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The importance of downstream δ-factor binding elements for the activity of the rpL32 promoter

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ABSTRACT

A salient feature of mammalian ribosomal protein genes is the location of promoter elements downstream, as well as upstream, of the transcriptional start point. Previous functional studies of the mouse rpL32 gene (Chung and Perry, Mol. Cell. Biol. 9, 2075; 1989) indicated that the first intron of this gene contains such an element. We show here that this element encompasses a binding site for a zinc finger nuclear protein known as δ (YY-1, μE1, UCRBP). The intronic δ site (δ_i) is located 32 bp downstream of another δ site in the first exon (δ_e). Transfection experiments with genes containing deleterious mutations in one or both δ sites or having alterations in the spacing between the sites indicate that the two δ elements function independently and contribute additively to the overall strength of the rpL32 promoter. Moreover, the contribution of the δ_i element is the same whether it is oriented parallel or antiparallel to the δ_e element. Together, the two δ elements raise the expression level about 10-fold over that attained by the upstream and initiator portions of the promoter. The positive role of the δ factor in rpL32 expression contrasts strikingly with its repressive role in various other genes.

INTRODUCTION

Mammalian ribosomes contain over 80 distinct structural proteins. The genes encoding these proteins must be coordinately expressed in all tissues in order to satisfy the requirements of growth, proliferation and maintenance. Although the ribosomal protein (rp) genes are widely dispersed in the genome, they are transcribed at remarkably uniform rates, presumably because their promoters are basically equipotent (1,2). The mammalian rp promoters analyzed to date have several common features, namely, (i) lack of a canonical TATA box, (ii) precise transcriptional initiation within an oligopyrimidine tract that is flanked by GC-rich sequences and (iii) four or five binding sites for ubiquitous transcription factors, which are located both upstream and downstream of the transcriptional start-point (1,3–9).

Two downstream elements appear to be required for optimal expression of the mouse rpL32 gene. One of these elements has been tentatively localized to a region of the first exon between +12 and +45 on the basis of transient transfection experiments with a deletion mutant of rpL32 that lacks this region (5). This exonic segment contains a binding site for δ factor (aka YY-1, NF-E1, UCRBP), a zinc finger protein that has been implicated in the transcriptional regulation of several other genes (5,10–13). The second downstream element of rpL32 is located in the first intron. Transfection experiments with mutants containing or lacking various intron 1 segments localized this element to a region between +47 and +73 (14). Interestingly, this region contains a sequence that is very similar to the exonic δ factor binding site. However, the binding properties and the functional importance of this intronic sequence have not heretofore been analyzed.

In the experiments reported here we demonstrate that the intronic element does indeed contain a second δ factor binding site. Moreover, we have used site directed mutagenesis to assess the relative contributions of the exonic and intronic δ elements and to evaluate the importance of spacing and orientation to overall promoter function. Our results indicate that these two downstream elements work independently and contribute additively to the strength of the rpL32 promoter.

MATERIAL AND METHODS

Plasmid construction

Various rpL32 mutants were produced by standard cloning procedures from a construct (designated as the wild type rpL32) that contains 160 bp of 5′ flanking sequence, the entire transcribed region of the gene, and 300 bp of 3′ flanking sequence (15) (Fig. 1A). This rpL32 construct contains all of the elements required for optimal expression of the rpL32 gene (5,16). Synthetic oligonucleotides representing the +11 (*Hae* II site) to +52 (*Hinf* I site) or the +50 (*Hinf* I site) to +77 (*Sac* II site) regions, and containing mutations (Fig. 1B) in the +28 to +37 or +60 to +69 regions respectively, were substituted for the corresponding wild-type sequences in the L32 construct by a directional cloning protocol. For the spacing mutants, IM/5 and

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IM/10, the sequences from +54 to +58 were repeated in tandem once or twice, respectively (Fig. 1B). Using a previously described (14) L32 construct Δ1.A(+11) (Fig 2A), a pair of mutants designed to examine the orientation effect of the intron element were produced as follows (Fig. 7). A unique *Sna* B1 site was engineered into the boundary of exon 1 and intron 1 of Δ1.A(+11). The resulting construct was digested with *Sna* B1 and *Sac* II, the fragment containing +46 (*Sna* B1) to +79 (*Sac* II) was removed, and the *Sac* II site was blunted with T4 DNA polymerase. This vector was then ligated to a double-stranded oligonucleotide consisting of sequences from +46 to +79 in either sense or antisense orientation. The resulting constructs were called Δ1.A.a and Δ1.A.ar, respectively. All the mutations were confirmed by restriction enzyme digestion and by sequencing through the altered regions. The plasmids were amplified in *Escherichia coli* HB 101 and purified by banding twice in cesium chloride. The DNA preparations used for transient transfection experiments were more than 80% in the supercoiled form as judged by agarose gel electrophoresis.

DNA transfection, RNA isolation and analysis

As detailed previously (14), cultured COS-7 cells were transfected by the DEAE-dextran-chloroquine procedure and harvested from plates 40 hrs. later by trypsinization. The test plasmids were always cotransfected with a wild-type rpS16 gene which served as a control for transfection efficiency and RNA yield. S1 nuclease assays of cytoplasmic RNA were carried out with an internal rpL32 probe (Fig. 1A) and a 5'-terminal rpS16 probe (14).

