Cooperation between CCAAT and octamer motifs in the distal sequence element of the rat U3 small nucleolar RNA promoter

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ABSTRACT

Mammalian U3 small nucleolar RNA promoters possess a highly conserved distal sequence element (DSE) consisting of CCAAT and octamer motifs separated by 11–12 base pairs. We show here that both motifs are required for transcription of a rat U3D gene in *Xenopus* oocytes. Deletion of the CCAAT motif leaves residual DSE activity, while removal of the octamer motif does not. Changing the conserved spacing between the two motifs generally inhibits transcription less than deletion of either motif, but increasing the spacing between the motifs by one helical turn of DNA preserves normal levels of transcription. We also show that the rat U3D DSE is functionally equivalent to the human U2 snRNA DSE, which consists of adjacent GC and octamer motifs, and that elements from the Herpes Simplex Virus thymidine kinase promoter can replace part or all of the U3D DSE. These data are apparently paradoxical; despite high evolutionary conservation, the U3 DSE is relatively insensitive to mutation, and other upstream motifs are also able to drive transcription from the U3 basal promoter. We suggest that the conserved structure of the U3 DSE may be required for regulation rather than efficiency of U3 transcription.

INTRODUCTION

The U small nuclear RNAs (snRNAs) are small, highly conserved RNAs found in all eukaryotic nuclei (reviewed in ref. 1). The snRNAs are packaged together with a set of proteins to form small ribonucleoprotein particles (snRNPs) which are involved in nuclear RNA processing reactions. The major snRNAs, U1–U6, are all involved in pre-mRNA splicing (2) with the exception of U3 snRNA, which is localized to the nucleolus where it helps to process rRNA precursors (3–5).

The vertebrate U1–U5 genes are transcribed by RNA polymerase II (6, 7) but lack TATA boxes and polyadenylation signals. Instead, both initiation of snRNA transcription and snRNA 3' end formation occur by mechanisms which are

apparently unique to snRNA genes. Formation of U snRNA 3' ends is directed by a sequence called the 3' box, which has so far only been found downstream of snRNA genes (8, 9). Remarkably, utilization of this 3' box is dependent on initiation from an snRNA promoter (10–12). The snRNA promoters contain two conserved elements in the 5' flank: a proximal sequence element (PSE) located around –55 and a distal sequence element (DSE) located around –220 (reviewed in ref. 7). The 11–16 nucleotide PSE (11, 13, 14) functions like a TATA box in fixing the site of transcription initiation (15–17). The DSE markedly increases the level of transcription from the PSE, and it contains an octamer sequence (7, 17–20) which is believed to bind the ubiquitous transcription factor Oct-1 (21). The DSE also has one or more binding sites for other transcription factors, and these sites can differ between different snRNA genes. For example, the human U2 gene DSE contains an Sp1 binding site (22, 23), the human U4C DSE contains CREB and AP2 binding sites (24), and chicken U1 and U4 gene DSEs contain binding sites for a transcription factor called SBF (25, 26). Although other 5' flanking regions in the snRNA promoters may be involved in snRNA transcription, these regions are not conserved between snRNA genes and appear to play a less important role than the DSE and PSE (11, 15, 27, 28).

We decided to study the transcriptional control of U3 snRNA genes for two reasons. First, because U3 is the only major snRNA involved in rRNA processing rather than mRNA splicing, transcription of U3 may be regulated differently from that of the spliceosomal snRNAs (29). Conceivably, transcription of U3 snRNA and the rRNA precursor must be coordinated though the two are products of different RNA polymerases. Second, consistent with the possibility that transcription or regulation of U3 snRNA differs from that of spliceosomal snRNAs, all mammalian U3 genes sequenced to date contain a highly conserved DSE called the 'U3 box' (30–33) in which CCAAT and octamer motifs are separated by 11–12 base pairs. Here we show that both the CCAAT and octamer motifs are necessary for full activity of the rat U3D gene in *Xenopus* oocytes, and mutating the highly conserved spacing between the two motifs

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generally inhibits transcription less than deletion of either motif. We also find that part or all of the U3 DSE can be replaced by the human U2 snRNA DSE or by Herpes Simplex Virus thymidine kinase promoter elements. The relative insensitivity of the U3 DSE to mutation, as well as the ability of other upstream motifs to drive transcription from the U3 PSE, suggest that evolutionary conservation of the U3 DSE is required for regulation rather than the efficiency of U3 transcription.

MATERIALS AND METHODS

Construction of the marked U3D gene

Standard cloning techniques used have been described (34). All blunting and filling in reactions were performed with Klenow polymerase, which was a gift from Cathy Joyce. Oligonucleotide-directed mutagenesis was performed using a kit from Amersham. All mutations and constructs were confirmed by restriction mapping and, where necessary, by DNA sequencing (35).

An AluI fragment of the rat U3D gene (30), cloned into the SmaI site of mp9, was a gift from Ilana Stroke. The fragment, which includes 548 nucleotides (nts) of 5' flank and 79 nts of 3' flank, was excised with EcoRI and HindIII, and the resulting 840 base pair (bp) fragment was cloned between the EcoRI and HindIII sites of pUC13. The U3D gene was then cut with NaeI at nt 117 in the coding region, and an XbaI linker (5'-CTCTA-GAG-3') was ligated into this site to create the -548 plasmid.

