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Multiple Functional Motifs in the Chicken U1 RNA Gene Enhancer

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The DNA sequence requirements of chicken U1 RNA gene expression have been examined in an oocyte transcription system. An enhancer region, which was required for efficient U1 RNA gene expression, is contained within a region of conserved DNA sequences spanning nucleotide positions −230 to −183, upstream of the transcriptional initiation site. These DNA sequences can be divided into at least two distinct subregions or domains that acted synergistically to provide a greater than 20-fold stimulation of U1 RNA synthesis. The first domain contains the octamer sequence ATGCAAAT and was recognized by a DNA-binding factor present in HeLa cell extracts. The second domain (the SPH domain) consists of conserved sequences immediately downstream of the octamer and is an essential component of the enhancer. In the oocyte, the DNA sequences of the SPH domain were able to enhance gene expression at least 10-fold in the absence of the octamer domain. In contrast, the octamer domain, although required for full U1 RNA gene activity, was unable to stimulate expression in the absence of the adjacent downstream DNA sequences. These findings imply that sequences 3′ of the octamer play a major role in the function of the chicken U1 RNA gene enhancer. This concept was supported by transcriptional competition studies in which a cloned chicken U4B RNA gene was used to compete for limiting transcription factors in oocytes. Multiple sequence motifs that can function in a variety of *cis*-linked configurations may be a general feature of vertebrate small nuclear RNA gene enhancers.

The small nuclear RNAs (snRNAs) of the U family are a special class of metabolically stable RNA molecules which have been highly conserved through evolution. In metazoans, these RNAs (U1 to U10) range in length from 60 to 216 nucleotides, are abundant RNA molecules (10^4 to 10^6 copies per cell), and are encoded by multigene families (for a review, see reference 37). In vivo, the snRNAs are associated with proteins as components of small nuclear ribonucleoprotein particles (snRNPs). Considerable evidence now exists that the snRNPs containing the U1, U2, U4, U6, and probably U5 snRNAs are involved in the splicing of mRNA precursors (3–5, 7, 23, 51).

With the exception of U6, the snRNAs are synthesized by an RNA polymerase II-like activity (19, 29, 32, 38), but they have an unusual 2,2,7-trimethyguanosine cap structure and are not polyadenylated. Moreover, the formation of the 3′ end of the snRNAs is dependent on the initiation of transcription from an snRNA gene promoter (10, 17, 34). Interestingly, vertebrate snRNA genes lack two promoter elements most commonly associated with RNA polymerase II transcription units, the so-called TATA and CCAAT boxes.

Our laboratory is interested in understanding the regulatory mechanisms involved in the expression of snRNA genes. We have previously reported the characterization of four chicken U1 RNA genes, a chicken U2 RNA gene, and two chicken U4 RNA genes (13, 19, 22). As with other vertebrate snRNA genes (1, 9, 20, 24, 26, 29, 44, 46, 47) the 5′-flanking DNA of these genes contains two distinct and evolutionarily conserved regions: a proximal region located at position −55 (relative to the transcriptional initiation site) and a distal region located near position −200 (13, 19, 22). The conservation of these DNA sequences implies that they constitute essential components of the snRNA gene promoter. In fact, it has been demonstrated that alterations in either of these conserved regions significantly decrease transcription of human and frog U1 and U2 RNA genes (1, 9, 24, 25, 28, 33, 44, 47). Two general conclusions have been drawn from the snRNA gene expression studies. First, the proximal control element is essential for accurate initiation of snRNA transcription (9, 33, 44). Second, the distal control region, which has properties similar to those of a classic enhancer element (24, 25, 28, 33), is required for maximal snRNA gene expression and facilitates the formation of a stable transcription complex (28). Additional DNA sequences in the 5′-flanking DNA may also be involved in modulating the expression of specific snRNA genes (e.g., elements A, C, and E of the human U1 RNA gene [33]).

To functionally define the DNA sequences important for chicken snRNA gene expression, we have prepared a family of deletion mutations in the 5′-flanking DNA of a cloned chicken U1 RNA gene. These mutant templates, when assayed for transcriptional activity by microinjection into *Xenopus laevis* oocytes, reveal that the chicken U1 RNA gene enhancer is composed of at least two subregions or domains which are both required for full enhancer activity. The first, the octamer domain, contains the octamer sequence ATGCAAAT and is recognized by a specific protein factor present in HeLa cell extracts. The other, the SPH domain, consists of a highly conserved region approximately 24 base pairs (bp) in length which contains DNA sequences crucial for efficient U1 RNA gene expression in the oocyte assay. Transcriptional competition studies between the chicken U1 and U4B snRNA genes reveal that these two snRNA genes require common limiting transcription factors even though the structural organization of their respective enhancers is different. Multiple regulatory elements, which together constitute the transcriptional enhancer, may be a general feature of vertebrate snRNA gene promoters.

MATERIALS AND METHODS

Construction of mutant U1 DNA templates. The U1 RNA gene used in this study was derived from a λ phage clone that contained three chicken U1 RNA genes within a 14-kilobase insert as reported previously (13). A 570-bp *Ava*I-*Mbo*II DNA fragment, which contained the U1-52a gene, was

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subcloned into the *Ava*I-*Hin*cII polylinker sites of pSP65 (Promega Biotech, Madison, Wis.) after the *Mbo*II end was made flush with T4 DNA polymerase. The resulting plasmid, named pU1Δ(−388), contained 388 bp of 5′-flanking DNA, the U1-52a gene, and 28 bp of 3′-flanking DNA. This was the parental plasmid from which the remainder of the U1 constructions were derived. Plasmids containing deletions from the 5′ direction are named by their endpoints within the 5′-flanking DNA of the U1-52a gene.

