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Positive and negative functional interactions between promoter elements from different classes of RNA polymerase III-transcribed genes

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Consensus tRNA gene promoter elements, A and B boxes, were introduced into the coding sequence of a *Xenopus* U6 gene. Combinations in which A and B boxes were coupled to wild-type or mutant U6 promoters were made. In this way information about both the functions of individual promoter elements and functional relationships between different classes of RNA polymerase III promoter element were obtained. Mutants in which the U6 PSE was non-functional were rescued by the presence of a B box, indicating a degree of functional relationship between these two elements. Moreover, the B box acted to increase the transcriptional activity and competitive strength of the wild-type U6 promoter. In contrast, no evidence was obtained to suggest that a tRNA A box can interact productively with U6 promoter elements in the absence of a B box. Data obtained suggest that the U6 PSE functions as an 'adaptor', being necessary to enable the basal U6 promoter to respond to upstream enhancement. Certain combinations of U6 and tRNA promoter elements are shown to be mutually antagonistic by a mechanism which is likely to involve blockage of transcription initiation. In summary, the U6 and tRNA promoters are shown to consist of functionally related, but distinct, promoter elements whose interactions shed new light on their normal roles in transcription.

Key words: RNA polymerase III/transcription/tRNA gene/U6 gene

Introduction

RNA polymerase III (pol III) synthesizes a wide variety of small nuclear and cytoplasmic RNAs (for reviews, see Geiduschek and Tocchini-Valentini, 1988; Ciliberto *et al.*, 1983; Murphy *et al.*, 1989). The genes transcribed by pol III can be loosely grouped into three classes according to their promoter structure. 5S, tRNA and U6 genes are characteristic members of each group. All members of the tRNA and 5S classes have promoters which are at least partly gene internal. Genes in both of these classes contain a gene-internal A box which is highly conserved. This element is linked to a second functional domain which is different for each class. 5S genes contain an A box and a second promoter region consisting of an intermediate domain and a C box (Pieler *et al.*, 1987). This region is recognized by the 5S-specific transcription factor TFIIIA (Engelke *et al.*, 1980; Fairall *et al.*, 1986). Genes in the tRNA class are quite diverse and include the viral VA and EBER genes as well as members of the Alu gene family (Guilfoyle and Weissman, 1981; Perez-Stable *et al.*, 1984; Howe and Shu, 1989). Box B is the second gene-internal promoter element of genes in the tRNA class (Hall *et al.*, 1982; Ciliberto *et al.*, 1983). Although functional gene internal promoter elements are essential for the transcription of genes in the 5S and tRNA classes, sequences in the 5'-flanking region can strongly influence the rate of transcription from a given promoter (e.g. Larson *et al.*, 1983; Selker *et al.*, 1986). In some cases these upstream elements resemble binding sites for pol II transcription factors such as TFIID, Sp1 and ATF (Howe and Shu, 1989; Ullu and Weiner, 1985).

In contrast to the genes in the 5S and tRNA classes, the transcription of U6 and 7SK genes is entirely dependent upon a complex upstream promoter (for reviews, see Murphy *et al.*, 1989;Sollner-Webb, 1988). The whole of the mouse U6 gene coding region downstream of +1 can be removed with no apparent effect on the efficiency of transcription from its promoter (Das *et al.*, 1988). Although U6 is transcribed by pol III its promoter has striking similarity to other snRNA gene promoters which are transcribed by pol II. In fact, both pol II and pol III transcripts are produced from the *Xenopus* U6 gene promoter in oocyte microinjection experiments (reviewed in Dahlberg and Lund, 1988; Parry *et al.*, 1989a). In common with other snRNA gene promoters U6 has a functional DSE, or enhancer, which contains an octamer motif (Bark *et al.*, 1987; Carbon *et al.*, 1987; Das *et al.*, 1988; Kunkel and Pederson, 1988) and an essential PSE-like sequence (Carbon *et al.*, 1987; Mattaj *et al.*, 1988; Kunkel and Pederson, 1988; Parry *et al.*, 1989b; Lobo and Hernandez, 1989). The PSE functions together with a TATA-like A/T-rich element, centred on position −30, to form the basal U6 promoter. Removal of the A/T-rich element from a U6 promoter leaves a functional pol II-type snRNA promoter and addition of the A/T-rich element to a pol II-type snRNA promoter creates a pol III promoter. Thus, the TATA-like box plays the major role in determining the polymerase specificity of the U6 gene (Mattaj *et al.*, 1988; Lobo and Hernandez, 1989; Kunkel and Pederson, 1989). Finally, sequences at or near the site of transcription initiation play a major role in U6 promoter efficiency (Mattaj *et al.*, 1988).

Since the promoter elements required for pol III transcription of the U6 gene are very different to those required by the genes in the 5S and tRNA classes, it was of interest to determine if any of the promoter elements of these classes are functionally related. The approach followed was to introduce, by site-directed mutagenesis, consensus tRNA gene promoter elements (A and B boxes) into the coding sequencing of the U6 gene. Such artificial A and B boxes have previously been shown to be functionally active (Murphy and Baralle, 1984). The tRNA promoter elements, singly or in combination, were analysed together with both the wild-type U6 promoter and mutant derivatives thereof. We present evidence that either a tRNA B box or a U6 PSE can combine with the U6 TATA-like element to form a

functional pol III promoter, suggesting that the PSE and B box are functionally related. The A box and TATA-like element are shown to play similar roles in the selection of the site of transcription initiation. Thus the two classes of promoter consist of related, but distinguishable, elements. Different combinations of pol III elements are shown to have both synergistic and antagonistic effects on transcription activity. Implications for the normal functions of the different pol III promoter elements are discussed.

