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Comparison of the Mouse L32 Ribosomal Protein Promoter Elements in Mouse Myoblasts, Fibers, and L Cells

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Abstract The sequences required for the maximal expression of the mouse L32 ribosomal protein gene and the binding of nuclear factors to L32 promoter elements were analyzed in mouse myoblasts, fibers, and L cells. Various L32 r-protein promoter sequences were linked to the chloramphenicol acetyltransferase gene (CAT), and the expression of the chimeric genes was measured transiently or after their incorporation into the genome. The sequence requirements for maximal expression of the L32 gene are very similar among the various cells and include the previously identified L32 core promoter from ≈ −150 to +75. Only the promoter region between −45 and +11 displays significant cell type specific differences. Relative to the maximal activity in each cell type, the expression of the L32-CAT gene containing the −45 to +11 region is greater in L cells than in myoblasts or fibers. This difference is correlated with the increased activity of an L cell nuclear factor(s) that binds to this fragment. In addition, our results show that deletion of sequences between −981 and −141 causes a 50–70% reduction of the expression of the L32-CAT gene in myoblasts, fibers, and L cells. The transcription of all the L32-CAT genes examined decreases after myoblasts differentiate into fibers in a manner similar to the endogenous L32 gene, but we were unable to distinguish between sequences involved in controlling the expression of the L32 gene during myoblast differentiation and those sequences required for maximal promoter activity. However, gel mobility shift assays showed differences in the binding of myoblast and fiber factors to the four promoter fragments examined. The possible role of these factor binding differences in controlling L32 transcription is discussed. © 1992 Wiley-Liss, Inc.

Key words: ribosomal protein, promoter elements, nuclear factors, myoblasts, L cells

The promoter architecture of mammalian ribosomal protein (r-protein) genes has several features in common. These r-protein promoters span a relatively small region which extends up to 200 nucleotides upstream and 50–100 nucleotides downstream of the cap site [Atchison et al., 1989; Dudov and Perry, 1986; Hariharan et al., 1989; Hariharan and Perry, 1989, 1990; Rhoads and Roufa, 1987; Chen and Roufa, 1988]. They have a single cap site within a polypyrimidine sequence flanked by GC-rich islands, and particularly degenerate CAAT and TATA boxes. Although some of these aspects of promoter structure are shared with other housekeeping genes, the combination of these features makes the mouse r-protein promoters rather uncommon.

R-protein genes are constitutively expressed at relatively high levels due to the high activity of their promoters, which are comparable in strength to the SV40 promoter [Atchison et al., 1989]. Furthermore, cell differentiation, environmental cues, and variation in cell division rate in mammalian cells produce relatively modest changes in the transcription of r-protein genes or r-protein mRNA levels which are likely controlled at the transcriptional level [Faliks and Meyuhas, 1982; Flusser et al., 1989; Geyer et al.,

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1982; Jacobs et al., 1985; Meyuhas et al., 1987]. For example, r-protein gene transcription is reduced only about two- to threefold during the differentiation of mouse myoblasts into skeletal muscle fibers [Agrawal and Bowman, 1987], whereas the transcription of other housekeeping genes such as β-actin is reduced seven- to eightfold [DePonti-Zilli et al., 1988; Seiler-Tuyns et al., 1984]. It is not known how r-protein genes maintain a high activity in different cells, or how their transcription is modulated during myoblast differentiation.

There are a number of possible mechanisms for the high and constitutive expression of the r-protein genes. One possibility is that the activities of trans-acting factors which control r-protein gene transcription do not significantly change in response to environmental cues and variations in cell division rate. An alternate possibility is that the r-protein genes contain a diverse group of cis-acting elements which can utilize a variety of trans-acting factors and thereby compensate for changes in the concentration or activity of any particular factor. In this case, the promoter sequences required for maximal expression of the L32 gene might vary from one cell type to another. This latter hypothesis is consistent with the fact that r-protein genes contain a number of distinct sequence elements [Atchison et al., 1989].

We have analyzed the sequences required for maximal expression of the L32 r-protein gene and the binding of trans-acting factors to promoter sequences in mouse MM14DZ myoblasts, fibers, and dividing mouse L cells. These experiments were undertaken to determine if there are cell type specific differences in these parameters and to determine what factors or sequences might modulate the transcription of the L32 gene during myoblast differentiation. Mouse L cells are a non-differentiating cell type derived from connective tissue. Myoblasts are rapidly dividing cells that differentiate into skeletal muscle fibers following depletion of growth factors from the culture medium [Hauschka et al., 1979]. Fibers are terminally differentiated, nondividing cells which express skeletal muscle specific genes. Overall, the contribution of various sequence elements to L32 promoter activity is similar among the various cells, although one promoter region is more active in L cells than in myoblasts or fibers. In contrast, large differences were detected in the binding of nuclear factors to promoter sequences in the three cell types. One of these differences is correlated to differences in the activity of a promoter sequences elements. In addition, our results indicate that sequences further upstream of the previously identified minimal promoter are required for maximal L32 promoter activity in myoblasts, fibers, and L cells.

METHODS

Cell Culture

MM14DZ mouse myoblasts [Hauschka et al., 1979] were grown as described previously [Bowman, 1987a,b]. Mouse L cells (NCTC clone 929) were grown in Eagle's Minimum Essential Medium supplemented with 5% newborn calf serum. The mouse L cells used in these experiments were growing, nonconfluent cells that had the medium changed the previous day.

Plasmid Constructs

L32-CAT plasmids were constructed by replacing sequences between the AccI and HindIII sites of the pSV2-CAT vector [Gorman et al., 1982] with L32 restriction endonuclease fragments. The 5′ limit of the L32 sequences in each construct corresponds to restriction sites as follows: −981 HaeII, −689 SspI, −458 NruI, −301 DraI, −141 SpeI, −45 DdeI, and −16 BssHII, while the 3′ limit of the +11 and +116 constructs corresponds to the HaeII and BglI sites in the L32 gene. The 5′ end of the L32 fragments containing 11 nucleotides of transcribed sequences was cleaved from the vector p3A HaeII 5′; 5 [Dudov and Perry, 1984] which contains the 1 kb HaeII L32 fragment inserted into the SmaI site in pUC12. This vector was then cleaved with HindIII which cleaves downstream of the SmaI site and ligated into the AccI-HindIII cut pSV2-CAT vector. The chimeric gene containing L32 sequences −981 to +116 was created by ligating the L32 AccI-BglI fragment (−159 to +116) from p3AR2.8 [Dudov and Perry, 1984] into the −981/+11 construct, using the AccI site at −159 of the L32 gene and the blunt ended SalI site in the pUC12 polylinker. The −458/+116, −141/+116, and the −16/+116 constructs were made by deleting the SacI-NruI, SacI-SpeI, and the SacI-BssHII fragments, respectively, from the −981/+116 construct. The SacI site is in the polylinker just upstream of the −981. The −45/+116 construct was made by

ligating the DdeI-BglI fragment into the SacI-SalI sites of the pUC12 polylinker. In the above experiments, overhanging ends were blunt ended using S1 nuclease.

