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Myoglobin Expression: Early Induction and Subsequent Modulation of Myoglobin and Myoglobin mRNA during Myogenesis

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We showed that myoglobin gene transcription and the appearance of myoglobin occur very early in myogenesis, in both humans and mice. In contrast to the contractile protein genes, there is a subsequent increase of 50- to 100-fold in myoglobin mRNA and protein levels during later muscle development. Myoglobin and myoglobin mRNA are present at elevated levels in fetal heart and are also detectable at low levels in adult smooth muscle. The absolute level of myoglobin mRNA in highly myoglobinized seal muscle is very high [2.8% of the total population of poly(A)$^+$ RNAs]. Levels of myoglobin in seal skeletal muscle and in various human muscle types appear to be determined by the size of the myoglobin mRNA pool. In contrast, low levels of myoglobin in mouse skeletal muscle are not apparently correlated with low levels of myoglobin mRNA. As expected from the early appearance of myoglobin mRNA in embryonic skeletal muscle, both rat and mouse embryonic myoblasts accumulate myoglobin mRNA on fusion and differentiation in vitro.

The expression of the muscle contractile protein genes is an attractive system for the study of the induction of tissue-specific genes in cellular differentiation, and can be modeled in culture by differentiation of skeletal muscle myoblasts to form multinucleate contractile myotubes. Many studies have shown that contractile protein genes are activated after myoblast fusion (see reference 9). These include skeletal and cardiac muscle α-actins (5), myosin light and heavy chains (42), and troponins and tropomyosins (15). Most of these proteins are encoded by multigene families (23, 46), individual members of which exhibit tissue-specific and developmentally regulated expression. For example, fetal- and neonatal-specific isoforms have been found for myosin heavy chain (42), and there are in total at least 10 isoforms of this protein expressed in mammalian tissues (10).

In contrast to the muscle-specific contractile protein genes, much less is known about the expression of other muscle-specific genes during myogenesis. An example of such a protein is myoglobin, the principal hemoprotein of vertebrate muscle, which serves to facilitate diffusion of oxygen to the muscle mitochondria (43). Elevated levels of myoglobin are found in skeletal muscle of diving mammals and birds and in some mammals adapted to hypoxic subterranean or high-altitude environments (11, 26, 34). Levels in some aquatic species are high enough to act as a significant oxygen store during diving (44) and therefore represent an evolutionary adaptation to a diving physiology. There is also a correlation between muscle type and myoglobin content, such that slow (or red) muscles contain a higher concentration of myoglobin than fast (white) muscles (36). Little is known about the regulation of myoglobin synthesis during muscle development. In humans and sheep, myoglobin accumulates in cardiac muscle early in fetal development, but was not thought to appear in skeletal muscle until later in gestation (27, 38).

Myoglobin genes are distant members of the globin gene superfamily, the duplication event leading to the divergence of myoglobin and hemoglobin genes having preceded the divergence of α- and β-globin genes (14). Myoglobin alone of the globin genes is expressed in the myogenic developmental system, rather than the erythroid system. We have previously cloned and characterized the myoglobin genes from the grey seal (7), humans (41), and mice (A. Blanchetot, M. Price, and A. J. Jeffreys, manuscript in preparation) and showed that myoglobin in each species is specified by a single gene. Each has the three-exon and two-intron structure found in α- and β-globin genes (7) but differs markedly from all characterized α- and β-globin genes in having very long introns and 3′-nontranslated mRNA sequences.

As a prelude to studying the molecular basis of myoglobin gene regulation during myogenesis and the nature of myoglobin adaptation in diving mammals, we now describe the analysis of myoglobin gene expression during development in vivo and in differentiating embryonic myoblasts in vitro.

MATERIALS AND METHODS

Measurement of myoglobin protein levels. Soluble protein extracts were made from human and mouse muscle by homogenizing frozen tissue in 2 volumes of ice-cold water and centrifuging at 12,000 × *g* for 10 min. Proteins were analyzed by electrophoresis on 16% sodium dodecyl sulfate (SDS)-polyacrylamide vertical slab gels (25). Gels were stained with Coomassie brilliant blue or electroblotted by the procedure of Towbin et al. (39). Western blots were blocked in a phosphate-buffered saline–Tween solution (PBS-Tween; 0.14 M NaCl, 0.25 M KCl, 10 mM sodium phosphate [pH 7.5], 0.15% Tween) and then incubated overnight with 25 μg of rabbit anti-human myoglobin immunoglobulin G (Dakopatts) per ml. After washing in PBS-Tween, filters were incubated with swine anti-rabbit immunoglobulin G (Dakopatts) diluted 1:1,000 for 3 h, washed, and then stained with hydrogen peroxide-diaminobenzidine (50 μl of H_2O_2 and 50 mg of diaminobenzidine in 50 ml of 0.2 M Tris hydrochloride [pH 8.0]). Approximate levels of myoglobin protein in adult muscle samples were assessed by comparing

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protein staining intensities of a series of dilutions of muscle extract (1:2 to 1:40, vol/vol) with standard amounts of purified myoglobin (5 to 0.1 μg). Myoglobin in fetal and smooth muscle samples was quantified by a similar comparative assessment after Western blotting and immunodetection.

Myoglobin from human and grey seal muscle was purified by isoelectric focusing as described previously (45), and myoglobin concentrations were estimated from A_{543} values.

Actin and myoglobin hybridization probes. The mouse skeletal α-actin cDNA probe, containing the sequence for amino acid residues 30 to 374 plus approximately 300 nucleotides (nt) of 3′ noncoding sequence, was isolated in a *Pst*I fragment from plasmid pAM91 (31). Myoglobin gene probes from the 3′-nontranslated regions of the human and seal genes were a 285-base-pair (bp) *Hpa*II-*Hin*fI fragment from pHM.17.E1 (41) and a 269-bp *Sau*96I-*Hin*fI fragment from pSM.1.17 (7), respectively (see Fig. 5). These three probes were separated from vector and other gene sequences by restriction and agarose gel electrophoresis onto DE81 paper (16) before labeling by nick translation (41).

An M13 recombinant, M13HEX2, contained a 253-bp *Bst*EII-*Hae*III human exon 2 fragment which had been isolated from plasmid pHM.27.B1.1 (41), blunt ended, and cloned into the *Sma*I site of M13mp18 (48). A ^{32}P-labeled, single-stranded, complementary antisense human myoglobin probe containing all but the 5′-most 11 bp of the exon 2 coding sequence, plus 42 bp of intron 2, was generated from M13HEX2 by the method of Jeffreys et al. (24). Similarly, a mouse exon 1 probe containing 119 bp of exon 1 plus 16 bp of the 5′-flanking sequence was generated from M13MEX1, isolated during sequence analysis of the mouse myoglobin gene (Blanchetot et al., in preparation).