The mutant construction pU1Δ(−206) was made by digesting the parental plasmid with *Bss*HII restriction endonuclease and recircularizing the plasmid with T4 DNA ligase. This resulted in the deletion of U1 DNA sequences from positions −370 to −206. This plasmid retained 28 bp of upstream U1 DNA sequences (normally found between positions −388 and −371 in the wild-type clone) moved adjacent to position −206. However, since four different chicken U1 RNA genes bear no homology with each other in that region, those sequences were not expected to contribute to U1 RNA gene activity. For simplicity, this plasmid was therefore named pU1Δ(−206).

The remaining mutants were constructed by first digesting the parental plasmid, pU1Δ(−388), with *Hin*dIII endonuclease, which cuts 3′ of the gene in the pSP65 polylinker. This was followed by a second digestion with a restriction endonuclease that cuts within the 5′-flanking DNA of the U1-52a gene. The resulting DNA fragments, which differed in the length of U1 5′-flanking DNA remaining, were subcloned into the *Hin*dIII site and a second appropriate polylinker site of the pUC19 vector. This method generated the following 5′ deletion plasmids: pU1Δ(−246) with *Kpn*I; pU1Δ(−197) with *Sph*I; pU1Δ(−188) with *Bsp*1286; pU1Δ(−90) using *Nla*IV; and pU1Δ(–48) with *Rsa*I. In all these constructions, the U1 gene was cloned into the same transcriptional orientation in pUC19.

The plasmid pU1Δ(−211/−182) contained an internal deletion within the U1 enhancer. It was constructed by digesting pU1Δ(−246) with *Bss*HII and *Sph*I, followed by a very heavy digestion with S1 nuclease (2 U/μl for 30 min) and recircularization with T4 DNA ligase. Sequencing of a plasmid from one of the resultant transformants revealed that the 28 bp of DNA between positions −211 and −182 had been deleted. This construction, named pU1Δ(−211/−182), was used for further analysis.

Oocyte nuclear injection and RNA analysis. Microinjection of DNA and extraction and analysis of RNA were done essentially as described by Yuo et al. (49). Each oocyte was injected with approximately 30 nl of buffer containing 88 mM NaCl, 10 mM Tris chloride (pH 7.5), 400 ng of U1 gene plasmid per μl, 1 ng of *Xenopus* 5S gene plasmid (pXbsF1[15]) per μl, and 8 μCi of [α-^{32}P]GTP (410 Ci/mmol) per μl. For transcriptional competition experiments, the injection mixture also included the plasmid pU4B(Sst), which contains a cloned chicken U4B RNA gene that is efficiently expressed in oocytes (19). When required, this plasmid was added to the injection mixture at a concentration of 40 ng/μl. Following injection, oocytes were incubated in modified Barth saline at 19°C for 18 to 25 h. Surviving oocytes were homogenized in 20 μl of homogenization buffer (0.3 M NaCl, 50 mM Tris chloride [pH 7.5], 1 mM EDTA, 2% sodium dodecyl sulfate, 1 mg of proteinase K per ml) per oocyte and incubated at room temperature for 30 min. RNA was extracted twice with phenol-chloroform (1:1, vol/vol) and precipitated with ethanol.

The RNA pellet was dissolved in loading buffer (99% formamide, 20 mM EDTA), and a sample was run in a 40-cm-long 10% polyacrylamide gel containing 7 M urea. Size markers, consisting of total nuclear RNA isolated from chicken liver, were run in adjacent lanes of the gel. After electrophoresis, the lanes containing the markers were excised and stained with ethidium bromide. The remainder of the gel was subjected to autoradiography (4°C overnight). U1 RNA was quantitated by densitometric scanning of various exposures of the resulting X-ray films.

Gel mobility shift assays. DNA fragments (indicated in Fig. 1 as probes A, B, C, and D) were end labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP and isolated by polyacrylamide gel electrophoresis. The isolated DNA fragments were incubated in a HeLa cell nuclear extract (generously provided by Sophia Tsai, Baylor College of Medicine) and assayed for protein binding by mobility shift analysis essentially as described by Singh et al. (41) with minor modifications. Each binding reaction mixture (20 μl) contained 10 mM Tris chloride (pH 7.5), 5 mM NaCl, 11 mM dithiothreitol, 5% glycerol, 1 mM EDTA, 6.3 μg of poly(dI-dC) · poly(dI-dC), ~0.5 ng (20,000 cpm) of labeled DNA fragment, and 0 to 10 μg of protein from a HeLa cell nuclear extract. After a 30-min incubation at 20°C, the binding reactions were run in a low-ionic-strength 4% polyacrylamide gel (30:1, acrylamide-bisacrylamide) containing 6.7 mM Tris chloride (pH 7.5), 3.3 mM sodium acetate, and 1 mM EDTA. Pre-electrophoresis (40 min) and electrophoresis were carried out at 11 V/cm with constant buffer recirculation. The gel was autoradiographed at 4°C overnight with an intensifying screen. For in vitro competition experiments, the same conditions were used except that the binding reaction mixtures additionally contained 25 or 100 ng of unlabeled competitor DNA fragments which either possessed or lacked the octamer sequence.

DNase I footprint analysis. A DNA fragment for footprint analysis was isolated from a plasmid pU1(−318/3), which contains 318 bp of 5′-flanking DNA and the first 3 bp of the U1-52a gene cloned into the polylinker of pSP64. This fragment, bounded by a polylinker *Bam*HI site (at the 5′ end with respect to U1 gene transcription) and an *Hin*dIII site (at the 3′ end), was isolated by gel electrophoresis. It was labeled at the *Bam*HI site on the nontemplate strand with T4 polynucleotide kinase and [γ-^{32}P]ATP or on the template strand with DNA polymerase I (Klenow fragment) and [α-^{32}P]dGTP. After labeling, the DNA fragment was digested with *Sau*3A, and the 177-bp *Bam*HI-*Sau*3A fragment (containing U1 gene 5′-flanking DNA sequences from position −318 to position −154) was isolated by polyacrylamide gel electrophoresis.