Results

Functional interactions between a B box and a U6 promoter

The effect of adding tRNA promoter elements to deletion derivatives of the U6 promoter was tested by injection of template DNAs into *Xenopus* oocyte nuclei together with a 5S maxigene which serves as an internal standard. Similar experiments were carried out either by single injection of templates or by co-injection of templates with competitor U6 mini- or maxi-gene templates with comparable results (data not shown). The constructs used are shown in Figure 1a. As previously reported, deletion or mutation of the PSE and TATA-like elements of the U6 promoter result in the loss of detectable transcription in this assay (Figure 1b, lanes 2 and 3). Removal of the DSE results in a 10- to 20-fold drop in transcription (Figure 1b, lanes wt and 1). This drop is partly compensated by the presence of a B box within the U6 coding region (Figure 1b, lane 4). To ensure that this was not due to a fortuitous effect of vector sequences brought into proximity with the promoter as a result of the deletion, a similar construct was made without deletion of the DSE (Figure 2a, construct 5′B). This construct was transcribed at a slightly higher level than wt U6 (Figure 2b, lanes wt and 3). To further investigate the strength of the agonistic interaction between the B box and the U6 promoter, the 5′B construct was co-injected with an equal concentration of either a U6 minigene or a U6 maxigene (Figure 2c, lanes 5′B), both of which have a wild-type U6 promoter but have different alterations in the U6 coding sequence (Carbon *et al.*, 1987). In both cases the 5′B construct competitively inhibited the co-injected template to a greater extent than did the wt U6 gene (Figure 2c, lanes wt). Neither a 5S gene (Carbon *et al.*, 1987) nor a wt tRNA gene (data not shown) inhibited the U6 maxigene when injected at the same molar ratio. Thus, a tRNA B box appears to be able to act synergistically with U6 promoter elements to increase relative promoter strength and the ability to compete for limiting transcription factors.

A B box can partly functionally replace the U6 PSE

Mutation of the PSE results in the loss of detectable transcription (Figure 1b, lane 2). Removal or mutation of the PSE was, however, partly compensated by the presence of a B box (Figure 1b, lane 5, Figure 2b, lane 4). Thus, combination of the TATA-like element of the U6 promoter with either a PSE or a B box results in the production of an active pol III promoter. The functional interaction of the B box and the TATA-like element does not depend on a particular spatial arrangement. This was shown by altering the sequence of the PSE in eight out of 10 positions in order to convert it into a B box (Figure 3a). Comparison with previous PSE mutagenesis (Parry *et al.*, 1989b) predicts that these changes

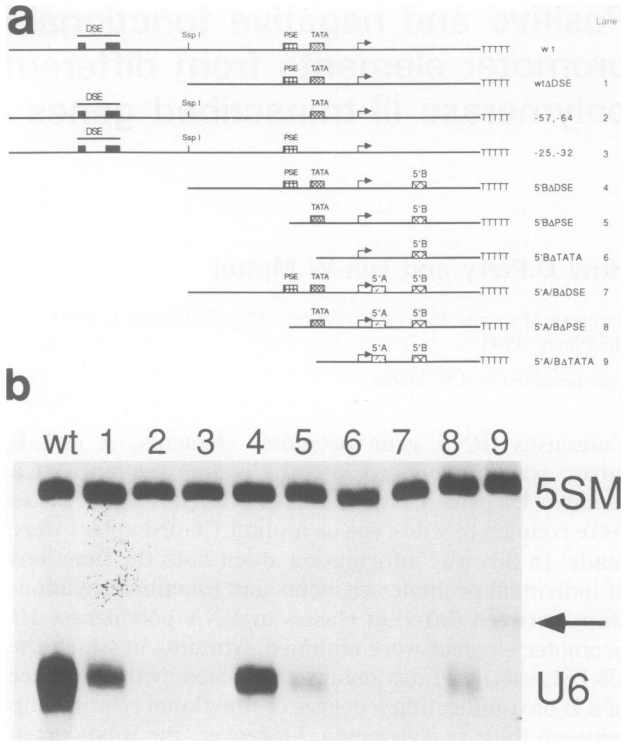

Fig. 1. Analysis of U6 promoter deletion mutants. (**a**) Structure of the constructs used in this analysis. The drawings are not to scale. Constructs containing gene internal B boxes, or an A and B box combination, had upstream sequences removed as shown. Constructs designated ΔPSE and ΔTATA have only 56 and 24 bp respectively of the wt U6 5′-flanking region. ΔDSE constructs have the promoter sequences upstream of −158 deleted. The −57, −64 and −25, −32 constructs are clustered point mutants in which the PSE and TATA-like elements are inactivated (Mattaj *et al.*, 1988). (**b**) Injection of the constructs shown in **a** into the nuclei of *Xenopus* oocytes together with a 5S maxigene internal control. The lane numbers in the figure correspond to the construct numbers given in **a**. The transcripts indicated are U6 and the 5S maxigene (5SM). The faint arrowed transcripts **lanes 8** and **9** are discussed in the text.

would completely inactivate the PSE, and, in fact, making the same mutations in a *Xenopus* U2 (pol II) PSE inactivates the pol II promoter (data not shown). The U6 PSE/B box exchange mutant was, however, weakly active (Figure 3b, compare lanes 1 and 2) when injected alone. Co-injection of either a wild-type U6 or 5S maxigene competitor with the exchange mutant abolished its transcription (data not shown), further emphasising the low activity of this construct.

A possible reason for the low transcriptional level of the TATA-B box combination promoter (725′B) was suggested by comparison of Figure 1b, lane 5 and Figure 2b, lane 4. The effect on transcription of removal of all sequences upstream of −57 from 725′B, including the DSE, to generate 5′BΔPSE, appeared to be small. This was investigated by direct comparison of the constructs shown in Figure 3d. Deletion of the DSE from the wt promoter results in a 10- to 20-fold reduction in transcription (Figure 3c, lanes 1 and 2). The same deletion has no effect on the activity of the TATA-B box combination promoter (Figure 3c, lanes 3 and 4). Thus, the PSE is required for the response of the basal U6 promoter to the DSE. This is discussed further below. In addition, comparison of lanes 2 and 4 shows that the basal

