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Optimal tRNA$^{(Ser)Sec}$ gene activity requires an upstream SPH motif

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ABSTRACT

The X. laevis tRNA$^{(Ser)Sec}$ gene is different from the other tRNA genes in that its promoter contains two external elements, a PSE and a TATA box functionally equivalent to those of the U6 snRNA gene. Of the two internal promoters governing classical tRNA gene transcription, only subsists the internal B box. In this report, we show that the tRNA$^{(Ser)Sec}$ contains in addition an activator element (AE) which we have mapped by extensive mutagenesis. Activation is only dependent on a 15 bp fragment residing between −209 and −195 and containing an SPH motif. In vitro, this element forms a complex with a nuclear protein which is different from the TEF-1 transcriptional activator that binds the SV40 Sph motifs. This AE is versatile since it shows capacity of activating a variety of genes in vivo, including U1 and U6 snRNAs and HSV thymidine kinase. Unexpectedly for an snRNA-related gene, the tRNA$^{(Ser)Sec}$ is deprived of octamer or octamer-like motifs. The X.laevis tRNA$^{(Ser)Sec}$ gene represents the first example of a Pol III snRNA-type gene whose activation of transcription is completely octamer-independent.

INTRODUCTION

Transcription of tRNA genes by RNA polymerase III (Pol III) necessitates in most cases two promoter elements situated inside the coding region. These constitute the Internal Control Regions (ICR) box A and box B. The transcription complex made on these promoters directs the Pol III to initiate transcription upstream of the mature 5′ end of tRNAs (reviewed in ref. 1).

The selenocysteine tRNA$^{(Ser)Sec}$ gene must be considered separately. This tRNA is the carrier molecule upon which selenocysteine is synthesized and serves as a donor of selenocysteine to the nascent polypeptide in response to specific UGA codons (reviewed in ref.2). Genes coding for tRNA$^{(Ser)Sec}$ are ubiquitous in the animal kingdom (3) and are also found in enterobacteria (4). Transcription of the tRNA$^{(Ser)Sec}$ gene also deserves special interest. It was previously shown that, unlike classical tRNA genes i) the start of transcription coincides with the mature 5′ end (5) ii) box A is debilitated by a two nucleotide insertion iii) a TATA sequence is required for efficient transcription (6). In a recent work, we reported the detailed analysis of the tRNA$^{(Ser)Sec}$ gene transcription by RNA polymerase III and showed that the promoter of this gene is tripartite (7). It is constituted by two upstream elements, a PSE (Proximal Sequence Element) and a TATA motif, in addition to the internal box B as in the other tRNA genes. The PSE and the TATA motifs are also two essential promoter elements of the vertebrate U6 and 7SK RNA genes which together constitute another class of Pol III genes with external promoter elements (reviewed in 8−10). The interplay of these two promoters confers the Pol III specificity to the vertebrate U6 gene (11−16). However, the PSE which is also found in Pol II snRNA genes, although similar in sequence and required for efficient and accurate Pol II transcription (reviewed in 8) is not positionally equivalent to the Pol III PSE (16).

Based on the fact it shares promoter elements with U6 and classical tRNA genes, the tRNA$^{(Ser)Sec}$ gene constitutes the paradigm of an intermediate class of Pol III genes. Upstream of the PSE, at about −220/−260 bp, is found in U6 and Pol II snRNA genes an element termed the DSE (Distal Sequence Element) which functions as an activator element always containing the octamer sequence ATGCAAAT as an obligatory motif. The activator element of the human 7SK gene, whose transcription functions similarly to U6 genes (17), requires a CACCC motif (18). It is unclear, however, whether this sequence could cooperate with an octamer sequence in vivo. A number of Pol II snRNA gene DSEs have been dissected, leading to the conclusion that other motifs always functionally cooperate with the octamer. Protein Sp1 binding sites were found in the human and X. laevis U2 gene DSEs (19−21), SPH motifs in the chicken U1 and U4B gene DSEs (22−25) and CREB and AP2 motifs in the human U4C DSE (26). As yet unknown motifs were also mapped in addition to Sp1 and octamer motifs in the X. laevis U2 DSE (21) and in the X. laevis U5 DSE in conjunction with the octamer (27). Despite the resemblance of these motifs with those known to activate protein coding gene transcription, snRNA and mRNA gene activator elements are functionally unrelated. This is exemplified by experiments showing that an snRNA

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octamer motif is unable to activate mRNA coding genes (28, 29). In fact, in Pol II snRNA genes, the DSE has a stabilizing effect on the transcription complex which is different from that established on protein coding genes (30–32). Among the set of proteins that presumably bind to the various snRNA gene DSEs, the ubiquitous transactivator OTF-1 has been shown to be the octamer motif binding protein (32). In the case of the chicken U4B DSE, another protein, termed SBF, mediates with OTF-1 the stimulatory effect of the SPH motif (25).

Here, we report that the optimal in vivo expression of the tRNA$^{(Ser)Sec}$ gene requires the presence of an upstream activator element. Surprisingly, however, and in marked contrast to what was described for Pol II and Pol III snRNA or snRNA-like genes thus far, the tRNA$^{(Ser)Sec}$ gene activator element, which contains an SPH motif, functions in a self-sufficient manner, independently of any octamer or octamer-like motifs.

