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ARTICLES

GA-Binding Protein Is Involved in Altered Expression of Ribosomal Protein L32 Gene

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Abstract Differentiation of BC_3H1 myoblasts to myocytes is accompanied by a 67% drop in the rate of rpL32 gene transcription. Addition of high concentrations of serum to resting myocyte populations stimulates cell growth and subsequent dedifferentiation to proliferating myoblasts with a return to the normal rate of rpL32 gene transcription. During these growth rate changes the binding activities of previously identified factors (β, γ, δ) which interact with the rpL32 gene promoter were examined by mobility shift assays. Binding of the β factor (an Ets related protein) to an oligonucleotide containing the β element was reduced significantly in myocyte nuclear extracts, but subsequent dedifferentiation increased binding within 30 min in either the presence or absence of the cycloheximide. Binding of the γ and δ factors to their respective elements changed only slightly during these processes. Dephosphorylation of either myoblast or myocyte extracts resulted in increased binding of the β factor suggesting that binding activity of the β factor is modulated by phosphorylation during the changes in BC_3H1 myoblasts growth rate. In addition, mobility shift assays with recombinant GABP α and β proteins and their specific antibodies revealed that GABP proteins bind to the rpL32 gene promoter in a sequence dependent manner, and that similar proteins are present in BC_3H1 myoblast/myocyte extracts. These results support the premise that the GABP heterodimer is the rpL32 β factor. Furthermore, during BC_3H1 myoblast differentiation and dedifferentiation neither the levels of the GABP α and β proteins nor their respective mRNAs change. These results suggest that GABP is a constitutively expressed protein and is involved in regulating rpL32 gene by post-transcriptional modifications. J. Cell. Biochem. 65:287–307.

Key words: GA-binding protein; rpL32 gene promoter; ribosomes; differentiation/dedifferentiation

The eukaryotic ribosome is a complex organelle composed of four rRNA species and more than 80 different proteins. Ribosome production changes in response to modulation of cellular growth rate and is regulated by a variety of mechanisms operating at several levels of gene expression. To understand how these mechanisms operate, special attention has been paid to identify factors which control production of the ribosomal components at the transcriptional level.

In the current studies we have investigated mechanisms controlling ribosomal protein L32 (rpL32) gene expression using mouse BC_3H1 myoblasts which, in response to a reduced concentration of serum in the culture medium, cease dividing, withdraw from the cell cycle, and synthesize a family of muscle-specific proteins [Olson et al., 1983; Glacer and Wice, 1989; Taubman et al., 1989]. However, unlike many muscle cell lines, such as rat L6 myoblasts, mouse BC_3H1 myoblasts fail to fuse during differentiation to myocytes. Following addition of high levels of serum to the medium, the differentiated (non-proliferating) myocytes reenter the cell cycle and start to proliferate with a concomitant loss of both the morphological and biochemical characteristics of the differentiated muscle-cell phenotype [Glacer and Wice, 1989; Taubman et al., 1989]. Thus, by manipulating growth conditions, mouse BC_3H1 myoblasts can move from a proliferative state to a differentiated phenotype and back to an actively growing myoblast population.

The rpL32 gene has been cloned and analyzed [Meyuhas and Perry, 1980; Dudov and Perry, 1984; Hariharan et al., 1989]. Its promoter region exhibits several distinct features, some of which are similar to constitutively ex-

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pressed genes, and therefore, rpL32 has been classed as a house-keeping gene [Dudov and Perry, 1986]. These features include the lack of a canonical TATA box and the location of the transcriptional initiation site within an oligopyrimidine tract flanked by blocks of high G + C content [Dudov and Perry, 1984; Wagner and Perry, 1985]. Although these GC-rich sequences do not represent Sp1-binding sites, typical for house-keeping genes, they may serve as a binding site for transcriptional regulatory proteins.

Cis-acting elements, designated β, γ, δ1, and δ2, are located both upstream and downstream of the transcription initiation site [Hariharan et al., 1989] (Fig. 1). Using a cell-free transcription system, studies from our laboratory have established the requirement for β, γ, and δ trans-acting factors in transcription of the rpL32 gene and confirmed their interaction with specific sequences in the promoter region [Yoganathan and Sells, 1991, 1992c; Yoganathan et al., 1992b]. The β factor binds to a DNA element (−79 to −69) which contains a core purine-rich sequence (5′-GGAA-3′) designated the Ets sequence motif [Karim et al., 1990; Koizumi et al., 1990]. In previous studies we reported that the β factor contains Ets epitopes and is immunologically related to the GA-binding protein (GABP) [Yoganathan et al., 1992a]. Molecular characterization of GABP revealed the involvement of two distinct polypeptides, GABP α and GABP β, both of which are required for avid interaction with DNA [LaMarco et al., 1991; Thompson et al., 1991]. The amino acid sequence of the GABP α subunit exhibits similarity to the Ets family of nuclear proteins. Amino acid sequence analysis of the GABP β subunit revealed four 33-amino acid repeats (ankyrin repeats) on the N-terminus which constitute surfaces for interaction with the GABP α protein [LaMarco et al., 1991; Thompson et al., 1991].

While the rpL32 gene promoter lacks a canonical TATA element, the TATA-binding protein (TBP) interacts with a region 30 base pairs upstream of the cap site without the aid of other factors [Yoganathan et al., 1992b, 1993]. A factor designated γ also interacts with a similar sequence (−36 to −17) in the rpL32 gene promoter and may play a role similar to TBP in initiating transcription [Yoganathan et al., 1993].

The δ1 and δ2 elements are located downstream of the transcription start site and contain the binding site for the δ factor [Hariharan et al., 1989; Chung and Perry, 1993]. Molecular cloning of a cDNA encoding the δ factor has revealed that it is a zinc finger protein of the

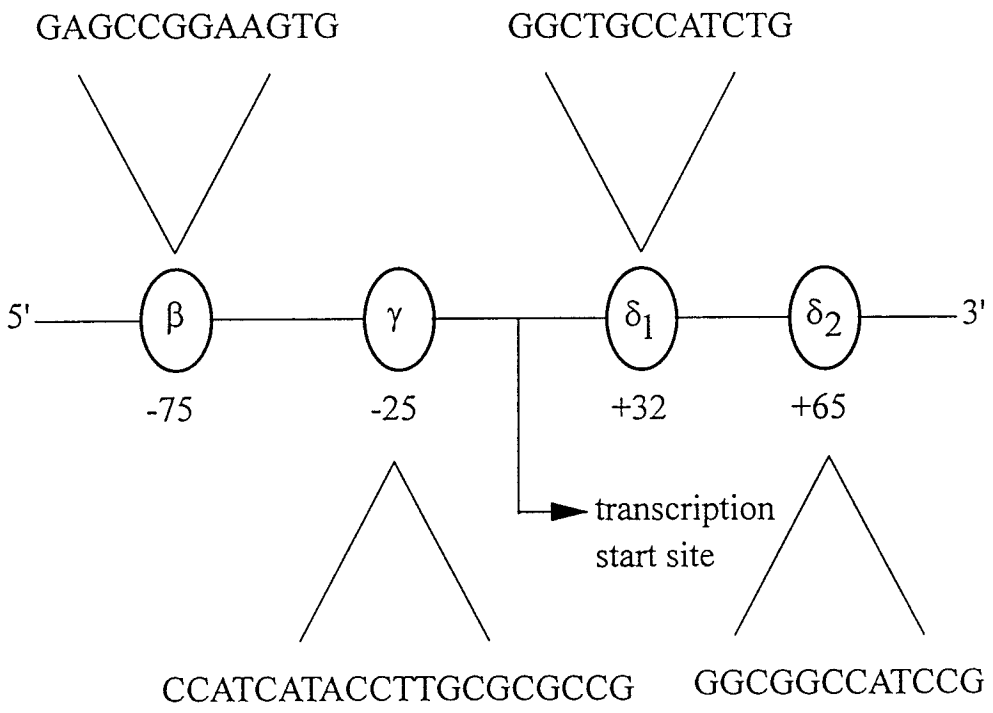

Fig. 1. RpL32 gene promoter. Schematic representation of the rpL32 gene promoter showing the locations and sequences of the cis-acting elements β, γ, δ_1, and δ_2.

Cys-Cys-His-His variety [Hariharan et al., 1991]. Transfection studies with site and spacing mutants indicated that both δ1 and δ2 work independently and contribute in an additive manner to the strength of the rpL32 promoter [Chung and Perry, 1993].

In this study we describe the mechanism by which rpL32 gene transcription is modulated during the transition between proliferating myoblasts and non-proliferating myocytes. Our results identify changes in the binding of proteins to specific sequences in the rpL32 gene promoter. Specifically, our data indicate that binding of the β factor to the rpL32 gene promoter is altered during the changes in BC_3H1 myoblasts growth rate, resulting from differentiation and dedifferentiation, and suggest that this alteration is modulated by phosphorylation. This study also reveals the relationship between the β factor, as a positive regulator of rpL32 gene transcription, and GABP α and β proteins.

MATERIALS AND METHODS

Cell Culture

Mouse BC_3H1 myoblasts [Schubert et al., 1974], from the American Type Culture Collection (Rockville, MD), were grown on 15-cm diameter tissue culture dishes in Dulbecco's modified Eagle's medium supplemented with 20% fetal bovine serum, at 37°C and 5% CO_2. Myoblasts were induced to differentiate to myocytes at 70% confluency by reducing the serum concentration to 0.5%. After 4 days in differentiation medium, reversal of the differentiation process was accomplished by increasing the serum concentration to 20%. The number of cells/cm^2 was determined by trypsinizing monolayers, collecting the cells by centrifugation (5 min at 2,000*g*) and resuspending them in 1 ml of medium. The concentration of cells was measured with a haemacytometer. Cell viability was assessed by adding an equal volume of 0.4% trypan blue to freshly trypsinized cells used for determining the cell number.

