Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 7933–7936, September 1993
Cell Biology

Coordinated decreases in rRNA gene transcription factors and rRNA synthesis during muscle cell differentiation

DAWN E. LARSON*, WENQIN XIE†, MARIJA GLIBETIC*, DANIEL O'MAHONY†‡, BRUCE H. SELLS*§, AND LAWRENCE I. ROTHBLUM†

*Department of Molecular Biology and Genetics, University of Guelph, Guelph, ON, Canada N1G 2W1; and †Weis Center for Research, Geisinger Clinic, Danville, PA 17822

Communicated by Oscar L. Miller, May 24, 1993 (received for review January 26, 1993)

ABSTRACT **rRNA synthesis decreases significantly during the differentiation of rat L6 myoblasts to myotubes. Nuclear run-on assays demonstrated that the decrease was attributable to decreased rates of rRNA gene transcription. Immunoblot analysis indicated a marked reduction in amounts of the RNA polymerase I transcription factors UBF1 and UBF2 (upstream binding factors 1 and 2, respectively). The levels of these factors dropped in parallel with the down-shift in rRNA gene transcription. The amount of UBF does not fall due to a general decrease in cellular protein, as myosin heavy-chain protein accumulates markedly during this same time. RNA blots of total RNA isolated from myoblasts and differentiating myotubes showed a decrease in the mRNA for UBF, at the same time the mRNA for myogenin was accumulating. The down-shift in UBF mRNA levels preceded the decrease in the protein levels for UBF. There have been reports that the acute response of the rRNA gene transcription system to physiological signals in many systems involves an RNA polymerase I-associated factor. However, our results imply that the regulation of rRNA gene DNA transcription in response to physiological processes, such as differentiation, may involve multiple regulatory pathways.**

During terminal differentiation of rat L6 myoblasts into myotubes, the rate of ribosome accumulation drops by 75% with a concomitant decrease in the rate of rRNA gene transcription (1, 2). However, the transcription rate of the ribosomal protein L32 and 5S rRNA genes, measured in isolated nuclei and by specific cell-free transcription assays (2–4), remains unchanged. This rate results in the continuous production of ribosomal proteins and 5S rRNA, which are rapidly degraded. Thus, ribosome biogenesis is limited by the rRNA supply and, therefore, is directly coupled with those mechanisms regulating rRNA gene transcription (5).

Three transcription factors or activities have been identified as essential for efficient rRNA gene transcription by RNA polymerase I *in vitro* (6, 7). One DNA-binding protein, designated factor D or the RNA polymerase I promoter selectivity factor SL-1, is functionally defined as the factor required for the recognition of homologous promoters. As such, factor D or SL-1 can reprogram heterologous extracts—i.e., mouse factor D directs human extracts to transcribe mouse rDNA (8–11). Rat SL-1 can bind both to the core promoter element and the 5′ boundary of the upstream promoter element of the rat 45S rDNA promoter, as well as binding to both the upstream promoter element and core promoter element of the rat spacer promoter (6, 12). SL-1 may have a molecular role similar to that of the transcription factor TFIID complex (7). Furthermore, TATA-binding protein is one component of SL-1 (13).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

A second factor, upstream binding factor (UBF), originally identified by DNA footprinting studies, binds to, at least, the upstream promoter element of the rDNA promoter (6, 12, 14, 15). UBF has been purified from several vertebrate sources, and cDNAs encoding the human, rat, mouse, and *Xenopus* forms of UBF have been cloned and sequenced (16–20). Vertebrate cells code for two forms of UBF, designated UBF1 and UBF2. The two mammalian forms differ in one of the four DNA-binding domains, or high-mobility group (HMG) boxes (17, 20). UBF1 and UBF2 also differ in both their DNA-binding and dimerization properties (21). Recently, it has been demonstrated that UBF1 and UBF2 are phosphorylated proteins and that the extent of phosphorylation of UBF is regulated in response to serum deprivation (22). Thus, the phosphorylation state of UBF may be important in regulating its ability to affect transcription by RNA polymerase I.