MATERIALS AND METHODS

Mutagenesis and constructs

Construction of the altered tRNA$^{(Ser)Sec}$, U6, U1 and tk CAT genes was done by using standard recombinant techniques or by site-directed mutagenesis with the in vitro Amersham kit. Constructs were verified by DNA sequencing and double-stranded DNAs served as mutated templates.

tRNA$^{(Ser)Sec}$ constructs

Genes truncated at positions −300, −280, −262, −240, −220 were obtained as follows: fragments −300,+103 ; −280,+103; −262,+103 ; −240,+103 ; −220,+103 were amplified from tRNA$^{(Ser)Sec}$−318,+103 by PCR using primer P2 (7) and P4 to P8 complementary to positions −300,−284 ; −280,−263 ; −262,−242 ; −240,−221 ; −220,−205 of the non coding strand, respectively. The 9 non-complementary bases at the 5′-end of P4 to P8 contain a BamHI site. The amplified fragments were digested with EcoRI and BamHI and then ligated to EcoRI-BamHI cut pBluescribe (+) (pBS+, Stratagene). The LS-80,−73 mutant (7) was digested with BamHI and BglII and self-ligated in diluted conditions to produce tRNA$^{(Ser)Sec}$ −72,+103.

Constructs with moved and flipped AEs

Constructs C342 and C343 were obtained by creating a BglII site at position −123 on tRNA$^{(Ser)Sec}$ −262,+103 and at position −169 on tRNA$^{(Ser)Sec}$ −318,+103 (7) respectively. AE was moved 46 bp upstream and placed in the reversed orientation by ligating in the wt and inverted orientations, respectively, the 143 bp BamHI-BglII fragment of C342 to the BamHI-BglII cut and phosphatased C343 construct. This BamHI-BglII fragment contains sequences from −262 to −122 along with 3 nucleotides arising from the BamHI restriction site. AE was moved 46 bp downstream by ligating in the wt orientation the 157 bp BamHI-BglII fragment of C343 to the BamHI-BglII cut and phosphatased C342 construct. This BamHI-BglII fragment contains sequences from −318 to −168 along with 7 nucleotides of 5′ sequences deriving from the polylinker.

U6 constructs

BglII sites were created at positions −342 and −196 of wt U6 (33) to yield C115 which was digested with BglII and self-ligated in diluted conditions to produce U6(−DSE). The 157 bp BamHI-BglII fragment of C343 (see above) was cloned in the wt orientation into the BglII site of U6(−DSE) to yield U6(−DSE+AE).

U1 constructs

The Xl U1B1 gene (34) contained in the 1148 bp PstI fragment from pXl U1-AB(P) (35) was cloned clockwise into the PstI site of pUC19 to yield Xl U1B1. The 462 bp HindIII fragment of Xl U1B1 was cloned clockwise into the HindIII site of pUC19 to yield Xl U1B1(−DSE). The 157 bp BamHI-BglII fragment of C343 was cloned in the wt orientation into the BamHI site of Xl U1B1(−DSE) to yield Xl U1B1(−DSE+AE).

tk CAT constructs

The 157 bp BamHI-BglII fragments of C343 and C343 bearing S−210/−207 (see Figure 1B) were inserted in the wt orientation into the BamHI site of pBLCAT8+ (36) to generate (wt AE)-tkCAT and (mut.AE)-tkCAT, respectively. Other constructs were previously described (7,11)

Oocyte microinjections

X. laevis oocyte nuclei were microinjected with 20 nl of DNA at a final concentration of 800 μg ml^{-1}. In the experiments shown in Figures 1A, 2, 4A, and 5 concentration of the injected templates was 400 μg ml^{-1}. Concentration of the coinjected maxi-tRNA$^{(Ser)Sec}$ (in Figures 1A, 2 and 5) was 400 μg ml^{-1}. In the experiment shown in Figure 4B, concentration of the injected templates was 125 μg ml^{-1}. The 5S maxigene was added at a concentration of 5 μg ml^{-1}. pUC19 DNA was added to maintain the final DNA concentration at 800 μg ml^{-1}. [α-^{32}P] GTP (400 Ci/mmol, 0.2 μCi per oocyte) was coinjected with the DNA. Oocytes were incubated and RNA extracted from batches of 10 oocytes as previously described (35) and 1/2 oocyte equivalent for tRNA$^{(Ser)Sec}$ or 1 oocyte equivalent for U6 and U1 was loaded onto 10% sequencing gels. The relative transcription efficiencies of the tRNA$^{(Ser)Sec}$, U6 and U1 mutants were measured by scintillation counting using the 5S maxigene transcript as the standard. In the experiment shown in Figure 4C, the templates and the pCH110 vector (37) were coinjected at a concentration of 300 μg ml^{-1} and the final DNA concentration was maintained to 800 μg ml^{-1} by addition of pUC19 DNA.

CAT activity in Xenopus oocytes

Extracts from 10 microinjected oocytes incubated at 19°C for 20 h were prepared as described in (38). The β-galactosidase activity used to monitor the injection efficiency was measured in the extracts as described in (39). The quantity of the extract used for the CAT activity was adjusted according to the injection efficiency indicated by the β-galactosidase activity. The volume of extract was completed to 100 μl with 250 mM Tris-HCl (pH 8). 20 μl of 4mM acetylcoenzyme A and 0.2 μCi of ^{14}C chloramphenicol (60 mCi/mmol) were added and the reaction was carried out as described in (39).