DNase I footprint analysis was performed essentially as described by Singh et al. (41). Each footprint reaction mixture (195 μl) contained 10 mM Tris chloride (pH 7.5), 5 mM NaCl, 11 mM dithiothreitol, 5% glycerol, 1 mM EDTA, 64 μg of poly(dI-dC) · poly(dI-dC), 200,000 cpm of the *Bam*HI-*Sau*3A fragment described above, and 7 μl of HeLa cell nuclear extract (70 μg of total protein). After incubation for 30 min at 20°C, 5 μl of 0.1 M $MgCl_2$ was added, followed by the addition of DNase I to a final concentration of 0.2 to 1.0 μg/ml. Following a 2.5-min digestion at room temperature, EDTA was added to achieve a concentration of 5 mM. The total reaction was loaded on a 4% polyacrylamide low-ionic-strength preparative mobility shift gel. The DNA bands corresponding to the free and bound fragments were excised from the gel and eluted in 0.5 mM sodium acetate (pH 7.5)–0.1% sodium dodecyl sulfate–1 mM EDTA with shaking at 37°C overnight. The supernatants were extracted sequentially with phenol-chloroform-isoamylalcohol (25:24:1,

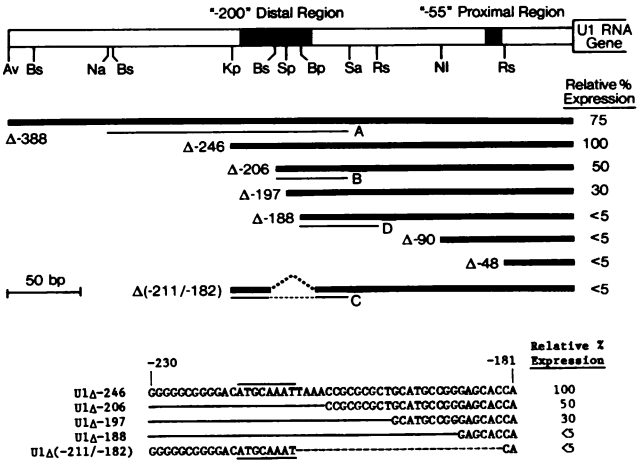

FIG. 1. Deletion mutations used to map the 5'-flanking DNA sequences required for chicken U1 RNA gene expression. The structure of the promoter region of the U1-52a gene is shown at the top. The solid boxes represent two regions of sequence conserved among chicken and other vertebrate snRNA genes. The deletion templates that were constructed are depicted as solid bars in the middle section of the figure. The endpoints of the deletion templates are indicated to the left of the solid bars, and the relative percent expression is shown to the right. The DNA probes used for band shift assays (A, B, C, and D) are indicated as thin lines under the deletion mutants from which they were derived. The DNA sequence of the U1 RNA gene enhancer region and the sequences of deletion mutants that contain endpoints within this region are shown at the bottom. The octamer motif is overlined or underlined. Solid lines represent vector DNA. Dashed lines represent an internal deletion of U1 sequences. Restriction enzyme abbreviations: Av, *Ava*I; Bp, *Bsp*1286; Bs, *Bss*HII; Kp, *Kpn*I; Na, *Nae*I; Nl, *Nla*IV; Rs, *Rsa*I; Sa, *Sau*3A; Sp, *Sph*I.

vol/vol/vol) and chloroform-isoamyl alcohol (24:1, vol/vol) and ethanol precipitated in the presence of yeast transfer RNA as carrier (5 μg/ml). After a reprecipitation step, the products were analyzed in an 8% polyacrylamide gel (19:1, acrylamide-bisacrylamide) in the presence of 8 M urea, followed by autoradiography at −70°C with an intensifying screen. Maxam and Gilbert (30) sequencing ladders of the same DNA fragment were run alongside as DNA sequence markers.

RESULTS

DNA sequence requirements of chicken U1 RNA gene expression. To functionally define the DNA sequences important for chicken U1 RNA gene expression, we prepared a series of deletion mutations in the 5'-flanking DNA of a cloned chicken U1 RNA gene (designated the U1-52a gene in reference 13). The structure of the U1-52a gene promoter and a partial restriction enzyme map are represented at the top of Fig. 1. The two conserved regions of DNA sequence expected to play a role in the expression of chicken U1 RNA genes are indicated by solid boxes. The middle section of Fig. 1 depicts deletion mutations that were constructed by using restriction enzyme sites in the 5'-flanking DNA of the U1 gene. Deletion constructions with endpoints that fall within the "−200" distal conserved region are shown in more detail at the nucleotide level near the bottom of Fig. 1.

The transcriptional activity of the U1 deletion templates was determined by measuring the accumulation of chicken U1 RNA after microinjection of the various constructions into *X. laevis* oocytes together with [α-^{32}P]GTP. A *Xenopus* 5S RNA gene was coinjected as an internal positive control to normalize for the efficiency of injection into the oocyte nuclei. Figure 2 shows typical results obtained with the different U1 templates. The relative transcriptional efficiencies of the various templates used in this set of experiments are summarized in the right-hand column of Fig. 1. Although this is a heterologous expression system, the synthesis of chicken U1 RNA was easily detected from a DNA template consisting of 246 bp of 5'-flanking DNA, the U1 RNA coding region, and 28 bp of 3'-flanking DNA. This U1 template, pU1Δ(−246), accumulated at least 20-fold more U1 RNA than control templates (compare lanes a and f) and was assigned a relative expression efficiency of 100%. A plasmid construction that contained a greater amount of U1 gene 5'-flanking DNA (to position −388) was also efficiently expressed in oocytes, but at only 75% of the level observed with the pU1Δ(−246) template (compare lanes g and h). This result may indicate that DNA sequences between positions −388 and −246 have a minor inhibitory effect on U1 expression. However, since the effect was small and because these DNA sequences are not well conserved among different chicken U1 RNA genes, we have not investigated this possibility further.