The response of rRNA gene transcription to a variety of physiological signals has been reported to involve an RNA polymerase I-associated activity (23) that has been referred to as factor C (24), transcription initiation factor IB (TIF-IB) (11), or transcription initiation factor C (TFIC) (25, 26). Operational differences—i.e., purification schemes and assays—leave it unclear whether these factors are the same. However, in all systems studied, this factor is found tightly associated with RNA polymerase I and appears to mediate the interaction of RNA polymerase I with the preinitiation complex over the transcription initiation site. TFIC may also be subject to posttranslation modification (26).

We have examined the hypothesis that upon entry of myoblasts into the noncycling, differentiated state, the levels of rDNA transcription factors, specifically UBF1 and UBF2, decrease. Although the reduced rate of transcription of rRNA genes in terminally differentiated myotubes has been attributed to changes in concentration of RNA polymerase I transcription factors (3), a definitive connection has not been made. We now report that a significant decrease occurs in the amounts of UBF1 and UBF2 concomitant with muscle cell differentiation and that the decrease in UBF protein is accompanied by a decrease in the mRNA levels for UBF. Our results indicate that at least part of the process by which cells regulate rDNA transcription is by regulating the levels of UBF1 and UBF2.

Abbreviations: factor D (or SL-1), transcription initiation factor for rRNA genes; UBF1 and UBF2, upstream binding factors 1 and 2, respectively, involved in rRNA gene transcription; factor C (or TIF-IB or TFIC), transcription initiation factor for rRNA genes; PABP, poly(A)-binding protein.

‡Present address: Centre for Cardiovascular Science, Department of Medicine and Experimental Therapeutics, University College Dublin, Mater Hospital, Dublin, 7, Ireland.

§To whom reprint requests should be addressed at: Dean's Office, College of Biological Science, University of Guelph, Guelph, ON, Canada N1G 2W1.

MATERIALS AND METHODS

Cell Culture. Rat L6 myoblasts were cultured in α minimum essential medium/10% fetal bovine serum and induced to differentiate at 70% confluency by supplementing the medium with 2.5% horse serum. Myotubes were harvested after 4 days when >95% of the cells had fused.

Nuclear Run-On Assay. Nuclei (5×10^6), isolated from rat myoblasts and differentiating myotubes as described (4), were incubated with 50 μCi of [^{32}P]UTP (1 Ci = 37 GBq) in buffer containing 500 μM CTP/500 μM GTP/500 μM ATP and human placental RNase inhibitor in a total volume of 100 μl for 20 min at 30°C. Specific RNA transcripts were detected by hybridization of the ^{32}P-labeled RNA (1×10^6 cpm) to the corresponding immobilized DNA sequences. DNA probes used were as follows: p2.0, containing a *Sal* I fragment of the rat 45S rRNA gene (27), and pHu73 (28), containing a 2.9-kbp cDNA insert coding for the human poly(A)-binding protein (PABP) mRNA (provided by R. Pictet, Institut Jacques Monod du Centre National de la Recherche Scientifique, Paris).

Immunoblot Analysis. The amounts of UBF1 and UBF2 in cell lysates from proliferating rat L6 myoblasts and differentiating myotubes were measured by using a monospecific-anti-UBF antisera, essentially as described (22). Before immunodetection, equal amounts of protein from each cell lysate were separated by SDS/PAGE (29).

RNA Isolation and Analysis. RNA was isolated by using either the guanidine isothiocyanate procedure, as described by Chirgwin *et al.* (30), or by the rapid protocol of Xie and Rothblum (31). RNA samples were denatured and applied to nitrocellulose by using a Schleicher & Schuell Minifold II slot blotter system. Alternatively, equal amounts of the RNA samples were separated by agarose-formaldehyde gel electrophoresis and blotted to Zeta-Probe filters. To visualize the UBF mRNA, the blots were hybridized with a probe for UBF, p405rUBF (17) labeled by random priming (29). Myogenin mRNA sequences were detected by using ^{32}P-nick-translated pEMCIIs (32), which contains cDNA sequences for myogenin (provided by W. E. Wright, University of Texas, Southwestern Medical Center, Dallas). All standard protocols were done as described (29).