Bandshift

The gel retardation assay was performed essentially as described in (40) with the following modifications. 8 μg of nuclear extracts (41) prepared from Hela cells or from Hela cells infected with a recombinant vaccinia virus (42) were mixed with 3 μg of poly dI.dC and 200 ng of plasmid pBS as non specific competitors in a 10 μl reaction volume containing 10 mM Hepes-KOH (pH 7.9), 1 mM DTT, 1mM $MgCl_2$, 30 mM KCl and 12% glycerol and incubated for 15 min at 0°C. The DNA template (20 fmoles, 10^4 cpm) was added and incubation continued for 20 min at 20°C. The resulting complexes were resolved in a 4% non denaturing polyacrylamide gel (29:1) in 0.25×TBE. The gel was

preelectrophoresed for 90 min at 11V cm^{-1}. Electrophoresis was carried out at the same voltage for 3 hours. The gel was then dried and fluorographed. For competition assays, competitor DNA and ^{32}P 5′ end-labeled probe were added simultaneously to the preincubation mix.

RESULTS

An activator element lying between −209 and −195 contains an SPH motif

In the recent report in which we provided a detailed analysis of the X. laevis tRNA$^{(Ser)Sec}$ gene transcription, we mentioned that it may contain an upstream activator element (7). In order to characterize it, the following experiments were carried out. Knowing that the upstream boundary of the gene maps at position −318, a series of 5′ truncated mutants extending from −300, −280, −260, −240 and −220 were prepared by PCR. The effect on transcription of these deletion mutants was measured by Xenopus oocyte nuclei microinjections in the presence of a tRNA$^{(Ser)Sec}$ maxigene as a competitor to obtain a more stringent assay. None of these mutants affect the template activity, as shown in Figure 1A, lane 3 for the −220,+103 construct, while truncation at −206 (construct −206,+741) substantially reduced transcription (lane 4). This reduced activity can only be accounted for by the upstream truncation since we have previously shown that regions comprised between +103 and +741 are transcriptionally inert (7). Activities of the wt (−318,+103) tRNA$^{(Ser)Sec}$ gene without (lane 1) or with (lane 2) competitor are shown for comparison. These experiments allow localization of the element downstream of −220. To identify the position, 8 clustered point mutations (S−219/−211,S−210/−207, S−206/−201, S−200/−198, S−197/−195, S−194/−192, S−191/−188, S−187/−181) were introduced which span positions −219 to −181 (Figure 1B). The template activity of these substitution mutants is displayed in Figure 1A. Only three of these, S−210/−207 (lane 11), S−206/−201 (lane 12), S−197/−195 (lane 6) affect the transcriptional activity and induce a 20-fold drop effect (see also for comparison in lane 9 the transcriptional activity of a construct carrying only 72 bp of 5′ flanking sequences, i.e. still retaining the PSE and TATA promoter elements). Remarkably, the wt transcription level is recovered past position −195 (compare lanes 7, 13, 14 with lane 2). Substitutions S−210, S−209, and S−195 were used to define with precision the borders of the element. Figure 1A shows that S−210 was without effect (lane 15) while S−209 and S−195 led to a significant drop in transcription efficiency (lanes 16 and 17).

Altogether, the results of this mutagenesis experiment allow us to conclude that another element spanning 15 bp between −209 and −195 participates in the tRNA$^{(Ser)Sec}$ gene transcription. Inspection of the DNA sequence between −209 and −195 shows that a perfect match with the SPH motif GCATGC contained in the SV40 enhancer and chicken U1 and U4 DSEs (43,44,24,25) resides between −206 and −201 (Figure 1B). The tRNA $^{(Ser)Sec}$ SPH motif is 5′ extended by an essential CCA sequence and contains 3′ to it the sequence GCG (−197/−195) whose abolition also induces a down effect on transcription activity. The tRNA$^{(Ser)\ Sec}$ motif appears to be modular in structure since the CTC to AGA substitution (S−200/−198 in Figure 1B) has no incidence on the template activity which remains at the wt level (Figure 1A, lane 5). Therefore the SPH motif which we have identified in the X.laevis tRNA$^{(Ser)Sec}$ gene resembles the chicken U4B SPH motif (25) in structure much more than the SV40 Sph-I motif (Table I).

The nature of this element was further elucidated by an experiment in which the 15 bp DNA motif was flipped or moved 46 bp upstream or downstream of its normal position. Figure 2, lanes 3−5, shows that the templates retain a substantial amount of activity in the presence of a competitor even when the element is moved downstream, inducing the more pronounced effect (lane 3). That this element can function closer (lane 3) or farther (lane 4) from its normal position or in the reverse orientation

