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Identification of a polypeptide bound to the β region of the mouse r protein L32 promoter

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Studies have been initiated to identify the protein component(s) which interact with the β regulatory region of the mouse ribosomal protein L32 gene promoter. By the combined use of the mobility shift assay and UV cross-linking, a factor specific for the upstream transcriptional control sequence of the β region of the ribosomal protein L32 promoter has been detected in mouse L1210 nuclear extracts. A mutation (GT→TC at −71 to −70) in this sequence eliminates the binding. β factor is identified as a 55 kDA polypeptide by UV cross-linking. Addition of excess β element (double-stranded oligonucleotide) to a cell-free transcription system reduces transcription of the ribosomal protein L32 gene. Our results provide evidence that the interaction between the β element and the β factor is involved in ribosomal protein L32 transcription.

Transcription factor, Ribosomal protein L32.

1. INTRODUCTION

Initiation of transcription by RNA polymerase II an important step in gene expression in eukaryotes requires both general and promoter specific factors [1–3] in addition to distinct DNA sequences or elements in the vicinity of the transcription initiation site. These sequences are recognized by protein factors that influence transcription by interacting with the transcriptional apparatus. In general, promoter elements, such as TATA, GC and CCAAT boxes are common to many genes transcribed by polymerase II [4]. The ribosomal protein L32 (rpL32) gene has a promoter with several distinctive features, including the lack of both a TATA box and a Sp1 binding site [5]. It is reasonable therefore to assume that the factors associated with the ribosomal protein L32 promoter are different from other promoter specific transcription factors. Hence, the identity of factors involved in ribosomal protein L32 promoter function is of considerable interest.

Using transient expression assays Perry's group has demonstrated that maximum (rpL32 gene) expression requires a sequence of 150 base pairs spanning the transcription start site [6–9]. Previous reports from this laboratory have demonstrated that cell-free extracts from either L6 myoblast or myotube nuclei initiate accurate transcription of the rpL32 gene [10–12]. The current investigations are designed to identify the factor(s) interacting with the β element of the mouse rpL32 gene promoter. More specifically, using UV cross-linking we have established 55 kDa as the molecular weight of the DNA binding protein that interacts specifically with the β element of this promoter. Our studies further suggest that interaction between the β element and the β factor is necessary for the efficient transcription of the rpL32 in vitro.

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2. MATERIALS AND METHODS

Mouse L1210 nuclear extract and cell-free transcription were performed as previously described [12,13]. Cell-free transcription was carried out at 30°C for 60 min using 4 μl extract and 250 ng of *Hin*dIII digested plasmid harbouring rpL32 promoter and coding region [5]. Gel mobility shift assay and UV cross-linking experiments were performed essentially as described [15]. Double-stranded oligonucleotides were made as described [14] oligonucleotides:(5′-CCCAGAGCCGGAAGTG-3′) and (5′-TGGGCACTTCCGGCTC-3′). A mutant double-stranded oligonucleotide that contains a 2-bp change in the β recognition site was prepared from synthesized oligonucleotides: (5′-CCCAGAGCCGGAATCG-3′) and (5′-TGGGCGATTCCGGCTC-3′). The plasmid P3AR2.8 was kindly provided by Dr. R.P. Perry (Fox Chase Cancer Center, Philadelphia, PA).

3. RESULTS

To define the protein binding to this region a gel mobility shift assay was performed using a [^{32}P]-end-labelled fragment (−80 to −69) and varying amounts of nuclear extract. As seen in Fig. 2A a band representing a protein-DNA complex appeared. The band increased in intensity as more extract was added to the reaction mixture suggesting that the 12 bp double-stranded DNA fragment was sufficient for binding the β factor. When excess poly [d(I-C)] was added to the reaction mixture as a non-specific competitor, binding to the labelled fragment still occurred (Fig. 2B). When