RESULTS

Down-Shift in rRNA Synthesis During Muscle-Cell Differentiation. Terminal differentiation of L6 myoblasts to myotubes represents an example of a rapidly proliferating population of cells being converted into a nondividing, fused population. We have shown (4, 33) that the transcription rates of the genes coding for ribosomal protein L32 and 5S rRNA are similar in nuclei isolated from myoblasts and myotubes. During differentiation, the rate of ribosome accumulation decreases by ≈75% (1). This down-shift has been reported (2, 34) to be from reduced gene-transcription rates. To measure the rate of rRNA gene transcription during muscle cell differentiation, transcription was measured in isolated nuclei, an assay that reflects the activity of genes being transcribed before cell lysis. Nuclei, isolated from proliferating myoblasts, and 2- and 4-day myotubes were incubated with [^{32}P]UTP for 20 min. The radiolabeled RNA was then purified and hybridized to plasmids containing gene sequences coding for pre-rRNA and PABP. Quantitation of band intensities from at least four independent experiments showed that the transcription rate of the rDNA decreased 30% by 2 days into the differentiation process (Fig. 1 and Table 1). This trend was continued such that by day 4 the synthesis of pre-rRNA dropped by 70%, compared with the rate seen in nuclei isolated from proliferating myoblasts. The reduced synthesis of pre-rRNA in nuclei isolated from myotubes is not a general phenomenon for all genes because transcription of the gene that codes for PABP, which is translationally regulated (35, 36), remained unchanged during the differentiation period (Fig. 1).

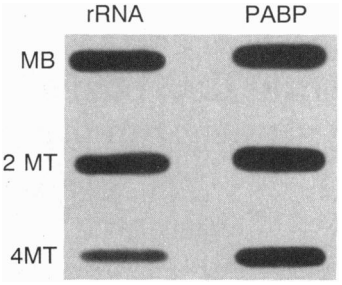

FIG. 1. Transcription of pre-rRNA and PABP mRNA in nuclei isolated from myoblasts and differentiating myotubes. Transcriptional activity of each gene was measured by using nuclear run-on assays. MB, myoblast; 2 MT and 4 MT, myotubes 2 or 4 days, respectively, after initiation of differentiation.

UBF Levels Decrease Significantly During Muscle Cell Differentiation. Accurate and efficient transcription of rat rDNA requires the interaction of RNA polymerase I with at least two DNA-binding proteins, factor D (SL-1) and UBF (6, 12). When the elongation activity of cell-free extracts prepared from proliferating myoblasts or terminally differentiated myotubes was measured by using calf thymus DNA, we observed no differences in the RNA polymerase I levels in myoblast and myotube extracts (3). However, when the myotube and myoblast extracts were assayed by using specific template, the myotube extracts supported lower levels of specific transcription from the rDNA promoter (3). Specific molecular and immunoassays have been recently developed to quantify UBF (22) and its mRNA. UBF has been purified from rat and consists of two distinct proteins of 94 and 97 kDa (6, 12). The cDNAs coding for UBF1 and UBF2 have been cloned and sequenced (17), and antibodies have been produced that recognize both forms (22). Thus, it is now possible to determine whether the changes in the rate of rRNA synthesis correlate with the amounts of rRNA gene-specific transcription factors UBF1 and UBF2.

The amounts of UBF1 and UBF2 in cell lysates from proliferating myoblasts and differentiating myotubes were measured by immunoblot analysis. Equivalent amounts of protein from each type of cell lysate were fractionated by SDS/PAGE and electroblotted onto Immobilon P; the relative amounts of UBF1 and UBF2 were detected by treating the membranes with rabbit anti-UBF and ^{125}I-labeled goat anti-rabbit IgG. Muscle cell differentiation in the same samples was monitored by the appearance of myosin heavy chain (Fig. 2*A*). Two days after the initiation of muscle cell differentiation, the amount of UBF1 and UBF2 started to decrease (Fig. 2*B*), in parallel with the rate of rRNA gene transcription in isolated nuclei. By day 4, when the rate of synthesis of pre-rRNA had decreased 70% (Fig. 1), the amounts of UBF1 and UBF2 had both decreased significantly (Fig. 2*B*). In

Table 1. Gene transcription in nuclei isolated from myoblasts and differentiating myotubes

Gene	Relative transcription rate by isolated nuclei, %		
	Myoblasts	2-Day myotubes	4-Day myotubes
PABP mRNA	100	98 ± 7	95 ± 9
Pre-rRNA	100	62 ± 8	34 ± 6

RNA synthesized by myoblast, 2-day myotube nuclei, and 4-day myotube nuclei *in vitro* was isolated, and the presence of pre-rRNA and PABP mRNA was detected by slot-blot analysis, as described for Fig. 1. Each value represents the average ± SE of at least four independent experiments.