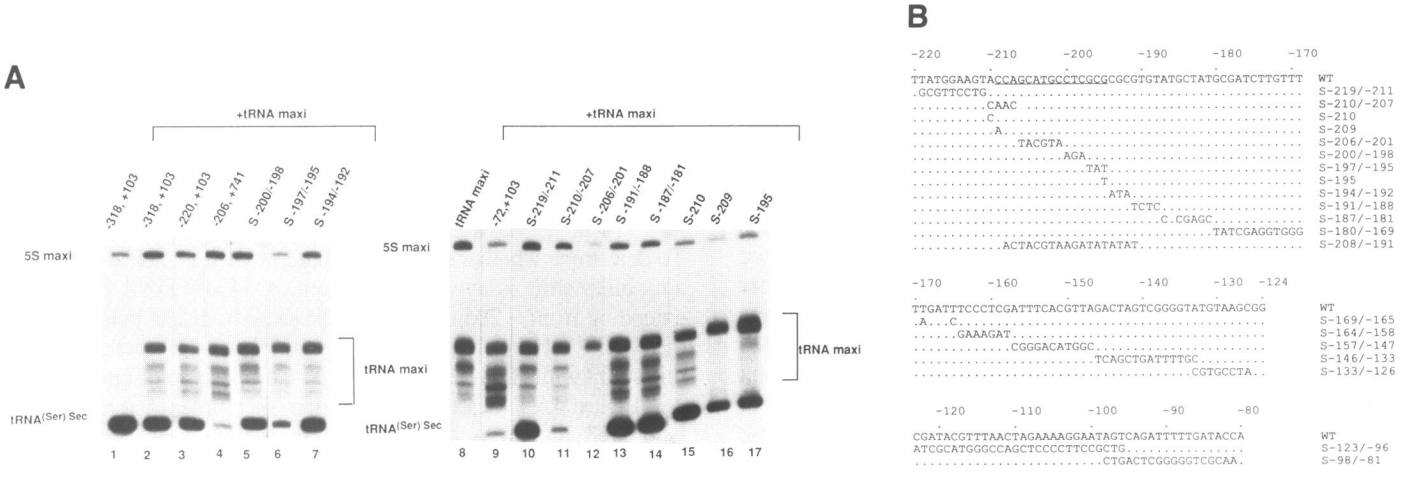

Figure 1. Mapping of the upstream sequences required for optimal in vivo transcription of the X. laevis tRNA$^{(Ser)Sec}$ gene. **A)** Template activity of various truncated or substituted tRNA$^{(Ser)Sec}$ genes. Transcription in oocyte nuclei of the constructs containing 318 (lane 2), 220 (lane 3), 206 (lane 4) and 72 (lane 9) bp of 5′-flanking sequences in competition with the tRNA$^{(Ser)Sec}$ maxigene. Lanes 5 to 7 and 10 to 17 show the transcription activity of various substituted (S) tRNA$^{(Ser)Sec}$ genes whose sequences are given in **B**. The 5S maxigene is microinjected as an internal control. Lanes 1 and 8 : tRNA$^{(Ser)Sec}$ and tRNA$^{(Ser)Sec}$ maxigene injected singly, respectively. Positions of tRNA$^{(Ser)Sec}$, tRNA$^{(Ser)Sec}$ maxi and 5S maxi are indicated. **B)** Nucleotide sequences of the substituted (S) tRNA$^{(Ser)Sec}$ genes used in this analysis. The wt non coding strand sequence is shown between positions −220 and −80 (top line). Each substitution (S) is named according to its coordinates in the substituted sequence. For each mutation only the substituted nucleotides are indicated. The mapped 15 bp element is underlined. The complete sequence of the wt gene is given in (3).

Table 1. Sequence comparison of the X. laevis tRNA$^{(Ser)Sec}$ gene activator element with various SPH motifs. SPH and octamer motifs are underlined.

-210	ACCAGCATGCCTCGCGCGCGTGTATG	-189	X.laevis **tRNA(Ser)Sec**	(3)
-226	CCCAGAATGCGCGGCGCTATGCAAAT	-201	bovine **tRNA(Ser)Sec**	(46)
-203	CCCAGGCATGCCTCGCGGCCGCC	-182	Chicken **U4B**	(25)
-178	CCCGGCATGCAGCGCGCGGTTT	-217	Chicken **U1**	(24)
-235	TTTAGCATGCCCCACCCATCTG	-214	humar. **7SK**	(18)
-214	GTATGCAAAGCATGCATCTCAA	-193	**SV40** Sph-II,Sph-I motifs	(43)

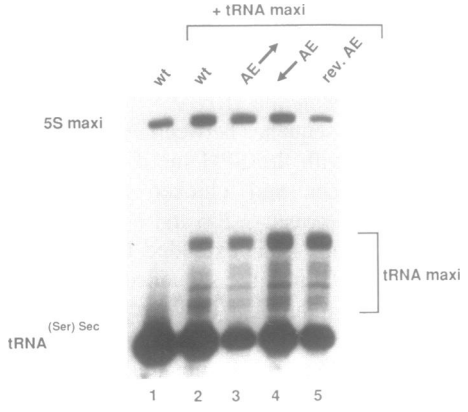

Figure 2. The 15 bp element located at positions −209 to −195 of the X. laevis tRNA$^{(Ser)Sec}$ gene acts as an activator element (AE). Transcription in oocyte nuclei of the wt tRNA$^{(Ser)Sec}$ or mutant tRNA$^{(Ser)Sec}$ genes in competition with the tRNA$^{(Ser)Sec}$ maxigene. Lane 2 : the 15 bp element in its wt position; lanes 3 and 4 : the 15bp element positioned 46 bp downstream and upstream from its wt position, respectively; lane 5 : the 15 bp element in the reversed orientation and located at positions −236 to −219. Lane 1 : wt tRNA$^{(Ser)Sec}$ gene injected singly.