Preparation and electrophoresis of RNA and filter hybridization. Embryonic myoblast cell lines L6 (rat [47]) and G8 (mouse [12]) were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. Fusion of myoblasts to produce multinucleate myotubes was carried out by the method of Walsh and Phillips (40), and poly(A)$^+$ RNA was prepared from pre- and postfusion cultures as described previously (3). Adult human tissues were obtained from autopsy, and fetal material was from abortions. Tissue was homogenized thoroughly in 3 M LiCl–6 M urea–10 mM sodium acetate (pH 5.6)–0.1% SDS with a Waring blender. RNA was prepared after precipitation for 24 to 48 h at 4°C by the method of Minty et al. (31). Poly(A)$^+$ RNA was isolated by three passages through an oligo(dT)-cellulose column (4), each passage being preceded by heat shocking the RNA in water at 65°C for 3 min. RNA concentrations were determined spectrophotometrically and confirmed by ethidium bromide staining of RNA electrophoresed in agarose gels. Northern blot analysis of RNA was performed by electrophoresis in 1% agarose gels containing 2.2 M formaldehyde and 10 mM sodium phosphate (pH 7.6). RNA was transferred to nitrocellulose and hybridized to ^{32}P-labeled probes as described by Thomas (37). Washed, dried filters were exposed to Kodak X-Omat X-ray film.

S1 nuclease protection mapping. S1 nuclease protection mapping was carried out as previously described (6). The human myoglobin gene probe fragment, a 1.0-kilobase *Sst*I-*Nco*I fragment extending upstream from an *Nco*I site at the initiation codon in the human myoglobin gene, was kinase labeled at the *Nco*I terminus with [γ-^{32}P]ATP. Total RNA (10 to 100 μg) was mixed with 30,000 cpm of probe DNA (approximately 100 ng) in 40 μl of 40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] (pH 6.4)–0.4 M NaCl–1 mM EDTA–2.5 mg of tRNA per ml–80% formamide, denatured at 85°C for 12 min, and then hybridized at 52°C for 15 h. Each hybridization mix was then diluted into 4 × 300 μl of S1 buffer to which between 20 and 400 U of S1 nuclease were added, and tubes were incubated at 37°C for 30 min. Nucleic acids were precipitated with ethanol, denatured, and electrophoresed in an 8% polyacrylamide–8 M urea sequencing gel against a Maxam-Gilbert sequence ladder of the probe fragment (28).

Measurement of myoglobin produced by in vitro translation of poly(A)$^+$ RNA. Seal and human muscle poly(A)$^+$ RNAs (3 μg) were translated in a final volume of 35 μl containing 22.5 μl of rabbit reticulocyte lysate (Amersham Corp., Arlington Heights, Ill.) plus [^{35}S]methionine (50 TBq/mmol; Amersham) at a final concentration of 37 MBq/ml at 30°C for 60 min. Reactions were chased by adding methionine to 10 mM and pancreatic RNase to 0.1 mg/ml. A 7-μl sample was removed to analyze total translation products and to measure total ^{35}S incorporation. The remainder was loaded on a preprepared isoelectric-focusing acrylamide gel (Ampholine PAGplate pH 3.5 to 9.5; LKB Instruments, Inc., Rockville, Md.) and focused with purified human and seal myoglobin as markers. Gel slices containing labeled myoglobin were excised and boiled in 62.5 mM Tris hydrochloride (pH 6.8)–2% SDS–10% glycerol–5% 2-mercaptoethanol. The proteins present were analyzed in an 18% SDS-polyacrylamide gel, together with total in vitro translation products. The gel was stained with Coomassie brilliant blue and fluorographed (8). Incorporation of [^{35}S]methionine in labeled myoglobin in the gel was measured by liquid scintillation spectrometry (2).

Measuring the absolute level of myoglobin mRNA. ^{32}P-labeled single-stranded DNA containing 253 nt of the human myoglobin exon 2 antisense strand was generated from M13HEX2 by an adaptation of the method of Jeffreys et al. (24). Approximately 5 μg of single-stranded M13HEX2 DNA was annealed with 50 ng of M13 universal primer (17) in 20 μl of 10 mM $MgCl_2$–10 mM Tris hydrochloride (pH 8.0), and primer extension was carried out as described previously (24), using a ratio of [α-^{32}P]dCTP (110 TBq/mmol; Amersham) and unlabeled dCTP calculated to give a specific activity of 7×10^3 cpm/ng of antisense DNA. Labeled cDNA was separated by agarose gel electrophoresis as described previously (24). The cDNA was eluted from the gel slice and recovered by ethanol precipitation, and the yield was measured by Cerenkov counting.

^{32}P-labeled cDNA was hybridized with seal poly(A)$^+$ RNA in 10 μl of 1 × SSC (0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0)–100 μg of tRNA per ml at 65°C for 5 to 41 h. Samples were electrophoresed directly in 1.5% agarose gels which were dried and autoradiographed. The amount of cDNA hybridized to RNA was determined by Cerenkov counting regions of the dried gels.

RESULTS

Presence of myoglobin early in fetal development. The semiquantitative analysis of myoglobin levels in adult human skeletal muscle by comparison of protein staining intensities with standard myoglobin samples gives an estimate of 7.5 mg of myoglobin per g (wet weight) of muscle. This value is in good agreement with previous estimates (1, 33). Western blotting combined with immunoanalysis allows the detection of very low levels of myoglobin, down to 3 ng (data not shown), and was used to assess levels of myoglobin in human fetal skeletal muscle extracts in which myoglobin protein cannot be detected by conventional protein staining