A deletion of the 5'-flanking DNA to nucleotide position −206, which removed a GC box and the highly conserved ATGCAAAT octamer sequence, reduced gene expression to approximately 50% of the wild-type level (lanes b and i). This result suggests that the octamer contributes to but is not essential for a reasonably high level of chicken U1 RNA

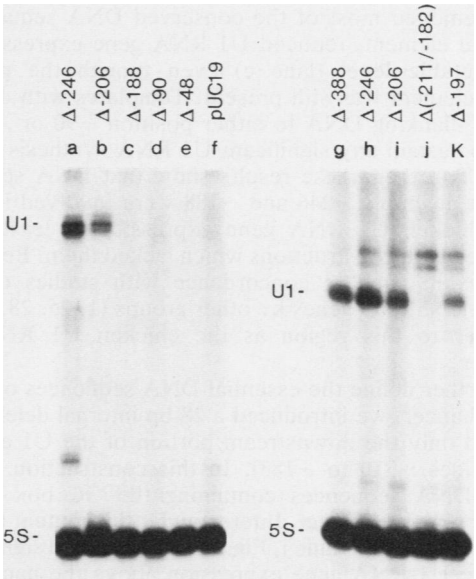

FIG. 2. Expression of U1 RNA gene 5'-flanking DNA deletion templates in *X. laevis* oocytes. Oocytes were injected with the wild-type or mutant templates shown in Fig. 1 and with [α-^{32}P]GTP. Total RNA was isolated 18 to 24 h later, run on 10% polyacrylamide gels, and subjected to autoradiography. The injected plasmids which gave rise to the corresponding RNA samples were pU1Δ(−246) (lanes a and h), pU1Δ(−206) (lanes b and i), pU1Δ(−188) (lane c), pU1Δ(−90) (lane d), pU1Δ(−48) (lane e), pU1Δ(−388) (lane g), pU1Δ(−211/−182) (lane j), and pU1Δ(−197) (lane k). Lane f shows the result obtained when the pUC19 vector, without a U1 insert, was injected. In each case, an *X. laevis* 5S RNA gene plasmid was coinjected to normalize for the efficiency of injection into the oocyte nuclei. Lanes a through f and g through k represent different sets of injection experiments. The bands corresponding to U1 RNA and 5S RNA are indicated.

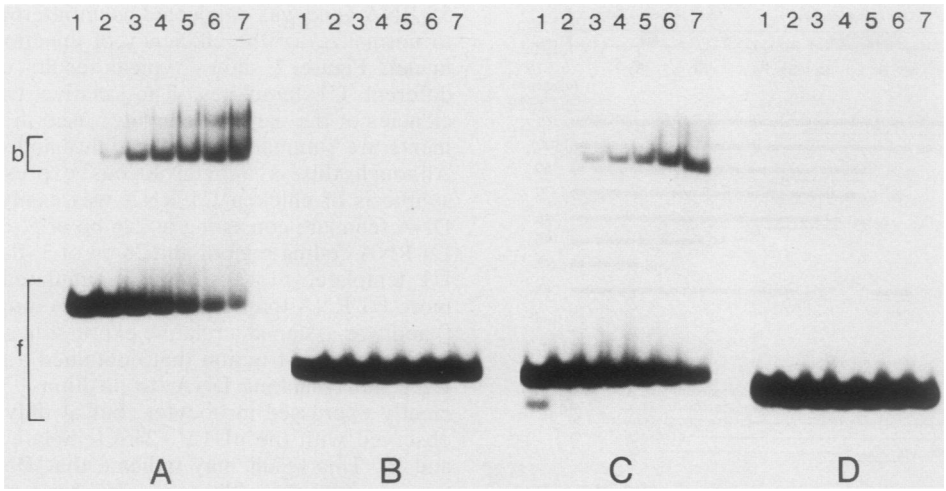

FIG. 3. Binding of a protein factor to the octamer domain of the chicken U1 RNA gene enhancer. A mobility shift assay was used to detect sequence-specific DNA-protein interactions as described in Materials and Methods. The end-labeled DNA fragments indicated as probes A, B, C, and D in Fig. 1 were assayed for protein binding, and the results for each fragment are shown in panels A, B, C, and D, respectively. Each labeled fragment was incubated with increasing amounts of HeLa cell extract and analyzed in low-ionic-strength polyacrylamide gels. The probes used contained U1 enhancer sequences as follows: (A) wild-type enhancer; (B) octamer domain deleted, SPH domain present; (C) octamer domain present, SPH domain deleted; and (D) both domains deleted. In each panel, lanes 1 through 7 contained 0, 1.5, 3.0, 4.5, 6.0, 7.5, or 10.0 μg of HeLa cell extract, respectively. The mobilities of the protein-bound (b) and protein-free (f) DNA bands are indicated.

gene expression in oocytes. The removal of an additional 9 bp to position −197 further decreased expression to about 30% (lane k). Interestingly, a 5′ deletion to position −188, which removed most of the conserved DNA sequences of the distal element, reduced U1 RNA gene expression to a nondetectable level (lane c) even though the proximal "−55" element was still present. Templates with deletions of the 5′-flanking DNA to either position −90 or −48 also failed to sustain any significant U1 RNA synthesis (lanes d and e). Together, these results show that DNA sequences between positions −246 and −188 were involved in stimulating chicken U1 RNA gene expression at least 20-fold compared with constructions which lacked them. Because of these results, and in concordance with studies on other vertebrate snRNA genes by other groups (1, 25, 28, 33), we will refer to this region as the chicken U1 RNA gene enhancer.