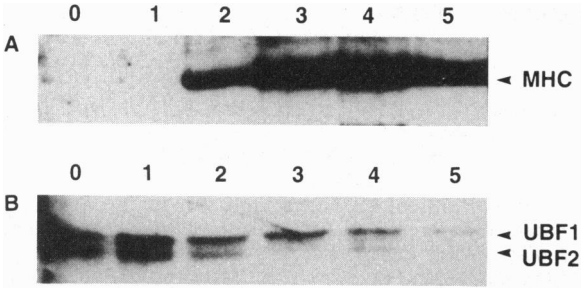

FIG. 2. UBF levels decrease during muscle cell differentiation. Cell lysates were prepared from proliferating myoblasts (0 days) and differentiating myotubes at 1–5 days after addition of differentiation medium. Equal amounts of protein (20 μg) from each lysate were fractionated by SDS/PAGE and transferred to Immobilon-P. Myosin heavy chain (MHC) and UBF1 and UBF2 were detected by immunoblot analysis in *A* and *B*, respectively.

contrast, as the cells differentiated, the levels of myosin heavy chain increased (Fig. 2*A*). In the same protein samples that showed decreased UBF1 and UBF2 levels, fibrillarin and tubulin levels remained constant (data not shown). These results show that a down-shift in rRNA gene transcription is accompanied by corresponding changes in one of the specific rDNA gene trans-acting factors, UBF.

We then examined the hypothesis that the decreased amount of UBF in myotubes resulted from a drop in the amount of UBF mRNA. Equal amounts of whole-cell RNA from myoblasts and myotubes were fractionated by denaturing agarose gel electrophoresis (Fig. 3*A*), transferred to nylon membranes, and probed for UBF sequences (Fig. 3*B*) and for myogenin mRNA (Fig. 4) by using ^{32}P-labeled probes. This latter probe was used to verify that differentiation had occurred at the transcription level (32) and was not from a general decrease in the mRNA levels in the myotubes. After autoradiography, the UBF blots were quantitated with an Ambis analyzer. After 1 day in differentiation medium, a 50% reduction in the UBF mRNA level was seen. After 4 days, the level was reduced to ≈10–20% of that in myoblasts. Myogenin mRNA, on the other hand, increased from essentially 0 (Fig. 4, Myb) to significant levels (Fig. 4, day 2 and 4 MT) during muscle cell differentiation, concurrent with cell fusion.

DISCUSSION

We have demonstrated that the down-shift in the rate of rRNA gene transcription that occurs during differentiation of rat L6 myoblasts to myotubes is accompanied by absolute decreases in the amounts of UBF1 and UBF2, by using antibodies that recognize both forms of the protein. UBF1

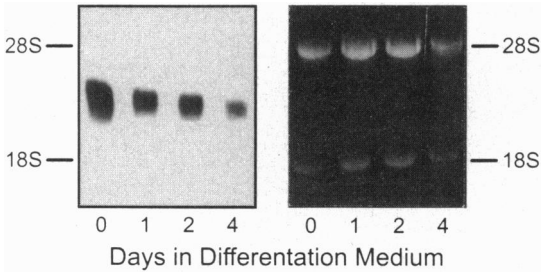

FIG. 3. UBF mRNA levels decreased during muscle cell differentiation. Aliquots of total cellular RNA (25 μg) were size-fractionated by agarose-formaldehyde gel electrophoresis and blotted; UBF mRNA sequences were detected by hybridizing with UBF cDNA sequences and radiolabeled by random priming. (*Left*) Autoradiogram showing UBF mRNA levels. (*Right*) Ethidium bromide-stained RNA before transfer.