Cell Transfection

DNA was stably introduced into mouse myoblasts and L cells by the calcium phosphate precipitation method [Wigler et al., 1977] with some modifications [Bowman, 1987a]. The calcium phosphate precipitate contained 10 μg of L32-CAT plasmid and 1 μg of pSV2-NEO [Southern and Berg, 1982]. Plasmid DNA was ethanol precipitated, then redissolved in 1 ml HEPES-PO_4 buffer (137 mM NaCl, 21 mM HEPES (pH 7.12), 0.5 mM Na_2PO_4). The DNA-calcium phosphate precipitate was formed by the addition of 50 μl of 2.5 M $CaCl_2$ per ml of DNA buffer solution. The precipitate was added to the cells then aspirated off after 4 h. Myoblasts were shocked for 5 min at 20°C in medium containing 15% glycerol, and L cells were shocked for 2 min in medium containing 10% glycerol. Selection of stable transformants began 24–30 h after glycerol shock in medium containing 400 μg/ml of G418. The resultant 20–200 colonies from each transfection were pooled. The calcium phosphate method was also used for the transient assays for myoblasts and fibers, except that fibers were glycerol shocked for only 1 min and the calcium phosphate precipitate contained 10 μg of L32-CAT plasmid and 1 μg of pSV2L which contains the luciferase gene [DeWet et al., 1987]. Myoblasts were harvested 24 h after transfection and fibers 48 h after transfection. Mouse L cells were transfected by the DEAE-Dextran method [Lopata et al., 1984] for transient assays. The cells were shocked 4 h after DNA addition in medium containing 10% DMSO, and cells were harvested 48 h later.

Nucleic Acid Isolation and Hybridization Analysis

RNA and DNA were isolated from myoblasts, fibers, and L cells by phenol-chloroform and LiCl extractions as described previously [Agrawal and Bowman, 1987]. Southern and RNA blot analysis were performed using Hybond-N (Amersham) paper as described previously [Bowman, 1987a]. The RNA gels were stained with ethidium bromide before blotting to insure that equal amounts of RNA were loaded onto each lane. Hybridizations of T7 RNA probes to Northern blots were performed at 65°C and rinsed at 65°C in 2 × SSC buffer, 75°C in 5 × SSC buffer, and 70°C in 0.1 × SSC buffer (1 × SSC contains 0.15 M NaCL and 0.015 M sodium citrate, pH 7.0).

Nuclear Run-On Transcription

Nuclei from myoblast and fiber cultures were isolated and incubated in transcription buffer containing 2 mCi/ml of [α-^{32}P] UTP (800 Ci/mmol) and heparin as described previously [Agrawal and Bowman, 1987]. After the 8 min incubation period, 25 μg of carrier tRNA was added and nuclei were digested with 40 μg/ml DNase at 33°C for 10 min, and RNA was isolated as described previously [Agrawal and Bowman, 1987]. Unincorporated nucleotides were removed from the RNA by spin column chromatography (Biogel P-30), then ethanol precipitated. Isolated RNA was then hybridized to nylon filters containing T7-transcribed RNA probes [Melton et al., 1984]. Filters were prepared by denaturing T7-transcribed RNAs in 6 × SSC, 7% formaldehyde at 60°C for 10 min, then applying the RNAs to Hybond-N paper (Amersham) with a slot blot apparatus. Filters were prehybridized at 65°C for 2 h in a 400 mM NaCl, 50 mM PIPES (pH 6.8), 5mM EDTA, 1% SDS solution in a buffer containing 60% formamide, 150 μg/ml tRNA, 1 mg/ml heparin, and 1× Denhardt's solution. Hybridization was performed at 65°C for 72 h in the same solution containing 1–2 × 10^6 cpm of ^{32}P-labeled RNA from the nuclear run-on transcriptions. The filters were rinsed once in 0.3 M NaCl, 10 mM Tris-HCl (pH 7.4), 2 mM EDTA, and 0.1% SDS for 15 min at room temperature, twice at 65°C for 60 min, and finally at 75°C for 60 min.

Luciferase and CAT Enzyme Assays

The luciferase activity in each extract was determined as described previously [DeWet et al., 1987], except that luciferase activity was monitored in a scintillation counter. Light emission was monitored with an open window at 10 successive 0.1 min intervals immediately following addition of substrate. The CAT activity was measured as described previously using ^{14}C-chloramphenicol [Lopata et al., 1984]. The percent of acetylation in each CAT reaction was determined by liquid scintillation counting [Lopata et al., 1984]. To normalize for transfection efficiency, the amount of luciferase activity in each cell extract was quantitated prior to performing CAT assays. The amount of cell extract added to each CAT enzyme reaction was ad-

justed accordingly to account for small differences in luciferase activity.

Gel Mobility Shift Assays

Nuclear extracts were isolated from myoblasts, fibers, and L cells as described previously [Dignam et al., 1983] except that the extraction and dialysis buffers contained 1 μg/ml pepstatin and 1 μg/ml leupeptin to inhibit protease activity. Dialyzed nuclear extracts were concentrated to 0.5–3.0 μg/ml by centrifugation in Centricon 10 microconcentrators. Total protein concentrations were determined by the Bradford method [Bradford, 1976]. Each 15 μl binding reaction was performed at 4°C for 30 min, and contained 2 μg of nuclear extract, 2 μg of poly dIdC, 0.1–0.2 ng ($1–2 \times 10^4$ cpm) of labeled L32 fragment, 60 mM KCl, 5% v/v glycerol, 10 mM Tris (pH 7.5), and 1 mM EDTA. Competition reactions were performed by adding 50–100-fold excess of unlabeled fragment to the binding reaction 10 min prior to the addition of labeled fragment. The binding reactions were fractionated by electrophoresis in 4% polyacrylamide (30:1 bis) gels at 4°C in 0.25× TBE buffer (1 × TBE = 89 mM Tris, 89 mM boric acid, and 2 mM EDTA) for all fragments except the −141 to −41 fragment which was fractionated in buffer containing 0.5 × TBE.

Calculations

Calculations were made using standard equations [Palmiter, 1973, and references therein] to determine if the increased stability of the L32-CAT mRNA is sufficient to account for the increased level of L32-CAT in fibers. The following equation was used to predict the steady state amount of L32-CAT mRNA (R) in fibers as compared to myoblasts, assuming that the rate of transcription (S) of the L32-CAT mRNA is 2.5 times greater in myoblasts as compared to fibers, the half-life ($T_{1/2}$) of the L32-CAT mRNA is 2 h in myoblasts and 6 h in fibers, and the doubling time (T_D) is 13 h in myoblasts and infinite in fibers:

$$S = (1/T_D + 1/T_{1/2})R \ln 2.$$

This analysis predicts that the level of L32-CAT mRNA should increase 1.4-fold after myoblasts differentiate into fibers which agrees with the 1.4-fold increase actually measured. Interestingly, if the transcription of the L32-CAT gene does not decrease following myoblast differentiation, then the level of L32-CAT mRNA should increase 3.5-fold after myoblasts differentiate into fibers.

