Structural requirements for the functional activity of a U1 snRNA gene enhancer

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ABSTRACT

The transcriptional enhancer of a chicken U1 small nuclear RNA (snRNA) gene contains a GC-box, an octamer motif, and an SPH motif that are recognized by the transcription factors Sp1, Oct-1, and SBF respectively. Previous work indicated that the octamer and the SPH motifs were both required for U1 gene enhancer activity in frog oocytes when the U1 gene was coinjected with a competing snRNA gene template. Here we show that neither two copies of the octamer motif, nor two copies of the SPH motif, can effectively substitute for the natural combination of octamer and SPH. Furthermore, neither the octamer nor the SPH motif (in the absence of the other) functioned efficiently in combination with a GC-box. Alteration of the spacing between the octamer and SPH motifs also reduced U1 template activity. Several potential cis-acting elements other than the SPH motif, with one possible exception among those tested, were unable to cooperate with the octamer motif to effectively enhance U1 gene expression. These results indicate that rather stringent structural requirements exist with respect to the essential cis-acting motifs present in the U1 enhancer, possibly reflecting the unique properties of the transcription complexes assembled on snRNA gene promoters.

INTRODUCTION

The U1, U2, U4, U5, and U6 small nuclear RNAs (snRNAs) are involved in splicing pre-messenger RNAs in eucaryotic cells. With the exception of U6, the snRNAs are synthesized by RNA polymerase II, yet their genes have a promoter structure distinct from that of genes encoding mRNAs (1,2). A proximal sequence element (PSE) located about 50–60 base pairs (bp) upstream of vertebrate snRNA genes specifies the transcription initiation site and, in addition, plays a role in the 3' end-formation of snRNAs (1,2). Further upstream near position –200 there exists a second regulatory region that acts as a transcriptional enhancer. Like the enhancers of mRNA genes, snRNA enhancers are composed of multiple functional motifs that cooperate to achieve a high level of gene expression (3–12). In addition, the snRNA

enhancer is required for the formation of a transcription complex that is stable to challenge by a second competing snRNA gene transcription unit (4,6–9,11–14).

Although enhancer structure varies among different snRNA genes, the chicken U1-52a gene (15) has an enhancer structure very typical of vertebrate snRNA genes. Fig. 1A shows the sequence of the enhancer of this chicken U1 gene and the location of the closely spaced GC-box, octamer motif, and SphI Post-octamer Homology motif (SPH motif) that comprise it. The GC-box and octamer motifs are recognition sites for the well-characterized transcription factors Sp1 and Oct-1, respectively. The SPH motif of the U1 snRNA gene is recognized by a novel SPH motif Binding Factor (termed SBF) that has been identified and partially characterized in our laboratory (8).

In mRNA enhancers, different functional motifs can often be freely interchanged with only minimal effects on enhancer activity, as long as the appropriate trans-acting factors are active within a cell. In contrast, transient expression studies in *Xenopus* oocytes suggested that the three motifs in the U1 enhancer are not qualitatively equivalent, since deletion or mutation of the individual motifs had dissimilar effects on transcriptional activity. For example, in a non-competitive assay the SPH motif was able to activate U1 transcription at least 10-fold even in the absence of the octamer and GC-box, whereas the octamer and GC-box together had no detectable enhancer activity in the absence of the SPH motif (7,8). These results point to a central role of the SPH motif for U1 gene enhancer activity in the oocyte expression system.

Deletion of the octamer motif had notably different effects on transcription levels depending upon the conditions of the assay. In the absence of a competing snRNA gene template, transcription levels were similar from templates either containing or lacking the octamer motif (as long as the SPH motif was present) (7,8). However, when a second snRNA gene template was co-injected into the oocytes as a competitor, the octamer and SPH motifs were both required together for the U1 gene to be transcriptionally active (8). The GC-box, on the other hand, was not essential for U1 enhancer activity under either competitive or non-competitive conditions, but it did provide a 2-fold stimulation of transcription when the octamer and the SPH motifs were also both present in the same construction (8).

