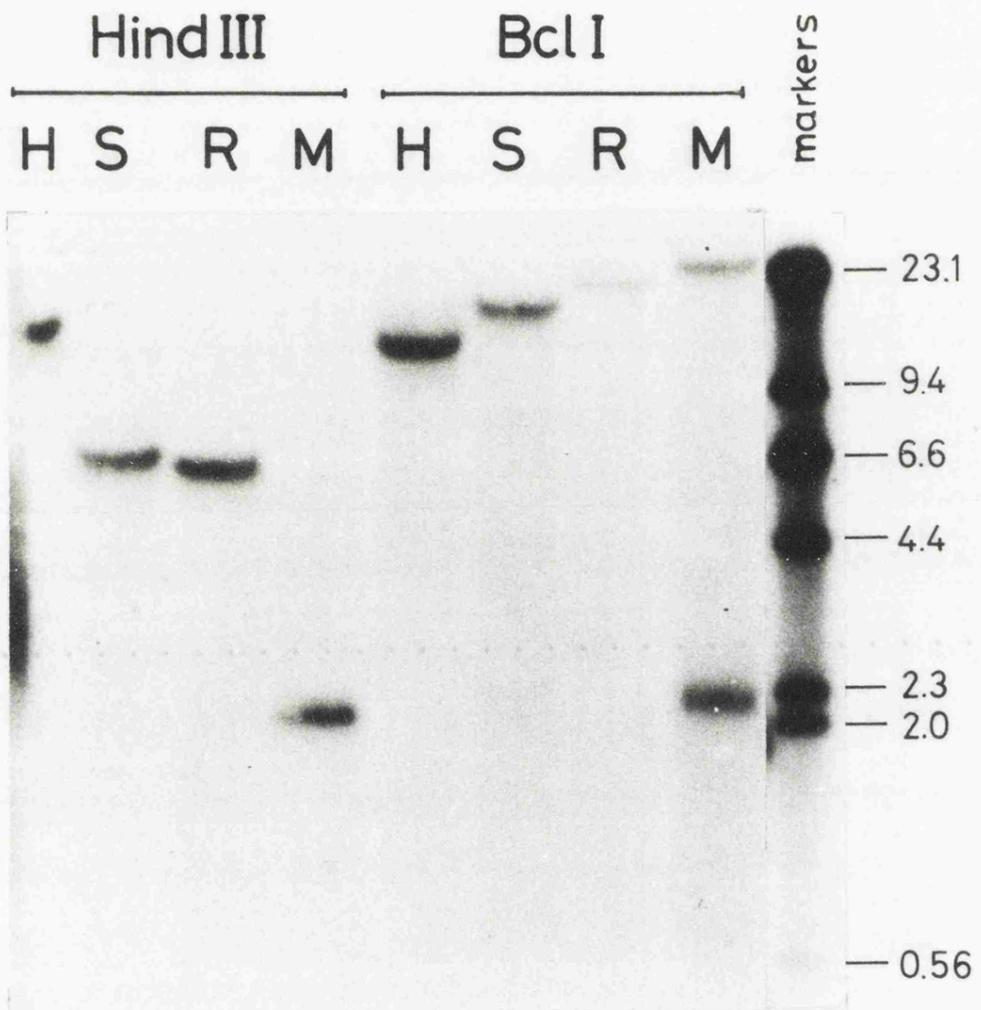
Figure 4.5   Myoglobin mRNA levels in mouse skeletal and cardiac muscles compared with that in human skeletal muscle

A. Relative levels of myoglobin mRNA in mouse skeletal and cardiac muscle

10, 5 and 2.5µg of each of mouse skeletal (a-c) and cardiac (d-f) total RNAs were denatured, electrophoresed and transferred to nitrocellulose by Northern blotting as described (2.5.6). The filter was hybridised with the 316nt single-stranded human exon 2 probe (Figure 4.2). A single myoglobin mRNA transcript about 1200nt is detected in both tissues.

B. Myoglobin mRNA in human and mouse muscle detected by various probes

4µg of each of human and mouse skeletal muscle poly(A)$^+$ RNAs were denatured and prepared for loading onto a 1% agarose-formaldehyde gel, each in a total volume of 80µl. Four identical pairs of tracks (H,M) were electrophoresed and the RNA transferred to nitrocellulose. The filter was cut into four strips, each of which was hybridised with a different myoglobin gene exon probe. The four probes were the nick translated seal exon 2 probe (S2), the 268nt single-stranded antisense human exon 2 probe (H2) and human and seal single-stranded antisense exon 3 probes (H3 and S3, respectively). Details of the origins of and the sequences included in these probes are shown in Figure 4.2. Filters were washed to a stringency of 0.25xSSC at 50°C. Autoradiographic exposures were overnight for the single-stranded probes and 48 hours for the nick translated seal exon 2 probe. Human and mouse myoglobin mRNAs are detected with all four probes

Underneath each track in the Figure is given the percentage sequence divergence between the probe and the homologous sequence in the mRNA with which it hybridises. These figures are derived from the DNA sequences of the human and seal myoglobin genes (Blanchetot et al.,1983, Weller et al.,1984) and the recently completed sequence of the mouse myoglobin gene (Blanchetot et al., in preparation).
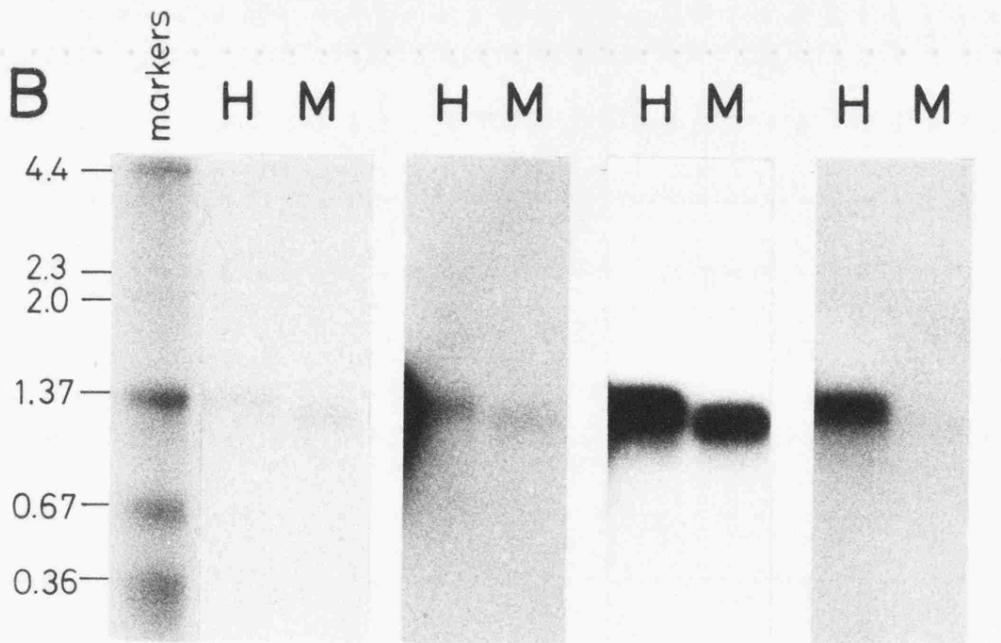
myoglobin gene had not been cloned at this time and so mouse myoglobin

gene probes were not available. The mouse myoglobin mRNA migrates at

approximately 1200nt, and is about 200nt shorter than the human message.

Recent sequence analysis of the mouse myoglobin gene has shown that this

is due to a shorter 3' non-translated region in the mouse gene (Blanchetot

et al., in preparation). The hybridisation signal is approximately the

same in tracks a and f, indicating that there is about four times as much

myoglobin mRNA in mouse cardiac muscle than in skeletal muscle. This is

in contrast with the situation in man, where the myoglobin mRNA levels in

these tissues are approximately the same.

4.6   Comparison of myoglobin mRNA levels in human and mouse skeletal

muscles

        The level of myoglobin protein in human skeletal muscle has been

estimated in this thesis, and elsewhere, at approximately 7mg/g wet weight

(Chapter 7; Weller et al., in preparation). In contrast, the level in

mouse skeletal muscle is much lower, and has been estimated at 0.3-0.5mg/g

wet weight (Weller et al., in preparation). There is therefore a

difference in protein levels of approximately 20-fold. It was therefore

decided to look at myoglobin mRNA pool levels in mouse muscle to see

whether there was a correspondingly low level of myoglobin mRNA.

        Direct comparison of myoglobin mRNA levels in human and mouse

muscle, however, cannot be made accurately from Northern blots without a

probe which is equally homologous to human and mouse mRNAs. Figure 4.5B

shows human and mouse skeletal muscle poly(A)$^{+}$ RNAs hybridised with four

different myoglobin gene probes (seal exons 2 and 3 and human exons 2 and

3). All four probes cross-hybridise to both human and mouse myoglobin

mRNAs. The sequence of the mouse myoglobin gene has since been completed

(Blanchetot et al., in preparation). The percentage sequence divergence

between each probe and its homologous sequence in the human and mouse

myoglobin mRNAs is given in Figure 4.5B. The probes which probably give

the most accurate indication of the relative myoglobin mRNA levels are

those from the seal myoglobin gene, as these are approximately equally

diverged from human and mouse myoglobin mRNAs.

Myoglobin mRNA concentrations in human and mouse skeletal muscle are

surprisingly approximately the same, despite the 20-fold difference in

protein levels. This therefore suggests that there may be differences in

the translational capacities of the two myoglobin mRNAs, although this

would need to be tested further by measuring amino acid incorporation into

myoglobin, in in vitro translations of human and mouse RNAs, for example.

4.7 Is the myoglobin gene expressed in non-muscle mouse tissue?

To test the possibility that myoglobin may also be expressed in

non-muscle tissue, Northern blot analysis was carried out on mouse

(outbred) liver, kidney and spleen RNAs, using pure coding sequence

myoglobin probes. Two identical Northern blot filters were prepared with

quite large amounts (20µg and 3µg, respectively) of total and poly(A)$^+$

RNAs. Mouse (IR) fibroblast poly(A)$^+$ RNA was also included. One filter

was hybridised with the 261nt human myoglobin single-stranded antisense

exon 2 probe, which contains no non-coding sequence (see Figure 4.2). The

other filter was hybridised with a mouse myoglobin single-stranded

antisense exon 1 probe (containing 119bp of exon 1, plus only 13 bp of 5'

flanking sequence) generated from M13.MEX1, which was recently isolated

during sequencing of the mouse myoglobin gene. The autoradiographs are

shown in Figure 4.6.

The patterns of hybridisation with the two probes are very

different. The mouse exon 1 probe detects a faintly hybridising species

in liver poly(A)$^+$ RNA the same size as the myoglobin transcript, which may

represent myoglobin mRNA from smooth muscle in the blood vessel walls in

the liver. There is also a hybridising species (approximately 1500nt)

Figure 4.6   Is myoglobin expressed in non-muscle tissue of the mouse?

     Two identical Northern blot filters were prepared with mouse
non-muscle RNAs; 3µg mouse IR fibroblast cell line poly(A)$^+$ RNA (F) and
20µg and 3µg total and poly(A)$^+$ respectively (T, A$^+$) from mouse liver,
kidney and spleen. One set of filters was hybridised with the 261nt pure
coding sequence single-stranded human exon 2 probe (A). The other set was
hybridised with a mouse exon 1 single-stranded probe containing only 13nt
of 5' flanking sequence in addition to 119nt of exon 1 sequence (B) see
Figure 4.2 for details of probes. Apparent discrete hybridising
components are marked with open triangles and one component which may
hybridise with both probes with a filled triangle. Autoradiographic
exposures were for 3 days (A) and 7 days (B), with an intensifier screen.

A

liver   kidney   spleen

F   T   A⁺   T   A⁺   T   A⁺

9.4 —
6.6 —
4.4 —
2.3 —
2.0 —
1.37 —
0.67 —
0.36 —

B

9.4 —
6.6 —
4.4 —
2.3 —
2.0 —
1.37 —
0.67 —
0.36 —
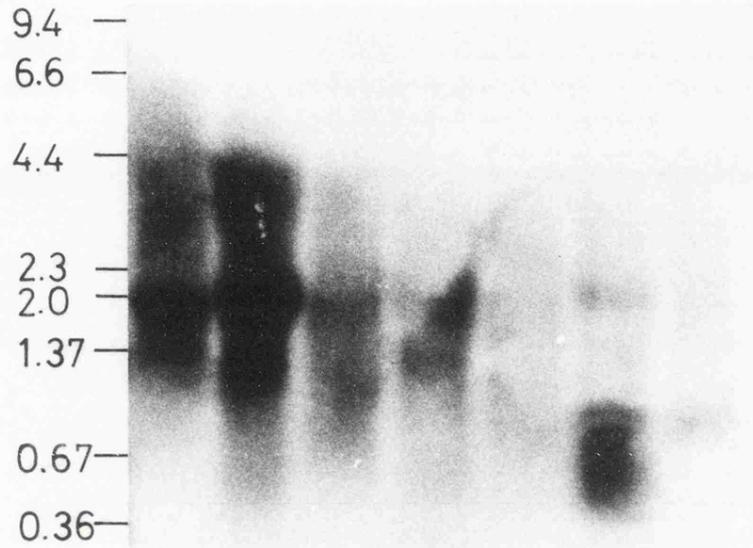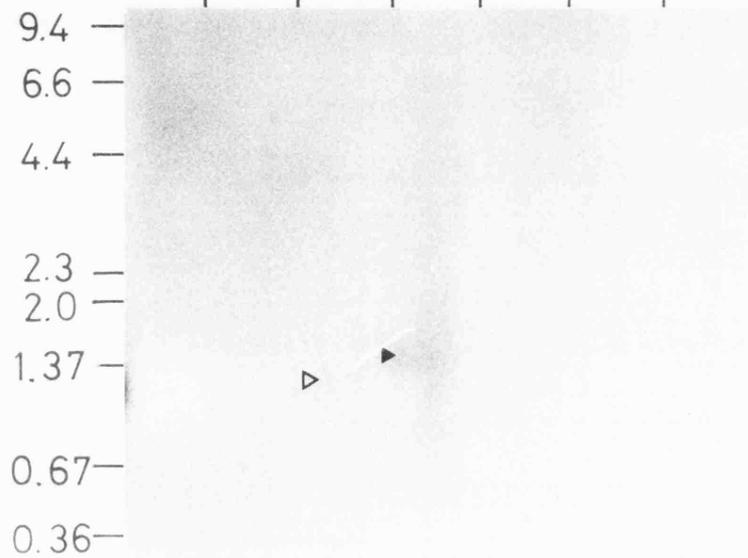
in total, but not polyadenylated, RNA from kidney. This fragment may also

be present in the hybridisation with the human myoglobin exon 2 probe

(Figure 4.6A). The human myoglobin exon 2 probe, in contrast, gives a

smear in all tracks in which a few more prominently hybridising species

appear. Particularly predominant is an approximately 2000nt component in

all tracks.

Southern blot analysis of human, mouse and chinese hamster DNAs with

a 0.8kb human exon 2 probe and also of human DNA with a seal exon 2 probe

detected a complex smear of fragments. These could be simplified to a

single component corresponding to the myoglobin gene by increasing the

post hybridisation wash stringency (Jeffreys et al., 1984; Weller et al.,

1984). Both probes contained some intron sequence in addition to coding

sequence. In contrast, Southern blot analysis of human, seal, rat and

mouse DNAs with a human myoglobin exon 2 probe containing no non-coding

sequence detected a single fragment corresponding to the myoglobin gene

under both 'Northern' (Figure 4.4) and 'Southern" (not shown)

hybridisation conditions. These results suggest that the complex

hybridisation pattern seen with exon 2 probes containing non-coding

sequence can be attributed to this non-coding sequence.

Hybridisation of a pure exon 2 probe to multiple components of

non-muscle RNAs was therefore an unexpected result. In addition, neither

a pure coding sequence exon 2 probe, nor a probe containing 42bp of intron

2 sequence, detected any additional components in any muscle RNAs (eg.

Figure 4.3). In view of these results, it is unlikely that there is

expression of DNA sequences genuinely related to the second exon of the

myoglobin gene specifically in non-muscle mouse tissues.

4.8 Summary

Myoglobin gene probes have been used in Northern blot analysis to

detect myoglobin mRNAs in human and mouse muscle RNAs. The two myoglobin

mRNAs migrate at approximately 1400 and 1200nt respectively, consistent with predicted mRNA sizes from sequence data from the cloned genes (Chapter 3; Blanchetot et al., in preparation). No myoglobin mRNA precursors were ever detected.

Northern blot analysis shows that myoglobin mRNAs are present at approximately equal concentration in human skeletal and cardiac muscles. It has been established that the myoglobin gene is indeed expressed in smooth muscle. Mouse skeletal muscle, which has low myoglobin protein levels, surprisingly has a myoglobin mRNA level similar to that of human skeletal muscle, and a level four times this in cardiac muscle, suggesting that there may be a reduction in the translational capacity of myoglobin mRNA in the mouse.

Hybridisation of mouse non-muscle tissue RNAs with myoglobin gene exon probes shows that these tissues do not contain the authentic myoglobin transcript, but might possibly contain RNA sequences related to the central exon of the myoglobin gene.

Chapter 5

EXPRESSION OF MYOGLOBIN DURING MUSCLE DEVELOPMENT

5.1 Introduction

Transcription of a large number of genes, including the contractile

protein genes, occurs very early in myogenesis following myoblast fusion,

which occurs after only 5-6 weeks gestation in man. In contrast, the

muscle-specific protein myoglobin has not been detected until later in

foetal development; myoglobin cannot be detected in aqueous protein

extracts of foetal skeletal muscle of 24 weeks by SDS-polyacrylamide gel

electrophoresis and staining with Coomassie brilliant blue (Tipler et al.,

1978). However, there are some reports of myoglobin detected by

spectrophotometric techniques at very low levels in early foetal skeletal

muscle (Kagen and Christian, 1966; Longo et al., 1973). The myoglobin

gene is therefore appears to be different from the contractile protein

genes, which are generally expressed at a high level following myoblast

fusion. Moreover, the developmental profile of myoglobin gene expression

is not the same in cardiac and skeletal muscles, as myoglobin is observed

to appear earlier and at higher concentrations in the foetal heart than in

skeletal muscle (Tipler et al., 1978).

This chapter describes experiments designed to determine how early,

and at what level, the myoglobin gene is expressed during development, and

also to compare the developmental expression of the myoglobin gene with

that of the contractile proteins actin and myosin light chain. Because of

the difficulty in obtaining late foetal, neonatal and juvenile human

muscle samples, a full developmental profile of skeletal muscle myoglobin

expression has been constructed only for the mouse. As in chapter 4, the

myoglobin mRNA pool levels were measured by Northern blot analysis. In

addition myoglobin protein concentrations in some of these tissues have

been determined by Western blotting and the results are presented

elsewhere (Weller et al., in preparation; see Chapter 9).

5.2   Myoglobin mRNA levels in human foetal skeletal muscle

Poly(A)$^+$ RNA samples were prepared as described in section 2.5.5

from human foetal and adult skeletal muscle. Five different poly(A)$^+$ RNAs

ranging from 10 to 20 weeks gestation and two adult poly(A)$^+$ RNAs were

analysed by Northern blotting. Filters were hybridised with the probe

from the 3' non-translated region of the human myoglobin gene, H3' (Figure

4.2). Because RNA from abortus material might have been degraded, a probe

from the 3' non-translated region of the myoglobin gene, near to the

poly(A) addition site, was used. In this way even very severely degraded

myoglobin transcripts would be detected by hybridisation, whereas if a

probe from the 5' end of the gene were used, homologous sequences in the

degraded RNA would be likely to be lost in oligo(dT)cellulose

chromatography (Figure 5.1A). Filters were then rehybridised with a mouse

skeletal muscle α-actin cDNA probe (Figure 5.1B) and with a human myosin

light chain cDNA probe (Figure 5.1C).

Myoglobin mRNA with the same mobility as in adult muscle is found at

low levels in poly(A)$^+$ RNA from all five foetal skeletal muscle samples.

As in adult muscle, there is no evidence for any precursors of myoglobin

mRNA. In contrast to the low concentration of myoglobin mRNA in foetal

skeletal muscle, the levels of muscle α-actin and myosin light chain 1 and

3 mRNAs do not differ significantly between foetal and adult skeletal

muscle. This experiment therefore shows that myoglobin is expressed in

human skeletal muscle as early as 10 weeks gestation, but the level of

expression at this early stage is very low, unlike that of that of the

contractile protein genes.

In order to determine the concentration of myoglobin mRNA in foetal

skeletal muscle, relative to that in adult muscle, Northern blots of

Figure 5.1   Expression of muscle-specific genes in human foetal and skeletal muscle

1 µg of each of five foetal skeletal muscle poly(A)$^+$ RNAs from foetuses of 10, 10-14, 14-16, 16 and 20 weeks gestation and 1 µg of each of two adult skeletal muscle poly(A)$^+$ RNAs (A1 and A2) were denatured, electrophoresed and transferred to nitrocellulose as described (2.5.6). Filters were hybridised with three different probes in succession and between each hybridisation were washed in water at 42°C and/or stored until virtually all ^{32}P had decayed to eliminate traces of the previous probe. All three probes were labelled with ^{32}P by nick translation.

A.   Human myoglobin gene 3' non-translated region probe, H3' (Figure 4.2). The exposure was for 4 days without an intensifier screen.

B.   Mouse skeletal muscle α-actin cDNA probe. The mouse muscle α-actin probe was isolated as a PstI fragment from pAM91, which contains a 1350bp muscle α-actin cDNA insert consisting of approximately 90% of the coding sequence plus approximately 300nt of 3' non-translated region (Minty et al., 1981). The migration positions of β and γ cytoplasmic actin mRNAs, approx 2000nt (open triangle) and muscle actin mRNAs, approx 1600nt (filled triangle) are indicated. Exposure was for 48 hours without an intensifier screen.

C.   Human myosin   light chain (MLC) probe. The myosin light chain probe was a ~600bp PstI fragment from a MLC cDNA cloned in pBR322. Separate migration of MLC_1 and MLC_3 cannot be distinguished on this blot. The appearance of an additional band above MLC mRNA is the remainder of the actin cDNA probe from the previous hybridisation which failed to wash completely off the filters. Exposure was for 5 days without an intensifier screen.
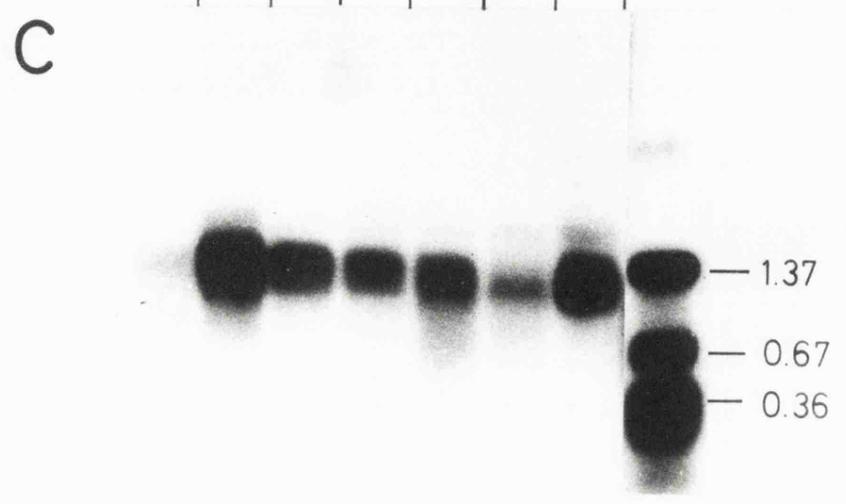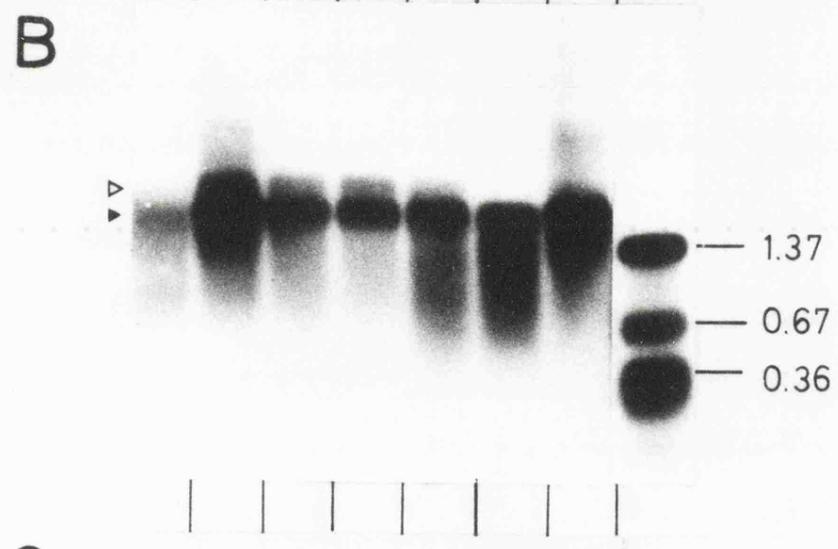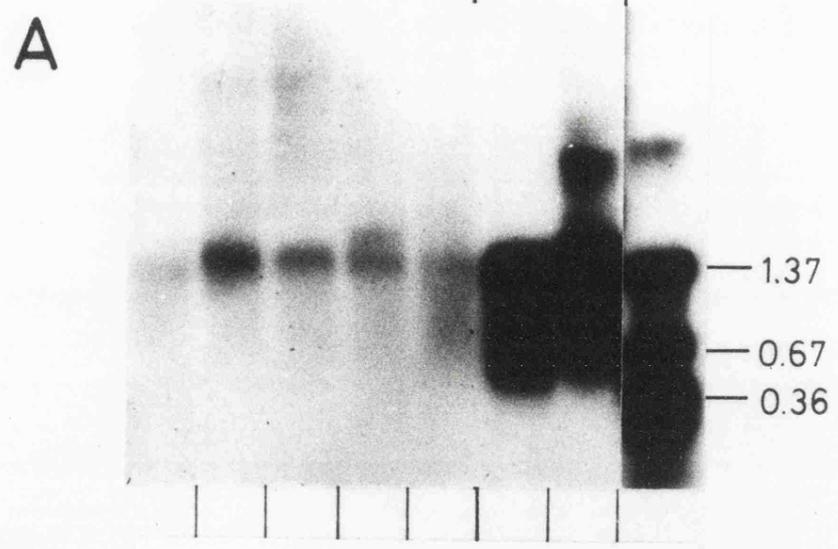
foetal muscle poly(A)$^+$ RNA were compared with those of serial dilutions of adult poly(A)$^+$ RNA and quantified by scanning densitometry of autoradiographs (Figure 5.2). An alternative approach; measuring relative myoglobin mRNA pool levels by spot hybridisations proved to be inaccurate as the concentration of myoglobin mRNA in foetal muscle RNA samples was low and background hybridisation, which could be avoided on Northern blots, was high (not shown). From scanning data, the level of myoglobin mRNA in the foetal RNA samples was calculated to be about 2.3% of that in the adult samples; range 0.6–5.4%, due to variation between foetal samples (Figure 5.2). There is therefore a difference of approximately 40–fold in concentrations of myoglobin mRNA between adult and foetal skeletal muscle, although there is considerable variation between samples.

5.3   Myoglobin mRNA levels in human foetal cardiac muscle

Myoglobin concentrations have been observed to be higher in foetal cardiac muscle than in foetal skeletal muscle (Longo et al., 1973; Tipler et al., 1978). To test whether the myoglobin mRNA concentration is also elevated in foetal cardiac muscle, Northern blot and spot hybridisations of poly(A)$^+$ RNAs from these tissues (both 20 weeks gestation) were carried out. Both the Northern blot filter and the spot filter were hybridised with the 316nt single–stranded human exon 2 antisense probe generated from M13.HEX2 by primer extension (Figure 5.3).

Unfortunately, the cardiac RNA is partially degraded and the hybridisation signal is rather weak. However, it appears that at 20 weeks gestation there is indeed a significantly higher concentration of myoglobin mRNA in foetal heart than in skeletal muscle. From the spot hybridisations this difference appears to be approximately 8–fold. As the myoglobin mRNA level in foetal skeletal muscle is about 2.3% of the corresponding adult level, the level in foetal heart is therefore

Figure 5.2   Determination of the relative concentration of myoglobin mRNA
in human foetal skeletal muscle

1μg of each of the five human foetal skeletal muscle poly(A)$^+$ RNAs
spanning 10 to 20 weeks gestation and dilutions of two adult samples
containing 0.2, 0.1, 0.05 and 0.025μg poly(A)$^+$ RNA (a-d) were transferred
to nitrocellulose by Northern blotting (2.5.6). The filter was hybridised
with the human myoglobin gene 3' non-translated region probe, H3' (Figure
4.2), [^{32}P]labelled by nick translation. Signal intensity in all tracks
was measured using a LKB 2202 ultroscan laser densitometer with 2220
recording integrator.