Construction of U3D promoter deletions and mutations

The U3D promoter deletions were all derived from the -548 parental construct. The -375 deletion was generated by cloning the XmaI/HindIII fragment from -548 into pUC13. The -350 and -271 deletions were generated by partially digesting -548 with BspMII, filling in, digesting with HindIII, and ligating this fragment into pUC13 cut with HincII and HindIII. The -144 and -24 deletions were made in the same way, except the -548 plasmid was first cut with FokI or AatII, respectively, before Klenow treatment. The -223 deletion was made by filling in the Fnu4HI/NsiI fragment from -375, cutting with BstBI, and ligating into -548 cut with SmaI and BstBI. The -195 deletion was made by isolating the MaeII/NsiI fragment from the -548 promoter, filling in, cutting with BstBI, and ligating the resulting MaeII/BstBI fragment into -375 cut with SmaI and BstBI. To make the -113 deletion, the -548 plasmid was cut with NsiI and HindIII, and the fragment containing U3 was ligated into pUC13 cut with PstI and HindIII.

-548B was made by digesting the -548 plasmid with BstBI, filling in, and religating. The -213B deletion was made by isolating the TaqI/NsiI fragment from -548B, treating with Klenow, and ligating into -548 cut with EcoRI and BstBI and filled in. The Δ PSE mutation was made by cutting -548 with AatII, blunting, cutting with HindIII, and cloning into -548 plasmid that was cut with NsiI, blunted, and recut with HindIII. The resulting plasmid is identical to -548 except that promoter sequences between the NsiI and AatII sites have been removed.

The -Oct mutation was created by Klenow treatment of the SacI/TaqI fragment from -375 and ligating this into -195 that was cut with EcoRI and filled in. This replaces a 15 bp octamer-containing sequence in the -375 promoter with a 14 bp linker sequence. The -CCAAT mutation was made by oligonucleotide-directed mutagenesis of the -375 deletion using the oligo 5'-A-CACTGGCGTATGGAATTCTAAGTTCTTCGATATGT-3'. The Δ CCAAT mutation was made by first creating two -375

clones with new EcoRI sites using oligonucleotide-directed mutagenesis. One clone, U3R1, had an EcoRI site inserted 13 bp upstream of the U3 enhancer using the oligo 5'-GGGCGAC-TGAATTCATGCGTAT-3'. The other clone, U3R2, had an EcoRI site inserted between the CCAAT box and octamer sequence in the U3 enhancer using the oligo 5'-GATTGGC-TGCCGAATTCGATATGTTA-3'. Both of these clones were then cut with EcoRI, and the small promoter fragment was isolated from U3R1 and cloned into the vector fragment from U3R2. The resulting Δ CCAAT clone is identical to -375 except for a 27 bp deletion which includes the CCAAT box.

The -375inv and -271inv mutations were created by cutting -548 with XmaI and NsiI, or BspMII and NsiI, respectively, treating with Klenow, and ligating this fragment into -548 that was cut with EcoRI and NsiI, and treated with Klenow.

The DSE spacing mutations all have wild type U3 sequences from -375 to +79 except for sequences between the CCAAT box and the octamer sequence. The A17 spacing mutation was created by treating the SacI/Fnu4HI fragment from -375 with Klenow, then ligating this fragment into -223 that was cut with SacI and blunted. The A27 and A37 mutations were created by cloning the blunted and filled in SacI/Fnu4HI and SacI/TaqI fragments from -375, respectively, into -223 that was cut with EcoRI and filled in. The A22 and A23 mutations were created by cutting A27 with SacI, blunting, and religating. A32 was made from A37 in the same way.

Construction of U3/U2 hybrid promoters

The U2 clone NE (22) was a gift from Manny Ares. To make 3NE, the -548 U3D clone was cut with NsiI, blunted, recut with EcoRI, and the U3 promoter fragment was isolated and cloned into NE cut with EcoRI and Eco47III. 3B-NE was made by cutting 3NE with BstBI, filling in, and religating. 3MNE was created by ligating the EcoRI/MaeII promoter fragment from -548 into NE cut with EcoRI and BstBI. N3E was made by ligating the Klenow-treated EcoRI/NsiI fragment from -195 into NE cut with SmaI and Eco47III.

The plasmid 2U3 was made by cloning the EcoRI/Eco47III U2 DSE fragment from NE into -548 plasmid that was cut with NsiI, blunted, and recut with EcoRI.

Construction of tk/U3 hybrid promoters

The plasmid pHEBo1 (36), which contains the HSV tk gene, was a gift from John L. Kolman. To make the tk/U3 plasmid, pHEBo1 was cut with BsmI and EcoRI, filled in, and the 190 bp fragment containing the tk promoter was ligated into the -195 deletion which was cut with EcoRI and filled in. Cloning of the Klenow-treated 59 bp MboII/EcoRI fragment from pHEBo1 (which contains the HSV tk Sp1 and CCAAT boxes) into the same -195 vector created tkU3-Oct. U3tkA31 was made by cloning the above MboII/EcoRI tk fragment into -223 which was cut with EcoRI and filled in.