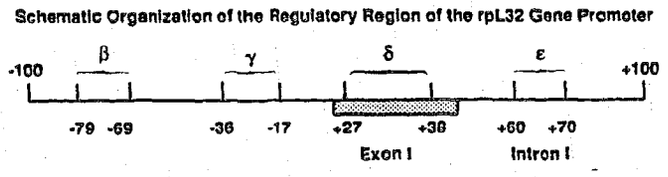

Fig. 1. Schematic organization of the 5′ regulatory region of the rpL32 gene promoter. Nucleotides are numbered beginning from the transcriptional start site. Segments containing specific binding site positions are indicated. Nuclear factors that bind to the specific region of the rpL32 promoter are designated β, γ, δ and ϵ. These functionally important regions were localized by transfection experiments and gel shift assay (Atchison et al. [6]; Hariharan et al. [9]).

the nuclear extract was treated with protease-K and/or heat inactivated (70°C for 3 min) it failed to complex with the labelled DNA fragment. These binding assays indicate that a protein is capable of binding to the 12 bp DNA fragment used in these studies.

To confirm the specificity of protein binding we constructed a mutant in which the wild-type (GT→TC) sequence was specifically altered at positions −71 and −70. The binding specificity of the wild-type and mutant type was then tested by a gel mobility shift assay (Fig. 3A). While the wild-type fragment was bound by the nuclear factor, no binding was detected with the mutated fragment (compare Fig. 3C, lanes 2–4 and lanes 13–15). To further assess the specificity of protein interaction with the probe a competition experiment was performed. Molar excesses of unlabelled wild-type and mutant-type fragment were added to the gel mobility shift reaction mixture (Fig. 3A). Molar excess of the unlabelled wild-type sequence efficiently competed for the specific binding whereas the mutant-type fragment failed to have any effect (compare Fig. 3A, lanes 5–8 and lanes 9–12).

To identify the molecular weight of the β factor, nuclear extracts were incubated with the labelled probe under the conditions for the gel mobility shift assay. Following incubation samples were irradiated with UV light and digested with DNAse I and micrococcal nuclease. The resultant material was fractionated by electrophoresis on an SDS-polyacrylamide gel. When the β element was UV irradiated in the presence of nuclear extract the most prominent polypeptide band detected had an apparent molecular weight of 55 kDa. To confirm the specificity the UV cross-linking was per-