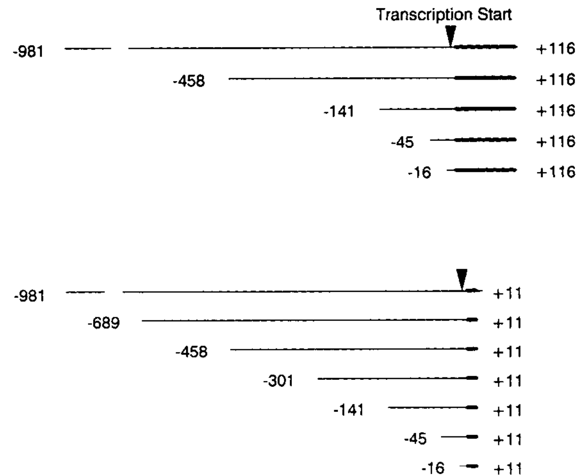


Fig. 1. The L32 promoter deletions used in these experiments. The indicated sequences were linked to the chloramphenicol acetyl transferase coding region. The thick line represents transcribed sequences and the thin line upstream, nontranscribed sequences. The transcription start site is indicated.

RESULTS

The relative contribution of various L32 sequence elements to promoter activity was analyzed in different mouse cell types by standard deletion analysis. Chimeric plasmids that contained 5′ and/or 3′ deletions of L32 promoter sequences linked to the chloramphenicol acetyltransferase (CAT) reporter gene were constructed (Fig. 1). The expression of the L32-CAT gene was assayed in stable transformants or transiently, 24–48 h after transfection. In order to minimize the variability due to the integration site in the stable transformants, 20–200 colonies from each transfection experiment were pooled, and 2–5 independent pools for each construct were examined. The variability among pools was low enough to allow the detection of relatively small differences in the expression of various L32-CAT genes.

The 5′ ends of the L32-CAT mRNAs produced by these genes were examined by primer extension and S1 nuclease mapping techniques. In line with previous results [Moura-Neto et al., 1989], these experiments show that the 5′ end of the L32-CAT mRNAs maps to the previously identified L32 transcription start site with the exception of the L32-CAT mRNA derived from the gene that contains only 16 nucleotides of

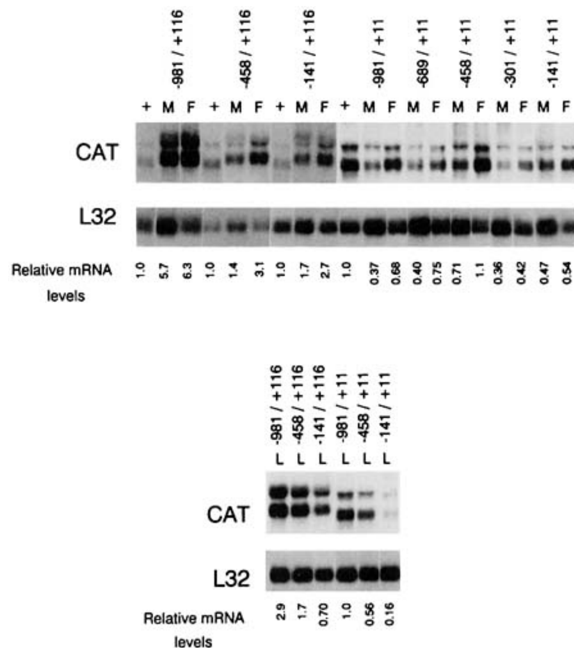


Fig. 2. Northern analyses of total cellular RNA from stable transformants. A composite of several Northern analyses performed with RNA isolated from the various stable transformants. Three micrograms of total cellular RNA from stably transformed myoblasts (M), fibers (F), and L cells (L) were denatured with glyoxal, fractionated on agarose gels, then blotted to nylon filters. Filters were hybridized to an RNA probe complementary to CAT sequences (the 250 N EcoRI-HindIII insert from pSV2-CAT) and a nick translated L32 cDNA probe [Agrawal and Bowman, 1987]. The intensity of the hybridization signal was quantitated by densitometric scanning of autoradiograms. Each filter contained a standard control (+) RNA sample from a −981 to +11 transformant to facilitate comparison of the data obtained from different gels. The L32-CAT mRNA levels normalized to that from the −981 to +11 fiber or L cell transformant are shown. The sizes of hybridized mRNAs were determined by comparison to ethidium-stained 18S and 28S rRNAs. The intensity of 18S and 28S rRNA staining in each lane was also used to confirm equal loading of RNA samples and to examine the integrity of the RNA samples (see Methods).

upstream sequences. This gene did not produce any transcripts initiating at the L32 start site (data not shown).

The expression of transfected L32-CAT genes in the stable transformants was measured using RNA gel blot analyses (Fig. 2). The level of the endogenous L32 mRNA was measured in each experiment to insure that the transformants regulate the expression of the endogenous L32 mRNA normally. To allow comparison of mRNA levels measured on different gels, a known amount of RNA isolated from the −981/+11 transformant was included on each gel. The predominant species of L32-CAT mRNA transcribed from the chimeric genes containing the 11 and 116 nucleotides of transcribed sequences are 1.5 and 1.6 kb in size, respectively (Fig. 2). The sizes of these RNAs correspond to transcripts that terminate near the SV40 polyadenylation signal within the vector. In addition, minor species of L32-CAT mRNA which are 0.3 kb larger are detected. These mRNA species probably contain additional 3′ sequences, since upstream transcription start sites were not detected in primer extension experiments and because the presence of different lengths of L32 upstream sequences in the chimeric genes has no effect on the size of these mRNAs. The proportion of the minor species to the predominant L32-CAT mRNA species was constant among the various transformants, and these minor species do not interfere with the analysis of the steady state levels of L32-CAT mRNAs.

To compare the expression of the L32-CAT genes in the different pools of transformants, the steady state level of L32-CAT mRNA in each pool was normalized to the L32-CAT gene copy number. The gene copy number was determined using quantitative Southern blot techniques (Fig. 3 and legend). The L32-CAT gene containing the largest amount of L32 sequence (from −981 to +116) gave the highest level of expression per gene copy number and was designated as the maximum value (100%) in each cell type. The L32-CAT mRNA levels per gene copy for the other L32-CAT genes were expressed as a percent relative to the −981/+116 L32-CAT gene (Fig. 4A,B).

Sequences Upstream of −141 Are Required for L32 Gene Expression

Deletion of sequences upstream of −141 results in a 50–70% reduction in L32-CAT mRNA levels in mouse myoblasts, fibers, and L cells (Fig. 4A,B). Because deletion of these upstream sequences does not alter the structure of the transcript, this reduction in mRNA levels must be due to a decrease in the activity of the L32 promoter. A stimulatory role for these upstream sequences has not previously been detected in COS or mouse plasmacytoma cells [Atchison et al., 1989; Dudov and Perry, 1986; Moura-Neto et al., 1989]. There appears to be a small difference in the effect of sequences upstream of −458 on L32 promoter activity in L cells as compared to myoblasts and fibers (Fig. 4A,B). However, this difference is dependent on whether the L32-CAT genes contain 11 or 116 nucleotides of transcribed sequences, and the magnitude of the differences is at the limit of detection for these assays.

