The Synthesis and Stability of Cytoplasmic Messenger RNA During Myoblast Differentiation in Culture

[poly(A)-containing RNA/poly(U)filters/chase experiments/polysome distribution/complementary DNA]

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ABSTRACT The synthesis of poly(A)-containing cytoplasmic RNA was examined in primary myoblast cultures prepared from skeletal muscle of fetal calves. After a period of cell division, these cells undergo fusion, with concomitant appearance of acetylcholine receptor and subsequent myosin synthesis. In the dividing myoblast there is a high level of messenger RNA synthesis, including a 26S RNA, the size of a putative messenger for the large subunit of myosin. In the transition period prior to fusion, there are quantitative changes in RNA synthesis. At this time, there is a pronounced production of 26S RNA, which diminishes during fusion. The possibility that 26S RNA is accumulated in the dividing myoblast was investigated by chase experiments. At fusion, there is a marked increase in the half-lives of a number of messenger RNA species, including 26S, which increases from about 10 hr in the dividing cell to a value of more than 50 hr. The identity of the more rapidly turning over 26 S in the myoblasts, compared to that of the 26 S at fusion, was examined in terms of polysomal distribution, migration on gels, and hybridization with complementary DNA for the myosin message. The results of these analyses suggest that the 26S species are identical. Thus, it would appear that in a predetermined cell like the myoblast, the transition to the differentiated state of myotube that is synthesizing muscle specific proteins is effected by the stabilization of messenger already being actively transcribed: terminal differentiation, with respect to myosin synthesis, is preceded by the stabilization of 26S RNA.

Muscle cells grown in vitro provide a model system in which to study certain aspects of terminal differentiation. After a defined time in culture, mononucleated myoblasts fuse to form multinucleated myotubes. This morphological event is characterized biochemically by the synthesis of muscle specific proteins (1–3). The lag period preceding fusion appears to be a necessary requisite for the conversion of myoblasts into myotubes (4). According to Holtzer (5), this period is linked to a requirement for at least one cell division, followed by passage through a postmitotic state after which the myoblast is competent to fuse. Little is known of what transcriptional modifications may be necessary before this state of competency is reached, nor the extent of control exerted at the level of translation in determining the expression of the muscle phenotype. Yaffe and Dym (6) have found that actinomycin D added to, or just prior to, fusion does not immediately block the appearance of muscle-specific proteins. This observation raises the question of possible accumulation of muscle-specific messenger RNAs in the myoblast before fusion. Reduced nucleolar activity (7) and minor changes in certain messenger RNAs (2) have been described at fusion in rat myoblast cell lines. We report in this paper a more detailed examination of the synthesis and properties of messenger RNA during myogenesis.

MATERIALS AND METHODS

Cell Culture. Skeletal muscle from 3-month-old fetal calves was used as a source of material, both because it was available in adequate quantity and because the resulting myoblasts grew well on the homologous serum. The culturing procedure was essentially that described by Yaffe and Fuchs (7). The cells were grown on gelatin-coated petri dishes. A preliminary plating for 30 min at 37° removed most of the more rapidly attaching fibroblasts. The culture medium was a mixture of Dulbecco's modified Eagle's medium and medium 199 (1:4), containing either 2% or 10% fetal calf serum and 1% chick-embryo extract. Cells were plated at a density of 2 to 5 million cells per 10-cm petri dish.

Protein Synthesis. Cultures were labeled for 2 hr with 10 μCi/2.5 ml of medium of 3H-labeled amino-acid hydrolysate (100 mCi/mmol). Total cytoplasmic protein, or myosin partially purified by repeated precipitation at low ionic strength (2), was analyzed by electrophoresis on 7.5% acrylamide-sodium dodecyl sulfate (SDS) gels (8). Myosin, prepared from fetal calf skeletal muscle, was used as a standard.

Acetylcholine Receptor. This was detected (9) by the binding of [3H]α-isotoxin from Naja nigriloriclis. Control assays were preincubated with 10−4 M decamethonium.

Labeling and Extraction of RNA. Cultures were labeled for 2 hr with 100 μCi of [3H]uridine (20 Ci/mmol) per 2.5 ml of medium. After harvesting, cells were lysed in a solution containing 0.5% NP40, 0.14 M NaCl, 0.02 M Tris-HCl (pH 7.5), and 0.005 M MgCl2 for 10 min. After centrifugation to remove the nuclei, cytoplasmic RNA was depurinated at least three times with an equal volume of chloroform-phenol (1:1), 1% isomyl alcohol, 0.2% SDS, and 0.01 M EDTA (10). RNA was precipitated overnight with 2 volumes of ethanol at −30°, using tRNA as carrier.