The means and standard deviations for all foetal and adult samples
(corrected for the amount of RNA present) were determined and the mean
level of myoglobin mRNA in foetal RNA was calculated with respect to the
adult level. The results of this show that the mean level of myoglobin
mRNA present in foetal skeletal muscle poly(A)$^+$ RNA is 2.3% of the adult
level (minimum 0.6%, maximum 5.4%) The large difference between minimum
and maximum estimates is due mainly to variation between the foetal
samples.
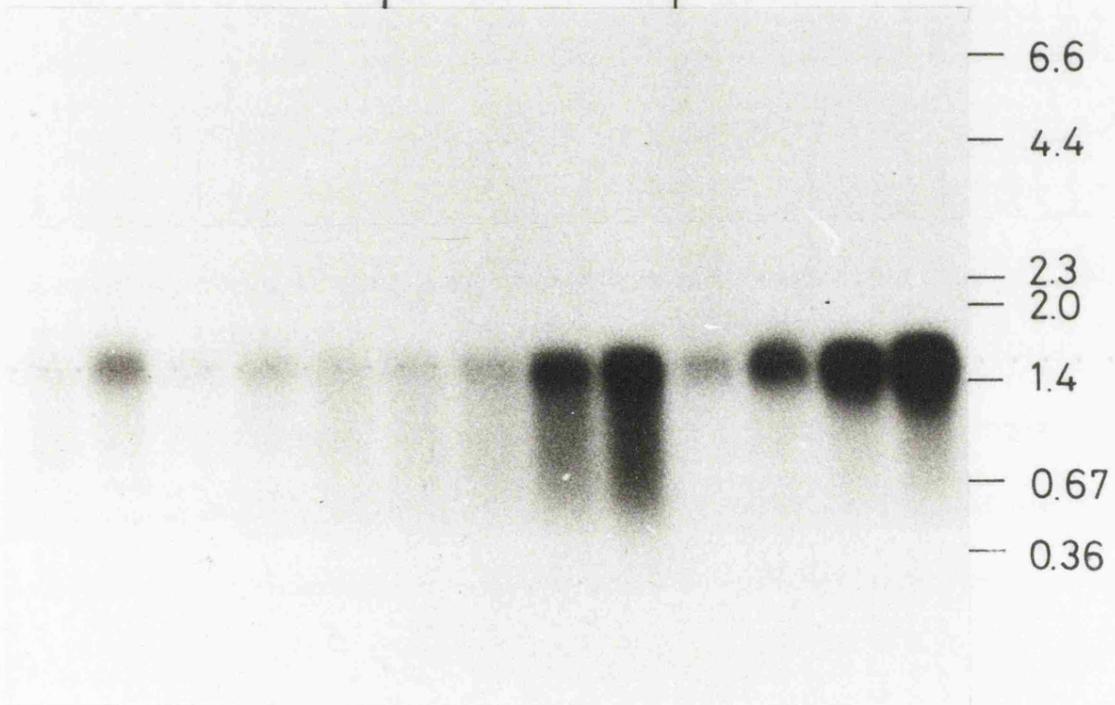
Figure 5.3   Myoglobin mRNA concentrations in human foetal cardiac muscle

0.5μg and 1.0μg (a and b) of each of foetal skeletal (sk) and foetal cardiac (car) muscle poly(A)$^+$ RNAs (both 20 weeks gestation) were denatured, electrophoresed and transferred to nitrocellulose. A spot hybridisation filter was also prepared from a 1 in 2 dilution series of the same RNAs, with 0.33μg in the first spot (see section 2.6.1). Both filters were hybridised with the 316nt single-stranded human myoglobin exon 2 probe (Figure 4.2).

A.   Northern blot hybridisation. Exposure for 5 days without an intensifier screen. The cardiac RNA is partially degraded.

B.   Spot hybridisation. Exposure for 7 days without an intensifier screen. The signals with spots containing 0.33μg foetal skeletal muscle mRNA and 0.04μg foetal cardiac RNA are similar.
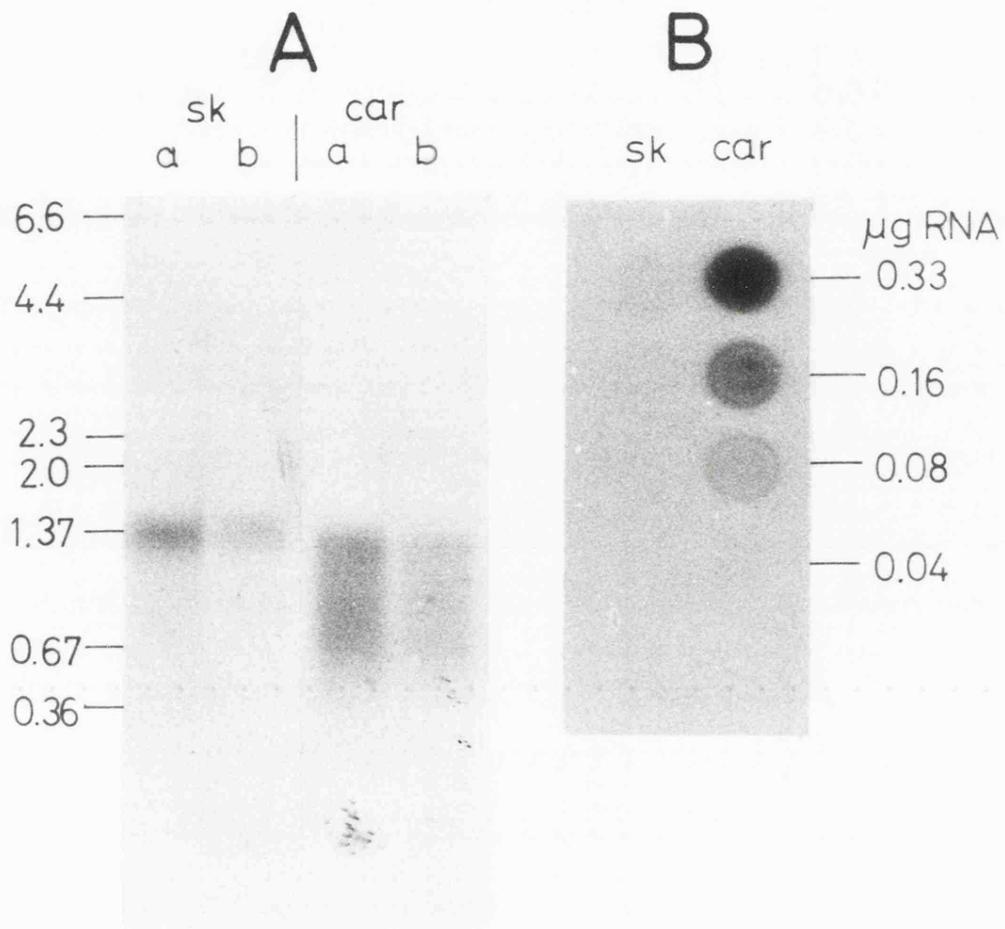
approximately 18% of the adult skeletal muscle level. Lack of RNA

unfortunately did not allow this quantitation to be checked further.

5.4   Changes in levels of myoglobin mRNA during skeletal muscle

development in the mouse

Northern blot analysis described in section 5.2 showed that there

was no consistent change in myoglobin mRNA concentration with increasing

gestational age over the limited period of 10-20 weeks gestation. In

order to find out at which stage in skeletal muscle development expression

of the myoglobin gene increases it was decided to look at developmental

expression of the myoglobin gene in the mouse.

Skeletal muscle tissue was obtained from outbred mice ranging in age

from 15 days gestation to adult. Muscle was separated as far as possible

from non-muscle tissue, though this proved to be difficult with early

embryos. The heart was excluded in all cases. Tissue was frozen directly

in liquid nitrogen and stored at -80°C. Poly(A)$^+$ RNA was prepared from

muscle from each developmental stage. Northern blots of these RNAs were

hybridised with myoglobin, skeletal α-actin and myosin light chain (MLC)

probes in succession (Figure 5.4).

Figure 5.4A shows the levels of myoglobin mRNA throughout skeletal

muscle development. The myoglobin message is just detectable at 15 days

gestation. At this stage, which is equivalent to 7 weeks gestational age

in man, the embryos are 12mm long and the skeletal muscles have just

become contractile (Rugh, 1968). There is a steady increase in myoglobin

mRNA concentration with increasing age, during both pre- and post-natal

development. There appears to be of the order of 5% of the adult level at

birth and much less, probably less than 1% at 15 days gestation. These

estimates are approximate and are based only on this Northern blot

hybridisation.

For comparison, Figure 5.4B and C show the same filter hybridised

Figure 5.4   Myoglobin, skeletal muscle actin and myosin light chain mRNA
levels in developing skeletal muscle in the mouse

2μg of poly(A)$^+$ RNA from skeletal muscle samples ranging from 15
days gestation to adult (ad) and from G8 myotubes (G8 MT) were denatured,
electrophoresed and transferred to nitrocellulose. The filter was
hybridised with three probes in succession. Filters were washed in
0.25xSSC (Human probes) or 0.1xSSC (mouse probe) at 50°C. Exposures to
X-ray film were without an intensifier screen. The signal from RNA from
19 days gestation is low for all three probes and may represent an error
in RNA estimation.

A.   Human myoglobin 316nt single-stranded antisense exon 2 probe
generated from M13.HEX2. Exposure was for 14 days.

B.   Mouse skeletal muscle α-actin cDNA probe (see Figure 5.1) labelled
with ^{32}P by nick translation. Exposure was overnight. The migration
positions of β and γ cytoplasmic actins ~2000 nt (open triangle) and
muscle actin ~1600 nt (filled triangle) are indicated.

C.   Human myosin light chain (MLC) cDNA probe (see Figure 5.1), labelled
by nick translation. Exposure was for 4 days. The migration positions of
MLC_1 and MLC_3 (~1050 and ~900nt) are indicated with open and filled
triangles, respectively.
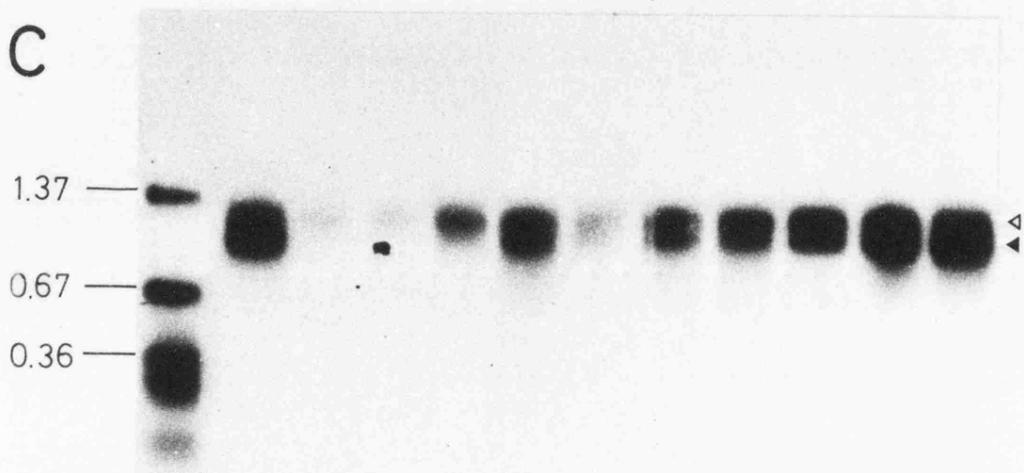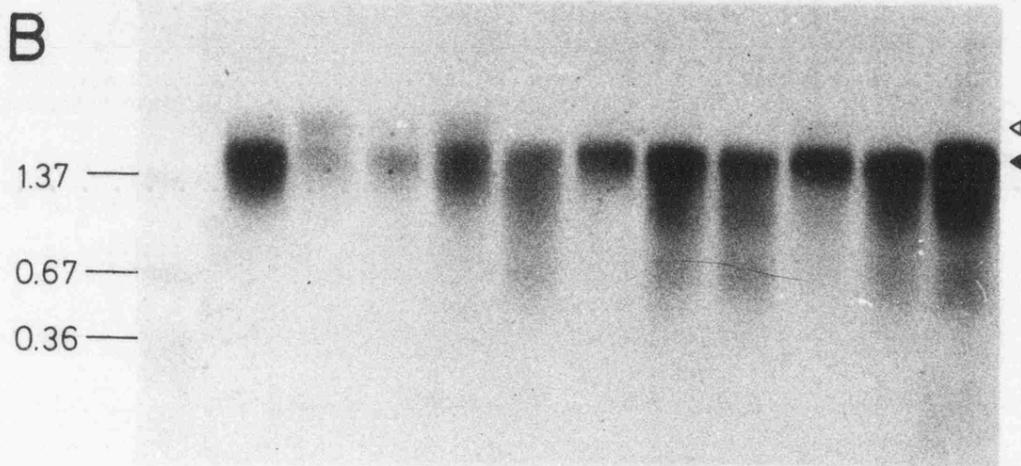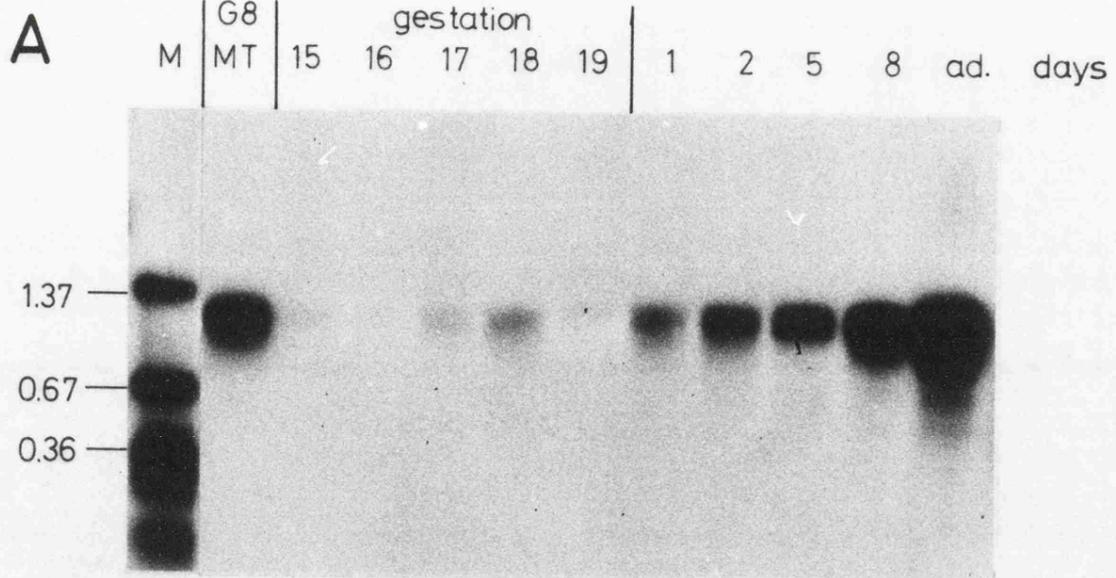
with mouse skeletal muscle α-actin and human myosin light chain (MLC) probes. Levels of muscle α-actin and MLC mRNAs vary less during development than myoglobin mRNA, although there appears to be a small increase in mRNA levels with age. This is probably due in part to the difficulty in obtaining embryo muscle samples uncontaminated with non-muscle tissue. The cytoplasmic actin (β and γ) mRNAs are most prominent in embryonic muscle, up to 18 days gestation. Also, over this early developmental period there appears to be a larger amount of MLC_1 than MLC_3 mRNA, though the levels are apparently equivalent in later development stages and in adult. The main conclusion from these results is that there is a large, gradual increase in levels of myoglobin mRNA between embryo and adult, apparently spanning the whole period between early myogenesis and the time of completion of fibre differentiation. This gradual but dramatic increase is not seen with the contractile protein genes.

5.5 Expression of the myoglobin gene in embryonic myoblasts following fusion and differentiation in vitro

The finding that the myoglobin gene is expressed in embryogenesis as early as 10 weeks gestation in man and 15 days in mouse shows that the myoglobin gene is indeed expressed very early in development, and suggests that it might also be expressed in differentiating myoblast cell lines as are the contractile protein genes (see Pearson, 1980). Poly(A)$^+$ RNA from pre- and post-fusion cultures of myogenic cell lines L6, rat (Yaffe, 1968) and G8, mouse (Christian et al., 1977) were therefore analysed by Northern blot hybridisation with myoglobin gene probes.

The filter was hybridised with the human myoglobin exon 2 BstEII-RsaI fragment (Figure 5.5A). A hybridising component with the same mobility as mouse myoglobin mRNA can be seen in tracks containing myotube RNA from both L6 and G8. This component is absent from myoblast RNA, even

Figure 5.5   Expression of myoglobin mRNA in embryonic myoblasts following
fusion and differentiation in culture

      Two identical Northern blot filters were prepared: 2µg of poly(A)$^+$
RNA from L6, rat (Yaffe et al., 1968) and G8, mouse (Christian et al.,
1977) myoblasts (MB) and myotubes (MT) were denatured, electrophoresed and
transferred to nitrocellulose by Northern blotting and hybridised with:

A.   Human exon 2 BstEII-RsaI fragment (HE2) [^{32}P]labelled by nick
translation. 14 day exposure.

B.   Mouse myoglobin single-stranded antisense probe generated from
M13.MEX1 (see Figure 4.2). Exposure was for 7 days.

C.   Mouse skeletal muscle α-actin cDNA probe. Exposure was for 2 days.
(Cytoplasmic, β and γ (~2000nt) and muscle α-actin (~1600nt) mRNAs are
indicated by open and filled triangles, respectively).

D.   Human myosin light chain (MLC) cDNA probe. Exposure for 4 days.
The migration position of MLC mRNA is indicated. Other bands on this
autoradiograph are remnants of the actin probe hybridisation in C, which
failed to wash completely off the filters.
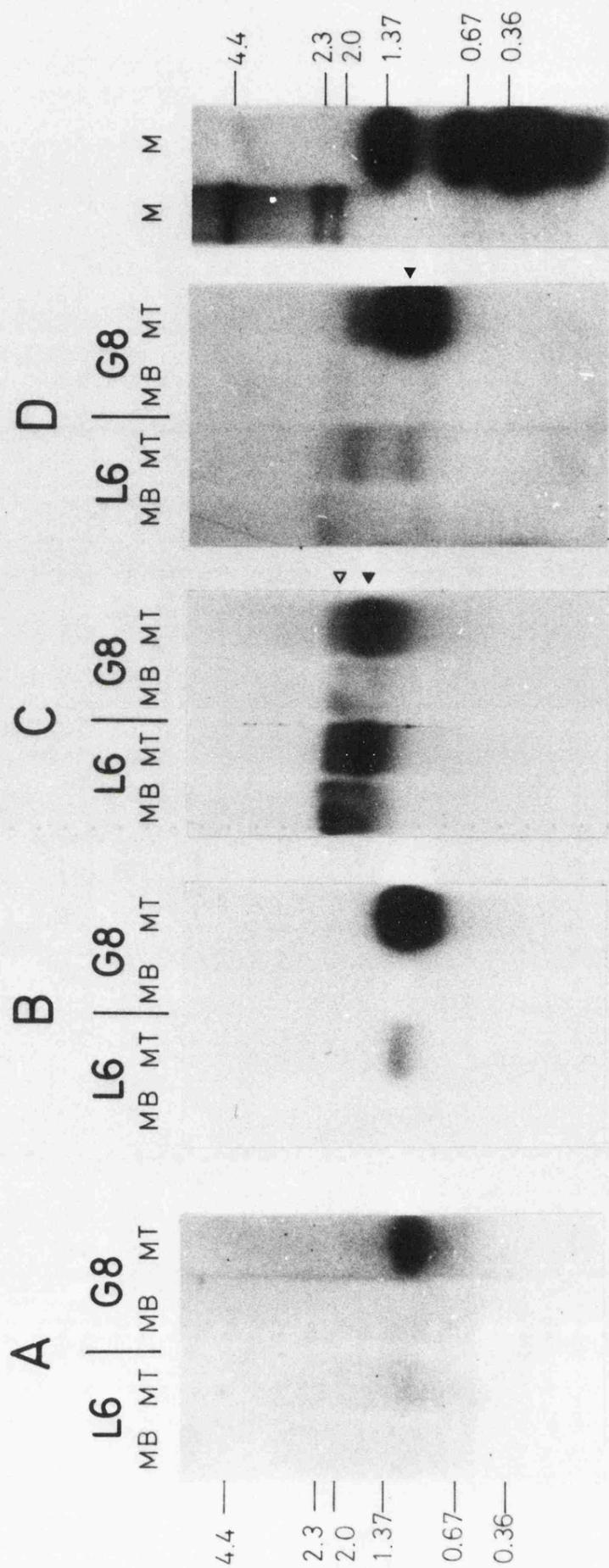
on long exposure autoradiographs (not shown). In order to confirm that this hybridising component was indeed myoglobin mRNA, a second identical filter was hybridised with the mouse myoglobin exon 1 single-stranded antisense probe (see Figure 4.2), generated from M13.MEX1 (Figure 5.5B). The pattern of hybridisation is exactly the same as with the human myoglobin gene probe, thus confirming that myoglobin mRNA is present in differentiated cells from these cell lines.

L6 is a line which has been used extensively for work on muscle gene expression in myogenesis. G8, however, is less well known. The contractile protein genes have long been known to be expressed following fusion and differentiation of myogenic cell lines in vitro (see Buckingham, 1977). The induction of transcription of contractile protein genes in this particular cell fusion was tested by rehybridising one of the filters with the mouse skeletal muscle α-actin cDNA probe, and then with the human MLC cDNA probe [Figure 5.5C (actin) and 5.5D (MLC)]. Cytoplasmic (β and γ) actin mRNA detected by this probe is present in both myoblast and myotube poly(A)$^+$ RNAs. Muscle α-actin mRNA, however, is present at a high level in myotubes only, of both L6 and G8. MLC mRNA is also present only in the myotube RNAs. The apparent low level of MLC mRNA in L6 myotube RNA is due to poor cross-hybridisation between the probe and the main MLC isoform expressed in L6 myotubes, which is the embryonic isoform (Buckingham et al., 1982). Thus genes for muscle isoforms of actin and myosin are also induced following fusion and differentiation of L6 and G8 myoblasts, the same myoblasts in which the myoglobin gene is also induced. These observations are therefore indicative of "normal" myogenic differentiation in these cell lines.

The level of expression of the myoglobin gene in myotubes was compared with that in embryonic muscle (Figure 5.4). The level of expression of myoglobin, α-actin and MLC mRNA is higher in G8 myotubes than in the earliest embryonic muscle. However, as mentioned in section

5.4, this is probably due in part to contamination of embryonic muscle samples with non-muscle tissues. Taking this into account, levels of α-actin and MLC mRNAs in G8 myotubes are probably similar to those in developing muscle in vivo, which moreover, do not vary greatly with age (section 5.4). The level of myoglobin mRNA in G8 myotubes, however, is quite high, similar to that in 8 day old mice. This suggests that the size of the myoglobin mRNA pool accumulated in fusing myoblasts in vitro might exceed that seen in early embryonic muscle in vivo. It is important to note that although the apparent level of induction of a gene following myoblast differentiation is likely to vary between different batches of fused cells, myoglobin mRNA has been found to accumulate reproducibly on a number of independent fusions of G8 myoblasts (M.Price, unpublished results).

To test the possibility that induction of expression of the myoglobin gene in embryonic myoblast differentiation might be associated with gene rearrangement or amplification, Southern blot analysis was carried out using G8 myoblast and myotube DNAs, and also mouse (DBA/2) liver DNA, hybridised with the 261nt pure human exon 2 probe generated from M13.HEX2 (Figure 5.6). A single hybridising component corresponding to the myoglobin gene is detected in each digested DNA. There is no variation in the apparent mobilities of the hybridising fragments, nor in the intensity of their hybridisation, between DBA/2 mouse liver, G8 myoblast and G8 myotube DNAs. This indicates that the induction of expression of the mouse myoglobin gene in mouse myoblasts is not accompanied by gene amplification or major rearrangement.


5.6   Summary

Myoglobin mRNA was detected at low levels in human skeletal muscle from as early as 10 weeks gestation. At 20 weeks gestation there is a significantly higher level of myoglobin mRNA in cardiac muscle than in

Figure 5.6   Southern blot analysis of the myoglobin gene in pre- and post-fusion G8 DNAs

5µg aliquots of DNA from G8 myoblasts (MB) and myotubes (MT) and from mouse liver (DBA/2) were digested with HindIII (H) or BamHI (B) and electrophoresed in a 0.5% agarose gel. The DNA was denatured in situ and transferred to nitrocellulose by Southern blotting (2.5.6).

The filter was hybridised with the 261nt human pure exon 2 single-stranded probe generated from M13.HEX2 under RNA (Northern) blot hybridisation conditions and were washed in 0.25xSSC at 50°C before autoradiography. Exposure was for 7 days without an intensifier screen.
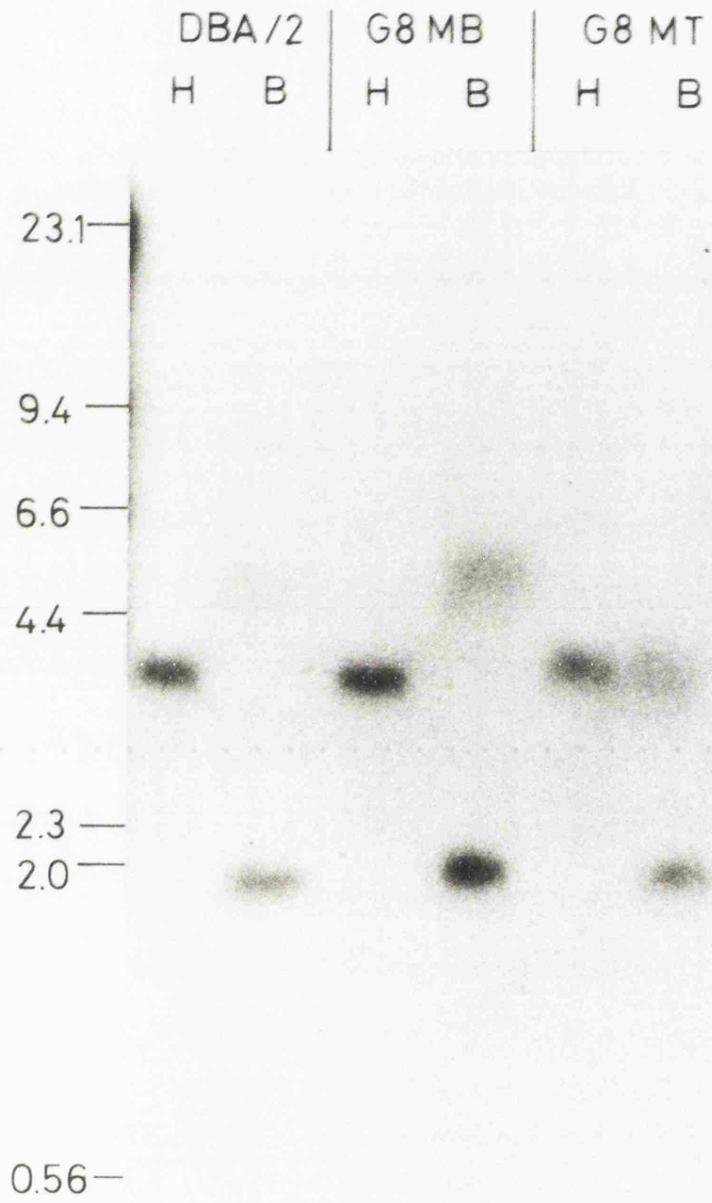
skeletal, correlated with higher levels of myoglobin (Tipler et al., 1978). The concentration of myoglobin mRNA in 10–20 week foetal skeletal muscle was estimated at ~2.3% of that in adult skeletal muscle by titration of RNA in Northern blot hybridisations. In contrast, levels of α-actin and myosin light chain mRNAs in foetal skeletal muscle were not significantly different from those in adult.

The increase in levels of myoglobin mRNA during development was analysed in the mouse. Myoglobin message was detected at very low levels at 15 days gestation and the level was found to increase steadily approximately 50 to 100–fold during both pre– and post–natal development. In contrast, levels of α-actin and myosin light chain mRNAs do not show this dramatic increase during development.

The possible induction of expression of the myoglobin gene in differentiating embryonic skeletal muscle myoblast cell lines was investigated. Myoglobin gene expression was indeed found to be induced, (as was expression of muscle actin and myosin light chain genes) on fusion and differentiation of two such cell lines; a mouse line, G8 and, to a lesser extent, a rat line, L6. The level of myoglobin mRNA in G8 myotubes was significantly higher than the level in embryonic skeletal muscle. Southern blot hybridisation showed that the induction of expression of the mouse myoglobin gene in G8 myotubes is not accompanied by gene amplification, nor major DNA rearrangement. The myoglobin gene is therefore expressed at the myoblast fusion stage of myogenesis, much earlier than previously thought and can be modelled by fusion of embryonic myoblast cell lines in vitro.

Chapter 6

ADAPTATION IN THE EXPRESSION OF THE MYOGLOBIN GENE IN THE GREY SEAL

6.1   Introduction

    Skeletal muscles of many diving mammals have greatly elevated levels
of myoglobin when compared with terrestrial species (see Wittenberg, 1970
and also Chapter 1). Myoglobin expression therefore provides an
attractive system for studying at the DNA level the evolutionary changes
which are associated with physiological adaptation, in this case elevated
levels of myoglobin. There are several possible levels at which this
adaptation could occur, including gene amplification, changes in rates of
gene transcription, changes in mRNA stability and translational control or
changes in regulatory loci or factors affecting myoglobin gene expression.
The adaptation may have resulted from one or a few changes, or may be the
result of a larger number of individual cumulative adaptations. However,
before such a study can be undertaken, it is first necessary to describe
the basis of elevated myoglobin levels in a diving mammal in vivo, about
which nothing is yet known.

    Although there are data available for myoglobin levels in some
Phocinae species, there is none for the grey seal, from which species the
myoglobin gene has been cloned and characterised. This chapter therefore
deals first with quantifying the myoglobin level in grey seal and human
skeletal muscle by isoelectric focusing and absorption spectroscopy. It
then goes on to compare myoglobin mRNA levels in seal and human muscles
and to quantify the relative translational capacities of these two
myoglobin mRNAs in rabbit reticulocyte lysates.

6.2 Measurement of myoglobin concentrations in grey seal and human skeletal muscle

Myoglobin concentrations have been measured in a number of ways, including spectrophotometrically (e.g. Weber et al., 1974) and immunologically (e.g. Kagen and Christian, 1966) and with varying degrees of pre-purification and precaution. However, only an approximate estimate was required here, as the difference between human and seal myoglobin concentrations was more important than their absolute values.

Myoglobin had previously been partially purified from homogenates of grey seal skeletal muscle by isoelectric focusing (Wood et al., 1982). This simple method yielded myoglobin judged greater than 90% pure by SDS-polyacrylamide gel electrophoresis and staining with Coomassie brilliant blue. It was therefore decided to make estimates of concentration by spectrophotometric analysis of myoglobin partially purified in this way.

Extracts from equal masses of human and seal skeletal muscle were partially purified by isoelectric focusing (Figure 6.1). Myoglobin is resolved into two components; red, oxygenated myoglobin (MbO_2) and brown metmyoglobin (metMb). In both seal and human extracts, myoglobin is by far the most abundant haemoprotein. It is interesting that there is significantly more non-myoglobin haemoprotein in seal extracts than human. Myoglobin levels have been correlated with cytochrome oxidase activity (Lawrie 1953a). Isoelectric focusing eliminates most additional proteins so that myoglobin purified in this way is >90% pure, as judged by SDS-polyacrylamide gel electrophoresis (Figure 6.1B). This myoglobin was used as a marker in subsequent gel electrophoresis.