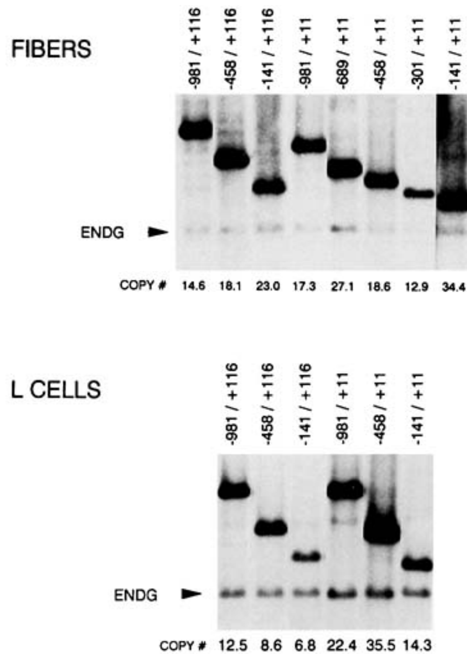


Fig. 3. Southern analysis of stably transformed fibers and L cells. A composite of several selected Southern analyses are shown. DNAs isolated from stable transformants were digested with EcoRI and BglI restriction enzymes. Five microgram and 0.5 μg (not shown) samples of digested DNA were fractionated by agarose gel electrophoresis, blotted to nylon filters, then hybridized to a nick translated NruI-HaeII (−458 to +11) L32 fragment. The number of transfected genes per haploid genome in each transformant was determined by comparison to the single copy endogenous L32 gene (ENDG) by densitometric scanning of autoradiograms. It is assumed that the gene copy number is identical in myoblasts and fibers, because these cell lines were grown for several weeks prior to initiating these experiments. The gene copy numbers in the transformants containing 301 and 141 nucleotides of upstream sequences were corrected for the fact that they contain fewer nucleotides complementary to the hybridization probe by multiplying the raw value for these S16-CAT genes by a factor reflecting the fraction of probe nucleotides complementary to the S16-CAT gene. The same relative values for gene copy number among the cell lines were obtained using a probe derived from the CAT gene (HindIII-EcoRI restriction fragment) which is 100% complementary to all the S16-CAT genes.

Sequences Located in the First Exon and Intron Stimulate Transcription of the L32 Gene

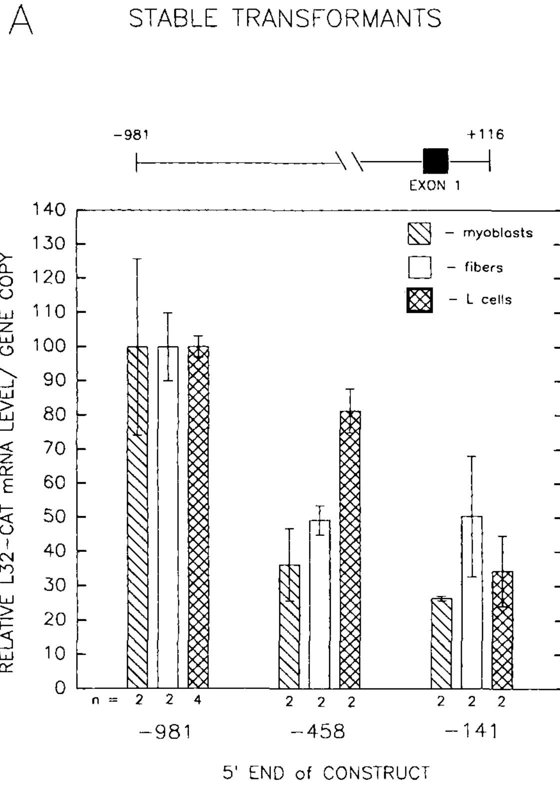
Previous experiments indicate that sequences within the first exon and intron of the L32 gene are required for maximal promoter activity in both mouse plasmacytoma cells and COS cells [Atchison et al., 1989; Chung and Perry, 1989; Moura-Neto et al., 1989]. Comparison of Figure 4A,B indicates that deletion of sequences between +11 and +116 results in a 70–90% reduction in the steady state levels of L32-CAT mRNA in stably transformed myoblasts, fibers, and L cells. The magnitude of the decrease is greater than that detected for the deletion of sequences upstream of −141. There is no detectable difference in the importance of these transcribed sequences for maximal promoter activity among the various cells.

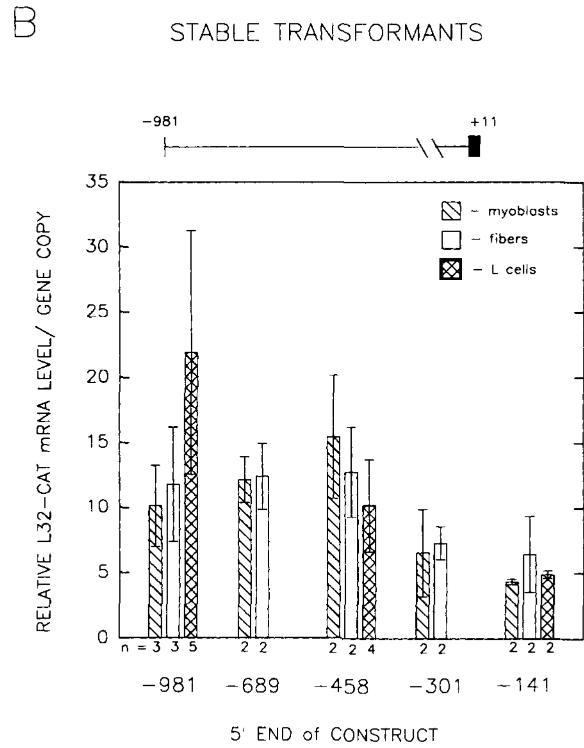
Proximal 5′ Sequences Are Required for Minimal L32 Promoter Activity

The contribution of proximal 5′ sequences (−141 to −16) to promoter activity in the presence and absence of the downstream sequence element was examined in transiently transfected myoblasts, fibers, and L cells. To control for possible differences in the transfection efficiency of the different constructs the L32-CAT plasmids were cotransfected along with the pSV2-L plasmid, which contains the luciferase gene. The amount of chloramphenicol acetyl transferase activity in each cell extract was normalized to the activity of the luciferase enzyme in each corresponding cell extract. Figure 4C shows that deletion of sequences between −141 and −45 results in a 50–70% reduction in CAT activity in myoblasts and fibers, and a slightly smaller decrease in mouse L cells regardless of the amount of transcribed sequences in the gene. Furthermore, the relative expression of the −45 to +11 L32-CAT gene is higher in L cells than in myoblasts or fibers. This difference in the activity of the −45 to +11 L32-CAT gene in L cells is the only cell type specific difference detected in these transient assays. Further deletion of upstream sequences to −16 resulted in the elimination of all detectable promoter activity in all cells. No differences were detected between myoblasts and fibers in either the stable or the transient transfection experiments.