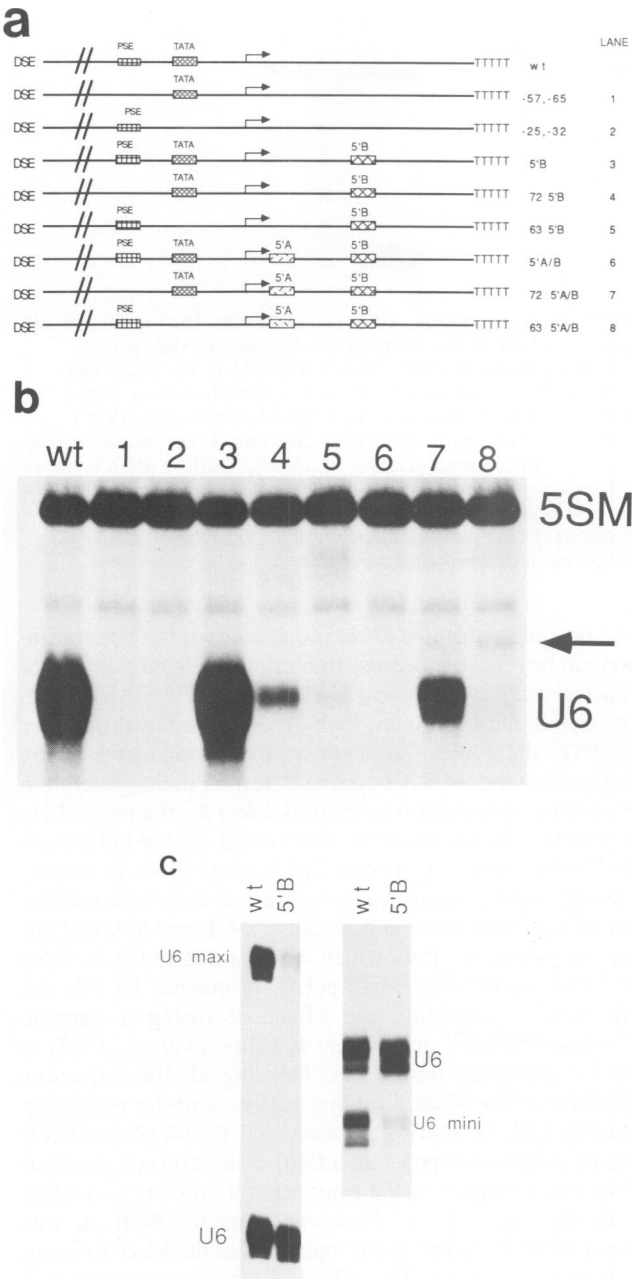

Fig. 2. Analysis of U6 mutants containing synthetic gene internal A and B boxes. (**a**) Structure of the mutants used in this analysis. The drawings are not to scale. All of the constructs also retain the DSE. A and B box sequences were introduced into individual constructs as shown in the figure. Where no TATA-like box or PSE is shown on an individual drawing, the element has been substituted by a *BglII* restriction site as detailed in Materials and methods. (**b**) Injection of the mutant constructs shown in **a** together with the 5S maxigene. (**c**) Microinjection of the wt U6 or the 5'B mutant together with the U6 maxi- or mini-gene. The templates were injected in equal molar quantities. The U6 maxi- and mini-gene transcripts are indicated. The transcripts labelled U6 arise either from wt U6 or 5'B.

PSE–TATA combination is not markedly more active than the TATA–B box combination. In other words, the TATA–B box combination makes a basal level pol III promoter which is, in terms of transcriptional activity, as efficient as the basal U6 promoter but is refractory to upstream activation.

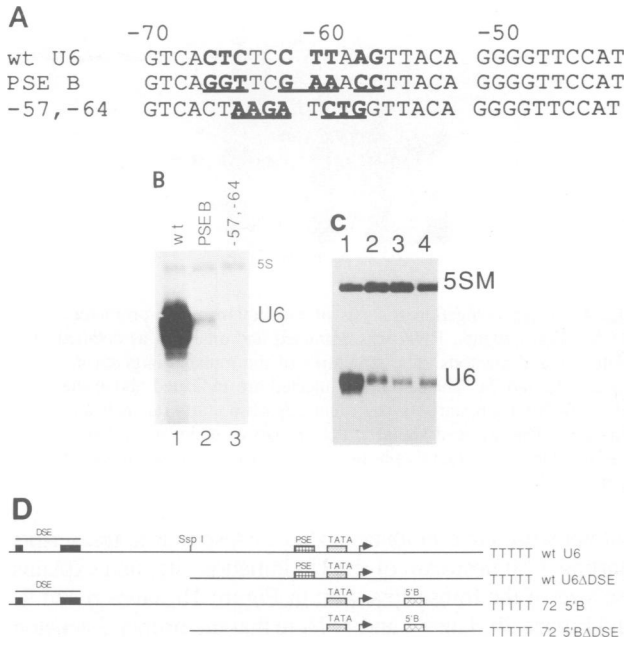

Fig. 3. Effect of replacing the PSE with a B box. (**a**) The wt U6 sequence in the PSE region was changed to that of a consensus B box by making the point changes shown. The sequence of the inactive −57, −64 mutant is shown for comparison. Previous analysis of the functionally interchangeable *Xenopus* U2 PSE suggests that the U6 PSE extends from −66 to −55. The mutations at positions −64 and −57 would, individually, be expected to inactive the PSE and those at −66, −65 and −61 to be strongly deleterious (Parry *et al.*, 1989b). (**b**) Single injection of mutants containing a B box in place of the PSE. **Lane 1**: wt U6; **lane 2**: the PSE-B box exchange construct; **lane 3**: the −57, −64 mutant. U6 and endogenous 5S transcripts are indicated. (**c**) The effect of DSE deletion. **Lane 1**: wt U6; **lane 2**: wtU6ΔDSE; **lane 3**: 72 5'B; **lane 4**: 72 5'BΔDSE. All constructs were co-injected with a 5S maxigene. (**d**) Schematic of the constructs used in **c**. The diagrams are not to scale.