Monitoring of myoglobin by absorption spectroscopy (2.7.1) during its purification by isoelectric focusing showed that there was a progressive oxidation of MbO_2 to metMb (see Figure 6.2) and also a

Figure 6.1   Partial purification of myoglobin by isoelectric focusing

A.   Soluble protein extracts from human (H), grey seal (S) and fin whale
(W) skeletal muscles were loaded onto a pre-prepared polyacrylamide
isoelectric focusing gel and focused at 1,500V, 50mA for about an hour
until the coloured proteins were resolved (2.7.1) The amount of extract
loaded is from an equivalent mass of tissue from each species. Oxygenated
myoglobin (red) and metmyoglobin (brown) are indicated by filled and open
triangles, respectively. Human haemoglobin (Hb) was also focused as a
marker.

B.   Total soluble protein extract from seal muscle and oxygenated
myoglobin, partially purified by isoelectric focusing, was electrophoresed
in an 18% SDS-polyacrylamide gel which was then stained with Coomassie
brilliant blue. The migration position of myoglobin is indicated.
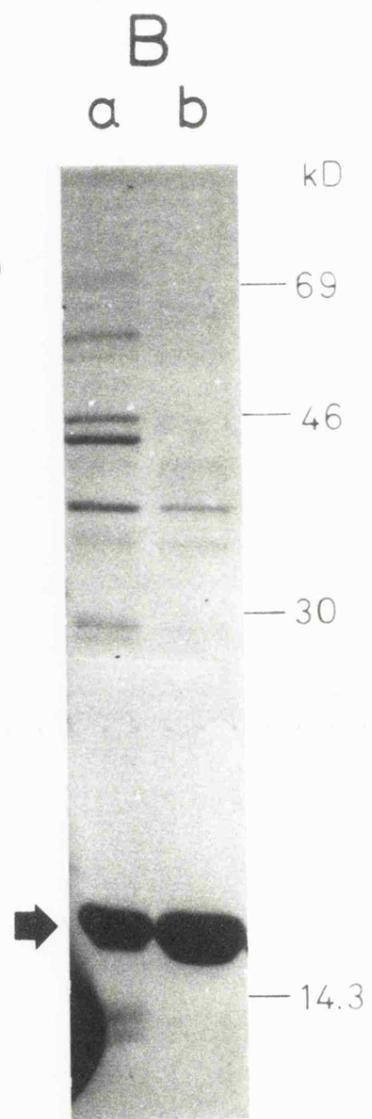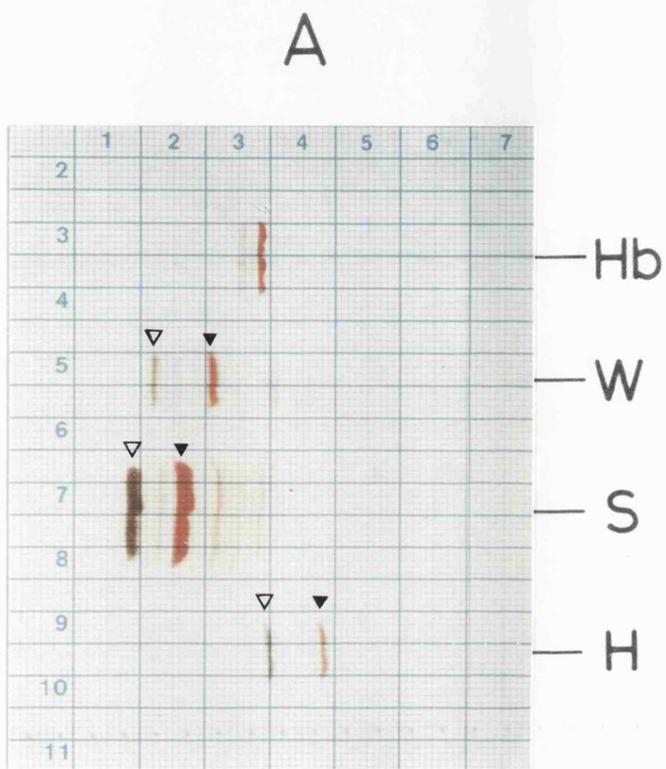
considerable loss of myoglobin, particularly from human muscle extracts. At the start of the purification procedure, however, virtually all myoglobin was in the oxygenated form in both seal and human extracts (Figure 6.2).

Consequently, the relative concentration of myoglobin in seal and human skeletal muscle was estimated from the absorbance at 543nm of fresh muscle homogenates from which cell debris had been removed by centrifugation. Using data from two experiments, it was found that seal muscle contained six to seven times as much myoglobin as human.

6.3 The absolute concentration of myoglobin in human and grey seal skeletal muscle

The concentration of myoglobin in skeletal muscle extracts can be calculated using the published extinction coefficient values for myoglobin. In fresh human and seal muscle extracts virtually all myoglobin present is in the oxygenated form, and there is relatively little contribution to the absorption by non-myoglobin haemoproteins (see above). Using a value of ε at 543 nm for oxygenated myoglobin at pH 7 equal to 1.36×10^4 (Antonini and Brunori, 1971) and the molecular weight of myoglobin as 17,000, myoglobin concentrations in two separate experiments were estimated to be 47 and 52 mg myoglobin/g wet weight for seal muscle and 7.1 and 8.2 mg/g for human muscle. Although these estimates are only approximate, the figures for the concentration of myoglobin in human muscle agree well with the estimate of 7.5 mg myoglobin/g wet weight, made by Western blotting (Weller et al., in preparation). In addition, this also confirms that the grey seal has high levels of myoglobin, similar to those in other members of the family Phocinae (see Chapter 9).

Figure 6.2 <u>Absorption spectra of myoglobin</u>

     Spectra were measured as described in section 2.7.3 and were found to be virtually identical for seal and human extracts.

A.   Absorption spectrum of whole muscle extract from which cell debris had been removed by centrifugation.

B,C. Absorption spectra of oxygenated myoglobin (B) and metmyoglobin (C), purified by isoelectric focusing of concentrated extracts.
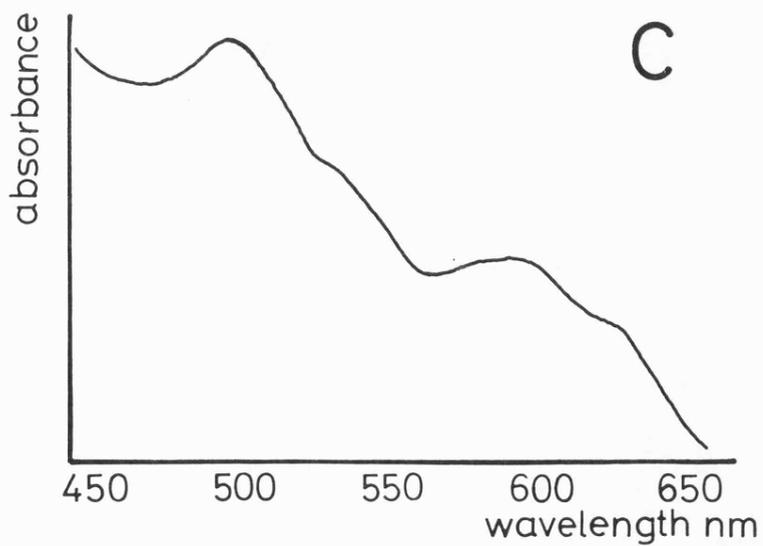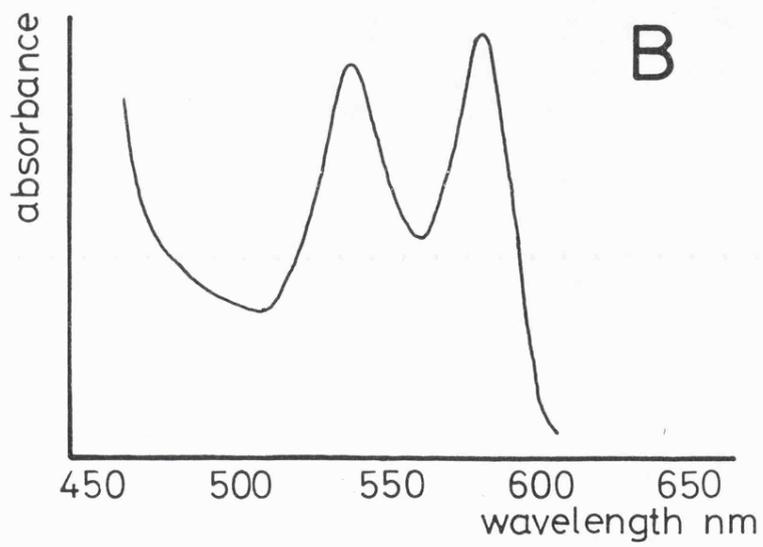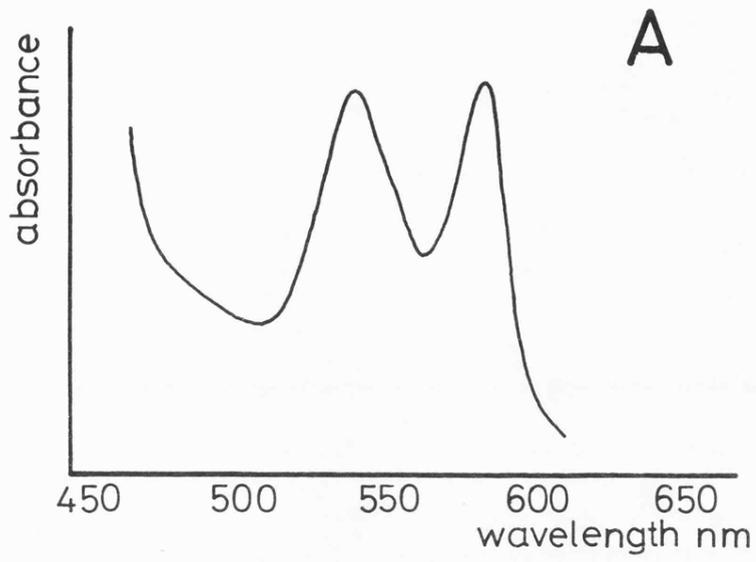
6.4 Measurement of myoglobin mRNA levels in seal and human muscle

To determine whether the high level of myoglobin in seal skeletal muscle is associated with a parallel elevation in the level of myoglobin mRNA, the relative concentrations of myoglobin mRNA (in the same human and seal muscle samples used for myoglobin protein measurements) were determined using Northern blot analysis and spot hybridisations.

Poly(A)$^+$ RNA was prepared from human and seal muscle (see section 4.2). Four identical Northern blot filters were prepared and each was hybridised with a different myoglobin probe (seal exons 2 and 3 and human exon 2 and 3) (Figure 6.3A-D). The seal myoglobin mRNA is indistinguishable in length from the human myoglobin message, as predicted from sequence data (Blanchetot et al., 1983; Chapter 3). All four probes show that there is considerably more myoglobin mRNA in seal muscle than in human, though the apparent difference in levels depends greatly on the probe.

An accurate estimate of the relative concentrations of myoglobin mRNA in seal and human muscle requires a probe which has equal homology to the two mRNAs. Such a probe is not available, so to obtain an estimate of the difference in myoglobin mRNA concentrations unbiased by the probe used, it was decided to use a mixed probe containing equimolar amounts of two equivalent fragments from the 3' non-translated regions of the seal and human myoglobin genes (see Figure 4.2). The 3' non-translated region is not sufficiently conserved between the seal and human genes for such fragments to cross-hybridise (not shown). On Northern blots and in spot hybridisations each component will therefore hybridise only to its corresponding mRNA. Equal labelling of seal and human components in nick translations of the mixed 3' probe was confirmed by making a spot hybridisation filter of dilutions of the unlabelled probe fragments and hybridising this filter at the same time (not shown).

Quantitation of the difference in myoglobin mRNA pool levels in

Figure 6.3   Estimation of relative myoglobin mRNA levels in human and seal skeletal muscle

A-E. Five identical pairs of tracks were electrophoresed with 1µg of human (H) and seal (S) poly(A)$^+$ RNAs. After transfer to nitrocellulose, the filter was cut into five strips, each of which was hybridised with a different probe. The probes were as follows:

A.   S2: Seal exon 2 (nick translated)

B.   S3: Seal exon 3 probe (single-stranded antisense probe generated from M13.SEX3)

C.   H2: Human exon 2 probe (single-stranded 316nt antisense probe generated from M13.HEX2)

D.   H3: Human exon 3 probe (single-stranded antisense probe generated from M13.HEX3)

E.   S3' + H3': Mixed probe containing equimolar amounts of equivalent fragments from human and seal myoglobin gene 3' non-translated regions, [^{32}P]labelled by nick translation.

Hybridisation was under standard conditions and filters were washed in 0.25xSSC at 50°C (except E, washed in 0.1xSSC at 50°C). Autoradiographic exposure, in all cases without an intensifier screen, was either overnight (B,C,D) or for 48 hours (A,E)

F.   Spot hybridisation filter of 1 in 2 dilution series of seal and human poly(A)$^+$ RNAs prepared as described in section 2.6.1, starting with 0.5µg in the first spot. The probe was the mixed 3' probe described above. The filter was washed in 0.25xSSC at 50°C and exposed without an intensifier screen for 3 days.
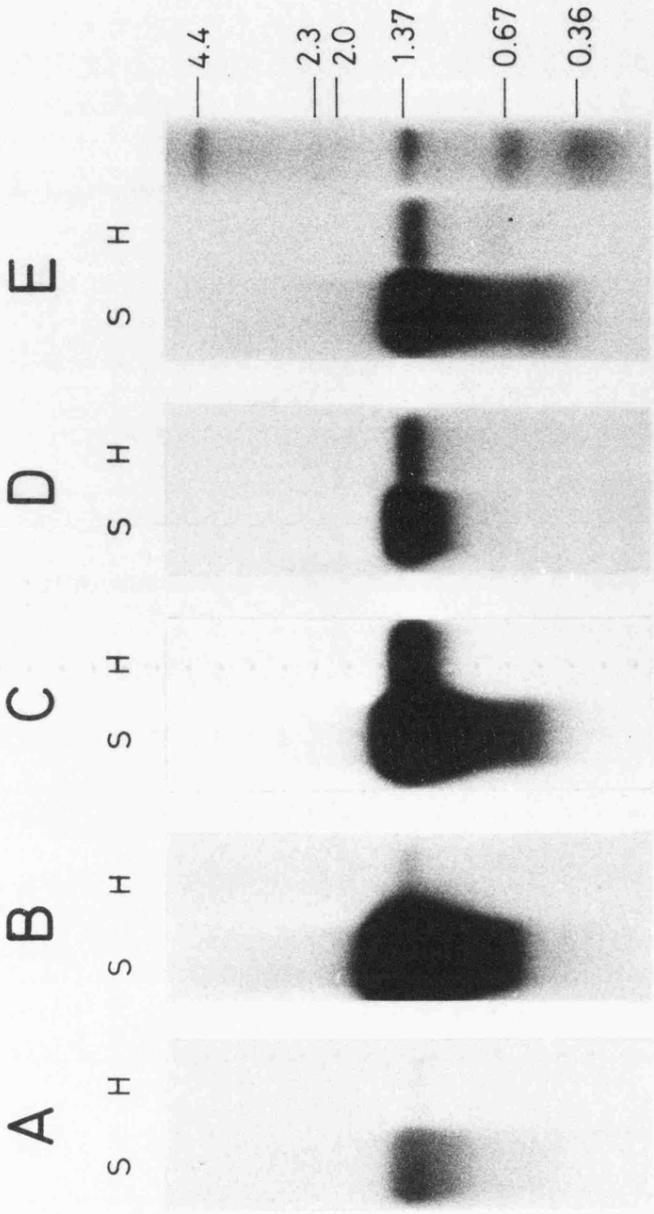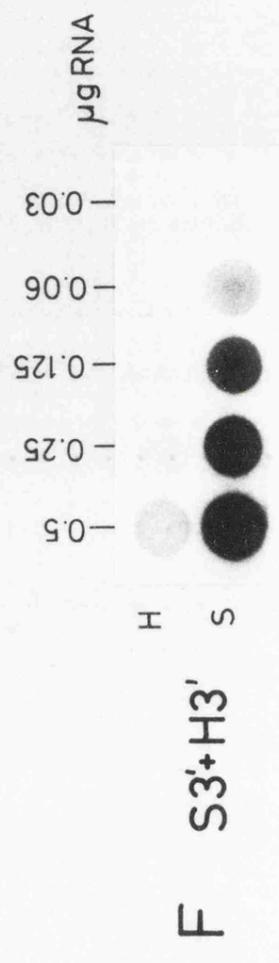
human and seal muscle with the mixed 3' probe shows a difference in myoglobin mRNA levels of approximately 8-fold (Figure 6.3E and F). This correlates with the 6 to 7-fold difference in myoglobin protein concentrations and suggests that the elevated myoglobin level in seal results primarily from a correspondingly elevated level of myoglobin mRNA. However, to confirm that this is so, the efficiency of translation of myoglobin mRNA from seal and human muscle was compared by in vitro translation.

6.5   Translational efficiency of seal and human myoglobin mRNAs

The efficiency of translation of seal and human myoglobin mRNAs was compared by in vitro translation in the rabbit reticulocyte lysate system. 0.5μg of each poly(A)$^+$ RNA was translated as described (2.7.2). Reactions were electrophoresed in an SDS-polyacrylamide gel which was fluorographed (see Figure 6.4A). Myoglobin was identified as a prominent band co-migrating with purified myoglobin in in vitro translations of seal poly(A)$^+$ RNAs, but could not be resolved in translations of human poly(A)$^+$ RNAs. In order to quantitate myoglobin synthesised in this system, it was therefore neccessary to separate the myoglobin from the remainder of the in vitro translation products. Previous work had shown that translation of myoglobin mRNA in this system produces native myoglobin complexed with haem (Wood et al., 1982). Newly translated myoglobin was therefore purified by isoelectric focusing using purified myoglobin as a marker and was then electrophoresed in an SDS-polyacrylamide gel, stained and fluorographed (Figure 6.4B,C).

Approximately equal amounts of synthesised myoglobin copurify with oxygenated myoglobin and metmyoglobin from both seal and human translations. Myoglobin can now clearly be resolved in seal and human in vitro translation products recovered from the isoelectric focusing gel. Direct quantitation of myoglobin synthesis is now possible.

Figure 6.4   Measurement of the efficiency of translation of seal and
human myoglobin mRNAs

3µg of each of seal and human skeletal muscle poly(A)$^+$ RNAs were
translated in in vitro translation reactions of total volume 35µl (2.7.5).
A sixth of each reaction was removed to analyse total protein products and
to measure [^{35}S] methionine incorporation (2.7.4). The remainder of each reaction
was focused on an isoelectric focusing gel with purified oxygenated
myoglobin (MbO$_2$) and metmyoglobins (metMb) from the appropriate species as
markers. Gel slices containing myoglobin produced in in vitro
translations were loaded directly into the slots of an 18%
SDS-polyacrylamide gel, together with input samples of total in vitro
translation products. After electrophoresis, the gel was stained with
Coomassie brilliant blue, photographed and fluorographed (2.7.3).
Mobility markers were a mixture of [^{14}C]methylated polypeptides.

A.   Fluorograph showing total protein products from in vitro
translations described above. The migration position of myoglobin is
indicated by a track containing myoglobin purified by isoelectric focusing
and stained with Coomassie brilliant blue (Mb).

B.   Photograph of Coomassie blue stained gel of proteins recovered from
isoelectric focusing gel with the same pI as human and seal myoglobins.
There is a certain amount of contaminating globin from the lysate present
(open triangle) and a small amount of one or two other unidentified
proteins. The migration position of purified myoglobin is indicated, but
no myoglobin can be detected by this staining method.

C.   Fluorograph of the gel shown in B. The migration position of
myoglobin, by far the most abundant in vitro translation product with this
pI, is indicated.
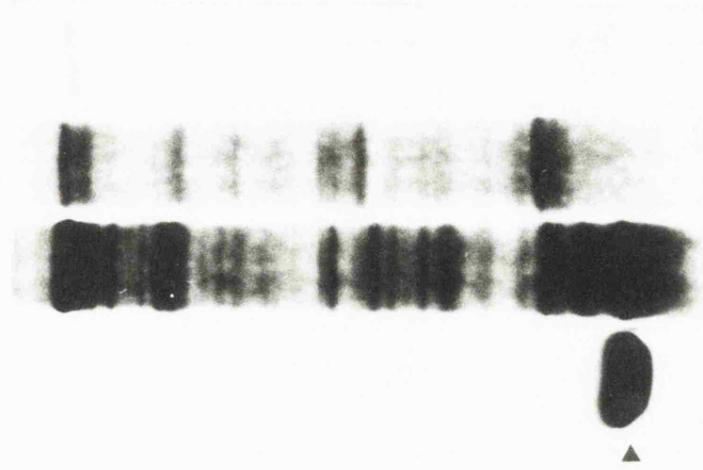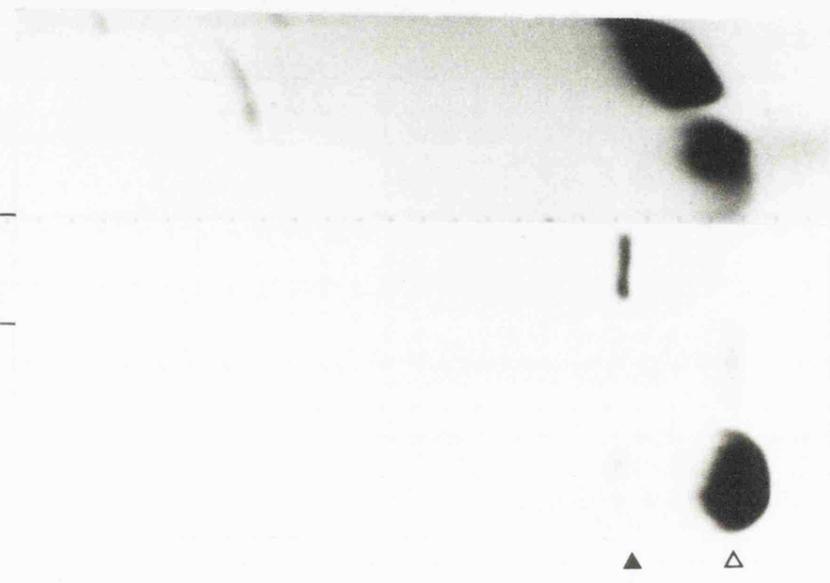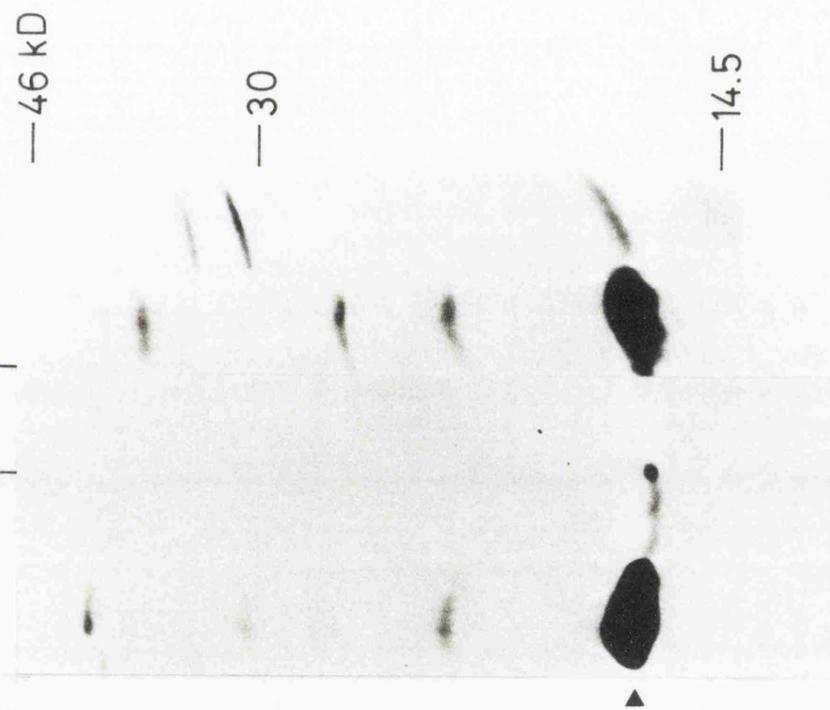
The relative incorporation of ^{35}S into seal and human myoglobin was
measured by scintillation counting of the myoglobin bands cut from the gel

(2.7.5). Taking into account the different incorporation of ^{35}S

methionine into total translation products from seal and human mRNA and

the number of methionine residues in each, 10.2 times as much seal

myoglobin was synthesised as human. This is from equal quantities of

poly(A)$^+$ RNA which corresponds approximately to RNA derived from an equal

mass of tissue (Table 4.1). This result correlates well with the

difference observed in myoglobin levels in skeletal muscle in vivo, and

also with the difference in myoglobin mRNA levels. The higher ^{35}S

incorporation into myoglobin in in vitro translations of seal RNA was

found to be independent of RNA concentration in translations with varying

amounts of seal and human RNAs (not shown). There is therefore no

significant difference in the efficiency of translation of seal and human

myoglobin mRNAs.

6.6 Summary

To define the basis for the elevated expression of myoglobin in

diving mammals, myoglobin and myoglobin mRNAs levels were measured in grey

seal and human skeletal muscles. The 6 to 7-fold difference in myoglobin

levels is reasonably consistent with an 8-fold difference in myoglobin

mRNA levels. Moreover, to confirm that the elevated myoglobin level in

seal results primarily from an elevated level of myoglobin mRNA, the

translational efficiency of myoglobin mRNA in seal and human RNA samples

was compared by in vitro translation. Seal RNA directed the synthesis of

10 times as much myoglobin as human RNA. No significant difference in

efficiency of translation of the two myogobin mRNAs was therefore found.

These results indicate that the elevated myoglobin level in the grey seal

results primarily from a correspondingly elevated level of myoglobin mRNA.

MEASUREMENT OF THE ABSOLUTE CONCENTRATION OF MYOGLOBIN mRNA

7.1 Introduction

Chapters 4-6 describe the measurement of the relative concentrations of myoglobin mRNA in various muscle tissues. However, the results from this work give no indication of the absolute concentration of the myoglobin message relative to the total population of poly(A)$^+$ RNAs.

Following cDNA cloning of seal myoglobin mRNA (Wood et al., 1982), the myoglobin cDNA clone was hybridised to the whole cDNA library and 4% of the clones were found to give a positive signal, suggesting that the abundance of myoglobin mRNA in the total population of seal muscle poly(A)$^+$ RNAs might be as high as 4%. However, this figure assumes that polyadenylated RNA sequences are represented in the cDNA library in proportion to their concentrations in the poly(A)$^+$ RNA from which the library was generated, and there are instances where this need not be the case. For example, the cDNA cloning of globin mRNA yields a significantly higher proportion of β-globin than α-globin cDNAs (Maniatis et al., 1976; Rabbitts, 1976). The work described in this chapter was therefore carried out in an attempt to measure the abundance of seal myoglobin mRNA and hence the concentrations of myoglobin mRNAs in the other muscle tissues discussed in the preceding chapters.

I describe a simple, novel method for the determination of the concentration of abundant mRNAs, using single-stranded DNA containing sequences homologous to the message, generated from M13 recombinants.

7.2 Strategy of the abundance assay

The strategy of the assay involves hybridisation of poly(A)$^+$ RNA with [^{32}P]labelled single-stranded antisense DNA generated from the

recombinant M13.HEX2 (which contains 254bp of human myoglobin sequence) by primer extension. Hybridisations were carried out in small volumes (usually 10μl) of 1xSSC, 0.1mg/ml calf liver tRNA, at 65°C overnight. Initially, after hybridisation, the remaining single-stranded molecules were digested with S1 nuclease, and the amount of ^{32}P remaining in the S1-resistant DNA/RNA hybrids was determined by Cerenkov counting after centrifugation through spun columns of Sephadex G50 to remove S1 nuclease digestion products.

During preliminary experiments, however, it was found to be impossible to digest adequately single-stranded antisense DNA with S1 nuclease without significant simultaneous digestion of DNA/RNA hybrids. This was most likely due to S1 digestion at the positions of mismatch between the human exon 2 sequence of the antisense DNA and the exon 2 region of the seal myoglobin mRNA.

To avoid using S1 nuclease digestion, it was decided to assess how much labelled antisense DNA had hybridised to RNA directly, by agarose gel electrophoresis and autoradiography. Unhybridised antisense DNA migrates very much faster in agarose gels than antisense DNA hybridised with myoglobin mRNA and hence the two can be distinguished. The fraction of labelled antisense DNA which had hybridised with RNA was measured by Cerenkov counting of appropriate regions of the gel. It was therefore possible to measure the amount of seal poly(A)$^{+}$ RNA which contains enough myoglobin mRNA just to hybridise with a certain amount of single-stranded antisense DNA, and thus to calculate the abundance of myoglobin mRNA in the population of seal muscle poly(A)$^{+}$ RNAs.

In order to test the applicability of this approach, a pilot experiment was carried out hybridising antisense DNA to its parent single-stranded M13.HEX2 molecule. 1μg [^{32}P]labelled single-stranded antisense human exon 2 DNA (specific activity 7x10^{3} cpm/ng) was hybridised with increasing amounts of the parent M13.HEX2 molecule (6 to 200ng).

Hybridisation was allowed to continue at 65°C overnight and then the reactions were loaded, without further treatment, into a 2% agarose gel which was dried and autoradiographed after electrophoresis (Figure 7.1).

The length of M13.HEX2 is 7496nt and the length of the antisense DNA generated from it is 316nt (made up from 254nt myoglobin sequence plus an additional 62nt primer and polylinker sequence). 1ng antisense DNA would therefore be expected to hybridise exactly with 7496/316 = 24ng of M13.HEX2 DNA. It can be seen from Figure 7.1 that approximately three quarters of the antisense DNA (0.75ng) hybridises with 25ng M13.HEX2 DNA. This is within the limits of accuracy imposed by the small volumes and the scintillation (Cerenkov) counting methods used and confirms the feasibility of this approach for measuring mRNA concentration.