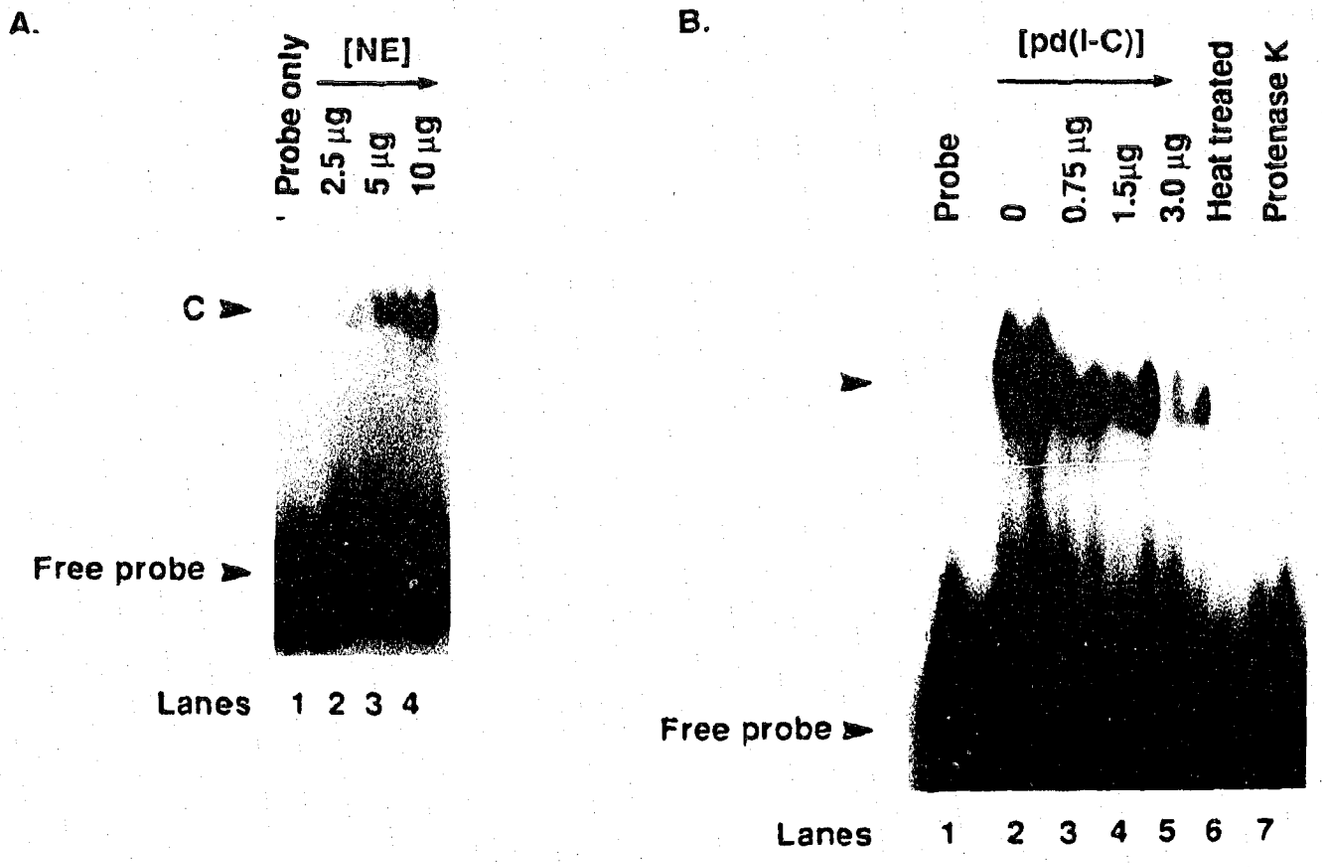

Fig. 2. (A) Gel mobility shift experiments. To determine whether the 12 bp oligonucleotide sequence spanning (rpL32) −80 to −69 is sufficient to bind to a factor a gel mobility shift assay was performed using a [^{32}P]-end-labelled β element with varying amount of mouse L1210 nuclear extract. As seen in Fig. 2A, a characteristic binding pattern emerges. The amount of the complex (C) increases as more extract is added to the reaction mixture. (B) The β element specifically binds a polypeptide. The double-stranded oligonucleotide containing the β region was [^{32}P]-end-labelled and used in a gel shift assay with mouse L1210 nuclear extract using excess poly[d(I-C)] as a non-specific competitor (lanes 3–5). Proteinase-K and heat-treated (70°C for 3 min) nuclear extracts were used in lanes 6 and 7, respectively.