The A box and TATA-like element both function in start site selection

Introduction of an A box alone into the U6 coding region together with any of the wt or mutant U6 promoters had no detectable effect (data not shown). Two different effects of the A box were observed when it was introduced in combination with a B box. The first was on the site of transcription initiation. The presence of an A box together with a B box led to the production of a novel transcript. (Figure 2b, compare lanes 5 and 8, faint arrowed transcript. Figure 1b, compare lanes 6 and 9, faint arrowed transcript; these longer transcripts are also present in Figure 2b, lane 7 and Figure 1b, lane 8, but are barely visible in the photographs. Their existence is confirmed in the experiment shown in Figure 4 below.) The transcripts observed were longer than U6. The reason for this was investigated by primer extension using a selection of the mutant constructs. In this experiment α-amanitin was co-injected with template DNAs at 1 μg/ml to prevent interference from the pol II transcripts of the U6 promoter. Two major primer extension products were seen (Figure 4). The lower (arrowed) band corresponds to the length expected for transcripts which start at the wt U6 initiation site. In this experiment this band results from primer extension products of endogenous U6 RNA as well as from transcripts of injected templates. A second product, marked with an arrowhead, is seen in all lanes where templates containing both an A and a B box were injected

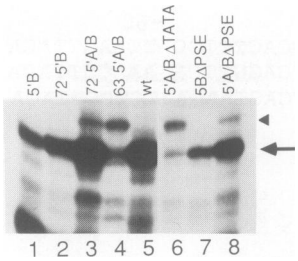

Fig. 4. Primer elongation analysis of a selection of the promoter combination mutants. RNA was extracted and analysed as detailed in Materials and methods. The structures of the mutants is given in Figures 1a and 2a. The templates injected are indicated above the lanes. Both wt U6 starts (arrow) and upstream starts (arrowhead) are indicated. The arrowed signal is due in part to endogenous U6 snRNA. Only constructs with an A and a B box showed upstream starts.

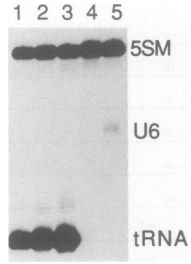

Fig. 5. Analysis of tRNA−U6 fusion constructs. The construction and sequences of the fusion constructs are described in Materials and methods. **Lane 1**: wt tRNA (Mcet 1; Ciliberto *et al.*, 1982); **lane 2**: tRNAΔCfr I in which all 5′-flanking regions have been deleted from Mcet 1 by digestion with *Cfr*I; **lane 3**: tRNA−*Bgl*, Mcet 1 mutated to form a *Bgl*II site at its 5′ end; **lane 4**: U6−tRNAΔ7; **lane 5**: −1,−8 mutant from Mattaj *et al.* (1988). The U6−tRNA construct in **lane 4** was made by the fusion of the wt U6 promoter fragment (**lane 5**) to the tRNA gene fragment (**lane 3**). This is diagrammed in Figure 6b. DNAs were co-injected with a 5S maxigene. tRNA, U6 and 5S maxigene transcripts are indicated.

but not with other templates. This corresponds to transcripts starting 7 bp upstream of the U6 initiation site, and explains the size of the transcripts seen in Figure 1b, lanes 8 and 9, and Figure 2b, lanes 7 and 8. Note that the primer extension experiment is qualitative, not quantitative. This does not affect the conclusion that the A box, in combination with a B box, selects a novel transcription initiation site. The TATA-like element of the U6 promoter has a similar function. The major transcripts arising from 5′BΔPSE and 5′A/BΔPSE (Figure 1b, lanes 5 and 8) are the same size as U6. Primer extension (Figure 4) shows that they initiate at the same nucleotide as wt U6. Removal of the TATA-like element in 5′A/BΔTATA (Figure 1b, lane 9) results in the displacement of the major site of transcription initiation from +1 to −7. Analogous results were obtained with a construct in which the TATA-like element was altered by site-directed mutagenesis (Figure 2b, lane 8). Thus, the TATA-like element must be functional in the selection of the initiation sites of the major transcripts of 5′A/BΔPSE and 725′A/B.

The second effect of the addition of an A box to constructs containing a B box was to augment transcription. This is best seen in the clustered point mutant series (Figure 2b, compare lane 7 with lane 4). It is interesting to note that the transcripts whose synthesis is stimulated are those for which the TATA-like element is involved in start-site selection. Thus, all three elements play a role in the generation of these transcripts, indicating that the TATA-like element and the A box do not mutually exclude each other's action.

The weakness of the combination promoter formed by joining consensus A and B boxes (Figure 1b, lane 9) was unexpected, and indicates the subtle nature of the structure of even 'simple' tRNA-type promoters. There are many reports of additional gene internal (e.g. Wilson *et al.*, 1985; Allison *et al.*, 1983) or 5′-flanking sequences (e.g. Larson *et al.*, 1983) being required to raise tRNA promoter activity above a basal level.

Antagonistic interactions between promoter elements

In the light of the positive and neutral interactions described so far a surprising observation was that shown in Figure 1b, lane 7 and Figure 2b, lane 6. When both an A and a B box are combined with either the complete or the basal U6 promoter the resulting construct is transcriptionally inactive. The possibility that this effect is due to transcript destabilization can be ruled out because transcripts of identical predicted structure are produced from 5′ABΔPSE and 72 5′AB (Figure 1b, lane 8 and Figure 2b, lane 7). Since removal of either the PSE, the TATA-like element, the A box or the B box singly from the wt 5′AB construct results in the generation of an active promoter (see Figures 1 and 2), the most likely explanation for the inactivity was mutual inhibition between the U6 promoter and the artificially created tRNA promoter. The only other explanation possible was that the combination of mutations made in generating 5′A/B and 5′A/BΔDSE had an inhibitory effect which was not due to the creation of a U6-internal tRNA-like pol III promoter. To rule out this second possibility the effect of fusing a genuine *C.elegans* $tRNA^{Pro}$ gene (Mcet 1, Ciliberto *et al.*, 1982) to the U6 promoter was tested. Deleting all the sequences upstream of the Mcet 1 coding region with the restriction enzyme *Cfr*I, or altering positions +1 to +6 of the tRNA coding sequence to generate a *Bgl*II restriction site, resulted in an augmentation of the transcription efficiency of Mcet 1 (figure 5, lanes 1−3). However, when the *Bgl*II site was used to fuse the U6 promoter upstream of the Mcet 1 coding sequences to generate U6−tRNAΔ7, this construct showed very low transcriptional activity (Figure 5, lane 4). Thus, fusion of a U6 promoter and either a genuine or an artificial tRNA-like promoter resulted in mutual inhibition.

