Transcriptional signals of a U4 small nuclear RNA gene

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ABSTRACT

The signals controlling the expression of a chicken U4 small nuclear RNA (snRNA) gene have been studied by microinjection into *Xenopus* oocytes. At least two distinct regions in the 5'-flanking DNA contribute to U4B RNA gene expression. The proximal regulatory element, which is inactivated by a 5'-flanking DNA deletion to position -38, provides a basal level of U4B RNA synthesis. The distal regulatory region, centered near position -200, acts as a transcriptional enhancer. It provides a 4-5 fold stimulation of U4B RNA gene expression above the basal level, and, like mRNA enhancers, is composed of multiple functional motifs. One of these, the octamer sequence ATGCAAAG, has previously been recognized as an important element of U1 and U2 snRNA gene enhancers, as well as being involved in the expression of a number of mRNA genes. However, the octamer sequence is not sufficient for U4B enhancer activity. An additional element, an "Sph motif," is located 12 base pairs downstream of the octamer and is an essential component of the U4B enhancer. Transcriptional competition studies indicate that the U4B and U1 snRNA genes utilize a common set of transcription factors.

INTRODUCTION

The small nuclear RNAs (snRNAs) of the U-family are evolutionarily conserved RNAs present in the nuclei of eukaryotic cells. They exist in vivo as components of small nuclear ribonucleoproteins (snRNPs). The U1, U2, U4, U5, and U6 snRNPs are involved in the splicing of mRNA precursors (1-6). With the exception of U6 RNA, the snRNAs are synthesized by an RNA polymerase II-like activity (7-11). However, they are not polyadenylated and they have a 2,2,7-trimethylguanosine cap. Moreover, the genes that encode the snRNAs contain no introns and they lack the TATA and CCAAT regulatory elements commonly found upstream of mRNA transcription units.

Nevertheless, vertebrate U1 and U2 RNA genes contain two conserved regions in their 5'-flanking DNAs which are required for proper gene expression (12-18). The proximal element, located near position -55 with respect to the RNA cap site, is analogous to a TATA box in fixing the site of transcriptional initiation (12,16,19). The distal element, located near

position -200, functions as a transcriptional enhancer (15,17,19-21).

Our group has recently described the cloning and characterization of two chicken U4 RNA genes (11). One of these, the chicken U4B RNA gene, is efficiently expressed when microinjected into frog oocytes (11). The other chicken U4 RNA gene, denoted the U4X gene, is not efficiently expressed in frog oocytes (11) although it is expressed in vivo in the chicken (Korf and Stumph, unpublished data). DNA sequence comparisons suggested that the U4 RNA genes also contain distal and proximal promoter elements analogous to those of the U1 and U2 RNA genes (11). Although U1 and U2 enhancers are structurally similar and highly homologous to each other (18), the analogous U4 enhancer regions appear to have an altered sequence arrangement (11). Interestingly, the altered arrangement of the chicken U4 enhancer is evolutionarily conserved in human U4 RNA genes (22).

In experiments described in this paper, we have used the frog oocyte expression system to investigate the functional significance of the distal and proximal promoter elements of the chicken U4B RNA gene. We find that the distal enhancer region is composed of multiple functional motifs which together are responsible for a 4-5 fold stimulation of U4B RNA gene expression. Expression of the U4B gene is reduced to a non-detectable level when both the enhancer region and the proximal regulatory element are deleted. Competition studies reveal that the chicken U1 and U4B RNA gene enhancers, although arranged differently with respect to sequence motifs, compete for a (set of) transcription factor(s) that is limiting in oocytes.

MATERIALS AND METHODS

Plasmid Constructions

The parental plasmid containing the chicken U4B RNA gene has been previously described (11). Briefly, a 698 base pair (bp) SstI fragment, which contained the U4B RNA gene, 280 bp of 5'-flanking DNA, and 283 bp of 3'-flanking DNA, was cloned into the SstI site of pUC19. This plasmid, previously named pU4B(Sst) (11), will be referred to in this paper as pU4BΔ(-280), based upon its content of 5'-flanking DNA. In this plasmid, the direction of transcription of the U4B RNA gene is the same as that of the bacterial lac Z gene in the vector. In all deletion mutant plasmids derived from this clone, the gene was maintained in the same transcriptional orientation.