Oocyte injections

Oocyte isolation, injections, and RNA extractions were performed as previously described (37). For each sample, 10-20 oocytes were injected and the RNA from these was pooled. To normalize for injection efficiency, all samples were coinjected with 0.5 ng of a *Xenopus borealis* 5S rRNA +20 maxigene (38). After RNA isolation, one oocyte equivalent of RNA from each sample was run on a 6% sequencing gel. After autoradiography, the amount of 5S maxigene transcript was estimated visually and a second

gel was run normalizing each sample for the amount of 5S transcript. The autoradiographs from normalized gels were scanned with a densitometer and the ratio of U3 (or U2) to 5S transcript was determined. For samples involving the U2 gene, the sum of the signals from the U2 and U2+10 transcripts was used to calculate the amount of U2 transcription, since the 3' end processing of U2 from U2+10 precursor varies between batches of oocytes (39). Each sample was injected in 1–5 separate experiments, and the values from these were averaged to determine the final value of the relative transcription of each sample.

For competition experiments, the amount of competitor and test DNAs was either 200ng + 200ng, or 300ng + 100ng per microliter of sample.

RESULTS

5' flanking sequence requirements for rat U3D gene transcription

We (30) and others (31–33) have noted a highly conserved region located about 220 nucleotides upstream of the 5' start site of all vertebrate U3 snRNA genes sequenced to date. This region, previously called the 'U3 box' (30), consists of two motifs (Fig. 1): a perfectly conserved eight base pair (bp) sequence containing a CCAAT box, and a nine bp octamer motif, which is also perfectly conserved except for a C to T change at one position in the rat U3D sequence. The spacing of 11–12 bp between these two motifs is also conserved, although the sequence of the spacer is not.

To study the role of this and other 5' flanking sequences in U3 transcription, we created a marked rat U3D gene by inserting an eight bp linker into the Nae I site at nt 116 in the U3 coding region. This marked gene contains 548 bp of upstream flank and 79 bp of downstream flank, and is as efficiently transcribed as the wild type rat U3D gene when microinjected into *Xenopus* oocytes (data not shown), where it gives rise to a 228 nt transcript which is not seen upon injection of pUC13 vector alone (Fig. 2B, lanes 1 and 2). This product is about nine nts longer than the expected marked U3D gene product, and probably represents a precursor elongated at the 3' end. This precursor, along with mature U3 RNA, is also seen when the wild type U3D gene is injected into oocytes (30). The lack of a mature U3 product from the marked U3D gene suggests that the 8 bp linker we have inserted interferes with an RNA secondary structure or snRNP structure needed for posttranscriptional 3' end processing, as it

Fig. 1. Sequence of conserved elements upstream of mammalian U3 snRNA genes. Conserved CCAAT and octamer motifs are boxed, and the conserved spacing between the two elements is indicated (30–32). The rat U3D sequence shown here corrects two errors in the published sequence (30) which were discovered upon resequencing.

Fig. 2. Progressive 5' deletions through the promoter of the rat U3D gene. (A) Sequence of the deletions. The sequence of the 5' flank of the rat U3D gene is shown. The conserved CCAAT and octamer motifs are boxed. The probable PSE, as determined by analogy with other snRNA genes, is also boxed. Endpoints of the 5' deletions are indicated above the sequence, and the relative transcription of each deletion mutant compared to the full promoter (-548) is noted above the deletion endpoint. The location of a CG dinucleotide insertion introduced into the B mutants (-548B and -213B) is indicated by a triangle below the sequence. (B) Expression of U3 genes with promoter deletions. Oocytes were injected with [α - 32 P]GTP, 5S maxigene plasmid, and the indicated templates. Labelled RNA was isolated, normalized for 5S maxigene transcript, and resolved on a 6% sequencing gel. The intensities of the U3 and 5S bands were determined by densitometry, and the relative levels of expression of the U3 mutants were calculated and are summarized in Fig. 2A. The Δ PSE mutation is an internal deletion in the -548 promoter from nucleotides -113 to -25. The structure of the -Oct mutation is shown in Fig. 3A. The markers are kinased Hpa II fragments of pBR322; the sizes are indicated on the left in nucleotides.

Fig. 3. Mutations in the U3 DSE. (A) Structure of deletion and substitution mutations in the U3 DSE. Conserved CCAAT and octamer motifs are boxed. Nucleotides in capital letters are wild type sequences, lowercase letters indicate linker sequences, and dashes indicate deleted nucleotides. The relative transcription of each mutant is indicated on the right. (B) Structure of inverted DSE mutations. Boxes represent the conserved CCAAT and octamer motifs and the PSE element, and the larger open box at the right denotes the U3 coding region. The mutation -375inv inverts the region from -375 to -114 in the U3 promoter, and the mutation -271inv inverts the sequences from -271 to -114 in the U3 promoter. The relative transcription of each mutation, compared to the -375 promoter, is indicated at the right. (C) Expression of U3 DSE mutations. Microinjections, markers, and analysis as in Fig. 2.

has been found for U2 snRNA that packaging into a mature ribonucleoprotein particle is necessary for correct 3' end maturation of the U2 precursor (40).

To determine the transcriptional role of the sequences in the marked U3D promoter, we created a series of progressive 5' deletions. The endpoints of these deletions, created by restriction site cleavage, are shown in Fig 2A. The deletion mutants were tested for transcriptional activity by microinjection into *Xenopus laevis* oocytes together with $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ and a *Xenopus borealis* 5S RNA maxigene (38). The coinjected 5S maxigene served as an internal control for injection efficiency, and has been shown not to compete with snRNA genes for transcription factors (39). Each deletion template was injected into 10–20 oocytes, labelled RNA from each set of oocytes was isolated and pooled, and the products were separated on a 6% sequencing gel after normalizing for transcription of the 5S maxigene. The results are shown in Fig. 2B. The relative transcription of each deletion was calculated compared to the -548 parent clone (Fig. 2A).