Stable transformants were produced using G418 selection for a cotransfected neomycin resistance gene. Expression of the transfected genes in pools of 20 independent transformants was examined by Northern blot, S1 nuclease protection and nuclear run-on analyses as described in an earlier publication (14). Southern blot analyses of transformant DNA demonstrated that the wild-type and mutant pools contained similar numbers of integrated gene copies and that each pool represented multiple integration events (14 and data not shown).

Electrophoretic-mobility-shift assays

Nuclear extracts from mouse plasmacytoma cells S194 were prepared by the method of Dignam *et al.* (17) with additional protease inhibitors (leupeptin and pepstatin) added to all solutions at 1 μg/ml. Gel-mobility-shift assays with nuclear extracts and DNA fragments were performed as described by Singh *et al.* (18). Each assay contained 8 μg of nuclear extract protein, 0.1 to 0.5 ng of the ^{32}P-labeled DNA fragment, and 3 μg of poly(dI-dC) (Pharmacia). The binding reactions were allowed to proceed for 30 min. at room temperature and then resolved on a 5% polyacrylamide gel in TAE buffer (6.7 mM Tris.HCl, pH7.5, 3.3 mM sodium acetate, 1 mM EDTA). For competition experiments, the unlabeled DNA fragments were added in a 20- or 100-fold molar excess over the labeled DNA probes. The competitor was incubated with the reaction mixture for 5 min. prior to the addition of the labeled DNA probes.

For the experiment with recombinant δ protein, binding was carried out for 20 min. at room temperature in the presence of 2 μg poly (dI-dC) and 10 μM $ZnSO_4$, and complexes were resolved on a 4% polyacrylamide gel in 0.25 × TBE buffer (22.5 mM Tris-borate, pH 8.3, 0.65 mM EDTA). Recombinant δ, a generous gift of Dawn Kelley, was derived from a glutathione-S-transferase (GST)-δ fusion protein produced in *E.coli* by a plasmid containing δ cDNA (TC, ref. 10). The cDNA was inserted in frame behind segments of the pGEX-2T vector (Pharmacia) that encode GST and a thrombin cleavage-site. After purification on a glutathione-Sepharose column, the fusion protein was cleaved with thrombin and concentrated with a centrifugal membrane (Amicon, Inc.).

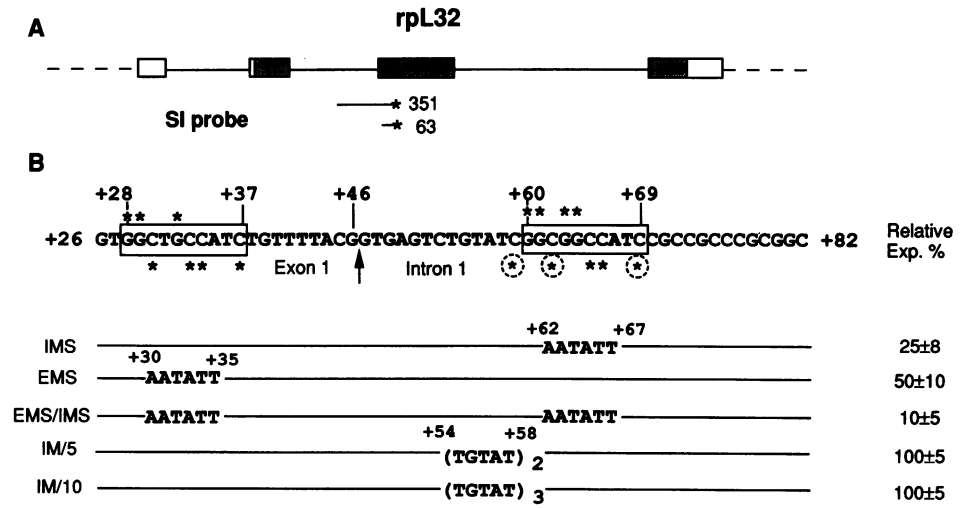

Figure 1. Structure of the mouse ribosomal protein gene rpL32 and various mutants. **A.** Schematic representation of the complete rpL32 gene. The locations of the S1 nuclease probe and protected fragment are indicated. Stippled boxes represent coding regions: open boxes represent 5' and 3' untranslated regions; solid lines represent introns; dashed lines represent 5' and 3' flanking sequences. **B.** Sequences of the +26 to +82 region. The two δ elements are boxed, and contact residues identified by methylation interference assays are marked with an asterisk. Encircled asterisks indicate that the contact residues have not been definitively identified. The junction of exon 1 and intron 1 is indicated with a vertical arrow. Sequences identical to the wild-type are represented by solid lines. The coordinates of the margins of δ elements in the wild-type and of altered regions in mutants are indicated. The relative expression of mutants as percent of wild-type is shown at right. These values were calculated from scanning of densitometric data from two or more independent transfection experiments.

Methylation interference assay

End-labeled DNA fragments were partially methylated with dimethylsulfate as described (19). The partially methylated DNA was then incubated with nuclear extract in a standard gel-mobility-shift assay. The bound and unbound fragments were separated by electrophoresis on a 5% polyacrylamide gel, collected by electroelution, extracted with phenol/chloroform, and precipitated with ethanol. The DNA was treated with 0.5 M piperidine at 90°C for 30 min., lyophilized twice with H_2O, and resolved on a sequencing gel containing 8% polyacrylamide and 7 M urea.