Two sets of plasmids containing deletions in the 5'-flanking DNA of the U4B RNA gene were constructed from the parental plasmid described above. Members of the first set of deletions were made by digesting pU4BΔ(-280) with

a single restriction enzyme that cut within the 5'-flanking DNA of the U4B RNA gene and once further upstream within the polylinker of pUC19. In each case the larger fragment, lacking a specific amount of 5'-flanking DNA, was re-ligated using T4 DNA ligase and used to transform *E. coli* TBl cells. This strategy was used with the enzymes SphI, KpnI, and SmaI to generate the deletion mutants pU4BΔ(-199), pU4BΔ(-117), and pU4BΔ(-38) respectively.

The second set of deletions was designed to localize the functional motifs of the U4B enhancer. These constructions were made by first isolating a 1120 bp AatII-AatII fragment from the parental plasmid (pU4BΔ[-280]). This fragment contained sequences extending from position -227 in the U4B gene 5'-flanking DNA to the Aat II site at position 2617 in pUC19. After blunting the ends with T4 DNA polymerase, it was ligated into a similarly blunted 2215 bp vector fragment derived by digesting pUC19 with AatII and SphI. This procedure generated the clone pU4BΔ(-227) which lacks AatII and SphI sites in the vector sequences.

Two clones containing short internal deletions within the enhancer region were subsequently constructed from the above plasmid. The pU4BΔ(-200/-194) construction was prepared by digesting pU4BΔ(-227) with SphI and blunting the ends with T4 DNA polymerase; whereas pU4BΔ(-190/-183) was prepared by digesting first with NotI restriction endonuclease followed by S1 nuclease to remove the 5'-protruding ends. After recircularization with T4 DNA ligase, the mutant plasmids were used to transform *E. coli*. All deletion mutations were confirmed by direct DNA sequencing of the double stranded plasmids using the enzymatic termination method of Sanger (23).

For the transcriptional competition experiments, other plasmids containing the chicken U4B and U4X genes were constructed as shown in Figure 4 and described in its legend. The chicken U1 RNA gene used as a reference gene in the competition experiments was the U1-52a gene (13). Its construction is described elsewhere (24). The pSV2AC plasmid, which contains the SV40 enhancer and bacterial chloramphenicol acetyl transferase gene, but lacks the SV40 early promoter and origin of replication (25), was obtained from Suresh Subramani of the University of California, San Diego.

Oocyte Nuclear Injections and RNA Analysis

Microinjection of plasmid DNA into *Xenopus laevis* oocytes, extraction of RNA from pooled oocytes, and the analysis of the labeled RNA in denaturing polyacrylamide gels were performed as previously described (11). In all injections a *Xenopus laevis* 5S RNA gene was coinjected as a positive internal control to normalize for the efficiency of injection into the oocyte nuclei.

Each oocyte was injected with 30 nl of buffer containing 88 mM NaCl, 10 mM Trischloride (pH 7.5), 400 ng of U4B gene plasmid per ul, and 7 uCi of [α - 32 P]GTP (410 Ci/mmol) per ul. For the transcription competition experiments, the injection mixture contained the above components except that the U1 RNA reference gene plasmid was included at a concentration of 40 ng per ul, and the competitor DNAs were at a concentration of 320 ng per ul. The relative transcriptional efficiencies of the various constructions were determined by densitometry of autoradiographic films exposed for varying periods of time. The numbers obtained are estimated to be accurate to within $\pm 10\%$.