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In this communication we report the results of experiments designed to further define the roles of individual protein binding sites within the U1 enhancer. We have found that enhancers with altered spacing between the octamer and SPH motifs can activate U1 expression, but less effectively than an enhancer with the wild type spacing. In addition, several other combinations of enhancer motifs among those tested were unable to generate significant enhancer activity comparable to the natural combination of octamer and SPH. These results indicate that, in comparison to mRNA promoters, unusually stringent structural requirements exist for the efficient enhancement of transcription from the U1 promoter.

MATERIALS AND METHODS

Construction of U1 DNA templates altered in the enhancer region

Plasmid template U1 Ψ (O,S) was previously named U1 Ψ and has been previously described (8). It lacks the GC-box of the wild type U1 enhancer and instead contains an EcoRI restriction site immediately upstream of the octamer sequence. Other features include an engineered KpnI/Asp718 site between the octamer and SPH motifs, natural BssHII and SphI sites within the SPH motif, and an engineered SstI site at the 3' end of the SPH motif. In oocyte expression assays, this construction has approximately 50% of the activity of a template containing the wild-type U1 enhancer (8).

U1 Ψ (S) (Fig. 1B) was constructed by digesting U1 Ψ (O,S) with EcoRI and Asp718, filling in the overhanging ends with Klenow DNA polymerase, and religating the blunted ends. To construct the mutant U1 Ψ (O,-5,S), U1 Ψ (O,S) was digested with KpnI and treated with T4 DNA polymerase in the presence of dATP, dCTP, and dTTP but no dGTP. The resulting linear DNA was recircularized, and one of the clones obtained was determined to contain a deletion of the five nucleotides GGTAC between the octamer and SPH motifs.

To construct the templates with 5, 10, 16, and 21 base pairs inserted between the octamer and SPH motifs, the DNA between the Asp718 and SphI sites of U1 Ψ (O,S) was replaced with a synthetic oligonucleotide sequence that generated an insertion of an extra 21 base pairs [template U1 Ψ (O,+21,S)]. The insertion of this sequence also introduced unique SmaI, EcoRV, and HincII restriction sites into the clone. To construct the U1 Ψ (O,+5,S), U1 Ψ (O,+10,S), and U1 Ψ (O,+16,S) templates, short segments were removed from U1 Ψ (O,+21,S) by digesting with two appropriate blunt-cutting restriction enzymes followed by religation. For example, U1 Ψ (O,+16,S) was obtained from U1 Ψ (O,+21,S) using SmaI and EcoRV; U1 Ψ (O,+10,S) using EcoRV and HincII; and U1 Ψ (O,+5,S) using SmaI and HincII.

The templates U1 Ψ (GC[A],S) and U1 Ψ (GC[C],S), in which the octamer sequence was replaced with two variants of the GC-box, were constructed by replacing sequences between the EcoRI and SphI sites of U1 Ψ (O,S) with oligonucleotides degenerate (A or C) at the single position of variability. U1 Ψ (O,S,S), which contains a duplication of the SPH motif, was prepared by replacing the sequence between the EcoRI and KpnI sites of U1 Ψ (O,S) with a synthetic oligonucleotide containing octamer and SPH motif sequences. U1 Ψ (S,S) was constructed from U1 Ψ (O,S,S) in a manner analogous to the derivation of U1 Ψ (S) from U1 Ψ (O,S) (see above).

A series of SPH motif substitution templates (Fig. 1C) were

constructed by replacing the DNA sequences between the Asp718 and SstI sites of U1 Ψ (O,S) with various synthetic oligonucleotides. In the first of these, U1 Ψ (O,NS), the U1 SPH motif was replaced by nonsense sequence; this construction was previously named U1 Ψ s7 and was shown to lack detectable template activity (8). All other enhancer mutations shown in Fig. 1C were constructed in a similar way but using different synthetic oligonucleotides of the appropriate sequence. Spacing was maintained constant in all cases except two. In the clone U1 Ψ (O,CU4BSPH1), an extra 5 bp were included to ensure insertion of a complete U4B SPH motif. Another clone, U1 Ψ (O,CU4BSPH3) had a two-base (AG) deletion which was obtained as an apparent cloning artifact during construction of U1 Ψ (O,CU4BSPH2). The enhancer sequences of all clones were confirmed by the dideoxy DNA sequencing method prior to oocyte injection.

