Insulin Stimulates the Translation of Ribosomal Proteins and the Transcription of rDNA in Mouse Myoblasts*

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Insulin stimulates the translation of ribosomal protein (r-protein) mRNAs and the transcription of rDNA in mouse MM14DZ myoblasts. Analysis of the distribution of S16, L18, and L32 r-protein mRNAs in polysome gradients indicates that the increased translation of these mRNAs in insulin-treated myoblasts is due to the recruitment of mRNAs that were not previously translated. In contrast, the translational efficiencies of β-actin, c-myc, and p31 mRNAs are not affected by insulin. Hybridization analysis of RNA transcribed in nuclear run-on reactions indicates that insulin also stimulates the transcription of rDNA. Both the increases in r-protein translation and rDNA transcription occur coordinately and are maximal within 15 min of insulin treatment of myoblasts. However, insulin has no effect on the rate of cell division or the steady state levels of r-protein mRNAs. Surprisingly, after myoblasts differentiate into fibers, insulin does not affect the r-protein mRNA translation or rDNA transcription. These experiments indicate that the synthesis of the macromolecular components of ribosomes is tightly and coordinately controlled in myoblasts.

Eukaryotic ribosomes are comprised of equimolar amounts of 70–80 different ribosomal proteins (r-proteins) and 4 rRNAs. To understand the mechanisms regulating the biosynthesis of eukaryotic ribosomes it must be determined how the equimolar accumulation of rRNA and r-protein is coordinated. In some cases either rRNA or r-protein is produced in excess, and the accumulation of rRNA and r-protein is coordinated by the turnover of the overproduced species (Craig and Perry, 1971; Clissold and Cole, 1973; Cooper, 1973; Abelson et al., 1974; Bowman and Emerson, 1977; Warner, 1977; Krauter et al., 1980; Wolf et al., 1980; LaMarca and Wasserman, 1984; Jacobs et al., 1985; Pierandrei-Amaldi et al., 1986; ElBaradi et al., 1986; Bell et al., 1988). In many eukaryotic systems equimolar accumulation is accomplished by the synthesis of rRNA and r-protein in approximately equal amounts. However, the mechanisms involved in coordinating their synthesis are not known (DePhilip et al., 1980; Faliks and Meyuhas, 1982; Pierandrei-Amaldi et al., 1982; Bozsoni et al., 1984; Al-Alia et al., 1985; Schmitt et al., 1985; Agrawal and Bowman, 1987; Meyuhas et al., 1987).

The possibility that the synthesis of eukaryotic r-proteins is regulated by feedback inhibition as in bacteria (Nomura et al., 1984) has been examined by introducing extra copies of r-protein genes or mRNAs into cells (Pearson et al., 1982; Bozsoni et al., 1984; Abovich et al., 1985; Warner et al., 1985; Bowman, 1987b; Rhoads and Roufa, 1987; Pierandrei-Amaldi et al., 1986; Baum et al., 1988). In these cases, the r-proteins are overproduced in proportion to their gene copy number and rapidly turn over. However, in two notable cases the overproduction of an r-protein inhibits the processing of its own mRNA, but the synthesis of other r-proteins is not affected (Bozsoni et al., 1984; Daheva et al., 1986). Despite these examples, autogenous regulation appears not to be a general mechanism for regulating r-protein synthesis. Only a fraction of the r-proteins has been examined in this manner, and it is still possible that the overproduction of some unstudied r-proteins may inhibit the synthesis of other r-proteins.

The differentiation of mouse MM14DZ myoblasts in cell culture provides an excellent system for studying the mechanisms coordinating rRNA and r-protein formation. Myoblasts divide rapidly in culture until the medium is depleted of growth-promoting factors. Then they withdraw from the cell cycle and fuse into fibers. As the cells withdraw, the synthesis of the muscle-specific contractile proteins initiates (Hauschka et al., 1979; Linkhart et al., 1981; Gospodarowicz et al., 1984). We have previously shown that after mouse myoblasts differentiate into fibers the rate of ribosome formation decreases 2–3-fold due to a decrease in the translation of rDNA and to a decrease in the translation and steady state levels of r-protein mRNAs (Agrawal and Bowman, 1987; Bowman, 1987a). However, other laboratories obtain different results when studying ribosome formation during the differentiation of rat myoblasts (Krauter et al., 1980; Jacobs et al., 1985). In rat only the transcription of rDNA decreases following myoblast differentiation (Krauter et al., 1979; Jacobs et al., 1985); neither the steady state levels nor the translational efficiencies of r-protein mRNAs decrease (Jacobs et al., 1985).