To further define the essential DNA sequences of the U1 gene enhancer, we introduced a 28-bp internal deletion that removed only the downstream portion of the U1 enhancer (nucleotides −210 to −183). In this construction, the upstream DNA sequences containing the GC box and the octamer remained intact. Interestingly, this mutant template [pU1Δ(−211/−182); lane j, Fig. 2] was unable to significantly stimulate U1 RNA gene expression above the background level. Together, the results shown in Fig. 1 and 2 indicate that the chicken U1 enhancer is composed of at least two functional subregions which are both required to fully activate U1 RNA gene expression. The first enhancer subregion, termed the octamer domain, consists of a GC box and the conserved octamer sequence ATGCAAAT. The other enhancer subregion, termed the SPH domain (for *Sph*I postoctamer homology), consists of a second conserved region about 24 bp in length located immediately downstream of the octamer. The two enhancer domains, however, were not functionally equivalent when assayed in the oocyte expression system. The SPH domain, consisting of DNA sequences 3′ of position −206, was able to enhance U1 RNA synthesis at least 10-fold even in the absence of the octamer domain (Fig. 2, lanes b and i). In contrast, the octamer domain (sequences upstream of −210), although required for full U1 RNA gene activity, was unable to significantly stimulate expression in the absence of the DNA sequences of the SPH domain.

The octamer domain of the U1 enhancer interacts with a sequence-specific DNA-binding factor. Since eucaryotic gene expression is mediated through the interaction of *trans*-acting factors with *cis*-acting DNA regulatory elements, we made use of a gel mobility shift assay to detect a factor that interacted with the U1 enhancer. The DNA fragments indicated in Fig. 1 as probes A, B, C and D were endlabeled, incubated with increasing amounts of HeLa cell extract, and analyzed for retarded electrophoretic mobility in native polyacrylamide gels. An excess of poly(dI-dC) · poly(dI-dC) was added to all reaction mixtures to compete for nonspecific DNA-binding proteins. Figure 3 shows the results of mobility shift assays with four different DNA fragments. One of these fragments (Fig. 3A) contained all of the DNA sequences of the wild-type U1 enhancer. Fragments B and C specifically lacked either the octamer domain (panel B) or the SPH domain (panel C). The fourth DNA fragment (panel D) lacked most of the evolutionarily conserved DNA sequences of the chicken U1 enhancer region. Fragments A and C, which contained the octamer domain, were recognized by a specific protein factor, as evidenced by an altered electrophoretic mobility (panels A and C). On the other hand, DNA fragments B and D, which lacked the octamer domain, failed to exhibit retarded electrophoretic mobility under identical conditions. This suggests that a factor specifically recognizes DNA sequences within the octamer domain.

To ensure that the observed protein-DNA interaction was indeed specific for the octamer domain, we compared the ability of the different DNA fragments to compete with the wild-type fragment for binding the HeLa factor in the mobility shift assay. Unlabeled DNA fragments A and C, which contained the octamer sequence, were very effective competitors for factor binding, whereas DNA fragments B

and D, which lacked the octamer sequence, were not efficient competitors (data not shown). Together, these band shift analyses localized the region of factor binding to DNA sequences between positions −246 and −211 near or within the chicken U1 RNA gene enhancer.

The DNA-binding factor specifically recognizes the octamer sequence ATGCAAAT. To identify the specific nucleotides interacting with the HeLa factor, a DNase I footprint experiment was performed. A DNA fragment encompassing the enhancer region of the U1 RNA gene was labeled on either the nontemplate strand or the template strand as described in Materials and Methods. After incubation with HeLa cell extract, the DNA fragment was subjected to limited DNase I digestion. Protein-bound and free DNA fragments were separated on a mobility shift gel, excised, and analyzed in sequencing gels. Maxam and Gilbert A+G and C+T reactions were run alongside as sequence markers.

Figure 4 shows the results for both the nontemplate strand (panel A) and the template strand (panel B). The arrows point out a total of five DNA bands that were fully protected from DNase I digestion. Of these, four corresponded to nucleotides within the octamer sequence and the remaining one was located only three nucleotides downstream of the octamer. In addition, some of the other DNA bands within the octamer exhibited a pattern of partial protection. Since this was the only region in the DNA fragment over which protection was observed, the results clearly indicate that the HeLa cell factor detected by the DNA-binding assay was specifically recognizing the octamer sequence in the U1 RNA gene enhancer. Other groups have shown that a HeLa cell factor recognizes the identical octamer sequence upstream of immunoglobulin, histone H2B, and U2 RNA genes (6, 31, 41, 43). Presumably, the same or a related factor is involved in the expression of U1 RNA genes.

DNA sequences in the U1 enhancer are required for transcriptional competition with the U4B snRNA gene. In the experiments described above, we detected a factor that bound to the octamer domain of the U1 RNA gene enhancer. When vertebrate U1 and U2 RNA genes are coinjected into oocytes, they compete for expression (1, 28, 47). Presumably, the U1 and U2 RNA genes compete for a similar set of limiting transcription factors. Undoubtedly, one possible factor is the octamer-binding protein. Nevertheless, the transcriptional assays shown in Fig. 2 clearly demonstrate that additional DNA sequences immediately downstream of the octamer were also essential elements of the U1 enhancer. It seems likely that these DNA sequences of the SPH domain are also recognition sites for specific transcription factors even though such interactions were not detected in the band shift and footprinting assays with extracts from HeLa cells.