Gel Mobility Shift Assays

A 168 bp fragment was isolated for mobility shift analysis by digestion of pU4B Δ (-280) with Asp718 (Boehringer Mannheim, Indianapolis, IN). This fragment contains the 5'-flanking DNA of the U4B RNA gene from position -117 to position -280 (and extends 10 bp further to the Asp718 site in the polylinker of pUC19). The fragment was labeled using T4 polynucleotide kinase and [γ - 32 P]ATP. Aliquots were then digested with one of four different restriction enzymes (AatII, HaeII, SphI, or NotI) that cut near or within the enhancer region. All eight labeled fragments resulting from the digestions (see Figure 3) were isolated and used for mobility band shift assays to detect sequence-specific DNA-binding proteins in extracts prepared from HeLa cells. (The HeLa cell extracts were a generous gift of Sophia Tsai, Baylor College of Medicine). The band shift assays were performed essentially as described by Singh et al. (26) with minor modifications. Each binding reaction (20 ul) contained 10 mM Trischloride (pH 7.5), 50 mM NaCl, 11 mM dithiothreitol, 5% glycerol, 1 mM EDTA, 6.3 ug (poly dI-dC)·(poly dI-dC) (Pharmacia), approximately 0.5 ng (20,000 cpm) 32 P labeled fragment, and 0 to 10 ug of protein from a HeLa cell nuclear extract. After a 30-minute incubation at 20°C, the binding reactions were run on low ionic strength 4% polyacrylamide gels (30:1 acrylamide:bis) containing 6.7 mM Trischloride (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.

As a positive control for binding, a 178 bp fragment containing the enhancer region of the chicken U1-52a RNA gene was employed. We have shown by band shift analysis and DNase I footprinting that HeLa cell extracts contain a factor that recognizes the octamer sequence ATGCAAAT located within the enhancer region of the U1 RNA gene (24). This U1 gene-derived fragment

Fig. 1 - Deletion mutations used to map the 5'-flanking DNA sequences required for chicken U4B RNA gene expression.

The structure of the promoter region of the U4B gene is diagrammed at the top of the figure. 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 end-points of the deletion templates are indicated to the left of the solid bars, and the relative % expression is shown to the right. The DNA sequence of the U4B RNA gene enhancer region and the sequences of deletion mutants that contain endpoints within the enhancer region are shown at the bottom of the figure. The octamer motif is overlined and underlined. Solid lines represent vector DNA. Dashed lines represent internal deletions of U4B sequences. Restriction enzyme abbreviations: Ss, SstI; Aa, AatII; Sp, SphI; No, NotI; Kp, KpnI; Sm, SmaI.

(20,000 cpm, ~ 0.5 ng) was incubated with 7 ug of the HeLa extract and run in a lane of the band shift gel adjacent to each of the experimental assays. The band shift gels were autoradiographed at 4°C overnight.

RESULTS

Cis-acting DNA Sequences Required for Chicken U4B RNA Gene Expression

To define the locations of the cis-acting DNA sequences required for the functional activity of the chicken U4B RNA gene promoter, a series of deletion mutants in the 5'-flanking DNA were constructed. A representation of the U4B gene promoter and a map showing pertinent restriction enzyme sites are shown at the top of Figure 1. The two regions of the promoter depicted as solid boxes are homologous to sequences required for the proper expression of

Fig. 2 - Expression of U4B RNA gene 5'-flanking DNA deletion templates in *Xenopus laevis* oocytes.

Oocytes were injected with the wild type or mutant templates shown in Figure 1 and with [α - 32 P]GTP. Total RNA was isolated 20h later, run on 8% polyacrylamide gels, and subjected to autoradiography. The injected plasmids which gave rise to the corresponding RNA samples are shown above each of the respective lanes: pU4B Δ (-280), lanes a, f, and g; pU4B Δ (-199), lane b; pU4B Δ (-117), lane c; pU4B Δ (-38), lane d; pU4B Δ (-227), lane h; pU4B Δ (-200/-194), lane i; pU4B Δ (-190/-183), lane j. Lane e shows the result obtained when the pUC19 vector, without a U4B 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 j represent different sets of injection experiments. The bands corresponding to U4 RNA and 5S RNA are indicated. The light band that appears between the U4 and 5S RNA bands is an RNA polymerase III transcript that is derived from the 3'-flanking DNA of the U4B RNA gene (unpublished data).

vertebrate U1 and U2 RNA genes. The center section diagrams the deletion mutants constructed. Deletions contained within the -200 distal region are shown at the nucleotide level at the bottom of the figure. The transcriptional activity of each deletion mutant was assayed by microinjection of the plasmid constructions into *X. laevis* oocytes together with [α -³²P]GTP. The accumulation of chicken U4B RNA was assayed by electrophoresis of the labeled RNA products in denaturing polyacrylamide gels. The expression level of a co-injected 5S RNA gene was used to normalize for the efficiency of injection into the oocyte nuclei.