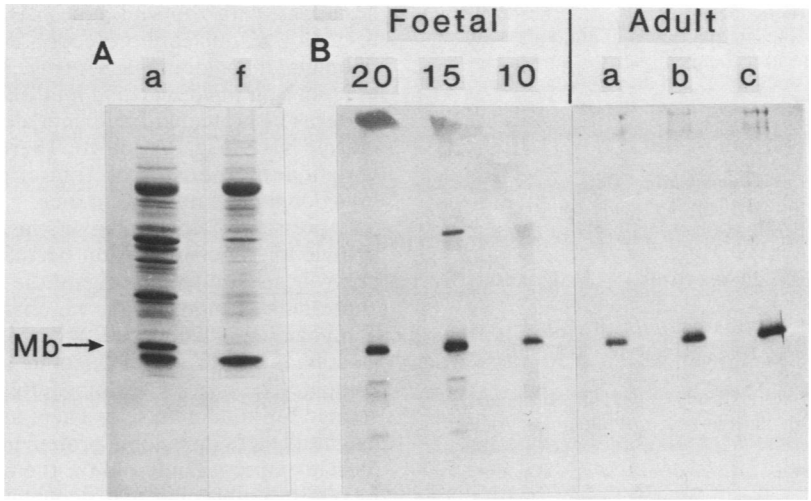

FIG. 1. Myoglobin levels in human adult and fetal skeletal muscle. (A) Protein profile of human adult (lane a) and fetal (lane f; 15 weeks of gestation) skeletal muscle soluble protein extracts. Samples were analyzed in 16% SDS-polyacrylamide gels and stained with Coomassie brilliant blue. The position of myoglobin (Mb) is marked). Both samples were prepared from the same wet weight of tissue. (B) Western blot of adult and fetal skeletal muscle preparations. Proteins were separated on 16% SDS-polyacrylamide gels, transferred to nitrocellulose, and reacted with rabbit anti-human myoglobin antiserum. Extracts from equal wet weights of fetal muscle from 10, 15, and 20 weeks of gestation were run undiluted. Lanes, a, b, and c are from an adult sample diluted 1 in 512 (a), 1 in 256 (b), and 1 in 128 (c). In the fetal muscle samples in which the protein concentration was high, some components of higher and lower molecular size than myoglobin could be detected. These are not thought to be myoglobin-related proteins, but arise owing to a slight impurity of the antibody.

(38) (Fig. 1A). Analysis of extracts of limb muscle from fetuses of between 10 and 20 weeks of gestation showed that myoglobin is present in the developing muscle as early as 10 weeks at about 0.2% (15 μg/g [wet weight]) of the adult level. This rises to between 0.4 and 1% (30 to 75 μg/g) in the second trimester (Fig. 1B; Table 1).

Estimates of myoglobin levels in adult and fetal cardiac muscle and also in adult smooth muscle samples (Table 1) were obtained in the same way. Levels in adult heart are similar to those in skeletal muscle, while levels in smooth muscle are extremely low at around 0.2 to 0.4% of the skeletal muscle level. Myoglobin occurs at a significantly higher level in fetal heart at 20 weeks of gestation than in skeletal muscle, as previously reported (38).

To confirm that myoglobin is expressed early in fetal development, poly(A)$^+$ RNA was prepared from adult and

TABLE 1. Myoglobin protein and mRNA levels in various muscle tissues

Species	Muscle type	Protein levels[a]		mRNA levels[b]		Mb mRNA[d]
		Concn (mg/g)	% Adult	% Adult	μg/mg of poly(A)$^+$ RNA[c]	
Human	Adult skeletal	7.5, 6–8*	(100)	(100)	3.7	2.1
	Adult cardiac	~7.5	~100	~100	~4	~2.0
	Adult smooth (bladder, gut, uterus)	0.015–0.030	0.2–0.4	~1	~0.04	~0.6
	Fetal skeletal					
	1st trimester (10 wks)	0.015	0.2	1–4	0.04–0.2	~0.6
	2nd trimester	0.030–0.075	0.4–1.0			
	Fetal cardiac (20 wks)	0.375	5	~10	~0.4	~1.2
Mouse	Adult skeletal	0.3–0.5	(100)	(100)	3.7	0.1
	Adult cardiac	1.0	200–300	400	15	0.1
	Fetal skeletal					
	14/15 days of gestation	0.003	1	1	0.04	0.1
	Birth	0.075–0.090	25–30	10	0.4	0.2
Seal	Adult skeletal	40–50*	(100)	(100)	28	1.6

[a] Approximate myoglobin protein concentrations, given in milligrams per gram (wet weight), were assessed by comparative protein staining and immunoanalysis as described in the text. Protein concentrations marked with an * were derived from absorbance measurements (543 nm) of myoglobin purified by isoelectric focusing. Myoglobin levels are also given as a percentage of the appropriate adult skeletal muscle level.

[b] 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. Estimates were derived from the signal intensity of RNA samples titrated on Northern blots and, in the case of human fetal skeletal muscle, confirmed by measurement of autoradiographic signal intensity by scanning densitometry.

[c] Micrograms of myoglobin mRNA per milligram of poly (A)$^+$ RNA.

[d] Milligrams of myoglobin per microgram of myoglobin mRNA.

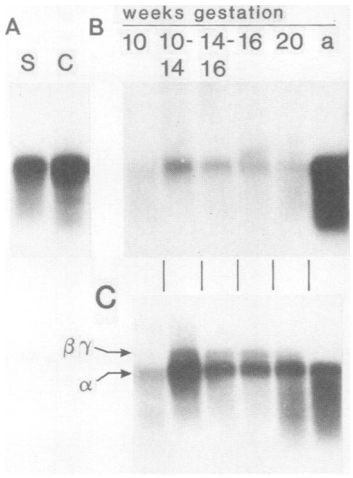

FIG. 2. Myoglobin mRNA levels in adult human skeletal and cardiac muscle and fetal skeletal muscle. (A) Northern blot of 0.25 μg of adult human skeletal (lane s) and cardiac (lane c) poly(A)$^+$ RNAs hybridized with a ^{32}P-labeled single-stranded human myoglobin exon 2 probe. The human myoglobin mRNA migrates at approximately 1,400 nt. (B) Northern blot of 1 μg of poly(A)$^+$ RNA from adult (lane a) and fetal skeletal muscle ranging in age from 10 to 20 weeks of gestation, hybridized with a nick-translated human myoglobin 3′-nontranslated-region probe. (C) The same blot shown in panel B after removal of the myoglobin probe, hybridized with the mouse skeletal α-actin cDNA probe. The positions of 2,000-nt cytoplasmic (β, γ) and 1,600-nt muscle (α) actin mRNAs are indicated. Minor variations in myoglobin mRNA levels between different fetal skeletal muscle poly(A)$^+$ RNA samples are largely reflected in corresponding changes in α-actin mRNA levels and presumably reflect variability in poly(A)$^+$ RNA purity and variation among individual fetuses.

fetal skeletal and cardiac muscle and also from adult smooth muscle and analyzed by Northern blot hybridization with human myoglobin gene probes. RNA samples from fetal skeletal muscle were also hybridized with a mouse α-actin cDNA probe, which cross-hybridizes with both human skeletal muscle α-actin mRNA and with cytoplasmic β- and γ-actin mRNAs. The myoglobin probes detected similar levels of 1,400-nt-long myoglobin mRNA in adult skeletal and cardiac muscle (Fig. 2A). As predicted, much lower levels of myoglobin mRNA could also be detected in adult smooth muscle RNA (data not shown) and in all RNA samples tested from fetal skeletal muscle ranging from 10 to 20 weeks of gestation (Fig. 2B). In contrast, the level of α-actin mRNA (Fig. 2C) (18) and myosin light chain mRNA (data not shown) in fetal skeletal muscle is not significantly different from that in adult muscle.