Incorporation of Radioactive Precursors Into DNA and RNA

To measure the incorporation of precursors into DNA and RNA, cells were seeded at a density of 1×10^4 cells/well in a 24 well plate. Thirty min prior to harvesting the cells at each time point, the growth medium, supplemented with 5 μCi/ml [^{3}H]-thymidine (specific activity 80.8 Ci/mM) or 5 μCi/ml [^{3}H]-uridine (specific activity 42.8 Ci/mM), was added to triplicate wells. After the labelling period, the medium was removed and cells washed with cold phosphate-buffered saline (10 mM KH_2PO_4, pH 7.2, 150 mM NaCl). Cells were lysed in buffer containing 10 mM Tris-HCl pH 7.6, 1 mM EDTA, 1% SDS, and 10 mM unlabelled thymidine or uridine. Trichloroacetic acid was added to a final concentration of 10%. Following incubation on ice for 30 min, the precipitates were collected on Whatman GF/C filter paper, dried, and the radioactivity measured.

Nuclear Transcription Assay

To isolate nuclei from proliferating myoblasts, myocytes, and dedifferentiated myoblasts, monolayers were trypsinized and cells collected by low-speed centrifugation (2,000*g*, 5 min) and washed twice with cold phosphate-buffered saline. Cells were suspended in 1 ml lysis buffer (10 mM Tris, pH 7,4, 10 mM NaCl, 3 mM $MgCl_2$, and 0.5% Nonidet P-40), vortexed for 10 s and allowed to swell on ice for 5 min. Cells were disrupted with 15 strokes of a Dounce homogenizer (B pestle). Cell lysis was monitored using phase contrast optics. Nuclei were collected following centrifugation for 5 min in a microfuge, washed once with lysis buffer, resuspended at a concentration of 5×10^6 nuclei/ml storage buffer (40% glycerol, 50 mM Tris, pH 8.0, 5 mM $MgCl_2$, 0.1 mM EDTA, and 1 mM dithiothreitol) and stored at −80°C for up to 3 months.

For the transcription reaction nuclei (5×10^6) were collected by centrifugation for 2 min in a microfuge. Pelleted nuclei were incubated with 50 μCi [α^{32}P]-UTP in buffer containing 25 mM Hepes, pH 7,9, 500 μM CTP, GTP, and ATP, 65 U/ml RNAguard (Pharmacia, Baie d'Urfe, Québec, Canada), 200 U/ml creatine phosphokinase in a total volume of 100 μl for 30 min. The labelled RNA was isolated by phenol/chloroform extraction following DNaseI and proteinase K digestions. After the final ethanol precipitation, the RNA pellet was resuspended in Tris/EDTA buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0) and the incorporated radioactivity measured. The relative incorporation of radioactive [α^{32}P]-UTP in all cell populations tested was approximately the same indicating that the overall level of RNA synthesis was comparable in the isolated nuclei. Specific RNA transcripts were detected by hybridization of the ^{32}P-labelled RNA to the appropriate DNA probe bound to nitrocellulose. Five μg of cesium

chloride-purified plasmid DNAs containing sequences coding for rpL32 mRNA and α-actin mRNA were linearized and immobilized on a nitrocellulose membrane. Equal amounts (1×10^6 cpm) of labelled nuclear RNA were used for the hybridizations, which were performed at 42°C for 48 h in 50% formamide and 5× NaCl/Cit (1× NaCl/Cit is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0), following 24 h prehybridization under similar conditions. Membranes were washed at 60°C for 2 h in 0.1× NaCl/Cit and 0.1% SDS. The radioactivity associated with each plasmid DNA was measured as described above. No radioactivity was associated with the control plasmids, pBR322, and pBluescript, demonstrating that non-specific hybridization was absent under the experimental conditions used.

RNA Isolation and Analysis

Following removal of medium, monolayers were washed twice with cold phosphate buffered saline. RNA was isolated using the guanidine isothiocyanate procedure as described by Chirgwin et al. [1979], with the exception that RNA was pelleted by centrifuging for 24 h at 150,000*g* in a Beckman SW50.1 rotor. Aliquots of RNA from equal numbers of cells were denatured at 60°C for 15 min in 50% formamide, 7% formaldehyde, and 25 mM Mops, pH 7.0, and size-fractionated by gel electrophoresis in a 1% agarose gel. RNA was transferred to Hybond-N (Amersham, Oakville, Ontario, Canada) and hybridized with radiolabelled probes using conditions described above. Alternatively, RNA samples were applied to Hybond-N nylon membrane using a Schleicher and Schuell Minifold II slot blot system. Following hybridization, the radioactivity associated with specific RNAs was visualized by autoradiography and the relative amount determined by scanning bands with a IS-1000 digital imaging system using the one-dimensional multi-lane scan analyst program. The band intensities were quantitated from four independent experiments for each plasmid DNA.

Plasmids

The following plasmids were used: plasmid p3AR2.8 [Dudov and Perry, 1984], containing a fragment of the murine rpL32 gene (kindly supplied by R.P. Perry, Institute for Cancer Research, Fox Chase, PA); plasmid pHFBA13 (ATCC 37854), containing human cDNA for eukaryotic translation factor 1α; pJN2CK-M (ATCC 57678), containing human cDNA coding for muscle creatine kinase; pUC65-2 [Wright et al., 1989], containing cDNA sequences for myogenin (generously supplied by W.E. Wright, Univ. Texas, Southwestern Medical Center, Dallas, TX); plasmid pAC269, containing chicken skeletal muscle α actin cDNA (generously supplied by Dr J. Haron, Roche Institute of Molecular Biology, Nutley, NJ); cDNA clones of GABP α and β [LaMarco et al., 1991] were kindly supplied by Thomas A. Brown, Central Research Division, Pfizer Inc. Plasmids were isolated by alkaline-SDS lysis of the bacteria and purified by cesium chloride density gradient centrifugation. For Northern and Slot blot analyses, all probes were radiolabelled by nick-translation using [α-^{32}P]dCTP (Boehringer-Mannheim, Laval, Québec, Canada).

Antibodies

Antibodies to GABP α and β proteins were generously provided by Thomas A. Brown. Antibodies were produced in rabbits by immunization with mouse GABP subunits purified after expression in *E. coli*. Bacterially expressed and purified murine GABP α and β proteins were obtained from Thomas A. Brown. Ets-2 affinity purified rabbit polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was raised against the carboxyl terminus of human Ets-2 protein.

Gel Mobility Shift Assay

Nuclear extracts were prepared according to the procedure of Dignam et al. [1983] with modifications for plate-grown cells as previously described [Zahradka et al., 1989]. In addition, the serine protease inhibitor AEBSF (4-(2-Aminoethyl)-benzenesulfonyl fluoride, ICN-Flow) was added to the initial lysis buffer at a final concentration of 0.1 mM. For some experiments, cell populations were treated with 5 µg/ml cycloheximide for up to 4 h prior to preparation of nuclear extracts.

Synthetic oligonucleotides representing the various rpL32 promoter elements: β (5′-CCCAGAGCCGGAAGTG-3′), γ (5′-AATTATCATACCTTGCGCG-3′), and δ (5′-GAGGTGGCTGCCATCTGTTT-3′) were purchased from general Synthesis & Diagnostics, Toronto. Double-stranded oligonucleotides, containing the specific binding sequence for either the β, γ, or δ elements, were prepared by annealing the two opposite strands and 5′-end labelling with [γ-^{32}P]ATP and T4 polynucleotide kinase. The ability of proteins present in nuclear extracts (5

μg) to bind to radiolabelled oligonucleotides (10^5 cpm) was measured in a buffer containing 12 mM Hepes, pH 7.9, 70 mM KCl, 0.4 mM EDTA, 0.5 mM DTT, 10% glycerol, 5 mM $MgCl_2$, and 1.5 μg poly[d(I-C)]. Binding reaction mixtures were incubated at 30°C for 30 min and the resulting protein-DNA complexes resolved by electrophoresis in 4% polyacrylamide gel and 0.5× TBE. Gels were then vacuum dried and visualized by autoradiography at −80°C with the aid of an intensifying screen. For samples containing antiserum, 1 μl of antiserum was added to the reaction mixture at the end of a 30 min incubation period for an additional 15 min at room temperature.

Phosphatase Treatment

Nuclear extracts prepared from myoblast and myocyte cell populations were treated with 400U and 1,600U of lambda protein phosphatase (New England BioLabs, Mississauga, Ontario, Canada) in a buffer provided by the manufacturer at 30°C for 20 min. Following dephosphorylation, extracts were diluted 2.5 folds with the binding buffer (described above) and incubated for an additional 30 min at 30°C in the presence of labelled oligonucleotide and poly[d(I-C)]. To ensure that phosphatase treatment had no effect on the β-oligonucleotide probe, labelled oligonucleotide was incubated in the same buffer with lambda protein phosphatase. Gel mobility shift assays were performed and protein-DNA complexes visualized as described above.

Western Blot Analysis

Nuclear extracts (4 μg) were incubated at 95°C in an equal volume of 2× SDS sample buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 10% β-mercaptoethanol, 20% glycerol) and size-fractionated on a 12.5% SDS polyacrylamide gel [Laemmli, 1970]. Following transfer to a nitrocellulose membrane [Schleicher and Schuell, Keene, NH] protein bands were detected using a horseradish peroxidase-conjugated secondary antibody and ECL detection system (Amersham, Oakville, Ontario, Canada).