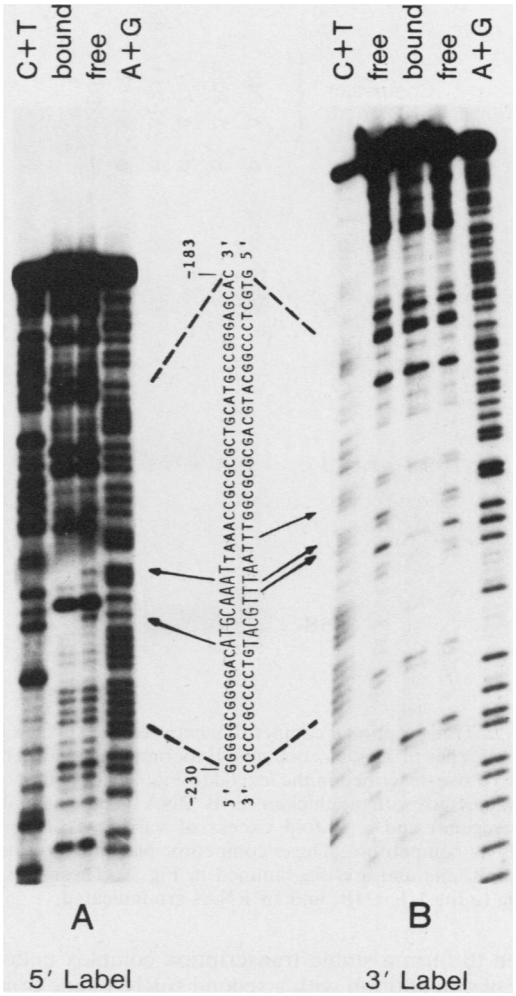

FIG. 4. DNase I footprint analysis of the interaction of a factor with the octamer sequence of the chicken U1 RNA gene enhancer. An end-labeled DNA fragment containing the U1 RNA gene enhancer region was labeled on either the nontemplate strand (A) or the template strand (B), incubated with HeLa cell extract, and treated with DNase I as described in Materials and Methods. The DNA sequence shown between the two autoradiogarms is the U1 enhancer region from nucleotide positions −230 to −183. The arrows point out five fully protected DNA bands corresponding to two nucleotides in the nontemplate strand and three nucleotides in the template strand. The octamer sequence is depicted in large capital letters.

To further define the relationships between the enhancer domains of the snRNA genes, we performed a series of transcriptional competition experiments between the U1 RNA gene and a chicken U4B snRNA gene recently characterized in our laboratory (19). In the experiment shown in Fig. 5, the U4B RNA gene was coinjected into oocytes with a 10-fold excess of either the wild-type U1 template or various U1 deletion templates. The wild-type U1 template, pU1Δ(−246), which contained an intact enhancer, was itself efficiently expressed and was able to substantially reduce the expression of the U4B test gene (lane a). A U1 template, pU1Δ(−206), which lacked the octamer domain, although not itself expressed in the competition assay, was almost as effective as the wild-type template in its ability to inhibit U4B RNA gene expression (lane b). This result indicates that the octamer domain was not essential for a reasonable level of competition in the oocyte assay. In contrast, the U1 template pU1Δ(−197), which had the 5′ portion of the SPH domain deleted, was neither detectably expressed nor able to act as an efficient transcriptional competitor (lane c). U1 templates that had the remaining portion of the SPH domain deleted also failed to appreciably inhibit U4B RNA gene expression (lanes e and f). Interestingly, of the various U1 templates assayed, only the wild-type U1 plasmid was detectably expressed in the competition experiments. In contrast, when injected alone, the U1 templates with deletions to positions −206 and −197 were expressed at 50 and 30% of the wild-type activity, respectively (Fig. 2, lanes i and k). These results suggest that an intact U1 enhancer is

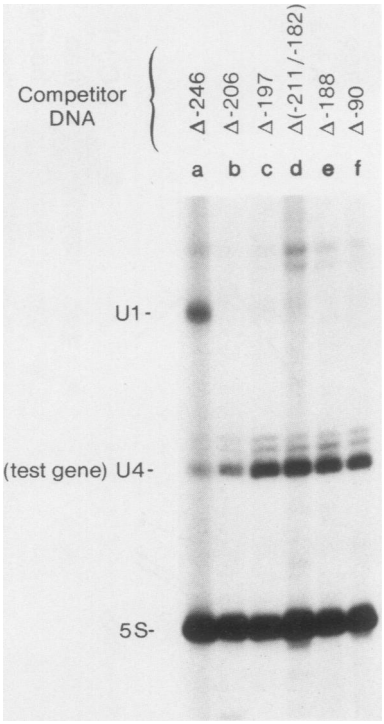

FIG. 5. Transcriptional competition between the chicken U1 and U4B RNA genes in *Xenopus* oocytes. Experimental conditions were similar to those described in the legend to Fig. 2 except that oocytes were coinjected with a chicken U4B RNA gene plasmid (as a reference gene) and a 10-fold excess of various U1 RNA gene plasmids as competitors. These competitor plasmids are indicated above each lane and are diagrammed in Fig. 1. The bands corresponding to the U1, U4B, and 5S RNAs are indicated.

required to form a stable transcription complex under conditions of competition with a second snRNA gene transcription unit.

We also examined the competitive ability of the internal deletion mutant pU1Δ(−211/−182), which lacked the SPH domain of the enhancer but retained the octamer domain. This U1 template was unable to significantly inhibit U4B gene expression (Fig. 5, lane d). A possible explanation is that the octamer-binding protein is not limiting in oocytes under our experimental conditions. An alternative explanation (favored by the data in Fig. 2) is that the octamer motif is not absolutely required for a relatively high level of enhancer activity. In contrast, the enhancer sequences of the SPH domain were able to appreciably inhibit U4B gene expression in the absence of the octamer domain, and these sequences were required for both expression and competition in the oocyte assay. This suggests that there is a limiting transcription factor (or factors) in oocytes that interacts with the SPH domain of the chicken U1 enhancer.

DISCUSSION

The distal conserved region has a greater than 20-fold stimulatory effect on chicken U1 RNA gene expression. We reported previously that four distinct copies of the chicken U1 RNA gene contain two regions of evolutionarily conserved sequence in their 5′-flanking DNA: a proximal region located from positions −59 to −1 and a distal region spanning approximately 50 bp near position −200 (13). Those findings implied that DNA sequences contained within those two separate regions constitute important control elements of the chicken U1 RNA gene promoter. Using a frog oocyte expression system, we have now demonstrated that DNA sequences within the distal conserved region (−230 to −183) are essential for efficient transcriptional activity of the chicken U1-52a RNA gene. When DNA was deleted from the 5′ direction to position −188, the transcriptional activity of the gene was reduced to a level no longer detectable above the background of the system. Since this corresponded to less than 5% of the wild-type activity, DNA sequences within the distal conserved region have at least a 20-fold stimulatory effect on chicken U1 RNA gene expression in the heterologous oocyte system. The presence of such an enhancer element near position −200 appears to be a general feature of vertebrate snRNA gene promoters (1, 25, 28, 33, 44, 47).