Analysis of RNA. RNA was layered onto 5–20% sucrose gradients in 0.05 M NaCl, 0.02 M Tris-HCl (pH 7.5), 0.01 M EDTA, 0.2% SDS, and centrifuged in the SW 56 rotor at 50,000 rpm. for 1 hr 40 min at 22°. The optical density profile of the gradient was analyzed and 0.1 ml fractions collected using an Isco gradient collector; 30 μl of each sample was counted immediately for radioactivity. The poly(A)-containing RNA in the remaining 70 μl was determined by hybridization to poly(U) glass-fibre filters (Caput, D., Lebleu, B., and Nudel, U., unpublished results; and ref. 11). Slow initial filtration (0.5 ml/min), followed by extensive washing gave reproducible results with no contamination by 28S ribosomal RNA. A similar type of analysis was carried out after migration of the total RNA on 0.5% agar, 2.4% acrylamide gels (12), and elution of the RNA from the gel slices.

Polysome Preparation. Cells were treated with cycloheximide (100 μg/ml) for 3 min before harvesting, and the antibiotic was retained in wash and extraction buffers. The cells

Abbreviations: cDNA, complementary DNA; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid.
were treated as described above except that the NaCl was 0.25 M. Cytoplasmic extracts were analyzed on 10–40% sucrose gradients (SW 50 rotor at 38,000 rpm for 35–50 min). The A_{260} profile was analyzed and fractions taken for RNA extraction and analysis. Ribonucleoprotein particles were obtained from the polysome fraction by treatment with EDTA at about 15 μmol/mg of ribosomes. The ribosomal subunits were separated by centrifugation on 5–20% sucrose gradients (0.15 M KCl, 0.01 M Tris-HCl (pH 7.5)) at 50,000 rpm for 1 hr 30 min in the SW 50 rotor.

RNA Chase Experiments. After labeling, the cultures were incubated for 45 min in new medium containing 10 mM glucosamine chloride (19), and uridine (100 μg/ml), then in the same medium without glucosamine, before incubation in medium alone.

Estimation of Pool Size. Cultures were labeled for 24 hr with 25I (100 μCi/mmol, 10 μCi/ml) and for the last 2 hr with [3H]uridine (100 μCi/2.5 ml). [3H]:[32P] ratios were estimated in the UTP, UDP, and UMP pools after passage of trichloroacetic acid (TCA)-soluble cell extracts over Dowex I (14).

dNA Preparation. Poly(A)-containing RNA sedimenting at 28S was prepared by poly(U)-Sepharose chromatography of total cytoplasmic RNA from embryonic calf muscle (manuscript in preparation). Complementary DNA (cDNA) was prepared from this RNA fraction using the RNA-directed DNA polymerase purified from avian myeloblastosis virus (15). The DNA product was labeled with [3H]dGTP and [3H]dCTP at low concentration (50–60 μM) and sedimented at 4–5 S in alkaline sucrose, thus corresponding to a molecular weight of approximately 6 × 10^4 (16).

Materials. Tissue culture medium and serum were from Grand Island Biological Co., U.S.A.; radio-isotopes were from C.E.A., Saclay, France; NP40 detergent was from Shell Oil Co.; cycloheximide was from Sigma Chemical Co.; d-glucosamine chloride was from Carlo Erba, Italy; and poly(U) was from Miles, U.S.A. [3H]α-isotinin (5410 cpm/μmol) was kindly supplied by J. P. Changeux.

RESULTS

Myoblasts prepared from fetal calf skeletal muscle remained stationary for the first 24 hr in culture. After a change of medium (taken as 0 hr), the cells grew exponentially for the
subunit of myosin (18). In Fig. 2, the results of this analysis are shown. It is remarkable that during logarithmic growth there is considerable synthesis of a 26S messenger RNA. Dividing fibroblasts (mouse, 3T3 cells), similarly analyzed, did not show any pronounced synthesis of an RNA of this size. In the period before fusion when the myoblasts cease to divide, ribosomal RNA synthesis is much reduced. The actual quantity of ribosomal RNA per cell remains approximately constant. The synthesis of poly(A)-containing RNA is also reduced, but increases just prior to fusion with an initial synthesis of lighter poly(A)-containing RNA, immediately followed by an increased synthesis of larger molecules, including the 26S species. Synthesis of this latter RNA is particularly high in the period 4–8 hr before fusion. This subsequently decreased, until by 75 hr when long multinucleated fuses appear, the synthesis of all poly(A)-containing RNA is much reduced.