RESULTS

Identification of a δ factor binding site in the functionally important region of intron 1

In a previous study of rpL32, we demonstrated the presence of a transcriptional regulatory element in the 5′ portion of intron 1 (14). The importance of this element for rpL32 expression is illustrated by an experiment (Fig. 2) in which the activity of the wild-type gene (L32) in stably transfected COS 7 cells is compared to that of a mutant gene lacking the entire 789 bp intron 1 segment (Δ1). Measurements of cytoplasmic mRNA accumulation by either Northern blot (Fig. 2B, top) or S1 nuclease protection assays (Fig. 2B, bottom) and analysis of gene transcription by a nuclear run-on analysis (Fig. 2C) show a striking decrease in activity when intron 1 is absent. When 129 bp of the 5′-most portion of intron 1 is reinserted into the deletion mutant [Δ1.A(+11)], transcriptional activity is restored to near wild-type levels (Fig. 2C). As described below, an experiment with a mutated form of this construct (Δ1.A.IMS, Fig. 7) demonstrated that the restoration of activity is due to the reinstatement of an intronic element and not to the duplicated exon 1 segment (1′, Fig. 2A). The presence of the unsplicable intron 1 fragment does not interfere with normal downstream splicing. However, it appears to cause a small reduction in transcript stability, as judged by the relative steady-state levels of Δ1.A(+11) and wild-type L32 mRNA.

Nuclear factor binding near the 5′ end of intron 1 was detected by Atchison *et al.* (16), but the exact location of the binding site was not determined. To localize this site more precisely, we prepared a series of fragments with the 3′ end fixed at +101 and the 5′ ends at +53, +59 and +79 (intron 1 starts at +47), incubated them with nuclear extract prepared from mouse plasmacytoma cells, and analyzed the complexes by a standard electrophoretic-mobility-shift assay. A retarded band clearly observed with the +53 to +101 fragment was significantly reduced in intensity when the +59 to +101 fragment was used as a probe and was not detectable with the +79 to +101 fragment probe (Fig. 3A). This suggests that a nuclear factor binding site is located between +59 and +79, in agreement with the result of the earlier analysis. Examination of the sequence of this region (Fig. 1B) reveals a motif (+60, GGCGGCCATC, +69) which is almost identical to the δ factor binding site (+28, GGC-TGCCATC, +37) in the first exon of rpL32 [the δ_e motif, (1,5,10)]. To determine if the intronic nuclear factor binding site coincides with this sequence, methylation interference analysis was used to identify the guanine contact residues in the binding site. As shown in Fig. 4, several contact residues within this sequence were identified, indicating that it does indeed comprise the binding site (designated as the δ_i motif). The footprint pattern is similar to that obtained using a fragment containing the δ_e motif (5) (Fig. 1B), suggesting that the δ_i and δ_e motifs both bind the same nuclear factor. Consistent with this supposition, we observed a single retarded band of identical mobility with fragments containing either the δ_e or the δ_i motif (Fig. 3B, lanes 3 and 4). When fragments spanning both motifs were used, two retarded bands of different intensities were observed (Fig. 3B, lanes 1 and 2). We presume that the more intense lower bands represent single occupancy of either the δ_i or δ_e motifs and the less intense upper band represents double occupancy of both motifs.

