Evidence for transcriptional regulation of the myosin heavy chain gene during myogenesis

Ian J.F. Wiid, Charles D. Boyd, Andre J. Bester, Paul D. Van Helden

M.R.C. Research Unit for Molecular and Cellular Cardiology, Department of Medical Biochemistry, University of Stellenbosch Medical School, P.O. Box 63, Tygerberg 7505, South Africa

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ABSTRACT
One of the changes accompanying skeletal muscle cell (myoblast) fusion is a dramatic increase in synthesis of muscle specific proteins, one of which is myosin. The underlying mechanism for this burst in synthesis is not yet understood but may occur by two mechanisms: (a) gradual storage of mRNA and translational control as found by others or (b) gene activation and rapid synthesis of mRNA for immediate translation. In this paper we show that the myosin gene changes its organization such that postfusion skeletal muscle cells show an increased susceptibility to DNase I, a recognized probe for gene activation. We also show that this change accompanies an increase in rate of transcription and an increased cell content of myosin heavy chain mRNA. This work shows that transcriptional control is an important mechanism during muscle cell development in addition to the translational control shown by other workers.

INTRODUCTION
The process of myogenesis is characterized by apparent morphological and biochemical changes. The most obvious morphological change is the fusion of mononucleated myoblast cells to form multinucleated myotube structures (1). During this event the contractile proteins become highly organized to form the contractile apparatus (sarcomeres). Fusion is accompanied by accumulation of muscle specific proteins and mRNA (2). These include the acetyl choline receptors (3), α-actin (4), creatine kinase (5), muscle specific myosin heavy chain (6), myosin light chains, tropomyosin and troponin (7). Evidence shows that the muscle specific contractile proteins are coded for by multigene families and different forms of these proteins may be expressed in various organisms or tissues (8,9). Skeletal, cardiac and smooth muscle have myosin heavy chains that are tissue specific and differ from non-muscle myosin heavy chain (10). There is evidence that post-transcriptional and post-translational controls may regulate the appearance of muscle specific proteins, such as myosin (11, 12), but the rapid increase in myosin during fusion cannot be accounted for by the amount of mRNA stored in mRNP particles alone and thus there is probably some form of transcriptional control regulating the rapid increase in
synthesis of these proteins. This may involve transcriptional activation of these genes. It has been shown that the conformation of chromatin in active genes may be such that these genes are rendered more susceptible to nuclease digestion than their inactive counterparts in other cell types (13). The nuclease DNAse I may be used as a probe for gene activity during cell development, since it preferentially degrades active gene sequences to non-hybridizing fragments more rapidly than inactive sequences (13). The nitrocellulose blot hybridization approach of Wu et al (14) has been used to analyze the nuclease susceptibility of specific regions of the genome by studying the preferential disappearance of specific restriction enzyme fragments as a function of nuclease sensitivity (15).

In addition to chromatin (DNA) conformational changes, several authors have suggested that methylation of DNA at target sites plays a role in the regulation of gene expression (16 - 19). The use of methylation sensitive restriction endonucleases has shown that methylation of DNA sequences in some genes correlates with a concomittant decrease in expression of that gene (16, 17). In this study, we use DNase I and a methylation sensitive endonuclease to examine the regulation of gene expression in an in vivo system, namely, chicken skeletal muscle myosin heavy chain.

MATERIALS AND METHODS

Skeletal muscle tissue was dissected from 10 day old and 15 day old chicken embryo leg muscle. Adult White Leghorn chickens were purchased from a local farm, and rendered anaemic by subcutaneous injection of Phenylhydrazine essentially as described by van der Westhuyzen (20).

Nuclei were prepared from homogenized erythrocytes and skeletal muscle by centrifuging the cellular homogenates through 2,3 M STM (2.3 M Sucrose, 5 mM Tris pH 7.5, 5 mM MgCl₂) in a Beckman SW41 rotor at 23 000 rpm for 90 minutes (21). Nuclei were finally suspended in 0.25 M STM (0.25 M Sucrose, 5 mM Tris pH 7.5, 5 mM MgCl₂).