Levels of myoglobin mRNA in different human muscle tissues were estimated by titration on Northern blots and compared with the corresponding levels of myoglobin protein in these muscle samples (Table 1). Tissue-specific differences in levels of myoglobin are consistently reflected in myoglobin mRNA levels, with very low levels in smooth muscle and fetal skeletal muscle (~1 to 4% of adult levels) and a relatively high level (~10% adult) in fetal cardiac muscle.

Same transcriptional initiation site is used in the myoglobin gene in different muscle tissues. S1 nuclease protection mapping has previously been used to determine the 5′ terminus of seal myoglobin mRNA (7), and the corresponding cap site in the human myoglobin gene was provisionally located by homology with the seal gene (41). This location was confirmed by S1 nuclease protection mapping of the 5′ terminus of human myoglobin mRNA (Fig. 3C). S1 nuclease mapping of human adult cardiac and fetal skeletal muscle RNAs gave patterns of protected fragments identical with adult skeletal muscle RNA (Fig. 3A and B). There is therefore no apparent variation in the site of transcriptional initiation in the myoglobin gene in these tissues.

Expression of myoglobin in the mouse. To confirm that myoglobin gene expression occurs at a low level early in myogenesis and to investigate the developmental profile of expression in more detail, we characterized the expression of myoglobin in the mouse. Myoglobin levels were quantified in adult skeletal muscle and in adult cardiac muscle by comparative protein staining after electrophoresis as described for human muscle. There appeared to be an order of magnitude less myoglobin protein in the adult mouse skeletal muscle extract than found for the human samples (Table 1). Levels in embryonic and neonatal samples were assessed

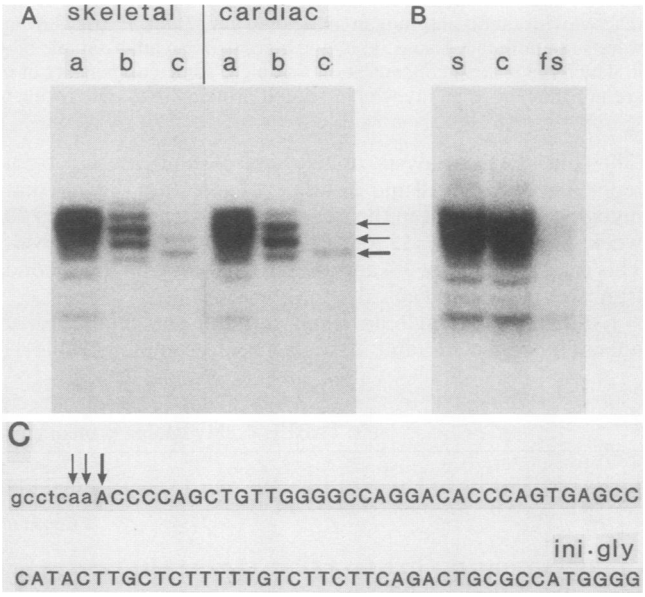

FIG. 3. The same myoglobin gene transcriptional start site is used in different human muscle RNAs, as shown by S1 nuclease protection mapping. (A) Autoradiograph from S1 nuclease protection mapping experiment with adult human skeletal and cardiac total RNA. A 50-μg sample of each RNA was hybridized with 30,000 cpm (approximately 100 ng) of probe fragment kinase labeled with [γ-^{32}P]ATP. The probe was an *Nco*I-*Sst*I fragment extending 1.0 kilobase upstream from the initiation codon of the human myoglobin gene. After hybridization, reaction mixtures were treated with 20 (lane a), 80 (lane b), or 320 (lane c) U of S1 nuclease, and the protected fragments were electrophoresed in an 8% polyacrylamide–8 M urea sequencing gel which was then dried and autoradiographed. (B) Long-exposure autoradiograph from S1 nuclease protection mapping experiment with 10 μg each of human adult skeletal (lane s) and cardiac (lane c) RNAs and 100 μg of 10- to 14-week fetal skeletal RNA (lane fs). The procedure was carried out as described above, except reaction mixtures were treated with 50 U of S1 nuclease. The intensity of signal obtained for the three different RNAs is consistent with estimates of myoglobin mRNA levels obtained from Northern hybridizations. (C) Sequence of part of the human myoglobin gene (41) showing the position of the cap site (arrows), determined by comparison of protected fragments with a Maxam-Gilbert sequence ladder of the probe fragment.

after Western blotting by comparison of staining intensity with adult samples and making use of the cross-reactivity between anti-human myoglobin antiserum and mouse myoglobin. These analyses indicated that myoglobin was also present in embryonic mouse muscle (14 days of gestation) at about 1% of the level found in the adult and that myoglobin levels reach 25 to 30% of the adult level at birth (Table 1).

Poly(A)$^+$ RNA was prepared from mouse skeletal muscle ranging from 15 days of gestation to adult. Northern blots of these RNAs were hybridized with the single-stranded antisense human myoglobin exon 2 probe or with the mouse skeletal muscle α-actin cDNA probe (Fig. 4). In adult muscle the myoglobin probe detected a mouse myoglobin mRNA at approximately the same concentration as the human myoglobin mRNA; this mRNA is about 1,200 nt long, 200 nt shorter than the human myoglobin mRNA owing to a shorter 3′-nontranslated region (Blanchetot et al., in preparation). Myoglobin mRNA was just detectable at 15 days of gestation. Levels showed some increase during fetal development and continued to increase substantially after birth before attaining the adult level (Table 1). In contrast, α-actin and myosin light chain mRNA levels showed relatively little variation, although levels of cytoplasmic (β, γ) actin mRNA decreased during embryonic muscle development. Slightly lower apparent mRNA levels early in development may result from the difficulty of obtaining pure skeletal muscle from early embryos.

Adaptation of myoglobin expression in a diving mammal. To define the basis for the elevated expression of myoglobin in diving mammals, we measured the myoglobin and myoglobin mRNA levels in grey seal skeletal muscle. Human and grey seal myoglobins were purified from skeletal muscle extracts by isoelectric focusing and quantified by absorption spectroscopy. The level of myoglobin in human muscle estimated by this method was in good agreement with the data from the comparative protein staining and Western blot analysis (Table 1). There was a six- to sevenfold-elevated level of myoglobin in seal skeletal muscle compared with human muscle.

Myoglobin mRNA levels in seal and human muscle were compared by Northern blot analysis of poly(A)$^+$ RNA, using a probe containing equimolar amounts of equivalent fragments from the 3′-nontranslated sequences of the grey seal and human myoglobin genes (Fig. 5A and B). An eightfold difference in myoglobin mRNA levels exists between human and seal skeletal muscle, comparable to the difference in myoglobin protein levels. This difference was confirmed by spot hybridizations and Northern blot analysis with additional human and seal myoglobin gene probes (data not shown).