In this paper we show that insulin stimulates the translation of r-protein mRNAs and the transcription of rDNA in mouse myoblasts but not in fibers. Maximal stimulation occurs within 15 min, making this an excellent system for studying the coordination of r-protein and rRNA metabolism.

**EXPERIMENTAL PROCEDURES**

Cell Culture—MM14DZ mouse myoblasts (Hauschka et al., 1979) were grown as described previously using partially purified fibroblast growth factor (Bowman, 1987b). Cultures were fed fresh media 24 h after passage. Myoblast fusion initiates after 72 h, and by 120 h greater than 95% of the nuclei are within fibers. Myoblasts were harvested at 48 h and at 120 h after passage.

Bovine insulin (Collaborative Research, Inc.) was dissolved in water and added to myoblast cultures to a final concentration of 4 μg/ml 24 h after passage. Experimental fiber cultures received an additional insulin dosage added directly to the plates 96 h after passage unless otherwise indicated.

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1 The abbreviation used is: r-protein, ribosomal protein.
Polysome and RNA Isolation—Myoblast and fiber cultures were made 90 μg/ml in cycloheximide immediately prior to harvesting. Cultures were harvested in 0.3 M NaCl, 10 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, 1% Triton X-100, 90 μg/ml cycloheximide and gently homogenized with four strokes in a loose Dounce homogenizer. Lysates were centrifuged at 15,000 × g for 5 min, and the supernatant was layered onto an 11-m10.5-1.5 M sucrose gradient with a 1-mL pad of 2 M sucrose made in the above buffer without Triton X-100. Gradients were centrifuged at 40,000 rpm for 165 min in an SW 41 rotor, fractionated, and the absorbance at 254 nm monitored (Agrawal and Bowman, 1987).

Sucrose gradient fractions were made 0.7% in sodium dodecyl sulfate. 20 μg of bacterial rRNA and 20,000 cpm of tritium-labeled bacterial RNA of a high specific activity was added to each fraction to act as carrier and recovery control RNA, respectively. RNA was isolated by phenol-chloroform extraction and ethanol-precipitated twice prior to gel fractionation.

RNA was isolated from myoblast and fiber cultures by phenolchloroform and LiCl extraction as described (Agrawal and Bowman, 1987; Bowman, 1987a).

Hybridization Analysis—RNAs isolated from both polysome gradients and cultures were denatured with glyoxal (McMaster and Carmichael, 1977). A small aliquot from the gradient fractions was denatured RNA onto different 1% agarose gels. The agarose gels were blotted and hybridized (Agrawal and Bowman, 1987) to nick-translated, labeled CDNA inserts for S16, L18, L32 (Meyubahs and Perry, 1986; Agrawal and Bowman, 1987), p31 (Theodor et al., 1985), and β-actin (Ponte et al., 1983) mRNA, pM c-myc54 plasmid for c-myc mRNA (Stanton et al., 1983), and p5B plasmid for 18 S rRNA (Bowman et al., 1981). Filters were rinsed as described (Agrawal and Bowman, 1987) and exposed to Kodak X-Omat AR x-ray film with an intensifying screen at −70°C. The exposed autoradiographs were scanned in a Helena Quick Scan densitometer to determine the relative hybridized signal in each lane.

Nuclear Transcriptions—Nuclei from myoblast and fiber cultures were isolated and incubated in transcription reaction buffer containing heparin as described (Bowman, 1987a). RNA was isolated and hybridized to the external transcribed spacer probe (ps) (Bowman et al., 1981) as described (Bowman, 1987a).

Filters were rinsed in 0.3 M NaCl, 10 mM Tris-HCl, pH 7.4, 2 mM EDTA, pH 7.4, and 0.1% sodium dodecyl sulfate for 24 h at 4°C. Hybridized RNA was eluted from the filters and quantitated by scintillation counting (McKnight and Palmiter, 1979).

DNA Quantitation—Cell cultures were harvested in a buffer containing 0.5 M NaCl and 2 mM EDTA, pH 7.4, homogenized with a syringe and a 26-gauge needle, and frozen at −20°C. Thawed samples were made 2.0 M NaCl, 50 mM NaPO₄, pH 7.4, and 0.1 μg/ml Hoechst 33258 dye (Labarca and Paigen, 1980). The fluorescence was determined using a Turner model 111 fluorometer. The DNA content of each sample was then determined from a standard curve derived from dilutions of a calf thymus DNA standard.