A likely explanation for this inhibition was steric hindrance (Figure 8, iii). If this explanation were correct, altering the distance between the U6 and tRNA promoter elements might be expected to restore transcriptional activity (Figure 8, iv). A series of U6/Mcet 1 fusions were made in which the distance between U6 and tRNA promoters was altered. The distance between the TATA-like element and +1 of the tRNA coding sequence and between +1 and the start of the A box in the various fusions are shown in Figure 6b. The transcriptional activity of the fusions is shown in Figure 6a. While the original fusion, in which the TATA−A box distance is 26 nucleotides, is not detectably transcribed (Figure 6b,lane 2), two derivatives in which the distance between the TATA and A boxes is increased by either seven or 16 nucleotides are strongly transcribed (Figure 6b, lanes 3 and 4). Comparison of the distance between the inactive

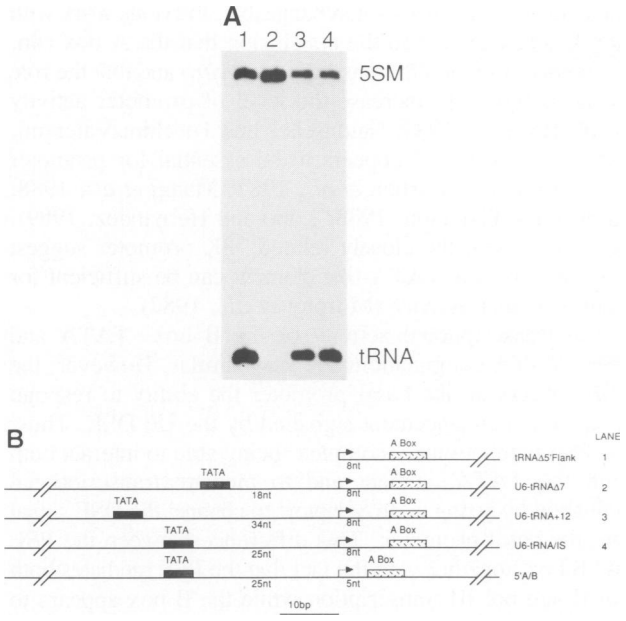

Fig. 6. Spacing dependence of fusion construct transcription. **(a)** U6−tRNA fusions in which the spacing between the U6 and tRNA promoters was varied were analysed by co-injection with a 5S maxigene into *Xenopus* oocyte nuclei. **Lane 1**: tRNAΔ5′flank; **lane 2**: U6−tRNAΔ7; **lane 3**: U6−tRNA+12; **lane 4**: U6−tRNA/1S. **(b)** Schematic of the relevant parts of the constructs used in **a**. The sequences of the fusion constructs are given in Materials and methods. The different shading of the arrow in the 5′A/B diagram is indicative of the fact that the sequences downstream of the start site in this construct arises from the U6 and not the tRNA coding region.

5′A/B construct and the active U6−tRNA/IS fusion (Figure 6b) suggests that altering the distance between the TATA-like element and the A box by as little as three nucleotides can decisively affect the properties of the resulting fusion promoter.

DNA-binding transcription factors are in excess over microinjected template DNA

The 5′AB fusion promoter was inactive, but could be reactivated by mutation of any of the four basal promoter elements (PSE, TATA, A box, B box; Figures 1 and 2). Together with the results of altering the spacing between the U6 and tRNA promoter elements, a model for inactivity can be proposed. In this model, transcriptional inactivity results from the formation of two mutually inhibitory transcription complexes on the same template (Figure 8, iii). This can arise in one of two situations. In the first (Figure 7a, A), DNA-binding transcription factors are limiting, but bind cooperatively to the fusion promoter templates such that templates are either fully occupied or unoccupied (or occupied by incomplete, and therefore inactive, transcription complexes). In the second, DNA-binding transcription factors are in excess over the template (Figure 7A, B). The predicted effects of adding a competitor template are different in the two cases. If factors are in excess (Figure 7a, B) the competitor template will be transcribed (indicated by arrows) while the fusion template remains inactive. If factors are limiting (Figure 7a, A) two different outcomes are possible. If the fusion promoter has a higher affinity for tRNA promoter binding factors than the tRNA gene, then both templates will remain inactive (Figure 7a, A, left panel).

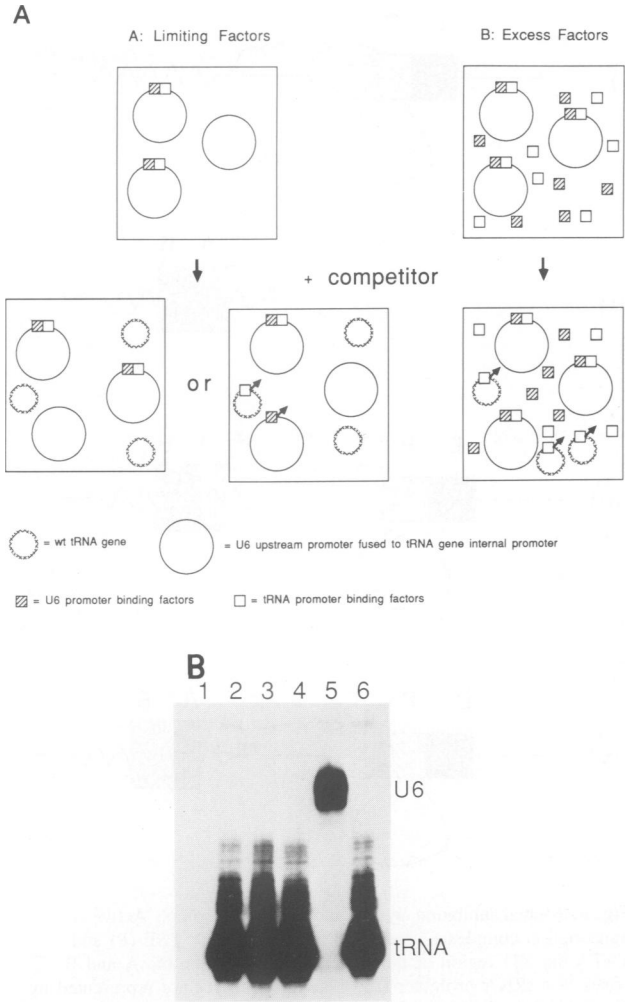

Fig. 7. Effect of competition on the expression of 5′AB. **(a)** Schematic of possible outcome of the competition experiment. **(b)** Injection of 5′AB either alone (**lane 1**) or in the presence of increasing molar ratios of Mcet 1 competitor (**lanes 2−4**). The 5′AB concentration was always 100 μg/ml. **Lane 5**: 5′B injected alone as a size marker; **lane 6**: Mcet 1 injected alone.