L32 Gene Expression During Myoblast Differentiation

The steady state level of endogenous L32 mRNA decreases about twofold after myoblasts differentiate into fibers, owing to the two- to threefold decrease in the transcription of the L32 gene [Agrawal and Bowman, 1987]. In contrast, the steady state level of L32-CAT mRNA in the stable transformants increases 1.4-fold after myoblasts differentiate into fibers (Fig. 2). This could be due to differences in the pattern of transcription of the L32-CAT gene during myoblast differentiation as compared to the endogenous L32 gene or to differences in the processing or stability of the L32-CAT mRNA as compared to the endogenous L32 mRNA.





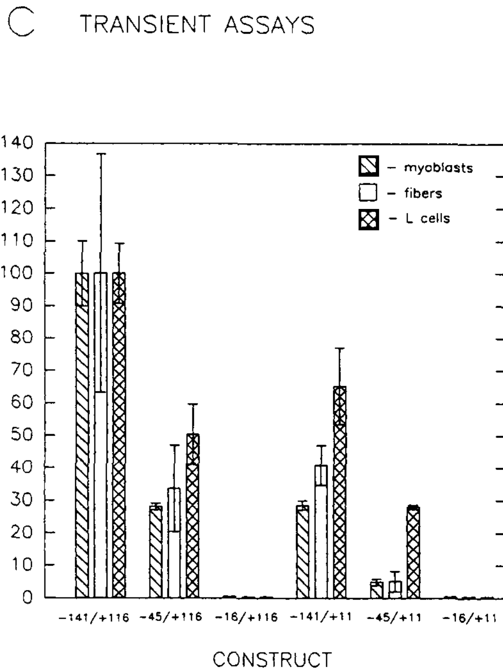


Fig. 4. Summary of the expression of the L32-CAT gene in the stable transformants and in the transient assays. **A:** Relative levels of L32-CAT mRNA per gene copy in stable transformants containing 116 nucleotides of transcribed sequences. **B:** Relative levels for genes containing 11 nucleotides of transcribed sequences. **C:** Chloramphenicol acetyl transferase activity normalized to the luceriferase activity for constructs assayed transiently. The height of each bar represents the mean value. The number of pools assayed for the stable transformants and the number of trials for the transient assays is indicated below each bar (n) in A and B and equals 2 for all constructs shown in C. Error bars indicate the range of values rather than the standard error of the mean. The −981 to +116 gene was the most active and was designated 100% in each cell type.

Nuclear Run-On Transcription Assays of the L32-CAT Genes in Myoblasts and Fibers

Nuclear run-on transcription assays were performed to determine if the pattern of transcription of the L32-CAT genes in myoblasts and fibers was different from the endogenous L32 gene. Nuclei were isolated from myoblasts and fibers containing L32-CAT genes having either −981 to +116 or −981 to +11 nucleotides of the L32 gene and incubated in transcription buffer containing [α-^{32}P] UTP. The labeled RNAs were hybridized to filters containing probes for CAT

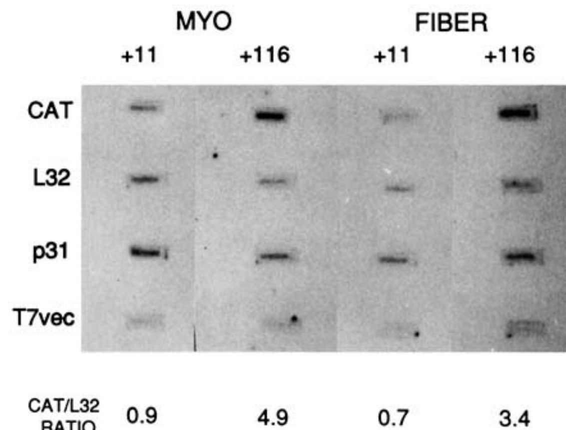


Fig. 5. Nuclear run-on transcription analysis. Nuclei isolated from myoblast (MYO) and fiber stable transformants containing the −981 to +11 (+11) or the −981 to +116 (+116) construct were incubated in transcription reaction buffer containing [α-^{32}P] UTP. The transcription reaction products were hybridized to nylon filters containing RNA probes complementary to the 250 N EcoRI-HindIII fragment from pSV2-CAT, to the 299 N EcoRI-SmaI fragment from intron 3 of the L32 gene, to the mouse p31 mRNA ($\approx$ 530 N) and to the 334 N region from the T7 promoter to the SspI site in the vector pT3/T7. The amount of RNA hybridizing to each probe was measured by densitometric scanning of autoradiograms. Hybridization to the CAT probe was normalized to the L32 hybridization and gene copy number per haploid genome. The +11 transformant gene copy number is 17 and the +116 gene copy number is 21. The CAT/L32 ratio for each transformant is indicated below each autoradiogram.

and intron 3 of the L32 gene. To internally control for differences in input counts and hybridization efficiency, a filter containing p31 basic protein sequences [Theodor et al., 1985] was included in each hybridization. Figure 5 shows that the fraction of total transcription devoted to L32 decreases reproducibly about 1.4-fold after myoblasts differentiate into fibers. This is less than the difference detected in earlier experiments [Agrawal and Bowman, 1987]. However, the overall rate of transcription decreases twofold after myoblasts differentiate into fibers [Bowman, 1987b]. Thus, the transcription of these r-protein genes decreases about two- to threefold after myoblast differentiation.

To determine if the transcription of the L32-CAT gene is regulated similarly to the endogenous L32 gene, hybridization to the CAT probe was normalized to the hybridization to the L32 probe. In order to compare the expression from the gene containing 11 nucleotides to those containing 116 nucleotides of transcribed sequences, differences in gene copy number were factored into this ratio. Figure 5 shows that there is little or no change in the CAT/L32 ratio after myoblasts differentiate into fibers for the transformant containing 11 or the one containing 116 nucleotides of transcribed sequences. This suggests that the transcription of the L32-CAT is regulated similarly to the endogenous L32 gene.

These experiments also show that the CAT-L32 ratio in the +11 transformants is about one-fifth that in the +116 transformants in both myoblasts and fibers, confirming that the higher expression of the L32-CAT genes containing 116 nucleotides of transcribed sequences is due to their increased transcription.