Nuclease digestion of nuclei. Nuclei were pelleted at 5 000 rpm in a Sorvall HB4 rotor and resuspended in RSB buffer (0.01 M Tris pH 7.5; 0.01 M NaCl; 4 mM EDTA) to a final concentration of 1 mg DNA/ml. Nuclei were digested with DNAse I (pancreatic, Worthington) at concentrations ranging from 0 - 45 units DNAse I/mg DNA/ml. Digestions were carried out in RSB buffer (pH 7.5) at 25°C for 10 minutes and were stopped by the addition of EDTA (pH 7.5) to a final concentration of 0.1 M. Five volumes of RSB were added to each incubation and SDS was added to a final concentration of 0.1%.

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teinase K was added to each incubation to give a final concentration of 100 μg/ml and the solution incubated at 37°C for 2 - 3 hours. DNA was extracted twice with phenol saturated with TE (10 mM Tris pH 7.5; 1 mM EDTA), ethanol precipitated and redissolved in TE buffer.

Restriction endonuclease digestions were carried out at 37°C for 12 hours at 3 units enzyme/μg genomic DNA.

**Analysis of DNA.** Restricted DNA fragments were electrophoresed in 0.6% agarose gels. DNA fragments were transferred onto nitrocellulose using the technique described by Southern (22). pMHC25, myosin heavy chain cDNA in pBR322 from rat muscle (23), was used as a myosin heavy chain probe and pCAβ GI, chicken β globin cDNA in pBR322, was used as a globin probe (24). Probes were nick translated (25) to a specific activity of at least 3 x 10^8 cpm/μg DNA with ^32^P-dCTP.

The protocol described by Jeffreys and Flavell (26) was used essentially for the washing and hybridization of nitrocellulose filters. Filters were incubated in a specially prepared perspex chamber at 65°C for 48 hours in 10 ml hybridization solution [3 x SSC (1 x SSC is 0.15 M NaCl; 0.015 M Trisodium citrate), 0.1% SDS, 10 μg/ml Poly A, 50 μg/ml sonicated heat denatured herring sperm DNA, ^32^P-labelled heat denatured DNA]. Filters were washed after hybridization at 65°C in 0.1 x SSC, 0.1% SDS, for the globin probe and 1 x SSC, 0.1% SDS for myosin probe.

**Analysis of RNA.** Total Poly A⁺ RNA was isolated from prefusion and post-fusion chicken embryo skeletal muscle as described by Pemberton (27). RNA samples were made up in a series of dilutions ranging from 0.25 - 6.0 μg poly A⁺ RNA. The final volumes were made up to 6.0 μl with 3 x SSC, the RNA spotted onto nitrocellulose and the filters dried at 80°C for 2 hours. Washes and hybridization with ^32^p-labelled pMHC25 were carried out exactly as described before.

Quantitative analysis of dot blots was done by cutting out the radioactive nitrocellulose spots and counting in a liquid scintillation counter.

For RNA-agarose glyoxal gel electrophoresis, RNA samples were treated prior to electrophoresis as described by McMaster (28). RNA was incubated at 50°C for 60 minutes in 6 M glyoxal, 50% DMSO, 0.5 M sodium phosphate (pH 7.0). Samples were electrophoresed on a 0.6% agarose gel and transferred to nitrocellulose according to the method described by Southern (22), excluding alkali pretreatment of gels. Nitrocellulose blots were washed at 45°C in hybridization solution for 4 hours (50% formamide, 0.1% SDS, 1 x Denhardt's solution, 2 x SSC, 50 μg/ml heat denatured sonicated herring sperm DNA). Heat dena-
tured $^{32}$P-labelled pMHC25 was added and the hybridization carried out at 45°C for 24 hours. Incubated filters were finally washed twice in 2 x SSC at 23°C and once in 1 x SSC at 68°C.