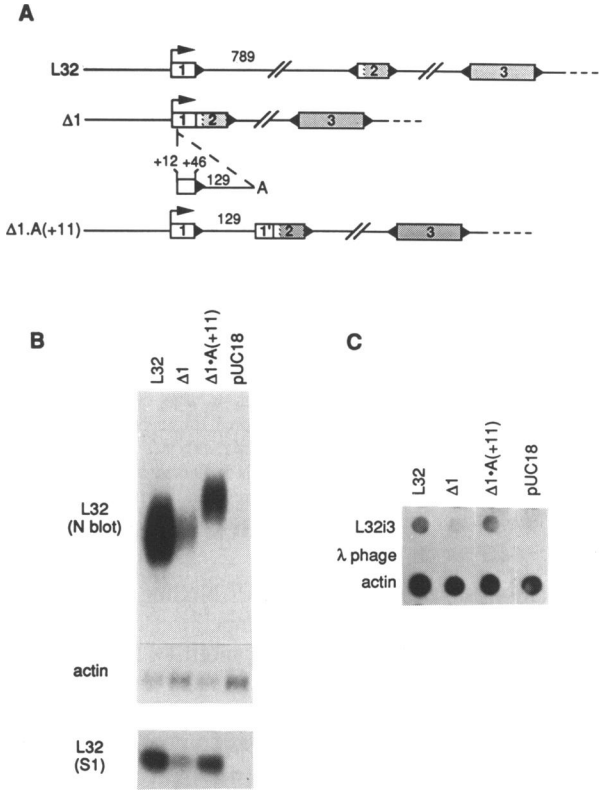

Figure 2. The contribution of intron 1 sequences to rpL32 expression. **A.** Schematic representation of relevant portions of the wild-type and mutant rpL32 genes. Open and stippled boxes respectively represent noncoding and coding exonic sequences. Thin lines represent flanking and intronic sequences. The site of transcriptional initiation is indicated by a horizontal arrow; filled triangles indicate splice junctions. Δ1 lacks the 789 bp intron 1 segment. In Δ1.A(+11), a fragment (A), consisting of the +12 to +46 portion of exon 1 and the first 129 bp of intron 1, is inserted at the +11 position of Δ1. **B.** RNA analyses of stably transformed COS cells containing either the wild-type rpL32 gene (L32), the Δ1 mutant, the Δ1.A(+11) mutant or a pUC vector (mock transfection). Upper panel shows a Northern blot of polyadenylated cytoplasmic RNA prepared from these transformants, which was hybridized with an rpL32 exon probe. The blot was stripped and rehybridized with a mouse β-actin probe to monitor the amount of RNA loaded in each lane (middle panel). The bottom panel shows S1 nuclease protection assays of the same RNAs with an internal probe (see Fig. 1A). **C.** Transcriptional activity of these transformants determined by nuclear run-on analysis. The source of immobilized DNA is shown at the left. $L32i_3$, actin and λ phage measure the signals from the transfected L32 genes, endogenous β-actin genes and nonspecific background hybridization, respectively. $L32i_3$ is a 920 bp *Pvu* II-*Eco* RI fragment from the third intron of rpL32. To avoid the influence of integration site differences, all measurements were made with pools of 20 independent transformants as described in the Materials and Methods Section. Relative to wild-type, the mRNA content of the Δ1 and Δ1.A(+11) mutants was approximately 15% and 70%, respectively, as judged by the S1 nuclease protection analysis; the relative transcription rates of Δ1 and Δ1.A(+11) were about 15% and 100% of the wild-type value.

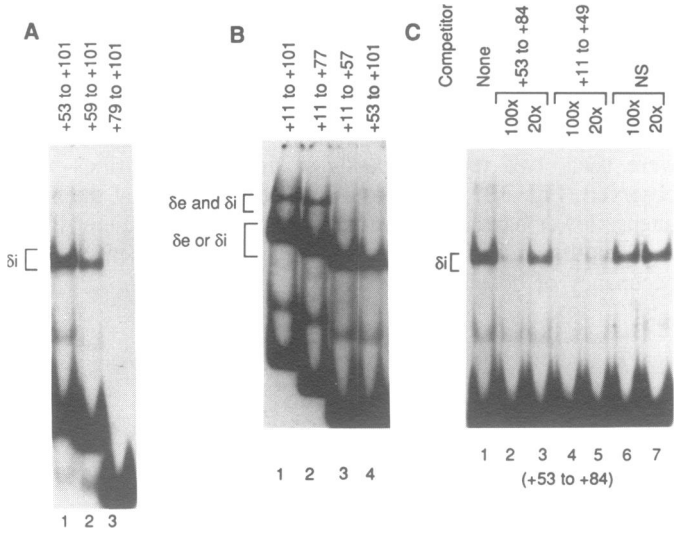

Figure 3. Electrophoretic-mobility-shift assays of rpL32 fragments covering the +11 to +101 region. **A.** Localization of nuclear factor binding sites in the 5′ region of the first intron. The boundaries of fragments used are indicated above lanes. **B.** Comparison of fragments containing factor binding sites in exon 1 and/or intron 1. The complexes corresponding to the retarded bands are indicated at left: the upper band represents occupancy at both sites; the lower band represents single occupancy at either the exonic or intronic site. The differences in mobility of the retarded bands are due to differences in size of the DNA probes. **C.** Competition binding assays. A labeled intron 1 fragment (+53 to +84) was incubated with nuclear extract in the presence of the indicated unlabeled competitor DNA. The molar excess of each competitor is indicated above each lane. NS (non-specific competitor) is a 50 bp fragment prepared from the polycloning sites (*Eco* RI to *Hind* III) of the pUC18 vector.