Stability Measurements of L32-CAT mRNAs in Myoblasts and Fibers

To determine if the increased levels of L32-CAT mRNAs in fibers are due to an increased stability of this mRNA, the stabilities of L32-CAT and the endogenous L32 mRNAs were estimated in actinomycin D chase experiments of the −981/+11 and −981/+116 transformants. Given the numerous side effects on cellular metabolism attributed to actinomycin D, these experiments provide a relative rather than an absolute measurements of mRNA half-lives. RNA was isolated at various times after treating myoblasts and fiber cultures with actinomycin D, and the relative concentrations of L32-CAT and the endogenous L32 mRNA were measured by RNA gel blot analysis (Fig. 6). Analysis of semi-log plots suggests that the L32-CAT mRNA has a half-life of 1.5–2.0 h in stably transformed myoblasts and 5.0–5.5 h in fibers of both the −981/+11 and the −981/+116 transformants. It was not possible to derive an accurate half-life of the endogenous L32 mRNA because it decayed little during the chase. Calculations described in the Methods section show that the increased stability of the L32-CAT mRNA in fibers is sufficient to account for the increased level of L32-CAT mRNA in fibers given that the transcription of the L32-CAT is decreased by a factor of 2.5. Therefore, the nuclear run-on and the mRNA stability measurements suggest that the transcription of the L32-CAT genes is regulated like the endogenous L32 gene during myoblast differentiation, and that the increased level of the L32-CAT mRNAs in fibers as compared to myoblasts is due to their increased stability in fibers.

Nuclear Factor Binding to L32 Promoter Sequences Varies Dramatically Among Myoblasts, Fibers, and L Cells

To determine if there are differences in the binding activity of nuclear factors to L32 pro-

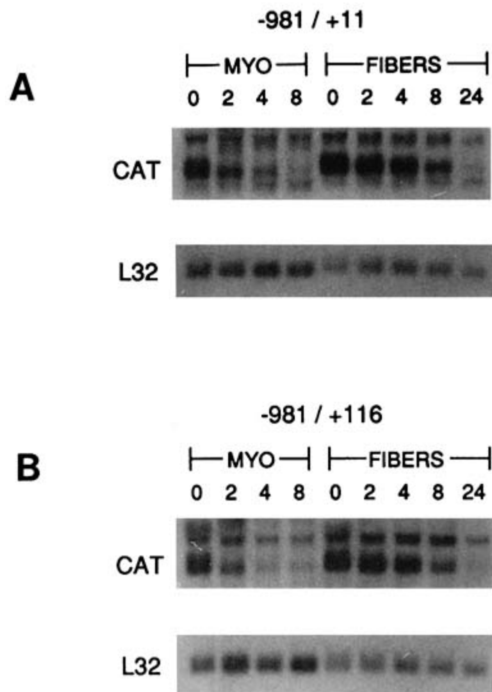


Fig. 6. Estimation of mRNA stability of actinomycin D chase. Stably transformed myoblasts and fibers containing the −981/+11 (**A**) and −981/+116 (**B**) constructs were treated with 1 μg/ml actinomycin D. Previous experiments indicate that this concentration was sufficient to reduce mRNA synthesis 95%. Total cellular RNA was isolated at time points after the addition of actinomycin D, fractionated by glyoxal agarose gel electrophoresis, and blotted to nylon filters. Filters were hybridized to probes complementary to CAT and L32 mRNAs as in Fig. 2. L32-CAT mRNA concentration was determined by densitometric scanning of autoradiograms. L32-CAT mRNA half-lives were estimated by semi-log plots of the data obtained from the Northern analyses.

moter sequences among various cell types, gel mobility shift assays were performed with crude nuclear extracts isolated from myoblasts, fibers, and L cells using four ^{32}P-labeled L32 DNA fragments that span the region from −141 to +116 (Fig. 7). Three different extracts from each cell type were tested and the binding patterns obtained were highly reproducible. Equal amounts of protein from each extract were used in the reactions, and the yield of nuclear extract from each type of cell was similar. We cannot be certain that factors are extracted with equal efficiency from the various cells. Thus, small differences in the binding activity of a particular factor might not actually reflect a difference in vivo. However, rather large differences are detected in our experiments, and these involve changes in the relative activities of the factors. The binding specificity was determined by testing the ability of a 50–100-fold molar excess of unlabeled probe or nonspecific fragment (CAT coding sequence) to compete for the binding of factors. Proteins specifically binding to a 5′ labeled fragment should be competed by an excess of the same unlabeled fragment, but not by the unrelated CAT fragment.

The binding of myoblast, fiber, and L cell factors to these fragments displays a strikingly large number of differences (Fig. 7; Table I). There are differences in the binding of factors to all four fragments between myoblasts and fibers. The binding of fiber factors to fragments −141/−41, −41/+11, and +45/+116 is extremely low as compared to the binding of myoblast factors (Fig. 7A,B,D). In contrast, the binding of fiber factors to the +11/+45 fragment was relatively high compared to myoblasts (Fig. 7C). Thus, the decreased transcription of the L32 gene in fibers is correlated with differences in the binding of factors to all of these fragments. The binding of L cell factors to fragments −141/−41 and +49/+116 is similar to that of the binding of myoblast factors to these fragments (Fig. 7A,D). The rate of transcription of the L32 gene in L cells is unknown, but it is likely to be similar to the transcription rate in myoblasts, as both of these cells divide rapidly. Thus, it is not surprising that the binding of factors to these fragments is similar. On the other hand, there are significant differences in the binding of myoblast and L factors to the −41/+11 and +11/+45 fragments (Fig. 7B,C). Interestingly, one of these fragments, −41/+11, is part of a slightly larger fragment that shows a cell type specific differences in its importance for the maximal expression of the L32-CAT gene in L cells. Thus, there is a correlation between the increased importance of this sequence in L cells and the increased binding of factors to these sequences. However, the differential binding to the +11/+45 fragment is not associated with any detectable cell type difference in the importance of these sequences for promoter function.

DISCUSSION

With one exception, the relative contribution of various L32 promoter elements to maximal gene expression is remarkably similar in the different cells studied here. Sequences within the first exon and intron of the L32 gene have previously been shown to stimulate transcription in COS and mouse plasmacytoma cells [Atchison et al., 1989; Dudov and Perry, 1986; Moura-Neto et al., 1989]. We show that these