To confirm that the elevated level of myoglobin in seal muscle results primarily from a correspondingly elevated level of myoglobin mRNA, we compared the efficiency of translation of myoglobin mRNA in seal and human muscle RNA samples by in vitro translation in rabbit reticulocyte lysates. As predicted, much less myoglobin was synthesized from human than from seal poly(A)$^+$ RNA, although the difference could not be directly quantified from fluorographs of SDS-polyacrylamide gels in which total translation products had been electrophoresed (Fig. 5C). Subsequent purification of newly synthesized myoglobin by isoelectric focusing showed that translation of seal poly(A)$^+$ RNA yielded 10.2 times as much myoglobin as translation of an equal amount of human poly(A)$^+$ RNA (Fig. 5D). This difference correlates well with the eightfold difference in myoglobin mRNA levels and indicates that there is no significant difference in efficiency of translation of seal and human myoglobin mRNAs.

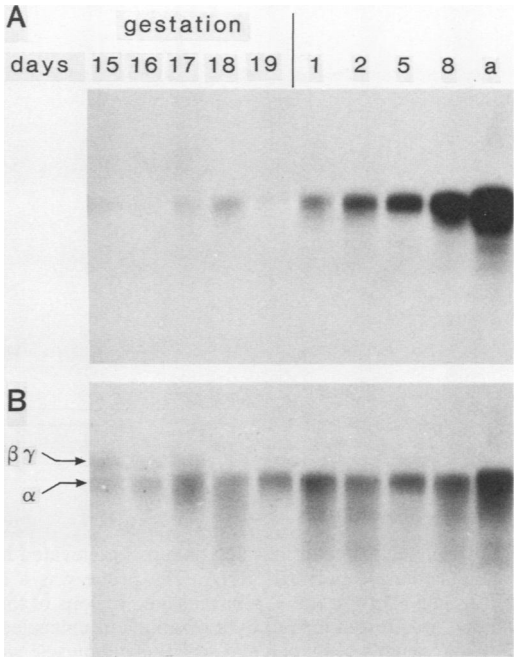

FIG. 4. Myoglobin mRNA levels in developing mouse muscle. (A) Northern blot of 2 μg of poly(A)$^+$ RNAs from skeletal muscle of mice of ages varying from 15 days of gestation to adult (lane a), hybridized with the single-stranded human myoglobin exon 2 antisense DNA probe. (B) The same blot as in panel A after removal of the myoglobin probe, hybridized with the mouse skeletal α-actin cDNA probe. The positions of cytoplasmic (β, γ; approximately 2,000 nt) and muscle (α; approximately 1,600 nt) actin mRNAs are indicated.

Absolute level of myoglobin mRNA. The absolute level of myoglobin mRNA within the total population of seal muscle poly(A)$^+$ RNA was determined by hybridizing a known, constant amount of ^{32}P-labeled antisense, single-stranded DNA complementary to exon 2 of human myoglobin mRNA with increasing amounts of seal poly(A)$^+$ RNA. High homology between seal and human myoglobin exon 2 sequences allows the two to hybridize (41). After hybridization, the samples were electrophoresed directly in an agarose gel which was then dried and autoradiographed. Figure 6 shows the result of such an experiment with seal poly(A)$^+$ RNA and with *Aspergillus nidulans* RNA as a negative control. The absolute level of myoglobin mRNA can be determined from the hybridization reaction in which all the labeled single-stranded antisense DNA just hybridizes with the seal RNA. Detailed titrations (Fig. 6, legend) showed that 100 ng of seal poly(A)$^+$ RNA contains just enough myoglobin mRNA to hybridize with 1 ng of exon 2 antisense DNA. This gives the absolute level of myoglobin mRNA as 2.8% of the total population of poly(A)$^+$ RNAs in seal skeletal muscle. This figure is in good agreement with the estimate of 4% made during cDNA cloning of the grey seal myoglobin gene (45). From this estimate, absolute myoglobin mRNA levels in all human, seal, and mouse tissues could be calculated (Table 1).

Induction of myoglobin gene expression in embryonic myoblasts during fusion and differentiation in vitro. The finding that myoglobin is expressed early in embryogenesis

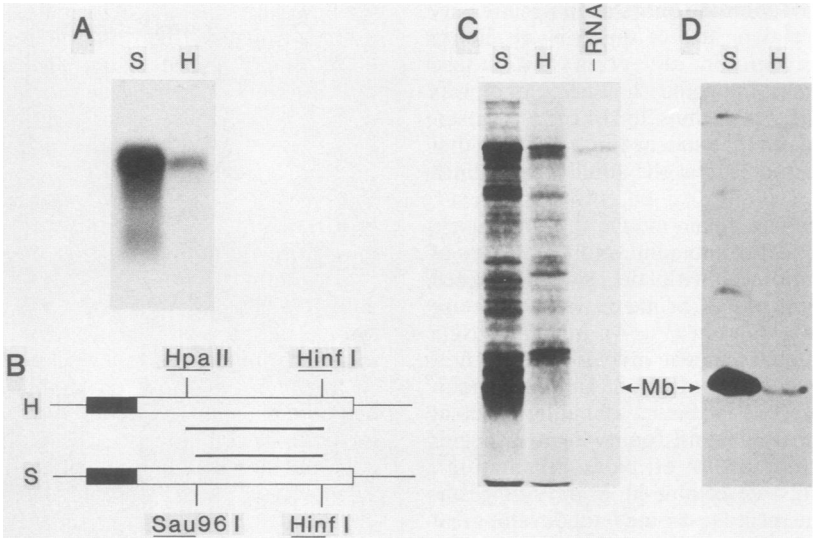

FIG. 5. Grey seal skeletal muscle contains an elevated level of myoglobin mRNA. (A) Autoradiograph of a Northern blot of seal and human skeletal muscle poly(A)$^+$ RNA. The probe was a mixed nick-translated probe containing equimolar amounts of almost exactly equivalent fragments from the 3′-nontranslated regions of the human (lane H) and grey seal (lane S) myoglobin genes. Equal labeling of the two fragments was tested in spot hybridizations of unlabeled probe DNAs (data not shown). A 1-μg sample of poly(A)$^+$ RNA representing equivalent wet weights of human and seal skeletal muscle was electrophoresed in each track. (B) Probe fragments from seal (S) and human (H) myoglobin gene 3′-nontranslated regions used in Northern blot hybridization shown in panel A. Filled box, Exon 3 coding sequence; open box, 3′-nontranslated region; bars, probe fragments. (C) Fluorograph showing total in vitro translation products produced by translating 3 μg of seal (S) and human (H) poly(A)$^+$ RNA in rabbit reticulocyte lysate in the presence of [^{35}S]methionine. A fifth of each reaction was electrophoresed in an 18% SDS-polyacrylamide gel which was stained and fluorographed. The migration position of myoglobin (Mb) is indicated. The overall level of incorporation of [^{35}S]methionine for human poly(A)$^+$ RNA was 70% that of seal poly(A)$^+$ RNA. (D) Myoglobin was partially purified by isoelectric focusing from the remainder of the in vitro translation products in the reaction described in panel C. Labeled myoglobin was electrophoresed in an 18% SDS-polyacrylamide gel and fluorographed. The migration position of myoglobin (Mb) is indicated.