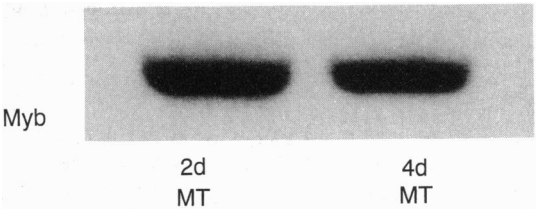

FIG. 4. Myogenin mRNA levels increased during muscle cell differentiation. Aliquots of total cellular RNA (10 μg) were applied to nitrocellulose and hybridized with ^{32}P-labeled nick-translated pEMCIIs. The Myb lane (myoblast) contained no signal, whereas significant amounts of myogenin mRNA were present 2 days after initiation of differentiation. 2d MT and 4d MT, myotubes 2 or 4 days, respectively, after initiation of differentiation.

and UBF2 are trans-acting factors involved in RNA polymerase I-directed gene transcription. A drop in rRNA gene transcription does not occur until 2 days after the initiation of muscle cell differentiation, concomitant with decreases in UBF levels. In addition, both UBF levels and rRNA gene transcription continue to decrease significantly during differentiation. These observations contrasts with what occurs upon serum deprivation of Chinese hamster ovary (CHO) cells and hypertrophic, neonatal cardiac myocytes. When CHO cells are serum-arrested, the levels of UBF1 and UBF2 remain unchanged, but the extent of phosphorylation is dramatically reduced in comparison with the phosphorylation of UBF in proliferating cell populations (22). In contrast, when neonatal cardiomyocytes hypertrophy (growth in the absence of mitosis), the mass of UBF increases (37). It appears, therefore, that multiple mechanisms exist by which UBF is involved in modulating rDNA transcription.

Myogenesis appears to be controlled by the interplay of soluble proteins that coordinately regulate the expression of proliferation-related and muscle-specific genes (38). Decreases in UBF expression during myogenesis may be related to the concomitant arrest of cell proliferation rather than to differentiation. This form of RNA polymerase I regulation may be exclusive to myogenic or other forms of terminal differentiation because the inhibition of rRNA gene transcription during myotube formation is a slow process when compared with the TFIC-mediated changes described (23, 39). Our results are consistent with the possibility that the method by which a cell regulates transcription depends upon the specific cell type and the stimulus to which that cell type responds.

These studies were supported in part by grants from the Medical Research Council (Canada) and the Natural Sciences and Engineering Research Council of Canada and by grants from the National Institutes of Health (GM46991 and HL47638) and the Geisinger Foundation. W.Q.X. and M.G. were supported by postdoctoral fellowships from the American Heart Association and International Juvenile Diabetes Foundation, respectively.

1. Krauter, K. S., Soeiro, R. & Nadal-Ginard, B. (1979) *J. Mol. Biol.* **134,** 727–741.
2. Jacobs, F. A., Bird, R. C. & Sells, B. H. (1985) *Eur. J. Biochem.* **150,** 255–263.
3. Zahradka, P. & Sells, B. H. (1988) *Eur. J. Biochem.* **171,** 37–43.
4. Zahradka, P., Larson, D. E. & Sells, B. H. (1989) *Eur. J. Biochem.* **184,** 261–266.
5. Larson, D. E., Zahradka, P. & Sells, B. H. (1991) *Biochem. Cell Biol.* **69,** 5–22.
6. Smith, S. D., Orahi, E., Lowe, D., Yang-Yen, H.-F., O'Mahony, D., Rose, K., Chen, K. & Rothblum, L. I. (1990) *Mol. Cell. Biol.* **10,** 3105–3116.
7. Bell, S., Jantzen, H.-M. & Tjian, R. (1990) *Genes Dev.* **4,** 943–945.
8. Mishima, Y., Financsek, I., Kominami, R. & Muramatsu, M. (1982) *Nucleic Acids Res.* **10,** 6659–6670.