Deletion of sequences from -548 to -271 has no major effect on transcription (Fig. 2A and 2B, lanes 2, 4–6). Deletion to -223 , which eliminates most of the conserved CCAAT box sequence, reduces transcription slightly to 77% of wild type (lane 7). Deletion of an additional 10 bp (-213 ; lane 8) decreases transcription to 63% of wild type. Deletions eliminating both the CCAAT and octamer sequences (-195 , -144 , -113 ; lanes 9–11) all consistently transcribe at about 30% of wild type, indicating that the DSE is an essential promoter element. A deletion to -24 which eliminates the PSE is transcriptionally inactive (lane 12). An internal deletion of the -548 promoter between nucleotides -113 and -25 , which eliminates the PSE element but retains the CCAAT and octamer motifs, is also transcriptionally inactive (ΔPSE , lane 14).

In order to construct the deletion mutant -213B , we first inserted a CG dinucleotide at position -118 in the U3D promoter to destroy a *TaqI* site (Fig. 2A). When this CG insertion was made in the context of the -548 promoter, the transcriptional ability of the resulting gene (-548B) was unusually variable from one injection to the next, ranging from 89% to 239% of wild type (the latter illustrated in Fig. 2B, lane 3). -548B was the only construct in this study whose transcriptional activity varied so widely between different sets of injections; the activity of other templates usually varied by no more than 10–20%. The -213B template, which contains the same CG insertion as -548B , was consistently transcribed at about 65% of wild type. However, because the transcriptional activity of -548B was frequently greater than wild type, we cannot rule out the possibility that the transcriptional activity of -213B is higher than it would be without the CG insertion. We currently have no explanation for the erratic behavior of the -548B mutant.

In order to more closely examine the role of the CCAAT and octamer motifs in U3D gene transcription, we made three additional mutations in the -375 deletion template. Two of these mutations, called $-CCAAT$ and $-Oct$, replaced the CCAAT or octamer motifs respectively with linker sequences, and the third mutation, ΔCCAAT , deletes 27 bp which includes the entire CCAAT box but none of the octamer motif. The sequences of these constructs, and their transcriptional efficiencies after injection into oocytes, are shown in Fig. 3A; the data are shown in Fig. 3C (lanes 1–4). Elimination of either the CCAAT or octamer motif significantly reduces transcription, although deletion of the octamer motif has a somewhat greater effect than removal of the CCAAT motif. The $-CCAAT$ and ΔCCAAT mutants are both transcribed less efficiently than the -223 and -213B mutants (Fig. 2) which delete part or all of the CCAAT

Fig. 4. Mutations in the spacing between the CCAAT and octamer motifs of the U3 DSE. (A) Sequences of the spacing mutations in the U3 DSE; the mutants are identical to the wild type promoter (–375) except for the changes shown here. The CCAAT and octamer motifs are boxed, and the sequences between them have been aligned to show homology between the various mutations. The relative transcription of each mutant is listed on the right. (B) Expression of the spacing mutations in the U3 DSE. Microinjections, markers, and analysis as in Fig. 2.

motif while leaving the octamer intact. This could indicate that vector sequences upstream of the deletion endpoints in –223 and –213B can partially substitute for the CCAAT motif when placed immediately upstream from the octamer.

We also made two mutations inverting the entire CCAAT and octamer region of the U3D promoter (Fig. 3B). These mutations, –375inv and –271inv, invert the region upstream of nt –114 in the –375 and –271 promoters, respectively. Both templates were transcribed less efficiently than the wild type U3 promoter (Fig. 3C, lanes 5 and 6), but the –271inv mutation was especially severe and was transcribed at the same low level as a template from which the entire DSE had been deleted. This may indicate that the inverted DSE is sensitive to the distance from the PSE. These results resemble those of Ares *et al.* (39), who found that an inverted U2 DSE could not function in *Xenopus* oocytes, and only at low levels in transfected HeLa cells (18).

Changing the conserved spacing in the U3 DSE

In order to test whether the conserved spacing of 11–12 bp between the CCAAT and octamer motifs of the U3 DSE is necessary for full transcriptional activity, we generated a series of mutations that increase the spacing between the two motifs. These mutations, whose sequences are shown in Fig. 4A, are derived from the –375 promoter and differ from wild type only in the spacing between the CCAAT and octamer motifs. The

Fig. 5. Structure and expression of chimeric U3/U2 promoters. (A) Structure of the U3/U2 chimeric promoters. Thin lines indicate U2 promoter sequences and thick lines indicate U3 promoter sequences. Boxes indicate conserved transcriptional control elements. The position of the U2 or U3 structural gene is denoted by the large open box on the right. Nucleotide numbers above each construct indicate nucleotides from the U2 gene, while numbers below each map indicate nucleotide numbers from the U3 gene. The relative transcription of each construct is listed on the right; the values given for the promoters driving the U2 gene are relative to the wild type U2 construct (NE), while the values for constructs driving the U3 gene are relative to the wild type U3 promoter (–548). (B) Expression of the U3/U2 chimeric promoter constructs. The position of the U2, U3, and 5S maxigene transcripts are indicated on the right. Microinjections, markers, and analysis as in Fig. 2.