If, on the other hand, the tRNA gene has an equal, or higher, affinity for its cognate transcription factors then some of the DNA-binding transcription factors will be sequestered from the fusion template onto the tRNA template resulting in transcription of both templates (Figure 7a, A, right panel).

These models were tested experimentally by co-injection of the 5′A/B template with the wt Mcet 1 tRNA gene. Control experiments showed that the wt U6 gene was transcribed in the presence of an equal quantity or a 5-fold excess of Mcet 1 but was inhibited by a 9-fold excess of Mcet 1 competitor (data not shown, but see Carbon *et al.*, 1987 for an analogous experiment with a 5S gene). Single injection of Mcet 1 or of 5′B (as a marker for the size of transcripts expected from 5′AB) gave rise to transcripts, while single injection of 5′AB did not (Figure 7B, lanes 6, 5 and 1 respectively). Co-injection of an equal concentration of Mcet 1 with 5′AB, or of a 2-fold or a 5-fold excess of Mcet 1 (Figure 7b, lanes 2−4) failed to reactivate 5′AB. Nevertheless Mcet 1 was actively transcribed in all cases. This result indicates (Figure 7a, B) that DNA-binding transcription factors must be in excess over template DNA

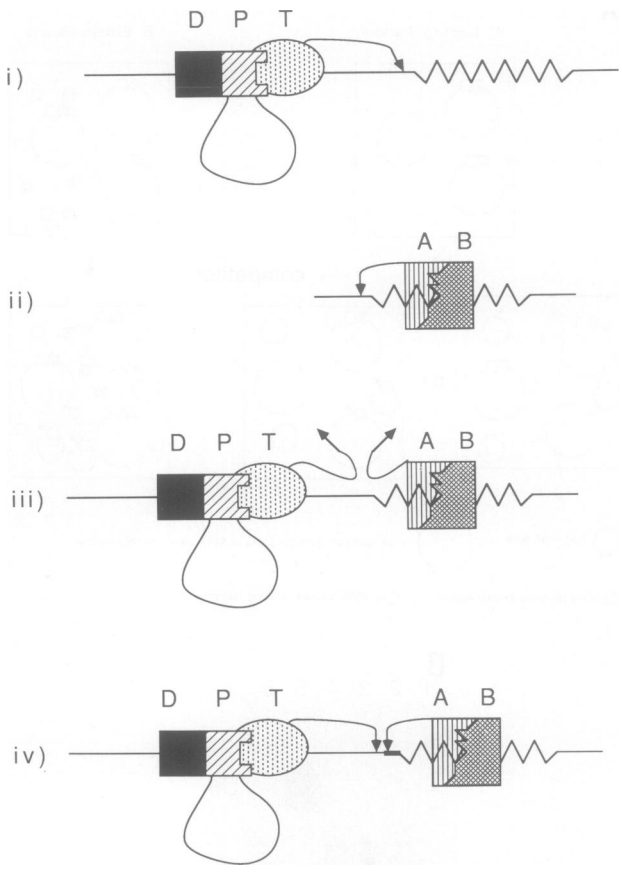

Fig. 8. Mutual inhibition by U6 and tRNA promoters. Active transcription complexes assembled on the DSE (D) PSE (P) and TATA-like (T) region of the U6 promoter (i) or on the A and B boxes of a tRNA promoter (ii). Coding sequences are represented by zig-zag lines, other DNA sequences by single lines. The DNA between the PSE and DSE regions is drawn as a loop. The arrows represent the transcription activating signals generated by the assembled transcription complexes which ultimately lead to transcription initiation. If the two complexes are too close to one another inhibition of transcription activation occurs (iii). This effect is relieved by increasing the spacing between the two promoters (iv).

under the condition of our experiments, and supports the idea that the U6–tRNA fusion templates are fully occupied with DNA-binding transcription factors. Similar competition experiments were carried out with the U6 maxigene (data not shown) and a 5S maxigene (data not shown but see Figure 6a) with identical results. The co-injected template was always transcribed while the fusion promoter remained inactive.

Discussion

Functional relationships between promoter elements from two different classes of RNA polymerase III-transcribed genes have been investigated. The results obtained allow new insights into the functions of parts of the U6 promoter in transcription initiation and in determining promoter strength. These will be discussed briefly.

The B box and the PSE

Either of these elements, when combined with the A/T-rich TATA-like element and transcription initiation regions from U6 will form a basal pol III promoter. They are thus, at some level, functionally interchangeable. Previous work with tRNA genes has led to the conclusion that the A box can, on its own, function in transcription *in vitro* and that the role of the B box is to increase the level of promoter activity (Ciliberto *et al.*, 1983; Geiduschek and Tocchini-Valentini, 1988). The U6 PSE appears to be essential for promoter function *in vivo* (Carbon *et al.*, 1987; Mattaj *et al.*, 1988; Kunkel and Pederson, 1988; Lobo and Hernandez, 1989), but results with the closely related 7SK promoter suggest that, *in vitro*, the TATA-like element can be sufficient for transcriptional activity (Murphy *et al.*, 1987).

The transcriptional activity of the B box–TATA and PSE–TATA combinations are very similar. However, the PSE confers on the basal promoter the ability to respond to upstream enhancement signalled by the U6 DSE. Thus, the PSE is functionally complex, being able to interact both with the TATA element and to mediate transcriptional activation by acting as an 'adaptor' to channel the DSE signal into the basal promoter. This difference between the PSE and B box, together with the fact that the PSE mediates both pol II and pol III transcription while the B box appears to be confined to pol III promoters, suggests that these two elements, although to some extent functionally similar, will bind to different transcription factors. It will be interesting to determine whether other pol III genes capable of responding to upstream binding sites for 'pol II' transcription factors (e.g. Howe and Shu, 1989) also require an 'adaptor'. Further, it is known that snRNA DSEs do not function with TATA-type pol II promoters and that at least some enhancers from non-snRNA genes cannot stimulate transcription through a PSE (Ciliberto *et al.*, 1987; Dahlberg and Schenborn, 1988; Tanaka *et al.*, 1988). Thus the PSE-based activation pathway must act through a specialized mechanism. It will be of interest to determine how much of this pathway is conserved between the two (pol II and pol III) types of PSE-containing promoter.