suggests that it might also be expressed in differentiating embryonic myoblast cell lines. Poly(A)$^+$ RNAs from pre- and postfusion cultures of the myogenic rat cell line L6 (47) and mouse cell line G8 (12) were analyzed by Northern blot hybridization with myoglobin gene probes and also with probes from other muscle-specific protein genes, including the mouse skeletal muscle α-actin cDNA probe. Myoglobin gene transcription was indeed induced after fusion and differentiation of both cell lines, but consistently more strongly in G8 myotubes (Fig. 7A). Longer exposure of this autoradiograph failed to show any trace of myoglobin mRNA in myoblasts (data not shown). Other muscle-specific protein genes were also induced: Figure 7B shows postfusion expression of muscle α-actin mRNA, and induction of myosin light-chain mRNA expression also occurred (data not shown).

The level of myoglobin mRNA obtained in G8 myotubes was consistently higher than that seen in embryonic muscle poly(A)$^+$ RNA and while variable from fusion to fusion was comparable to that seen in skeletal muscle from 1- to 8-day-old mice (data not shown).

Southern blotting showed that the myoglobin gene hybridization pattern is identical in mouse (DBA/2) liver, G8 myoblast, and G8 myotube genomic DNA (data not shown). This indicates that the induction of expression of the mouse myoglobin gene in G8 myotubes is not accompanied by gene amplification or major rearrangement.

DISCUSSION

Early work suggested that detectable levels of myoglobin only appear in skeletal muscle toward the end of gestation (27, 38). However, using more sensitive techniques, we showed that the myoglobin gene is expressed at low levels in myogenesis as early as 10 weeks of gestation in humans and 14 days in the mouse. During subsequent pre- and postnatal development, levels of myoglobin mRNA in skeletal muscle increase by 50- to 100-fold before reaching adult levels. This is in marked contrast to the muscle contractile proteins, the mRNA levels of which do not vary significantly during later myogenesis. We confirmed that myoglobin and myoglobin mRNA levels are elevated in the fetal heart (38), presumably reflecting its early activity in utero. We also showed that myoglobin and myoglobin mRNA are present at low levels in adult human smooth muscle, from which myoglobin was previously thought to be absent (19), although chicken gizzard smooth muscle has been shown to contain high levels of myoglobin (22). Since myoglobin is associated with slow muscle fibers (red) in adult muscle (36), low levels of myoglobin in fetal skeletal muscle and smooth muscle might therefore be correlated with the relative lack of slow fibers in these tissues (13).

Levels of myoglobin in embryonic-fetal versus adult skeletal muscle, as well as in different human adult muscle tissues, appear to be largely determined by the size of the myoglobin mRNA pools (Table 1). Similarly, grey seal skeletal muscle, which contains approximately seven times as much myoglobin as human skeletal muscle as an adaptation to diving, contains a correspondingly elevated level of myoglobin mRNA. It therefore seems likely that this adaptation to high myoglobin levels has arisen through enhanced transcriptional activity of the myoglobin gene or through reduced turnover of myoglobin mRNA or both. In contrast,

mouse skeletal and cardiac muscle contain very low levels of myoglobin, yet the level of myoglobin mRNA in these tissues is comparable to those seen in humans (Table 1). This difference might reflect a decreased translational efficiency of mouse myoglobin mRNA, reduced by at least an order of magnitude compared with human and seal mRNA, or might instead arise through increased turnover of myoglobin protein in mouse muscle. Since purified mouse myoglobin is not available, it is not yet possible to distinguish between these alternatives directly by in vitro translation experiments such as those shown in Fig. 5.

Myoglobin gene expression in skeletal muscle appears to be regulated at two distinct developmental phases, first, a low-level expression in early myogenesis and, second, an elevation of myoglobin mRNA pools in later myogenesis possibly linked to the development of slow fibers. The first phase can apparently be modeled in vitro in embryonic myoblast cell lines, which accumulate myoglobin mRNA upon cell fusion and differentiation to form contractile myotubes. Since several contractile protein genes are also transcriptionally activated upon differentiation in vitro (9), this raises the possibility that there may be some regulatory similarities in the initial induction of myoglobin genes and contractile protein genes. However, mouse G8 embryonic myoblasts consistently accumulate higher levels of myoglobin mRNA per unit mass of poly(A)$^+$ RNA than are found in embryonic mouse skeletal muscle. This might suggest that the regulation of myoglobin gene expression in vitro does not fully reflect that seen in vivo.

