Persistence of Chromosomal Proteins HMG-14/-17 in Myotubes following Differentiation-dependent Reduction of HMG mRNA* 

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James M. Pashté, Jaswant S. Bhorjee, Bruce M. Patterson, and Michael Bustin

From the Laboratories of Molecular Carcinogenesis and Biochemistry, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892 and the Department of Biomedical Sciences, Meharry Medical College, Nashville, Tennessee 37208

The expression of chromosomal proteins HMG 14 and HMG-17 during cellular differentiation was studied in cultured mouse myoblasts. During myogenesis the level of both HMG-14 and HMG-17 mRNA decreased to less than 20% of that found in myoblasts. The down-regulation of HMG-14/-17 mRNA occurred simultaneously with activation of muscle-specific actin mRNA and was not linked to DNA synthesis, indicating that it is a differentiation-related event.

The expression of these two classes of nucleosomal proteins was similar to that into the major histone fractions in that it was significant in myoblasts and undetectable in myotubes. The decrease in mRNA and protein synthesis did not affect the cellular levels of HMG proteins. These results indicate that the regulation of HMG-14/-17 mRNA levels is different from that of the histones and is linked to differentiation rather than to DNA synthesis.

Chromosomal proteins HMG-14 and HMG-17 are among the most abundant nonhistone proteins found in the nuclei of higher eukaryotes (1). The two proteins, which have higher affinity for nucleosomes than for DNA (2), bind to the inner side of the nucleosomal DNA (3) potentially altering the interaction between the DNA and the histone octamer. The limited amount of HMG proteins in the nucleus (4, 5) confines their presence to a subset of the nucleosomes. So far, the cellular role of these proteins is not understood. The data obtained from nucleosome digestion (6, 7), nucleosome reconstitution (2, 8), immunofluorescence (9), immunofractionation (10-12), and antibody microinjection (13) are consistent with the proposition (6, 7) that the two proteins may be involved in determining certain properties of the chromatin structure of transcriptionally active genes.

Myogenic differentiation involves an ordered sequence of molecular events in which relatively homogeneous populations of rapidly dividing myoblasts differentiate into myotubes (14). The process involves a switch in gene expression resulting in both activation and suppression of certain sets of genes (14). Therefore, this system is suited for studies on the cellular role and mode of action of putative regulatory proteins such as HMG-14/-17.

The present study on the levels of HMG-14 and HMG-17 mRNA and protein during various stages of myogenesis is the first examination of the pattern of HMG transcription in a differentiating system. The results indicate that during myogenesis both the mRNA levels and protein synthesis rate decrease without a significant alteration in the cellular protein concentration. Interruption of DNA synthesis with hydroxyurea resulted in immediate down-regulation of histone mRNA levels without having any effect on HMG synthesis, indicating that the expression of these two classes of nucleosomal proteins is independently regulated.

MATERIALS AND METHODS

Cell Culture—Mouse myoblast cultures (C2C12) were obtained from the American Type Culture Collection. Cells were grown in 80-cm2 flasks obtained from Nuclon. Cell culture media, sera, and reagents were from Gibco. Actinomycin D, cytosine arabinoside, and hydroxyurea were from Sigma. Cultures were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Myoblast differentiation medium consisted of medium plus 10% horse serum. Nonfusing cells were removed by treatment of cultures with 1 mg/ml cytosine arabinoside. Labelling of cell cultures was done by incubating cultures with lysine-free minimal essential medium containing 10% dialyzed serum, removing medium, and incubating with lysine-free medium plus 3.9 μCi/ml [3H]-labeled lysine (270 μCi/ml).

Biochemical Procedures—RNA extraction and Northern blot analysis were done as described previously (15). Mouse HMG-17 and HMG-14 mRNA were probed using the entire human HMG-17 cDNA (15) and human HMG-14 cDNA (16) respectively. Mouse α- and β-actin mRNAs were probed with a chimeric plasmid that contains both actin forms. Glyceraldehyde-3'-phosphate dehydrogenase was probed with a plasmid (Ref. 17; a gift from Dr. B. Gerwin, National Cancer Institute, National Institutes of Health) derived from the corresponding rat gene. Nuclear protein was obtained by scraping monolayer cultures into phosphate-buffered saline containing 1 mM phenylmethylsulfonyl fluoride and 0.1 mg/ml aprotinin (Sigma), followed by centrifugation (500 × g) and homogenization of cell pellets in nuclei isolation buffer (1 mM Tris, pH 7.4, 10 mM NaCl, 3 mM MgCl2, 0.5% Triton X-100). The nuclei were pelleted (500 × g), washed and resuspended in distilled water. Nucleosomal proteins were extracted with either 0.4 M HClO4 for histone or 1.25 N HClO4 for HMG followed by centrifugation (10,000 × g) and precipitated in 0.3 N HCl and 6 volumes of acetone. Proteins were separated by polyacrylamide gel electrophoresis (18) and visualized by Coomassie Blue and or silver staining. Mouse HMG proteins were identified by comparing their migration with that of purified mammalian HMG-14/-17 standards and by Western blot analysis using anti-HMG-14 (9) and anti-HMG-17 antibodies (19, 20). Radioactive proteins were visualized by autoradiography (1 week, -70 °C, Kodak XAR-5 film).

To ensure reproducibility, the procedures were performed several times with different batches of cells.