In vitro transcription. Nuclei were isolated from prefusion and post-fusion chicken embryo skeletal muscle and suspended in 0.25 M STM at a concentration of 5 μg DNA/60 μl nuclear suspension. Transcription reactions were done with 60 μl nuclear suspension. The incubation medium was as follows: 100 mM Tris pH 7.9; 4 mM MgCl$_2$; 0.1 mM EDTA; 0.1 mM dithiothreitol; 1 mM each of cold ATP, GTP, CTP; 12% (v/v) glycerol and 40 mM ammonium sulphate (29). For labelling, 50 μl $^3$H-UTP (54 Ci/mmol) was added to 200 μl incubation volume. The reaction was carried out at 37°C for 60 minutes. Reactions were terminated by addition of an equal volume of 10% TCA. Acid insoluble material was collected on Whatman GFC filter discs, dried and counted in a liquid scintillation counter.

RESULTS

DNAse I sensitivity of myosin heavy chain gene. Nuclei from immature and mature erythrocytes were digested with DNAse I at concentrations ranging from 0 - 45 units DNAse I/mg DNA/ml suspension. The DNA was extracted from the nuclei, restricted with EcoRI restriction endonuclease and the fragments separated on a 0.6% agarose gel. Blot hybridization of the restriction fragments with pCACBG I resulted in the appearance of a 6.2 and a 9 Kb fragment which are known to contain β globin gene sequences (30). (Fig. 1). The increased sensitivity of the active β globin genes in the immature erythrocytes (Fig. 1B) is evident, as the bands representing globin genes rapidly decrease in intensity at DNAse concentration above 10 units/mg DNA/ml whereas those in mature (inactive) erythrocytes remain visible throughout the entire concentration range of DNAse I and are very much more intense (Fig. 1A). This applies particularly to the 6.2 Kb band which is known to be the adult β-globin gene.

Nuclei were isolated from 11 day and 15 day old chicken embryo skeletal muscle and will be referred to as prefusion and postfusion nuclei. The nuclei were digested under the same conditions as for erythrocyte nuclei, blotted and hybridized to $^{32}$P-labelled myosin heavy chain cDNA insert of pMHC25. This insert corresponds to a 650 bp section at the 3' region of myosin heavy chain mRNA from L6E9 myotubes (31). Figure 2 shows the DNAse I digestion pattern of myosin heavy chain gene. pMHC25 hybridizes to a number of restriction fragments in the genomic DNA restricted by Hind III which range in size from 23 Kbp
FIGURE 1
Autoradiograph of (A) normal and (B) anaemic chicken erythrocytes DNA digested with 0, 5, 10, 15, 20, 25, 30, 35 units of Pancreatic DNAse I/mg DNA/ml (Fig. 1 A and B, lanes 1 - 8). DNA was restricted to completion with EcoRI restriction endonuclease and the blotted DNA probed with $^{32}$P-labelled embryonic chicken β-globin cDNA.

FIGURE 2
Autoradiograph of (A) prefusion and (B) postfusion chicken embryo skeletal muscle DNA digested with 0, 5, 10, 15, 20, 25, 35 units of Pancreatic DNAse I/mg DNA/ml (Fig. 2 A and B, Lanes 1 - 7). DNA was restricted to completion with Hind III restriction endonuclease and the blotted DNA probed with $^{32}$P-labelled pMHC25 (myosin heavy chain cDNA).
FIGURE 3
Autoradiograph of (A) prefusion and (B) postfusion chicken embryo skeletal muscle DNA digested with 0, 5, 10, 15, 20, 25, 30, 35 units of Pancreatic DNase I/mg DNA/ml (Fig. 3A and B, lanes 1 - 8). DNA was restricted to completion with EcoR1 restriction endonuclease and the blotted DNA probed with 32P-labelled pCABGI (chicken β globin cDNA).

to less than 2 Kbp. The bands generated represent most of the myosin heavy chain genomic sequence (32).

The myosin heavy chain genomic DNA fragments show increased sensitivity to DNAse I digestion in postfusion nuclei (Fig. 2B). In prefusion nuclei (Fig. 2A), strong hybridization is evident at a DNAse I concentration of 15 units/mg DNA/ml, whereas these bands show greater nuclease susceptibility in postfusion nuclei at the same DNAse I concentration.