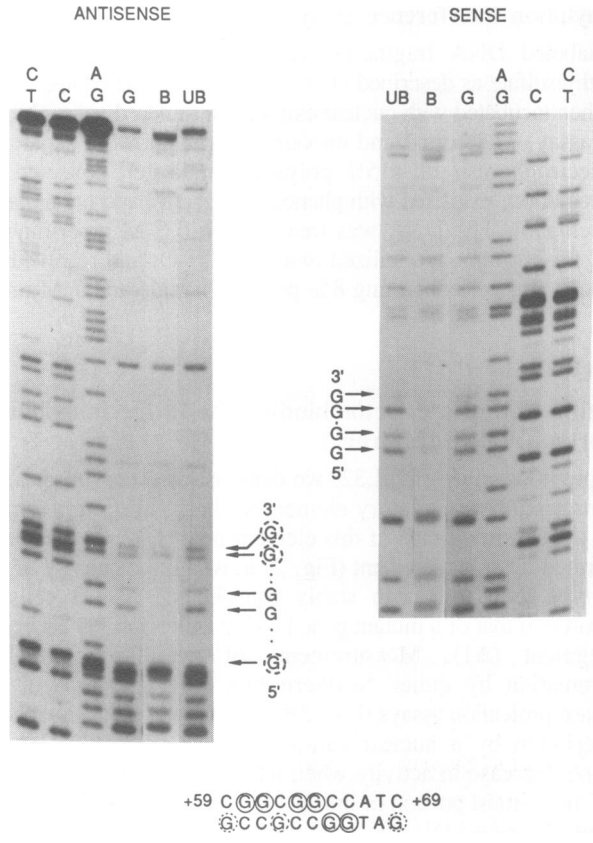

Figure 4. Methylation interference analysis of the nuclear factor binding site in the first intron. A standard electrophoretic-mobility-shift assay was performed using a partially methylated fragment corresponding to +53 to +101, end-labeled on either sense or antisense strand. The bound (B) and unbound (UB) fragments were isolated, cleaved with piperidine, and analyzed on a sequencing gel together with (A+G)-, G-, C- and (C+T)-cleaved marker fragments. The positions of the uncleaved G residues in the bound DNA are indicated by arrows. The sequence of the δ element at the bottom shows contact guanine residues (encircled). The dashed circles identify residues for which the assignment is not definitive because of gel compression problems.

The relationship between the factors binding to the δ_i and δ_e motifs was further assessed by a competition assay (Fig. 3C). A labeled fragment containing the δ_i motif (+53 to +84) was used as a probe in a mobility-shift assay. A 20- or 100-fold molar excess of the same unlabeled fragment or a fragment containing the δ_e motif (+11 to +49) was used as competitor. To monitor the effect of nonspecific competition, a fragment of similar size spanning the polycloning site of the pUC18 vector (NS) was assayed in parallel. The fragment containing the δ_e motif was able to compete efficiently with the labeled fragment containing the δ_i motif (lanes 4 and 5). In fact, δ_e appears to be a better competitor than δ_i itself (compare lanes 3 and 5). As expected, the addition of the nonspecific competitor did not affect the binding of the labeled fragment (lanes 6 and 7).

To demonstrate conclusively that the δ_i motif is a *bona fide* δ factor binding site, we carried out experiments with a bacterially synthesized protein encoded by δ cDNA. As shown in Fig. 5, the recombinant δ protein froms a complex with a δ_i-containing oligonucleotide that is indistinguishable from that formed with the nuclear extract protein (lanes 1 and 2). The specificity of this complex is demonstrated by differential competition with homologous (lane 3) vs. heterologous (lane 4) oligonucleotides. Taken together, the foregoing results clearly indicate that the δ factor binds to two closely spaced regions that are required for optimal expression of rpL32.

The functional relationship between the δ_e and δ_i motifs

Since the δ_e and δ_i motifs are separated by 32 base pairs or about three helical turns, the proteins bound to these sites should be in relatively close proximity on the same face of the DNA

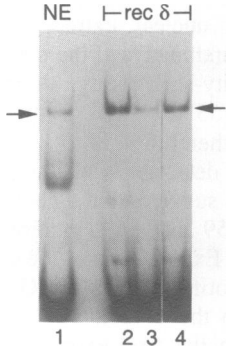

Figure 5. The δ_i motif recognizes recombinant δ protein. Electrophoretic mobility shift assays of an oligonucleotide representing the +45 to +80 region of rpL32. Lane 1: nuclear extract protein; lanes 2–4: recombinant protein. A 100× molar excess of unlabeled +45 to +80 oligonucleotide or an oligonucleotide representing the −46 to −8 region of rpL32 was added to the binding reactions of lanes 3 and 4, respectively. Arrows indicate the δ factor complexes.