Xenopus oocyte nuclear microinjection and RNA analysis

Microinjection of supercoiled plasmid DNA into *Xenopus laevis* oocytes, extraction of RNA from injected oocytes, and analysis of RNA were performed as previously described (7,16). For each template being analyzed, approximately 30 nl of DNA solution was injected into the nucleus of each of approximately 40 oocytes. For the injections with U4 competitor template, the injection mixture contained U1 template DNA (320 μ g/ml), U4 competitor template DNA (40 μ g/ml), a *X.laevis* 5S RNA gene plasmid as an internal control (400 ng/ml), and [α - 32 P]GTP (8 μ Ci/ml). The U4 competitor template was the previously described plasmid U4B Ψ WT-A3 (9), which is a transcriptionally compromised U4 template that permits a higher level of U1 expression from coinjected U1 templates in the competition assays. For injections without U4 competitor, other conditions remained the same except that the concentration of U1 plasmid was adjusted to 400 μ g/ml. All constructions were injected on multiple occasions, and typical results are shown in the figures.

RESULTS

Spacing alterations between the octamer and SPH motifs reduce enhancer activity

Previous results had shown that the SPH motif by itself could enhance transcription of the chicken U1 snRNA gene under non-competitive conditions (7,8). However, under competitive conditions (i.e., coinjection into oocytes of a U4 snRNA gene competitor template), detectable transcription of the U1 gene required the presence of both the octamer and SPH motifs. Thus it was clear that the octamer and SPH motifs functionally cooperate in some manner to promote the formation of a stable, active, U1 transcription complex.

To investigate the molecular basis of this functional cooperativity, U1 templates were constructed in which the spacing between the octamer and SPH motifs was altered (Fig. 1B). These constructions were injected into oocytes together with a competing U4B template and α - 32 P[GTP]. Transcription products were resolved by polyacrylamide gel electrophoresis and detected by autoradiography (Fig. 2). The pseudo-wild-type template, U1 Ψ (O,S), contained the wild-type spacing between the octamer and SPH motifs and was efficiently expressed (lane 1). Deletion of 5 bp between the octamer and SPH motifs resulted in a marked decrease in U1 template activity [U1 Ψ (O,-5,S), lane 2]. Similarly, the insertion of 5, 10, 16, or 21 bp of random sequence

Figure 1. Plasmid templates constructed for U1 transcription studies. (A) The wild type U1 gene enhancer sequence is shown, and the GC-box, octamer, and SPH motifs are indicated by underlining. Dashed underlines indicate some ambiguity in the exact boundaries of the SPH motif. (B) Constructions with altered spacing between the octamer and SPH motifs or containing substitutions for the octamer motif. The italicized nucleotides in the U1Ψ(O,S) sequence represent changes from the wild type sequence that create KpnI and SstI sites in the enhancer region. (C) Constructions with substitutions for the SPH motif. Dashes and asterisks both indicate identity with the U1Ψ(O,S) sequence shown in the top line. Asterisks furthermore indicate that certain flanking nucleotide positions are also identical (fortuitously) in the DNA from which the substituted sequence was obtained. [For example, in the *X. laevis* U5 enhancer region, the following sequences are present: ACCCATGC-TGCATTAAGAGCGCTC and CGGCTCGGTGCGCGCCGGTAGCT, where the bold-face nucleotides correspond to the positions denoted by asterisks in the U1Ψ(O,XLU5pocs1) and U1Ψ(O,XLU5pocs2) constructions respectively]. Two underlined nucleotides in the U1Ψ(O,XLU5pocs2G) sequence indicate changes relative to the XLU5pocs2 sequence that increase its similarity to the wild type chicken U1 SPH motif. Delta symbols (Δ) indicate deletions of nucleotides. The U1Ψ(O,CU4BSPH1) sequence shown at the bottom contains the five additional nucleotides CGGCC inserted at the position indicated by the caret.

between the octamer and SPH motifs resulted in expression levels significantly lower than that observed for the pseudo-wild-type template. Thus the wild-type spacing between the octamer and SPH motifs was required for optimal template activity. Nonetheless, it is important to note that all of the templates with insertions and deletions between the octamer and SPH motifs were expressed at levels significantly higher than the negative control template that completely lacked the octamer motif (lane 7). Thus when the distance was altered between the octamer and SPH motifs, functional cooperativity was still possible but was in general less efficient, implying that a stereospecific alignment of Oct-1 and SBF on the U1 enhancer is important for optimal activity.