The first set of promoter mutants studied contained 5' serial deletions extending over a large part of the promoter region of the U4B RNA gene. The results of those injections are shown in the left panel of Figure 2. The DNA template containing 280 bp of 5'-flanking DNA (lanes a and f) was considered to have wild type activity (11) and was assigned an expression efficiency of 100%. The right-hand column of Figure 1 shows the relative expression efficiency of each of the deletion templates.

A deletion of sequences to position -199 (Figure 2, lane b) reduced U4B RNA gene expression to approximately 27% of the wild type activity. Thus, DNA sequences between positions -280 and -199 are required for an approximately 4-fold enhancement of U4B gene activity. One prominent element that is deleted in this construction is a variant (ATGCAAAG) of the so-called octamer sequence (26-28). The position of this octamer motif is indicated by overlining and underlining in the sequence diagram at the bottom of Figure 1.

A more extensive deletion of sequences to position -117 had no further effect on gene activity (lane c), indicating that sequences between positions -199 and -117 are insufficient for enhancing U4B gene expression. A template which contained only 38 bp of 5'-flanking DNA was incapable of being expressed at a detectable level in frog oocytes (lane d). This result indicates that DNA sequences between positions -117 and -38 are required for a detectable level of U4B RNA gene expression. Although we have not functionally further localized these essential sequences, this last template lacks the proximal -55 regulatory element which is a highly conserved feature of chicken and other vertebrate snRNA genes and is required for accurate initiation of snRNA transcription (12,16,19).

To localize the functional sequences in the distal -200 region that are required for enhancer activity, a second set of deletion templates were assayed. Typical results are shown in the right-hand panel of Figure 2. A template with 227 bp of 5'-flanking DNA had full transcriptional activity (compare lanes g and h), indicating that DNA sequences upstream of position

Fig. 3 - Mobility shift assay demonstrating the binding of a factor to the octamer region of the chicken U4B RNA gene enhancer.

The diagram in the center of the figure represents a DNA fragment containing the U4B gene 5'-flanking DNA sequences between positions -117 and -280. The large open rectangle represents the enhancer region of the U4B gene near position -200, and the solid box depicts the location of the octamer sequence ATGCAAAG within the enhancer region. Various ^{32}P -labeled DNA fragments (A through H at the bottom of the figure) were isolated, incubated with increasing amounts of HeLa cell extract and assayed in native mobility shift gels in the panels at the top of the figure. In each panel (A-H), lanes 1 through 5 contained 0, 4, 6, 8, or 10 μg respectively of HeLa cell extract incubated with the correspondingly labeled fragment (A-H) derived from the 5'-flanking DNA of the U4B RNA gene. Lane 6 in each panel contained a DNA fragment derived from the enhancer region of a chicken U1 RNA gene which contains the octamer sequence ATGCAAAT which by DNaseI footprinting is recognized by a protein in the HeLa cell extracts (24). This fragment was included in each case as a positive control for binding. Some of the U4B gene fragments are slightly contaminated with other DNA fragments of unknown origin, but these do not interfere with the analysis. Restriction enzyme abbreviations: As, Asp718; Ss, SstI; Aa, AatII; Ha, HaeII; Sp, SphI; No, NotI.

-227 are not required for enhancer function in the oocyte transcription assay. Interestingly, a short internal deletion of 5 bp (at an SphI site) in the -200 region resulted in the reduction of U4B RNA gene expression to a level that was comparable to that observed when the entire enhancer region had been deleted (compare lane 1 with lane c in Figure 2). This complete loss of enhancer activity in the pU4BΔ(-200/-194) template was observed in spite of the fact that the octamer sequence was perfectly intact in that construction.

A second short internal deletion, pU4B Δ (-190/-183), had only a minor effect on expression (Figure 2, lane j). In this case, the synthesis of U4B RNA was reduced to approximately 71% of that obtained with a wild type template. Although the effect of this deletion was small, it may be significant since it has been reproducible in three separate injection experiments (data not shown). This mutation disrupts a potential binding site for the transcription factor Spl (29). In conclusion, the DNA sequences most important for the activity of the U4B enhancer lie between positions -227 and -189. DNA sequences between -189 and -184 may also contribute to enhancer activity, but their role appears to be of lesser importance.