The A box and the TATA-like element

As discussed above, these two elements are sufficient, when tested *in vitro*, to direct transcription. The A box is also involved in the choice of the initiation site (Ciliberto *et al.*, 1983) although there is not an absolutely defined distance upstream of the A box at which transcription initiates. For example, the distance between the A box and the site of transcription initiation differs substantially in tRNA and 5S genes. The combination promoters studied here show that the TATA-like U6 element has a role in the selection of the initiation point. This can be best seen by comparing the major start sites of the 72 5′AB and 63 5′AB constructs (Figure 2b, lanes 7 and 8). The construct which retains the TATA element starts mainly at the wt U6 initiation site, while the construct in which the TATA is mutant starts 7 bp further upstream. Previous experiments (Mattaj *et al.*, 1988) have also shown an important role for the sequence around the initiation point in the definition of the U6 transcription start site and, perhaps as a consequence, in promoter efficiency. The choice of the U6 initiation site therefore depends on at least two components.

In summary, the results discussed so far indicate that the two classes of promoter are constructed of functionally similar elements which, presumably, bind functionally related but different transcription factors. The major difference seen between the two promoters seem to be the

degree of flexibility of the TATA-like element compared to the A box. In addition to its interaction with the PSE, the TATA element can be combined with the B box, positioned either upstream or downstream, to form a functional promoter, whereas the only functional *in vivo* combinations containing the A box also contained a B box. This may indicate that the factor(s) binding to the TATA-like element has more interacting surfaces, which enable it to interact with several other factors, while the A box-binding factor(s) may not have a surface capable of interacting with the PSE-bound factor(s). Much progress has been made in the characterization of tRNA and 5S gene transcription factors (Geiduschek and Tocchini-Valentini, 1988). A similar effort with genes of the U6 class is now required in order that the structural basis of the similarities and differences can be resolved.

Antagonistic interactions between two adjacent promoters

A surprising result was that combination of all four basal promoter elements, i.e. PSE, TATA, A box and B box (for simplicity, the initiation region is ignored in this discussion) led to transcriptional inactivity, or at least to marked mutual inhibition between the two linked promoters. This was true both when an artificial tRNA-like promoter was generated within the U6 coding sequence and when a *C.elegans* $tRNA^{Pro}$ gene was linked to the U6 promoter. Two experimental observations are particularly relevant to the mechanism of this mutual antagonism. First, removal of any of the four basal promoter elements from the construct relieves the inhibitory effect (Figures 1 and 2). Second, inhibition depends critically on spacing. A difference of 3 bp in the distance between the TATA and A box elements changed the activity of the resulting construct from undetectable to high. This is diagrammed in parts iii and iv of Figure 8. These results strongly suggest that inhibition occurs as a result of the assembly of two mutually inhibitory transcription complexes on the same template, which may prevent transcription initiation by steric interference.

This explanation can only be valid in one of two circumstances which are diagrammatically represented in Figure 7a. In the first all microinjected templates are fully occupied by DNA binding transcription factors (Figure 7a, B). In the second (Figure 7a, A) there are two populations of templates, one of which is fully occupied and which sequesters essential (DNA bound or non-DNA bound) transcription factors in inactive complexes, thereby preventing transcription from partly occupied or unoccupied templates. 5S (Figure 6a), U6 (unpublished data) and tRNA genes (Figure 7b) are all expressed when co-injected with the U6/tRNA combination promoter, although the combination promoter always remains inactive. The combination template does not, therefore, irreversibly sequester any factors necessary for pol III transcription, and it cannot be activated by co-injection of another pol III promoter. It follows (Figure 7a, lower panels) that transcription factors which stably associate with DNA are in excess over the number of injected U6/tRNA combination templates ($\sim 10^9$ per oocyte), and that the microinjected templates are fully occupied.

This conclusion is unexpected and, at first sight, seems to be in contradiction to earlier electron microscopic data, which suggested that only a small proportion of the DNA templates microinjected into oocytes were assembled into active chromatin (e.g. Trendelenburg *et al.*, 1980). There is, however, no real contradiction since only actively transcribing templates with nascent RNA chains and inactive templates are distinguishable in the electron microscope. Our data suggest that many templates with bound transcription factors, but from which no transcription is taking place, can exist in the oocyte, and such templates would be unlikely to be distinguishable from templates without bound transcription factors in the electron microscope.

The proposal that DNA-binding transcription factors are in excess over template DNA is relevant for the interpretation of the mechanism of competitive inhibition between microinjected templates. The 5'B construct, in which the wt U6 promoter is attached to a U6 coding region containing a B box, is a stronger template than either the U6 minigene or the U6 maxigene (Figure 2c). There are two possible explanations for this. Either the two templates compete for limiting DNA-binding transcription factors (analogous to Figure 7a, A) or the assembled transcription complexes compete for limiting, essential soluble factors (e.g. RNA polymerase). If all templates are fully occupied by DNA binding transcription factors then the explanation for the difference in competitive ability must be that the combination of factors bound to the U6/B box combination promoter has a higher affinity for limiting soluble factors required for transcription than does the wt U6 promoter on its own. It will be of interest to determine whether this is the mechanism by which natural pol III genes like U6 or the EBER genes have created strong promoters by combining elements normally found in different promoter classes.

Materials and methods

Mutagenesis and promoter constructs

Oligonucleotides were synthesized with the phosphoramidite method (Sinha *et al.*, 1984) on an Applied Biosystems synthesizer and used for mutagenesis by the method of Kramer *et al.* (1984) or by using a site-directed mutagenesis kit (Amersham International PLC, Amersham, UK). Template DNAs were cloned into an M13 mp9 vector in order to carry out the mutagenesis reaction. All oligonucleotides used for mutagenesis had 12–15 nucleotides complementary to the wt U6 sequence on either side of the non-complementary mutagenic region. In cases where more than one region was mutated in the wt U6 DNA, the mutagenesis was carried out stepwise. The wt *Xenopus* U6 DNA used for all mutagenesis reactions was the 977 bp *Bam*HI fragment reported in Krol *et al.* (1987).