As a control to show that the DNAse I susceptibility of the myosin heavy chain gene family is altered during myogenesis, an EcoR1 digest of prefusion and postfusion DNAse I digested nuclear DNA was hybridized to pCABGI (Fig. 3A and B). The β globin gene is essentially inactive in muscle tissue and the results show that under the same experimental conditions, the myosin genes become very much more rapidly degraded (Fig. 2A and B). This is an indication of gene activation in the myosin heavy chain genes.

Dot-blot hybridization of myosin cDNA to poly A+ mRNA from prefusion and postfusion cells and measurement of rate of transcription. It is known that there is a dramatic increase in myosin production concomitant with myoblast fusion (2). If this is accompanied by a change in the DNAse I sensitivity of the myosin gene, then presumably this increase in myosin synthesis is a result of gene activation, or increased mRNA production.

In order to quantify RNA synthesis, prefusion and postfusion chicken
TABLE 1
Endogenous transcriptional activity of nuclei isolated from prefusion and postfusion chicken embryo skeletal muscle. Reactions were carried out at 37°C for 60' and terminated by adding an equal volume of 10% TCA (see Materials and Methods). Acid insoluble material was collected on Whatman GFC filter discs and counted in a liquid scintillation counter.

### ENDOGENOUS TRANSCRIPTIONAL ACTIVITY OF ISOLATED NUCLEI

<table>
<thead>
<tr>
<th>MATERIAL</th>
<th>INCORPORATION OF ³H-UMP (dpm/µg DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PREFUSION</td>
<td>83</td>
</tr>
<tr>
<td>POSTFUSION</td>
<td>124</td>
</tr>
</tbody>
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*prefusion and postfusion chick embryo skeletal muscle nuclei were isolated and the transcriptional activity was determined making use of the endogenous DNA-dependent RNA polymerase. Table 1 shows 124 cpm ³H-UTP incorporated / µg DNA in postfusion nuclei whereas the amount of ³H-UTP incorporated with prefusion nuclei was 83 cpm / µg DNA. This gave an increase of 49% ³H-UTP incorporated in postfusion nuclei.

In order to establish that this increased synthesis included myosin mRNA,

**FIGURE 4**
Dot blot hybridization of (A) prefusion and (B) postfusion chicken embryo skeletal muscle poly A⁺ RNA. RNA samples were spotted onto nitrocellulose at increasing concentrations (1 - 24) and hybridized with ³²P-labelled pMHC25 (myosin heavy chain cDNA).
Quantitative analysis of Dot-blot of poly A$^+$ RNA isolated from prefusion (●) and postfusion (○) chicken embryo skeletal muscle. After hybridization with $^{32}$P-labelled pMHC25, dots were cut out from the hybridized nitrocellulose blot, dried and counted in a liquid scintillation counter. For each series, the lowest count (106 cpm in both cases) was allocated a number of 1 and the rest of the counts calculated as a factor of 1 (hybridization index).

Although quantitative differences in the myosin heavy chain messenger population could be detected it was also necessary to determine whether any qualitative changes occurred in the myosin heavy chain message during myogenesis. Figure 6 shows no difference in the size of the messenger between prefusion and postfusion myosin heavy chain mRNA and also shows the quantitative increase observed in postfusion cells.

**Methylation of myosin genes.** Several authors have reported alterations in the methylation pattern of cytosine residues of a gene (12, 33, 34). Developmental changes in myosin heavy chain gene expression may arise as a result of changes in methylation of such cytosine residues and therefore we made use of the isochizomeress Hsp I and Hpa II to digest DNA isolated from prefusion and postfusion cells. The DNA was electrophoresed on a 0.6% agarose
FIGURE 6
Autoradiograph of RNA-Agarose glyoxal gel electrophoresis of (1) postfusion and (2) prefusion chicken embryo skeletal muscle poly A+ RNA. The RNA was probed with 32P-labelled pMHC25 (myosin heavy chain cDNA).

gel and blotted, then hybridized with labelled pMHC25. The results (Figure 6) show no difference in the methylation pattern of the myosin heavy chain gene in prefusion and postfusion nuclei.