The size of the RNA uridine precursor pools was measured during the period in culture. Under standard culture conditions, the nucleotide pools remained approximately constant, indicating that the changes observed in RNA synthesis are not primarily a reflection of precursor availability. Similarly, it has been shown that amino-acid pools do not undergo major changes at fusion (3). The general pattern of messenger RNA synthesis correlates with what has been described for protein synthesis. The earlier appearance of smaller poly(A)-containing RNA, just before fusion, is reflected in the initial synthesis of smaller (<50,000) proteins after fusion.

**RNA Chase Experiments.** The synthesis of a 26S RNA in the dividing myoblast raises the question of whether it is accumulated, and more generally whether messengers produced prior to fusion are used during fusion. One approach to this is to look at the stability of different messenger species. Chase experiments in eukaryotic cells are not very satisfactory because of the large uridine precursor pools. We have found that treatment of the cells with glucosamine chloride, with consequent conversion of uridine to UDPG, as described by Scholtissek (13), is satisfactory as a means of reducing the pool size. A criterion for the efficiency of the uridine chase was provided by the labeling of ribosomal RNA. The specific activity of ribosomal RNA increased by 5–10% of its value at the end of the labeling period in the first 2–3 hr after the beginning of the chase and remained constant thereafter eventually declining slightly. The fact that this percentage increment was similar at different times during the period in culture, suggests that the efficiency of the chase did not change and hence that valid comparisons can be made between the apparent stability of messengers at different times. The results of two such chase experiments are shown in Fig. 3. This shows that in the dividing myoblast the majority of poly(A)-containing RNAs have a short half-life, relative to the situation in cells labeled during the transition period prior to fusion, or at fusion. The results of a series of these experiments are summarized in Table I. There is a certain category of small messenger RNAs of very short half-life (<6 hr) present at all times. In the dividing myoblast, approximately 75% of all messengers have a half-life of about 10 hr; only a few species, one of 23 S and two, present in small quantities, of 30 and 32 S have a sufficiently long half-life to be present for utilization at fusion. In the nondividing myoblast and in the myotube, most messengers have a half-life of 20–25 hr and a certain number have a very much longer half-life. Among the latter is the 26S RNA. The difference in half-life of this species before and

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**Table 1.** Half-lives of classes of poly(A)-containing RNA before and after fusion

<table>
<thead>
<tr>
<th>RNA Species (S)</th>
<th>Myoblast (12–36 hr) Approximate half-life (hr)</th>
<th>Fused myoblast (48–72 hr) Approximate half-life (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;18</td>
<td>&lt;6</td>
<td>&lt;18</td>
</tr>
<tr>
<td>19–21</td>
<td>≈10</td>
<td>16–21</td>
</tr>
<tr>
<td>26S</td>
<td></td>
<td>23</td>
</tr>
<tr>
<td>30</td>
<td>25–30</td>
<td>30</td>
</tr>
<tr>
<td>32</td>
<td></td>
<td>32</td>
</tr>
<tr>
<td>(+ few species</td>
<td></td>
<td>≈10</td>
</tr>
<tr>
<td>&lt;18S, &gt;18S</td>
<td></td>
<td>≈15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>≈20</td>
</tr>
</tbody>
</table>

FIG. 3. In chase experiments on cytoplasmic RNA before and after fusion, cells were labeled with [3H]uridine (100 μg/2.5 ml) for 2 hr and the label was chased, as described in Materials and Methods, for varying periods. The left-hand column of gradients represents an experiment on RNA labeled before fusion (24 hr), and the right-hand column RNA labeled in the transition period (48 hr) or during early fusion (60 hr).

the first of the muscle-specific proteins to appear; biochemical modification of the membrane thus apparently accompanies the early morphological events of fusion, which precede the formation of muscle protein and muscle fibres.

**RNA Synthesis.** The synthesis of cytoplasmic RNA was examined in the myoblast cultures. After separation on sucrose gradients the labeling of total RNA was determined and part of the same fraction was also analyzed for poly(A)-containing RNA which comprises most classes of messenger RNA (17). This method clearly distinguished messenger RNA in relation to the main peaks of 18 and 28S ribosomal RNA. Among the species of poly(A)-containing RNA, 26S RNA was distinguished as the putative messenger RNA for the large
after fusion is demonstrated in Fig. 4. The half-life of the 26S RNA increases from about 10 hr in the dividing myoblast to a value of about 56 hr in the myoblast committed to fusion during the transition period, and in the myotube.