Effect of replacing the octamer motif with a GC-box or with a second copy of the SPH Motif

Because the octamer motif is evolutionarily conserved in the enhancers of vertebrate snRNA genes, a series of experiments was designed to investigate whether the octamer motif in the U1 enhancer could be functionally replaced by binding sites for other transcription factors. To address this question, the octamer was replaced in the U1 enhancer with two different versions of the

GC-box (GGGGCGGGGA, found naturally in the U1 enhancer, or GGGGCGGGGC, a consensus GC-box) or with a second complete copy of the U1 SPH motif. The sequences present in these constructions, U1 Ψ (GC[A],S), U1 Ψ (GC[C],S), and U1 Ψ (S,S), respectively, are shown in Fig. 1B. In oocyte expression assays, each of these constructions was transcribed with a considerably lower efficiency than the pseudo-wild-type template (Fig. 3, cf. lanes 2, 3, and 4 with lane 1). Thus, neither two tandem copies of the SPH motif, nor a GC-box and an SPH motif together, could fully substitute for the wild type combination of an octamer and an SPH motif. Nonetheless, it is again important to note that these constructions each supported a level of expression higher than that supported by the negative control template that contained only a single SPH motif (lane 6). From these results we can conclude that two tandem copies of the SPH motif, or a GC-box and an SPH motif together, can act to establish a stable U1 transcription complex, even though the activity is severely reduced when the octamer motif is absent. As a positive control, a template that contained two copies of the SPH motif plus an octamer was readily transcribed (lane 5) at a level only slightly reduced in comparison to the pseudo-wild-type construction.

Figure 2. Effect on U1 gene expression of altering the spacing between the octamer and SPH motifs in the U1 enhancer. *Xenopus laevis* oocytes were injected with [α - 32 P]GTP, a 5S RNA gene template (as an internal control), a U4 snRNA gene plasmid (as a competitor template), and various U1 RNA gene templates with altered spacing between the octamer and SPH motifs (constructions shown in Fig. 1B). Total RNA was isolated 18 to 20 hours later from the injected oocytes, run in an 8% denaturing polyacrylamide gel, and subjected to autoradiography. The U1 plasmids injected are indicated above each lane. The bands corresponding to U1, U4B, and 5S RNA are indicated beside the panel.

Replacement of the SPH motif with other cis-acting regulatory elements

To define the role of the SPH motif in the U1 enhancer, we examined whether other sequences of known and unknown functional significance from the enhancer regions of other vertebrate snRNA genes could functionally substitute for the chicken U1 gene SPH motif. We also wished to determine whether binding sites for other known RNA polymerase II transcription factors could functionally replace the SPH motif in the U1 gene enhancer. A number of SPH motif substitution templates (Fig. 1C) were therefore constructed and injected into oocytes. The results are presented in Fig. 4A-C. In each panel, lane 1 shows the level of expression of the pseudo-wild-type template U1 Ψ (O,S). Substitution of a nonsense sequence for the SPH motif resulted in the complete loss of detectable template activity (lane 5 in each panel). This confirms that the SPH motif is essential for U1 enhancer function even under the non-competitive conditions utilized in these assays.

In an initial set of experiments (Fig. 4A), templates were injected in which the U1 SPH motif was replaced with the homologous SPH motif from the chicken U4B enhancer. [Like U1, the U4B enhancer contains an octamer sequence followed by an SPH motif that is recognized by SBF. In the U4B enhancer, however, each motif is in the orientation opposite to its direction in the U1 enhancer (9,16)]. A synthetic 23 bp sequence corresponding to the U4B SPH motif stimulated U1 expression as efficiently as the U1 SPH motif (cf. lanes 1 and 2 in Fig. 4A). Thus the U4B SPH motif can fully substitute for the U1 SPH motif even when placed into the U1 enhancer in the (opposite)

Figure 3. Effect on U1 gene expression of replacing the octamer motif in the U1 enhancer with a GC-box or a second copy of the SPH motif. Oocytes were injected with [α - 32 P]GTP and various U1 mutant templates shown in Fig. 1B. Experimental conditions were as described in the legend of Fig. 2.