RESULTS

The translational efficiencies of several r-protein mRNAs were inferred in insulin-treated and control mouse MM14DZ myoblasts from their distribution in polysome gradients. Cell extracts were fractionated by sucrose gradient sedimentation. The gradients were fractionated into polysomal and nonpolysomal fractions as shown in Fig. 1. RNA was isolated from fractions of the gradient, and the relative amounts of specific mRNAs in each fraction were determined by RNA gel blot analysis. A small amount of Escherichia coli [3H]RNA was added to each fraction just after fractionation to monitor the recovery of RNA during isolation. The recovery was usually greater than 85%, and little normalization of the hybridization signals was required. Differences as small as 25–30% in the fraction of mRNA associated with polysomes was accurately measured using this recovery control.

Fig. 2 shows the results of the RNA gel blot analysis of polysome gradients from insulin-treated and control myoblast cultures. Consistent with previous results only 40–50% of the mRNAs for r-proteins S16, L18, and L32 are associated with polysomes in control myoblasts, indicating that these mRNAs are translated very efficiently in both control and insulin-treated myoblasts. In addition, these results indicate that our
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 Autoradiographs, such as those shown in Figs. 2 and 3, were analyzed by densitometry. The percentage of each RNA located in the polysomal fractions is indicated below. The increase in the fraction of r-protein mRNAs associated with polysomes in insulin-treated myoblasts is statistically significant at 0.05 level for S16 and L18 mRNAs and the 0.01 level for L32 mRNA as determined using the t test.

<table>
<thead>
<tr>
<th>Probe</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Experiment 3</th>
<th>Average of experiments</th>
<th>Ratio of insulin-treated to control</th>
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<tbody>
<tr>
<td>S16</td>
<td>58 54 47</td>
<td>53</td>
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<td></td>
<td>82 69 77</td>
<td>76</td>
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<td></td>
<td>35 35 33</td>
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<td></td>
<td>38 39 33</td>
<td>37</td>
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<td>L18</td>
<td>36 37 50</td>
<td>41</td>
<td>1.3</td>
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<td></td>
<td>47 47 73</td>
<td>56</td>
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<td></td>
<td>30 40 28</td>
<td>33</td>
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<td></td>
<td>32 39 23</td>
<td>31</td>
<td></td>
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<tr>
<td>L32</td>
<td>55 43 56</td>
<td>51</td>
<td>1.5</td>
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<td>78 69 79</td>
<td>75</td>
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<td></td>
<td>49 54 41</td>
<td>48</td>
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<td></td>
<td>48 42 36</td>
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<td>P31</td>
<td>&gt;95 &gt;95 &gt;95</td>
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<td>β-actin</td>
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<tr>
<td>18 S</td>
<td>66 44 72</td>
<td>61</td>
<td>0.9</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>51 53 65</td>
<td>57</td>
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<td></td>
<td>46 44 32</td>
<td>41</td>
<td>0.9</td>
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<td></td>
<td>41 30 36</td>
<td>36</td>
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</table>

*Not determined.

This possibility was examined by adding fresh medium with and without insulin to fiber cultures 24 h prior to harvesting. Fig. 3 shows that insulin in the presence of fresh media still does not stimulate the translation of r-protein mRNA. Consequently, the lack of response in fiber cultures is not due to a deficiency in the culture medium; rather, fibers and myoblasts respond differently to insulin.

**The Effect of Insulin on the Distribution of Ribosomes in Polysome Gradients**—The fraction of ribosomes associated with polysomes was measured by hybridizing gradient fractions to a probe complementary to 18 S rRNA (Bowman et al., 1981). Figs. 2 and 3, and Table I show that the percentage of ribosomes in polysomes is higher in myoblasts than in fibers. This overall distribution is not affected by insulin in either myoblasts or fibers, suggesting that insulin does not stimulate a general recruitment of RNA into polysomes. Furthermore, examination of polysome profiles (Fig. 1) indicates a slight difference in the ratio of 40 S to 60 S subunits in insulin-treated myoblasts as compared to control myo-
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**Fig. 3. RNA gel blot analyses of fiber polysome density gradients.** In the experiments shown in the column labeled control, no insulin was added to the cultures; in the column labeled + insulin, insulin was added at 24 and 96 h after passage; in the column labeled fresh media, no insulin was added, but the medium was replaced with fresh medium at 96 h; and in the column labeled fresh media + insulin, insulin was added at 24 h, and the medium was replaced with fresh medium containing insulin at 96 h. The fiber cultures were then harvested at 120 h. Polysome gradients were fractionated into nonpolysomal and polysomal fractions as shown in Fig. 1. RNA was isolated from gradient fractions and separated on parallel agarose gels. The resulting duplicate gel blots were hybridized to the indicated probes. Representative gel profiles are shown; these are not all derived from the same experiment. This figure does not reflect the relative concentrations of r-protein mRNA in fibers and insulin-treated fibers (see Fig. 6) as different amounts of cells and/or probe are used in some cases. This figure only shows the distribution of mRNA in polysome gradients.

blasts. This difference was not detected in fibers.