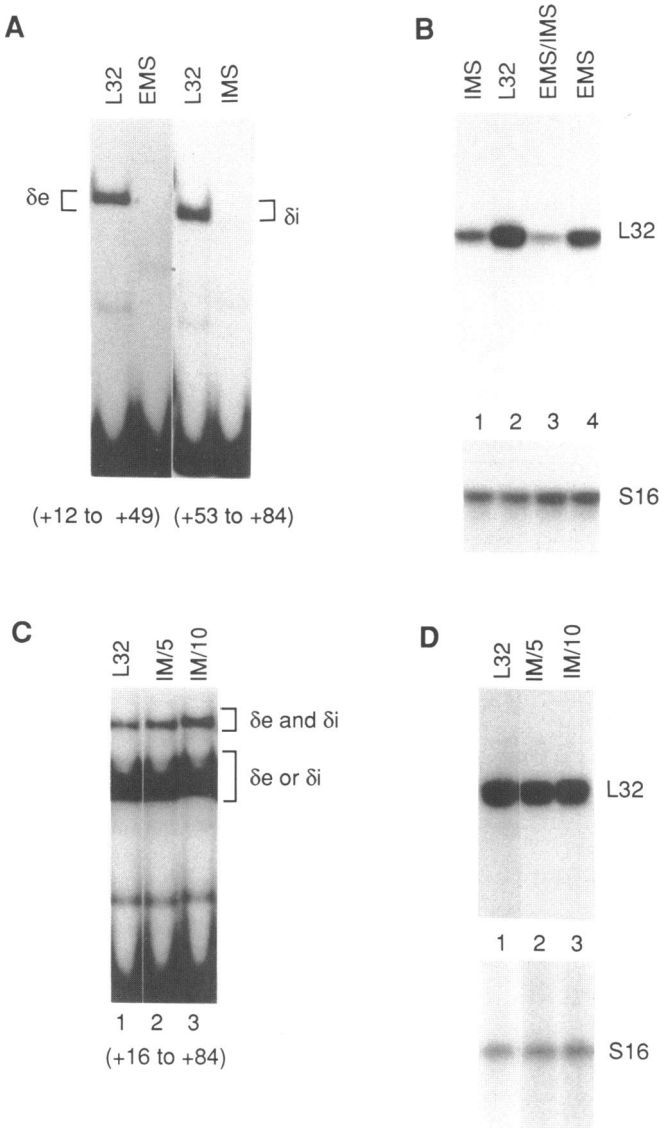

Figure 6. The consequences of mutations that destroy one or both δ factor binding sites or that alter the spacing between them. **A.** Electrophoretic-mobility-shift assays of the exon 1 (EMS) and intron 1 (IMS) mutants with exon 1 (+12 to +49) and intron 1 (+53 to +84) probes. **B.** Comparison of the expression of the wild-type rpL32 gene (L32) with mutants bearing mutations at intronic (IMS), exonic (EMS) or both δ factor binding sites (EMS/IMS). Tested genes were cotransfected into COS cells with an rpS16 gene and cytoplasmic RNA was analyzed by an S1 nuclease protection assay with either the internal rpL32 probe (top panel) or the rpS16 probe (bottom panel). The expression of rpS16 was used to monitor transfection efficiency and RNA yield. **C.** Electro-phoretic-mobility-shift assay of spacer mutants IM/5 and IM/10 (Fig. 1) in a fragment (+12 to +84) spanning both the exon 1 and intron 1 δ sites. D. Expression in transfected COS cells of spacer mutants (top panel) and a cotransfected rpS16 gene (bottom panel).

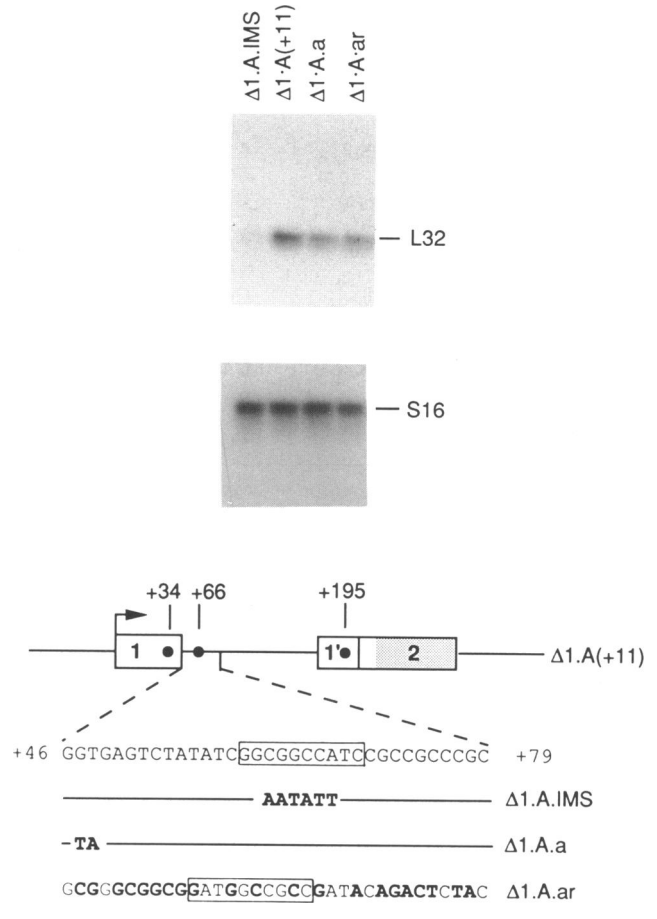

Figure 7. The effect of orientation on the activity of the intronic δ factor binding site. The bottom diagram schematically shows the Δ1.A(+11) construct as in Fig 2 with the positions of the δ binding sites (filled circles) indicated. The sequence alterations in the mutants, including those produced as a result of the inversion, are shown in bold face beneath the wild-type sequence. Δ1.A.a is identical to Δ1.A(+11) except for a two bp substitution, which created a *Sna* B1 restriction site at the 5′ end on intron 1. Boxed sequences mark the δ factor binding site. The expression of a cotransfected rpS16 gene was used to monitor the transfection efficiency and RNA yield. Transient transfection and S1 nuclease protection assays were as described in Fig. 6.