U4B orientation. Two shortened versions of the chicken U4B SPH motif stimulated U1 expression but at a lower level (lanes 3 and 4). This was not surprising as nucleotides omitted in these constructions were recently found to be required for the full activity of the U4B SPH motif (9).

The next set of experiments (Fig. 4B) tested whether sequences from the enhancer region of a *X.laevis* U5 gene could substitute for the SPH motif in the context of the U1 enhancer. One of these post-octamer sequences, or 'pocs' (denoted XLU5pocs1), contained DNA sequences that are located immediately 3' of the conserved octamer motif in the *X.laevis* U5 gene enhancer (12). Although it has no known enhancer activity, the sequence bears some resemblance to the SPH motif. When substituted for the SPH motif in the chicken U1 enhancer, a very weak band of U1 transcription, marginally above background, was observed on the original autoradiogram (Fig. 4B, lane 4).

The second *X.laevis* U5 sequence tested (denoted XLU5pocs2) lies immediately 3' of the XLU5pocs1 sequence in the native U5 enhancer. This XLU5pocs2 sequence has also been termed the D5 (formerly the DSE2) region and is an important element required for the full activity of the *X.laevis* U5 gene enhancer (4,12). Importantly, it has a quite extensive sequence similarity to the chicken U1 SPH motif (Fig. 1C). When substituted for the SPH motif, it clearly stimulated U1 template activity (Fig. 4B, lane 2), although somewhat less effectively than the original chicken sequence. Interestingly, a pair of T to G base changes (lane 3), which made the *Xenopus* U5 sequence more similar to the chicken U1 SPH motif, surprisingly created an activator motif reproducibly more potent than either the original chicken U1 sequence or the original frog U5 sequence (Fig. 4B, cf. lanes 1, 2, and 3).

Three additional sequences of interest were tested for their ability to substitute functionally for the SPH motif in the U1 enhancer (Fig. 4C). One of these (denoted HU2pocs) is highly

Figure 4. Effect on U1 gene expression of replacing the SPH motif in the U1 enhancer with other cis-acting regulatory elements. U1 constructions (shown in Fig. 1C) were assayed for transcriptional activity by injection into oocytes similarly to the experiments in Figures 2 and 3, except that no competing snRNA gene template was coinjected. Each panel represents a separate set of oocyte injections. The U1 SPH motif was replaced with: (A) variants of the SPH motif found in the chicken U4B enhancer; (B) sequences from a *X.laevis* gene enhancer region; and (C) potential cis-acting sequences from other sources.

conserved just downstream of the octamer in human and rat U2 RNA genes (17,18), but it has little sequence similarity to the SPH motif. To our knowledge, definitive experiments to assess its activity in the human or rat U2 enhancers have not been reported. Interestingly, it had a very weak, but nonetheless detectable, ability to activate U1 transcription in the oocyte expression assay (lane 2). Another construction, U1Ψ(O,SV40sph/oct) (sequence shown in Fig. 1C), contained a region of the SV40 enhancer that includes a functional octamer sequence [recognized by Oct-1 (19–21)] flanked by two tandem sph motifs recognized by the transcription factor TEF-1 first identified in HeLa cells (22). [These sph motifs of the SV40 enhancer, although sharing some sequence similarity to the U1 SPH motif, are not recognized by SBF (ref. 10 and C. Cheung, unpublished data).] Interestingly, the construction containing this SV40 sequence was completely inactive (lane 3). This result revealed two important points. First, two copies of the octamer motif (the natural site plus the synthetic site) were insufficient to generate U1 enhancer activity. Second, the octamer sequence was unable to cooperate with tandem TEF-1 binding sites to enhance U1 transcription in the oocyte assay. However, we do not know if TEF-1 and its required coactivator (23) are present in *X.laevis* oocytes. Because of this, we also replaced the SPH motif with another activator element that is known to have functional activity in frog oocytes; this sequence [in U1Ψ(O,USFsite)] was taken from the 5'-flanking DNA of the *X.laevis* gene that encodes the mRNA for TFIIIA. It is recognized by a transcription factor present in *X.laevis* oocytes that is homologous to the mammalian transcription factor USF or MLTF (24–26). Furthermore, this USF site is a functional cis-acting element that has been shown to stimulate expression of the TFIIIA

gene in frog oocyte injection assays (24,25). Despite this, the element was unable to activate expression of the U1 snRNA gene when substituted for the SPH motif (Fig. 4C, lane 4).