7.3   Measurement of the concentration of myoglobin mRNA in seal poly(A)$^+$ RNA

Hybridisations were carried out containing 1ng of labelled antisense single-stranded DNA and between 4ng and 3µg of seal poly(A)$^+$ RNA and between 40ng and 3µg of RNA from Aspergillus nidulans as a control (Figure 7.2). Single-stranded DNA hybridises to seal RNA, but not to RNA from Aspergillus nidulans, confirming authentic hybridisation to myoglobin mRNA. Hybridised antisense DNA/myoglobin mRNA does not migrate as a discrete component because some degradation inevitably occurs after prolonged hybridisation. Tighter bands with a similar mobility to that of myoglobin mRNA were seen on other gels with samples which had been hybridised for a shorter time (see Figure 7.4). From Figure 7.2 it can be seen that 120ng of seal poly(A)$^+$ RNA contains just enough myoglobin mRNA to hybridise with 1ng of exon 2 antisense DNA. If the length of the seal myoglobin mRNA is approximately 1300nt (1066nt predicted from the gene sequence plus ~200nt adenosine residues) and the length of the antisense DNA is 316nt, then 120ng of seal poly(A)$^+$ RNA must contain approximately

Figure 7.1   Titration of M13.HEX2 DNA against single-stranded DNA generated from M13.HEX2

A series of hybridisations was set up between single-stranded antisense human exon 2 DNA generated from M13.HEX2 by primer extension (2.1) and the parent M13.HEX2 molecule. All hybridisations were carried out in 10μl 1xSSC, 0.1mg/ml tRNA and contained 1ng antisense DNA, as measured by Cerenkov counting, and between 6ng and 200ng M13.HEX2 DNA. After hybridisation at 65°C overnight under liquid paraffin, samples were extracted twice with ether to remove the paraffin and electrophoresed directly in a 2% agarose gel with single-stranded mobility markers.

The gel was dried onto a glass plate and exposed to X-ray film overnight. The migration positions of M13.HEX2 and [^{32}P]labelled single-stranded antisense DNA are indicated. The identity of the component indicated by a triangle is unknown but is always present in single-stranded antisense DNA preparations. It may represent a double-stranded form, perhaps created by foldback during primer extension, since it does not hybridise either to M13.HEX2 DNA or to myoglobin mRNA (see Figure 7.2).
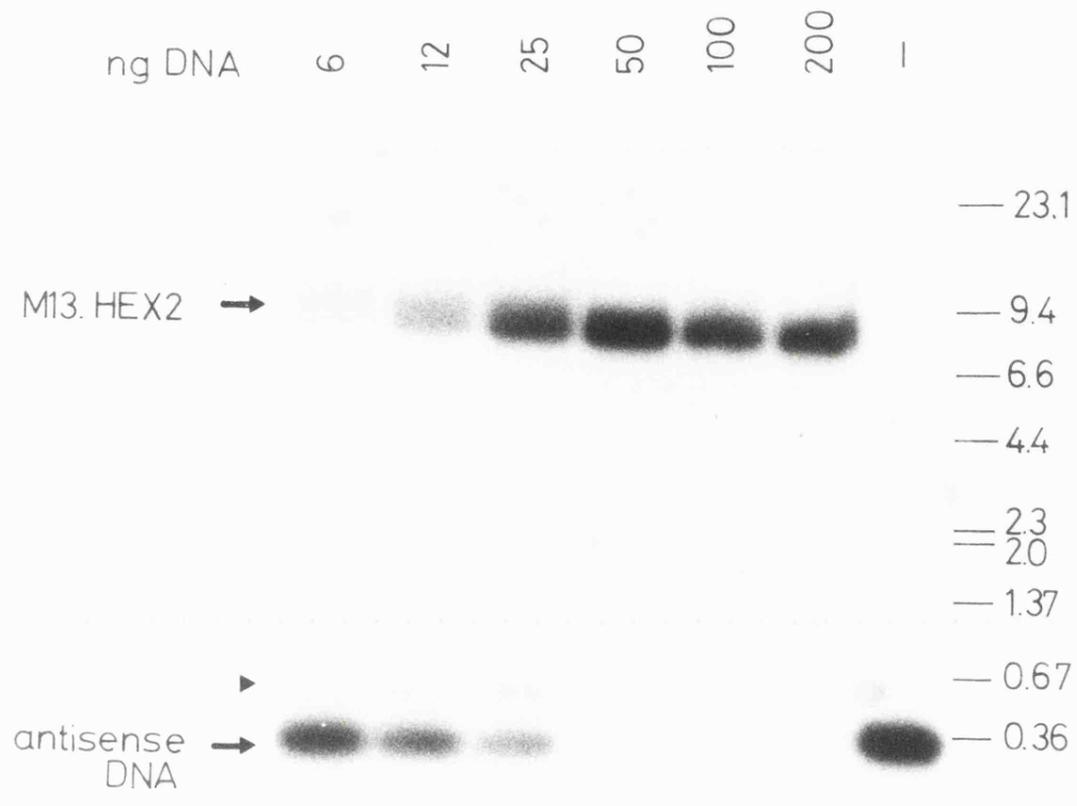
Figure 7.2   Measurement of the concentration of myoglobin mRNA in seal
poly(A)$^+$ skeletal muscle RNA

Hybridisation of increasing amounts of seal poly(A)$^+$ RNA (4ng to
3.15µg) and Aspergillus nidulans RNA (40ng to 3.15µg) were carried out as
described in the legend to figure 7.1. All hybridisations contained 1ng
[^{32}P]labelled antisense DNA. After hybridisation the reactions were
electrophoresed with single-stranded mobility markers (M) in a 2% agarose
gel, which was photographed, dried and exposed to X-ray film overnight,
without an intensifier screen. A photograph of the ethidium-bromide
stained gel is shown in A, and the autoradiograph in B.

The migration position of the 18S and 28S rRNAs and tRNA are
indicated in A, and positions of unhybridised antisense DNA and myoglobin
mRNA in B. Antisense DNA hybridised with myoglobin mRNA does not run as a
discrete component because of the inevitable partial degradation of RNA
after prolonged incubation at high temperatures.
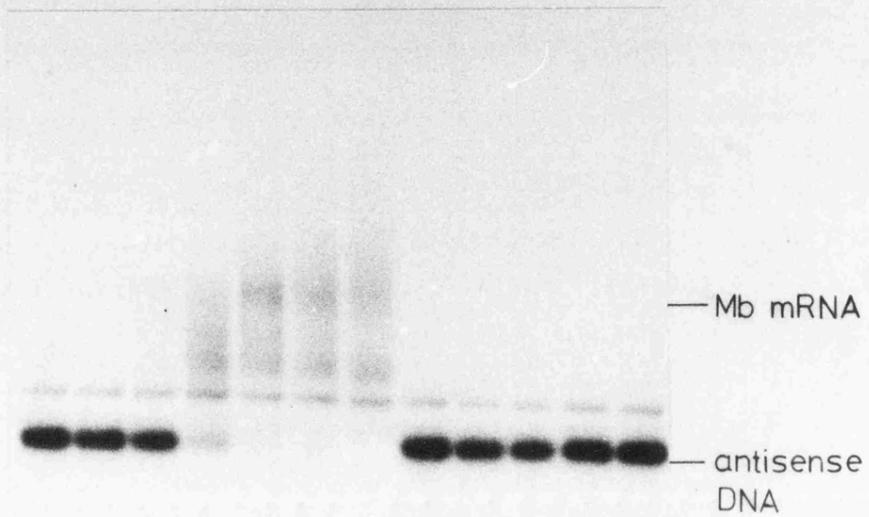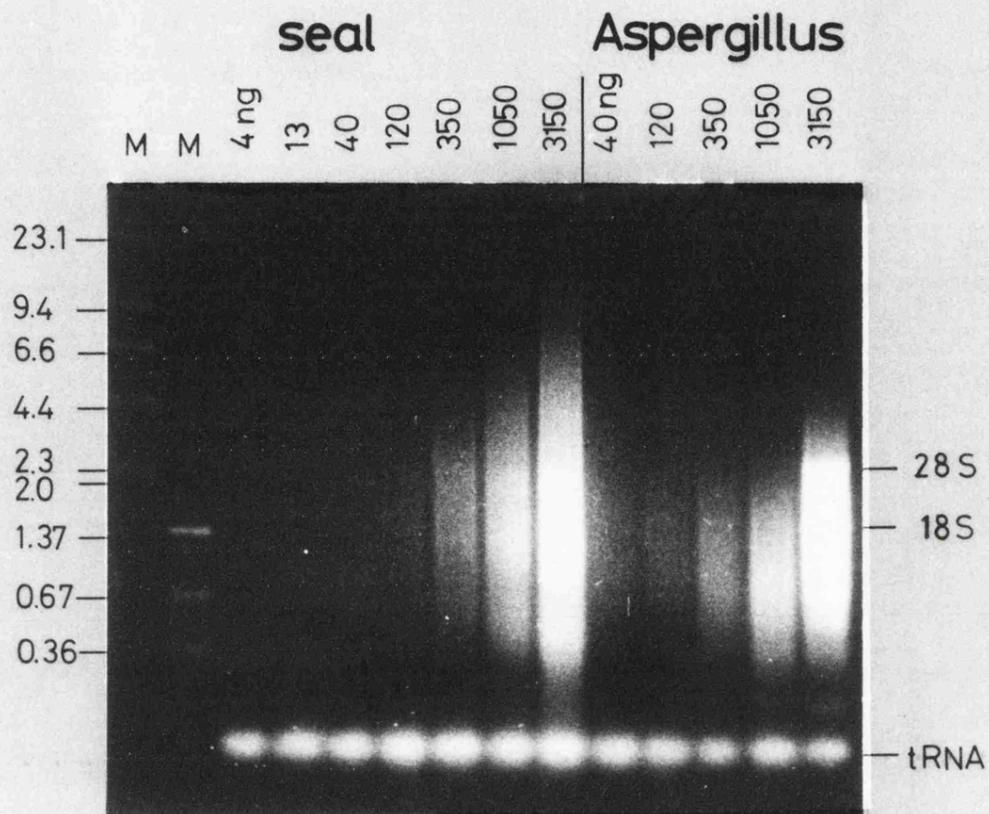
1x1300/316 = 4.1ng myoglobin mRNA. This corresponds to a myoglobin mRNA concentration of 3.4% of the mass of the total population of seal muscle poly(A)$^+$ RNAs. This is in good agreement with the figure of 4% estimated during cDNA cloning of seal myoglobin mRNA (Wood et al., 1982).

To show that this result is valid, it is necessary to ensure that hybridisation is complete under the conditions used. This is considered in the next section.

7.4 Tests and controls

i) Titration of antisense DNA against RNA.

In the experiment described above there is no way of telling that the hybridisation reaction has reached completion. With such an experiment it would be more conventional to set up a DNA driven reaction, varying the antisense DNA concentration rather than that of the RNA. This should not only give the same result in terms of the myoglobin mRNA concentration in seal poly(A)$^+$ RNA, but would also indicate whether hybridisation had reached completion if, beyond a certain titration point, no further antisense DNA was taken up into hybrid.

Hybridisations were set up under the usual conditions, but all contained the same amount (120ng) of seal poly(A)$^+$ RNA and between 0.125 and 4ng of [^{32}P]labelled antisense DNA (Figure 7.3A). As found previously, 120ng of seal RNA hybridised almost exactly with 1ng cDNA. There appears to be little increase in the incorporation of [^{32}P]labelled DNA into hybrids with amounts of antisense DNA greater than 1ng. To check this, regions of the dried gel corresponding to hybridised and unhybridised antisense DNA were cut from the glass plate and Cerenkov counted.

The incorporation of [^{32}P]labelled antisense DNA into RNA can be expressed graphically in terms of the cpm in hybridised and unhybridised DNA plotted against the total cpm recovered from each track: a measure of

Figure 7.3   Titration hybridisation of [^{32}P]labelled antisense DNA against seal poly(A)$^+$ RNA

A.   A series of hybridisations was carried out under standard conditions as described in Figure 7.2. Each hybridisation contained 120ng of seal mRNA and between 0.125 and 4ng of [^{32}P]labelled antisense exon 2 DNA. Samples were electrophoresed in a 2% agarose gel which was dried and exposed to X-ray film overnight.

B.   Regions of the dried gel containing unhybridised antisense DNA and antisense DNA hybridised with myoglobin mRNA were cut from the gel and Cerenkov counted. The unidentified 'foldback' component was not included in either region. The data are plotted as counts per minute (cpm) in hybridised or unhybridised antisense DNA against input of antisense DNA (as measured by total cpm). Approximately 75% of the label is taken up by the seal mRNA at low concentrations of antisense DNA. After the titration end point is reached, little more label is incorporated into hybrid, but appears as unhybridised antisense DNA. The titration end point represents the point at which all the myoglobin mRNA in the seal poly(A)$^+$ RNA is hybridised with antisense exon 2 DNA.

     The cpm in the hybridised fraction does not reach a plateau. This is because there is some contaminating labelled DNA of higher molecular weight in the antisense DNA preparation (the hybridisation is thought to have reached completion, see section 7.4ii). Extrapolation of this curve will give an estimate of the antisense DNA input at which all the myoglobin mRNA is hybridised with antisense DNA, taking account of this background. This is estimated to be ~2.5x10^3 cpm. 1ng of antisense DNA from a control with no RNA cut from the dried gel in this way contains 3.47 x 10^3 cpm. The titration end point is therefore reasonably consistant with the previous observation that 1ng of antisense DNA hybridises exactly with myoglobin mRNA in 120µg of seal mRNA (Figure 7.2).

A 0.125 0.25 0.5 1 2 4 ng antisense DNA

Mb mRNA

antisense DNA

B
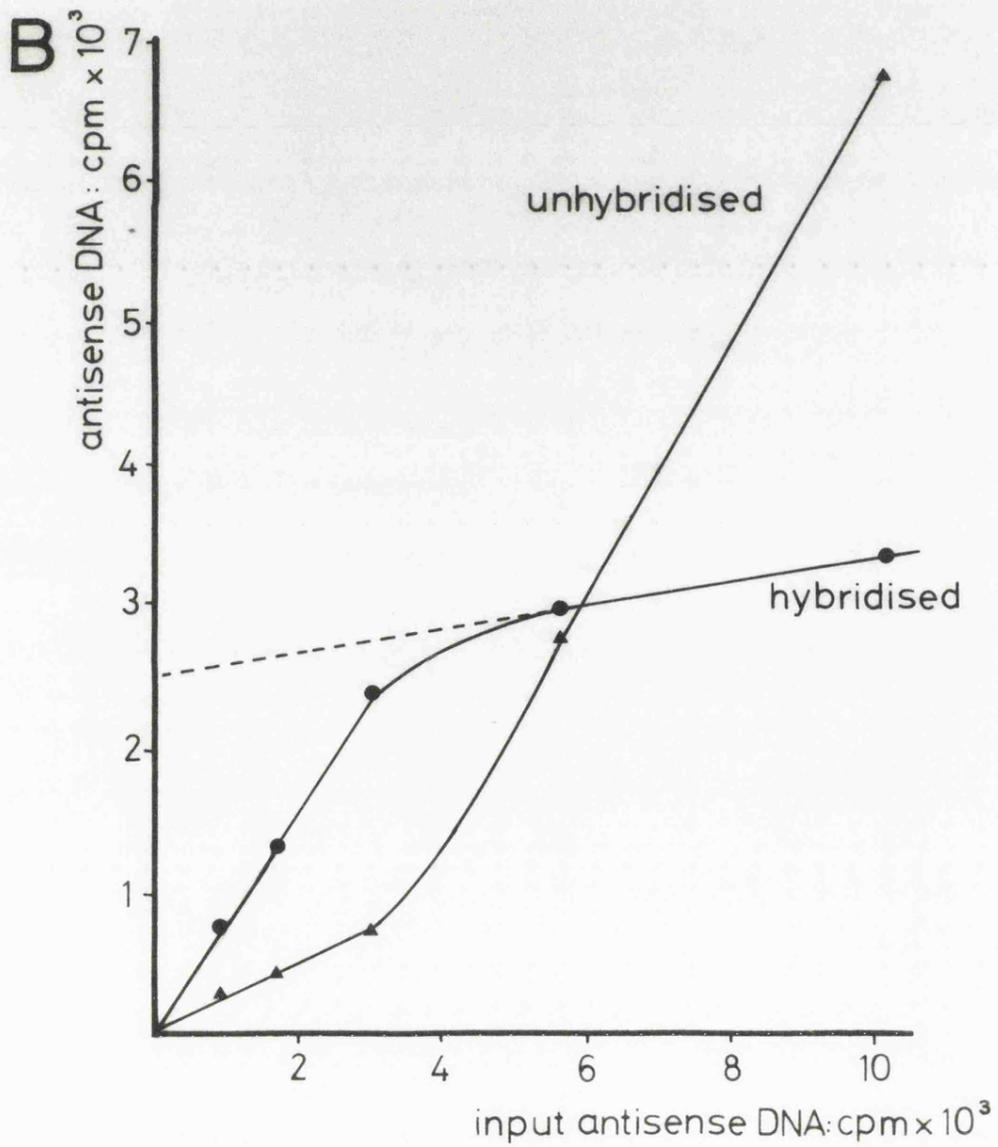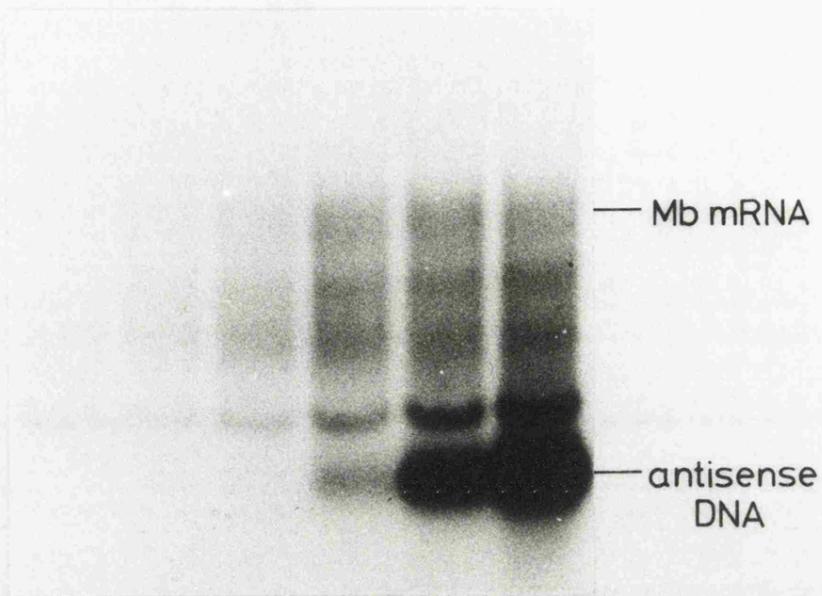
the input of antisense DNA into each hybridisation (Figure 7.3B). Before

the point at which all myoglobin mRNA in seal poly(A)$^+$ RNA is hybridised

with exon 2 antisense DNA, the majority of the [^{32}P]labelled antisense DNA

is taken up into hybrids (approximately 75%). At higher concentrations of

antisense DNA, when all the myoglobin mRNA is hybridised, additional

antisense DNA is not incorporated to any great extent. A small increase

in apparent incorporation is probably due to slight contamination of

antisense DNA during the preparation of antisense DNA by larger labelled

DNA fragments with no homology to myoglobin mRNA, or to non-specific

hybridisation. It is unlikely to be due to incomplete hybridisation (see

below). The end point, at which all myoglobin mRNA in 120ng poly(A)$^+$ RNA

is hybridised (corrected for this background) is compatible with the value

of 1ng estimated in section 7.3 (Figure 7.3B). The result obtained by

titrating RNA against a fixed amount of antisense DNA, described in the

previous section is therefore confirmed.

ii) Reaction kinetics and the extent of hybridisation over time.

To confirm that the hybridisation reactions do indeed reach

completion in the 15 hour (overnight) incubation, an approximate value for

$t^1/_2$, when the hybridisation is 50% complete, can be calculated for the

reaction.

Taking the molecular weight of a nucleotide to be 330, the initial

concentration, C_0, of antisense DNA (1ng in a 10μl volume reaction) is

3.3x10^{-7} M, and Cot is approximately 2x10^{-2}. If the value for Cot$^1/_2$ for

the human genome, (complexity 3x10^9) is ~10^4 (Britten and Kohne, 1968),

using this data as a standard, we can calculate the approximate value of

Cot$^1/_2$ for the hybridisation of antisense DNA (complexity 316):

$$\text{Cot}^{1}/_{2} \text{ (antisense DNA)} \quad = \quad \frac{10^{4} \times 316}{3 \times 10^{9}} \quad = \quad 1.05 \times 10^{-3}$$

$$\text{and } t^{1}/_{2} \text{ (antisense DNA)} \quad = \quad \frac{1.05 \times 10^{-3}}{3.3 \times 10^{-7}} \quad = \quad 3182 \text{ seconds} \quad (\text{or } 53 \text{ minutes})$$

This rough calculation therefore indicates that the hybridisation of antisense DNA with myoglobin mRNA should be complete within the 15 hour incubation time.

That the reaction really had gone to completion was confirmed experimentally by setting up two sets of four identical hybridisations and allowing the hybridisation reaction to proceed for varying lengths of time. The two sets of reactions were under standard conditions (see 7.2) with 1ng antisense DNA and either 50 or 80 µg of seal poly(A)$^{+}$ RNA (i.e. an excess of antisense DNA). Hybridisation samples were then electrophoresed in a 2% agarose gel which was dried and autoradiographed (Figure 7.4A). Regions of the dried gel containing unhybridised antisense DNA and DNA hybridised with RNA were cut out and Cerenkov counted. The proportion of antisense DNA hybridised to RNA was calculated and plotted against hybridisation time (Figure 7.4B). Proportionally more antisense DNA is taken up by 80ng of seal RNA than by 50ng, as expected. The graph shows that hybridisation is substantially complete after about 5 hours. It can therefore be concluded that hybridisation will have reached completion in previous experiments with overnight hybridisation incubations,.

Figure 7.4A also shows that after a short hybridisation time, antisense DNA is incorporated into a discrete band of slightly slower mobility than myoglobin mRNA, which corresponds to intact myoglobin mRNA hybridised with antisense DNA. With longer hybridisation times gradual breakdown of RNA causes smearing of the myoglobin mRNA band in agarose gel electrophoresis.

The experiments described in this section confirm the quantitation of myoglobin mRNA concentration in seal muscle, described in section 7.3,

Figure 7.4 <u>Time course hybridisation of [^{32}P]labelled antisense DNA with</u>
<u>seal poly(A)$^+$ RNA</u>

A.   Two sets of four hybridisations were set up, each with 1ng
[^{32}P]labelled antisense DNA, one set with 50ng seal RNA and the other with
80ng. Hybridisations were carried out at 5°C, as described previously,
and for durations from 0.5 to 41 hours. Samples hybridised for less than
the longest time were stored frozen. Samples were electrophoresed in a 2%
agarose gel together with single-stranded DNA size markers pAT153 x <u>Hinf</u>I,
end-labelled by a fill-in reaction using [^{32}P]dATP and reverse
transcriptase. The gel was dried and exposed to X-ray film overnight,
without an intensifier screen.

B.   Regions of the dried gel containing hybridised and unhybridised
antisense DNA were excised separately and Cerenkov counted. Because of
degradation of hybridised DNA/Mb mRNA , the unidentified 'foldback'
component was included in the 'hybridised DNA' gel slices, but was also
counted from the input track (i/p), and this value subtracted from the
others. The amount of hybridised antisense DNA is plotted (as a
percentage of the average total input) against hybridisation time in
hours. Proportionally more antisense DNA hybridises with 80ng of seal
poly(A)$^+$ RNA than 50ng, as expected. Hybridisation is substantially
complete after about 5 hours.

A

| | M | 50 ng RNA | | | | 80 ng RNA | | | | | |
| | | 0.5 | 5.5 | 17 | 41 | 0.5 | 5.5 | 17 | 41 | i/p | hours |

1.63 — Mb mRNA/cDNA

0.52
0.40
0.30 — antisense DNA
0.22
0.15
0.07

B
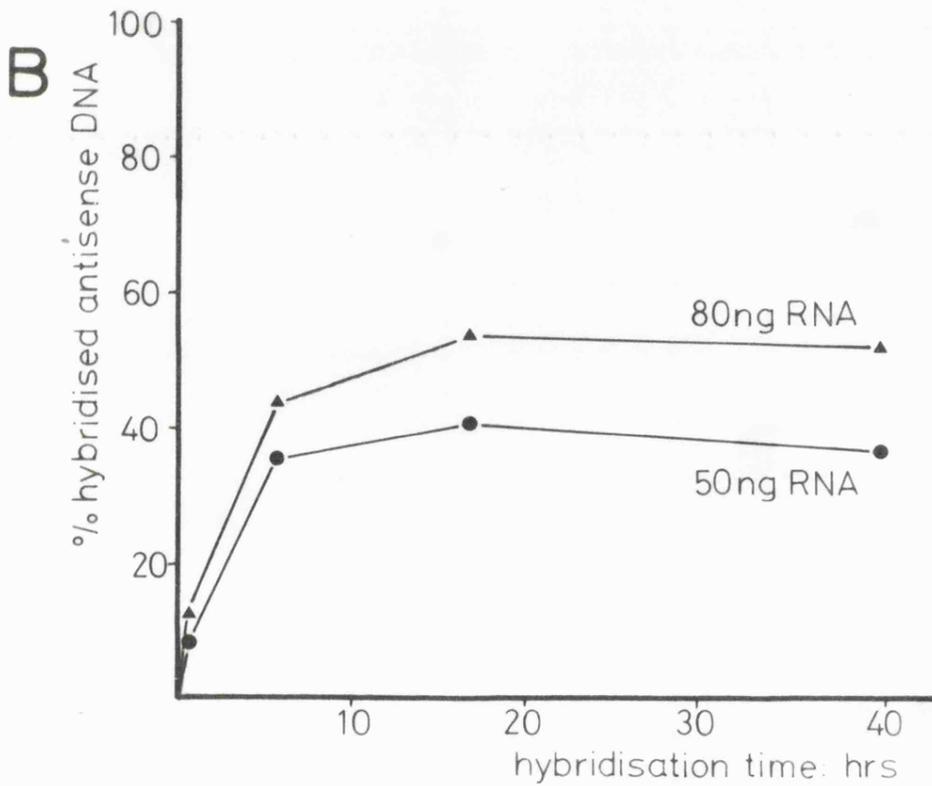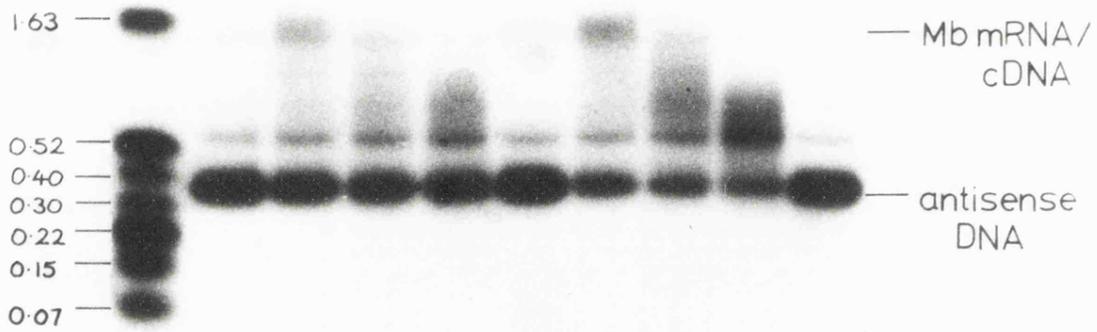
and indicate that this assay is a valid method for measuring the absolute concentration of mRNAs. It would, however, be more difficult with mRNAs of lower abundance, as the hybridisation kinetics would be less favourable and it would be necessary either to reduce the volume of the hybridisation reactions, or to increase the amount of RNA present and/or the hybridisation time to ensure that the hybridisation reached completion.

Having determined that myoglobin mRNA accounts for 3.4% of seal skeletal muscle poly(A)$^+$ RNA, it is possible to calculate its concentration in the range of human and mouse muscle tissues for which the relative myoglobin mRNA concentrations were determined in Chapters 4 and 5 (see Chapter 9).

7.5 Summary

This chapter describes the development of a novel hybridisation assay to measure the absolute level of myoglobin mRNA within the total population of seal muscle polyadenylated RNAs. The method involves titration of RNA against [^{32}P]labelled single-stranded antisense DNA and following the extent of hybridisation by agarose gel electrophoresis.

From this assay, the concentration of myoglobin mRNA in seal skeletal muscle was found to be approximately 3.4% of the total polyadenylated RNA. This corresponds well with the estimate of 4% made during cDNA cloning of the seal myoglobin gene (Wood et al., 1982).

Chapter 8

MAPPING OF THE HUMAN MYOGLOBIN GENE CAP SITE AND S1 NUCLEASE-SENSITIVE

SITES IN SUPERCOILED MYOGLOBIN GENE DNA.

8.1 Introduction

This chapter decribes two separate mapping experiments: localisation

of S1 nuclease sensitive sites in supercoiled plasmids containing

myoglobin sequences and conventional so-called Berk-Sharp S1 nuclease

protection mapping of the human myoglobin cap site, to determine the

position of the transcriptional start site.

DNase I hypersensitive sites in chromatin are correlated with

differential gene expression (Weintraub and Groudine, 1976) and correspond

to regions of chromatin which are digested preferentially by DNase I and

other endonucleases, including S1 nuclease. Such sites are indicative of

non-B-DNA structure, as indicated by their sensitivity to a number of

single-strand specific endonucleases, including S1 nuclease (see

Weintraub, 1985). Such structures may be important in sequence

recognition, for example, by factors required for initiation of

transcription, as sequences cleaved by S1 nuclease digestion often include

regions of regulatory or promoter elements (Larson and Weintraub, 1982;

Evans et al., 1984). In many cases it has been shown that regions

containing a site sensitive to S1 nuclease in active chromatin may also be

sensitive in supercoiled (but not relaxed) plasmid DNA (Larson and

Weintraub, 1982). Two such S1 nuclease-sensitive sites have been found in

the 5' flanking region of the human β-globin gene (Evans et al., 1984).

It was therefore decided to look for S1 nuclease-sensitive sites in

supercoiled plasmid DNAs containing myoglobin gene sequences, although as

yet, there are no data for the occurrence of S1 nuclease or DNase I

hypersensitive sites in chromatin in or near the myoglobin gene.