The analysis of a series of deletion mutations within the −200 region further revealed that there was a stepwise decrease in transcriptional activity as DNA sequences within the U1 enhancer were removed in a 5′ to 3′ direction. This indicated that enhancer activity was spread throughout the conserved distal region. Perhaps the most unexpected finding was that the deletion of a highly conserved motif, the octamer sequence ATGCAAAT, had only a minor effect (ca. twofold) on the ability of the enhancer region to activate chicken U1 RNA gene expression when assayed in oocytes. This octamer motif, or a close variant of it, is a highly conserved feature of the enhancer regions of vertebrate snRNA genes (13, 19, 20, 22, 24, 25, 28, 33, 45), and it is also a regulatory component involved in the expression of a number of cellular and viral transcription units (8, 14, 27, 36, 39, 42, 50). In several instances, it has been demonstrated to be a recognition site for a sequence-specific DNA-binding protein (or family of proteins) present in extracts prepared from a variety of sources (6, 16, 31, 41, 43, 48). Our results indicate that the octamer motif, in the context of the chicken U1 RNA gene enhancer, also can be recognized by a protein factor present in HeLa cell extracts. Although we have not pursued the characterization of this protein, it seems very likely that we have detected the same ubiquitous factor reported by the other groups.

The U1 enhancer contains multiple functional motifs. Earlier comparisons had revealed that DNA sequences were highly conserved among four different chicken U1 RNA genes for approximately 25 to 30 bp immediately downstream of the octamer motif (13). The functional importance of these additional conserved DNA sequences is evident from the results presented in Fig. 2. These downstream DNA sequences enhanced chicken U1 RNA gene expression at least 10-fold even in the absence of the octamer, whereas the reciprocal construction, which retained the octamer but lacked the downstream enhancer sequences (i.e., the SPH domain), was transcriptionally inactive. These results indicate that snRNA gene enhancers, like mRNA gene enhancers, are composed of multiple functional motifs which act synergistically to fully activate gene expression.

However, our results further suggest that the octamer and SPH domains are not functionally equivalent in the chicken U1 RNA gene enhancer; the conserved enhancer sequences 3′ of the octamer appear to play a more important role in stimulating expression of the chicken gene in *X. laevis* oocytes (Fig. 1 and 2). This idea is supported by the transcriptional competition studies shown in Fig. 5. A construction which retained the SPH domain but lacked the octamer motif was still an effective competitor, as demonstrated by its ability to inhibit expression of a coinjected

U4B snRNA gene. In contrast, a construction which lacked the SPH domain but retained the octamer motif was not an efficient competitor, even though the octamer motif in this construction was capable of being recognized in vitro by a DNA-binding protein present in HeLa cell extracts (Fig. 3). Although these results were obtained in heterologous systems, the most direct interpretation of the results is that the octamer and SPH domains act in concert to promote the formation of a stable transcription complex. The importance of DNA sequences 3′ of the octamer motif is supported by a recent study of the human U1 RNA gene promoter carried out by Murphy et al. (33). Their data suggested that the human U1 octamer motif, like the analogous chicken motif, is functionally inactive in the absence of downstream enhancer sequences. This indicates that the presence of multiple functional motifs may be a general feature of vertebrate snRNA gene enhancers. Our results are consistent with a model in which the ubiquitous octamer-binding protein does not act by itself to directly stimulate transcription, but rather facilitates the stable binding of additional transcription factors to the enhancer region (16).

In the case of the chicken U1 and U4B RNA genes, a prominent feature of the SPH domain of both enhancers is the existence of a recognition site for the restriction enzyme *Sph*I. The removal of the *Sph*I site (plus three additional nucleotides 3′ of the *Sph*I recognition site) reduced chicken U1 RNA gene expression from 30% of the wild-type activity to a nondetectable level (<5%) (lane c versus lane k in Fig. 2). In the case of the chicken U4B RNA gene, we have recently shown that the activity of the U4B RNA gene enhancer was abolished by a 5-bp deletion of DNA sequences at an *Sph*I site located 12 bp downstream of the octamer motif (K. J. McNamara, R. J. Walker, K. A. Roebuck, and W. E. Stumph, submitted for publication). Interestingly, a similar deletion of DNA sequences at an *Sph*I site within the human U2 RNA gene enhancer likewise eliminated the enhancer function of that gene (1, 25). However, in the human U2 RNA gene the *Sph*I site and the octamer sequence partially overlap (GCATGCAAAT), so that any potentially distinct functions of the two motifs could not be independently evaluated in that particular experiment.

The existence of partially overlapping *Sph*I and octamer motifs is not an uncommon occurrence in the enhancer regions of eucaryotic transcription units (e.g., simian virus 40 [12], the chicken β-globin gene [8], and the human U2 RNA gene [1, 25]). In other cases, an octamer sequence and a site recognized by the *Sph*I restriction endonuclease are found in proximity to each other, but are nevertheless distinct entities (e.g., within the enhancers for chicken U1, chicken U4B, and human U3 RNA genes [13, 19, 45]). The *Xenopus* U1B RNA gene enhancer is a particularly interesting case in that there exist three tandem *Sph*I restriction sites, with the terminal one partially overlapping an octamer sequence (9, 11). This structural arrangement may make the *Xenopus* U1B enhancer unusually simplified and compact (11).

