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

FOLLY A. WELLER,† MELANIE PRICE,† HARVEY ISENBERG,‡ YVONNE H. EDWARDS,‡ and ALEC J. JEFFREYS§

Department of Genetics, University of Leicester, Leicester LE1 7RH,† and MRC Human Biochemical Genetics Unit, The Galton Laboratory, University College, London NW1 2HE,‡ United Kingdom

Received 9 April 1986/Accepted 2 September 1986

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 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–Tweens 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 H₂O₂ 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...
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 gray seal muscle was purified by isoelectric focusing as described previously (45), and myoglobin concentrations were estimated from A_{435} 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 PsiI 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) HpaII-HinfI fragment from pHM.17.E1 (41) and a 269-bp Sau96I-HinfI 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 BstEII-HaeIII human exon 2 fragment which had been isolated from plasmid pHM.27.B1.1 (41), blunt ended, and cloned into the SmaI site of M13mp18 (48). A 32P-labeled, single-stranded, complementary antisense human myoglobin probe containing all but the 5'-most 11 bp of the exon 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 autopsies, and fetal material was obtained 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 precipitated with ethanol, redissolved in water, and recovered by ethanol precipitation, and the yield was measured by spectrophotometry. The remainder was loaded on a 3'-end-labeled cDNA filter blot and baked at 85°C for 2 h. The RNA was electrophoresed in 1% agarose gel, transferred to nitrocellulose (Nytran), and hybridized with 32P-labeled cDNA as described previously (4). The cDNA was eluted from the gel slice and recovered by ethanol precipitation, and the yield was measured by Cerenkov counting. 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 75 μg of myoglobin per g (wet weight) of muscle. This value is in good agreement with previous estimates (1, 33). Western blotting combined with immunohistochemistry 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.
VOL. 6, 1986

MYOGLOBIN GENE EXPRESSION 4541

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

* 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.

† 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.

§ Micrograms of myoglobin mRNA per milligram of poly (A)^+ RNA.

¶ Milligrams of myoglobin per microgram of myoglobin mRNA.
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 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 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% 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 (47), 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 mRNAs 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. 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 32P-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.

**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-32P]ATP. The probe was an NcoI-SstI fragment extending 1.0 kilobase upstream from the initiation codon of the human myoglobin gene. After hybridization, reaction mixtures were treated with 10 (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.

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 32P-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
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.

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 differentiation and fusion 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.

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 x 10^6 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 32P-labeled antisense DNA was hybridized to increasing amounts (6 to 200 ng) of the single-stranded M13HEX2 parent molecule in 10 μl of 1 x 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) 32P-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 32P-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.
ACKNOWLEDGMENTS

We thank Mike Webb and Frank Walsh for generous gifts of RNA samples and myoblast cell lines and for advice on cell culture. The mouse myoglobin clone M13MEX1 was kindly provided by Alain Blanchetot, and Aspergillus nidulans RNA was provided by John Clements.

A.J.J. is a Lister Institute Research Fellow. This work was supported by a grant from the Medical Research Council.

LITERATURE CITED

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