RESULTS AND DISCUSSION

Incubation of myogenic mouse C2 cells in mitogen-depleted medium induced differentiation and resulted in the formation of multinucleate myotubes. Microscopic examination revealed that within 4 days of treatment over 90% of the myoblasts fused into myotubes. Nonfused cells were then removed by treating cultures with cytosine arabinoside. Synthesis rates for DNA, protein, and RNA during various stages of myogen-
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Fusion of myoblasts into myotubes resulted in a 90% decrease in DNA synthesis and a 25% decrease in RNA synthesis with no significant change in the rate of incorporation of radioactive amino acids (data not shown). These changes in DNA, RNA, and proteins during myogenesis are consistent with those previously described (21).

Fig. 1 presents an example of results obtained by Northern analysis of mRNA isolated at various times following the switch into differentiation media. During differentiation, the HMG-14/-17 mRNA level increased slightly up to 39 h after removal of the growth factors and then decreased to 50% of maximum at 63 h and to 10% of maximum at 98 h after induction of differentiation. This decrease in HMG-14/-17 mRNA level paralleled the switch from synthesis of β-actin to α-actin and was not due to variation in amount of mRNA applied since the level of another marker, glyceraldehyde-3'-phosphate dehydrogenase, exhibited a marked elevation at 98 h (Fig. 1).

HMG and histone H1 protein levels were analyzed during myogenesis by acid urea-gel electrophoresis of 1.25 N perchloric acid cell extracts (Fig. 24). The location and relative amount of the protein bands were confirmed by Western analysis (data not shown). Densitometric analysis of the proteins resolved on either urea or SDS-containing gels, indicated that for each time point the HMG protein level varied

Fig. 3. Effect of hydroxyurea on HMG and histone H1 synthesis. Myoblasts were incubated with or without 10 mM hydroxyurea. At each time point cultures were radiolabeled with [3H]lysine for 1 h and cultures were harvested as described under “Materials and Methods.” A, sequential staining with Coomassie Blue and silver. B, autoradiogram of gel following a 1-week exposure to Kodak XAR film, −70 °C.

Fig. 4. Effect of hydroxyurea on HMG-14 and HMG-17 mRNA levels. Northern blot analysis of RNA isolated from control and hydroxyurea-treated cells. All tracks were loaded with 10 μg of total RNA and the blots sequentially probed with the various probes. A, myoblasts incubated with or without 10 mM hydroxyurea for the time indicated at the top of the figure. B, myoblasts incubated for 6 h with 10 mM hydroxyurea plus or minus 2 μM actinomycin D.
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less than 5\% when compared to histone H1 (data not shown). Previous studies have found that, although the relative proportion of the various H1 subfractions changes during myogenesis, the total amount of H1 does not (22, 23). Therefore, the constant ratio of H1 to HMG indicates that during myoblast differentiation the levels of HMG protein remain relatively constant.

Next we examined the relative synthesis of histone and HMG proteins in myoblasts and myotubes. Nuclei from 1-h pulse-labeled cell cultures were extracted with either 1.25 N perchloric acid to obtain a histone H1 and HMG fraction or 0.4 M \( \text{H}_2\text{SO}_4 \) to obtain a fraction containing all histones and HMGs. Protein extracts were then electrophoresed in an 18% polyacrylamide gel containing sodium dodecyl sulfate. Autoradiograms of the gels (Fig. 2B) show that in fused myotubes the synthesis of both histone and HMG protein was negligible as compared to that seen in myoblasts. These results suggest that the HMG-14 and -17 chromosomal proteins are synthesized in the myoblast stage of differentiation, and they support previous results that indicated the proteins have relatively long half-lives (4, 24). However, it is also possible that the rate of protein degradation changes during differentiation.

During myogenesis the synthesis of the nucleosomal histone is tightly coupled to DNA replication (22, 23). To test whether the synthesis of HMG-14 and -17 in myoblasts is similarly linked to DNA synthesis growing cultures were treated with 10 \( \mu\text{M} \) hydroxyurea. Under these conditions incorporation of radiolabeled thymidine into trichloroacetic acid-precipitated material was less than 20\% of that of untreated myoblasts indicating that synthesis of DNA was blocked. Whereas a 3-h incubation with hydroxyurea decreased the amount of histone H4 mRNA by more than 90\%, the HMG-14 and HMG-17 mRNAs were not affected even after a 9-h incubation with hydroxyurea (Fig. 3A). However, inhibition of RNA synthesis by addition of actinomycin D to the hydroxyurea-treated cells drastically reduced the amount of HMG-14/-17 mRNAs (Fig. 3B). These and other results indicate that HMG mRNAs are not unusually stable and their persistence during the hydroxyurea treatment reflects continued synthesis.

HMG and histone protein synthesis under these conditions followed a similar pattern similar to that detected for RNA synthesis (Fig. 4). A 1-h pulse labeling of several cultures with \(^{14}\text{C}\)lysine revealed that the synthesis of histone H1 was significantly inhibited in the presence of hydroxyurea within the first 3 h of incubation (Fig. 4B). In contrast, HMG-14 and -17 synthesis was unaffected during the entire 9-h incubation. Fig. 4A shows that equal amount of protein was applied to each lane. The data indicate that hydroxyurea treatment drastically reduced the synthesis of histone H1 without affecting the synthesis of the HMG proteins.

We conclude that the down-regulation of HMG-14/-17 synthesis observed during myogenesis is a consequence of differentiation and is distinct from that seen with histone during suppression of DNA synthesis and cell cycle inhibition.

REFERENCES

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