Identity of 26S RNA. The question remains as to whether the 26S RNAs of different stabilities are in fact the same molecule. Several lines of evidence, briefly summarized here, suggest that they may be. From a preparation of 26S RNA, purified in quantity from fetal skeletal muscle, a cDNA reverse copy has been made, using the reverse transcriptase. This cDNA hybridizes to the same extent with RNA isolated from sucrose gradients in the region of 26 S, prepared from dividing myoblasts and from fused myotubes (Fig. 5). RNA isolated from the same region of sucrose gradients but prepared from bovine liver, did not hybridize significantly with the cDNA. Secondly, both 26S RNA species migrate on gels more slowly than 28S ribosomal RNA: in a position expected of an RNA molecule of about 32 S (Fig. 6). This phenomenon has been described by Morris et al. (20) for the myosin messenger RNA and is presumably a result of secondary structure in the molecule.

Experiments designed to describe its cytoplasmic localization provide additional evidence that the early 26S species is not a messenger for another protein synthesized in the dividing myoblast. When polysomes are isolated from the cultures and the localization of different messenger RNAs is examined throughout a ribosome-polysome gradient, the 26S RNA is not found in the heavy polysomes before fusion. It appears to be in the form of a ribonucleoprotein particle cosedimenting with ribosomes and very small polysomes. In contrast, the stable 26S RNA, although initially during the transition period and early fusion in a similar ribonucleoprotein particle, eventually appears in the heavier polysomes during fusion. The timing of this relocation, just prior to and during the synthesis of myosin, reinforces the view that the 26S RNA is coding for the large subunit of myosin. The appearance in the heavy polysomes can be seen either in chase experiments, after a label in the transition period, or by labeling directly during fusion. The polysomal distribution findings are represented schematically in Fig. 7. EDTA treatment of the preparations confirms the apparent size of the ribonucleoprotein particle.
DISCUSSION

In any discussion of the results described, the identity of the 26S messenger RNA assumes considerable importance since, because of its relatively large size, it is the only messenger species easily identifiable in the myoblast system. From previous reports (18, 20, 21, 22) and our own experiments on the myoblasts, particularly those on the polysomal distribution of this RNA, it seems very likely that the 26S RNA synthesized in the cultures at all stages is predominantly the messenger for the large subunit of myosin. This being so, terminal differentiation in the myoblast apparently takes place in cells whose genome is already programmed for the expression of muscle-specific characteristics at the level of transcription, and control at a cytoplasmic level is primarily required for phenotypic expression. The changes in RNA synthesis during the transition period which follows active cell division and precedes fusion suggest that this pre-established transcriptional pattern is still open to finer modification. However, the important point is the synthesis of myosin 26S RNA already in the dividing myoblast. In the transition period when the myoblasts become committed to fusion (5), the most striking molecular event is the stabilization of classes of messenger RNA, including the 26S RNA. It is in this period just prior to fusion that the observations with actinomycin D have been made (6); the lack of effect on early myosin synthesis is in agreement with our observations on the timing of messenger stabilization.

The range of messenger half-lives described here is comparable to what has been observed recently in other eukaryotic cell systems where the use of metabolic inhibitors has been avoided and poly(A)-containing RNA examined. Singer and Pennan (23) report that in dividing Hela cells there are at least two different classes of messenger RNA, one with a half-life of about 7 hr, the other with a half-life approaching 24 hr. Our results on the dividing myoblast also indicate a range of half-lives of this order, although the major component is about 10 hr, perhaps related to the cell generation time of 18 hr, compared with 24 hr for Hela cells.

In the fused myoblast we continue to observe the class of small poly(A)-containing RNAs with a half-life of about 6 hr. This is a further indication, in addition to the criterion of ribosomal RNA specific activity already discussed, that the chase is operating with similar efficiency after fusion. There continues to be synthesis of classes of RNA with a half-life of about 24 hr, but now there is a further class of messenger RNA of very much longer half-life. The presence of very stable messenger RNAs coding for proteins characteristic of the differentiated state has been described in a number of differentiated cell types (24, 25); the encultured reticulocyte being an extreme example.

The significant feature of this description of the myoblast system, where the process of terminal differentiation takes place in vitro, is the demonstration of the transition from the synthesis of a relatively unstable messenger RNA coding for a protein, in this case myosin, typical of the differentiated tissue, to the production of this same messenger RNA as a highly stable RNA species utilized during differentiation. The mechanism of this stabilization is not directly related to the entry of the messenger into polysomes, since the stable 26S species appears in the cytoplasm at least 16 hr before it is found in the polysomes. It would thus appear to be linked to some characteristic of the ribonucleoprotein particles shown to be present before and after fusion. Either the RNA itself is modified in some way or the proteins associated with it are different. These possibilities are under investigation.

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