**Insulin Stimulates the Transcription of rDNA**—The transcription of rDNA was measured in isolated nuclei to determine if insulin also stimulates the transcription of rDNA. Heparin was included in these transcription reactions to inhibit transcription initiation and RNA degradation. RNA formed in the transcription reactions was isolated and hybridized to filters that contain sequences complementary to the external transcribed spacer of 45 S pre-rRNA. We estimate that we can measure a 25% difference in the transcription of rDNA between different samples within a single experiment, in part because 200–2000 cpm hybridized to the filters in these experiments. This amount of radioactivity is accurately measured by scintillation counting. To ensure that the filters were not being saturated, hybridizations containing different amounts of input [32P]RNA were performed for each transcription reaction. As noted previously (Bowman, 1987a), Table II shows that there is some variability in the absolute fraction of radioactivity that hybridizes to the filters from one experiment to the next. However, within each myoblast experiment insulin reproducibly increases the fraction of total transcription devoted to rDNA by 1.5-fold. In contrast, insulin does not increase rDNA transcription in fibers (Table II).

The increase in the fraction of total transcription devoted to rRNA in insulin-treated myoblasts could be due to an increase in the rate of rDNA transcription/nucleus or a decrease in the transcription of non-rDNA genes. If the transcription of the non-rDNA genes is reduced, the overall rate of transcription should be significantly reduced in insulin-treated myoblasts as non-rDNA transcription comprises 70–90% (Table II (Bowman, 1987a)) of the total. The overall rate of transcription in control and insulin-treated myoblasts was...
Table II

rDNA transcription rates

The relative rate of rDNA transcription is expressed as the percentage of total input counts/min that hybridized to the p3 external transcribed spacer probe (Bowman et al., 1981). In each experiment shown below the transcription reactions were performed side by side using the same batch of [32P]UTP filters, and hybridization and rinse solutions. Each hybridization reaction contained a pBR322 filter to monitor background hybridization which was always less than 10 cpm. It is estimated that rDNA transcription comprises 10-30% of the total based on the percent counts/min hybridized here and in Bowman, 1987a, the size of the probe and the size of 45 S pre-rRNA.

The insulin-stimulated increase in rDNA transcription in myoblasts is statistically significant at the 0.01 level as determined using the t test.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Input counts/min</th>
<th>Counts/min hybridized</th>
<th>Percent hybridized</th>
<th>Ratio of insulin-treated to control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>14,800</td>
<td>259</td>
<td>1.8</td>
<td></td>
</tr>
<tr>
<td>Myoblasts + insulin (15 min)</td>
<td>18,700</td>
<td>545</td>
<td>2.9</td>
<td>1.7</td>
</tr>
<tr>
<td>Myoblasts + insulin (60 min)</td>
<td>13,500</td>
<td>425</td>
<td>3.1</td>
<td>1.6</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>9,230</td>
<td>216</td>
<td>2.3</td>
<td></td>
</tr>
<tr>
<td>Myoblasts + insulin (24 h)</td>
<td>7,140</td>
<td>238</td>
<td>3.3</td>
<td>1.4</td>
</tr>
<tr>
<td>Experiment 3</td>
<td>14,700</td>
<td>327</td>
<td>2.2</td>
<td></td>
</tr>
<tr>
<td>Myoblasts + insulin (15 min)</td>
<td>14,500</td>
<td>431</td>
<td>3.0</td>
<td>1.3</td>
</tr>
<tr>
<td>Experiment 4</td>
<td>17,100</td>
<td>453</td>
<td>2.7</td>
<td></td>
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<tr>
<td>Fibers + insulin</td>
<td>15,000</td>
<td>373</td>
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<td>1.1</td>
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<tr>
<td>Experiment 5</td>
<td>14,600</td>
<td>331</td>
<td>2.3</td>
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<tr>
<td>Fibers + insulin</td>
<td>14,600</td>
<td>331</td>
<td>2.3</td>
<td>0.9</td>
</tr>
</tbody>
</table>

Temporal Coordination of rDNA Transcription and r-Protein mRNA Translation—The transcription of rDNA and the translation of r-protein mRNA were measured at various times after the addition of insulin to myoblast cultures to determine if these increases are temporally coordinated. Fig. 4 shows that the maximal increase in both the transcription and translation is reached within 15 min of insulin addition. Furthermore, the magnitude of the increases is similar.