The other experiment described in this chapter is the determination of the site of the start of transcription of the human myoglobin gene. S1 nuclease protection mapping has previously been used to determine the 5' terminus of seal myoglobin mRNA (Blanchetot et al., 1983) and the corresponding cap site in the human myoglobin gene was provisionally located by homology with the seal gene (Weller et al., 1984). That this location is correct therefore needed confirmation by direct S1 nuclease protection analysis of human myoglobin mRNA transcripts. The possibility that differences in myoglobin gene expression in different muscle tissues and during development, described in Chapters 4 and 5, may be correlated with alternative transcription start sites is also investigated.

8.2 Mapping the cap site of human myoglobin mRNA

The location of the start of transcription in the human myoglobin gene had previously been provisionally assigned by homology with the seal gene (Weller et al., 1984). The experiments described in this section were designed to confirm that this location was correct, and to investigate the possibility that an alternative transcriptional start site(s) may be utilised in cardiac muscle or early during skeletal muscle development.

S1 nuclease protection mapping involves the hybridisation of RNA with a uniquely end-labelled DNA fragment, which spans the putative RNA end-point, in this case the cap site. Single-stranded and remaining unhybridised DNA is removed by S1 nuclease digestion. The size of the DNA fragment protected from S1 nuclease digestion can be determined by electrophoresis in a sequencing-type polyacrylamide gel and autoradiography.

The fragment used for end-labelling was a 1026 bp fragment extending upstream from the NcoI site at the initiation codon to an SstI site. Use of a fragment with a 3' protruding terminus (i.e. SstI) eliminates the

necessity for a second cleavage of the fragment after end-labelling, as polynucleotide kinase, used for the labelling reaction, operates inefficiently on recessed 5' termini. pHM.27.B2.9 DNA was digested with SstI and NcoI, treated with alkaline phosphatase and end-labelled with [γ-^{32}P]ATP and polynucleotide kinase as described (section 2.13.2). The 1026bp fragment was recovered and 100ng was hybridised with 50µg of human muscle total RNA (section 2.13.3). Hybrids were then digested with increasing amounts of S1 nuclease and protected DNA fragments were analysed by electrophoresis in an 8% polyacrylamide sequencing gel with size markers prepared by carrying out Maxam-Gilbert G+A and T+C sequencing reactions on the probe fragment, (section 2.13.4).

Figure 8.1A shows an autoradiograph of such an experiment, showing DNA fragments protected from S1 nuclease by hybridisation with skeletal and cardiac muscle RNAs. There is no difference between their patterns of protected fragments at any of the concentrations of S1 nuclease. The main protected fragments are indicated and their positions in the sequence of the human myoglobin gene are shown in Figure 8.1C. The main protected fragment corresponds to initiation of transcription at the same base as predicted by homology with the seal myoglobin gene (Weller et al., 1984).

Figure 8.1B shows protected fragments from hybridisations of the same probe fragment with 10µg of skeletal and cardiac RNA, 100µg of foetal skeletal RNA and 30µg of Aspergillus nidulans RNA, as a control. All samples shown were treated with the same concentration of S1 nuclease. The pattern of protected fragments seen with adult skeletal and cardiac muscle RNAs can also be seen with foetal skeletal muscle RNA. There is no protection with Aspergillus nidulans RNA. The intensity of the protected fragments reflects the relative abundance of myoglobin mRNA in the different RNA samples (see Chapters 4 and 5). There is therefore no detectable utilisation of alternative sites of transcriptional initiation of the human myoglobin gene in adult cardiac or foetal skeletal muscle.

Figure 8.1   S1 nuclease protection mapping of the transcriptional start site of the human myoglobin gene

A.   Adult skeletal and cardiac muscle RNAs.   50µg of each of skeletal and cardiac total RNAs were hybridised with 2x 10^5cpm (100ng) NcoI-SstI probe fragment, end-labelled at the NcoI terminus with [γ-^{32}P]ATP by polynucleotide kinase (2.13).   After hybridisation, the reactions were each divided equally and treated with varying amounts of S1 nuclease: 20 units (a), 80 units (b) or 320 units (c).   After digestion nucleic acids were recovered by ethanol precipitation and were electrophoresed in a 0.35mm thick 8% polyacrylamide sequencing gel, together with two Maxam and Gilbert sequencing reactions (G+A and C+T), as size markers (not shown). The gel was fixed, dried and exposed to X-ray film for 10 days with an intensifier screen.

The main S1 nuclease-protected fragment and two minor ones are indicated with heavy and light arrows, respectively.

B.   Foetal skeletal muscle RNA.   10µg of each of adult skeletal (as) and adult cardiac (ac) muscle RNAs, 100µg of foetal skeletal (fs) muscle RNA and 30µg of Aspergillus nidulans RNA (An), as a control, were each hybridised with 20,000 cpm probe fragment.   Hybridisation was for 15 hours, as slightly stronger protection was seen after overnight incubation.   Reactions were each divided into four tubes as above and treated with varying amounts of S1 nuclease, before recovery of protected fragments and electrophoresis as above.   Only tracks digested with the smallest amount (50 units) of S1 nuclease are shown, as the signal from foetal RNA is very faint.   No protected fragments are seen with Aspergillus RNA.

C.   Location of the cap site in the human myoglobin gene.   The region of sequence upstream from the NcoI restriction site at the initiation codon is shown.   The three nucleotides corresponding to the terminal nucleotides of the protected fragments shown in A, and which represent the site(s) of initiation of transcription, are indicated in the same way; a heavy arrow indicating the major initiation site.   The sequence which is transcribed is shown in upper case.

A

skeletal | cardiac
a b c | a b c
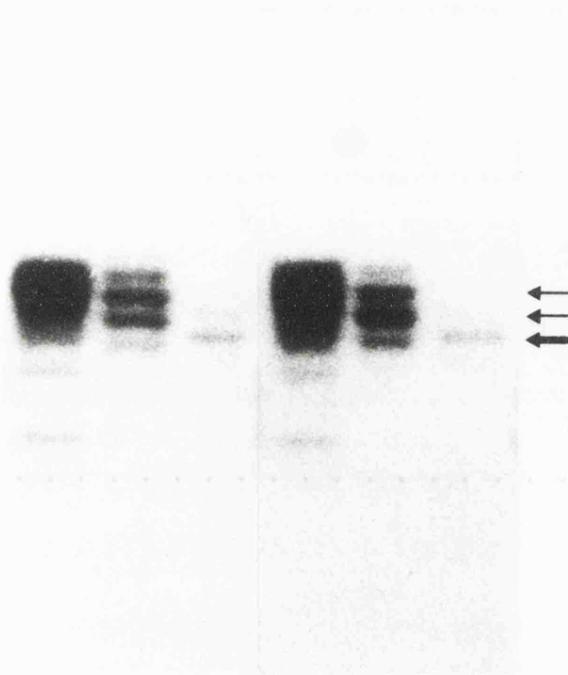
B

as ac fs A.n.
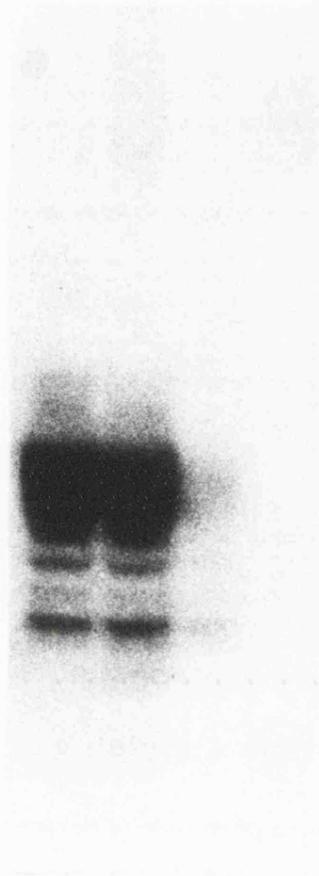
C

↓ ↓ ↓
gcctcaaACCCCAGCTGTTGGGGCCAGGACACCCAGTGAGCC

ini·gly

CATACTTGCTCTTTTTGTCTTCTTCAGACTGCGCCATGGGG

8.3   Investigation of S1 nuclease-sensitive sites in myoglobin recombinant plasmid DNAs

To detect S1 nuclease-sensitive sites in plasmid DNA, the DNA is first treated with S1 nuclease such that the majority of plasmid molecules are linearised. Supercoiled molecules are converted to open circles (by nicking) in a rapid reaction, followed by a slower conversion to linear molecules by cleavage of the strand opposite the initial nick (Lilley, 1980). Any preferential S1 nuclease cleavage sites can then be mapped by restriction endonuclease digestion. Because any S1·nuclease-sensitivity was expected to be in the 5' flanking region of the myoglobin gene, only plasmids pHM.27.B2.9 (see Figure 3.2) and pSM.19.5 (Blanchetot et al., 1983), both of which contain only the 5' flanking regions and 5' parts of the human and seal myoglobin genes respectively, were investigated.

S1 nuclease was titrated in a series of test reactions with pHM.27.B2.9 DNA (Figure 8.2A). Even at low concentrations of S1 nuclease, the supercoiled plasmid is converted to open circular (nicked) and linear forms. At higher concentrations DNA starts to be degraded. The presence of a band double the size of the linear plasmid (which is approximately 10kb) is probably due to a proportion of the plasmid DNA being present in a cointegrative dimer form, which is converted to a linear dimer form by S1 nuclease. An almost identical S1 nuclease digestion profile was obtained for pSM.19.5 (not shown).

With 0.05 units of S1 nuclease per µl, virtually all supercoiled plasmid DNA is converted to open circles/linear and there is little additional degradation of DNA. A bulk S1 nuclease digestion reaction was therefore carried out with each plasmid. A series of pairs of restriction endonuclease cleavage reactions was carried out, each with 0.75µg of DNA treated with S1 nuclease as described, or untreated as a control. The enzymes chosen were ones with few cleavage sites. Digested DNAs were electrophoresed directly in a 0.5% agarose gel (Figure 8.2B). The gel is

Figure 8.2 <u>S1 nuclease treatment and restriction endonuclease digestion</u>
<u>of pHM.27.B2.9 and pSM.19.5</u>

A.   Titration of S1 nuclease.   0.5µg aliquots of pHM.27.B2.9 (and
pSM.19.5, not shown) DNA were digested in 10µl S1 nuclease reaction buffer
with between 0.01 and 50 units of S1 nuclease (section 2.14.2), and were
electrophoresed directly in a 0.5% agarose gel. The migration positions
of covalently closed (supercoiled) circles (CCC), linear (L) and open
circles (OC) are indicated. Dimers are present, most likely cointegrates,
as they are not cleaved to the monomer linear form at high concentrations
of S1 nuclease. A virtually identical digestion profile was obtained for
pSM.19.5.

B.   Restriction endonuclease digestion of S1 nuclease-digested
pHM.27.B2.9 and pSM.19.5 DNAs.   A scaled up version of the digest with
0.05 units of S1 nuclease per µl, as shown in A, was carried out with the
two plasmids. A series of pairs of restriction endonuclease digestion
reactions were carried out with 0.75µg of DNA; S1-treated (+) and
untreated (-), as a control. Restriction enzymes which had a small number
of cleavage sites were chosen, these are <u>Hin</u>dIII (H), <u>Bgl</u>II (Bg), <u>Eco</u>RI
(E), <u>Bam</u>HI (B), <u>Kpn</u>I (K), <u>Sst</u>I, (S) and <u>Pst</u>I (P). The <u>Kpn</u>I digest failed
to cleave the DNA. Digestion reactions were electrophoresed directly in a
0.5% agarose gel. i/p and S1 i/p are plasmid DNAs, treated and untreated
with S1 nuclease, respectively.

     Additional fragments in S1 nuclease-treated DNA digests are
indicated, those resulting from S1 nuclease cleavage in the vector
sequence by open triangles, and those resulting from cleavage in the
myoglobin gene sequence by filled triangles (see Figure 8.2). The sizes
of restriction endonuclease and S1 nuclease-generated fragments and a map
for pHM.27.B2.9, showing S1 nuclease-sensitive sites, are shown in Figure
8.3.
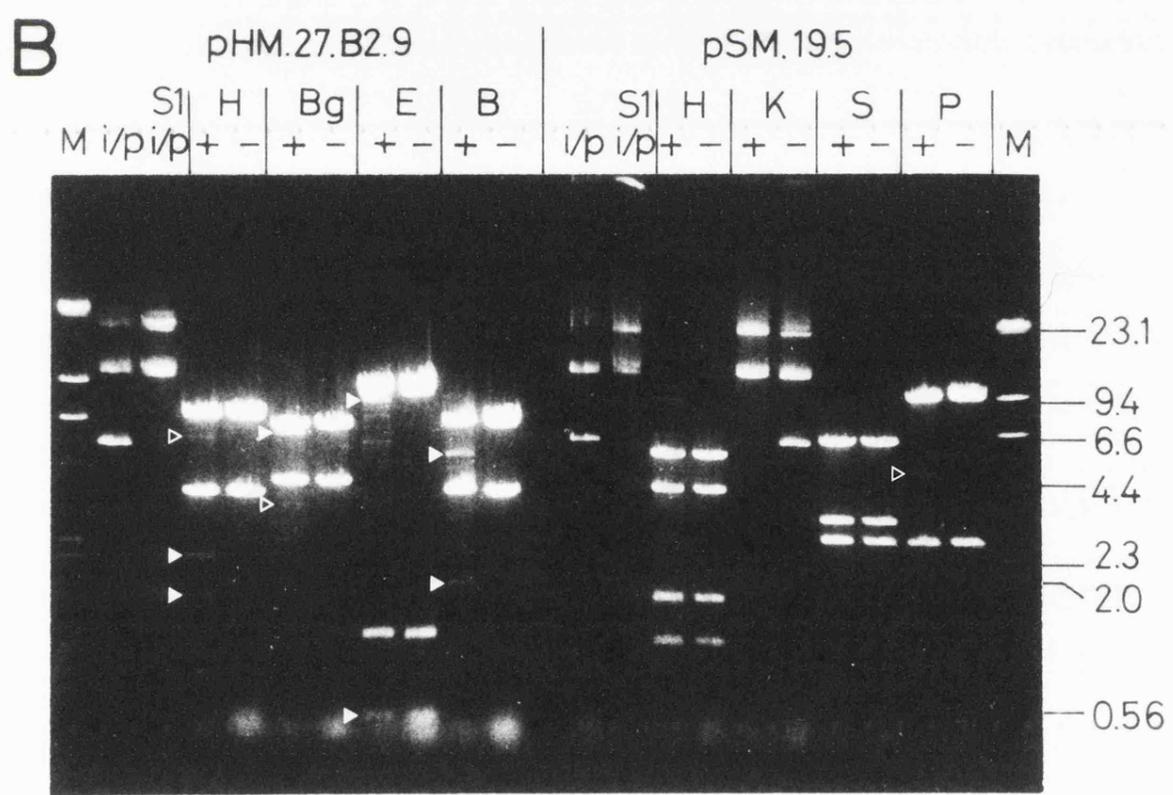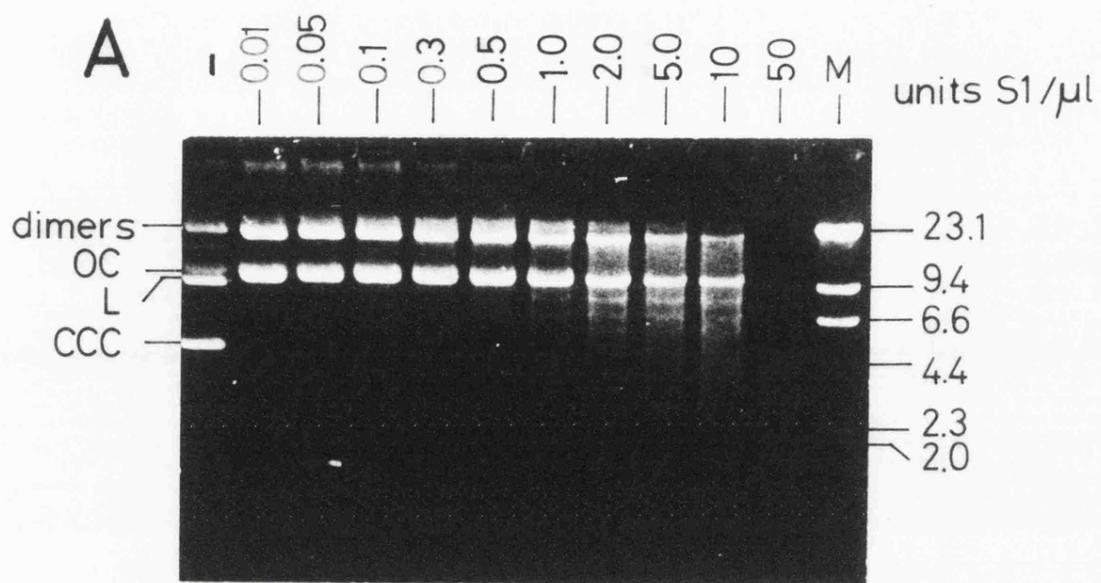
overloaded with DNA with respect to the main restriction endonuclease digestion fragments. Additional bands in S1 nuclease-treated DNA digests, however, are still relatively faint, though clearly visible in some digests. Principal additional S1 generated fragments are indicated in Figure 8.2B). There is only one clear S1 nuclease-generated fragment in restriction endonuclease digests of pSM.19.5.

The sizes of restriction endonuclease fragments and S1-generated fragments from pHM.27.B2.9, together with the resulting cleavage map indicating the approximate positions of the S1 nuclease-sensitive sites, are given in Figure 8.3. Two S1 nuclease-sensitive sites can be mapped. One is in the vector, pAT153, in a region previously shown to contain S1 nuclease-sensitive sites in pBR322 (Lilley, 1980). The other S1 nuclease-sensitive site in plasmid pHM.27.B2.9, stronger than the pAT153 site, maps unexpectedly approximately 800-900bp into the first intron.

As there are no strong S1 nuclease-generated fragments in digests of pSM.19.5 (Figure 8.2B), plasmid DNAs were treated with a higher concentration of S1 nuclease: 0.5u/µl, rather than 0.05u/µl, used previously. Figure 8.4A shows restriction endonuclease cleavage of pHM.27.B2.9 and pSM.19.5 by HindIII. No additional S1 nuclease-generated DNA fragments are seen at this concentration of S1 nuclease in digests of either plasmid. The only difference between HindIII digests in this Figure and those in Figure 8.2B is an increased smearing of DNA, attributable to the higher concentration of S1 nuclease.

S1-sensitivity of linear pHM.27.B2.9 was also tested. Plasmid DNA was first linearised by digestion with BamHI (which generates two fragments). Aliquots of digested DNA were treated with increasing concentrations of S1 nuclease, as described for supercoiled DNA above (Figure 8.4B). At no concentration of S1 nuclease is there any preferential S1 nuclease cleavage site, although severe degradation of DNA occurs at high S1 concentrations. The S1 nuclease-sensitive site in the

Figure 8.3 Mapping of S1 nuclease-sensitive sites in pHM.27.B2.9

A.   Table of DNA fragment sizes generated from pHM.27.B2.9 by treatment
with S1 nuclease and restriction endonucleases cleavage. The fragment
sizes (kb) given here are derived from the gel shown in Figure 8.2B. Only
the sizes of S1 nuclease-generated fragments indicated in that Figure are
shown. Those S1-generated fragments resulting from cleavage in the vector
are indicated 'v'.

B.   Restriction endonuclease cleavage map of pHM.27.B2.9 with S1
nuclease-sensitive sites indicated. This is the map constructed from the
data in A, and shows the positions of the S1 nuclease cleavage sites
relative to restriction sites (for HindIII (H), BglII (Bg), EcoRI (E), and
BamHI (B)), previously determined (see Figure 3.2). Two S1 nuclease-
sensitive sites can be mapped. One is in a region of the vector
previously shown to be S1-sensitive (Lilley, 1980). The other is in the
first intron of the human myoglobin gene and is mapped to this position by
data from all four restriction endonucleases.

| Enzyme	Endonuclease-generated fragments	Additional S1-generated fragments
HindIII	6.6	5.6_v
	3.4	2.05
		1.43
BglII	6.1	5.85
	3.8	3.15_v
EcoRI	8.5	8.1
	1.0	0.6
	0.4	
BamHI	6.5	5.0
	3.6	1.65
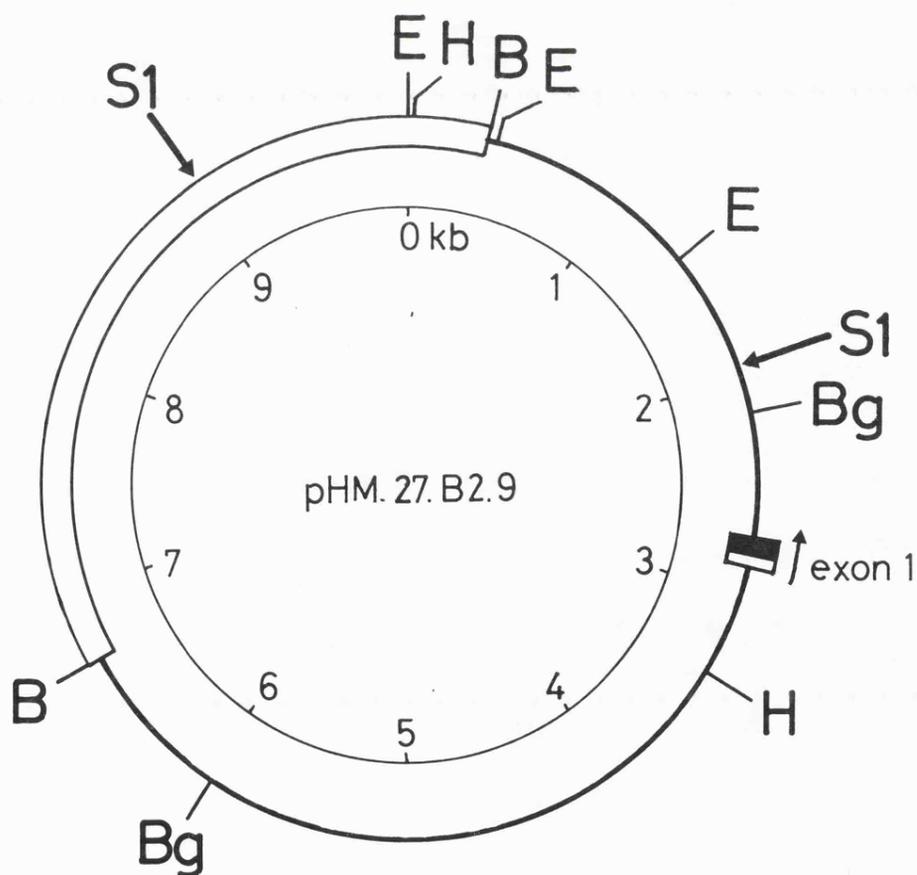
Figure 8.4   Treatment of pHM.27.B2.9 and pSM.19.5 with a high concentration of S1 nuclease and treatment of linear plasmid DNAs with S1 nuclease

A.   Treatment with a high concentration of S1 nuclease. Plasmid DNAs were digested with 0.5u/µl S1 nuclease, as described (2.14.1). DNA was recovered by phenol extraction and ethanol precipitation, then digested with HindIII (+), together with plasmid DNA which had not been S1-treated (-). These digests can be compared with those in Figure 8.2B. S1 nuclease-generated fragments are indicated and no additional S1-generated fragments are seen, although there is more non-specific degradation of DNA due to the increased concentration of S1 nuclease.

B.   S1 nuclease treatment of linear plasmid DNAs.   To test whether the S1 nuclease-sensitive site in the myoglobin intron sequence in pHM.27.B2.9 was also sensitive in the absence of supercoiling, plasmid DNA was first digested with BamHI, to generate two fragments, and then treated with a range of concentrations of S1 nuclease. Samples were electrophoresed directly in a 0.5% agarose gel.
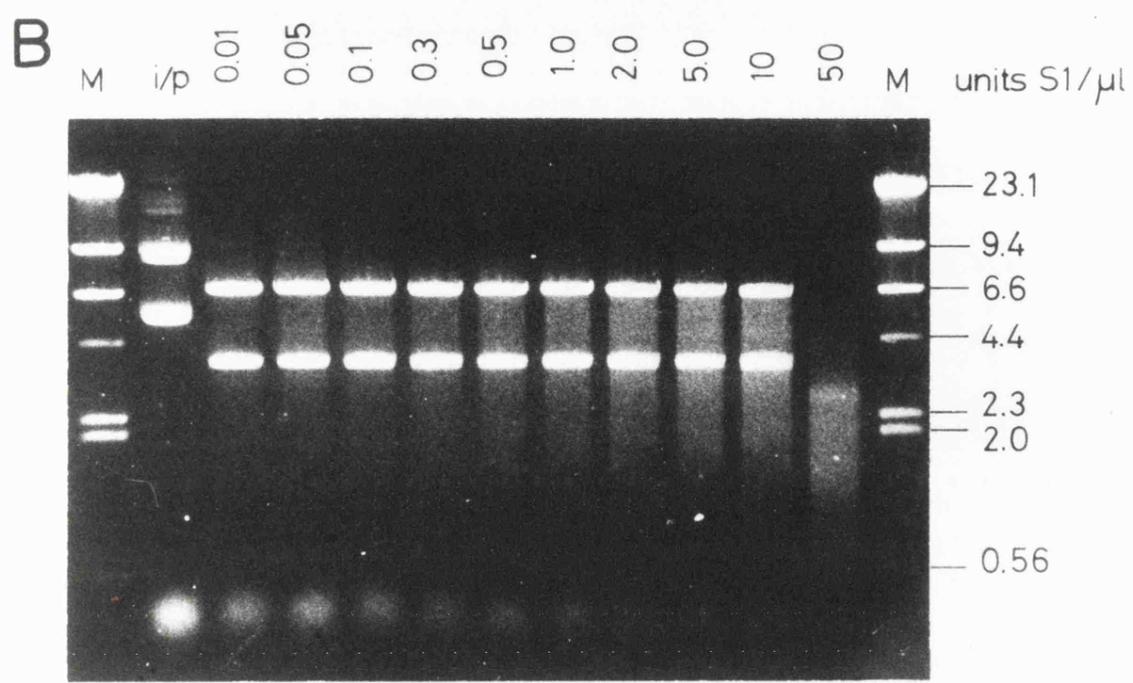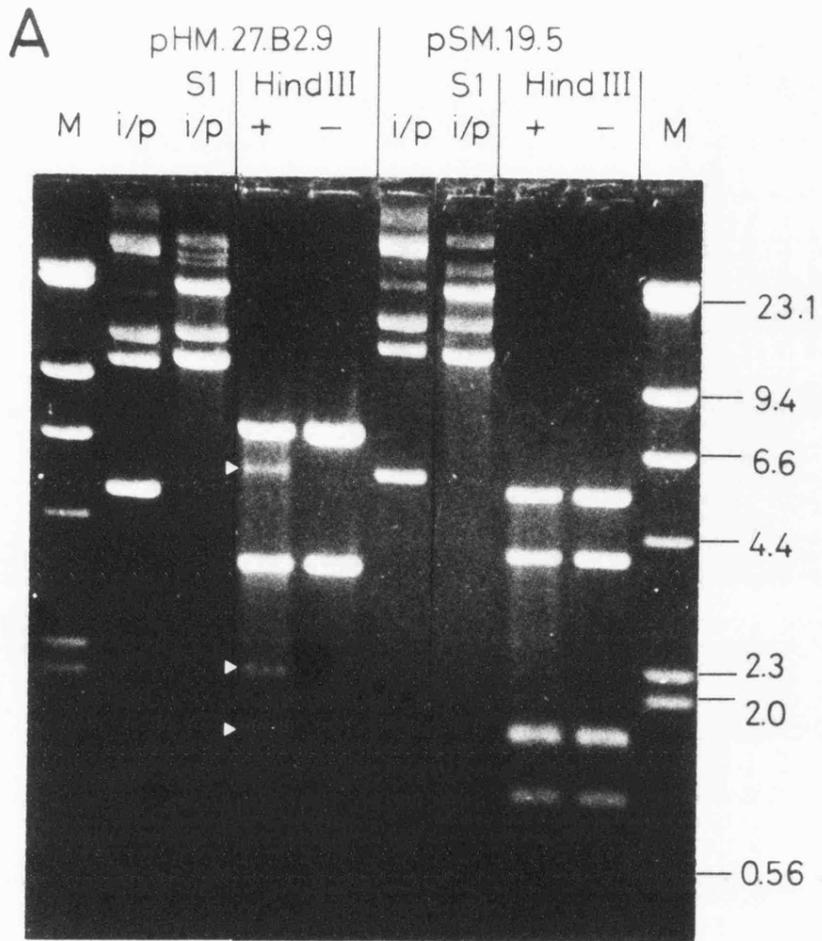
first intron of the myoglobin gene described above is therefore not S1-sensitive in linear DNA, i.e. in the absence of supercoiling.