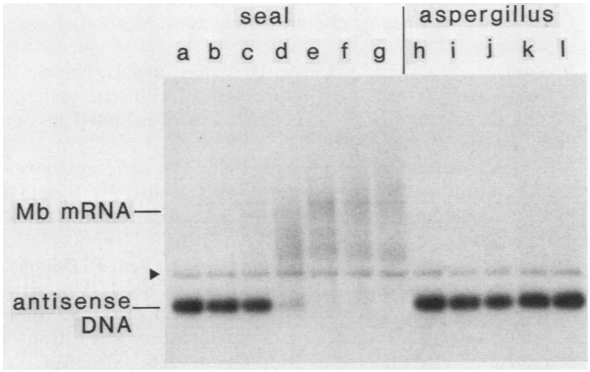

FIG. 6. Absolute level of myoglobin mRNA in grey seal skeletal muscle. Single-stranded human exon 2 antisense DNA (1 ng) with a specific activity of 7×10^3 cpm/ng, generated from M13HEX2 by primer extension, was hybridized for 15 h with increasing amounts of seal poly(A)$^+$ RNA (4, 13, 40, 120, 350, 1,050, and 3,150 ng [lanes a to g]) or *A. nidulans* RNA (40, 120, 350, 1,050, and 3,150 ng [lanes h to l]). Hybridization reactions were electrophoresed directly in a 1.5% agarose gel which was dried and autoradiographed. A total of 80% of exon 2 antisense DNA hybridized with 120 ng of seal poly(A)$^+$ RNA (lane d), giving an abundance estimate of 2.8% of myoglobin mRNA in seal muscle poly(A)$^+$ RNA. The migration positions of seal myoglobin mRNA (Mb mRNA) and unhybridized antisense DNA are indicated. Hybridized DNA-myoglobin mRNA does not migrate as a tight band because RNA inevitably degrades after prolonged incubation at 65°C. Tighter bands, with a mobility similar to that of myoglobin mRNA, were seen on other gels with samples which had been hybridized for a shorter time (data not shown). Various controls were used to confirm this quantitation. First, 1 ng of ^{32}P-labeled antisense DNA was hybridized to increasing amounts (6 to 200 ng) of the single-stranded M13HEX2 parent molecule in 10 μl of 1 × SSC at 65°C for 15 h. The titration endpoint estimated by gel electrophoresis and autoradiography was achieved with 30 ng of template, compared with 24 ng predicted from the relative sizes of probe and template. Second, 50 or 80 ng of seal poly(A)$^+$ RNA was hybridized to excess (1 ng) ^{32}P-labeled antisense DNA for 0, 5, 17, and 41 h. A hybridization plateau was achieved within 5 h, with 38 and 54% of DNA hybridized, respectively, giving estimates of myoglobin mRNA abundance of 3.1 and 2.8%, respectively. Third, a constant amount of seal poly(A)$^+$ RNA (120 ng) was hybridized to increasing amounts of ^{32}P-labeled antisense DNA (0.1 to 4 ng) for 15 h. The amount of DNA hybridized achieved a plateau of 0.72 ng at ≧1 ng of input antisense DNA, giving an abundance estimate of 2.5%. The component indicated by the arrowhead is always present in single-stranded antisense DNA preparations but is of unknown origin. It may represent a double-stranded form, perhaps created by foldback during primer extension, since it does not hybridize either to M13HEX2 DNA or to myoglobin mRNA.

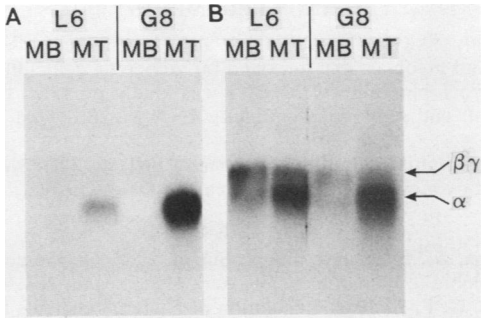

FIG. 7. Induction of myoglobin gene expression in differentiating embryonic myoblasts. (A) Northern blot of 2 μg of L6 and G8 myoblast (MB) and myotube (MT) poly(A)$^+$ RNA hybridized with a single-stranded mouse myoglobin exon 1 antisense probe. Myotubes were harvested after 5 days in fusion medium. The mouse myoglobin transcript detected after fusion is approximately 1,200 nt long. (B) The same blot in panel A hybridized with the mouse α-skeletal muscle actin cDNA probe. The migration positions of cytoplasmic actin (β and γ; approximately 2,000 nt) and α-muscle actin (α; approximately 1,600 nt) are indicated. Cytoplasmic (β and γ) actins are expressed both before and after fusion, whereas muscle α-actin expression is induced upon fusion and differentiation of myoblasts.

DNA transfection experiments have shown that exogenous contractile protein genes can be expressed at a very low level in myoblasts in vitro and that in most cases the level of expression is appropriately enhanced upon fusion and differentiation to form myotubes (21, 29, 30, 32, 35). Such experiments will allow the localization of *cis*-acting regulatory elements that confer tissue specificity and differentiation-linked inducibility on contractile protein genes. The substantial induction of myoglobin mRNA in G8 myoblasts upon differentiation should allow these studies to be extended to a muscle-specific gene that does not specify a contractile protein. Preliminary experiments involving transient expression analysis of ~950-bp segments of seal and mouse myoglobin gene 5′-flanking region fused to a reporter gene (chloramphenicol acetyltransferase) (20) indicate that the gene fusion is expressed in G8 cells and furthermore that expression is enhanced ~5- to 15-fold upon differentiation in vitro (M. Price and A. J. Jeffreys, unpublished data). This system might therefore permit the identification of sequences involved in myoglobin gene induction, particularly with respect to a highly conserved 250-bp region located ~120 bp upstream from the myoglobin cap site and shared by human, seal, and mouse myoglobin genes (41) (Blanchetot et al., in preparation). This system could also be used to determine whether the high level of myoglobin mRNA seen in seal muscle arises through increased promoter activity of the myoglobin gene and, if so, to define the molecular basis for this physiological adaptation.