additional spacer nucleotides are either polylinker sequences or a duplication of wild type spacer sequences. The transcriptional activities of these constructs were assayed by microinjection into *Xenopus* oocytes; the results are shown in Fig. 4B, and the relative transcription of each of the mutants is shown in Fig. 4A. Increasing the spacing between the two motifs by 6 bp (mutant A17; Fig. 4B, lane 2), or a little over half a helical turn of DNA, decreases transcription to 58% of wild type. Increasing the spacing by 11 bp (mutant A22; lane 3), or one helical turn, restores wild type levels of transcription. Increasing the spacing beyond one helical turn also decreases transcription, with the levels ranging from 81% of wild type for mutant A23 (lane 4) to 41% for mutant A32 (lane 6). This last mutation, which increases the wild type spacing by two helical turns, is the most severe, reducing transcription to levels found when the CCAAT motif is deleted altogether. The data suggest that the CCAAT motif cannot function when placed two turns upstream from the octamer motif; however, all the other spacing mutations have milder effects, which is surprising since the spacing between the CCAAT and octamer motifs is so highly conserved.

Functional equivalence of the U2 and U3 gene DSEs

Because the structure of the U3 DSE is so strikingly conserved, we decided to test whether this DSE might be specifically designed to drive transcription from the U3 gene, perhaps by binding factors that interact with other U3 promoter elements, or whether the U3 DSE was interchangeable with other snRNA DSEs. We made several constructs that replaced the human U2 DSE with the rat U3 DSE, and *vice versa* (Fig. 5A). The U2 DSE is quite different from the U3 DSE, consisting of an octamer motif and an Sp1 binding site 0–2 bases upstream (22, 23). The U3/U2 hybrid promoter constructs shown in Fig. 5A were all derived from the human U2 clone NE, which contains a 10 bp linker in the U2 coding region (22). The NE gene gives rise to two transcripts upon injection into *Xenopus* oocytes, a marked U2 snRNA of 200 bases and a marked U2 precursor of 210 bases that is elongated at the 3' end (8, 39, and Fig. 5B, lane 3). The NE clone contains –240 nts upstream from the U2 start site, a region which includes the U2 DSE (Fig. 5A). When we replaced the U2 DSE in the NE clone with the U3D DSE by fusing the U3 promoter sequences from –548 to –114 onto the U2 basal promoter at position –109, the resulting construct, called 3NE (Fig. 5A), was transcribed 4-fold less efficiently than the wild type U2 gene (Fig. 5B, lane 4). While this is a significant decrease, it is not as drastic as the 20- to 50-fold decrease seen when the U2 DSE is deleted from the U2 gene (39). Insertion of 2 bp at position –118 in the U3 sequences, between the U3 DSE and the U2 basal promoter of the 3NE clone, had no additional effect on transcription (3B-NE; lane 6). However, if the U3 sequences from –548 to –194 were fused to the U2 basal promoter at nt –212, so that sequences between the U3 DSE and U2 PSE are all derived from the U2 gene, the resulting construct (3MNE) is transcribed at 146% of the wild type U2 gene (lane 5). From these results we conclude that the U3 DSE can functionally replace the U2 DSE, but not every juxtaposition of the U3 DSE and the U2 PSE is equally compatible. Conceivably, U2 sequences between –212 and –109 are necessary for full levels of U2 transcription; deletion of sequences between the DSE and PSE of the human U2 promoter was reported to have no effect on transcription in transfected HeLa cells (18), but other data indicated that this deletion decreases transcription to 20–40% of wild type (11). Alternatively,

Table 1. Relative transcription of wild type and mutant U3 promoters under competitive conditions.

Test DNA	Competitor	Ratio*	Relative Transcription
–548	3MNE	1:1	100%
–Oct	"	"	15%
–CCAAT	"	"	26%
A27	"	"	131%
A22	"	"	84%
–548	3MNE	3:1	100%
–Oct	"	"	0%
–CCAAT	"	"	25%
A27	"	"	94%
–548	NE (U2)	1:1	100%
–Oct	"	"	0%
–CCAAT	"	"	54%

*Ratio = Mass of competitor : Mass of test template

The gels shown in Figs. 6A and 6B were scanned by densitometry and normalized for the amount of 5S maxigene transcript in order to control for injection efficiency. The relative transcription of each test template was determined by comparison with the wild type (–548) template under the same competitive conditions. The competitor genes used were NE, a human U2 gene extending from –240 upstream of the coding region to +152 downstream and containing a ten base pair linker inserted into the coding region (22), and 3MNE, the marked U2 gene from the clone NE in which the U2 DSE is replaced by the U3D DSE (see Fig. 5A). The mass ratio of competitor to test plasmid was either 1:1 or 3:1.

sequences between –194 and –114 of the U3 promoter could contain a negative regulatory element. To test this possibility, we replaced U2 sequences between –198 to –110 in the NE clone with the –195 to –109 region of the U3 promoter. The resulting construct, N3E (Fig. 5A), was transcribed at 74% of wild type (Fig. 5B, lane 7). This slight decrease is nowhere near the 4-fold difference seen with 3NE, indicating that these U3 sequences in themselves cannot account for the transcriptional difference between 3NE and 3MNE.