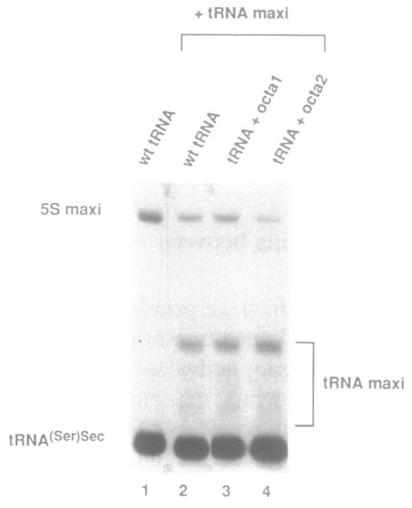

Figure 3. Analysis of an octamer motif containing tRNA$^{(Ser)Sec}$ gene. Coinjection with the tRNA$^{(Ser)Sec}$ maxigene of the wt tRNA$^{(Ser)Sec}$ (lane 2) or mutant tRNA$^{(Ser)Sec}$ genes (lanes 3 and 4). Lane 3 : tRNA +octa1, the octamer motif ATTTGCAT is placed between positions −246/−239; lane 4 : tRNA + octa 2, the octamer motif ATTTGCAT is placed between positions −225/−218. Lane 1: wt tRNA$^{(Ser)Sec}$ injected singly.

(lane 5), combined with the fact that a construct deprived of this element exhibits a 10−20-fold drop in transcription (Figure 1A, lane 9), means that the 15 bp fragment bears the properties of an activator element. This activator, spanning positions −209 to −195 will be further abbreviated as AE.

The SPH motif functions in vivo in the absence of octamer or octamer-like motifs

In Pol II snRNA gene DSEs, SPH or Sp1 motifs cooperate with the obligatory octamer ATGCAAAT sequence (see Introduction for detailed references). In a Pol III snRNA gene like Xenopus U6, an Sp1 motif, in addition to the octamer sequence, also participates in the activation by the DSE element (unpublished data). On the basis of this activation scheme, the effect of another unidentified motif working in concert with the AE could have escaped detection following inactivation of the 15 bp DNA sequence. To test this hypothesis, a series of 8 clustered point mutations were introduced which substituted variable lengths of DNA spanning positions −180 to −81 (Figure 1B). These mutants do not affect the transcription level, compared to the wt tRNA$^{(Ser)Sec}$ gene activity (not shown). Interestingly, even S−133/−126 which substitutes the almost perfect octamer sequence ATGTAAGC does not induce a reduction in transcription activity.

These experiments clearly demonstrate that the in vivo tRNA$^{(Ser)Sec}$ gene transcription is only dependent on, and highly stimulated by, an activator element containing an SPH motif GCATGC. Quite strikingly, however, and in marked contrast to what occurs in Pol II and Pol III snRNA gene DSEs, this AE functions alone without cooperating with octamer or octamer-like motifs.

We next asked whether a tRNA$^{(Ser)Sec}$ construct carrying in addition to the AE an octamer sequence could compete out transcription of a tRNA$^{(Ser)Sec}$ gene carrying only the AE element. The octamer motif in reverse orientation ATTTGCAT was placed either as in the Xenopus U6 gene (positions −246,-239) giving rise to a 29 bp spacing with the tRNA$^{(Ser)Sec}$ AE (construct tRNA+octa1 in Figure 3) or 8 bp upstream (positions −225,-218) of the AE motif (construct tRNA+octa 2 in Figure 3) to mimic the situation found in the chicken U4B gene DSE (23). The results of the coinjection with the tRNA$^{(Ser)Sec}$ maxigene as the reporter (compare lanes 2, 3 and 4 in Figure 3) show that an octamer motif does not provide a competitive advantage to a tRNA$^{(Ser)Sec}$ containing only the AE motif since both type of genes are transcribed equally well.

The SPH motif forms in vitro a specific complex with a nuclear protein different from TEF-1

To determine whether the AE can bind in vitro a nuclear protein, a mobility shift assay was carried out. A 143 bp DNA fragment extending from −265 to −123 or the same fragment but carrying the S-208/−191 substitution (listed in Figure 1B) were used in this study with Hela nuclear extracts. Comparison of lanes 2 and 4 in Figure 4A indicates that formation of complex A is abolished when the mutant is used instead of the wt DNA fragment. Formation of this complex A is competed when a double-stranded oligodeoxynucleotide corresponding to regions −210 to −189 and therefore containing the SPH motif is added in the binding reaction as a specific competitor (Figure 4A lanes 5 and 6). This clearly demonstrates that complex A does not result from the binding of a protein outside of the 15 bp fragment, particularly to the almost perfect octamer sequence ATGTAAGC (−133,−126). Addition of an unspecific competitor constituted by the oligodeoxynucleotide corresponding to the S−208/−191