UV Protein Cross-Linking

Binding reaction conditions for UV cross-linking were identical to the binding conditions used for gel mobility shift assays, except that 20 μg/ml bovine serum albumin was added to the reaction mixture. Following incubation at 30°C for 30 min, DNA-protein complexes were resolved by PAGE as described above. A UV light transilluminator was then used to irradiate the mobility shift gel for 30 min at 4°C and the binding complexes were viewed by autoradiography. The cross-linked products were recovered from the gel slices by electroelution at 200V and precipitated with 5 volumes of ice-cold acetone. Protein-DNA complexes were collected by centrifugation in Sorvall RC-5B superspeed centrifuge (10,000 rpm, 20 min), resuspended in 1× SDS sample buffer and resolved by electrophoresis as described above. To ensure equal recovery from all cell populations, gels were stained with Coomassie Blue (R-250). Cross-linked proteins were detected by assaying with GABP α and β specific antibodies as described above.

RESULTS

Differentiation and Dedifferentiation of Mouse Myoblasts

To investigate the mechanism by which ribosome production is regulated during differentiation and subsequent dedifferentiation of mouse BC_3H1 myoblasts, these processes were first defined both morphologically and biochemically. Proliferating mouse myoblasts appear as single cells with a flattened, stellate, fibroblast-like morphology. When the fetal bovine serum in the growth medium was reduced from 20 to 0.5% (differentiation medium), long spindle-shaped cells, characteristic of the muscle cell phenotype, developed after 4 days in differentiation medium. These differentiated muscle cells were defined as myocytes. When growth medium (containing 20% serum) was added to the myocyte population, the muscle cell phenotype began to change within 12 h and by 24 h reverted to that characteristic of proliferating myoblasts prior to differentiation. The population of cells in which the differentiation process had been reversed was designated as dedifferentiated myoblasts. The processes of differentiation and dedifferentiation had no affect on viability, which was determined to be >99% in all cell populations. Cell viability was measured by the ability of cells to exclude the stain trypan blue.

Synthesis of Muscle-Specific mRNAs During Differentiation and Dedifferentiation

The processes of differentiation and dedifferentiation were also monitored by the appear-

ance of muscle-specific mRNAs, to verify that the observed changes in morphology occurred in concert with changes at the molecular level. In proliferating myoblasts, muscle creatine kinase (MCK) and the myogenic regulatory protein myogenin (Mg) mRNA levels were both below the limits of detection by Northern blot analysis. MCK mRNA was first detected within 2 days following initiation of muscle cell differentiation and continued to increase during the 4 day differentiation period, at which time all the cells had assumed the myocyte phenotype (Fig. 2). Addition of growth medium to myocytes resulted in a decrease in MCK mRNA during the subsequent 36 h. Similar results were observed with Mg mRNA, first being detected within 1 day following initiation of differentiation and declining immediately following the signal to dedifferentiate (Fig. 2). In contrast, the level of elongation factor 1α mRNA (EF-1α), a translationally regulated mRNA [Loreni et al., 1993], remained constant during both the differentiation and dedifferentiation processes (Fig. 2).

Differentiation and dedifferentiation of BC_3H1 cells were also monitored by determining the number of cells in each population that contained myosin heavy chain protein. Immunochemical staining of myocyte monolayers using MF-20 monoclonal antibody revealed that >95% of individual myocytes exhibited a positive reaction. In contrast, proliferating myoblasts and dedifferentiated myoblasts (after 48 h in growth medium) displayed no staining (data not shown).

Rate of Cell Growth is Modulated by Differentiation and Dedifferentiation

To characterize the effect of differentiation and dedifferentiation on cell growth, a number of parameters were measured. To examine the effect of these two processes on the rate of cell division, cell number/cm^2 was measured for both myoblasts and myocytes. The number of cells remained relatively constant (8×10^6), varying by less than 15%, during the differentiation period (data not shown). Addition of growth medium to the myocyte population increased the number of cells/cm^2 by 40% and 75% after 24 h and 36 h, respectively. This increase in cell number, and thus growth rate of the dedifferentiated myoblasts, was similar to that observed in proliferating myoblasts prior to differentiation.

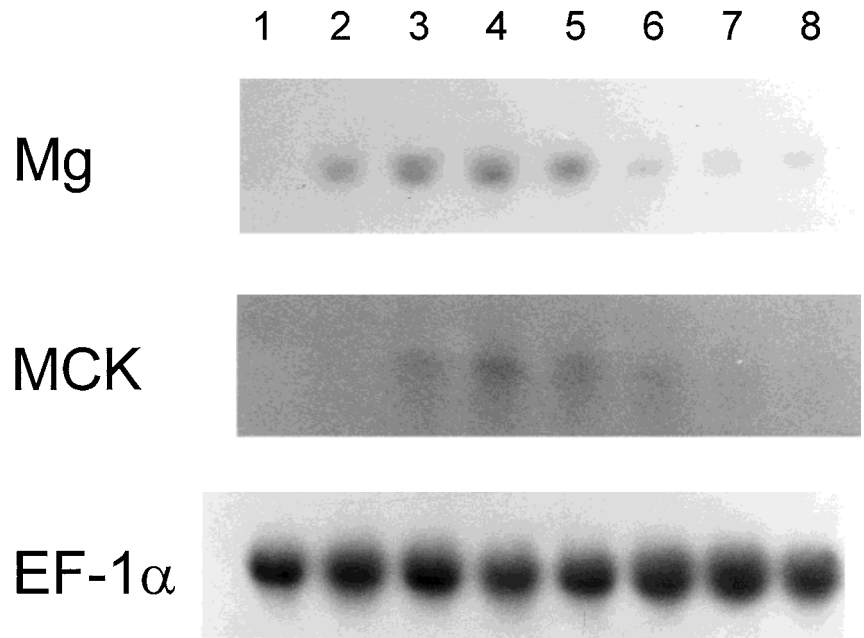

Fig. 2. Northern blot analysis of muscle-specific mRNA levels during differentiation and dedifferentiation. Aliquots of RNA from an equal number of cells were size fractionated as described in Materials and Methods and hybridized with radiolabelled DNA sequences coding for myogenin (Mg), muscle creatine kinase (MCK), and elongation factor 1α (EF-1α). The lanes represent RNA isolated from: **1:** proliferating myoblasts; **2–5:** differentiated myocytes for 1, 2, 3, and 4 days; **6–8:** myocytes induced to dedifferentiate for 6, 12, and 24 h.

The rate of DNA synthesis, another indicator of cell growth, was measured in proliferating myoblasts, myocytes, and dedifferentiated myoblasts. The incorporation of [^{3}H]-thymidine was assayed by adding [^{3}H]-thymidine to the culture medium 30 min prior to harvesting cells. The amount of [^{3}H]-thymidine incorporated into trichloroacetic acid precipitable material was measured and expressed relative to myoblasts which was set at 100% (Table I). The results show that the incorporation of [^{3}H]-thymidine decreased by 85% in 4 days myocytes. Following dedifferentiation of myocytes, incorporation of radiolabelled thymidine did not increase until 12 h after the addition of serum, returning to levels observed in proliferating myoblast populations by 24 h. Thus, the observed changes in rate of DNA synthesis in myocytes and dedifferentiated myoblasts reflected modulation of cell division and closely paralleled changes in cell number.

The effect of differentiation and dedifferentiation on RNA synthesis was measured in proliferating myoblasts, myocytes, and dedifferentiated myoblasts as described above for DNA synthesis except that the radioactive precursor [^{3}H]-uridine was used. The amount of [^{3}H]-uridine incorporated into RNA decreased in 4 days myocytes by more than 80%, compared to control myoblasts. Depression of RNA synthesis in myocytes was reversible since the incorporation of [^{3}H]-uridine also returned to control levels in populations of myocytes which were stimulated to dedifferentiate (Table 1). The rapid increase in RNA synthesis within 6 h may be indicative of an early stimulation of rRNA synthesis.

TABLE I. Relative Rates of Incorporation of Radioactive Precursors Into DNA and RNA During Differentiation and Dedifferentiation in BC3H1 Cells*

	Relative incorporation of radioactive precursor, %	
Cell population	[^{3}H]-thymidine	[^{3}H]-uridine
Myoblasts	100	100
Myocytes, 2 days	19.8 ± 4.0	71.0 ± 6.3
Myocytes, 4 days	12.9 ± 1.9	19.2 ± 2.6
Dedifferentiated, 6 h	3.9 ± 1.3	36.5 ± 1.2
Dedifferentiated, 12 h	57.4 ± 5.6	86.6 ± 2.6
Dedifferentiated, 24 h	95.6 ± 6.4	122.9 ± 18.1
Dedifferentiated, 36 h	103.6 ± 4.3	132.2 ± 7.1

*Thirty min prior to harvesting various cell populations, the medium was supplemented with 5 μCi/ml [^{3}H]-uridine or [^{3}H]-thymidine. The amount of radioactivity incorporated into trichloroacetic acid-precipitable material was measured and expressed relative to control myoblasts which was set at 100%. Growth medium was added to 4 days myocytes for up to 36 h, designated dedifferentiated in table. Each data point represents the average ± SE of three independent experiments.

Differentiation and Dedifferentiation Modulate rpL32 Gene Transcription

To establish whether the rate of rpL32 gene transcription changed during the differentiation and dedifferentiation processes the nuclei were isolated from proliferating myoblasts, myocytes, and from dedifferentiated myoblasts. A decrease of 67% in the rate of rpL32 mRNA synthesis was noted in nuclei isolated from myocyte, compared to myoblast cell populations (Fig. 3, Table II). Addition of growth medium to myocytes, on the other hand stimulated rpL32 gene transcription. Within 6 h a significant increase occurred in the rate of transcription (Fig. 3). This enhanced rate increased further such that by 12 h of growth stimulation the rate observed was similar to that observed in proliferating myoblasts and was maintained for 24 h. The processes of differentiation and dedifferentiation did not affect the rate of α-actin mRNA synthesis (Fig. 3, Table II) which was used as a control [Larson et al., 1991]. Additionally, no radioactivity was associated with the control plasmids, pBR322, and pBluescript, demonstrating that non-specific hybridization was absent under experimental conditions used.