We also replaced the U3D DSE in the –548 gene with the U2 DSE from the NE clone. This construct, named 2U3 (Fig. 5A), functioned at 158% of the level of the wild type U3 gene (Fig. 5B, lane 2), showing again that the U2 and U3 DSEs can be functionally equivalent.

Competitive ability of U3D promoter mutations

We were surprised that none of our mutations, with the exception of those that deleted the PSE, decreased transcription below 20–30% of wild type levels. Studies on chicken U1 and U4 gene expression have demonstrated that some promoter mutations which have only a mild effect in *Xenopus* oocytes have a much more severe effect when coinjected along with a competitor snRNA gene (19, 20, 25, 26). Competition presumably reflects a decrease in the ability of the mutants to compete for transcription factors which are needed to form a stable transcription complex. We therefore coinjected several of our mutant and wild type templates with two competitor snRNA genes.

We first tested the ability of U3 DSE mutants to compete against the 3MNE clone (Fig. 5A). Since 3MNE produces a U2 transcript, the products of the test and competitor genes are easily distinguished. We performed two sets of competitions with 3MNE: one with a competitor:test template ratio of 1:1, the other with a ratio of 3:1. The results are shown in Fig. 6A and in Table 1. The –Oct mutant is transcribed at very low levels in the 1:1 competition, and is not detectably transcribed in the 3:1 competition (Fig. 6A, lanes 2 and 7). This indicates that the

Fig. 6. Competition between wild type and mutant U3 promoters. (A) Competition between a U2 gene with a U3 DSE (3MNE) and wild type and mutant U3 genes. Oocytes were injected with 3MNE DNA (see Fig. 5A) and the indicated U3 templates at a mass ratio of 1:1 (lanes 1–5) or 3:1 (lanes 6–9). (B) Competition between a wild type U2 promoter (NE) and wild type and mutant U3 promoters. Oocytes were injected with a marked U2 gene (22) and the indicated U3 templates at a 1:1 ratio. The positions of the U3, U2, and 5S transcripts are indicated on the right. Microinjection, markers, and analysis were as in Fig. 2.

octamer sequence is necessary for the formation of a stable U3 transcription complex, similar to what has been found for other snRNA genes (15, 17, 19, 20, 25, 27). The –CCAAT mutant is transcribed in both competitions at about 25% of wild type (lanes 3 and 8), indicating that the CCAAT box sequence is not necessary for the formation of a stable transcription complex, and that the octamer motif alone can support a basal level of U3 transcription.

We also tested the ability of two of the DSE spacing mutants to compete against the 3MNE gene. The A27 mutant (Fig. 4A), which was transcribed at 52% of wild type under noncompetitive conditions, was transcribed at levels equal to or slightly higher than wild type in competition with 3MNE (Fig. 6A, lanes 4 and 9). Although we do not understand why this mutant functions better under competitive conditions, the result further illustrates that the spacing between the CCAAT and octamer motifs can be changed with little difference in transcriptional ability in oocytes. The A22 mutant is also transcribed at wild type levels in a 1:1 competition with 3MNE (lane 5), in agreement with results found under noncompetitive conditions.

We also tested the ability of two of the U3 promoter mutants to compete against the wild type U2 promoter (NE) at a ratio of 1:1. These results are shown in Fig. 6B and summarized in Table 1. The –Oct mutation was no more able to compete with the U2 gene (Fig. 6B, lane 2) than with 3MNE as described above (Fig. 6A, lanes 2 and 7). The failure of the –Oct mutant to compete with U2 probably cannot be ascribed to competition for the CCAAT motif binding factor, because the U2 promoter lacks a CCAAT motif; however, the possibility cannot be excluded that the U2 promoter sequesters the CCAAT binding

Fig. 7. Structure and expression of U3 genes containing Herpes Simplex Virus tk promoter elements. (A) Structure of U3 promoters containing tk promoter elements. Thin lines indicate U3 promoter sequences, thick black lines indicate HSV tk promoter sequences, and striped boxes indicate linker sequences. Empty boxes denote U3 promoter elements and black boxes denote HSV tk promoter elements. The 5' end of the U3 structural gene is represented by the open box at the right. The transcription of each mutant relative to the –375 template is listed at the right. (B) Expression of the tk/U3 constructs. Microinjection, markers, and analysis as in Fig. 2.

factor by protein/protein interactions rather than direct binding to DNA. The –CCAAT mutant is expressed at 52% of the level of wild type (lane 3), which is twice as high as when the 3MNE competitor is used. This may indicate that the U3 DSE in the 3MNE competitor is better able to compete with –CCAAT for the octamer factor than is the U2 DSE. This result also confirms that the octamer does not require the CCAAT motif to form a stable transcription complex.

Herpes Simplex Virus thymidine kinase promoter elements can functionally replace U3 promoter elements

mRNA promoters, such as the Herpes Simplex Virus thymidine kinase (HSV tk) promoter, cannot drive the synthesis of snRNA genes since 3' end formation directed by the 3' box requires a compatible snRNA promoter (10, 12). However, mRNA enhancers which contain the octamer motif, but no elements foreign to the U2 promoter, can stimulate transcription from the U2 PSE (11, 21); foreign promoter elements reduce U2 synthesis by inhibiting 3' end formation. Since the HSV thymidine kinase (tk) upstream promoter region contains both octamer and CCAAT motifs, as well as an Sp1 binding site (42), sequence elements that are all present in snRNA DSEs, we asked whether some or all of these elements could replace some or all of the conserved U3 DSE.