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LITERATURE CITED

1. **Akeson, A., G. Biorck, and R. Simon.** 1968. On the content of myoglobin in human muscles. Acta Med. Scand. **183**:307–316.
2. **Ames, G. F.-L.** 1974. Resolution of bacterial proteins by polyacrylamide gel electrophoresis on slabs. J. Biol. Chem. **249**:634–644.
3. **Auffray, C., and F. Rougeon.** 1980. Purification of mouse immunoglobulin heavy chain messenger RNAs from total myeloma tumour RNA. Eur. J. Biochem. **107**:303–314.
4. **Aviv, H., and P. Leder.** 1972. Purification of biologically active globin mRNA by chromatography on oligothymidilic acid-cellulose. Proc. Natl. Acad. Sci. USA **69**:1408–1412.
5. **Bains, W., P. Ponte, H. Blau, and L. Kedes.** 1984. Cardiac actin is the major actin gene product in skeletal muscle cell differentiation in vitro. Mol. Cell. Biol. **4**:1449–1453.
6. **Berk, A. J., and P. A. Sharp.** 1977. Sizing and mapping of early adenovirus mRNAs by gel electrophoresis of S1 endonuclease-digested hybrids. Cell **12**:721–732.
7. **Blanchetot, A., V. Wilson, D. Wood, and A. J. Jeffreys.** 1983. The seal myoglobin gene: an unusually long globin gene. Nature (London) **301**:732–734.
8. **Bonner, W. M., and R. A. Laskey.** 1974. A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. **46**:83–88.
9. **Buckingham, M. E.** 1977. Muscle protein synthesis and its control during the differentiation of skeletal muscle cells *in vitro*. Int. Rev. Biochem. **15**:269–332.
10. **Buckingham, M. E., and A. J. Minty.** 1983. Contractile protein genes, p. 365–396. *In* N. Maclean, S. Gregory, and R. Flavell (ed.), Eukaryotic genes: their structure, activity and regulation. Butterworths, London.
11. **Butler, P. J.** 1982. The comparative physiology of diving in vertebrates. Adv. Comp. Physiol. Biochem. **8**:179–363.
12. **Christian, C. N., P. G. Nelson, J. Peacock, and M. Nirenberg.** 1977. Synapse formation between two clonal cell lines. Science **196**:995–998.
13. **Colling-Saltin, A.** 1978. Enzyme histochemistry on skeletal muscle in the human foetus. J. Neurol. Sci. **39**:169–185.
14. **Czelusniak, J., M. Goodman, D. Hewett-Emmett, M. L. Weiss, P. J. Venta, and R. E. Tashian.** 1982. Phylogenetic origins and adaptive evolution of avian and mammalian haemoglobin genes. Nature (London) **298**:297–300.
15. **Devlin, R. B., and C. P. Emerson, Jr.** 1978. Coordinate regulation of contractile protein synthesis during myoblast differentiation. Cell **13**:599–611.
16. **Dretzen, G., M. Bellard, P. Sassone-Corri, and P. Chambon.** 1981. A reliable method for the recovery of DNA fragments from agarose and acrylamide gels. Anal. Biochem. **112**:295–298.
17. **Duckworth, M. L., M. J. Crait, P. Goelet, G. F. Hong, M. Singh, and R. C. Titmas.** 1981. Rapid synthesis of oligodeoxyribonucleotides. IV. Efficient mechanized synthesis of heptadecadeoxyribonucleotides by an improved solid phase phosphotriester route. Nucleic Acids Res. **9**:1691–1706.
18. **Edwards, Y. H., J. C. Lloyd, S. L. McMillan, and F. J. Benham.** 1985. Human glyceraldehyde-3-phosphate dehydrogenase: mRNA levels and enzyme activity in developing muscle. Mol. Cell. Biol. **5**:2147–2149.
19. **Fasold, H., G. Riedl, and F. Jaisle.** 1970. Evidence for an absence of myoglobin from human smooth muscle. Eur. J. Biochem. **15**:122–126.
20. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2**:1044–1051.
21. **Grichnik, J. M., D. J. Bergsma, and R. J. Schwartz.** 1986. Tissue restricted and stage specific transcription is maintained within 411 nucleotides flanking the 5'-end of the chicken α-skeletal actin gene. Nucleic Acids Res. **14**:1683–1701.
22. **Groschel-Stewart, U., U. Jaroschik, and H. Schwalm.** 1971. Chicken gizzard, a myoglobin-containing smooth muscle. Experientia **27**:512–513.
23. **Humphries, S. E., R. Whittall, A. Minty, M. Buckingham, and R. Williamson.** 1981. There are approximately 20 actin genes in the human genome. Nucleic Acids Res. **9**:4895–4908.
24. **Jeffreys, A. J., V. Wilson, and S. L. Thein.** 1985. Hypervariable "minisatellite" regions in human DNA. Nature (London) **314**:67–73.
25. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
26. **Lenfant, C.** 1973. High altitude adaptation in mammals. Am. Zool. **13**:447–456.
27. **Longo, L. D., B. J. Koos, and G. G. Power.** 1973. Fetal myoglobin: quantitative determination and importance for oxygenation. Am. J. Physiol. **224**:1032–1036.
28. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65**:499–560.
29. **Melloul, D., B. Aloni, J. Calvo, D. Yaffes, and U. Nudel.** 1984. Developmentally regulated expression of chimeric genes containing muscle actin DNA sequences in transfected myogenic cells. EMBO J. **3**:983–990.
30. **Minty, A., H. Blau, and L. Kedes.** 1986. Two-level regulation of cardiac actin gene transcription: muscle-specific modulating factors can accumulate before gene activation. Mol. Cell. Biol. **6**:2137–2148.
31. **Minty, A. J., M. Caravatti, B. Robert, A. Cohen, P. Daubas, A. Weydert, F. Gros, and M. E. Buckingham.** 1981. Mouse actin mRNAs: construction and characterization of a recombinant plasmid molecule containing a complementary DNA transcript of mouse α-actin mRNA. J. Biol. Chem. **256**:1008–1014.
32. **Minty, A., and L. Kedes.** 1986. Upstream regions of the human cardiac actin gene that modulate its transcription in muscle cells: presence of an evolutionarily conserved repeat motif. Mol. Cell. Biol. **6**:2125–2136.
33. **Moller, P., and C. Sylven.** 1981. Myoglobin in human skeletal muscle. Scand. J. Clin. Lab. Invest. **41**:479–482.
34. **Nevo, E.** 1979. Adaptive convergence and divergence of subterranean mammals. Annu. Rev. Ecol. Syst. **10**:269–308.
35. **Nudel, U., D. Greenberg, C. P. Ordahl, O. Saxel, S. Neuman, and D. Yaffe.** 1985. Developmentally regulated expression of a chicken muscle-specific gene in stably transfected rat myogenic cells. Proc. Natl. Acad. Sci. USA **82**:3106–3109.
36. **Peter, J. B., R. J. Barnard, V. R. Edgerton, C. A. Gillespie, and K. E. Stempel.** 1972. Metabolic profiles of three fibre types of skeletal muscle in guinea pigs and rabbits. Biochemistry **11**:2627–2633.
37. **Thomas, P. S.** 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc. Natl. Acad. Sci. USA **77**:5201–5205.
38. **Tipler, T. D., Y. H. Edwards, and D. A. Hopkinson.** 1978. Developmental changes in the protein profiles of human cardiac and skeletal muscle. Ann. Hum. Genet. **41**:409–418.
39. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets. Procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
40. **Walsh, F. S., and E. Phillips.** 1981. Specific changes in cellular glycoproteins and surface proteins during myogenesis in clonal muscle cells. Dev. Biol. **81**:229–237.
41. **Weller, P., A. J. Jeffreys, V. Wilson, and A. Blanchetot.** 1984. Organisation of the human myoglobin gene. EMBO J. **3**:439–446.
42. **Whalen, R. G., S. M. Sell, G. S. Butler-Browne, K. Schwartz, P. Bouveret, and I. Pinset-Harstrom.** 1981. Three myosin heavy-chain isozymes appear sequentially in rat muscle development. Nature (London) **292**:805–809.
43. **Wittenberg, B. A., J. B. Wittenberg, and P. R. B. Caldwell.** 1975.

Role of myoglobin in the oxygen supply to red skeletal muscle. J. Biol. Chem. **250**:9038–9043.

44. **Wittenberg, J. B.** 1970. Myoglobin-facilitated oxygen diffusion: role of myoglobin in oxygen entry into muscle. Physiol. Rev. **50**:559–636.
45. **Wood, D., A. Blanchetot, and A. J. Jeffreys.** 1982. Molecular cloning of seal myoglobin mRNA. Nucleic Acids Res. **10**:7133–7144.
46. **Wydro, R. M., H. T. Nguyen, R. M. Gubits, and B. Nadal-Ginard.** 1983. Characterisation of sarcomeric myosin heavy chain genes. J. Biol. Chem. **258**:670–678.
47. **Yaffe, D.** 1968. Retention of differentiation potentialities during prolonged cultivation of myogenic cells. Proc. Natl. Acad. Sci. USA **61**:477–483.
48. **Yanish-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33**:103–119.