DISCUSSION
Eukaryotic cells have been shown to control gene expression at a variety of levels, from transcription through to post-transcriptional control (35). Translational control has long been considered an important factor in muscle protein biosynthesis (11, 36, 37). This belief has centered around the discovery of stored mRNP particles in embryonic and cultured muscle cells (11, 36). However, reports have shown that the amount of mRNA stored as mRNP particles may be insufficient to code for the increase in synthesis of muscle specific proteins during development (37). It is therefore likely that there is also a transcriptional control mechanism involved in this process. Using cDNA probes for myosin heavy chain (plasmid pMHC25), we have examined the developmental changes in the genes coding for this protein.

Experiments done on pre- and postfusion chick skeletal muscle show the digestion pattern of the myosin heavy chain gene family (Figure 2A and B) after DNAse I digestion. The bands generated represent most of the myosin
heavy chain genomic sequences (32) which presumably code for the 8-9 known isoforms of myosin heavy chain (37). Our results show that in postfusion nuclei, the myosin heavy chain gene is more susceptible to DNAse I digestion than in prefusion nuclei.

The persistence of the high molecular weight β globin restriction fragments where the myosin fragments have disappeared shows the specific degradation and therefore activation of the myosin heavy chain genes.

These results point to a gene activation process and therefore presumably an increased transcription of myosin heavy chain messenger. However, increased nuclease sensitivity does not necessarily reflect the transcriptional activity of a gene. For example, the genes for both embryonic and adult β globin are DNAse I sensitive in embryonic red blood cells, but only embryonic globin is produced (39).

For this reason, it was necessary to test whether the DNAse I sensitivity of the myosin heavy chain corresponded to an increase in myosin messenger RNA. The results obtained (Table 1, Figure 4, 5) showed that there is not only an increase in transcription, but also an increase in myosin mRNA which is quantitatively related to the increase in transcription (49% and 43% increase respectively). These results agree with those obtained by other workers who have shown that the increase in poly A+ myosin heavy chain mRNA found in postfusion (differentiated) skeletal muscle cells is due to transcription of new messenger molecules (31, 37, 40 - 42).

Presumably the increase in transcription does not reflect only the activation of the myosin heavy chain gene, but also other muscle specific protein genes which make proteins in the correct stoichiometric amounts to form the sarcomere. From the results we conclude that the increased sensitivity of the postfusion myosin heavy chain gene to DNAse I digestion, reflects its increased transcribability. Similar results have been reported for other systems (43).

Methylation of DNA has also been ascribed an increasingly important role in the control of gene expression. From Figure 7 it is evident that there is no difference in the methylation pattern of the myosin heavy chain gene in prefusion and postfusion DNA, although there is evidence suggesting a definite correlation between undermethylation and increased DNAse I sensitivity of other genes (12). On the other hand, there is also evidence pointing to the fact that the methylation of certain areas within the gene is unaffected by the stage of differentiation (34). Our data suggest that there are specific sites in the myosin heavy chain gene that become methylated at a very early
FIGURE 7
Methylation pattern of the myosin heavy chain gene in prefusion and postfusion chicken embryo skeletal muscle. DNA was restricted with the isoschizomers Msp I and Hpa II. Blotted DNA was probed with $^{32}$P-labelled pMHC25. Lanes (1) Postfusion (Hpa II); (2) Postfusion (Msp I); (4) Prefusion (Hpa II); (5) Prefusion (Msp I). Lambda DNA was restricted with Hind III, labelled with $^{32}$P-dCTP and used as molecular marker (lane 7). To control for complete digestion Postfusion (lane 3) and Prefusion (lane 6) DNA was digested with Hind III.

stage of embryonic development and where we were unable to detect changes in methylation during the transition from myoblast to myotube.

We have presented evidence in this paper that the burst of myosin synthesis observed during myoblast fusion is at least partly due to an increased myosin messenger RNA content and that this mRNA synthesis results from an organizational change in the myosin heavy chain gene leading to increased synthesis of this messenger. This change is reflected by some structural alteration in the gene which makes it more susceptible to DNAse I digestion.

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$^1$To whom correspondence should be addressed
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