DISCUSSION

Earlier work had suggested that the GC-box, octamer, and SPH motifs present in the enhancer of a chicken U1 RNA gene were not functionally equivalent. Although all three motifs were important for full enhancer activity, the deletion or mutation of individual motifs affected transcription differently. This suggested that the proteins which recognize these motifs play distinct roles in the activation of U1 gene expression. To further define their roles in enhancer function, we tested the ability of various combinations of motifs to activate transcription from the U1 promoter.

Fig. 5 summarizes results obtained from the current data as well as from previously published studies (7,8). For constructions that contained an intact SPH motif (lines 1–3 and 5–7), the most meaningful data were obtained under competitive conditions since this eliminated the relatively high level of expression that resulted from activation by the SPH motif alone under non-competitive conditions (line 3) (7,8). On the other hand, since mutation of the SPH motif decreased expression to non-detectable levels (line 4), constructions with sequences replacing the SPH motif were primarily assayed under non-competitive conditions (lines 8–11).

Results shown in Fig. 2 indicated that the wild-type spacing between the octamer and SPH motifs was not essential to form a stable transcription complex, but was nonetheless required for optimal enhancer activity. Moreover, among the spacing mutants,

	Enhancer Motifs	U1 Gene Expression	
		non-competitive conditions	competitive conditions
1	GC — Oct — SPH	+	+
2	— Oct — SPH	+	+
3	— — — SPH	+	—
4	— Oct —	—	—
5	— Oct — — SPH	nt	(+)
6	— SPH — SPH	nt	+ / —
7	— GC — SPH	nt	+ / —
8	— GC — Oct	—	—
9	— Oct — Oct	—	nt
10	— Oct — USFsite	—	nt
11	— Oct — U5 D5	+	nt

Figure 5. Summary of the ability of various combinations of enhancer motifs to activate U1 gene expression in oocyte transcription assays. Only combinations of the octamer and the SPH motif or the octamer and the D5 motif were able to efficiently activate U1 expression. A plus sign is indicative that the construction retained at least 25% of the activity of the wild type enhancer. A minus sign indicates non-detectable activity. The plus sign in parentheses indicates that the level of activity was variable depending upon the distance between the octamer and the SPH motifs. The +/— symbol designates a very low but detectable level of transcription. The letters nt mean 'not tested.' In line 4, the diagonal lines symbolize that the SPH motif was rendered inactive by multiple point mutations. Data for lines 1–4 and 8 were reported previously in references (7) and (8).

the one with the highest level of activity contained a 10 bp insertion (one helical turn of the DNA) between the octamer and SPH motifs. This may reflect a requirement for stereospecific interactions between Oct-1 and SBF, or alternatively, a requirement for stereospecific interactions of these enhancer-bound proteins with general factors bound to the proximal region or transcription start site. Similar spacing requirements have been observed between the octamer and GC-boxes of the human U2 enhancer (5) and between the octamer and CCAAT boxes of the rat U3 enhancer (11).

In the assays done under competitive conditions (Figs. 2 and 3), a reduction in transcription from the U1 template did not always result in a corresponding increase in the level of transcription from the coinjected U4 competitor template. This implies that the full transcriptional activity of the U1 template is not required for competition with U4. Thus, it appears that stable complexes, which are capable of competing for required factors, can be formed in some cases on templates that are relatively inactive for transcription.

It was particularly interesting to find that neither a duplication of the octamer motif, nor a duplication of the SPH motif, produced an enhancer with functional activity comparable to the wild type (Fig. 5, lines 6 and 9). Thus Oct-1 and SBF seem to provide distinct, non-redundant, functions in the U1 enhancer with respect to transcriptional activation. This is consistent with studies in other systems indicating that Oct-1 requires a partner

to activate transcription from a distance. Similar to the result with two SPH motifs, a combination of GC-box and SPH motif provided only a very low level of activity (Fig. 5, line 7). On the other hand, constructions combining the octamer motif with either a GC-box or a USF site completely lacked detectable activity (lines 8 and 10). This further confirms the importance of the SPH motif in the U1 enhancer. The results of the duplication and substitution experiments, however, must be interpreted with a caveat in mind. Since spacing between motifs is a critical parameter for optimal activity (Fig. 2), it is possible that the spacing between the motifs in the artificial constructions may not be ideal for optimal enhancer activity. This has not been investigated in the current study.