To determine whether the changes in the rate of rpL32 gene transcription during differentiation and dedifferentiation reflected parallel alterations in rpL32 mRNA levels, total cellular RNA was isolated and the relative amount of rpL32 mRNA quantitated by slot blot analysis (Fig. 4). Myocyte formation resulted in a 70% decrease in the rpL32 mRNA level, which returned to that found in proliferating myoblasts within 12 h of dedifferentiation. Using EF-1α mRNA as a control, it was noted that the level of this mRNA was constant in myoblasts, myocytes, and dedifferentiated myoblasts (Fig. 4).

Binding Activity of Trans-Acting Factors Involved in rpL32 Gene Transcription

The rate of gene transcription is controlled by trans-acting factors binding to sequence-specific elements in the gene promoter. In the rpL32 gene promoter, cis-acting elements, β, γ, δ_1, and δ_2 have been identified to which factors, desig-

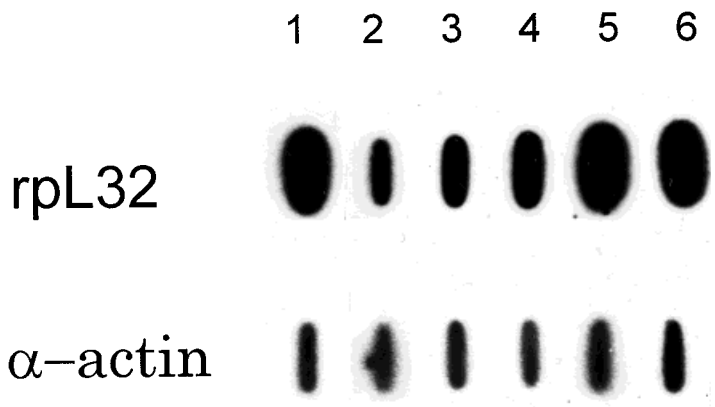

Fig. 3. RpL32 gene transcription in nuclei isolated from proliferating myoblasts, myocytes, and dedifferentiated myoblasts. Transcriptional activities were measured using nuclear run-on assays as described in Materials and Methods. The radioactivity associated with rpL32 and α-actin gene sequences were detected by autoradiography. The lanes represent nuclei isolated from: **1:** myoblasts; **2:** myocytes; **3–6:** myocytes induced to dedifferentiate for 3, 6, 12, and 24 h respectively.

TABLE II. Relative Rates of Gene Transcription During Differentiation and Dedifferentiation in BC3H1 Cells*

	Relative rate of gene transcription by isolated nuclei, %	
Cell population	rpL32	α-actin
Myoblasts	100	100
Myocytes, 4 days	33 ± 7	93 ± 8
Dedifferentiated, 3 h	49 ± 10	96 ± 7
Dedifferentiated, 6 h	53 ± 13	97 ± 9
Dedifferentiated, 12 h	104 ± 10	99 ± 7
Dedifferentiated, 24 h	105 ± 14	101 ± 6

*The levels of gene transcription were measured by nuclear run-on assays, as described in Materials and Methods. Nuclei were isolated from proliferating myoblasts, and differentiated myocytes for four days. Growth medium was added to 4 d myocytes for up to 24 h, designated dedifferentiated in table. Each data point represents the average ± SE of 3 independent experiments.

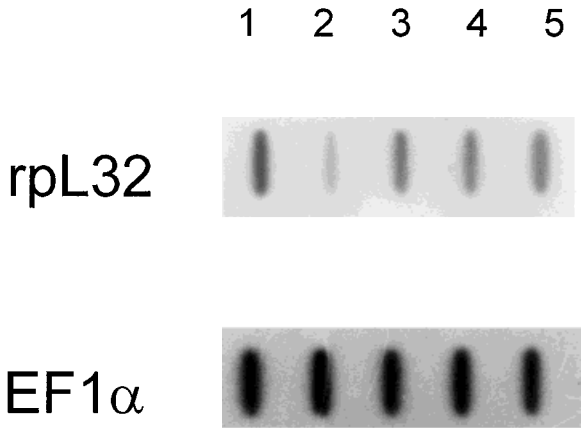

Fig. 4. Slot blot analysis of changes in specific RNA levels during differentiation and dedifferentiation. Aliquots of total cellular RNA (5 μg) were applied to nitrocellulose and hybridized with [^{32}P]-radiolabelled DNA containing sequences coding for rpL32 or EF-1α mRNA. The lanes represent RNA isolated from **1:** proliferating myoblasts; **2:** myocytes; and **3–5:** myocytes induced to dedifferentiate for 6, 12, and 24 h.

nated β, γ, and δ, respectively, bind (Fig. 1). To compare the binding activities of proteins associated with each of these elements, nuclear extracts were prepared from myoblasts, myocytes, and dedifferentiated myoblasts. Whether the binding of trans-acting factors required for rpL32 gene transcription changes in response to differentiation and dedifferentiation was determined by gel mobility shift assays. Radiolabelled oligonucleotides, representing the β, γ, or δ element, were incubated with nuclear extracts prepared from various BC_3H1 cell populations. Competition experiments with a 50-fold molar excess of unlabelled oligonucleotides to each element indicated that the observed shifts were specific for each element [Yoganathan and Sells, 1991, 1992c].

The binding of proteins to the β element was significantly reduced (to barely detectable levels) in nuclear extracts prepared from myocytes, as compared to myoblasts (Fig. 5). Following addition of growth medium to myocyte populations, the binding of protein to the β element increased within 30 min. In contrast, following the signal to differentiate smaller changes (~20%) were observed in the factor binding to the γ element, while no alteration was detected in binding of proteins to the δ element (Fig. 5), suggesting that factors bind-

ing to the δ element were present at similar levels in all cell populations tested. These results indicate that of the various established factors, only the β factor binding activity is significantly modulated during the down-shift in the rate of rpL32 gene transcription following BC_3H1 myoblast differentiation.

To determine whether new protein synthesis was required for increased binding of the β factor in response to the signal to dedifferentiate, myocytes were stimulated to dedifferentiate in the presence of cycloheximide (to inhibit de novo protein synthesis). The binding activity of factors present in the cycloheximide-treated nuclear extracts was then measured by gel mobility shift assays. Treatment of myocytes with 20% serum and cycloheximide for up to 4 h failed to inhibit the increase in β factor binding activity observed with serum alone (Fig. 6). These results indicate that the enhanced β factor binding does not require de novo protein synthesis but rather implies modification of a preexisting "inactive" factor in myocytes. Cycloheximide treatment did not affect the binding activity of either the γ and δ factors (data not shown).

Since changes in the level of the β factor failed to explain the increased binding to the β element observed during conversion of myocytes to dedifferentiated myoblasts, experiments were designed to assess whether phosphorylation/dephosphorylation was involved in modifying the binding activity of the β factor. Nuclear extracts were prepared from both myoblast and myocyte cell populations and treated with lambda protein phosphatase to release phosphate groups from serine, threonine or tyrosine residues in proteins [Zhuo et al., 1993]. Dephosphorylation of either myoblast or myocyte nuclear extracts with lambda protein phosphatase resulted in increased binding of the β factor to its corresponding element (Fig. 7). Two different concentrations of enzyme were used in an attempt to maximize the level of dephosphorylation and increase the level of binding. However, the level of binding failed to reach that observed in control myoblast extracts, perhaps due to the non-specific nature of phosphatase used. Although, at present it is unclear whether the β factor is a phosphoprotein, the data indicate that phosphorylation modulates interaction of the β factor with the β element and suggest that changes in this interaction leads to altered expression of the rpL32 gene.

GABP Antibodies Recognize the rpL32 β Factor

The observation that the rpL32 gene promoter contains an Ets binding element suggests that a member of the Ets family of oncoproteins may function to regulate rpL32 transcription. Previous studies have suggested that the heterodimer GABP, which contains an Ets related subunit, may be the rpL32 β factor [Yoganathan et al., 1992a; Genuario et al., 1993]. Experiments were designed, therefore, to determine whether the rpL32 β factor, present in the BC_3H1 nuclear extracts and which binds to the β element, is related to GABP α and β proteins. Thus, various BC_3H1 nuclear extracts were treated with antibodies specific to both GABP subunits and DNA-protein-antibody complexes were identified by mobility shift assays. If rabbit polyclonal antiserum to GABP recognizes proteins binding to the β element in the rpL32 gene promoter, a supershift would be observed.