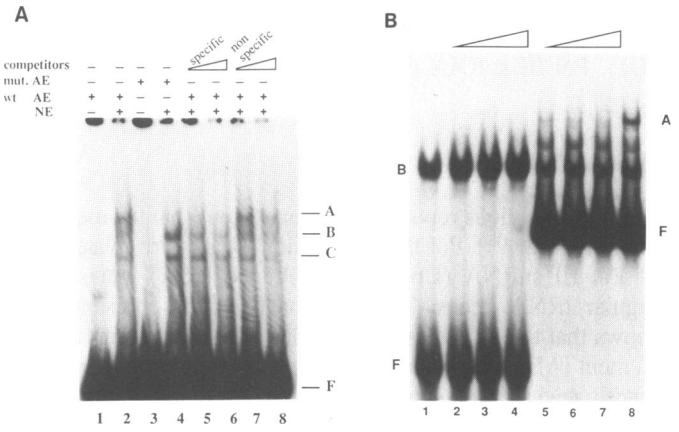

Figure 4. DNA binding analysis in Hela nuclear extracts. A)The DNA binding assays were performed with the ^{32}P 5'-end labeled 143 bp BamHI/BglII fragment (positions −265 to −123) containing the wild type (wt AE; lanes 1, 2, 5 to 8) or the mutated (S-208/−191, see Figure 1B) activator element (mut. AE; lanes 3 and 4) and 8 μg of Hela nuclear extracts. The same profile was obtained using 2 to 12 μg of protein per assay. Competitive experiments were performed in the presence of a 50 and 100-fold molar excess of specific competitor DNA (lanes 5 and 6, respectively) and in 200 and 400-fold molar excess of unspecific competitor DNA (lanes 7 and 8, respectively). The specific and unspecific competitors are double stranded oligodeoxynucleotides (34 bp) containing the wt AE (positions −209 to −195) or the mut. AE (S-208/−191, see Figure 1B), respectively. Lanes 1 and 3 : no extracts. A : specific complex. B and C : unspecific complexes. F : free DNA. NE : nuclear extracts. Open triangles denote the increasing concentration of the competitors. **B)** The assays were performed with the ^{32}P 5'-end labeled 25 bp GT-IIC motif which binds the TEF-1protein (43). Hela nuclear extracts containing the recombinant TEF-1 were mixed with GT-IIC alone (lane 1) or with a 50, 100 or 200-fold molar excess (lanes 2,3,4, respectively) of the 34 bp oligo containing the 15 bp SPH motif of the X.laevis tRNA$^{(Ser)Sec}$ gene. Lane 8 shows the bandshift obtained with the 5'-end labeled 143 bp BamHI/BglII fragment (position −265/−123) containing the X.laevis tRNA$^{(Ser)Sec}$ activating sequence. In lanes 5−7 a competitive bandshift assay was performed with a 50,100 or 200-fold molar exess of the 34 bp competitor oligo used in lanes 2−4. Complex formation was performed as described in (42). A : DNA-protein complex formed with the 143 bp fragment containing the tRNA $^{(Ser)Sec}$ AE, as in Figure 3A. B : DNA-protein complex formed between the SV40 GT-IIC oligo and the TEF-1 protein. F : free DNA. Open triangles depict increasing concentration of competitor DNA.

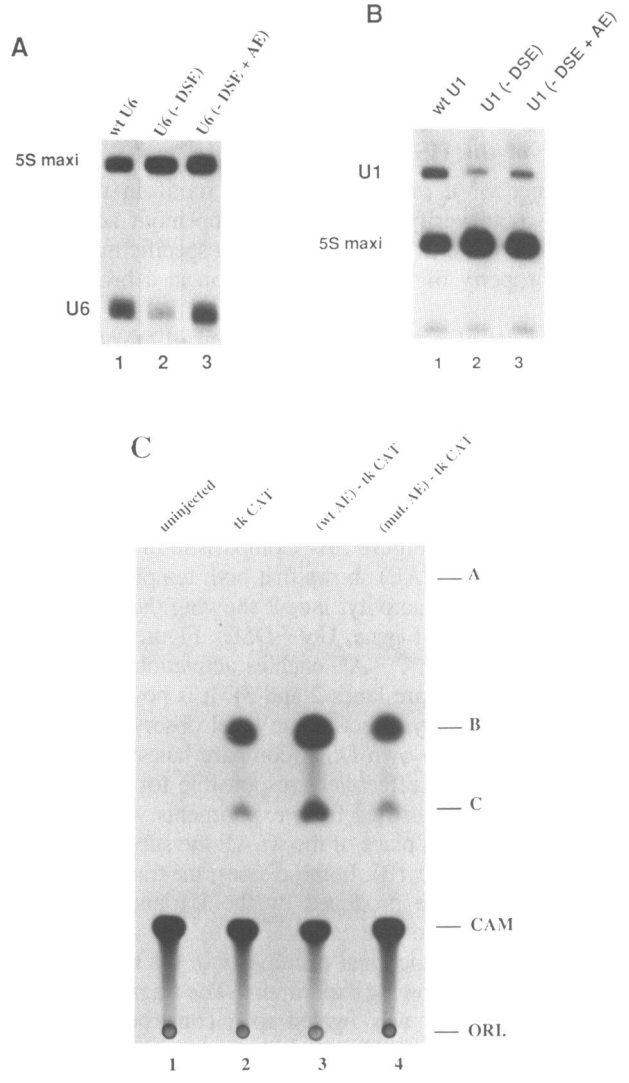

Figure 5. A) U6 transcription activation. The injected templates are indicated above the lanes. U6(−DSE) contains 195 bp of U6 5'-flanking sequence. U6(−DSE+AE) : U6 DSE-less construct containing the activator element of the tRNA$^{(Ser)Sec}$ gene (AE) at positions −236,−222. **B)** U1 transcription activation. The injected templates are indicated above the lanes. U1 wt corresponds to X. laevis U1B1 (34), U1(−DSE) contains 222 bp of U1 5'-flanking sequence, U1(−DSE +AE) : U1 DSE-less construct containing the activator element of the tRNA$^{(Ser)Sec}$ gene (AE) at positions −293,−279. **C)** Activation of thymidine-kinase transcription. CAT analysis from oocytes injected with tkCAT (lane 2), (wt AE)-tkCAT (lane 3) or (mut.AE)-tkCAT (lane 4). In the (wt AE)-tkCAT construct, AE is located in the wt orientation between positions −156 to −142 upstream of the tk cap site. (mut. AE)-tkCAT contains AE substituted at positions −210,−207 (see Figure 1B). The positions of chloramphenicol (CAM) and its acetylated forms (A, B, C) are indicated. Ori. : origin.

substitution of Figure 1B does not prevent the formation of the specific complex A (Figure 4A lanes 7 and 8).