The hybrid tk/U3 constructs are shown in Fig. 7A. In the construct tkU3, the U3 DSE was replaced with nts –200 to –76 of the HSV tk promoter, which contains the octamer and CCAAT motifs together with the Sp1 binding site. This construct transcribes at 84% of the wild type U3 gene (Fig. 7B, lane 2), indicating that the tk elements present in this construct can functionally replace the U3 DSE, although the arrangement of the octamer and CCAAT motifs is different in the two promoters and an additional transcription factor binding site is present. Also, it is not known whether the same factor binds to the U3 and tk

CCAAT motifs; the GCCAAT sequence is common to both motifs, but other bases which have been conserved among the U3 CCAAT sequences are not present in the tk CCAAT motif.

If the octamer motif is removed from the tkU3 construct, by truncating the tk sequences at nt -125, transcription is reduced to 35% of wild type (tkU3-Oct; Fig. 7B, lane 4), the level of transcription found for U3 constructs lacking the DSE (Fig. 2A). This indicates that the tk Sp1 site and CCAAT motifs require the octamer motif to stimulate U3 transcription.

We also made a construct, U3tkA31, in which the tk Sp1 site and CCAAT motif have replaced the CCAAT motif in the U3 DSE, while the U3 octamer motif is maintained (Fig. 7A). This construct is transcribed at 66% of wild type (Fig. 7B, lane 3), indicating that the tk elements can partially compensate for the lack of a U3 CCAAT motif. We wish to emphasize here that the spacing between the U3 octamer sequence and the tk CCAAT box is 31 bp, a spacing which, if present between the wild type U3 CCAAT and octamer motifs, would most likely be a moderate down mutation (see Fig. 4A).

DISCUSSION

We have analyzed the role of the conserved sequence elements in the rat U3D DSE. This DSE, which is highly conserved among all mammalian U3 genes characterized to date, consists of a CCAAT motif located 11–12 bp upstream from an octamer motif (Fig. 1). We found that both the CCAAT and octamer motifs are required for wild type levels of transcription after microinjection into *Xenopus* oocytes. Deletion of the CCAAT motif reduces transcription under competitive and noncompetitive conditions, but is not as severe as deletion of the octamer motif, which abolishes transcription under competitive conditions. The absolute requirement of an octamer motif for U3 DSE activity was confirmed by transcriptional analysis of hybrid genes in which HSV tk promoter sequences were fused onto the U3 basal promoter.

Our data suggest that the CCAAT and octamer motifs play different roles in producing U3 DSE activity. Since templates lacking a CCAAT box, but containing an octamer motif, are still transcribed under competitive conditions, we can conclude that the CCAAT element is not necessary for the formation of a stable transcription complex, although it does activate the low level of transcription seen with the octamer motif alone. Since templates lacking an octamer motif are not transcribed under competitive conditions, this element is necessary for the formation of a stable transcription complex. The requirement for the octamer sequence, which has been found for other snRNA genes as well (15, 17, 19, 22, 25, 26, 43), and the observation that the CCAAT and octamer motifs interact synergistically and not additively to stimulate transcription, suggest two possible models for the interaction of the CCAAT and octamer binding factors. First, the octamer factor, in addition to allowing the formation of a stable transcription complex, could facilitate the binding of the CCAAT transcription factor in a cooperative manner. This would resemble the cooperative interaction between Sp1 and the octamer binding factor in the U2 DSE (44). Second, the octamer factor may serve as an adaptor, interacting with the CCAAT box factor in such a way as to allow it to stimulate transcription from the basal complex formed at the PSE. This would resemble the role of the octamer factor in helping the Herpes Simplex Virus VP16 protein to stimulate transcription from TATA box-containing

promoters (45). Of course, neither of these models precludes the other, and both could occur simultaneously.

The inability of an octamer motif to generate full DSE activity except in conjunction with another transcriptional motif has been found in other snRNA DSEs (15, 20, 22, 24, 25, 26, 41, 43), although this is not always the case (17, 21, 46). A comparison of our results with extensive data on the chicken U1 and U4 gene DSEs is interesting (25, 26). In these DSEs, the octamer motif acts cooperatively with an SPH motif to produce DSE activity. As with the U3 gene, the octamer motif is necessary for the formation of a stable transcription complex. However, the SPH motif, in contrast with the U3 CCAAT motif, is also necessary for the formation of a stable transcription complex. Thus, in different snRNA DSEs the octamer and auxiliary factors may play different roles in producing DSE activity.

Although the 11–12 bp spacing between the two conserved motifs in the U3 DSE has been conserved in all mammalian U3 genes sequenced to date, we found that the spacing can be changed with only modest or little effect on transcription. Increasing the spacing by half a helical turn is moderately inhibitory while a further increase to one whole turn restores wild type levels of transcription. In general, increased spacing has only mild effects, and indeed an increase of one and one half turns allows wild type levels of transcription under competitive conditions. Recently, it was shown that the 0–2 bp spacing between the octamer and Sp1 motifs in the U2 gene DSE can be increased by one helical turn with little effect on transcription, while increases of one half or one and one half helical turns result in 50% and 20% of wild type transcription levels respectively (44). Thus the U3 DSE seems to be less sensitive to spacing changes of more than one helical turn than the U2 DSE, although the patterns seen with one half or one added helical turns are quite similar for the two genes.