The presence of the rabbit antiserum specific to GABP α protein in the binding reaction resulted in a supershift (Fig. 8, lane 3). This observation suggests that the protein(s) binding to the β element within the promoter of rpL32 gene contains the GABP α subunit. A supershift was also observed with antibodies raised against the GABP β protein, and in the reaction mixture where antibodies to both proteins were present (Fig. 8, lanes 4, 5). These results, again, indicate that GABP α and β proteins specifically interact with the β binding element of the rpL32 gene promoter. Control experiments in which rabbit pre-immune serum or antibody specific to Ets-2 protein was added to the binding reaction, failed to produce a supershift, suggesting that the antibodies to GABP specifically interacted with the GABP α and β proteins bound to the β element, but not with the region of the protein containing the highly conserved Ets binding domain (Fig. 8, lanes 6, 7). No supershift was observed in reactions where GABP antibodies were mixed with radioactive probe in the absence of nuclear extract, confirming the specificity of rabbit polyclonal antibodies for the β-site binding proteins (Fig. 8, lanes 8, 9). Similarly, pre-immune serum and Ets-2 antibodies by themselves did not react with the radioactive probe (Fig. 8, lanes 10, 11). A supershift was also observed when nuclear extracts prepared from BC_3H1 myocytes and dedifferentiated myoblasts were incubated with GABP antibodies in the presence of

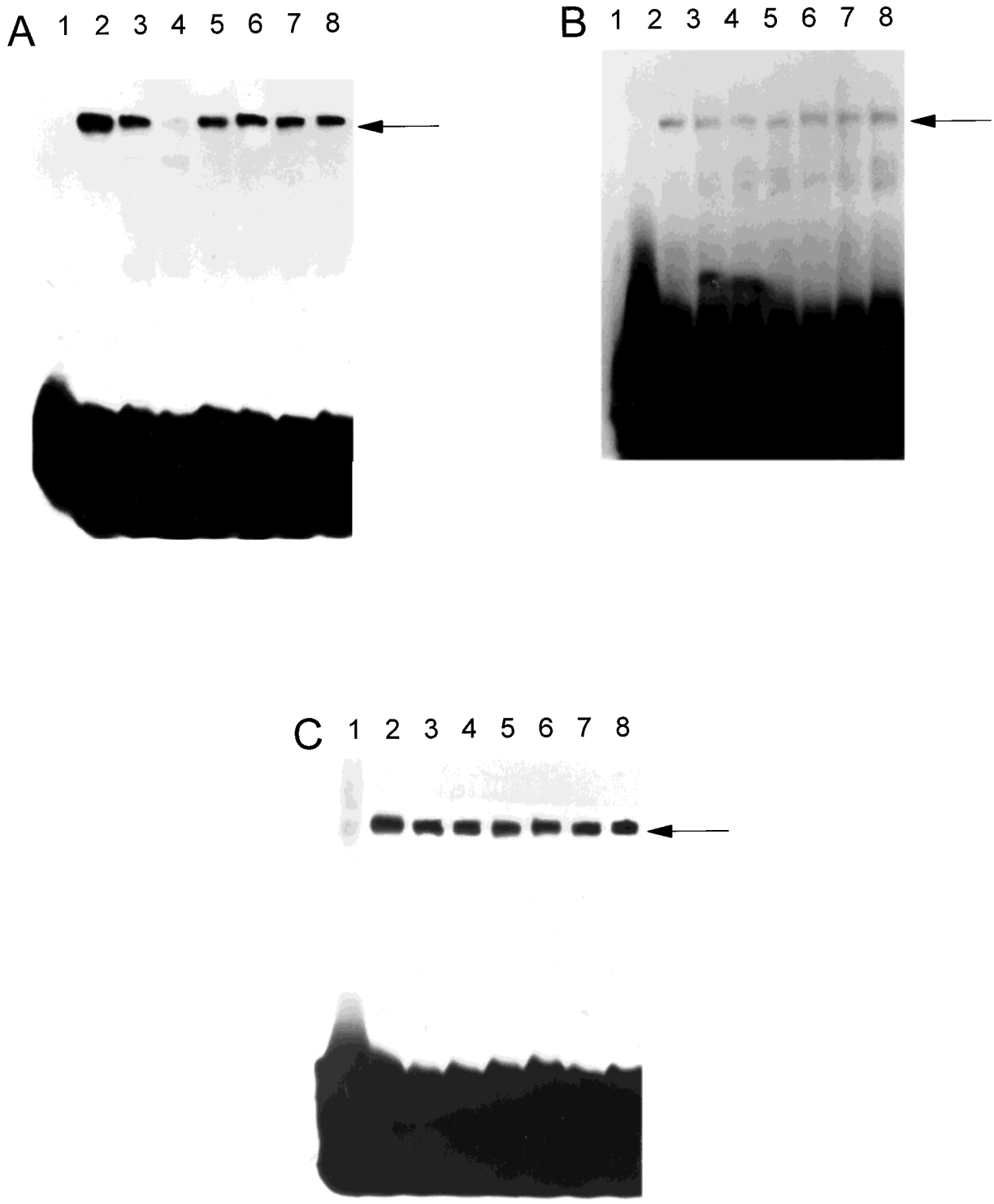

Fig. 5. Gel mobility shift analyses of the binding activity of proteins to the β, γ, and δ elements in the rpL32 gene promoter. Synthetic double-stranded ^{32}P-labelled oligonucleotides containing the β (**A**), γ (**B**), or δ (**C**) elements were incubated with equal amounts of nuclear extract prepared from different cell populations. The lanes represent: **1:** unbound probe; **2:** proliferating myoblasts; **3–4:** myocytes differentiated for 2 and 4 days; **5–8:** myocytes induced to dedifferentiate for 1, 4, 6, 12 h.

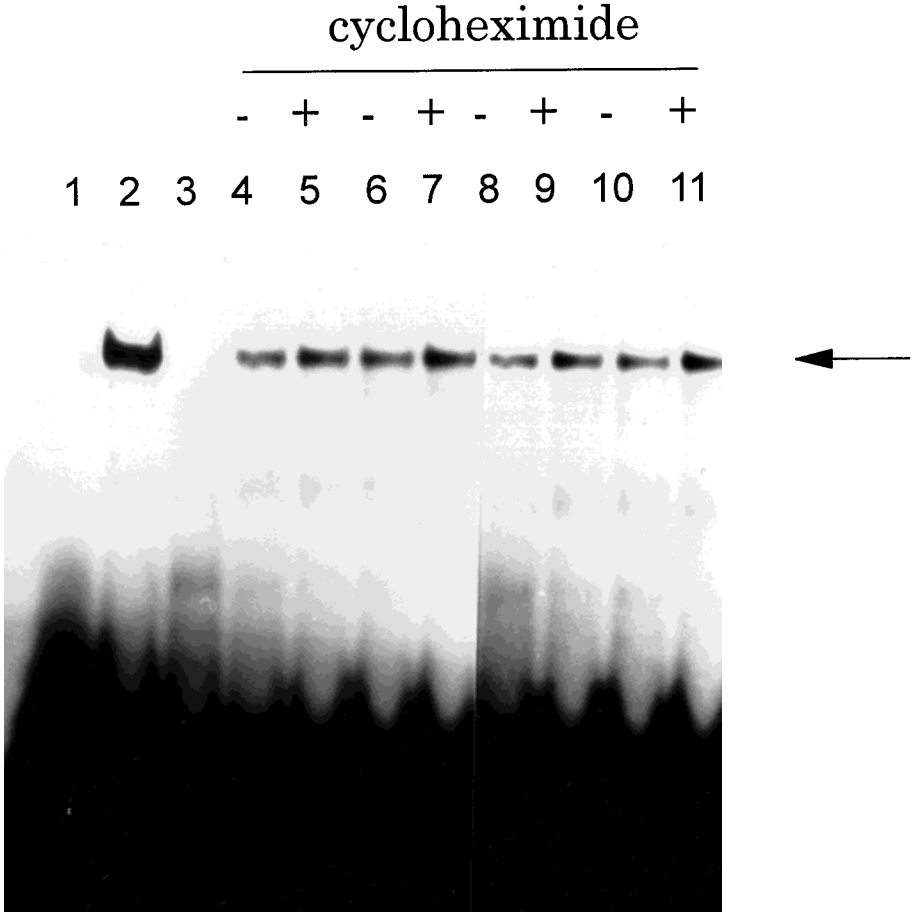

Fig. 6. Gel mobility shift analysis of the proteins binding to the β element in the presence of 5 μg/ml cycloheximide. The synthetic double-stranded [^{32}P]-labelled oligonucleotide containing the β element was incubated with equal amounts of nuclear extract prepared from myocyte populations dedifferentiated in the presence of cycloheximide. The lanes are: **1:** unbound probe; **2:** proliferating myoblasts; **3:** myocytes differentiated for 4 days, **4, 6, 8, 10:** myocytes induced to dedifferentiate for ½, 1, 2, and 4 h, **5, 7, 9, 11:** myocytes induced to dedifferentiate for ½, 1, 2, and 4 h with cycloheximide.

radiolabelled β element (data not shown). However, the level of supershift obtained with myocyte extracts was lower than that observed with myoblast extracts, supporting the previous observation that the binding activity of the β factor in myocyte extracts is reduced. Thus, these results indicate that following differentiation-dedifferentiation processes similar proteins bind to the β element within the rpL32 gene promoter and they are immunologically related to the GABP proteins.