A/B box-containing constructs

The A and B box oligonucleotides introduced the following changes into the wt U6 DNA. 5'A box oligo changes the wt sequence from +6 to +15 to GGCTTAGCCG. 5'B box oligo changes the wt sequence from +50 to +59 to GTTCGAAACC.

The −25,−32 and −57,−64 *Bgl*II mutants described in Mattaj *et al.* (1988) were used as templates in mutagenesis reactions to make constructs in which the 'TATA-like' or PSE functions have been eliminated. The structure of the mutants created by these site-directed mutagenesis reactions is shown diagrammatically in Figure 2a.

Constructs designated ΔDSE (see Figure 1a) were made by sub-cloning the *Ssp*I–*Eco*RI fragment (lacking the DSE) from the appropriate parent construct into pUC19 (*Sma*I/*Eco*RI cut).

ΔPSE and ΔTATA mutants shown in Figure 1a were made as follows: *Bgl*II–*Eco*RI fragment from 72 5'B (structure shown in Figure 2a) was subcloned into pUC 19 (*Bam*HI/*Eco*RI cut) to generate the 5'BΔPSE construct. Similarly, the *Bgl*II–*Eco*RI fragments were subcloned from the 63 5'B, 72 5'AB and 63 5'AB constructs shown in Figure 2a to generate the 5'BΔTATA, 5'ABΔPSE and 5'ABΔTATA mutants shown in Figure 1a. The upstream *Bgl*II sites used in the sub-cloning procedures were introduced in the mutagenesis reactions, described above, which eliminate the PSE and TATA-like sequence elements.

A comparison of wt U6 and a construct containing a B box in place of the PSE is shown in Figure 3a.

U6 – tRNA fusion constructs

The tRNAΔCfr construct was made by digesting Mcet 1 (see Ciliberto *et al.*, 1982) with Cfr I. The 5′ overhang was filled in using the Klenow enzyme and the plasmid recut with *Eco*RI. The fragment containing the tRNA coding region was then sub-cloned into pUC 19 cut with *Sma*I and *Eco*RI. The tRNAΔCfr construct has no wt tRNA sequence upstream of +1. The U6 *Bgl*/START mutant was generated by the insertion of AGATC between +1 and +2 of the U6 coding region. After *Bgl*II digestion, Klenow-mediated repair and *Hin*dIII digestion, the promoter fragment of U6 *Bgl*I/START was inserted into tRNAΔCfr digested with *Hin*dIII and *Sma*I to generate U6−tRNA+12.

To make the tRNA−U6Δ7 construct the sequence from +1 to +6 of the plasmid Mcet 1 was changed to AGATCT (to generate the construct tRNA−*Bgl*+1). The −1,−8 U6 construct (Mattaj *et al.*, 1988) was digested with *Bgl*II and *Eco*RI and treated with alkaline phosphatase. The *Bgl*II/*Eco*RI fragment containing the tRNA coding region from tRNA−*Bgl*I was then sub-cloned into this vector to generate the tRNA−U6Δ7 clone.

The tRNA−U6/1S fusion was generated in an analogous manner to the above constructs. The *Bgl*II/EcoRI fragment from tRNA−*Bgl* (+2), in which the Mcet 1 coding sequence from +2 to +7 was changed to AGATCT, was fused to the promoter fragment of *Bgl*/START at its *Bgl*II site (see above).

The sequences of the fusion plasmids are listed in comparison with the 5′AB constructs.

5′AB:	CGCTGGAGTTTCG TGCT	− A box
U6−tRNA+12:	CGCTGGAGTTTCGAGATCGGTGGCCGAAT	− A box
U6−tRNA/1S:	CGCTGGAGTTTCGAGATC TT	− A box
U6−tRNAΔ7:	CGCT AAGATC TAT	− A box

Oocyte microinjection

Oocytes were microinjected with the 50 nl of DNA at a total concentration of either 100, 250 or 500 μg/ml. Most figures show results obtained with injection at the highest concentration, but no qualitatively different effects were seen at lower concentrations. When U6 maxi- or mini-gene competitor DNAs were included in the DNA mixture they were mixed in a 1:1 ratio with the test construct. The 5S maxigene DNA (Bogenhagen *et al.*, 1980) was injected at a final concentration of 1 μg/ml (Figures 1, 2 and 3) or 100 μg/ml (Figures 5 and 6). [α-^{32}P]GTP (Amersham) was co-injected with the DNA at 0.1−0.5 μCi per oocyte. RNA was extracted and analysed as described (Mattaj and De Robertis, 1985). Oocytes were extracted and analysed in batches of 8−10. Half an oocyte equivalent (~2.5 μg) of RNA was loaded onto the gel.

Primer elongation

The oligonucleotide used for the primer elongation analysis was described previously (Mattaj *et al.*, 1988). The (single-stranded) oligonucleotide was labelled at the 5′ end using polynucleotide kinase. RNA to be analysed was extracted as described above. 10 μg of RNA (approximately two oocyte equivalents) was ethanol precipitated and resuspended in 90% dimethylsulphoxide, heated at 45°C for 20 min, ethanol precipitated and resuspended in 50 mM Tris−HCl pH 8.3, 5 mM $MgCl_2$, 50 mM KCl, 1 mM dithiothreitol with ~250 000 c.p.m. of the labelled primer. The sample was heated to 65°C for 5−10 min and slowly cooled to <40°C over several hours. The sample was then ethanol precipitated/washed and taken up in the buffer descibed above. Each dNTP was added to a 1 mM final concentration and 20 units of AMV reverse transcriptase was also added. The final reaction volume of 30 μl was incubated for 2 h at 42°C. After treatment with 0.3 M NaOH (10 min at 56°C) and neutralization, the sample was ethanol precipitated (twice) washed with 70% ethanol and loaded on a 15% denaturing polyacrylamide gel.

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