The bandshift analysis shows that the region comprised between −209 and −195, which constitutes the activator, specifically binds a protein contained in the Hela nuclear extracts. As formation of the resulting complex A is not inhibited by a competitor containing the U2 snRNA gene octamer sequence (11), this complex does not contain an octamer binding protein (data not shown).

We have shown that the 15 bp motif contains a perfect match with the GCATGC Sph-I motif contained in the SV40 enhancer (Table 1). This enhanson binds the transcriptional activator TEF-1 (40,42). We wished to determine whether this transcription factor is also able to bind in vitro the tRNA$^{(Ser)Sec}$ SPH motif . To do this, nuclear extracts prepared from Hela cells expressing the recombinant TEF-1 protein (a generous gift from I. Davidson and P. Chambon) were used in an in vitro binding experiment (Figure 4B). Lanes 1 and 8 show the bandshift obtained with the SV40 GT-IIC 25 bp oligo representing one of the TEF-1 binding sites (40) and with the 143 bp fragment (−265/−123) containing the tRNA$^{(Ser)Sec}$ AE, respectively. Lanes 2−4 indicate that the binding of TEF-1 to the GT-IIC sequence (complex B in Figure 4B) is not competed by a 50, 100 or even 200 fold molar excess of the 34 bp oligo containing the tRNA$^{(Ser)Sec}$ gene activator element (−208 to −191), respectively. In contrast, lanes 5−7 show that this same oligo, in similar concentrations, competes out the formation of a complex with the 143 bp fragment (complex A in Figure 4B), even at an as low as 50 fold molar excess (lane 5). The formation of complex A is totally abolished at the 100 fold molar excess (lane 6).

This competitive bandshift assay unambiguously establishes that the mobility shift observed in lanes 1−4 is the result of the binding of TEF-1 to its cognate DNA motif whereas that shown

in lanes 5–8 is not caused by TEF-1. Hence, the tRNA $^{(Ser)Sec}$ SPH motif binds in vitro a nuclear protein which is different from the transcriptional activator TEF-1.

The tRNA$^{(Ser)Sec}$ activator element also activates in vivo the promoter of the HSV thymidine kinase gene

The absence of a functional octamer motif in the activator prompted us to verify whether the 15 bp motif activates only transcription of the tRNA$^{(Ser)Sec}$ gene in a specific manner or has also the property of mediating activation in a broad range of genes.

The first set of genes to be tested were the Xenopus U1B1 (33, 34) and U6. Their respective DSEs were substituted by a DNA fragment originating from positions −318 to −168 which contains the tRNA$^{(Ser)Sec}$ AE. The 15 bp motif is thus placed at −236,−222 upstream of the U6 initiation start site and at −293,−279 upstream of the U1 start site. The template activity of the U6 construct carrying the tRNA$^{(Ser)Sec}$ AE, U6(−DSE +AE), is shown in Figure 5A. Comparison of lanes 1, U6 wt and 3, U6(−DSE +AE) shows that both templates display the same transcriptional activity, lane 2 showing the transcriptional level of a DSE-less U6 gene, U6(−DSE). Figure 5B shows that, although the tRNA$^{(Ser)Sec}$ AE enables activation of a U1 DSE-less construct (compare lanes 2 and 3), it is however unable to bring this stimulatory effect to the level observed with the wt U1 gene carrying its own DSE (compare lanes 1 and 3). That the AE element was effectively responsible for the stimulation of U1 and U6 was verified from experiments with U1 and U6 templates carrying in place of the wt AE the substitution mutant S−208/−191 (Figure 1B). In these cases, the transcription levels are reduced to those produced by the U1 and U6 DSE-less constructs, respectively (data not shown).

The unexpected functional peculiarity of the tRNA$^{(Ser)Sec}$ AE derives from the following experiment. The fragment containing the tRNA$^{(Ser)Sec}$ AE was ligated to a construct carrying the herpes simplex virus (HSV) thymidine kinase (tk) gene promoter which is positioned upstream of the E. coli chloramphenicol acetyltransferase (CAT) gene in plasmid pBLCAT8+ (36) to give construct (wt AE)-tkCAT (see Materials and Methods). The AE is thus located at −156,−142 relative to the tk cap site. This construct was injected into Xenopus oocyte nuclei and the result of the CAT assay is shown in Figure 5C. Comparison of lanes 2 and 3 shows that the presence of the AE element induces stimulation of the CAT activity by a factor 3 (in Xenopus oocytes, activity of the tk promoter is responsible for the relatively strong signal observed in lane 2; see also reference 45). In lane 4, is shown the CAT activity driven by the tk promoter upstream of which was hooked a mutant AE. This construct, (mut. AE)-tkCAT, carries in place of the wt AE the substitution mutant S−210/−207 which led to a drop in tRNA$^{(Ser)Sec}$ transcription in vivo (Figure 1A, lane 11). Comparison of lanes 2, 3 and 4 shows that (mut. AE)-tkCAT does not stimulate CAT activity which only yields the signal due to the tk promoter alone. The correlation between the absence of stimulation of the CAT activity (lane 4) and the use of the mutant AE which has lost its activation property in the tRNA$^{(Ser)Sec}$ gene context (Figure 1A, lane 11) is therefore a strong evidence which enables us to state that the stimulation observed in lane 3 effectively originates from activation of the tk promoter by the tRNA$^{(Ser)Sec}$ transcriptional activator.