The U4B enhancer interacts with a sequence-specific DNA-binding protein

To detect factors that interact with the enhancer region of the U4B RNA gene, we performed gel mobility shift assays. For these experiments, the 168 bp fragment diagrammed in the center of Figure 3 was employed. The U4B enhancer is situated near the midpoint of this fragment, which contains the U4B gene 5'-flanking DNA sequences from the SstI site at position -280 to the Asp718 site at position -117. When incubated with nuclear extracts prepared from HeLa cells, this fragment formed a complex of retarded mobility on native polyacrylamide gels (data not shown). To localize the site of interaction, the fragment was digested with one of four different restriction enzymes that cut near or within the enhancer region. The eight fragments that resulted from these digestions were individually incubated with increasing amounts of HeLa cell extract and analyzed on mobility shift gels (Figure 3). Fragments C, D, and E each formed a strong complex with a component of the HeLa cell extract. These complexes were identified as bands of retarded electrophoretic mobility (Panels C, D, and E). Fragment B weakly interacted to form a light band of similarly retarded mobility (Panel B). Each of these four fragments (B, C, D, and E) contains the octamer variant ATGCAAAG which is depicted as the solid box in Figure 3. In fragment B, the octamer is located within 3 nucleotides of the end of the fragment, undoubtedly accounting for the weakness of the interaction observed in that particular case. In contrast, none of the four fragments that lacked the octamer sequence formed a complex of retarded mobility (Panels A, F, G, and H).

The results in Figure 3 strongly suggest that the octamer variant ATGCAAAG in the U4B enhancer is a recognition site for a DNA-binding protein, probably NF-A (28). In some cases (e.g., Panels C and D), a second band of even slower electrophoretic mobility appeared above the major retarded band at the higher extract concentrations. This could indicate the existence of

Fig. 4 - Competition between the U4B enhancer and the U1 snRNA gene for transcription factors in oocytes.

Experimental conditions were similar to those described in Figure 2 except that oocytes were injected with a U1 RNA gene plasmid (as a reference or test gene) together with an 8-fold excess of various competitor DNA plasmids. The arrangement of the U4X and U4B RNA genes and their enhancer regions in the chicken genome is shown below the autoradiogram. The black bars underneath indicate the segments of chicken DNA cloned into pUC19 in the various plasmids used as competitors. The letters a through e in the lower part of the figure designate the competitor plasmids used in the correspondingly labeled lanes of the autoradiogram. The (+) and (-) signs indicate the degree of competition. Restriction enzyme abbreviations: S, SstI; P, PstI; K, KpnI.

additional protein/DNA or protein/protein interactions. However, the formation of this slower moving band was not always reproducible from experiment to experiment, and it may therefore be due to a non-specific interaction. Consequently, we have not further investigated its significance.

Competition Between the Chicken U4B, U1, and SV40 Enhancers for Transcription Factors

When vertebrate U1 and U2 RNA genes are co-injected into *X. laevis* oocytes, they compete for expression (14,15,20). This is interpreted to mean that U1 and U2 RNA genes require a similar set of transcription factors for expression. To determine whether the U1 and U4 RNA genes compete for transcription factors in oocytes, we performed the experiments shown in Figure 4, lanes a-d. In these experiments, a constant amount of a chicken U1 RNA reference gene (or test gene) was co-injected into oocytes together with an 8-fold excess of various U4 RNA gene constructions.

When the U1 RNA gene was co-injected with an excess of the wild type U4B RNA gene, chicken U4B RNA accumulated in the oocyte, but very little U1 RNA was synthesized (Figure 4, lane a). In contrast, when a plasmid containing the U4B gene with only 117 bp of 5'-flanking DNA was co-injected together with the U1 RNA gene template, a strikingly different result was obtained (lane b). In this case, U1 RNA was efficiently synthesized, but there was very little if any expression of the U4B RNA gene. Thus, the enhancerless U4B construction was unable to compete for expression even though it was present in 8-fold molar excess over the U1 RNA gene template. This result indicates that the wild type U1 RNA gene with its enhancer was capable of sequestering factors into a stable transcription complex, thereby inhibiting the expression of the enhancerless U4B RNA gene. Efficient competition for limiting factors has also been shown to be enhancer-dependent for the human (19) and chicken (24) U1 RNA genes and the human (14,15) and frog (20) U2 RNA genes.