We have noticed that immediately downstream and partially overlapping the CCAAT motif in the U3D DSE is the sequence TGCCA, which can function as a half-site for the transcription factor NF1 (47, 48). In our DSE spacing mutations, this sequence is always in its wild type position even though the CCAAT box has been moved further upstream. If this sequence does bind NF1, it may have a mitigating effect on the severity of the DSE spacing mutations. However, we have found that DSE spacing mutations that lack this sequence do not transcribe more poorly than those containing it (data not shown), nor is this sequence conserved among U3 genes.

In this work, we would not detect any transcriptional signal lying downstream of the U3 DSE because deletion of the DSE leaves only basal levels of transcription. For some snRNA promoters, transcriptional control elements are found downstream of the octamer motif; these can be DSE elements located on the 3' side of the octamer motif (20, 24, 25, 27, 43, 46) or uncharacterized sequences between the DSE and PSE whose deletion has a moderate, but not severe, effect on transcription (11, 15, 24). For other snRNA genes, however, deletion of sequences between the DSE and the PSE has no effect (39, 43, 46). We found that there is no decrease in the basal levels of transcription when the sequences between the DSE and the PSE are deleted, nor can we find any sequences in this region that are conserved between mammalian U3 genes. Fortunately, we found that insertion of a CG dinucleotide at nt -118 caused the resulting clone (-548B) to be transcribed at very different levels in different injections with different batches of oocytes. This inconsistency suggests that promoters bearing mutations in this

region are more sensitive to slightly different environmental conditions. One possibility is that the CG insertion could create, mutate, or abolish a transcription factor binding site; alternatively, the CG insertion could work in another, perhaps indirect, way. We also found that the transcriptional activities of constructs containing the U3D DSE fused onto the U2 basal promoter depend markedly on the sequences between the DSE and PSE. This dependence is not just an artifact of the oocyte system, since we see the same results when the U3/U2 constructs are transfected into HeLa cells (data not shown). Thus sequences between the DSE and the PSE appear transcriptionally important, but their precise role in transcription, and which sequences are involved, remain elusive.

We have shown that promoter sequences from the HSV tk promoter can functionally replace part or all of the U3 DSE, albeit with reduced transcriptional efficiency. Previous studies have shown that while mRNA promoters cannot substitute for snRNA promoters (10, 12), the U2 snRNA DSE can be replaced by polymerized SV40 enhancer motifs that contain an octamer motif (11, 18, 21). If these octamer motifs are mutated, enhancer activity is lost, and if the enhancer fragment contains motifs not found in the U2 DSE, then enhancer activity is decreased due to aberrant initiation and 3' end formation (11, 21). We show here that the tk promoter Sp1 binding site and CCAAT motif can activate transcription from the U3 basal promoter, provided that an octamer motif from either the tk promoter or U3 gene is present. Two caveats must be noted, however. First, since we assay only U3 RNA of the correct size, we cannot say if aberrant transcripts with altered 5' and 3' ends are also being produced. Second, since both the Sp1 site and CCAAT motif from the tk promoter are present in all our constructs, we cannot say if one or both of these motifs are activating U3 transcription. Nonetheless, the tk/U3 constructs, like the U3/U2 constructs, illustrate that the U3 DSE, while highly conserved, can be functionally replaced by other promoter sequences that differ markedly in structure.

We have shown throughout this work that the conserved structure of the U3 enhancer is not necessary to stimulate U3 transcription in *Xenopus* oocytes. The question then arises as to why this structure has been so conserved. First, mutations which have little or no effect on transcription in oocytes may have a greater effect in mammalian cells. We have found, however, that a number of our constructs behave similarly when transfected into HeLa cells (data not shown). Second, mutations which have little or no effect in tissue culture might have a more deleterious effect under more stringent physiological conditions. Third, nucleotides surrounding the CCAAT motif are absolutely conserved, strongly suggesting that a specific CCAAT box binding factor recognizes this site preferentially. A number of proteins are known to bind CCAAT motifs, and the sequence context of the motif helps determine which factor will bind to the element (49–52). The conserved U3 sequence, AGCCAAT-C, is also found in the human hsp70 promoter, where it binds a factor called CBF (53). The expression of hsp70 is cell-cycle regulated in HeLa cells, and CBF may be involved in this regulation. Thus the U3 CCAAT motif, and indeed the entire DSE structure, may have been conserved for regulatory reasons, and the U3 gene may be regulated during cell growth and/or development in ways that differ from genes encoding the spliceosomal snRNAs. Indeed, it has been shown that in mouse embryogenesis the synthesis of U3 snRNA follows a pattern that is different from that of the other major U snRNAs (29). There

is also evidence that different forms of U3 in the mouse may be regulated differently in different cell types (54). Furthermore, it has been found that U3 genes, unlike U1 genes, are responsive to the VP16 transcriptional activator protein of HSV, and this response is directed by a conserved difference in the U3 octamer sequence (55). The availability of U3 promoter mutations will allow us to investigate the role of the conserved DSE structure in the regulation of mammalian U3 biosynthesis.

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