8.4 Fine-scale mapping of the myoglobin first intron S1 nuclease-sensitive site

The strategy employed in fine-scale mapping of the S1 nuclease-sensitive site was to digest S1-treated and untreated plasmid DNAs with restriction endonucleases, as above, but then to end-label the restriction fragments with ^{32}P by a 'fill-in' reaction, using reverse transcriptase. Labelled DNAs could then be electrophoresed and the gel autoradiographed. Using restriction endonucleases with cleavage sites close to the S1-sensitive site, it is possible to visualise the small DNA fragments produced by S1 nuclease and to obtain a much more accurate position for the S1 nuclease-sensitive site.

pHM.27.B2.9 DNA was digested with 0.05u/μl S1 nuclease, as described previously. Aliquots of S1-treated and untreated DNAs were digested with the restriction endonucleases EcoRI, HpaII, BstEII and TaqI, all of which have cleavage sites within approximately 600bp of the S1 site, as mapped in the previous section. DNA was [^{32}P]end-labelled using reverse transcriptase and electrophoresed directly in a 2.5% agarose gel which was dried and exposed to X-ray film (Figure 8.5A). S1 nuclease-generated fragments are indicated, and their sizes are shown in Figure 8.5B. A map of the EcoRI- BglII fragment containing the S1-sensitive site is shown (Figure 8.5C,D). The sequence of this region has been completed and is shown in Figure 8.5E. Very unexpectedly this region is not AT-rich and contains no homocopolymer nor inverted repeat usually associated with S1 nuclease sensitivity [as tested by computer-mediated dot matrix analysis (Konkel et al., 1979)]. There are no notable features in this region of sequence and the basis of its S1 nuclease-sensitivity remains unknown. It is clear, however, that this site is S1-sensitive only in supercoiled DNA,

Figure 8.5 Fine scale mapping of the S1 nuclease sensitive site in the human myoglobin gene

A.   Autoradiograph of [³²P]end-labelled restriction endonuclease fragments of S1 nuclease-digested (+) and undigested (⁻) pHM.27.B2.9 DNA, separated by agarose gel electrophoresis in a 2.5% agarose gel. 0.5µg aliquots of DNA were digested with EcoRI, HpaII, BstEII or TaqI and were [³²P]end-labelled using reverse transcriptase by a 'fill in' reaction with [³²P]dCTP or [³²P]dATP, as appropriate. λ x HindIII plus pBR322 x Sau3A DNA fragments were also [³²P]end-labelled as size markers. Half of each digestion was then electrophoresed in a 2.5% agarose gel which was dried onto a glass plate. Exposure was for 2 hours. The digestion with HpaII is not complete. S1 nuclease-generated fragments are indicated by filled triangles.

B.   Estimated sizes of S1 nuclease-generated fragments indicated in A.

C.   Restriction endonuclease cleavage map of pHM.27.B2.9, showing cleavage sites for restriction endonucleases HindIII, BglII, EcoRI and BamHI as in Figure 8.3B. The approximate position of the S1 nuclease-sensitive site in the insert is shown. The bar represents the DNA fragment for which an enlarged scale map is given in D.

D.   Restriction endonuclease cleavage map of the BglII-EcoRI fragment indicated in C; EcoRI (E), HpaII (Hp), Taq(I), BstEII (Bs) and BglII (Bg). The approximate position of the S1 nuclease-sensitive site is shown as derived from data in B.

E.   DNA sequence of part of the first intron of the human myoglobin gene. No further sequencing into the intron has been carried out. The BstEII, TaqI and HpaII sites closest to the S1-sensitive site are indicated. The sequence available does not extend as far downstream as the EcoRI site, but Smith-Birnstiel mapping (3.7) shows that there are no additional BstEII or TaqI sites in this region. The region of sequence indicated by this experiment as being sensitive to cleavage by S1 nuclease is shown underlined.

A

EcoRI HpaII   BstEII TaqI
M | + | − | + | − | M | + | − | + | − | M

23,9,6 —
4.4 —
2.3 —
2.0 —
1.37 —
0.67 —
0.56 —
0.36,0.34 —
0.32 —
0.27,0.26 —
0.21 —
0.105 —
0.091 —
0.077 —

B
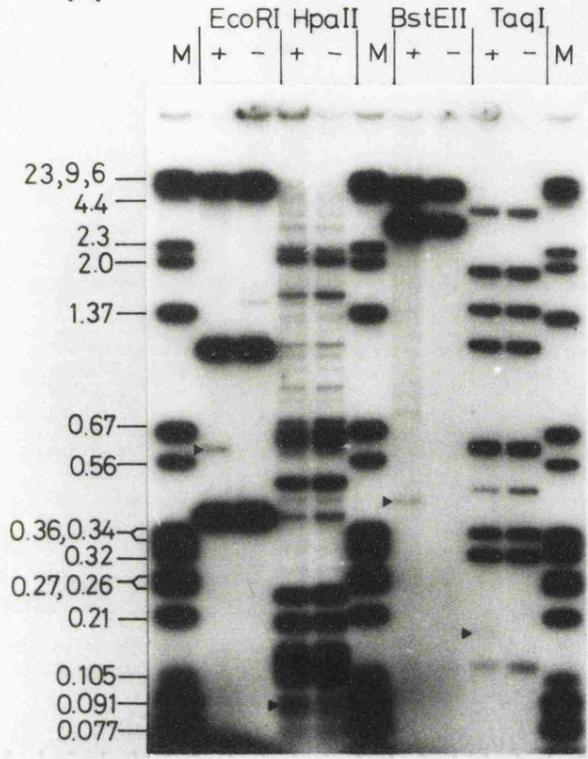
Restriction endonuclease	S1-generated fragment size /bp
EcoRI	615–620
HpaII	85–90
BstEII	440–450
TaqI	185–190

C

pHM.27.B2.9

E H B E
E
S1
Bg
B
Bg
H

D

E   Hp   S1   T   Bs Bg

100 bp

E
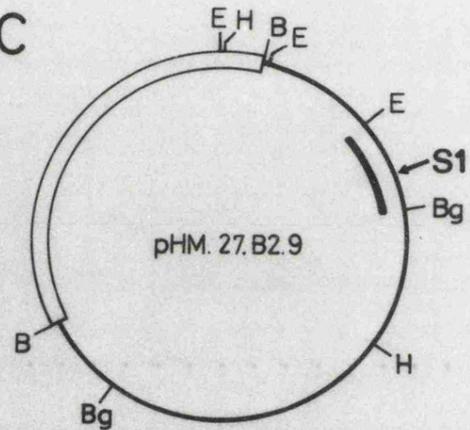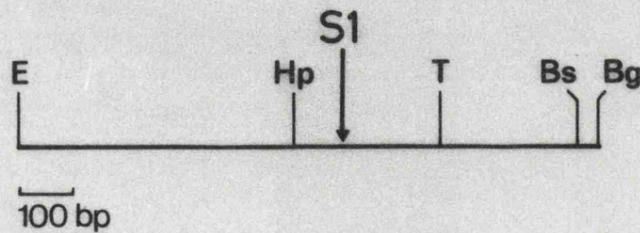
```
                                              BglII                                        BstEII
tgtcaaggttaagacatgctcttcaaggggagacagatctggttctagacttggctctgccactgagccactgggtgacc

tttgggaaggtactcaacctctcggagcctcaatttcctctcctgtacagtgaggggatatcctaatatctatatcctag

aggagagtgtgagaattaaataaaataatgcatgcaagaggcctggcatggttcctggcatatactgagtcctagaaatgt

tagtagctattactgatgaagcccaggctagggacctttcaaagaattgcaattagagaacagaagatagaggctcatta
     TaqI
ggatccttcgatgttgggtatgtctctagtttgagaggtctgaatgatgtggtctgcaagtatatcctgccttctaccac

aagggattccagaatacaccaaagaaaacaaaattctgaggtttgtaaatagagggtggctgtggtttgtacatagaagc

tcatctcctcgttgccttctatcccaaaggtgatacactcttctcttggcccccttcctcaccattctgagctggttccc
                                                        HpaII
tcagaagtctaataggttaggaatcaacgtttctgccaacgggaggaaggaagtgggcgccgg
```

and that the cleavage must occur at a fairly precise position as the small
S1-generated fragments used to map its position migrate as discrete bands
(see Figure 8.5A).

8.5 Summary

The transcriptional start site of the human myoglobin gene has been
located in adult cardiac and adult and foetal skeletal muscles using S1
nuclease protection mapping. The site is invariant in these tissues and
is at precisely the position predicted by homology with the seal gene.

Experiments designed to detect S1 nuclease-sensitive sites in
supercoiled plasmid DNAs detected an S1 sensitive site in the first intron
of the human myoglobin gene. Curiously, no sequence characteristics
obviously correlated with S1 nuclease-sensitivity were found in this
region.

Chapter 9

DISCUSSION

9.1 Myoglobin gene structure

The characterisation of the human myoglobin gene is described in

chapter 3, and the sequence of transcribed regions and considerable

lengths of 5' flanking and intron sequence is presented there. The amino

acid sequence from the cloned gene corresponds exactly with the published

human myoglobin sequence (Romero-Herrera and Lehmann, 1974), establishing

that the gene cloned is indeed the functional human myoglobin gene. The

grey seal also has a single myoglobin gene and, based on myoglobin protein

sequencing data, this is likely to be a general rule for the vertebrates.

The overall structures of the seal and human myoglobin genes are

very similar (Weller et al., 1984). The two introns interrupt the coding

sequence at precisely the same positions. These correspond to the intron

positions in all other vertebrate α- and β-globin genes. Like the seal

myoglobin gene, the human gene has remarkably elongated introns and

non-translated mRNA sequences when compared with those of α- and β-globin

genes. The first intron of the human gene is 5.8kb long, compared with

4.8kb in the seal gene, and 108-886 bp in α- and β-globin genes

(Blanchetot et al., 1983). The human myoglobin gene is therefore the

longest globin gene so far described. Characteristically elongated

non-coding regions are therefore not peculiar to the grey seal myoglobin

gene. The mouse myoglobin gene has also been cloned and sequenced

(Blanchetot et al., in preparation). The structure of the mouse gene is

similar to that of seal and human genes, but the second intron is

only ~1500bp, compared with ~3.5kb in human and seal genes, suggesting

that although myoglobin gene introns are characteristically long, their

precise length may be variable.

Figure 9.1 shows the sequence alignment of the 5' flanking region and the first exon of the seal and human myoglobin genes. The 5' flanking region of the human gene, like that of the seal, contains only one of the three conserved elements found in the 5' flanking regions of other globin genes and which are required for gene transcription. A normal TATA box is located 33bp upstream from the cap site (32bp upstream in seal). The CCAAT box, normally present at approximately -70 to -80, is absent. A sequence CCATT occurs at -67, but is not present in the seal gene and so is unlikely to be functionally important. The dimerised CACCC element in the -100 region of β-globin genes described by Dierks et al. (1983) is also absent. Instead there is a block of sequence, from -68 to -114 in the human gene, which is unusually purine-rich; 90% A and G. This purine-rich sequence also occurs in the seal gene, although there is no direct conservation of the precise sequence between them.

The overall level of sequence substitution in the non-coding regions of seal and human myoglobin genes is 35%, and shows no significant difference from the mean level of silent site divergence in coding sequences (Weller et al., 1984). Interestingly there is a conserved region extending upstream from the 'GAGA' sequence. Within this region, which is approximately 200bp long, the divergence falls from 35% to approximately 13%, a difference which is highly significant. Beyond this conserved region in the 5' direction, the divergence level returns to 35%. This conserved sequence box has also been found in the mouse gene (Blanchetot et al., in preparation). It is not known whether any of these 5' flanking region sequences are implicated either in the general control of myoglobin gene transcription or in maintenance of the different levels of myoglobin in human and seal muscle.

The cap site and polyadenylation site in the human myoglobin gene were originally located by homology with sites in the seal gene (Weller et al., 1984). The location of the cap site has also been directly

118

Figure 9.1 Sequence alignment of human and seal 5' flanking regions and exon 1

The position of the cap site is indicated and the regions which are transcribed into mature mRNA are in upper case. Substitutions are shown by asterisks. The TATA box and polypurine regions are indicated by boxes.

```
man    ccttctgtcggct-atgagagtccagacagt-gccaacctcctccc-cttcttccacacgcacaaccacccac--cccctgtgctgctgagctgtcc
          **    **    **        *              *        **  *  **              **    ****              *

seal   cccttctgctggcttctgagactccagacagtccagacctcctgctcttgccacatacacaaacaccccacgcccctggctgctgagttgtcc


man    tgcctcgccac-aatggcacctgcctaaaatagcttcccatgtgagggctagagaaaggaaaagattagacctccctg|gatgagagagagaaagtgaa|
          *          *           ***                  *       * * ***              *     *** ****   *

seal   tacctcccccaaatggcacctgcccaaaatagcttcc-atgtgagagctacagaaggaaaagattagacccgcat|gaaaagaggaggagggagga|

                                                                              CAP
man    |ggagggcaggggagggggacagcgag|ccattgagcgatctttgtcaagcatccagaag|tataaa|acgccctgggaccaggcagcctcaaACCCCAG
        *****  *  *  ***          ** * *  *     ***     *       *          ***                ***

seal   |gg-----aggagagagtgagcaag|ctgtcaagtgatctgtcaagcttctgggaaag|tataaa|tc-cctctgggccaggcgatctcaaACCCCAG

                                                              ini Gly Leu Ser Asp Gly Glu Trp Gln L
man    CTGTTGGGGCCAGGACACCCAGTGAGCCCCATACTTGCTCTTTTTGTCTTCTTCAGACTGCGCCATG.GGG.CTC.AGC.GGG.CTC.AGC.GGG.GAA.TGG.CAG.T
         **     *               ***                           *
                                                              ini Gly Leu Ser Asp Gly Glu Trp His L
seal   CTGTCAGAGCCAGGACACCGAGTCAGCCGGGACTTGCTCTTCCTCAGACTGCCTGCTCTTCCTCAGACTGCCACCATG.GGG.CTC.AGC.GGC.GAC.TGG.CAC.T


       eu Val Leu Asn Val Trp Gly Lys Val Glu Ala Asp Ile Pro Gly His Gly Gln Glu Val Leu Ar(q)
man    TG.GTG.CTG.AAC.GTC.TGG.GGG.AAG.GTA.GAG.GCT.GAC.ATC.CCA.GGC.CAT.GGG.CAG.GAA.GTC.CTC.ATC.AGtaaaggaag
                                            *   *           *
       eu Val Leu Asn Val Trp Gly Lys VAl Glu Thr Asp Leu Ala Gly His Gly Gln Glu Val Leu Ile Ar(g)
seal   TG.GTG.CTG.AAC.GTC.TGG.GGG.AAG.GTG.GAG.ACT.GAC.CTC.GCG.GGC.CAT.GGG.CAG.GAG.GTC.CTC.ATC.AGtaaaggaag
```

determined (see chapter 8). The sequence data predict a human myoglobin

mRNA 1066nt long, excluding the poly(A) tail. This is similar to the

1083nt seal myoglobin mRNA (Blanchetot et al., 1983). Human and seal

myoglobin mRNAs were found to be indistinguishable in size in Northern

blot analysis, both migrating at approximately 1400nt. In contrast, the

mouse myoglobin mRNA is smaller, migrating at approximately 1200nt and is

200nt shorter than the human myoglobin mRNA, due to a shorter 3'

non-translated region (Blanchetot et al., in preparation).

S1 nuclease-sensitivity of supercoiled myoglobin gene DNA was

investigated. S1 nuclease-sensitive sites in globin chromatin in chick

red cell nuclei have been found to parallel DNase I hypersensitive sites

in approximate location and developmental specificity (Weintraub et al.,

1982). Such regions which often, but not always, occur to the 5' side of

actively transcribed genes, have been hypothesised to be important in gene

control (Weintraub, 1983). Such S1-sensitive sites are retained following

transfection of DNA into a different cell type, suggesting that the

potential to form secondary structures in chromatin is a dominant

characteristic of the DNA sequence itself (Weintraub, 1983). These sites

were found to retain S1 nuclease-sensitivity in supercoiled, but not

linear plasmids (Larson and Weintraub, 1982). The experiments described

in chapter 8 failed to detect any S1 nuclease-sensitive sites in the 5'

flanking regions of either human or seal myoglobin genes. DNase I

sensitivity of myoglobin genes in chromatin, however, has not been

investigated, although the myoblast cell culture system would be ideal for

such experiments.

However, an S1 site was mapped to a ~20bp region approximately 900bp

into the first intron of the human myoglobin gene (Figure 8.5). A range

of sequence types has been shown to be sensitive to S1 nuclease in

negatively supercoiled DNA. These presumably can adopt conformations

susceptable to S1 cleavage and include closely spaced inverted repeats

(Lilley, 1980), direct repeats (Htun et al., 1984), AT-rich regions (Hofstetter et al., 1976), polypyrimidine.polypurine stretches (Htun et al., 1984) and junctions between B-DNA and Z-DNA (Singleton et al., 1982). The human myoglobin gene intron 1 S1 nuclease-sensitive site was not found to contain any characteristics likely to cause S1 nuclease-sensitivity. The location of the site within myoglobin gene intron sequence suggests that it may not be important in regulation of myoglobin gene expression. However, control elements including the immunoglobulin heavy and light chain enhancers have been found in intron sequence within their transcription units (Gillies et al., 1983; Picard and Schaffner, 1984).

9.2 Chromosome assignment of the human myoglobin gene

The divergence of myoglobin from the ancestral haemoglobin gene occurred 500-800 mya (Hunt et al., 1978; Czelusniak et al., 1982) and preceded the α/β globin gene duplication. The α- and β-globin gene clusters are unlinked in birds and mammals but a single globin gene cluster containing both α- and β-globin genes is found in Xenopus tropicalis (Jeffreys et al., 1980). This gene arrangement is consistent with the hypothesis that the α- and β- globin genes originally arose as the result of a tandem duplication. This linkage has been maintained in some amphibians, but the two genes became dispersed in the line leading to mammals and birds. It was therefore possible that the myoglobin gene, which also arose by gene duplication, may still be linked to the α- or β- globin gene cluster.

Chromosome assignment of the human myoglobin gene was therefore carried out using probes from the gene to detect its presence in a series of human/rodent somatic cell hybrids containing various complements of human chromosomes (Jeffreys et al., 1984). The myoglobin gene was found to cosegregate with chromosome 22. Cell hybrids containing translocations

of chromosome 22 were used to localise the myoglobin gene to 22q11-22q13. The myoglobin gene is therefore not linked with either the the α-globin cluster in the region 16p12-16pter (Barton et al., 1982), or the β-globin cluster in the region 11p15-11pter (De Martinville and Franke, 1983), and therefore represents a third dispersed globin locus. However, it is possible that other vertebrates retain linkage between myoglobin and haemoglobin genes, and it would be interesting to look at the location of the myoglobin gene in Xenopus tropicalis.

9.3 Tissue-specific and developmental expression of the myoglobin gene

Chapters 4 and 5 describe the estimation of myoglobin mRNA concentrations in various adult and foetal human muscles. In conjunction with this work, myoglobin protein levels were measured by Drs Yvonne Edwards and Harvey Isenberg at the MRC Mammalian Biochemical Genetics Unit, University College, London (Weller et al., in preparation).

The mRNA levels and corresponding myoglobin protein levels for a number of muscle types are shown in Table 9.1. Figures are also expressed as a percentage of the concentration of protein or mRNA in adult skeletal muscle. The measurement of the abundance of seal myoglobin mRNA, described in Chapter 7, allows calculation of the myoglobin mRNA concentration in all other tissues studied. An indication of the translational capacity of each myoglobin mRNA can also be made by comparing the myoglobin protein and mRNA levels.

Levels of myoglobin in different muscle tissues (and also throughout myogenesis) appear to be determined by the size of the myoglobin mRNA pools, suggesting that changes in levels of myoglobin may be largely under transcriptional control (Table 9.1). Myoglobin mRNA is a muscle-specific transcript and is not detectable in non-muscle tissues of the mouse (Chapter 4).

Table 9.1. Myoglobin protein and mRNA levels in various muscle tissues.

[1] µg of myoglobin mRNA / mg poly(A)$^+$ RNA

[2] mg of myoglobin / µg myoglobin mRNA

Approximate myoglobin protein concentrations, given in mg/g (wet weight), were assessed by comparative protein staining and immunoanalysis, as described (Weller et al., in preparation). Protein concentrations marked '*' were derived from absorbance measurements (543nm) of myoglobin purified by isoelectric focusing (see Chapter 6). Myoglobin levels are also given as a percentage of the appropriate adult skeletal muscle level. Myoglobin mRNA levels are given relative to levels in adult skeletal muscle of the species concerned, and also as absolute levels, derived from the abundance of seal myoglobin mRNA in poly(A)$^+$ RNA (see Chapter 7). Estimates were derived from signal intensity of RNA samples titrated on Northern blots, and in the case of human foetal skeletal muscle, confirmed by measurement of autoradiographic signal intensity using scanning densitometry (Chapter 4).

Table 9.1. Myoglobin protein and mRNA levels in various muscle tissues

Species	Muscle type	Protein levels		mRNA levels		
		conc.mg/g	% adult	% adult	μg/mg poly(A)+RNA[1]	mg Mb/ μg Mb RNA[2]
HUMAN	adult skeletal	7.5, 6→8*	(100)	(100)	4.5	1.7
	adult cardiac	~7.5	~100	~100	~5	~1.5
	adult smooth: bladder gut uterus	0.015→0.030	0.2-0.4	~1	~0.05	~0.5
	foetal skeletal					
	1st trimester (10 wks)	0.015	0.2	1→4	0.05→0.2	~0.5
	2nd trimester	0.030→0.075	0.4→1.0			
	foetal cardiac (20 wks)	0.375	5	~10	~0.5	~1.0
MOUSE	adult skeletal	0.3→0.5	(100)	(100)	4.5	0.1
	adult cardiac	1.0	200→300	400	18	0.1
	foetal skeletal:					
	14/15 days gestation	0.003	1	1	0.05	0.1
	birth	0.075→0.090	25→30	10	0.5	0.2
SEAL	adult skeletal	40→50*	(100)	(100)	3+	1.3

i) <u>Tissue-specific expression</u>

Measurement of myoglobin in human skeletal muscle, by Western

blotting and spectrophotometric methods (Weller <u>et al</u>., in preparation;

Chapter 8) are in good agreement (Table 9.1). A value of 7mg/g wet weight

of tissue is slightly higher than the previously published levels, which

average approximately 5mg/g (eg. Moller and Sylven, 1981). Contrary to a

previous report (Fasold <u>et al</u>., 1970), it is now established that the

myoglobin gene is expressed in human smooth muscle, although the levels

are very low, probably less than 1% of the skeletal muscle level. It is

not known whether myoglobin is expressed at this level in all smooth

muscle cells, or at a higher level in a sub-population of cells.

Myoglobin in a single cardiac muscle sample was approximately 2-fold

higher than found previously (e.g. Biorck, 1949) and similar to that in

skeletal muscle. Variation between cardiac myoglobin mRNA concentrations

in the two tissue samples was, however, considerable. Interestingly, both

cardiac myoglobin and myoglobin mRNA levels in mouse heart are

several-fold higher than in skeletal muscle. Wittenberg (1970) has

pointed out that mammalian cardiac myoglobin concentrations appear to be

invariant (approximately 3mg/g). The level in mouse cardiac muscle,

however, is three-fold less than this value (Table 9.1).

S1 nuclease protection mapping has shown that the differential

expression of the myoglobin gene in cardiac and skeletal (and also foetal

skeletal) muscles in man is not accompanied by transcription of the

myoglobin gene from any alternative promoter or cap site.

Myoglobin levels can be modulated by external factors which affect

the work load and/or physiology of the muscle. These include altitude

acclimatisation and increased exercise, where the myoglobin concentrations

have been shown to increase only in the exercised muscles (Pattengale and

Holloszy, 1967). The mechanisms by which levels of myoglobin in specific

tissues are controlled during development and adaptation are unknown. It

would be interesting to see whether myoglobin protein levels are paralleled by changes in mRNA levels in these instances.

It is interesting that adult smooth muscle appears structurally undifferentiated, lacking the orderly arrangement of the contractile apparatus seen in striated muscles. This may reflect a reduced requirement for facilitated oxygen diffusion. However, the metabolism of smooth muscle has been described as being similar to that of red skeletal muscle, although the levels of the oxidative enzymes are lower (Bass et al., 1969). It is possible that the expression of myoglobin at characteristically higher levels in striated muscle is in some way correlated with gene expression within the striated muscle phenotype.

ii) Developmental expression

Longo et al. (1973) compared myoglobin concentrations in different foetal lamb muscles and found that the myoglobin concentration in diaphragm was high: 20% of the adult diaphragm myoglobin level, compared with barely detectable foetal skeletal muscle myoglobin levels. The lamb foetal heart was found to contain 50% of the adult myoglobin level. This led to the suggestion that levels of myoglobin in foetal tissues are related to their use in utero. The foetal heart is functional from very early in development and the diaphragm has been found to contract periodically in utero in movements not unlike respiratory movements (Dawes et al., 1972). Skeletal muscle, however, although functional from approximately 8 weeks gestation in man (Arey, 1974), does not experience a significant work load until after birth.

The human myoglobin gene was found to be expressed in skeletal muscle at low levels (approximately 1% of adult) as early as 10 weeks gestation, which is considerably earlier than demonstrated previously. Muscle differentiation begins in the fifth week and muscles are capable of contraction by the eighth week (Arey, 1974). Kagen and Christian previously detected foetal skeletal muscle myoglobin at 0.5% of the adult

123

level at 20 weeks gestation, using an immunological assay (Kagen and Christian, 1966). In contrast, cardiac muscle of the same foetal age contains significantly more myoglobin (see Longo et al., 1973) and myoglobin mRNA (Table 9.1), which presumably reflects its higher work load in utero [the human heart is functional from the fourth week in gestation (Gilmour, 1941)]. The glyceraldehyde-3-phosphate dehydrogenase gene is also found to be expressed at relatively higher levels in the foetal heart than in skeletal muscle (Edwards et al., 1985).

The levels of myoglobin mRNA in skeletal muscle must increase by approximately 50 to 100-fold before attaining adult levels. Study of myoglobin and myoglobin mRNA levels in the mouse showed that this dramatic increase was gradual, with no abrupt increases, for example, at birth. Adult levels were not attained until some weeks after birth. This is in marked contrast to the muscle contractile proteins, the mRNA levels of which do not vary significantly (see Figure 5.4).

The developmental appearance of myoglobin can be described in relation to other changes occuring during myogenesis. Expression of the myoglobin gene has been shown to be induced following cell fusion in two embryonic muscle myoblast cell lines, indicating that myoglobin expression normally starts at the myoblast fusion stage, very early in myogenesis (see Chapter 5). Myoglobin protein was detected at very low levels in mouse skeletal muscle at 14 days gestation (Weller et al., in preparation) and myoglobin mRNA at 15 days gestation, which is equivalent to 6 to 7 weeks gestation in man (Chapter 5). Myoglobin mRNA levels were not investigated in muscle earlier than 15 days gestation. This is consistent with the embryological observations of muscle development; skeletal muscle myoblasts appear at 11 days gestation and myotubes at 14 to 15 days (Rugh, 1968). Myoglobin and myoglobin mRNA were detected at low concentrations (~1% adult) at the equivalent gestational stage in human skeletal muscle.

Levels of both myoglobin mRNA and protein increase steadily in the

mouse throughout pre- and post-natal development, reaching ~10-30% of

adult levels at birth and achieving the adult level some weeks later.

Similarly, other muscle characteristics are not apparent until some time

after birth, including complete histochemical differentiation of fibre

types (Briskey, 1970), contractile properties characteristic of fast

muscle (Brown, 1973; Buller et al., 1960a,b) and loss of polyneural

innervation (O'Brien et al., 1978). The time course of the appearance of

myoglobin in human muscle is not so clear, although it is likely to be

gradual, as in the mouse; Kagen and Christian (1966) found that skeletal
                myoglobin
muscle is approximately 10% of the adult level at birth and 36% at 17

months. Nothing is known of myoglobin mRNA levels at these stages. In

contrast to the situation in the mouse, human muscle fibre

differentiation, as judged by histochemical staining, is complete as early

as 20 weeks gestation (Briskey, 1970), when the levels of myoglobin and

myoglobin mRNA are very low. The developmental expression of the

myoglobin gene relative to the timing of other aspects of myogenesis in

these two species is therefore not the same. These observations support

the hypothesis that myoglobin concentration in developing muscle is

related to work load in utero (Longo et al., 1973), such that myoglobin

concentration increases to approximately adult levels at the same time as

fibre differentiation is completed in the mouse (~2 weeks postnatal, see

Dubowitz, 1970) but remains low in human foetal skeletal muscle, even

though fibre differentiation is complete.

9.4 The induction of myoglobin expression in early myogenesis

        Myoglobin gene induction was found to occur on differentiation of G8

and L6 rodent myoblast cell lines. Myoglobin mRNA was found at higher

levels in G8 than L6 and could not be detected in myoblasts from either

line. It is not known whether the myoglobin mRNA is translated in these

cells.

The metabolism of myoblasts changes on fusion and comes to rely increasingly on oxidative pathways: myotubes have been found to be more sensitive than myoblasts to antimycin A, an inhibitor of the electron transport system (Konigsberg, 1964). The metabolism of primary cultures of fused myoblasts has been shown to depend on the muscle from which the myoblasts were originally derived. Nougues and Bacou (1977) found a significantly higher level of the oxidative enzyme isocitrate dehydrogenase in muscle fibres formed by in vitro differentiation of ten day embryonic chick adductor magnus muscle (red) myoblasts than in fibres from pectoralis major (white) myoblasts. Heywood et al. (1973) found that myoglobin is synthesised in culture two days after the fusion of 11 day embryo chick leg muscle, but is not detected in breast muscle cultures. This shows that characteristic metabolic differences between red and white muscle, including myoglobin concentrations, are established very early in myogenesis.

There has been other work reported on the expression of the myoglobin gene in early chick myogenesis. Kagen et al. (1969) describe the use of an immunoprecipitation method using myoglobin antiserum to precipitate newly synthesised myoglobin from minced muscle preparations incubated with [^{14}C]lysine. They reported that preparations of embryonic chick heart synthesised myoglobin at an enormously high rate; 5 to 10% of TCA-precipitable material. Myoglobin synthesis in 1 day chick embryos was claimed to account for 7 to 10% of the synthesis of total soluble proteins, and this high level also increased with age (Kagen and Linder, 1970). Using the same assay, Kagen and Freedman (1974) claimed that myoglobin synthesis in differentiating primary cultures of embryonic chick myoblasts was related to culture calcium concentration in addition to cell fusion, and could be stimulated by cAMP to account for up to 20% of total protein synthesis. Use of this assay to estimate myoglobin synthesised in embryos in vivo, however, provided conflicting results. Incorporation of

[^{14}C]lysine into myoglobin was not found in cardiac muscle until after 6 days and much later in skeletal muscle (Kagen and Freedman, 1973). In contrast, using a different in vitro protein synthesis system to estimate myoglobin synthesis, Low and Rich (1973) found that myoglobin began to accumulate only after day 16, and was found to be around 15% of the adult level on hatching (at 21 days).

It is extremely unlikely that myoglobin synthesis occurs either as early as, or at levels as high as those reported by Kagen and coworkers. Fortunately, the necessity for an indirect method for measuring gene expression can be overcome to a large extent with the use of cloned gene sequences, whereby mRNA levels for a particular protein can be determined directly.