In summary, however, our results provide strong evidence that Oct-1 and SBF, bound to adjacent sites on the DNA, functionally synergize to satisfy a requirement for U1 enhancer activity that is absent from or not fully provided by several other combinations of factors tested. At the present time we do not know the basis of this functional cooperativity. One possibility would be that Oct-1 and SBF bind cooperatively to DNA. However, we have so far been unable to obtain any evidence for cooperative binding of these two factors to their adjacent sites in the DNA by using gel mobility shift assays (J.Miyake and C.Cheung, unpublished results). Alternatively, Oct-1 and SBF may together provide a unique surface that is required for effective interaction with factors bound at the PSE. A third possibility is that Oct-1 and SBF each interacts with a distinct component of the basal transcription machinery. Further experiments will be required to distinguish among these (and still other) possibilities.

The octamer has been recognized for some time as a motif universally found in the enhancers of vertebrate snRNA genes. However, it has not been obvious whether any other particular motif has been conserved among vertebrate snRNA gene enhancers. SBF binding sites (SPH motifs) are present in the enhancers of three chicken snRNA genes (U1, U4B, and U4X) that we have studied (8,9,27). Moreover, all variants of the SPH motif (> 15 tested) that are recognized by SBF are also functional in an expression assay (8,9). A *X.laevis* tRNA^{(Ser)Sec} gene, which possesses an snRNA-like promoter, also contains a functional SPH motif that is required for enhancer activity (10). Similar sequences have been noted in the enhancer regions of a number of snRNA genes from other vertebrates (9). One of these related sequences, D5 (originally called DSE2), exists in the enhancer of the *X.laevis* U5 gene (4,12) and can functionally replace the U1 SPH motif in the expression assay with only about a 2-fold reduction in activity. However, gel mobility shift assays indicate that it is not a high affinity target for chicken SBF (C.Cheung, unpublished data). Thus, the *Xenopus* homolog of SBF may have a slightly different sequence specificity, or the D5 sequence may be recognized by an entirely different factor.

Interestingly, a synthetic sequence (XLU5pocs2G) intermediate in primary structure between the chicken U1 SPH motif and the *Xenopus* D5 sequence was a better activator motif than either SPH or D5. There are at least two potential explanations for this phenomenon. One possibility is that it is a better binding site for the *Xenopus* homolog of SBF. Alternatively, if the *Xenopus* D5 sequence is recognized by a factor distinct from SBF, the 'hybrid' sequence may be recognized by more than one factor in *Xenopus* oocytes (i.e., SBF and a distinct D5 binding factor). This would likely lead to the establishment of a larger number of active transcription complexes on the injected DNA and the higher level of U1 expression that is observed.

In conclusion, our results indicate that the octamer motif is insufficient to activate U1 expression, even when duplicated or associated with certain other motifs that can activate mRNA promoters. Although other combinations of enhancer motifs tested can provide a low level of U1 activation, full activity of the U1 promoter seems to require synergism between octamer and SPH motifs. Furthermore, enhancer activity is reduced by spacing alterations between the two motifs. These results suggest that, in comparison to mRNA enhancers, the requirements for the full activity of an snRNA enhancer are more stringent. Similar conclusions have been reached in two studies by Tanaka et al. (28,29). In the first study, they showed that the GAL4 protein, which can activate mRNA promoters, cannot activate the U2 snRNA promoter (28). In the more recent study, they found that an evolutionarily conserved domain present in the Oct-1 and Oct-2B proteins is required to activate U2 gene transcription. In contrast, Oct-2 protein, which lacks this domain, cannot activate U2 transcription, even though it can activate mRNA promoters (29). These stringent requirements for activation of snRNA promoters may indicate that precise interactions occur between enhancer-bound proteins and the factors that are the targets of their activation during the formation and/or function of the unique transcription complexes that are assembled on snRNA genes.

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