molecule. The potential for interaction could lead to a synergistic effect on the δ factor function. To explore this possibility, we examined the *in vivo* activities of rpL32 genes that have deleterious mutations in one or both binding sites or that have altered spacing between the sites. A 6 bp mutation in the δ_e site (EMS, Fig. 1B) specifically abolished δ factor binding to the exonic site (Fig. 6A) and reduced rpL32 expression by a factor of two to three (Fig. 6B, lane 4). A 6 bp mutation in the δ_i site (IMS, Fig. 1B) abolished δ factor binding to the intronic site (Fig. 6A) and decreased expression by a factor of four (Fig. 6B, lane 1). When the EMS and IMS mutations were combined (EMS/IMS, Fig. 1B), the expression was reduced to about 10% of the wild-type level (Fig. 6B, lane 3), which is roughly an additive effect of the individual IMS and EMS mutations. Such additivity suggests that there is little or no synergism between proteins binding to the δ_e and δ_i sites. This conclusion is consistent with the results of experiments with the spacing mutants. Mutants in which the distance between the δ_e and δ_i binding site was altered by either one half or a full helical turn (IM/5 and IM/10, respectively, Fig. 1B) still effectively bound δ factor to both exonic and intronic sites (Fig. 6C) and were expressed at levels indistinguishable from that of the normal rpL32 gene (Fig. 6D). In the case of IM/5, the addition of a half helical turn between the δ motifs should place the proteins on the opposite faces of the DNA molecule as well as altering their

Table 1. Occurrence of putative δ factor binding sites downstream of the transcriptional start-point in vertebrate genes that encode ribosomal proteins

Gene	Sequence	C	e/i	strand
L30(m)	CGGCCATCTTG	19	e	+
L32(m)	CTGCCATCTGT	34	e	+
	CGGCCATCCGC	66	i	+
L13a(m)	CCGCCATCTTC	21	e	−
L7a(h)	CTGCCATCCTC	79/81	i	+
L7a(m)	CC t CCATCCGC	60/64	i	+
	t C a CCATCTTG	25/29	e−i	−
S17(h)	CCGCCATCCTC	53	i	+
	CC c CCATCTGC	73	i	+
L1(X)	CCGCCATCTCC	43	e	−
L14(X)	CAGCCATCCCC	119	i	−
	CCGCCATC a TG	37	e	+
L7(m)	t C t CCATCCCC	74	i	+
P2(r)	gG t CCATCCCT	108	i	−

The first 150 bp of transcribed sequence in eleven vertebrate rp genes was searched for tracts similar to the δ binding sites in rpL30 and rpL32. The listed sequences conform to the prototype CNGCCATCY in at least 7/9 positions on either + or − strand as indicated; mismatches are in lower case. The downstream positions of the underlined C residues are indicated. For the L7a genes, the position relative to both start points is given. e or i denotes that the sequence is in the first exon or intron, respectively. m, h, ×and r respectively indicate mouse, human, *Xenopus* and rat genes.

The sequences for L30(m), L32(m), L7a(m), L7a(h), S17(h), L1(X), L14(X), L7(m) and P2(r) are listed as such in the Genbank/EMBL compilations. L13a(m) is listed as tum$^-$ transplantation antigen. The rp genes that did not contain a recognizable downstream δ binding sequence were S16(m), S14(h) and ub-rp-52.

Table 2. Occurrence of δ factor binding sites in non-rp regulatory elements

Type	Gene*	Binding Site Sequence	Factor Synonym†
enhancers	IgH	CGGCCATCTTG	μE1
	Igκ(3′)	CCTCCATCTTG	μE1
upstream	AAV(P5)	GCGACATTTTG	YY1
(promoter)	MuLV(LTR)	ACGCCATTTTG	UCRBP
	IAP	GCGCCATCTTG	(δ)
	c-*myc*	CGACCATTTTC	CTF(μE1)
	sk-actin	TCGCCATATTT	CTF(μE1)
	EBV(BLZF1)	CAGCCATCTCC	(YY1)
	c−*fos*	TGTCCATATTA	(YY1/μE1)
downstream	DHFR (m)	CTGCCATCATG	−
(promoter)	Surf-1	CAGCCATCTTT	−
	LINE-1	CGGCCATCTTG	(UCRBP)
initiator	AAV(P5)	TCTCCATTTTG	YY1

* Abbreviations and references: IgH and Igκ(3′), immunoglobulin heavy chain and immunoglobulin κ 3′ enhancers, respectively (12); AAV(P5), P5 promoter of adeno-associated virus (11,21); MuLV (LTR), long terminal repeat of murine leukemia virus (13); IAP, intercisternal A particle (22); c-*myc* and sk-actin (23−25); EBV (BLZF1), BLZF1 promoter of Epstein-Barr Virus (26); c-*fos* (24); DHFR, dihydrofolate reductase (27); Surf-1, surfeit locus-1 (28); LINE-1, long interspersed repeat element-1 (29).

† Identity of factors in parentheses was established by binding of cloned factor or by competition with known binding sites; −, not tested.

spacing. Taken together, these results indicate that the exonic and intronic δ elements function independently to boost the overall activity of the rpL32 promoter.