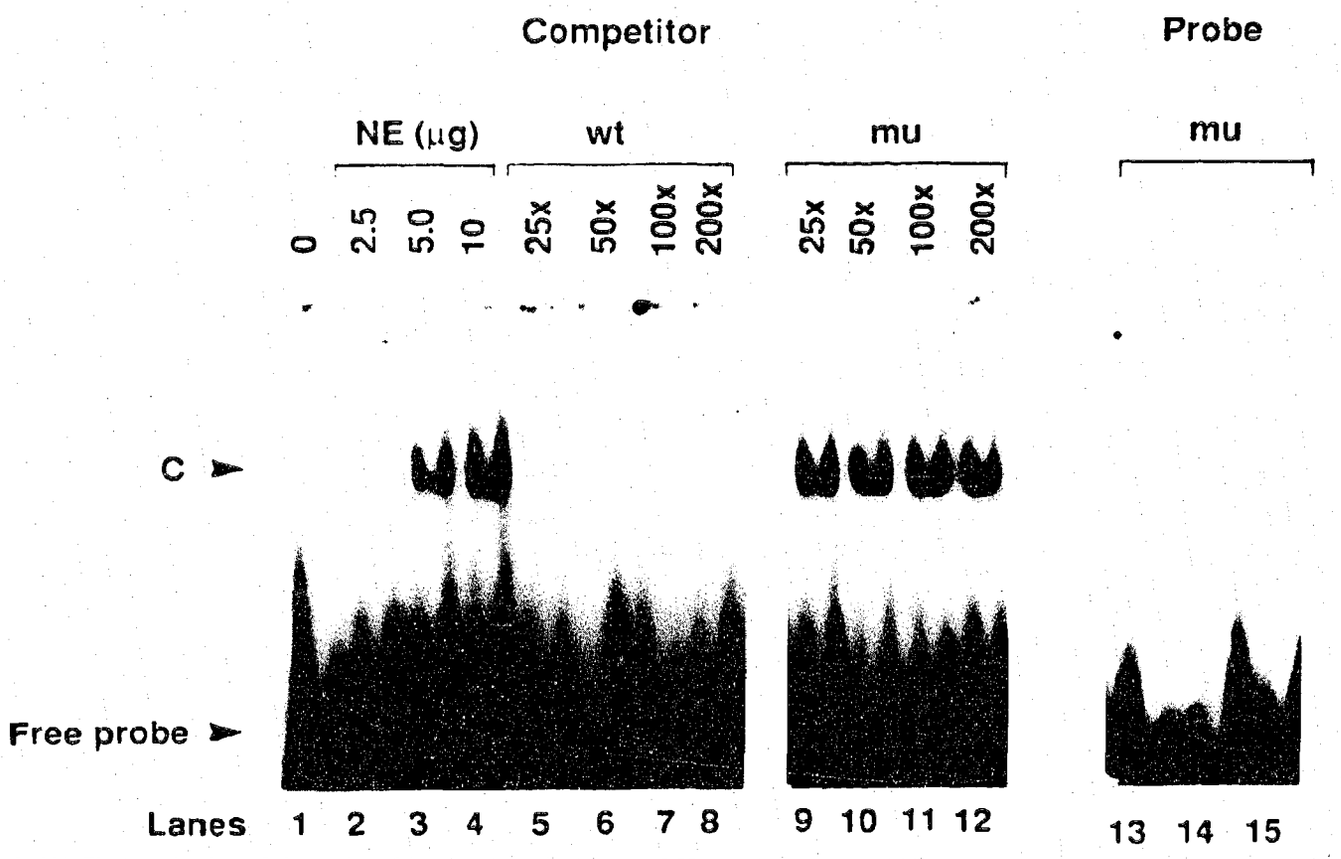

Fig. 3. (A) Characterization of the nuclear factor binding site in the rpL32 β region by gel mobility shift assay. Competition of wild-type and mutated-type double-stranded (β region) oligonucleotide with the rpL32 β element DNA for factor binding. Increasing amounts of unlabelled competitor oligonucleotide were added to the reaction mixture containing 0.1 ng of wild-type or mutant labelled probe. Binding reaction mixtures contained the ^{32}P-labelled β region, nuclear extract, poly[d(I-C)] as a non-specific competitor, increasing molar concentration of wild-type (lanes 5–8) and mutant (lanes 9–12) oligonucleotides as indicated. The normal β binding site probe (lanes 2–4) and mutated probe (lanes 13–15) were labelled and used in a gel mobility shift assay.

formed in the presence of an excess of unlabelled double-stranded competitor oligonucleotide containing or lacking a β binding site. The specific competitor abolished labelling of the 55 kDa polypeptide whereas the non-specific competitor fragment lacking the β binding site failed to affect the photo-labelling of the 55 kDa polypeptide (Fig. 4). We therefore conclude that the 55 kDa polypeptide binds specifically to the β element.

This study strongly suggests that a factor binds to the β element. To directly examine whether this factor is in fact required for transcription of rpL32 gene, we performed the following cell-free transcription experiment. Since the β element binds a 55 kDa component it should be possible to add excess β element to the cell-free transcription reaction mixture to sequester the 55 kDa factor and block or reduce transcription. Addition of a double-stranded oligonucleotide containing the β site to the cell-free transcription system specifically inhibited transcription in a concentration dependent manner (Fig. 5, lanes 2–4) whereas addition of an oligonucleotide containing a mutated β site sequence failed to significantly affect transcription (Fig. 5, lanes 5–7). An equivalent amount of an unrelated oligonucleotide pro-