Insulin Does Not Increase the Steady State Levels of r-Protein mRNAs or the Growth Rate of Myoblasts—RNA gel blot analysis (Fig. 5) shows that the concentrations of the r-protein mRNAs as well as p31 and c-myc RNAs are unaffected by insulin treatment in both myoblasts and fibers. With respect to myoblasts, similar results were obtained regardless of whether RNA was isolated 2 or 24 h after insulin treatment. Insulin does not rapidly and dramatically alter the transcription of r-protein genes or the stability of r-protein mRNAs because the steady state levels of r-protein mRNAs are unchanged after 2 h of insulin treatment. Our experiments are, however, not sensitive enough to eliminate the possibility that small changes in r-protein mRNA levels occur slowly over time due to a small increase or decrease in the transcription of r-protein genes or the stability of r-protein mRNAs.

To determine if insulin stimulates myoblast cell division, the amount of DNA/culture was determined for control and insulin-treated cultures. Fig. 6 shows that insulin does not affect the 15-h myoblast cell division time.
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DISCUSSION

The immediate result of insulin action on myoblasts is to stimulate the translation of r-protein mRNAs and the transcription of rDNA. In contrast, insulin does not rapidly and dramatically affect the transcription of r-protein genes or the stability of r-protein mRNAs. The probable long term result of insulin action is to increase the ribosome content of myoblasts 1.5-fold, although this was not directly demonstrated in this paper. A coordinated change in r-protein mRNA translation and rDNA transcription also occurs following mouse myoblast differentiation (Agrawal and Bowman, 1987) and in other cells (Brown and Litina, 1964; DePhilip et al., 1980; Pierandre-Amaldi et al., 1982; Geyer et al., 1982; Baum and Wormington, 1986; Al-Atia et al., 1985; Meyuhas et al., 1987) and may be a general mechanism for regulating ribosome formation in eukaryotic cells.

Previous experiments suggest that insulin stimulates rDNA transcription and r-protein mRNA translation in resting chick embryo fibroblasts (DePhilip et al., 1980; Ignotz et al., 1981). We draw similar conclusions from our experiments. We have, however, measured rRNA transcription directly using nuclear run-on reactions as opposed to in vivo pulse-labeling experiments, thereby reducing the possibility that RNA degradation confounds our results. Furthermore, we have shown that the increased translational efficiencies of r-protein mRNAs are due to the recruitment of previously untranslated r-protein mRNAs into polysomes and that an increase in r-protein mRNA levels is not an immediate consequence of insulin treatment.

It is not known if insulin is the real effector in resting chick embryo fibroblasts and mouse myoblasts. It is possible that a contaminant in the insulin is the real effector or that this response is actually mediated by the IGF-1 or IGF-2 receptor (Hintz et al., 1972; Zapf et al., 1981). IGF-1 has previously been shown to stimulate rDNA transcription (Surmacz et al., 1987). Synergistic effects may also play a role in the stimulation, since these experiments are performed in complex medium. We do not know why insulin does not affect rRNA and r-protein synthesis in cultured fibers. However, insulin does have known effects on polypeptide initiation in adult muscle (Harmon et al., 1984). Despite the lack of knowledge concerning the endocrinology of this phenomenon, the end effect of insulin on r-protein and rRNA synthesis is now well documented and provides an excellent system for studying the coordination of r-protein and rRNA formation.

One interesting possibility is that the insulin-stimulated increase in r-protein and rRNA synthesis is coordinated at the level of translation. In this scenario, the direct effect of insulin is to stimulate translation. As a consequence, the r-protein mRNAs, which are inefficiently translated, are then preferentially translated at a higher rate according to the models previously proposed (Lodish, 1976; Godefroy-Colburn
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and Thach, 1981). If rDNA transcription is controlled by a short-lived factor whose mRNA is also inefficiently transcribed, a general increase in protein synthesis would also preferentially increase the synthesis of this factor, thereby stimulating rDNA transcription. Previous experiments indicate that rDNA transcription in some cases is regulated by a short-lived factor (Mishima et al., 1979; Gokal et al., 1986; Tower and Sollner-Webb, 1987). However, it is clear that this is not the only mechanism for controlling rDNA transcription. For instance, in rat fibres the r-proteins are translated quite efficiently yet rDNA transcription is depressed (Jacobs et al., 1985).

Acknowledgment—We thank Marga Costan for her excellent technical assistance.

REFERENCES


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