We have shown that the activator element residing between −209/−195 and which functions in absence of an octamer motif has the potentiality to activate not only the tRNA$^{(Ser)Sec}$ gene but also a variety of different types of gene promoters : Pol II snRNA (U1), Pol III snRNA (U6) and Pol II mRNA (tkCAT).

DISCUSSION

From our previous report (7), it was obvious that the mechanism of the tRNA$^{(Ser)Sec}$ Pol III transcription is far more related to that of Pol III snRNA (U6) or snRNA-type (7SK) genes than to regular tRNA genes. The functional analysis presented here shows that the X. laevis tRNA$^{(Ser)Sec}$ gene contains an activator element (AE) constituted by a 15 bp fragment which specifically binds a nuclear protein in vitro and that activation of transcription is only AE-dependent. The AE element contains an SPH motif flanked by functionally important CCA and GCG sequences 5′ and 3′ to this motif, respectively. The hallmark of the AE is the absence of a perfect octamer sequence which is one of the universal motifs contained in snRNA gene DSEs (see Introduction). The X.laevis tRNA$^{(Ser)Sec}$ gene is therefore the first example of an snRNA-type gene which does not harbor an octamer motif in its activating sequence. Although a CACCC box constitutes an important distal sequence element of the human 7SK gene (18) (Table 1), this system is different from the X. laevis tRNA$^{(Ser)Sec}$ gene since the authors do not rule out the possibility that an octamer-like immediately adjacent to the C-ACCC box might also contribute to the activation effect. By comparing the sequences shown in Table 1, we observed that the human 7SK gene also contains a sequence matching perfectly the SPH motif (−231/−226) and which is preceded by TTA, a close match to the essential CCR sequence of the chicken U1 and U4 and X.laevis tRNA$^{(Ser)Sec}$ genes. Therefore, it might well be that this motif is also responsible for transcription activation of the 7SK gene in vivo.

Table 1 shows a sequence comparison between the X. laevis tRNA$^{(Ser)Sec}$ 15 bp element and the same lengths of DNA in the chicken U1 and U4B DSEs in which SPH motifs were also found. The sequence similarity is perfect with the U4B DSE. The only sequence variation in the U1 DSE occurs in the triplet sandwiched between the SPH motif and the GCG and which we have shown not to be important for the function of the X.laevis tRNA AE. We have shown that the CCA sequence preceding the SPH motif is important for the AE function and it is also absolutely required for the binding of the SBF factor to the chicken U4B DSE (25). This suggests that SBF might also mediate the stimulatory effect of the X.laevis tRNA$^{(Ser)Sec}$ AE. This is also corroborated by our data which established that TEF-1, the transcriptional activator which binds the SV40 Sph-I motif (42) is not able to form a complex in vitro with the tRNA AE.

The AE element showed capacity to activate U1 snRNA gene transcription. In the Pol II snRNA gene context, the tRNA$^{(Ser)Sec}$ AE could not stimulate U1 transcription as much as the U1 DSE did. This might be an effect of the remote position of the AE relative to the position of the octamer in the wt U1 gene. However, it is worthnoting that in the chicken U1 and U4B DSEs, the SPH motif cooperates with an octamer motif since either of these two elements taken separately does not productively stimulate transcription (see Introduction for references). Therefore, we favor the possibility that the lower ability of the tRNA$^{(Ser)Sec}$ activator to stimulate U1 gene transcription is due to the absence of an octamer motif which in chicken U1 and U4 snRNA genes must function in concert with an SPH motif. This

study also provided evidence that the AE element is versatile since it is capable of stimulating transcription not only of snRNA genes but also of the thymidine kinase gene.

Interestingly, the absence of an octamer both in the AE element and in the flanking regions, together with the observation that adding it does not provide a competitive advantage lead to the more general notion that an octamer binding protein is dispensable for activation of transcription of the Pol III snRNA-type genes. What could then be the mechanism by which the AE element fulfills its activation potentiality? It is very likely that transcription complexes made on X.laevis tRNA$^{(Ser)Sec}$ templates are extremely stable, suggesting that the stabilization of these by OTF-1 (as it is required in the U6 gene, our unpublished results) is unnecessary. The DNA sequence of the bovine tRNA$^{(Ser)Sec}$ (46) in a region homologous to that of the X.laevis tRNA$^{(Ser)Sec}$ is shown in Table 1. Eleven of the 12 important base pairs constituting the X.laevis tRNA$^{(Ser)Sec}$ AE are strictly similar in the bovine counterpart. Surprisingly, in the latter, 2 bp downstream of the SPH motif lies a perfect octamer sequence. The question which now arises is the following. Why in the bovine tRNA$^{(Ser)Sec}$ is the SPH motif followed by an octamer sequence while it is not in the X.laevis gene? We hypothesize that sequences residing at or near the X.laevis tRNA$^{(Ser)Sec}$ basal promoter are sufficient to allow the establishment of a stable transcription complex in an octamer-independent fashion. This is what we are currently investigating.

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