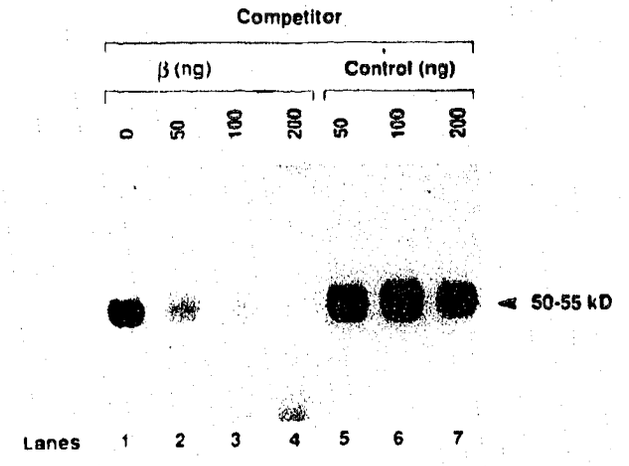

Fig. 4. Identification of the protein(s) binding to the β element. The labelled β element DNA probe was incubated with nuclear extracts in the presence of increasing amounts of an unlabelled oligonucleotide containing a β site or an unrelated oligonucleotide lacking β site followed by UV irradiation and separated by SDS/polyacrylamide gel electrophoresis. Bands are identified by autoradiography. The DNA competitor used and amount of DNA competitors, are indicated above the lanes. The arrow indicates the position of the 50–55-kDa band.

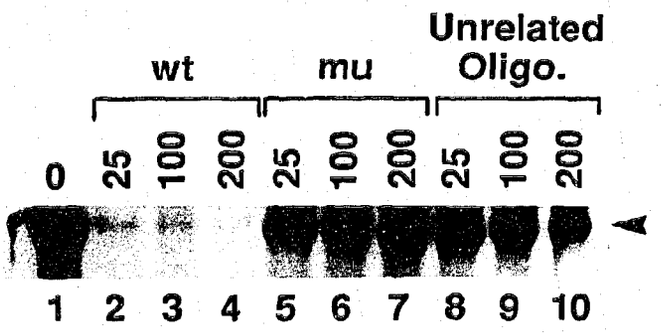

Fig. 5. β factor is required for cell-free transcription of the rpL32 promoter. Reaction mixture contained 0.25 μg of *Hin*dIII digested P3AR2.8 template, 20 μg nuclear extract and varying amounts of oligonucleotide and appropriate amounts of ribonucleoside triphosphates. Extracts were preincubated with oligonucleotide competitor for 15 min at 30°C prior to addition of template. The competitor used and amount of competitor (ng) are indicated above the lanes. Arrow indicates the accurately initiated transcripts from the *Hin*dIII digested P3AR2.8 templates.

duced no significant affect (Fig. 5, lanes 8–10). These results suggest that the β factor is directly involved in transcription of the rpL32 gene.

4. DISCUSSION

This study was designed to identify the factor interacting with the β element and to investigate whether this factor is functionally involved in transcription of the rpL32 gene, using a cell-free transcription system. Transient expression assays have suggested that sequence −79 to −69 in the rpL32 promoter region is required for rpL32 gene expression [6]. A DNA segment containing the −79 to −41 promoter sequence was used in the mobility shift assay and shown to be involved in the binding of a protein [6]. In our studies we have used a smaller DNA segment spanning −80 to −69 to detect the transacting factor in mouse L1210 nuclear extracts. Our results indicate that the 12 bp DNA sequence spanning −80 to −69 is sufficient to interact specifically with a protein. We have identified the binding site as the sequence 5′-GAGCCGGAAGTG-3′ which is located at position −80 to −69. A mutation in the −71 to −70 region (GT→TC) abolishes binding. Mutation analysis suggests that the sequence at −71 and −70 is critical in recognizing the β factor.

UV cross-linking experiments revealed that a 55 kDa polypeptide was specifically cross-linked to the β factor binding site. To establish the functional activity of the β element the influence of preincubating the 12 bp oligonucleotide with the nuclear extract upon its ability to transcribe the rpL32 gene was examined. The β element (−80 to −69) when added in molar excess dramatically inhibited transcription of the rpL32 gene. These data suggest that the β factor is indeed required for the efficient cell-free transcription of the rpL32 gene. The upstream (−80 to −69) sequence location is frequently occupied by an upstream transcription factor in other RNA polymerase II genes and activates or stimulates transcription [4]. It is reasonable to assume that the β factor might also act as an upstream transactivating factor in rpL32 gene transcription. Since incubation with the β element does not affect cell-free Ad MLP transcription (data not shown), the β factor appears not to be a general polymerase II transcription factor. We presume that the β element acts as an upstream transacting element although studies with purified β factor will be needed to conclusively establish its role in rpL32 gene expression.

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