When isolated as solitary motifs, DNA sequences within viral enhancers appear to lack significant activity (16, 18, 35, 39, 50). However, when duplicated or combined with other enhancer motifs, activity can be restored (16, 18, 35, 39, 50). Our data are consistent with a similar structural requirement for snRNA gene enhancers. The multiple functional motifs in the enhancers of snRNA genes may provide a mechanism to ensure that these housekeeping-type genes are adequately expressed in a wide variety of cell types. Because individual

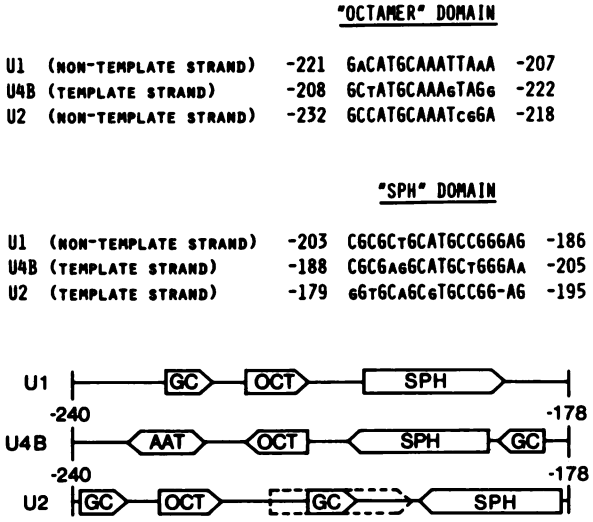

FIG. 6. Structural organization of chicken U1, U4B, and U2 RNA gene enhancers. A DNA sequence comparison between the octamer domains and between the SPH domains of the chicken U1, U2, and U4B snRNA genes is shown at the top. The nontemplate and template strands are compared as indicated, and the numbers designate the nucleotide positions relative to the transcriptional initiation sites. The DNA sequences shown are about 75 to 80% homologous among the genes. Nucleotide mismatches are shown as small capital letters. Note that most of the mismatches maintain purine-purine or pyrimidine-pyrimidine conservation. The lower part of the figure schematically diagrams the structural organization of sequence motifs within the enhancer regions of the three genes. Note that the octamer and SPH domains occur in the same relative order but can occur in different positions and orientations relative to one another. Also shown are the locations of four GC box core hexamers (GGGCGG), which are potential binding sites for the transcription factor Sp1 (21). The double-pointed box at the 5′ end of the U4B enhancer stands for a 10-bp dyad symmetry (containing a central *Aat*II restriction endonuclease site) which is perfectly conserved in DNA sequence at a similar location upstream of a human U4 RNA gene (2). The dashed box in the U2 enhancer represents an SPH-like homology in which the *Sph*I site has been replaced with a GC box. For the sequence of this region of the U2 enhancer, see reference 22. Abbreviations: GC, GC box; OCT, octamer motif; SPH, *Sph*I postoctamer homology; AAT, *Aat*II homology.

enhancer motifs can have different cell type specificities (12, 16, 35, 39), the relative importance assigned to the various motifs in snRNA gene enhancers may depend on the type of cell used to measure enhancer activity. In the oocyte expression system used in the present study, the SPH domain of the chicken U1 RNA gene enhancer seems to play the predominant role in elevating the expression of the gene. If a homologous expression system were used, it is conceivable that the relative activity of the two enhancer domains could differ from that observed in frog oocytes. Experiments in a homologous system will be required to satisfactorily address this question.

Chicken snRNA gene enhancers contain independently inverted functional domains. Our data have revealed that the chicken U1 and U4B RNA genes compete for a similar set of transcription factors (this paper; McNamara et al., submitted). We previously pointed out that the octamer motifs within the enhancers of the chicken U1 and U4B RNA genes have an inverted orientation (i.e., they are on opposite DNA strands) (19). Likewise, closer inspection reveals that the downstream subregions of the U1 and U4B enhancers also have the greatest homology (~80%) when opposite DNA

strands are compared. Figure 6 points out these features. The DNA sequence comparisons shown in the upper part of the figure indicate that the sequences of the octamer domain and of the SPH domain (which is partially composed of the *Sph*I motif) are both inverted in the U4B RNA gene enhancer relative to their orientations in the U1 RNA gene enhancer. Interestingly, the chicken U2 RNA gene also contains both an octamer motif (in the same orientation as the U1 octamer) and an SPH homology (in an inverted orientation relative to U1) (Fig. 6). Although the chicken U2 RNA gene does not contain an *Sph*I site in its enhancer region, it does contain the closely related DNA sequence GCGTGC. The lower part of Fig. 6 schematically diagrams the structural arrangements of various sequence motifs in the chicken U1, U2, and U4B RNA gene enhancers. In this model, the multiple sequence motifs are not restricted to any particular position or orientation within the enhancer region but can function in a variety of configurations.

The data of Fig. 3 and 4 demonstrate the existence of a factor present in HeLa cell extracts that binds sequence-specifically to the octamer motif in the U1 enhancer. Under identical conditions, we were unable to detect any DNA-binding activities that specifically recognized DNA sequences within the SPH domain. Several reasons may account for this. For example, the putative proteins that bind to the SPH domain of the enhancer may be of much lower abundance than the ubiquitous octamer-binding protein. Alternatively, they may require different conditions for binding, or they may be less stable than the octamer-binding factor. Finally, the factors may be absent from or inactive within HeLa cells, and it may be necessary to isolate these proteins from homologous chicken cell extracts. We are currently investigating these possibilities. In conclusion, the enhancers of snRNA genes, like viral and cellular mRNA enhancers (18, 39, 40, 50), appear to be composed of multiple sequence motifs which can function in a variety of *cis*-linked configurations. To further understand the relationship between the enhancer domains, it will be necessary to introduce more defined mutations into the various functional motifs of the enhancer and to purify and characterize the DNA-binding proteins that act individually and together to activate snRNA gene expression.

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