To determine whether it was necessary for the U4B enhancer to be linked to an active transcription unit for it to be an effective competitor, the injections shown in lanes c and d of Figure 4 were performed. The diagram at the bottom of Figure 4 shows the arrangement of U4 RNA genes in the chicken genome. The U4X RNA gene is closely linked to the U4B RNA gene; it codes for a variant U4 RNA, and the U4X gene is not detectably expressed when injected into *X. laevis* oocytes (11). When a plasmid containing the U4X RNA gene was co-injected in 8-fold excess over the U1 RNA reference gene, it did not compete for expression (Figure 4, lane d). However, when the U4B gene enhancer was included on the same plasmid with the U4X gene and used as a competitor, expression of the U1 RNA reference gene was significantly reduced,

even though expression of the U4X RNA gene was not detectable (Figure 4, lane c). This result indicates that the U4B enhancer can compete for factors even when it is not linked to an active transcription unit. This is in agreement with results reported by Murphy et al. (19) indicating that transcription is not required for the human U1 enhancer to act as an effective competitor.

The results presented in Figures 1 and 2 revealed that the chicken U4B RNA gene enhancer contains at least two distinct functional motifs: (1) the octamer region which is a binding site for a known protein factor, and (2) downstream DNA sequences (-195 to -199) additionally required for enhancer activity. These latter sequences occur within an SphI restriction endonuclease recognition site. A similar organization is found within the SV40 72 base pair repeats which contain both an identical octamer variant and also a so-called Sph motif (30-32). Furthermore, it has been shown that a plasmid containing SV40 DNA can compete with a human U1 RNA gene for transcription in oocytes (19). We therefore decided to test whether a plasmid (pSV2AC) containing the SV40 enhancer could compete when co-injected with the U4B RNA gene. Indeed, this plasmid was an effective competitor (lane e in Figure 4). Together, these results indicate that the U1, U4B, and SV40 enhancers can be recognized by a similar set of oocyte transcription factors.

DISCUSSION

In this paper we have investigated the DNA sequence requirements of chicken U4B RNA gene expression with particular emphasis placed upon mapping the functional elements of the distal regulatory region. In overall structure, the U4B promoter is similar to vertebrate U1 and U2 RNA gene promoters that have been previously characterized, but differs in several details as discussed further below. The distal regulatory region of the U4B RNA gene is centered near position -200 and stretches over a distance of approximately 24-40 nucleotides. It enhances U4B RNA gene expression 4-5 fold. A more proximal element, located between positions -117 and -38, is necessary to obtain a detectable level of U4B RNA synthesis. Presumably, this critical proximal regulatory element is a sequence located near position -55, first identified in U1 and U2 RNA genes, that is responsible for fixing the initiation site for snRNA transcription (12,16,19). At that position, the sequence CCGTG is absolutely conserved among the chicken U1, U2, and U4B RNA genes (11), and similar sequences are present in the snRNA genes of other vertebrates at the same location (12,14,16,17,33-36).

When compared to U1 and U2 RNA genes, the U4B RNA gene has a higher level of basal activity in the absence of an enhancer sequence. For example, the

U4B RNA gene construction with only 117 bp of 5'-flanking DNA is still quite active in the oocyte assay (~26% of the wild type activity). In contrast, expression of the chicken U1 RNA gene is reduced to <5% of the wild type activity by a deletion to either position -188 or -90 (24). Similar drastic reductions in efficiency have been noted by other groups after deleting the enhancers of frog or human U1 or U2 RNA genes (12,14-17,19-21). It is possible that a third unmapped element may exist in the U4B gene 5'-flanking DNA downstream of position -117 that could be responsible for elevating the basal level of U4B RNA gene expression (similar to element C in the human U1 RNA genes [19]). An examination of that question and further mapping of the proximal element will be the subjects of a future report.