GABP Binds to the rpL32 Gene Promoter

The following experiments were designed to establish whether recombinant GABP α and β proteins recognize and bind to the β element of the rpL32 gene promoter, and whether nuclear extracts prepared from exponentially growing BC_3H1 myoblasts contains similar DNA binding proteins. To evaluate the binding properties of purified GABP α and β proteins and those present in nuclear extracts, mobility shift assays were used. The recombinant GABP α protein recognized the Ets-motif within the β element binding site (Fig. 9, lane 3). However, this DNA-protein complex migrated faster on a nondenaturing acrylamide gel compared to the DNA-protein complex obtained when myoblast nuclear extract was incubated with the radioactive β element (Fig. 9, lane 2). The GABP β protein, on the other hand, failed to bind to the radioactive probe, a result which is in agreement with previous observations that the GABP β does not by itself bind to the DNA element containing the -GGAA- sequence [Thompson et al., 1991; Brown and McKnight, 1992]. When both GABP α and β proteins were present in the reaction mixture with the radiolabelled β

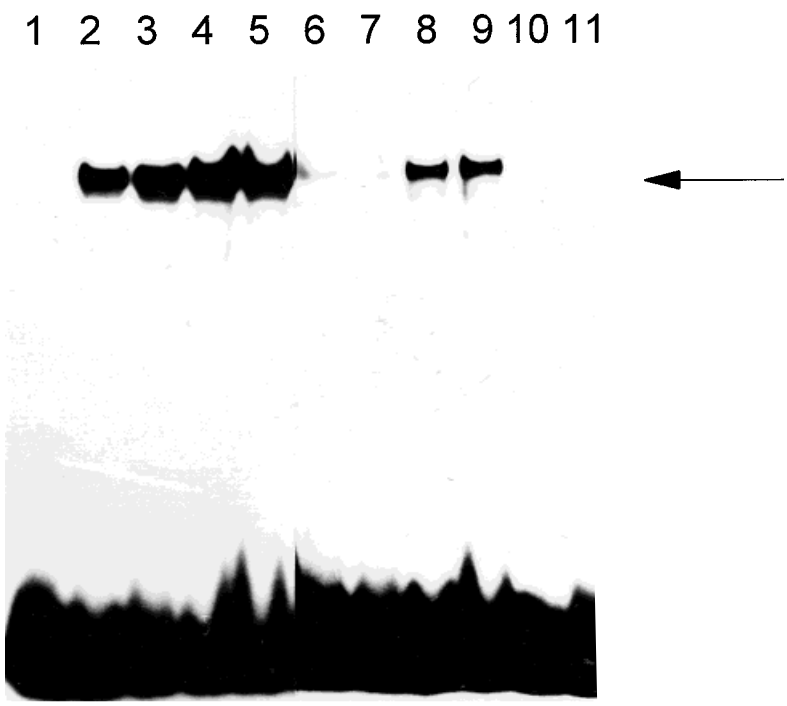

Fig. 7. Gel mobility shift analysis of the proteins binding to the β element following phosphatase treatment. The synthetic double-stranded [^{32}P]-labelled β element was incubated with equal amounts of nuclear extract prepared from different BC_3H1 cell populations, treated with 400 U and 1600 U of lambda protein phosphatase. The lanes represent: **1:** unbound probe; **2:** myoblasts; **3:** myoblasts incubated with buffer alone; **4, 5:** myoblasts incubated with 400 U and 1,600 U of lambda protein phosphatase; **6:** myocytes; **7:** myocytes incubated with buffer alone; **8, 9:** myocytes incubated with 400 U and 1,600 U of lambda protein phosphatase; **10, 11:** β element incubated alone with the 400 U and 1,600 U of lambda protein phosphatase.

element, a DNA-protein complex was observed migrating at a rate similar to that observed with myoblast nuclear extracts (Fig. 9, lane 5). These results suggest that the Ets motif of the β element in the promoter region of the rpL32 gene binds the GABP α and β proteins as a heterodimer and that the BC_3H1 nuclear extract contains similar binding protein(s). In addition, recombinant GABP proteins obtained following expression in *E. coli* are always unphosphorylated. The observation that recombinant GABP proteins recognize and bind to the rpL32 β element is in agreement with previous findings (Fig. 7) that the non-phosphorylated form of the β factor is the one which binds to the rpL32 gene promoter.

β Factor Comprises Both GABP Proteins

To assess the macromolecular composition of the β factor which binds to the rpL32 gene promoter and to further evaluate the similarity between the GABP proteins and the rpL32 β factor, UV protein cross-linking studies were employed. A radiolabelled oligonucleotide containing the β element was incubated with equal amounts of nuclear extracts prepared from various BC_3H1 cell populations and then resulting protein-DNA complexes were resolved by electrophoresis under conditions used for the mobility shift assay. Following radiation by UV light, the gel-shifted band was excised and subjected to SDS polyacrylamide gel electrophoresis to identify the components in the cross-linked complexes. The presence of GABP α and GABP β proteins within these DNA-protein complexes were determined by Western blot analysis using GABP α and β specific antibodies.

The UV cross-linking studies revealed that the rpL32 β factor is composed of both GABP α and β proteins (Fig. 10). Both exponentially growing myoblast and dedifferentiated myoblasts revealed the presence of GABP proteins associated with the β element, while this interactions was not observed with myocyte extracts (Fig. 10). These results are consistent with previous observations that following differentiation BC_3H1 myoblasts, binding of the β factor to the rpL32 β element is significantly reduced.

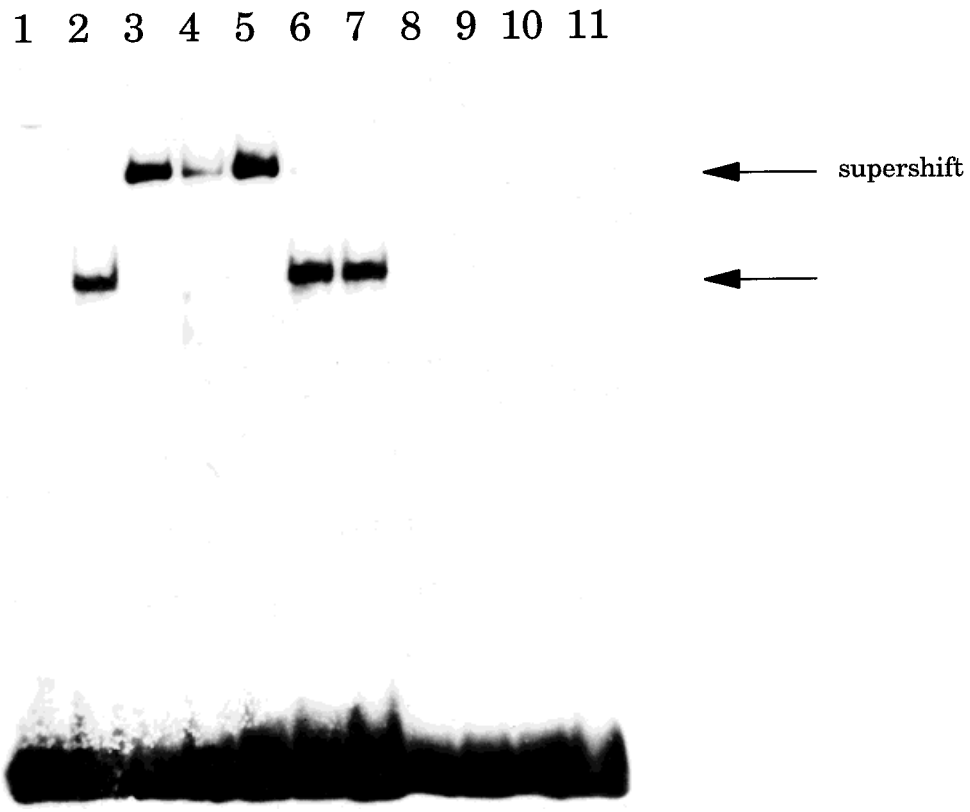

Fig. 8. Gel mobility shift analysis of the proteins binding to the β element in the presence of GABP α and β antibodies. The synthetic double-stranded [^{32}P]-labelled β oligonucleotide was incubated with nuclear extracts prepared from myoblasts in the presence of specific GABP antibodies and analyzed as described in Materials and Methods. The lanes represent: **1:** unbound probe; **2:** extract alone; **3–4:** extracts incubated with GABP α and GABP β antibodies respectively; **5:** extracts incubated with antibodies raised against both GABP subunits; **6, 7:** control experiments with pre-immune serum and Ets-2 antibody; **8–11:** β oligonucleotide probe incubated with GABP α, GABP β antibody, pre-immune serum and Ets-2 antibody respectively.

Thus, GABP proteins can not be detected in myocyte cell populations by their ability to interact with the β element.

Cellular Levels of GABP Proteins Are Not Affected by Growth Perturbations

Cellular growth perturbations as observed during BC_3H1 myoblasts differentiation to myocytes and their subsequent dedifferentiation to proliferating myoblasts result in alteration in both the rate of rpL32 gene transcription and its mRNA levels. Previous results indicated that the GABP α and β proteins are involved in regulating rpL32 gene transcription, thus, it is important to establish whether different growth conditions of BC_3H1 myoblasts affect the total cellular level of these proteins. The specific levels of GABP α and β proteins were determined by Western blot analysis.

Nuclear extracts prepared from exponentially growing myoblasts, myocytes, and dedifferentiated myoblasts were subjected to SDS polyacrylamide gel electrophoresis. Following the transfer to a nitrocellulose membrane, blots were incubated with rabbit polyclonal antibodies specific for GABP proteins. GABP β protein migrated as a single 37 kD protein and its level did not change following the signal to differentiate or when dedifferentiation occurred (Fig. 11). The GABP α subunit was detected as a 60 kD protein and, similarly, myoblast differentiation and subsequent dedifferentiation failed to affect its level. These results demonstrated that GABP proteins are abundant and that their level is not altered by the proliferative state of BC_3H1 cells. It should be noted that the purified GABP α and β proteins migrated at the same rate on SDS-polyacrylamide gel as the

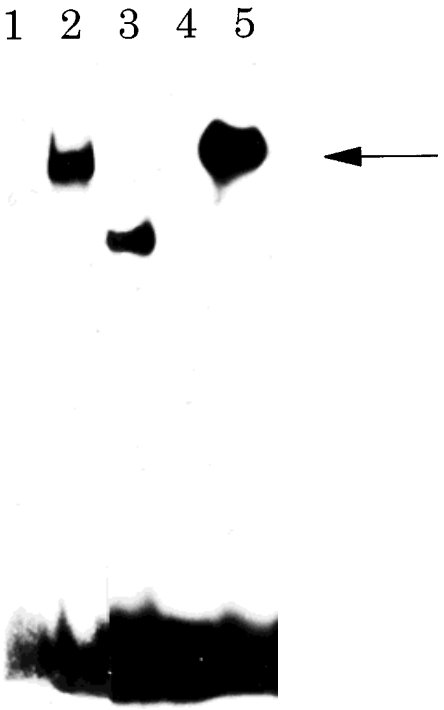

Fig. 9. Demonstration that GABP binds to the rpL32 gene promoter. Recombinant proteins GABP α and/or GABP β were incubated with radiolabelled β oligonucleotide under conditions described for the mobility shift assay. The lanes represent: **1:** unbound probe; **2:** myoblast nuclear extracts; **3:** GABP α protein; **4:** GABP β protein; **5:** GABP α and β proteins.