The discovery that expression of the myoglobin gene is induced, together with the contractile protein genes, in embryonic myoblast cell lines following cell fusion and differentiation, raises the possibility that there may be similarities in the regulatory mechanisms of myoglobin and contractile protein genes. Modulation of myoglobin expression during later myogenesis, however, does not parallel that of the contractile proteins (see Chapter 5). Regulation of muscle gene expression in myogenesis is complex, and as yet we know very little of the mechanisms of gene induction in myogenesis (see section 1.5). The contractile protein genes are not all expressed from the same time in myogenesis and there is switching of isoforms expressed, both during development and as a response to external stimuli. However, it would be interesting to look at the appearance of myoglobin mRNA for comparison with that of other characterised muscle gene transcripts over the period of myoblast fusion and differentiation.

The expression of the myoglobin gene in differentiating myoblasts in culture offers an attractive system, using transfection techniques, to define sequences involved in the induction of the myoglobin gene. The

127

role of a long, highly-conserved region found 5' to human, seal and mouse myoglobin genes (Blanchetot et al., in preparation) in particular could be investigated.

9.5 Adaptations in the expression of myoglobin

As discussed in Chapter 1, some animal species have elevated muscle myoglobin levels as an adaptation to a particular physiology or habitat. A comparison of in vivo myoglobin and myoglobin mRNA pool levels in seal and man was made in an attempt to determine at which level(s) the adaptation leading to high levels of myoglobin in the seal occurred.

It was first neccessary to quantify the myoglobin concentration in seal muscle, compared with human. Great precision was not required for a number of reasons: the difference in myoglobin concentrations between the two species was more important than their absolute values. In addition, the age and muscle type of the two species could not be matched. The seal was a juvenile and the muscle-type was unknown. The human sample was gastrocnemius, an amputation from an elderly individual. Approximate myoglobin concentrations were calculated, however, and are shown in Table 9.2, together with representative values from other species. The values determined are in good agreement with those previously published for both human muscle and muscle from a range of Phocinae species. The grey seal has 6 to 7 times as much myoglobin as man.

There is some evidence that myoglobin concentration is correlated with the number of type I fibres in a particular muscle (Morita et al., 1970). However, fibre ratios cannot be the only factor involved. The myoglobin concentration in ox muscle is 30 times that in rabbit [6mg/g and 0.2mg/g, respectively (Lawrie, 1953a)]. Type I fibres in these tissues, however, were only 5 to 6 times more abundant in ox muscle [22% and 4%, respectively, of muscle fibres in these species (Morita et al., 1970)]. Some of the observed difference in myoglobin levels must therefore be

Table 9.2   Skeletal muscle myoglobin levels for a range of species

Species	Mb mg/g wet weight	Reference
seals		
harbour seal	75	Robinson (1939),
	35	Hapner et al. (1968)
ribbon seal	81	Lenfant et al. (1970)
harp seal	7	George et al. (1971)
grey seal	50	This work
Other diving species		
sperm whale	90	Scholander (1940)
bottlenose whale	90	"
blue whale	10	Lawrie (1952)
porpoise	38	Blessing (1972)
dolphin	35	Eichelberger (1939)
sea otter	26	Lenfant et al. (1970)
pengiuns		
adelie, chinstrap	33	Weber (1974)
gentoo	44	"
Terrestrial species		
man	5	e.g. Moller and Sylven (1981)
man	7	this work, Weller et al. (in preparation)
pig	4.5	Lawrie (1950)
horse	7	"
guinea pig	1	Tappan and Reynafarje (1957)
rabbit	0.2	Lawrie (1952)
mouse	0.3-0.5	Weller et al. (in preparation)

attributable to an increased concentration within individual fibres. However, it would be interesting to know the distribution of type I and type II muscle fibres in the grey seal. In the harp seal, the increased level of myoglobin, although not great, can be attributed to increased staining of individual type I fibres for myoglobin, rather than to a significant increase in the number of type I fibres. Some variation was, however, found in fibre ratios in different muscles (George et $al.$, 1971).

Levels of myoglobin mRNA in the same seal and human muscle tissues used to measure myoglobin concentrations showed a difference of approximately 8-fold. Moreover, seal and human poly(A)$^+$ RNAs were found to direct the synthesis of myoglobin in an equivalent ratio (Chapter 6). This indicates that the level of myoglobin in the grey seal is correlated with, and presumably determined by, the size of the myoglobin mRNA pool, and that there is no difference in the translational capacities of seal and human myoglobin messages (see Table 9.1). The evolutionary basis of the adaptive increase in myoglobin levels in the grey seal might therefore be accounted for solely by an increase in the size of the myoglobin mRNA pool. This could be due either to increased transcriptional activity or enhanced myoglobin mRNA stability, or both. Similarly, an increase in myoglobin mRNA pool level in tissue as a whole could result from either an increase in the proportion of muscle fibres expressing the gene (Type I) or an increase in pool levels within individual fibres, or both.

That myoglobin mRNA is an extremely abundant muscle mRNA in grey seal skeletal muscle was first suggested following cDNA cloning of the seal muscle poly(A)$^+$ RNA, when 4% of clones were found to hybridise with the myoglobin cDNA (Wood et $al.$, (1982). This high myoglobin mRNA concentration has been confirmed using a novel hybridisation assay, described in Chapter 7. The advantages of this assay are that the hybridisation between the single-stranded DNA probe and the mRNA can be determined directly by agarose gel electrophoresis and autoradiography,

without the need for the S1 nuclease digestion, purification of S1 resistant RNA/DNA hybrids and scintillation counting required for other methods (e.g. Deely et al., 1977). It is possible to use this method to measure message abundance using a probe which has less than 100% homology with the mRNA, difficult with methods using S1 nuclease. The assay gave the value of the abundance of seal myoglobin mRNA as 3.4% of total poly(A)$^+$ RNAs, in good agreement with the 4% figure from the cDNA cloning. Myoglobin mRNA must therefore be one of the most abundant mRNAs in seal skeletal muscle. Having determined the concentration of myoglobin mRNA in one tissue, absolute myoglobin mRNA levels in all human, seal and mouse tissues could then be calculated (Table 9.1).

An interesting exception to the observation that different myoglobin levels in muscle are determined directly by myoglobin mRNA pool levels is expression of myoglobin mRNA in the mouse. Levels of myoglobin protein in the mouse are low (see Tables 9.1 and 9.2), as in other small rodents, which tend to have predominantly fast skeletal muscle motor units (James, 1968; Davies and Gunn, 1971) and lower numbers of type I fibres (Morita et al., 1970). Western blotting has shown that the level of myoglobin in mouse skeletal (limb) muscle is 0.3-0.5mg/g, approximately 20 times lower than in man (Weller et al., in preparation). Surprisingly, the level of myoglobin mRNA in mouse muscle is comparable to that in man (Chapter 4). This difference may be due to a low translational efficiency of the mouse myoglobin mRNA, reduced by at least an order of magnitude compared with man and seal (Table 9.1). However, a reduced translational efficiency has yet to be shown directly by in vitro translation experiments.

The basis for such a low translational efficiency of mouse myoglobin mRNA is unclear. There is no significant difference in the pattern of codon usage between the human and mouse myoglobin genes (not shown). Pelletier and Sonenberg (1985) have recently shown that the efficiency of translation of herpes simplex thymidine kinase mRNA can be

reduced by introducing inverted repeats into the 5' non-translated region of the gene to increase the secondary structure of the mRNA. It is possible that differences in 5' secondary structure may be found between the mouse and human myoglobin mRNAs. It has been shown that single base changes in the immediate vicinity of the initiation codon can alter the translational efficiency of the rat preproinsulin gene by as much as 15-fold (Kozak, 1984). Thus it is possible that only a single change may have occurred at the DNA level to account for the large observed difference in translational efficiencies of the human and mouse myoglobin mRNAs.

Two different evolutionary adaptations in myoglobin levels compared with that in man (elevated myoglobin in the grey seal and low myoglobin in the mouse) therefore appear to have been achieved in different ways. The seal has an elevated myoglobin mRNA level as a result of an increased mRNA pool level and the mouse, which has a myoglobin mRNA level similar to that in man, apparently as a result of a reduced translational efficiency of the myoglobin mRNA. It is interesting that each adaptation appears to have been achieved by a change in only one level of the myoglobin expression process and not by cumulative changes in more than one step. It is, however, possible that the increased mRNA levels in seal result from many individual changes at the DNA level.

The finding that high myoglobin levels in the seal are reflected in equally high mRNA levels suggests that it may be possible to determine the molecular basis of this elevated expression. Myoglobin gene expression is an ideal system in which to investigate evolutionary adaptation at the DNA level. By identifying sequences responsible for the elevated expression in seal, it might be possible to distinguish DNA sequence differences between human and seal genes which led to increased expression in seal (and which have presumably been acquired as a result of selection) from those which are neutral and have no effect on myoglobin expression. We

131

can ask whether the increased myoglobin expression in seal has resulted from a small number of stepwise changes at the DNA level, or from a larger number of small cumulative changes. In this way we may gain a greater understanding of the general process of adaptation at the molecular level.

The myoblast system provides a model in which this can be investigated. It should be possible to use transfection techniques to introduce human and seal myoglobin genes into these cells, induce expression by fusing the cells and assay transcriptional activity. The transcription from the human and seal myoglobin gene promoters, fused to the E.coli chloramphenicol acetyl transferase gene, could be measured to establish whether the observed difference in human and seal mRNA pools were due to a difference in transcriptional activity. If this were so, a series of seal/human myoglobin hybrid promoters could be made and assayed for their activity in the myoblast cell system. In this way it may be possible to identify specific sequences or promoter elements responsible for maintenance of the observed difference in human and seal myoglobin mRNA pool levels.

BIBLIOGRAPHY

Aaij,C and Borst,P. 1972. The gel electrophoresis of DNA. Biochem. Biophys. Acta 269:192-200.

Ames,G.F-L. 1974. Resolution of bacterial proteins by polyacrylamide gel electrophoresis on slabs. J.Biol.Chem. 249:634-644.

Anstrand,I., Anstrand,P., Christensen,E.H. and Hedman,R. 1960. Myohaemoglobin as an oxygen store in man. Acta Physiol.Scand. 48:454-460.

Antoine,M. and Neissing,J. 1984. Intron-less globin genes in the insect Chironomus thummi thummi. Nature 310:795-798.

Antonini,E and Brunori,M. 1971. Haemoglobin and myoglobin in their reaction with ligands. North Holland Publishing company, Amsterdam.

Arey,L.B. 1974. Developmental anatomy. W.B.Saunders Company, Philadelphia.

Aviv,H. and Leder,P. 1972. Purification of biologically active globin mRNA by chromatography on oligothymidilic acid-cellulose. Proc.Natl.Acad.Sci.USA 69:1408-1412.

Barr,L.,Dewey,M.M. and Berger,W. 1965. Propagation of action potentials and the structure of the nexus in cardiac muscle. J.Gen.Physiol. 48:797-823.

Barrie,P.A. 1982. A study on the β-globin gene cluster in man and the primates. PhD thesis, University of Leicester, 1982.

Barany,M. and Close,R.I. 1971. The transformation of myosin in cross-innervated rat muscles. J.Physiol. 213:455-474.

Barton,S., Malcolm,S., Murphy,C. and Ferguson-Smith,M.A. 1982. Localisation of the human α-globin gene cluster to the short arm of chromosome 16 (16p12-16pter) by hybridisation in situ. J.Mol.Biol. 156:269-278.

Basi,G.S., Boardman,M. and Storti,R.V. 1984. Alternative splicing of a Drosophila tropomyosin gene generates muscle tropomyosin isoforms with different carboxy-terminal ends. Mol.Cell.Biol. 4:2828-2836.

Bass,A., Brdiczka,D., Eyer,P., Hofer,S. and Pette,D. 1969. Metabolic differentiation of distinct muscle types at the level of enzyme organisation. Eur.J.Biochem. 10:198-206.

Batra,S. 1973. The role of mitochondrial calcium uptake in contraction and relaxation of the human myometrium. Biochem.Biophys.Acta 305:428-432.

Beatty,C.H. and Bocek,R.M. 1970. Biochemistry of red and white muscle. In The physiology and biochemistry of muscle as a food II. eds: Briskey,E.J., Kassens,R.G. and Marsh,B.B. University of Wisconsin Press.

Benton,W.D. and Davis,R.W. 1977. Screening λgt recombinant clones by hybridisation to single plaques in situ. Science 196:180-182.

Benvenisty,N., Mencher,D., Mehuhas,O.,Razin,A. and Reshef,L. 1985.
Sequential changes in DNA methylation patterns of the rat
phosphoenolpyruvate carboxykinase gene during development.
Proc.Natl.Acad.Sci.USA. 82:267-271.

van den Berg,J. 1978. Comparison of cloned rabbit and mouse β-globin
genes showing strong evolutionary divergence of two homologous pairs
of introns. Nature 276:37-44.

Berk,A.J. and Sharp,P.A. 1977. Sizing and mapping of early adenovirus
mRNAs by gel electrophoresis of S1 endonuclease-digested hybrids.
Cell 12:721-732.

Bestor,T.H., Hellewell,S.B. and Ingram,V.M. 1984. Differentiation of two
mouse muscle cell lines is associated with hypomethylation of their
genomes. Mol.Cell.Biol. 4:1800-1806.

Biggin,M.D., Gibson,T.J. and Hong,H.F. 1983. Buffer gradient gels and ^{35}S
label as an aid to rapid DNA sequence determination.
Proc.Natl.Acad.Sci.USA. 80:3963-3965.

Billeter,R., Heizmann,C.W., Howland,H. and Jenny,E. 1981. Analysis of
myosin light and heavy chain types in single human skeletal muscle
fibres. Eur.J.Physiol. 116:389-395.

Biorck,G. 1949. On myoglobin and its occurrence in man. Acta.Med.Scand.
(suppl 226).

Birnboim,H.C. and Doly,J. 1979. A rapid alkaline extraction procedure for
screening recombinant plasmid DNA. Nucl.Acids Res. 7:1513-1523.

Blanchetot,A., Wilson,V., Wood,D. and Jeffreys,A.J. 1983. The seal
myoglobin gene: an unusually long globin gene. Nature 301:732-734.

Blattner,F.R., Williams,B.G., Blechl,A.E., Denniston-Thompson,K.,
Faber,H.E., Furlong,L., Grunwald,D.J., Kiefer,D.O., Moore,D.D.,
Schumm,J.W., Sheldon,E.L. and Smithies,O. 1977. Charon phages:
safer derivatives of bacteriophage lambda for cDNA cloning. Science
194:161-169.

Blau,H.M., Chiu,C-P. and Webster,C. 1983. Cytoplasmic activation of human
nuclear genes in stable heterokaryons. Cell 32:1171-1180.

Blessing,M.H. 1972. Studies on the concentration of myoglobin in the sea
cow and porpoise. Comp.Biochem.Physiol. 41A:475-480.

Bonner,W.M. and Laskey,R.A. 1974. A film detection method for
tritium-labelled proteins and nucleic acids in polyacrylamide gels.
Eur.J.Biochem. 46:83-88.

Bouche,J.P. 1981. The effect of spermidine on endnuclease inhibition by
agarose contaminants. Anal.Biochem. 115:42-45.

Boulton,F.E., Huntsman,R.G., Lorkin,P.A. and Lehman,H. 1969. Abnormal
human myoglobin: 53(D4) glutamic acid - lysine. Nature 223:823-833.

Boulton,F.E., Huntsman,R.G., Romero-Herrera,A.E., Lorkin,P.A. and
Lehman,H. 1971a. A human myoglobin variant 133(H10) lysine →
asparagine. Biochem.Biophys.Acta. 229:871-876.

Boulton,F.E., Huntsman,R.G., Yawson,G.I., Romero-Herrera,A.E., Lorkin,P.A. and Lehman,H. 1971b. The second variant of human myoglobin. Brit.J.Haem. 20:69-73.

Boulton,F.E., Huntsman,R.G., Romero-Herrera,A.E., Lorkin,P.A. and Lehman,H. 1971c. The third variant of human myoglobin showing an unusual amino acid substitution 138(H16) arginine - tryptophan. Biochem.Biophys.Acta. 229:716-719.

Boyer,S.H., Fainer,D.C. and Naughton,M.A. 1963. Myoglobin: inherited structural variation in man. Science 140:1228-1231.

Breitbart,R.E., Nguyen,H.T., Medford,R.M., Destree,A.T., Mahdavi,V. and Nadal-Ginard,B. 1985. Intricate combinatorial patterns of exon splicing generate multiple regulated troponin T isoforms from a single gene. Cell 41:67-82.

Briskey,E.J., Kassens,R.G. and Marsh,B.B. (eds) 1970. Physiology and biochemistry of muscle as a food. University of Wisconsin Press.

Britten,R.J. and Kohn,D.E. 1968. Repeated sequences in DNA. Science 161:529-540.

Brooke,M.H. and Kaiser,K.K. 1974. The use and abuse of muscle histochemistry. Annals N.Y.Acad.Sci. 228:121-144.

Brown,M.D. 1973. Role of activity in the differentiation of slow and fast muscles. Nature 244:178-9.

Brown,M.D., Cotter,M.A., Hudlicka,O. and Vrbova,G. 1976. The effects of different patterns of muscle activity on capillary density, mechanical properties and structure of slow and fast rabbit muscles. Pfluegers Arch. 361:241-250.

Buckingham, M.E. 1977. Muscle protein synthesis and its control during the differentiation of skeletal muscle cells in vitro. In: Biochemistry of cell differentiation II, Vol 15. ed Paul,J. University Park Press, Baltimore.

Buckingham,M.E., Minty,A.J., Robert,B., Alonso,S., Cohen,A., Daubas,P., Weydert,A. and Caravatti,M. 1982. Messenger coding for myosins and actins: their accumulation during terminal differentiation of a mouse muscle cell line. In: Muscle development: molecular and cellular control. eds; Pearson, M.L. and Epstein,H. Cold Spring Harbor, New York.

Buckingham,M.E. and Minty,A.J. 1983. Contractile protein genes. In: Eukaryotic genes: their structure, activity and regulation. eds; Maclean,N., Gregory,S. and Minty,A.J. Butterworth.

Burke,R.E., Levine,D.N., Zajac III,F.E., Tsairis,P. and Engel,W.K. 1971 Mammalian motor units: physiological-histochemical correlation in three types of cat gastronemius. Science 174:709-712.

Buller,A.J., Eccles,J.C. and Eccles,R.M. 1960a. Differentiation of fast and slow muscles in the cat hind limb. J.Physiol. 150:399-416.

Buller,A.J., Eccles,J.C. and Eccles,R.M. 1960b. Interactions between motorneurones and muscles in respect of the characteristic speeds of their responses. J.Physiol. 150:417-439.

Burnstein,M. and Shainberg,A. 1979. Concanavalin A inhibits fusion of myoblasts and appearance of acetylcholine receptors in muscle cultures. FEBS Lett. 103:33-37.

Butler, P.J. and Jones,D.R. 1982. The comparative physiology of diving in vertebrates. Adv.Comp.Physiol.Biochem. 8:179-368.

Carmon,Y., Czosnek,H., Nudel,U., Shani,M. and Yaffe,D. 1982. DNaseI sensitivity of genes expressed during myogenesis. Nucl.Acids Res. 10:3085-3098.

Chamberlain,J.S., Jaynes,J.B. and Hauschka,S.D. 1985. Regulation of creatine kinase induction in differentiating myoblasts. Mol.Cell.Biol. 5:484-492.

Chambon,P., Dierich,A., Gaub,M-P., Jakowlev,S., Jongstra,J., Krust,A., LePennee,J-P., Oudet,P. and Reudelhuber,T. 1984. Promoter elements of genes coding for proteins and modulation of transcription by estrogens and progesterone. Rec.Prog.Hor.Res. 40:1-39.

Charnay,P., Triesman,R., Mellon,P., Chao,M., Axel,R. and Maniatis,T. 1984. Differences in human α- and β-globin gene expression in mouse erythroleukaemia cells: the role of intragenic sequences. Cell 38:251-263.

Chiquet,M., Eppenberger,H.M. and Turner,D.C. 1981. Muscle mophogenesis: evidence for an organising function of exogenous fibronectin. Dev.Biol. 88:220-235.

Chiu, C-P. and Blau,H.M. 1984. Reprogramming cell differentiation in the absence of DNA synthesis. Cell 37:879-887.

Chiu, C-P. and Blau,H.M. 1985. 5-Azacytidine permits gene activation in a previously noninducible cell type. Cell 40:417-424.

Christian,C.N., Nelson,P.G., Peacock,J. and Nirenberg,M. 1977. Synapse formation between two clonal lines. Science 196:995-998.

Close,R. 1967. Properties of motor units in fast and slow skeletal muscles of the rat. J.Physiol. 193:45-55.

Close,R.I. 1972. Dynamic properties of mammalian skeletal muscles. Physiol.Rev. 52:129-197.

Cohen,S.N., Chang,A.C.Y. and Hsu,L. 1972. Non-chromosomal antibiotic resistance in bacteria: genetic transformation of Escherichia coli by F-factor DNA. Proc.Natl.Acad.Sci.USA 69:2110-2114.

Collins and Weissman. 1984. The molecular genetics of human haemoglobin. Prog.Nucl.Acids Res.Mol.Biol. 31:312-462.

Couch.C.B. and Strittmatter,W.J. 1983. Rat myoblast fusion requires metalloendoprotease activity. Cell 32:257-265.

Czelusniak,J., Goodman,M., Hewett-Emmett,D., Weiss,M.L., Venta,P.J. and Tashian,R.E. 1982. Phylogenetic origins and adaptive evolution of avian and mammalian haemoglobin genes. Nature 298:297-300.

Davies,A.S. and Gunn,H.M. 1971. A comparative histochemical study of the mammalian diaphragm and M.semitendinosus. J.Anat. 110:137-139.

Dawes,G.S., Fox,H.E., Leduc,B.M., Liggins,G.C. and Richards,R.T. 1972. Respiratory movements and rapid eye movement sleep in the foetal lamb. J.Physiol. 220:119-143.

Deely,R.G., Gordon,J.I., Byrns,A.T.H., Mullinix,K.P., Binastein,M. amd Goldberger,R.F. 1977. Primary activiation of the vitellogenin gene in the rooster. J.Biol.Chem. 252:8310-8319.

Devine,C.E., Somlyo,A.V. and Somlyo,A.P. 1972. Sarcoplasmic reticulum and excitation-contraction coupling in mammalian smooth muscles. J.Cell Biol. 52:690-718.

Devlin,R.B. and Emerson,C.P.Jr. 1978. Coordinate regulation of contractile protein synthesis during myoblast differentiation. Cell 13:599-611.

Devlin,R.B. and Emerson,C.P.Jr. 1979. Coordinate accumulation of contractile protein mRNAs during myoblast differentiation. Dev.Biol. 69:202-216.

Dhoot,G.K., Gell,P.G.H. and Perry,S.V. 1978. The localisation of the different forms of troponin T in skeletal and cardiac muscle. Exp.Cell Res. 117:357-370.

Dierks,P., van OOyen,A., Cochran,M.D., Dobkin,C., Reiser,J. and Weissman,C. 1983. Three regions upstream from the cap site are required for efficient and accurate transcription of the rabbit β-globin gene in mouse 3T6 cells. Cell 32:695-706.

Doefler,W. 1983. DNA methylation and gene activity. Ann.Rev.Biochem. 62:93-124.

Dretzen,G., Bellard,M., Sassonec,P. and Chambon,P. 1981. A reliable method for the recovery of DNA fragments from agarose and acrylamide gels. Anal.Biochem. 112:295-298.

Dubowitz,V. 1970. Differentiation of fibre types in skeletal muscle. In: The physiology and biochemistry of muscle as a food. eds: Briskey,E.J., Kassens,R.G. and Marsh,B.B. University of Wisconsin Press.

Duckworth,M.L., Gait,M.J., Goelet,P., Hong,G.F., Singh,M. and Titmas,R.C. 1981. Rapid synthesis of oligodeoxyribonucleotides IV. Efficient, mechanised synthesis of heptadecaoxyribonucleotides by an improved solid phase phosphotriester route. Nucl.Acids Res. 9:1691-1706.

Ebashi,S. 1974. Regulatory mechanism of muscle contraction with special reference to the Ca-troponin-tropomyosin system. Essays in Biochem. 10:1-36.

Edge,M.B. 1970. Development of apposed sarcoplasmic reticulum at the T-system and sarcolemma and the change in orientation of triads in rat skeletal muscle. Dev.Biol. 23:634-650.

Edwards,Y.H., Lloyd,J.C., McMillan,S.L. and Benham,F.J. 1985. Human glyceraldehyde-3-phosphate dehydrogenase: mRNA levels and enzyme activity in developing muscle. Mol.Cell.Biol. 5:2147-2149.

Efstratiadis,A., Posakony,J.W., Maniatis,T., Lawn,R.M., O'Connell,C.,
    Spritz,R.A., De Riel,J.K., Forget,B.G., Weissman,S.M.,
    Slightom,J.L., Blechl,A.E., Smithies,O., Baralle,F.E.,
    Shoulders,C.C. and Proudfoot,N.J. 1980. The structure and evolution
    of the human β-globin gene family. Cell 21:653-668.

Endo,M. 1977. Calcium release from the sarcoplasmic reticulum.
    Physiol.Rev. 57:71-108.

Eichelberger,L., Fletcher,E.S., Geiling,E.M.K. and Vos,B.J. 1939. Muscle
    and blood haemoglobin in the dolphin. Science 90:443.

Eisenberg,B.R. and Kuda,A.M. 1976. Discrimination between fibre
    populations in mammalian skeletal muscle using ultrastructural
    parameters. J.Ultrastruct.Res. 54:76-88.

Enquist,L. and Sternberg,N. 1979. In vitro packaging of λDam vectors and
    their use in cloning DNA fragments. Meth.Enzymol. 68:281-298.

Emerson,C.P. and Beckner,S.K. 1975. Activation of myosin synthesis in
    fusing and mononucleated myoblasts. J.Mol.Biol. 93:431-447.

Eppenberger,H.M., Perriard,J.C., Rosenberg,U.B. and Streter,E.E. 1981.
    The M_r 165,000 M-protein myomesin: a specific protein of
    cross-striated muscle cells. J.Cell Biol. 89:185-193.

Evans,T., Schon,E., Gora-Maslak,G., Patterson,J. and Efstratiadis,A. 1984.
    S1-hypersensitive sites in eukaryotic promoter regions. Nucl.Acids
    Res. 12:8043-8058.

Fasold,H., Riedl,G. and Jaisle,F. 1970. Evidence for an absence of
    myoglobin from human smooth muscle. Eur.J.Biochem. 15:122-126.

Fischman,D.A. 1972. Development of striated muscle. In: The structure and
    function of muscle. Vol I. ed: Bourne,G.H. Academic Press.

Folkow,B. and Halicka,H.D. 1968. A comparison between 'red' and 'white'
    muscle with respect to blood supply, capillary surface area and
    oxygen uptake during rest and exercise. Microvasc.Res. 1:1-14.

Fritch,E.F., Lawm,R.M. and Maniatis,T. 1980. Molecular cloning and
    characterisation of the human β-like globin gene cluster. Cell
    19:959-972.

Gabbiani,G., Schmid,E., Winter,S., Chaponnier,C., Chastonary,C.,
    Vanderkerckhove,J., Weber,K. and Franke,W. 1981. Vascular smooth
    muscle cells differ from other smooth muscle cells: predomonance of
    vimentin filaments and a specific α-type actin. Proc.Natl.
    Acad.Sci.USA. 78:298-302.

Gauthier,G. 1969. On the relationship of ultrastructural and cytochemical
    features to colour in mammalian skeletal muscle. Z.Zellforsch.
    95:462-482.

Gauthier,G., Lowey,S., Hobbs,A.W. 1978. Fast and slow myosin in
    developing muscle fibres. Nature 274:25-29.

Gauthier,G., Lowey,S., Hobbs,A.W. 1979. Distribution of myosin isoenzymes
    among skeletal muscle fibre types. J.Cell Biol. 81:10-25.

George,J.C., Vallyathan,N.V. and Ronald,K. 1971. The harp seal Pagophilus groenlandicus (Erxleben,1777). VII. A histochemical study of certain skeletal muscles. Can.J.Zool. 49:25-30.

Gilfix,B.M. and Sanwal,B.D. 1980. Inhibition of myoblast fusion by tunicamysin and pantomysin. Biochem.Biophys.Res.Commun. 96:1184-1191.

Gillies,S.D., Morrison,S.L., Oi,V.T. and Tonegawa,S. 1983. A tissue-specific transcription enhancer element is located in the major intron of a rearranged immunuglobulin heavy chain gene. Cell 33:717-728.

Gilmour,J.R. 1941. Normal haemopoiesis in intra-uterine and neonatal life J.Path.and Bact. 52:25-45.

Go,M. 1981. Correlation of DNA exonic regions with protein structural units in haemoglobin. Nature 291:90-92.

Goldspink,G. 1980. Growth of muscle. In: Development and specialisation of skeletal muscle. ed: Goldspink,D.F. Cambridge University Press.

Gordon,H. and van Essen,D.C. 1985. Specific innervation of muscle fibre types in a developmentally polyinnervated muscle. Dev.Biol. 111:42-50.

Griffin,G.E., Williams,P.E. and Goldspink,G. 1971. Region of longitudinal growth in striated muscle fibres. Nature 232:28-29.

Groschel-Stewart,U., Jaroschik,U. and Schwalm,H. 1971. Chicken gizzard, a myoglobin-containing smooth muscle. Experientia 27:512-513.

Grosveldt,G.C., de Boer,E., Shewmaker,C.K. and Flavell,R.A. 1982. DNA sequences necessary for transcription of the rabbit β-globin gene in vivo. Nature 295:120-126.

Grunstein,M. and Hogness,D.S. 1975. Colony hybridisation: a method for the isolation of cloned DNAs that contain a specific gene. Proc.Natl.Acad.Sci.USA. 72:3961-3965.

Gurdon,J.B., Fairman,S., Mohun,T.J. and Brenner,S. 1985. Activation of muscle-specific actin genes in Xenopus development by an induction between animal and vegetal cells of a blastula. Cell 41:913-922.

Hamoir,G. 1977. Biochemistry of the myometrium. In: Biology of the uterus. ed: Wynn,R.M. Plenum Press, New York.