Our current data provide evidence that the U4B RNA gene enhancer region is composed of a minimum of two distinct functional motifs. One of these, the octamer motif, has been previously recognized and implicated as a functional element of snRNA gene enhancers (19-21,37,38). In agreement with these findings, a deletion (pU4BΔ[-199]) in the U4B gene enhancer which removes the octamer results in a reduction of U4B gene expression to a basal level. To further confirm the importance of the octamer, the specific binding of a protein factor to this region has been mapped by band shift analysis.

However, the octamer motif is not sufficient for enhancer activity, because a short internal deletion 12 bp downstream of the octamer also eliminates the activity of the enhancer. This deletion occurs within an SphI restriction endonuclease recognition site. Similar sequences, which have been called Sph motifs (30-32), are located within the SV40 enhancer and form part of the binding site for a sequence specific DNA-binding factor (31). Although we have not detected specific protein binding to the Sph motif of the U4B enhancer using HeLa cell extracts, the functional requirement for this motif in the oocyte transcription assay is nonetheless evident from the results shown in Figure 2.

In comparison to a construction lacking an enhancer, the wild type chicken U4B RNA gene, when injected in excess into oocytes, greatly reduces expression of a coinjected U1 RNA gene; this suggests that the U1 and U4B RNA genes compete for a common set of transcription factors. Moreover, the results shown in Figure 4 indicate that the competition is primarily due to sequences within the U4B enhancer. In fact, the chicken U1 and U4B RNA gene enhancers do contain homologous sequence elements. We have recently shown that the chicken U1 RNA gene enhancer consists of (1) an upstream domain containing an octamer motif and (2) an essential downstream domain which contains an Sph motif (24). However, in the U1 enhancer the octamer motif

(ATGCAAAT) is on the non-template strand, whereas it (ATGCAAAG) is on the template strand of the U4B enhancer (11). Moreover, DNA sequence comparisons suggest that the Sph motif (which probably consists of the SphI site plus additional non-palindromic flanking DNA sequences) is also inverted in the U4B RNA gene enhancer relative to its orientation in the U1 enhancer (24). Thus, distinct motifs in snRNA enhancers can apparently function in different orientations relative to one another.

Since the SV40 enhancer is an effective competitor in the transcription assay, our data suggest that snRNA gene enhancers are structurally similar to the enhancers of viral and cellular genes that encode mRNAs. It has been proposed by another group that the *Xenopus* U1B RNA gene enhancer is extraordinarily compact, and, in that respect, differs from mRNA gene enhancers (39). Although the reasons for our disparate conclusions are not immediately obvious, it should be recognized that the *Xenopus* U1B and human U2 RNA gene enhancers do contain both an octamer sequence and an SphI recognition site but in those two examples the two motifs overlap (GCATGCAAAT). Thus, in those particular cases, the sequences that compose the snRNA enhancer may be unusually compacted.

Finally, it is likely that other sequences in addition to the octamer and Sph motifs are involved in snRNA gene enhancer function. For example, the deletion of a GC-box in the construction pU4B Δ (-190/-183) had a small but detectable effect on U4B RNA gene expression (Figure 1 and Figure 2, lane j). This suggests that the transcription factor Spl plays a role in U4B enhancer function. Moreover, there is a 10 bp perfect palindromic sequence (GTGACGTCAC) present upstream of the octamer at positions -233 to -224 in the chicken U4B sequence. The identical sequence is present at nearly the same position upstream of a human U4 RNA gene (22). Its evolutionary conservation argues for its functional importance; however, we have not observed any transcriptional effect after deleting more than half of this potential regulatory element (construction pU4B Δ [-227]). Although this lack of an effect could result from the utilization of a heterologous system, it is also possible that the U4B enhancer contains functionally redundant sequence elements; it may be possible to delete an individual element and have little effect on expression. Since enhancer motifs can exhibit cell-type specificity (31,32,40), some elements also may be overlooked depending upon the type of cell used for the assay, regardless of whether the cells are derived from a homologous or heterologous species. In the case of the snRNA genes, multiple enhancer motifs are probably important to ensure that these housekeeping-type

genes can be adequately expressed in all cells irrespective of the cellular background.

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