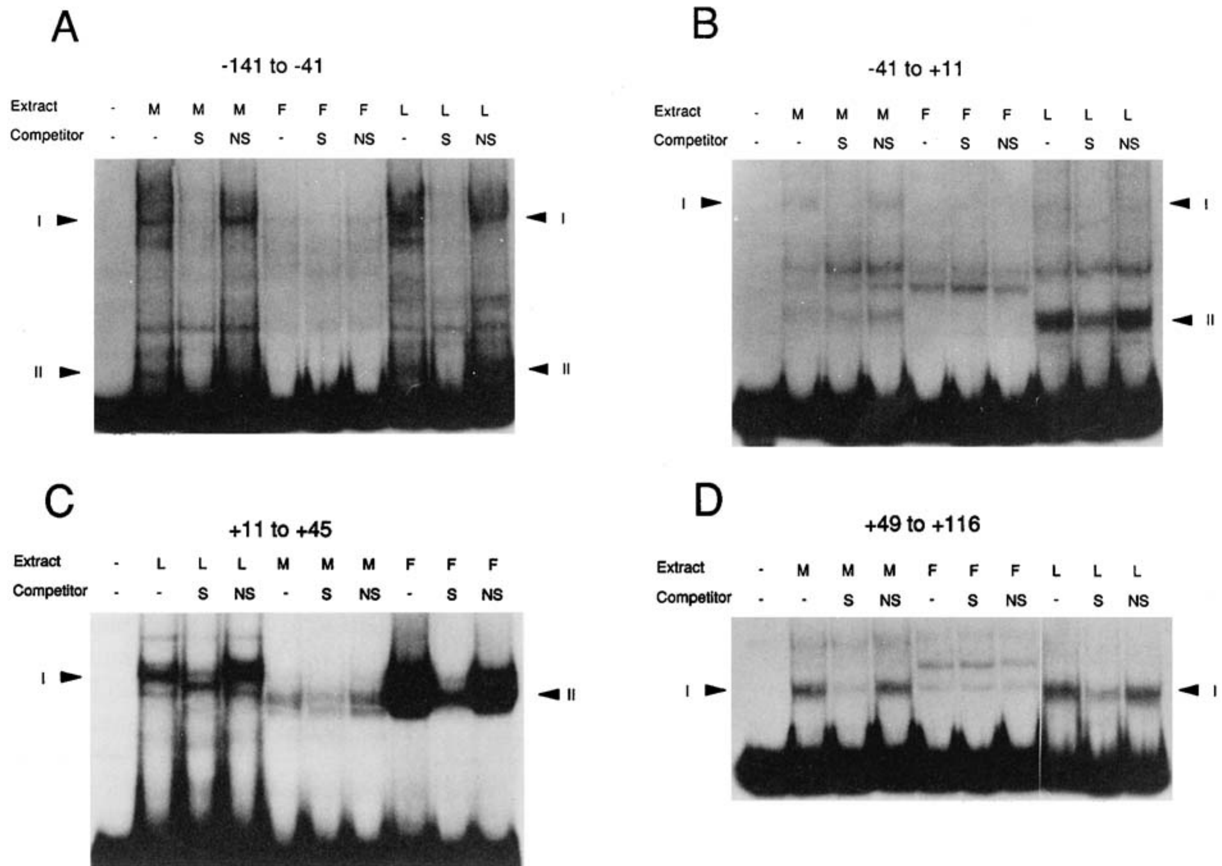


Fig. 7. Analysis of nuclear factor binding to L32 promoter fragments by gel mobility shift assay. Nuclear extracts were isolated from myoblasts (M), fibers (F), and L cells (L). Each extract was incubated in binding buffer containing the 5′ labeled −141/−41 (**A**), −41/+11 (**B**), +11/+45 (**C**), and +49/+116 (**D**) L32 DNA fragments. Competition reactions contained 50–100-fold excess of either the unlabeled fragment which is the specific competitor (S) or the 120 bp PvuII-Hind III fragment from the CAT coding region of pSV2-CAT which is the nonspecific competitor (NS). Shifted bands on each autoradiogram which were specifically competed out by the specific L32 competitor are indicated by arrows. In each autoradiogram the upper band was designated I and the lower band II.

TABLE I. Summary of Gel Mobility Shift Data

		Band intensity		
L32 fragment	Shifted band	M	F	L
−141 to −41	I (upper)	++	0	++
	II (lower)	++	0	++
−14 to +11	I (upper)	+/0	0	+/0
	II (lower)	0	0	+++
+11 to +45	I (upper)	0	0	++
	II (lower)	+	++++	0
+49 to +116	I	++	0	++

sequences are also stimulatory in mouse myoblasts, fibers, and L cells, and that there are no cell types specific differences in the importance of these sequences for maximal promoter activity. These exon and intron sequences are clearly affecting transcription, as indicated by nuclear run-on transcription assays (Fig. 5) [Chung and Perry, 1989] and mRNA stability measurements (Fig. 6). Similarly, previous experiments show that the sequences between −36 and −159 stimulate transcription [Atchison et al., 1989; Dudov and Perry, 1986; Moura-Neto et al., 1989]. Our experiments also indicate that these sequences stimulate transcription in myoblasts, fibers, and mouse L cells and that there are no cell type specific differences in the importance of these for maximal gene expression.

Our experiments also show that sequences upstream of −141 are required for maximal L32 promoter activity in myoblasts, fibers, and L cells. The contribution of these upstream sequences to the transcription of the L32-CAT gene is not as great as that of the sequences located between −141 and +116. Deletion of the sequences upstream of −141 reduce mRNA levels by 50–70%, whereas deletion of exon and intron sequences reduce transcription 70–90% and deletion of sequences between −45 and

−141 reduce transcription by about 75%. Previous experiments did not detect a significant stimulatory effect of these upstream sequences [Dudov and Perry, 1986; Moura-Neto et al., 1989]. In most of these previous experiments, the expression of the transfected gene was assayed transiently. We detected a more variable effect of these sequences in transient assays (data not shown). It is possible that the importance of these sequences is masked in transient assays because of the higher amount of transfected DNA per cell in the transiently transfected cells as compared to stably transformed cells. This could titrate out factors binding to this region resulting in only a fraction of the genes binding the appropriate factors.

It is not known how these upstream sequences stimulate transcription. These sequences could stimulate transcription directly by binding trans-acting factors that promote transcription. Alternately, these sequences could suppress read-through from upstream promoters. Such read-through could inhibit initiation from the L32 promoter.

One cell type difference in the importance of the different L32 promoter elements was detected. The relative activity of the −45 to +11 construct is considerably higher in L cells than in myoblasts or fibers. Furthermore, L cell extracts contain a higher concentration of factor(s) that bind to the −41 to +11 probe than do myoblasts or fibers. This raises the possibility that the activity of this factor mediates the increased activity of the −45 to +11 L32-CAT gene in L cells. However, genes having additional L32 sequences are not detectably expressed at relatively higher levels in L cells, probably because most of the stimulatory sequences are located either upstream or downstream of the −45 to +11 region and the inclusion of these other regions masks the effect of these sequences in L cells.

Nuclear run-on transcription assays and mRNA stability measurements indicate that the transcription of the L32-CAT genes, like that of the endogenous gene, decreases after myoblast differentiate into fibers, even though the steady state levels of the L32-CAT mRNAs increase after myoblast differentiation. If a specific sequence controls the decreased transcription of the L32 gene, then deleting this sequence from the L32-CAT gene should increase or decrease the expression of the L32-CAT gene in fibers as compared to myoblasts depending upon whether the element was inhibitory or stimulatory. However, the steady state level of L32-CAT mRNA increased an average of about 1.4-fold for all the constructs, suggesting that the transcription of all the L32-CAT genes are regulated similarly. We were therefore unable to identify a specific element controlling the decreased transcription of the L32 gene during myoblast differentiation. It is possible that sequences controlling transcription during myoblast differentiation are located within the smallest functional promoter sequence tested (−45 to +11) and further deletion eliminates both regulation and activity. Another possibility is that the decreased transcription is not mediated by a specific sequence, but is controlled by the modification of some general factor like RNA Polymerase II.