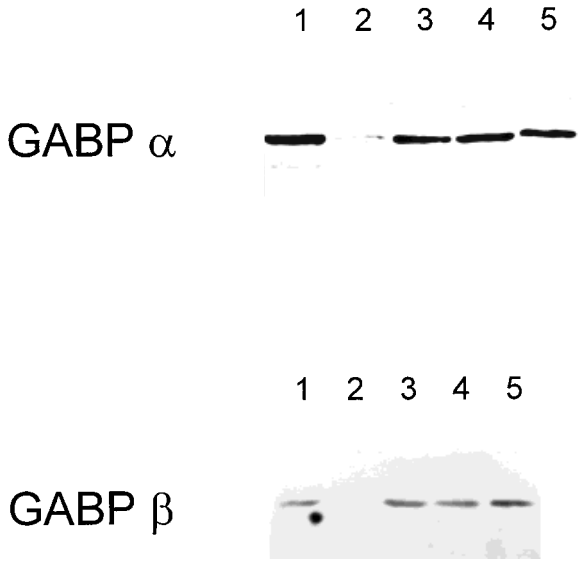

Fig. 10. UV-protein cross-linking and immunological analysis of proteins binding to the β element. The synthetic double-stranded [^{32}P]-labelled β oligonucleotide was incubated with equal amounts of nuclear extract prepared from various cell populations. Following electrophoresis the mobility shift gel was UV irradiated, and GABP α and β proteins were analyzed by Western blotting as described in Materials and Methods. The lanes represent: **1:** proliferating myoblasts; **2:** myocytes differentiated for 4 days; **3–5:** myocytes induced to dedifferentiate for 6, 12, and 24 h.

proteins in the nuclear extracts, confirming that polyclonal GABP antibodies specifically reacted with only GABP α and GABP β proteins (Fig. 11).

Cellular GABP mRNA Levels Are Not Affected by Differentiation and Dedifferentiation

To establish whether GABP mRNA levels change during the differentiation-dedifferentiation processes, total cellular RNA was isolated from different BC_3H1 cell populations and subjected to Slot blot analysis (Fig. 12). Myocyte formation resulted in no alteration in the level of GABP α and β mRNAs. Both the myoblast and myocyte cell populations contained the same level of these mRNAs (Fig. 12). Similarly, the dedifferentiation process produced no change in the level of GABP α and β mRNAs (Fig. 12) indicating that total cellular levels of these mRNAs are unaffected by different growth conditions of BC_3H1 myoblasts. It should be noted that, to the best of our knowledge, this is the first examination of the total cellular levels of GABP α and β mRNAs in a system exhibiting alteration in growth rates.

DISCUSSION

Muscle cell differentiation is known to involve an ordered sequence of molecular events in which proliferating, undifferentiated, myoblasts exit the cell cycle, synthesize an array of muscle-specific proteins, and cease dividing. The BC_3H1 cells used in these studies—unlike those of many other muscle cell lines—fail to fuse during the differentiation process. Furthermore, the BC_3H1 cell line has the ability to undergo reversible differentiation following exposure of myocytes to high levels of serum. With the transition of myocytes to myoblasts, synthesis of muscle-specific proteins is arrested and cells re-enter the cell cycle.

The failure of BC_3H1 myoblasts to fuse or commit to terminal differentiation during formation of myocytes is due to the lack of MyoD1 gene expression in these cells [Brennan et al., 1990]. Differentiated BC_3H1 cells express myogenin to a high level [Miller, 1990; Brunetti and Goldfine, 1990], and concurrently synthesize a wide variety of muscle-specific proteins [Strauch and Rubenstein, 1984; Glaser and Wice, 1989; Taubman et al., 1989]. Our results demonstrate that exponentially growing, sub-confluent myo-

Fig. 11. Western blot analysis of GABP α and β levels during differentiation and dedifferentiation. Cell extracts were prepared from various BC_3H1 cell populations and resolved by SDS-PAGE. GABP α **(A)** and GABP β **(B)** protein levels were determined immunochemically using GABP α and β specific antibodies as described in Materials and Methods. The lanes represent: **1:** proliferating myoblasts; **2:** myocytes differentiated for 4 days; **3–6:** myocytes induced to dedifferentiate for 6, 12, and 24 h, 6-GABP α **(A)** or GABP β **(B)** protein.

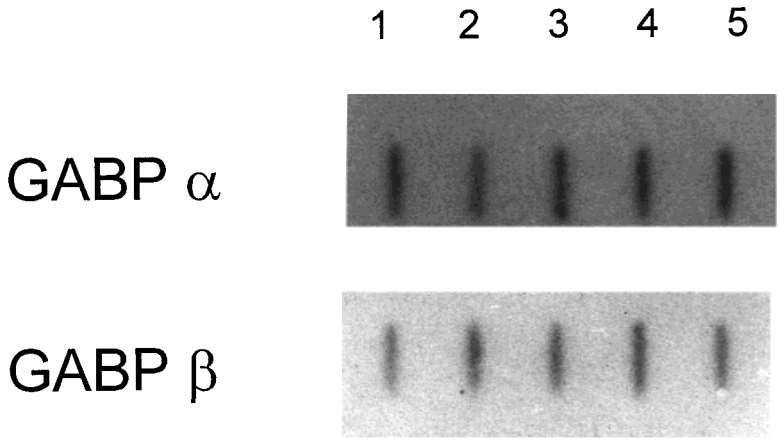

Fig. 12. Slot blot analysis of GABP α and β mRNA levels during differentiation and dedifferentiation. Aliquots of RNA from an equal number of cells were hybridized with radiolabelled DNA sequences coding for GABP α and GABP β. The lanes represent RNA extracted from: **1:** proliferating myoblasts; **2:** myocytes; **3–5:** myocytes induced to dedifferentiate for 6, 12, and 24 h.

blasts do not express the muscle specific regulatory factor, Mg, and MCK mRNAs, as previously documented [Miller, 1990]. Induction of their synthesis is closely coupled to the cessation of cell division found in differentiated myocytes. Following the signal to differentiate Mg and MCK mRNAs can be detected within 24 and 48 h respectively. On the other hand, myocytes exposed to high levels of serum re-initiate the growth process and cease synthesizing both Mg and MCK mRNAs. The gradual drop in Mg and MCK mRNA levels in dedifferentiated myoblasts reflects the half-life for these mRNAs which is ~10 h [Spizz et al., 1986; Brunetti and

Goldfine, 1990]. The presence of these mRNAs in 36 h dedifferentiated myoblasts is due to the confluent state of the cell monolayer, a phenomenon previously reported [Munson et al., 1982; Taubman et al., 1989].

This study employs for the first time the mouse BC_3H1 myoblast cell line to identify mechanisms which control expression of the genes required for ribosome biogenesis. One of the first events to occur following modulation of cell growth is altered synthesis of ribosome precursors. From the data presented here, the downshift in growth rate, as observed during myoblast differentiation, resulted in a decline in ribosome production. The rate of RNA synthesis decreased more than 80% in myocytes, but following the signal to dedifferentiate it returned quickly to levels observed in myoblasts.

Regulation of r-protein synthesis in eukaryotes involves diverse mechanisms at the level of transcription, splicing, mRNA translation and post-translational modification [Larson et al., 1991; Kaspar et al., 1993]. Often, multiple levels of regulation can occur in the same cell type. Although the r-protein genes are widely dispersed in the genome, their expression is coordinated in all tissues to satisfy the requirements for cell growth and proliferation. Any excess in production of ribosomal proteins is rapidly degraded, resulting in their equimolar levels in the cell. Coordination is achieved, at least in part, by similar promoter strengths of the murine rpL32, L30, and S16 genes [Hariharan et al., 1989].

An examination of the expression of the gene for rpL32 indicates that 4 days following initiation of differentiation, when BC_3H1 myoblasts are totally converted to myocytes, the rate of its transcription has dropped by ~70%, as measured by nuclear run-on assays. Transcriptional control of ribosomal protein gene expression, as observed during differentiation of BC_3H1 myoblasts, is only infrequently observed in eukaryotes [Tushinski and Warner, 1982; Agrawal and Bowman, 1987; Bowman, 1987b], while either protein turnover and/or translational control appear to be the favoured mechanisms for regulating the cellular levels of ribosomal proteins [Agrawal and Bowman, 1987; Amaldi et al., 1989; Kaspar et al., 1990, 1992; Amaldi and Pierandrei-Amaldi, 1990; Aloni et al., 1992].