9. Miesfeld, R. & Arnheim, N. (1984) *Mol. Cell. Biol.* **4,** 221–227.
10. Learned, R. M., Cordes, S. & Tjian, R. (1985) *Mol. Cell. Biol.* **5,** 1358–1369.
11. Clos, J., Buttgereit, D. & Grummt, I. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 604–608.
12. Smith, S. D., Oriahi, E., Yang-Yen, H.-F., Xie, W.-Q., Chen, C. & Rothblum, L. I. (1990) *Nucleic Acids Res.* **18,** 1677–1685.
13. Comai, L., Tanese, N. & Tjian, R. (1992) *Cell* **68,** 965–976.
14. Bell, S. P., Learned, R. M., Jantzen, H.-M. & Tjian, R. (1988) *Science* **241,** 1192–1197.
15. Pikaard, C. S., Smith, D., Reeder, R. & Rothblum, L. (1990) *Mol. Cell. Biol.* **10,** 3810–3812.
16. Jantzen, H.-M., Admon, A., Bell, S. P. & Tjian, R. (1990) *Nature (London)* **344,** 830–836.
17. O'Mahony, D. J. & Rothblum, L. I. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 3180–3184.
18. Bachvarov, D. & Moss, T. (1991) *Nucleic Acids Res.* **19,** 2331–2335.
19. McStay, B., Hwa, C., Pikaard, C. S. & Reeder, R. H. (1991) *EMBO J.* **10,** 2297–2303.
20. Hisatake, K., Nishimura, T., Maeda, Y., Hanada, K.-I., Song, C.-Z. & Muramatsu, M. (1991) *Nucleic Acids Res.* **19,** 4631–4637.
21. O'Mahony, D. J., Smith, S. D., Xie, W.-Q. & Rothblum, L. I. (1992) *Nucleic Acids Res.* **20,** 1301–1308.
22. O'Mahony, D. J., Xie, W.-Q., Smith, S. D., Singer, H. A. & Rothblum, L. I. (1992) *J. Biol. Chem.* **267,** 35–38.
23. Paule, M. R., Iida, C. T., Harris, G. H., Knoll, D. A. & D'Alessio, J. M. (1984) *Nucleic Acids Res.* **12,** 8161–8180.
24. Tower, J. & Sollner-Webb, B. (1987) *Cell* **50,** 873–883.
25. Cavanaugh, A. H., Gokal, P. K., Lawther, R. P. & Thompson, E. A. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 718–721.
26. Mahajan, P. & Thompson, E. (1990) *J. Biol. Chem.* **265,** 16234–16243.
27. Rothblum, L. I., Reddy, R. & Cassidy, B. (1982) *Nucleic Acids Res.* **10,** 7345–7362.
28. Grange, T., Martins de Sa, C., Oddos, J. & Pictet, R. (1987) *Nucleic Acids Res.* **15,** 4771–4787.
29. Ausubel, F., Brent, R., Kingston, R., Moore, D., Seidman, J., Smith, J. & Struhl, K. (1987) *Current Protocols in Molecular Biology* (Wiley-Interscience, New York).
30. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. & Rutter, W. J. (1979) *Biochemistry* **18,** 5294–5299.
31. Xie, W.-Q. & Rothblum, L. I. (1991) *BioTechniques* **11,** 324–329.
32. Wright, W. E., Sassoon, D. A. & Lin, V. K. (1989) *Cell* **56,** 607–617.
33. Glibetic, M., Larson, D. E., Sienna, N. & Sells, B. H. (1992) *Exp. Cell Res.* **202,** 183–189.
34. Krauter, K. S., Soeiro, R. & Nadal-Ginard, B. (1980) *J. Mol. Biol.* **142,** 145–159.
35. Maundrell, K., Imaizumi-Scherrer, M. T., Maxwell, E. S., Civelli, O. & Scherrer, K. (1983) *J. Biol. Chem.* **258,** 1387–1390.
36. Thomas, G. & Thomas, G. (1986) *J. Cell Biol.* **103,** 2137–2144.
37. Xie, W.-Q. & Rothblum, L. I. (1993) *Trends Cardiovasc. Med.* **3,** 7–11.
38. Hastings, K. E. M. & Emerson, C. P., Jr. (1984) in *Recombinant DNA and Cell Proliferation*, eds. Stein, G. & Stein, J. (Academic, New York), pp. 219–241.
39. Sollner-Webb, B. & Tower, J. (1986) *Annu. Rev. Biochem.* **55,** 801–830.