Previous experiments have identified four plasmacytoma cell nuclear factors, designated β, γ, δ, and ϵ, that bind to regions of the L32 promoter [Atchison et al., 1989; Hariharan et al., 1989]. Although the promoter elements we used contain the binding sites for these factors, we did not directly compare plasmacytoma cell nuclear extracts to myoblast, fiber, or L cell extracts. Therefore, we cannot be certain that these same factors bind in our experiments. However, our major aim was to determine if there are cell type specific differences in factor binding to the different promoter elements.

The activity of factors binding to L32 promoter elements is quite different in myoblasts as compared to fiber extracts. The binding of factors to three promoter fragments is considerably less in fibers as compared to myoblasts, whereas the binding to the +11/+45 is dramatically higher in fibers. The relationship of these changes in the activity of DNA binding factors to the decreased transcription of the L32 gene is unclear, especially considering the fact that we were unable to identify specific sequences modulating the transcription of L32-CAT gene. However, as mentioned above, it is possible that the controlling element is located between −45 and +11 and that the decreased activity of the factor binding to this region controls the decreased transcription of the L32 gene during myoblast differentiation. Further experimentation is required to elucidate the role of these factors in the transcription of the L32 gene.

In conclusion, our studies show that there is remarkable similarity in the relative importance of the L32 promoter elements to maximal expression in these different cells, whereas the activity of nuclear factors that bind to these promoter elements varies widely. This indicates that there

is not a simple relationship between the activity of nuclear factors and the expression of the L32 gene. Perhaps one factor is actually rate limiting and the others are always present in excess.

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REFERENCES

Agrawal MG, Bowman LH (1987): Transcriptional and translational regulation of ribosomal protein formation during mouse myoblast differentiation. J Biol Chem 262:4868–4875.

Atchison ML, Meyuhas O, Perry RP (1989): Localization of transcriptional regulation elements and nuclear factor binding sites in mouse ribosomal protein gene rpL32. Mol Cell Biol 9:2067–2074.

Bowman LH (1987a): The synthesis of ribosomal proteins S16 and L32 is not autogenously regulated during mouse myoblast differentiation. Mol Cell Biol 7:4464–4471.

Bowman LH (1987b): rDNA transcription and pre-rRNA processing during the differentiation of a mouse myoblast cell line. Dev Biol 111:152–163.

Bradford MM (1976): A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–255.

Chen IT, Roufa DJ (1988): The transcriptionally active human ribosomal protein S17 gene. Gene 70:107–116.

Chung S, Perry RP (1989): Importance of introns for expression of mouse ribosomal protein gene rpL32. Mol Cell Biol 9:2075–2082.

DePonti-Zilli L, Seiler-Tuyns A, Paterson BM (1988): A 40-base pair sequence in the 3′ end of the β-actin gene regulates β-actin mRNA transcription during myogenesis. Proc Natl Acad Sci USA 85:1389–1393.

DeWet JR, Wood KV, DeLuca M, Helsinki DR, Subramani S (1987): Firefly luciferase gene: Structure and expression in mammalian cells. Mol Cell Biol 7:725–737.

Dignam JD, Lebovitz RM, Roeder RG (1983): Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res 11:1475–1489.

Dudov KP, Perry RP (1984): The gene family encoding the mouse ribosomal protein L32 contains a uniquely expressed intron containing gene and an unmutated processed gene. Cell 37:457–468.

Dudov KP, Perry RP (1986): Properties of a mouse ribosomal protein promoter. Proc Natl Acad Sci USA 83:8545–8549.

Faliks D, Meyuhas O (1982): Coordinate regulation of ribosomal protein mRNA levels in regenerating rat liver: Study with the corresponding mouse cloned cDNA. Nucleic Acids Res 10:798–801.

Flusser G, Ginzburg V, Meyuhas O (1989): Glucocorticoids induce transcription of ribosomal protein genes in rat liver. Mol Cell Endocrinol 64:213–222.

Geyer PK, Meyuhas O, Perry RP, Johnson LF (1982): Regulation of ribosomal protein mRNA content and translation in growth-stimulated mouse fibroblasts. Mol Cell Biol 2:685–683.

Gorman CM, Moffat LF, Howard BH (1982): Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol Cell Biol 2:1044–1051.

Hariharan N, Kelley DE, Perry RP (1989): Equipotent mouse ribosomal protein promoters have a similar architecture that includes internal sequence elements. Genes Dev 3:1789–1800.

Hariharan N, Perry RP (1989): A characterization of the elements comprising the promoter of the mouse ribosomal protein gene RPS16. Nucleic Acids Res 17:5323–5337.

Hariharan N, Perry RP (1990): Functional dissection of a mouse ribosomal protein promoter: Significance of the polypyrimidine initiator and an element in the TATA-box region. Proc Natl Acad Sci USA 87:1526–1530.

Hauschka SD, Linkhart TA, Clegg CH, Merrill GM (1979): Clonal studies of human and mouse muscles. In Mauro A (ed.): "Muscle Regeneration." New York: Raven Press, pp 211–322.

Jacobs FA, Bird RC, Sells BH (1985): Differentiation of rat myoblasts. Regulation of ribosomal proteins and their mRNAs. Eur J Biochem 150:255–263.

Lopata MA, Cleveland DW, Sollner-Webb B (1984): High level transient expression of chloramphenicol acetyl transferase gene by DEAE-dextran mediated DNA transfection coupled with a dimethyl sulfoxide or glycerol shock treatment. Nucleic Acids Res 12:5707–5717.

Melton DA, Kreig PA, Rebagliati MR, Maniatis T, Zinn K, Green MR (1984): Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucleic Acids Res 12:7035–7056.

Meyuhas O, Thompson EA, Perry RP (1987): Glucocorticoids selectively inhibit the translation of ribosomal protein mRNAs in P1798 lymphosarcoma cells. Mol Cell Biol 7:2601–2699.

Moura-Neto R, Dudov KP, Perry RP (1989): An element downstream of the cap site is required for transcription of the gene encoding mouse ribosomal protein L32. Proc Natl Acad Sci USA 86:3997–4001.

Palmiter RD (1973): Rate of ovalbumin messenger ribonucleic acid synthesis in the oviduct of estrogen-primed chicks. J Biol Chem 248:8260–8270.

Rhoads DD, Roufa DJ (1987): A cloned human ribosomal protein gene functions in rodent cells. Mol Cell Biol 7:3767–3774.

Seiler-Tuyns A, Eldridge JD, Paterson BM (1984): Expression and regulation of chicken actin genes introduced into mouse myogenic and nonmyogenic cells. Proc Natl Acad Sci USA 81:2980–2984.

Southern PJ, Berg P (1982): Transformation of mammalian cells to antibiotic resistance with a bacterial gene under the control of the SV40 early region promoter. J Mol Appl Genet 1:327–341.

Theodor L, Peleg D, Meyuhas O (1985): P31, a mammalian housekeeping protein encoded by a multigene family containing a high proportion of pseudogenes. Biochem Biophys Acta 826:137–146.

Wigler MS, Silverstein S, Pellicer A, Cheng Y, Axel R (1977): Transfer of purified herpes virus thymidine kinase gene to cultured mouse cells. Cell 11:223–232.