Hapner,K.D., Bradshaw,R.A., Hartzell,C.R. and Gurd,F.R.N. 1968. Comparision of myoglobins from harbour seal, porpoise and sperm whale. J.Biol.Chem. 243:683-689.

Harris,S. 1985. A comparative study of β-globin pseudogenes in man and the primates. PhD thesis, University of Leicester.

Hartshorne,D.J. and Siemankowski,R.F. 1981. Regulation of smooth muscle actomyosin. Ann.Rev.Physiol. 43:519-530.

Henneman,E. and Olson,C.B. 1965. Relations between structure and function in the design of skeletal muscles. J.Neurophysiol. 28:581-598.

Henneman,E., Clamann,H.P., Gillies,J.D. and Skinner,R.D. 1974. Rank order
    of motoneurones within a pool: law of combination. J.Neurophysiol.
    37:1338-1349.

Heywood,S.M., Havaranis,A.S. and Herrmann,H. 1973. Myoglobin synthesis in
    cell cultures of red and white muscle. J.Cell Physiol. 82:319-322.

Hofstetter,H., Schambock,A., van den Berg,J. and Weissman,C. 1976.
    Specific expression of the inserted DNA segment from hybrid plasmids
    constructed by the poly(dA).poly(dT) method. Biochem.Biophys.Acta.
    454:587-591.

Hohn,B. 1979. In vitro packaging of λ and cosmid DNA. Meth.Enzymol.
    68:229-309.

Holloszy,J.O. and Booth,F.W. 1976. Biochemical adaptations to endurance
    exercise. Ann.Rev.Physiol. 38:273-291.

Htun,H., Lund,E. and Dahlberg,J.E. 1984. Human U1 RNA genes contain an
    unusually sensitive nuclease S1 cleavage site within the conserved
    3' flanking region. Proc.Natl.Acad.Sci.USA. 81:7288-7292.

Humphries,S.E., Whitall,R., Minty,A., Buckingham,M. and Williamson,R.
    1981. There are approximately 20 actin genes in the human genome.
    Nucl.Acids Res. 9:4895-4908.

Hunt,T.L., Hurst-Calderone,S. and Dayhoff,M.O. 1978. In: Atlas of protein
    sequence and structure. pp 229-257. ed: Dayhoff,M.O. National
    Biomedical Research Foundation, Washington.

Huxley,H. and Hanson,J. 1954. Changes in the cross-striations of muscle
    during contraction and stretch and their structural interpretation.
    Nature 173:973-77.

Ishikawa,H. 1983. Fine structure of skeletal muscle. In: Cell and muscle
    motility IV. eds: Dowbin,R.M. and Shay,J.W. Plenum Press.

James,N.T. 1968. Histochemical demonstration of myoglobin in skeletal
    muscle fibres and muscle spindles. Nature 219:1174-1175.

Jeffreys,A.J., Wilson,V., Wood,D., Simons,J.P., Kay,R.M. and Williams,J.G.
    1980. Linkage of adult α- and β-globin genes in X.laevis and gene
    duplication by tetraploidisation. Cell 21:555-564.

Jeffreys,A.J., Blanchetot,A. and Weller,P. 1983. Myoglobin genes. In:
    Brussels Haemoglobin Symposium pp487-508. eds: Schnek,A.G. and
    Paul,C. Presses Universitaires de Bruxelles, A.S.B.L.

Jeffreys,A.J., Wilson,V., Blanchetot,A., Weller,P., Geurts van Kessel,A.,
    Spurr,N., Solomon,E. and Goodfellow,P. 1984. The human myoglobin
    gene: a third dispersed globin locus in th human genome. Nucl.Acids
    Res. 12:3235-3243.

Jeffreys,A.J., Wilson,V. and Thein,S-L. 1985. Hypervariable
    "minisatellite" regions in human DNA. Nature 314:67-73.

Jensen,E., Paludan,K., Hyldig-Hielsen,J.J., Jorgensen,P. and Marcker,K.A.
    1981. The structure of a chromosomal leghaemoglobin gene from
    soybean. Nature 291:677-679.

Jolesz,F. and Streter,F.A. 1981. Development, innervation and
    activity-pattern induced changes in skeletal muscle.
    Ann.Rev.Physiol. 43:531-552.

Kagen,L.J. and Christian,C.L. 1966. Immunologic measurements of myoglobin
    in human adult and fetal skeletal muscle. Am.J.Physiol. 211:656-660.

Kagen,L., Linder,S. and Gurevich,R. 1969. Myoglobin synthesis of embryo
    and adult avian tissue studied immunologically. Am.J.Physiol.
    217:591-595.

Kagen,L., Linder,S. 1970 Myoglobin synthesis studied in vitro in the
    chick embryo. Dev.Biol. 22:200-212.

Kagen,L. and Freedman,A. 1973. Embryonic synthesis of myoglobin in vivo
    estimated by radioimmunoassay. Dev.Biol. 31:295-300.

Kagen,L. and Freedman,A. 1974. Studies on the effects of acetylcholine,
    epinephrine, dibutyryl cyclic adenosine monophosphate, theophyline
    and calcium on the synthesis of myoglobin in muscle cell cultures
    estimated by radioimmunoassay. Exp.Cell Res. 88:135-142.

Kalderon,N. and Gilula,N.B. 1979. Membrane events involved in myoblast
    fusion. J.Cell Biol. 81:411-425.

Karlsson,S. and Nienhuis,A.W. 1985. Developmental regulation of human
    globin genes. Ann.Rev.Biochem. 54:1071-1108.

Kaufman,S.J. and Foster,R.F. 1985. Remodelling of the myoblast membrane
    accompanies development. Dev.Biol. 110:1-14.

Kendrew,J.C., Bodo,G., Dintzis,H.M., Parrish,R.G., Wyckoff,H. and
    Phillips,D.C. 1958. A three dimensional model of the myoglobin
    molecule obtained by X-ray analysis. Nature 181:662-666.

Kendrew,J.C., Dickerson,R.E., Strandberg,B.E., Hart,R.G., Davies,R.D.,
    Phillips,D.C. and Shore,V.C. 1960. Structure of myoglobin: a three
    dimensional Fourier synthesis at 2A resolution. Nature 185:422-427.

Kendrew,J.C., Watson,H.C., Strandberg,B.E., Dickerson,R.E., Phillips,D.C.
    and Shore,V.C. 1961. The amino acid sequence of sperm whale
    myoglobin: A partial determination by X-ray methods, and its
    correlation with chemical data. Nature 190:666-670.

Kendrew,J.C. 1963. Myoglobin and the structure of proteins. Science
    139:1259-1266.

Konigsberg,I.R. 1964. Clonal and biochemical studies of myogenesis.
    Carnegie Inst. Washington Yearbook 63:516-517.

Konkel,D.A., Maizel,J.V. and Leder,P. 1979. The evolution and sequence
    comparison of two recently diverged mouse chromosomal β-globin
    genes. Cell 18:865-873.

Kozak,M. 1984. Point mutations close to the AUG initiator codon affect the
    efficiency of translation of rat preproinsulin in vivo. Nature
    308:241-246.

Kushner,S.R. 1978. An improved method for transformation of Escherichia coli with COL E1 derived plasmids. In: Genetic Engineering. pp17-23. eds: Boyer,H.W. and Nicosia,S. North Holland Biomedical Press. Amsterdam.

Laemmli,U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

Lane,B.P., Elias,J. and Drummond,E. 1977. Immunoelectronmicroscopic localisation of α-actinin in skeletal muscle cells. J.Histochem. Cytochem. 2569-72,

Larson.A and Weintraub,H. 1982. An altered DNA conformation detected by S1 nuclease occurs at specific regions in active chick globin chromatin. Cell 29:609-622.

Lawrie,R.A. 1952. Biochemical differences between red and white muscle. Nature 170:122-123.

Lawrie,R.A. 1953a. The activity of the cytochrome system in muscle and its relation to myoglobin. Biochem.J. 55:298-305.

Lawrie,A. 1953b. The relation of energy-rich phosphate in muscle to myoglobin and to cytochrome oxidase activity. Biochem.J. 55:305-309.

Lawrie,R.A. 1953c. Effect of enforced exercise on myoglobin concentration in muscle. Nature 171:1069-1070.

Lazarides,E. and Hubbard,B. 1976. Immunological characterisation of the 100A filaments from muscle cells. Proc.Natl.Acad.Sci.USA. 73:4344-4348.

Lenfant,C., Johansen,K. and Torrance,J.D. 1970. Gas transport and oxygen storage capacity in some pinnipeds and the seal otter. Resp.Physiol. 9:277-286.

Lenfant,C. 1973. High altitude adaptation in mammals. Am.Zool. 13:447-456.

Lilley,D.M. 1980. The inverted repeat as a recognisible structural feature in supercoiled DNA molecules. Proc.Natl.Acad.Sci.USA. 77:6468-6472.

Lipton,B.H. and Kinigsberg,I.R. 1972. A fine structural analysis of the fusion of myogenic cells. J.Cell Biol. 53:348-364.

Loenen,W.A.M. and Brammar,W.J. 1980. A bacteriophage lambda vector for cloning large DNA fragments made with several restriction enzymes. Gene 20:249-259.

Longo,L.D., Koos,B.J. and Power,G.G. 1973. Fetal myoglobin: quantitative determination and importance for oxygenation. Am.J.Physiol. 224:1032-1036.

Low,R.B. and Rich,A. 1973. Myoglobin biosynthesis in the embryonic chick. Biochem. 12:4555-4559.

Magram,J., Chada,K. and Costantini,F. 1985. Developmental regulation of a cloned adult β-globin gene in transgenic mice. Nature 315:338-340.

Mai,J.V., Edgerton,V.R. and Barnard,R.J. 1970. Capillarity of red, white and intermediate muscle fibres in trained and untrained guinea-pigs. Experientia 26:1222-1223.

Maniatis,T., Gek Kee,S., Efstratiadis,A. and Kafatos,F.C. 1976. Amplification and characterisation of a β-globin gene synthesised in vitro. Cell 8:163-182.

Maniatis,T., Fritch,E.F. and Sambrook,J. 1982. Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory.

de Martinville,B. and Franke,U. 1983. The c-Ha-rasI, insulin and β-globin loci map outside the deletion associated with aniridia-Wilms' tumour. Nature 305:641-643.

Maxam,A.M. and Gilbert,W. 1980. Sequencing end-labelled DNA with base-specific chemical cleavages. Meth.Enzymol. 65:499-560.

Mayer,Y., Czosnek,H., Zeelon,P.E., Yaffe,D. and Nudel,U. 1984. Expression of the genes coding for the skeletal muscle and cardiac actins in the heart. Nucl.Acids Res. 12:1087-1101.

McPherson,A. and Tokunaga,J. 1967. The effects of cross-innervation on the myoglobin concentration of tonic and phasic muscles. J.Physiol. 188:121-129.

Melloul.D., Aloni,B., Calvo,J., Yaffe,D. and Nudel,U. 1984. Developmentally regulated expression of chimeric genes containing muscle actin DNA sequences in transfected myogenic cells. EMBO J. 3:983-990.

Merrill,G.F., Hauschka,S.D. and McKnight,S.L. 1984. tk enzyme expression in differentiating muscle cells is regulated through an internal segment of the cellular tk gene. Mol.Cell.Biol. 4:1777-1784.

Messing,J., Crea,R. and Seeberg,P.H. 1981. A system for shotgun DNA sequencing. Nucl.Acids Res. 9:309-321.

Messing,J and Vieira. 1982. A new pair of M13 vectors for selecting either strand of double-digest restriction endonuclease fragments. Gene 19:269-276.

Minty,A.M., Caravatti,M., Robert,B., Cohen,A., Daubas,P., Weydert,A., Gros,F. and Buckingham,M.E. 1981. Mouse actin mRNAs: construction and characterisation of a recombinant plasmid molecule containing a complementary DNA transcript of mouse α-actin mRNA. J.Biol.Chem. 256:1008-1014.

Mohun,T.J., Brennan,S., Dathan,N., Fairman,S. and Gurdon,J.B. 1984. Cell type-specific activation of actin genes in the early amphibian embryo. Nature 311:716-721.

Moller,P. and Sylven,C. 1981. myoglobin in human skeletal muscle. Scand.J.Clin.Invest. 41:479-482.

Monster,A.W. and Chan,H. 1977. Isometric force production by motor units of extensor digitorum communis muscle in man. J.Neurophysiol. 40:1432-1443.

Morita,S., Cassens,R.G. and Briskey,E.J. 1970. Histochemical localisation of myoglobin in skeletal muscle of rabbit, pig and ox. J.Histochem. Cytochem. 18:364-366.

Morrison,P., Rosenman,M. and Sealander,J.A. 1966. Seasonal variation of myoglobin in the northern red backed vole. Am.J.Physiol. 211:1305-1306.

Moss,F.P. and Leblond,C.P. 1970. Nature of dividing nuclei in skeletal muscle of growing rats. J.Cell Biol. 44:459-462.

Nabeshima,Y., Fujii-kuriyama,Y., Muramatsu,M. and Ogata,K. 1984. Alternative transcription and two modes of splicing result in two myosin light chains from one gene. Nature 308:333-338.

Nadal-Ginard,B., Medford,R.M., Nguyen,H.T., Periasmy,M., Wydro,R.M. Hornig,D., Gubits,R., Garfinkel,L.I., Wikzorek,D., Bekesi,E. and Mahdavi,V. 1982. Structure and regulation of a mammalian sarcomeric myosin heavy-chain gene. In: Muscle development; molecular and cellular control. eds: Pearson,M.L. and Epstein,H. Cold Spring Harbour Laboratory.

Neely,J.R. and morgan,H.E. 1974. Relationship between carbohydrate and lipid metabolism and the energy balance of heart muscle. Ann.Rev. Physiol. 31:413-459.

Nevo,E. 1979. Adaptive convergence and divergence of subterranean mammals. Ann.Rev.Ecol.Syst. 10:269-308.

Nobbs,C.L., Watson,H.C. and Kendrew,J.C. 1966. Structure of deoxymyoglobin: a crystallographic study. Nature 209:339-341.

Nougues,J. and Bacou,F. 1977. Enzymatic activities of muscle fibres differentiated, in vitro, from pectoralis major (white) and adductor magnus (red) muscles of chick embryos. Experientia 33:714-716.

Nudel,U., Calvo,J.M., Shani,M. and Levy,Z. 1984. The nucleotide sequence of a rat myosin light chain 2 gene. Nucl.Acids Res. 12:7175-7186.

Nudel,U., Greenberg,D., Ordahl,C.P., Saxel,O., Neuman,S. and Yaffe,D. 1985. Developmentally regulated expression of a chicken muscle-specific gene in stably transfected rat myogenic cells. Proc.Natl.Acad.Sci.USA. 82:3106-3109.

O'Brien,R.A.D., Ostberg,A.J.C. and Vrbova,G. 1978. Observations on the elimination of polyneural innervation in developing mammalian skeletal muscle. J. Physiol. 282:571-582.

Offer,G., Moos,C. and Starr,R. 1973. A new protein of the thick filaments of vertebrate skeletal muscle. J.Mol.Biol. 74:654-676.

Ordahl,C.P. and Caplan,A.I. 1976. Transcriptional diversity in myogenesis. Dev.Biol. 54:61-72.

Ordahl,C.P. and Cooper,T.A. 1983. Strong homology in promoter and 3'-untranslated regions of chick and rat α-actin genes. Nature 303:348-349.

Papayannopoulou,T., Tatsis,B., Kurachi,S., Nakamotot,B. and
    Stamatoyannopoulos,G. 1984. A haemoglobin switching activity
    modulates hereditary persistance of foetal haemoglobin. Nature
    309:71-73.

Pattengale,P.K. and Holloszy,J.O. 1967. Augmentation of skeletal muscle
    myoglobin by a program of treadmill running. Am.J.Physiol.
    213:783-785.

Pearson,M.L. 1980. Muscle differentiation in cell culture: a problem in
    somatic cell and molecular genetics. In: The molecular Biology of
    Development. eds: Leighton,T. and Loomis,W.F. Academic Press.

Pelletier,J. and Sonenberg,N. 1985. Insertion mutagenesis to increase
    secondary structure within the 5' noncoding region of a eukaryotic
    mRNA reduces translational efficiency. Cell 40:515-526.

Periasmy,M., Strehler,E.E., Garfinkel,L.I., Gubits,R.M., Ruiz-Opazo,N, and
    Nadal-Ginard,B. 1984. Fast skeletal muscle myosin light chains 1
    and 3 are produced from a single gene by a combined process of
    differential RNA transcription and splicing. J.Biol.Chem.
    259:13595-13604.

Perkoff,G.T. and Tyler,F.H. 1958. Estimation and physical properties of
    myoglobin in various species. Metabolism 7:751.

Perry,S.V. and Dhoot,G.K. 1980. Biochemical aspects of muscle development
    and differentiation. In: Development and Specialisation of Skeletal
    Muscle. ed: Goldspink,D.F. Cambridge University Press.

Perutz,M.F. 1964. The haemoglobin molecule. Sci.Am. 211:64-76.

Perutz,M.F., Kendrew,J.C. and Watson,H.C. 1965. Structure and function of
    haemoglobin II. Some relations between polypeptide chain
    configuration and amino acid sequence. J.Mol.Biol. 13:669-678.

Pette,D., Smith,M.E., Staudte,H.W. and Vrbova,G. 1973. Effects of long
    term electrical stimulation on some contractile and metabolic
    characteristics of fast rabbit muscles. Pfluegers Arch. 338:257-272.

Pette,D. 1980. Plasticity of Muscle. Walter de Gruyter, Berlin.

Picard,D. and Schaffner,W. 1984. A lymphocyte-specific enhancer in the
    mouse immunoglobulin gene. Nature 307:80-82.

Pinset, C. and Whalen,R.G. 1985. Induction of myogenic differentiation in
    serum-free medium does not require DNA synthesis. Dev.Biol.
    108:284-289.

Ponte,P., Gunning,P., Blau,H. and Kedes,L. 1983. Human actin genes are
    single copy for α-skeletal and α-cardiac actin but multicopy for β-
    and Y-cytoskeletal genes: 3' untranslated regions are isotype
    specific but are conserved in evolution. Mol.Cell.Biol. 3:1783-1791.

Rabbitts,T.H. 1976. Bacterial cloning of plasmids carrying copies of
    rabbit globin messenger RNA. Nature 260:221-225.

Rash,J.E. and Staehelin,L.A. 1974. Freeze-thaw demonstration of gap
    junctions between skeletal myogenic cells in vivo. Dev.Biol.
    36:455-461.

Revel,J.P. 1962. The sarcoplasmic reticulum of the bat cricothyroid muscle. J.Cell Biol. 12:571.

Reynafarje,B. 1962. Myoglobin content and enzymatic activity of muscle and altitude adaptation. J.Appl.Physiol. 17:301-305.

Reynafarje,B. and Morrison,P. 1962. Myoglobin levels in some tissues from wild Peruvian rodents native to high altitude. J.Biol.Chem. 237:2861-2864.

Robert,B., Barton,P., Minty,A., Daubas,P., Weydert,A., Bonhomme,F., Catalan,J., Chazottes,D., Guenet,J-L. and Buckingham,M. 1985. Investigation of genetic linkage between myosin and actin genes using an interspecific mouse back-cross. Nature 314:181-183.

Robinson,D. 1939. The muscle haemoglobin of seals as an oxygen store in diving. Science 90:276-277.

Romera-Herrera,A.E. and Lehman,H. 1971. Primary structure of human myoglobin. Nature New Biology 232:149-152.

Romera-Herrera,A.E., Lehman,H., Joysey,K.A. and Friday,A.E. 1978. On the evolution of myoglobin. Phil.Trans.Royal Soc.Lon. 283:61-163.

Rosenmann,M. and Morrison,P. 1965. Seasonal augmentation of myoglobin in the snowshoe hare. J.Biol.Chem. 240:3353-3356.

Rowe,R.W.D. and Goldspink,G. 1969. Muscle fibre growth in five different muscles in both sexes of mice. I normal mice. J.Anat. 104:519-530.

Rozek,C.E. and Davidson,N. 1983. Drosophila has one myosin heavy chain gene with three developmentally reglated transcripts. Cell 32:23-34.

Rugh,R. 1968. The mouse, its reproduction and development. Burgess.

Ruiz-Opazo,N., Weinberger,J. and Nadal-Ginard,B. 1985. Comparison of a- tropomyosin sequences from smooth and striated muscle. Nature 315:67-70.

Salmons,S. and Vrbova,G. 1969. The influence of activity on some contractile characteristics of mammalian fast and slow muscles. J.Physiol. 201:353-349.

Salmons,S. and Henriksson,J. 1981. The adaptive response of skeletal muscle to increased use. Muscle and Nerve 4:94-105.

Sanger,F., Coulson,A.R, Barrell,B.G.m Smith,A.J.H. and Roe,B.A. 1980. Cloning in single-stranded bacteriophage as an aid to rapid DNA sequencing. J.Mol.Biol. 143:161-178.

Schaffner,W., Gross,K., Telford,J. amd Birnstiel,M. 1976. Molecular analysis of Psammachinus miliaris: the arrangement of the five histone coding and spacer sequences. Cell 8:411-478.

Schneiderman,L.J. 1962. Myoglobin in the human foetus. Nature 194:191-192.

Scholander,P.F. 1940. Experimental investigations on the respiratory functions in diving mammals and birds. Hvalradets Skrifter Norske Videnskaps-Akad.Oslo 22:5-131.

Schudt,C., van der Bosch,J. and Pette,D. 1973. Inhibition of muscle cell fusion *in vitro* by Mg^{2+} and K^{+} ions. FEBS Lett. 32:296-298.

Sessions,A. and Horwitz,A.F. 1981. Myoblast aminophospholipid asymmetry differs from that of fibroblasts. FEBS Lett. 134:75-78.

Sessions,A. and Horwitz,A.F. 1983. Differentiation-related differences in the plasma membrane phospholipid asymmetry of myogenic and fibrogenic cells. Biochem.Biophys.Acta 728:103-111.

Shani,M., Zevin-Sonkin,D., Carmon,Y., Czosnek,H., Nudel,U. and Yaffe,D. 1982. Changes in myosin and actin gene expression and DNase I sensitivity associated with terminal differentiation of myogenic cultures. In: Muscle Development: Molecular and Cellular Control. eds: Pearson,M.L. and Epstein,H. Cold Spring Harbor.

Shani,M., Admon,S. and Yaffe,D. 1984. The methylation state of 2 muscle-specific genes: restriction enzyme analysis did not detect a correlation with expression. Nucl.Acids Res. 12:7225-7234.

Shani,M. 1985. Tissue-specific expression of rat myosin light-chain 2 in transgenic mice. Nature 314:283-286.

Shoenberg,C.F. 1977. The contractile mechanism and ultrastructure of the myometrium. In: Biology of the Uterus. ed: Wynn,R.M. Plenum Press.

Simpson,F.O., Rayns,D.G. and Ledingham,J.M. 1973. The ultrastructure of ventricular and atrial myocardium. In: Ultrastructure of the Mammalian Heart. eds: Challice,C.E. and Viragh,S. Academic Press.

Singleton.C., Keysik,J., Stirdivant,S.M. and Wells,R.D. 1982. Left-handed Z-DNA is induced by supercoiling in physiological ionic conditions. Nature 299:312-316.

Smith,H.O. and Birnstiel,M.L. 1976. A simple method for DNA restriction site mapping. Nucl.Acids.Res. 3:2387-2398.

Smith Quinn,L., Holtzer,H. and Nameroff,M. 1985. Generation of chick skeletal muscle cells in groups of 16 from stem cells. Nature 313:692-694.

Squire,J. 1981. The structural basis of muscular contraction. Plenum Press.

Somlyo,A.V. and Somlyo,A.P. 1971. Strontium accumulation by sarcoplasmic reticulum and mitochondria in vascular smooth muscle. Science 174:995.

Southern,E.M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J.Mol.Biol. 98:503-617.

Staden,R. 1980. A new computer method for the storage and manipulation of DNA gel reading data. Nucl.Acids Res. 8:3673-3694.

Stockdale,F.E. and Holtzer,H. 1961. DNA synthesis and myogenesis. Exp.Cell Res. 24:508-520.

Stockdale,F.E., Baden,H. and Raman,N. 1981. Slow muscle myoblasts differentiating *in vitro* synthesise both slow and fast myosin light chains. Dev.Biol. 82:168-171.

Streter,F.A., Gerely,J., Salmons,S. and Romanul,F.C.A. 1973. Synthesis by fast muscle of myosin characteristic of slow muscle in response to long-term stimulation. Nature 241:17-19.

Strickland,N.C. and Goldspink,G. 1973. A possible indicator muscle for the fibre content and growth characteristics of porcine muscle. Animal Production 16:135-146.

Takano,T. 1977. Structure of myoglobin refined at 2.0A resolution. J.Mol.Biol. 110:537-568.

Tappan, D.V and Reynafarje,B. 1957. Tissue pigment manifestations of adaptation to high altitudes. Am.J.Physiol. 190:99-103.

Thomas,P.S. 1980. Hybridisation of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc.Natl.Acad.Sci.USA. 77:5201-5205.

Tipler,T.D., Edwards,Y.H. and Hopkinson,D.A. 1978. Developmental changes in the protein profiles of human cardiac and skeletal muscle. Ann.Hum.Genet. 41:409-418.

Turner,D.C., Wallimann,T. and Ebbenberger,H.M. 1973. A protein thet binds specifically to the M-line of skeletal muscle is identified as the muscle form of creatine kinase. Proc.Natl.Acad.Sci.USA. 70:702-705.

Twigg,A.J. and Sherratt,D. 1980. Trans-complementable copy-number mutants of plasmid ColEI. Nature 283:216-218.

Vaughan,B.E. and Pace,N. 1956. Changes in myoglobin content of the high altitude acclimatized rat. Am.J.Physiol. 185:549-556.

Wahl,G.M., Stern,M. and Stark,G.R. 1979. Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl paper and rapid hybridisation by using dextran sulphate. Proc.Natl.Acad.Sci.USA. 76:3683-3687.

Wakelam,M.J.O. 1983. Inositol phospholipid metabolism and myoblast fusion. Biochem.J. 214:77-82.

Wakelam,M.J.O. and Pette,D. 1983. Abstr. FEBS Meet. 15th S-12 MO-230.

Wakelam,M.J.O. 1985. The fusion of myoblasts. Biochem.J. 228:1-12.

Walsh,F.S. and Phillips,E. 1981. Specific changes in cellular glycoproteins and surface proteins during myogenesis in clonal muscle cells. Dev.Biol. 81:229-237.

Walsh,F.S., Moore,S.E., Woodroofe,M.N., Hurko,O., Nayak,R., Brown,S.M. and Dickson,J.G. 1984. Characterisation of human muscle differentiation antigens. Exp.Biol.Med. 9:50-56.

Weaver,R.F. and Weissman,C. 1979. Mapping of RNA by a modification of the Berk-Sharp procedure: the 5' termini of 15S β-globin mRNA precursor and mature 10S β-globin mRNA have identical map coordinates. Nucl.Acids Res. 7:1175-1193.

Weber,R.E., Hemmingson,E.A. and Johansen,K. 1974. Functional and biochemical studies of penguin myoglobin. Comp.Biochem.Physiol. 49B:197-214.

Weintraub,H. and Groudine,M. 1976. Chromosomal subunits in active genes
have an altered conformation. Science 193:848-856.

Weintraub,H., Beug,H., Groudine,M. and Graf,T. 1982.
Temperature-sensitive changes in the structure of globin chromatin
in lines of red cell precursors transformed by ts-AEV. Cell
28:931-940.

Weintraub,H. 1983. A dominant role for DNA secondary structure in forming
hypersensitive structures in chromatin. Cell 32:1191-1203.

Weintraub,H. 1985. Assembly and propagation of repressed chromosomal
states. Cell 42:705-711.

Weller,P., Jeffreys,A.J., Wilson,V. and Blanchetot,A. 1984. Organisation
of the human myoglobin gene. EMBO J 3:439-446.

Whalen,R.G., Sell,S.M., Butler-Browne,G.S., Schwartz,K., Bouveret,P. and
Pinset-Harstrom,I. 1981. Three myosin heavy-chain isozymes appear
sequentially in rat muscle development. Nature 292:805-809.

Whalen,R.G., Bugaisky,L.B., Butler-Browne,G., Sell,S.M., Schwartz,K. and
Pinset-Harstrom,I. 1982. In: Muscle Development: Molecular and
Cellular Control. eds: Pearson,M.L. and Epstein,H. Cold Spring
Harbor.

Wittenberg,J.B. 1959. Oxygen transport- a new function proposed for
myoglobin. Biol.Bull.1959 402-403.

Wittenberg,J.B. 1970. Myoglobin-facilitated oxygen diffusion: role of
myoglobin in oxygen entry into muscle. Physiol.Rev. 50:559-636.

Wittenberg,B.A., Wittenberg,J.B. and Caldwell,P.R.B. 1975. Role of
myoglobin in the oxygen sypply to red skeletal muscle. J.Biol.Chem.
250:9038-9043.

Wood,D., Blanchetot,A. and Jeffreys,A.J. 1982. Molecular cloning of sea
myoglobin mRNA. Nucl.Acids Res. 10:7133-7144.

Wood,D. 1984. The isolation of a mammalian myoglobin gene. PhD thesis,
University of Leicester.

Wooten,G.F. and Reis,D.J. 1972. Blood flow in red and white muscle in
early development. Int.J.Neurosci. 3:155-164.

Wright,W.E. 1984. Control of differentiation in heterokaryons and hybrids
involving differentiation-defective myoblast variants. J.Cell Biol.
98:436-443.

Wright,W.E. 1985. The amplified expression of factors regulating
myogenesis in L6 myoblasts. J.Cell Biol. 100:311-316.

Yaffe,D. 1968. Retention of differentiation potentialities during
prolonged cultivation of myogenic cells. Proc.Natl.Acad.Sci.USA.
61:477-483.

Yang,R.C-A., Lis,J. and Wu,R. 1979. Elution of DNA from agarose gels
after electrophoresis. Meth.Enzymol. 68:176-182.

Yanish-Perron,C., Vieira,J. and Messing,J. 1985. Improved M13 phage
    cloning vectors and host strains: nucleotide sequencs of the M13mp18
    and pUC19 vectors. Gene 33:103-119.

.

.