The δ_i element functions in both orientations

Since there appears to be little or no synergism between the δ_e and δ_i elements, we wondered whether the δ_i element would function if it were oriented in the opposite direction. Previous attempts to examine orientation dependence (14) employed a construct in which the δ_i and δ_e sites were concomitantly inverted and displaced more than 130 bp from the transcriptional start point. Although a decrease in expression was observed with this construct, the multiple alterations precluded any clear-cut interpretation. To address this question in a more rigorous fashion, we examined the activity of a mutant gene in which a segment that extends from +46 to +79 was inverted. This segment spans the δ_i motif (+60 to +69) and should have a sufficient amount of flanking sequence to preserve the intrinsic structural properties of the binding site. It also contains a GT→TA substitution at +47,+48, which was made in order to create a convenient restriction site (Fig. 7). Since the 5′ end of this segment comprises the exon 1-intron 1 boundary, its manipulation would be expected to interfere with the proper splicing of intron 1. To avoid complications due to alterations

in RNA splicing, we studied the effect of the inversion in the context of the variant rpL32 gene, Δ1.A(+11), which contains an unsplicable intron 1 fragment that encompasses the δ_i motif. As demonstrated earlier (Fig. 2), this variant is transcribed at essentially the wild-type level. Moreover, the IMS mutation of the δ_i motif, which reduces wild-type rpL32 expression to about 25% (Fig. 6B), has a similar effect on the Δ1.A(+11) variant (Fig. 7, compare lanes 1 and 2). This result indicates that the activity of Δ1.A(+11) is appropriately dependent on the integrity of the δ_i site and that the extra copy of the δ_e element at +191 to +200 cannot compensate for the loss of the δ_i element. When the +46 to +79 segment was inserted in either the sense (Δ1.A.a) or antisense (Δ1.A.ar) orientation, the level of expression was very similar to that of the parental Δ1.A(+11) gene (Fig. 7, lanes 2−4). From this result we conclude that the orientation of the δ factor on the intronic binding site is not critical for its functional activity. The validity of this conclusion rests on the presumption that δ_i is the sole functional element in the +46 to +79 segment. Although we cannot formally exclude the possibility that this segment contains an additional orientation-independent element that compensates for the loss of δ_i function, the presence of such an element seems unlikely because no additional protein binding sites in this region are detected by electrophoretic-mobility shift assays (Figs. 3,6).

DISCUSSION

The foregoing experiments demonstrate that the mouse rpL32 gene contains two functionally important binding sites for the transcription factor δ. Both δ sites are located downstream of the transcriptional start point. In addition to a previously characterized exonic site centered at +34 (the δ_e site) (5), we show here that there is also an intronic site centered at +66 (the δ_i site). The results of transfection studies with δ site and spacing mutants indicate that the two δ elements function independently to increase the overall activity of the rpL32 promoter. Thus, genes lacking functional δ_e or δ_i sites were, respectively, 50 <10% or 25<8% as active as the wild-type rpl32 gene, while genes lacking both δ sites were expressed at only 10 <5% the wild-type level. the roughly additive effects of the δ site mutations, together with the lack of any significant difference in expression when the $\delta_e-\delta_i$ spacing was changed by one half or one helical turn or the δ_i element was inverted, argues against any strong synergistic interaction between δ factors bound at the two downstream sites.

The δ factor is known to bind to a downstream element in another mouse rp gene, rpL30 (1). The rpL30 δ binding site (centered at +19 in exon 1) contains the sequence CNGCCATCY, which is common to both the δ_e and δ_i sites of rpL32. Moreover, this same sequence or a closely matching sequence is present at similar downstream locations in 8 out of 11 other vertebrate rp genes that were examined (Table 1). These putative δ sites occur in both exonic and intronic portions of the genes and in both orientations with respect to the direction of transcription. Our finding that normal and inverted δ_i elements have equivalent activities indicates that δ elements positioned in either orientation have the potential to be functionally important.

In a previous study from this laboratory (1), the rpL30 δ element was thought to be critical for the expression of this gene in transient transfection experiments. However, more recent experiments with newly prepared DNA samples did not confirm this observation, but rather indicated that this element contributes only slightly to the activity of an otherwise intact rpL30 promoter (20). Indeed, the effect of deleterious δ site mutations on rpL30 expression appears to be even less than the 50 <10% decrease observed for the de mutation in rpl32. Interestingly, the relative contribution of the δ element to rpL30 expression became readily detectable when the promoter activity was substantially decreased by mutations in two other elements located upstream of the start point (20). Thus, in the rpL30 gene, promoter element redundancy may mask the importance of the δ element, at least in the rapidly growing cells normally used for transfection experiments. In contrast, the contribution of each of the δ elements in the rpL32 gene is measurable and the combined contribution of both elements is substantial (about 10-fold). Thus, the role of any particular δ element within an rp promoter probably depends on its context within the constellation of other transcriptional activators.

The molecular cloning of a cDNA encoding the δ factor has revealed that it is a zinc finger protein of the *cys-cys-his-his* variety (10). This same protein was concurrently implicated in the regulation of a wide variety of cellular and viral genes (11−13,21). The sequences of the binding sites in these other genes conform to the consensus CNGCCATYTTG (Table 2), which closely resembles that of the rp δ site sequences (CCGCCATCYNC). However, the location of the binding sites and the regulatory role of the protein factor seem to vary considerably from gene to gene. In some locations, such as the downstream δ sites of rpL32 and the initiator element of the AAV P5 promoter the factor has a positive effect on transcription, whereas in other locations, such as upstream elements in the MuLV LTR or the AAV P5 promoter or the 3′-enhancer of Ig-κ genes, it appears to have a repressive function. In the skeletal α-actin and c-fos promoters, its repressive effect is modulated by competitive binding of the serum response factor (24,25). Understanding the mechanistic basis for the functional versatility of δ/YY1/μE1/UCRBP is an attractive goal for future research.

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