Modulation of rpL32 gene expression has been examined in many other mammalian systems exhibiting a change in cellular growth rate, however, regulation of its synthesis has been observed at different levels. Translational control of the rpL32 mRNA has been reported in serum stimulated 3T3 cells [Thomas and Thomas, 1986], mitogen-activated T-lymphocytes, and 3T3 fibroblasts [Kaspar et al., 1990, 1992; Levy et al., 1991], during *Xenopus laevis* development [Amaldi et al., 1989; Amaldi and Pierandrei-Amaldi, 1990], and in regenerating rat liver [Aloni et al., 1992]. An examination of rpL32 gene expression in two different muscle cell lines, rat L6 and mouse MM14, which both terminally differentiate and fuse into myotubes, revealed dissimilar modes of regulation. Although a downshift in the rate of ribosome production occurs and transcription of the rRNA gene is suppressed in both systems following myoblasts differentiation, balanced production of the ribosomal proteins is achieved by different mechanisms [Krauter et al., 1979; Jacobs et al., 1985; Agrawal and Bowman, 1987]. Decreased synthesis of rpL32 in mouse MM14 myotubes is controlled by a decrease in the steady state level and translational efficiency of rpL32 mRNA [Agrawal and Bowman, 1987; Harris et al., 1992]. In contrast, differentiation of rat L6 myoblasts to multinucleated myotubes display no change in the rate of transcription of the rpL32 gene [Jacobs et al., 1985]. Reduced accumulation of rpL32 in this system is achieved by rapid turnover of the newly synthesized protein [Krauter et al., 1979; Jacobs et al., 1985]. Why one strategy is chosen over another in a particular cell line or under a given set of conditions is currently unclear.

A study of the factors binding to the β, γ, and δ elements in the rpL32 gene promoter revealed that following BC_3H1 myoblast differentiation the greatest change occurred in protein(s) binding to the β element, while only slight changes were observed in protein(s) binding to the γ and δ elements. After 2 days of differentiation a drop of ~50% was noted in the binding of the factor(s) to the β element, although by this time complete differentiation had not yet occurred with a mixed population of phenotypes still evident. However, 4 days after induction of differentiation, at which time all the cells had assumed the differentiated phenotype, binding of the β factor was significantly reduced, as measured by mobility shift assays. Following

addition of growth medium to the 4 day myocyte population, transcription of the rpL32 gene increased gradually and by 12 h period reached the rate observed in control myoblast populations. In the same time interval, the binding of factor(s) to the β element increased within 30 min. Although these experiments failed to demonstrate a precise temporal relationship between the rate of transcription and the change in binding of the β factor, they suggest that altered expression of the rpL32 gene following the signal for differentiation or dedifferentiation results at least in part from differential binding of the factor(s) to the β element in the rpL32 gene promoter. This delay in transcription, implies, however, that other factor(s), beside those already identified, may limit rpL32 gene transcription. A report by Harris et al. [1992] indicated that binding of the factors to the rpL32 gene promoter were quite different in MM14 myoblasts, as compared to fiber extracts. In addition, they reported that sequences upstream of −141 are also required for expression of the rpL32 gene, since deletion of that region reduced mRNA levels by 50–70% in mouse MM14 myoblasts [Harris et al., 1992]. A stimulatory effect of these upstream sequences has not been detected previously in COS or mouse plasmacytoma cells [Dudov and Perry, 1986; Atchison et al., 1989; Moura-Neto et al., 1989]. Although it is still unknown how these elements stimulate transcription of the rpL32 gene, it seems that protein(s) binding to these upstream sequences could be a limiting factor.

The rapid change in protein binding to the β element observed early in the conversion of myocytes to myoblasts suggests that post-translational modification of the β factor is involved. Support for this postulate was strengthened by experiments in which myocytes were induced to dedifferentiate in the presence of cycloheximide. Despite inhibition of de novo protein synthesis, the increased binding of the β factor to its promoter element was observed during dedifferentiation, suggesting that inactive β factor was present in myocyte cell extracts. Thus, the ability of β factor to bind to the rpL32 gene promoter is due to a post-translational event, such as modification of the β factor, rather then an increase in the production of the protein factor.

Previous studies have reported that phosphorylation of specific transcription factors can play an important role in modulating their transcriptional activity [Marais et al., 1992; Rabault and Ghysdael, 1994]. Reversible phosphorylation is a major mechanism for influencing protein conformation, enzymatic activity, or protein-protein interactions. Therefore, we investigated whether phosphorylation was involved in modifying the factors binding to the β element. Our results reveal that the binding activity of the β factor was altered by a phosphorylation/dephosphorylation process. Dephosphorylation of either myoblast or myocyte nuclear extracts resulted in increased binding of the factor(s) to the β element. The precise model for this modulation in vivo is yet unknown, since there are several levels of regulation which can alter the activity of a transcription factor. Binding of a transcription factor to its DNA element can be affected either positively or negatively by phosphorylation. This same modification can also alter interaction with other components of the transcriptional apparatus. For example, phosphorylation of Ets-1 protein inhibits its DNA binding activity [Rabault and Ghysdael, 1994], whereas DNA binding of the serum response factor is stimulated by phosphorylation [Marais et al., 1992]. Alternatively, a transcription factor can be sequestered in the cytoplasm and rendered inactive either by lacking the nuclear target sequence or by its interaction with an inhibitor. Phosphorylation of the factor itself or its cytoplasmic anchor allows nuclear translocation and increased transcriptional activity as has been reported for NF-*k*B [Baeuerle, 1991].

Several pieces of evidence indicate that the β factor belongs to the Ets oncogene family [Yoganathan et al., 1992a; Genuario et al., 1993]. Ets proteins form a novel class of sequence-specific DNA-binding proteins which interact with purine rich sequences in promoters of various genes and contribute to their transcriptional activation. A number of properties of the Ets gene products suggest that they play an important role in gene regulation [Seth et al., 1992; Janknecht and Nordheim, 1993]. These include their nuclear localization and rapid expression, of at least some members of this family, in response to specific extracellular signals. Furthermore, Ets proteins are subject to rapid and transient phosphorylation in response to mitogenic stimuli [Koizumi et al., 1990; Rabault and Ghysdael, 1994]. Thus, as presented here, involvement of phosphorylation in modulated binding of the β factor and, in turn, altered

expression of the rpL32 gene is in accord with properties of members of the Ets protein family.

The β factor is immunologically related to GABP, a heterodimer of which one of the monomers is a member of Ets family of proteins [Yoganathan et al., 1992a; Genuario et al., 1993]. Recombinant GABP proteins bind to the corresponding β sequence in rpL30 gene promoter and give a DNase I footprinting, similar to that obtained with nuclear proteins [Genuario et al., 1993]. Point mutations within this element in the rpL30 gene promoter abolish GABP binding [Genuario et al., 1993].

In the work presented here, antisera against both GABP α and GABP β proteins specifically recognize proteins binding to the rpL32 β element, thus, inhibiting formation of the mobility shift observed with BC_3H1 myoblast nuclear extracts. In addition, purified GABP α and β proteins attach to this functionally important element in the rpL32 gene promoter in a sequence dependent manner. While GABP α protein binds directly to the rpL32 β element, GABP β does not by itself bind to this element, as previously documented [Thompson et al., 1991]. When both proteins are incubated with radiolabelled β element a larger complex forms, and is indistinguishable from that observed with BC_3H1 myoblast nuclear extracts. This observation implies that GABP α and β proteins specifically recognize and bind to the β element, and support the premise that this heterodimer is the rpL32 β factor.

UV-protein cross-linking studies revealed that the β factor comprises both GABP α and GABP β proteins. In addition, following differentiation of the BC_3H1 myoblasts binding of both components of GABP to the β element is significantly reduced. Accordingly, neither GABP α nor β protein binding to the rpL32 β element was detected by mobility shift assays in the myocyte extracts following their exposure to UV light. Given that the cellular levels of GABP α and β proteins were unchanged by the differentiation process, these observations suggest that the absence of protein binding to the β element in myocyte extracts resulted from post-translational modification. Although, many Ets proteins are phosphorylated [Koizumi et al., 1990; Rabault and Ghysdael, 1994], the results presented in this paper are the first indication that the GABP heterodimer may be a phosphorylated protein.

Differentiation-dedifferentiation of BC_3H1 cells failed to affect the production of GABP α and β proteins. Neither GABP mRNA nor protein levels changed when BC_3H1 cell underwent differentiation or dedifferentiation. Thus, these results support the contention that a post-translational modification, e.g., phosphorylation is responsible for altered binding of β factor to the rpL32 promoter. Therefore, GABP α and β proteins may play a role in controlling expression of the rpL32 gene since they appear to be ubiquitous in their tissue distribution and not altered by cellular growth perturbations. Not ruled out by these studies is the possibility that other factors and/or the extent of GABP factor phosphorylation are responsible for fine tuning transcription of the rpL32 gene.

Potential GABP binding sites are found in promoter regions of many other ribosomal protein genes, usually at locations similar to that in rpL32 [Genuario et al., 1993; Safrany and Perry, 1995; Genuario and Perry, 1996]. Closer evaluation of the effects of the GABP heterodimer on the expression of ribosomal protein genes has been reported only with rpL30 and rpS16 gene promoters [Genuario et al., 1993; Genuario and Perry, 1996]. The rpL32 gene promoter differs somewhat from these promoters. The rpL32 promoter possesses a solitary GABP binding site that can accommodate only one GABP dimer. In contrast, rpL30 and rpS16 promoters can accommodate tetrameric GABP complexes, since they are composed of two contiguous β binding sites [Safrany and Perry, 1995; Genuario and Perry, 1996].

A GABP-related factor also binds to the cytochrome c oxidase subunit IV (COXIV) and COXVb gene promoters, suggesting a possible role in mediating coordination of subsets of genes involved in mitochondrial respiration [Virbasius and Scarpulla, 1991; Carter et al., 1992; Virbasius et al., 1993; Carter and Avadhani, 1994]. Both COXIV and COXV are nuclear genes which encode mitochondrial proteins required for oxidative phosphorylation. Cloning of the murine COXIV and COXV genes identified promoter characteristics similar to that of rpL32 and other ribosomal protein genes [Basu and Avadhani, 1991]. Thus, considering the wide cellular distribution of GABP α and β proteins, they represent an attractive candidate for functional control of a set of house-keeping genes

involved in regulating vital processes in eukaryotic cells, such as translation and respiration.

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