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Analysis of the Selenocysteine tRNA$^{[SER]SEC}$ Gene Transcription *in Vitro* Using *Xenopus* Oocyte Extracts

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An *in vitro* transcription system was devised using a *Xenopus* oocyte S150 extract for analyzing expression of the Sec tRNA$^{[Ser]Sec}$ gene. The activator element, located at about −200 that is required for maximal expression of the tRNA$^{[Ser]Sec}$ gene *in vivo,* had no effect on tRNA$^{[Ser]Sec}$ transcription *in vitro. In vitro* transcription of this gene even tolerated mutations within or deletion of the PSE, showing marked contrast with the requirements observed *in vivo.* However, the TATA box was indispensable for basal level expression of the gene both *in vivo* and *in vitro.* The region spanning from the TATA box to the transcription start point (−33 to −1) was sufficient for Pol III to recognize the tRNA$^{[Ser]Sec}$ gene promoter *in vitro.*

The eukaryotic genes transcribed by RNA polymerase III (Pol III) may be divided into two types according to the organization of their promoter elements (1). Genes belonging to one type contain promoter elements within their coding regions and include tRNA, 5S rRNA and adenovirus VA RNA genes. Genes belonging to the other group contain controlling elements in the upstream region of their genes, which usually entails a TATA motif, and include the vertebrate U6 snRNA, 7SK, Epstein Barr virus EBER, and the unique selenocysteine (Sec) tRNA$^{[Ser]Sec}$ genes. Transcription of the tRNA$^{[Ser]Sec}$ gene is regulated by three distinct promoter elements located upstream of the gene (2,3): the TATA box located at about −30, a proximal sequence element (PSE) located at about −70, and an activator element (AE) located at about −200. *In vivo* transcription studies have shown that the distance between the TATA box and the coding region of the tRNA$^{[Ser]Sec}$ gene is a critical factor in determining the transcription start point (tsp; 4). To more fully examine the role of the sequence elements and the protein factors involved in transcription of the tRNA$^{[Ser]Sec}$ gene, an *in vitro* transcription system was prepared using a *Xenopus* oocyte extract. In contrast to previously observed *in vivo* transcription studies (2-5), only a short DNA sequence of approximately 30 nucleotides that span the region from the TATA motif to the tsp was sufficient for basal level transcription. This region was also sufficient for Pol III specificity of the reaction.

MATERIALS AND METHODS

Plasmid constructs. Plasmid pXEM3 encoding the *X. laevis* tRNA$^{[Ser]Sec}$ gene and other plasmids containing the promoter mutant derivatives were previously described (4). pPTG$^-$, pPG$^-$, and pTG$^-$ were constructed by subcloning PCR-derived fragments amplified from pXEM3 or plasmids encoding the TATA or PSE mutant into the T/A cloning

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Abbreviations: AE, activator element; AdMLP, adenovirus major late promoter; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; IE; intermediate element; PMSF, phenylmethylsulfonyl fluoride; Pol II, RNA polymerase II; Pol III, RNA polymerase III; PSE, proximal sequence element; PTF, proximal sequence element transcription factor; SDS, sodium dodecyl sulfate; TBP, TATA-binding protein.

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site of pCRII (Invitrogen). Oligonucleotides used for PCR amplifications were SECACT2 (5′-AAAAAAGTAAGT-AAGTAAGTAAGTAAGTAAGTAAATACCTCAGTACTCC-3′) in conjunction with PSE-S (5′-ATTTTTTGTCAC-CCCAATATATAAT-3′) for pPTG$^-$ and pPG$^-$ or TATA-S (5′-GTGGGAGGGGGTATAAAAGGAAATGGGA-3′) for pTG$^-$, respectively. p5GMLG$^-$ was obtained from R.G. Roeder and plasmids encoding genes for *X. borealis* somatic 5S rRNA, *X. laevis* tRNA^{Met} and satellite I RNA from A. Wolffe.

Preparation of transcription extracts. Ovaries from mature females of *X. laevis* (Nasco) were surgically removed and gently shaken for 30 min at room temperature in two volumes of OR-2 solution (5 mM Na-HEPES [pH 8.0], 80 mM NaCl, 2.5 mM Kcl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM Na_2HPO_4) containing 0.15% of collagenase (SIGMA). Released oocytes were washed eight times in two volumes of the OR-2 solution containing 1 mM phenylmethylsulfonyl fluoride (PMSF; SIGMA) and then washed eight times in two volumes of the buffer HP117 (33 mM K-HEPES [pH 7.9], 117 mM Kcl, 20% glycerol, 0.1 mM ethylenediaminetetraacetic acid [EDTA], 1 mM dithiothreitol [DTT]). Washed oocytes were suspended in one volume of buffer HP117 and centrifuged for 45 min at 28000 rpm and at 4°C in a SW41 rotor (Beckman). The supernatant of crushed oocytes (designated XOS150) were aliquoted and stored at −80°C.

In vitro transcription assays. Transcription reactions were performed for 2 hr at 22°C in a final volume of 25 μl that contained 15 μl of XOS150 (20 μg of protein), 10 mM $MgCl_2$, 10 mM phosphocreatine (SIGMA), 2 mM DTT, 1 mM ATP, GTP, and CTP, respectively, 50 nM UTP, 5 μCi [α-^{32}P]UTP (Amersham) and plasmid (50 ng for TATA-less class III genes and 3 μg for the tRNA$^{[Ser]Sec}$ gene and G-less templates, respectively). When G-less templates were transcribed, GTP was not included in the reactions and two units of RNase T1 (Pharmacia) were added 30 min before terminating the reactions. When *Xenopus* oocyte extracts were depleted of endogenous factors by addition of a DNA fragment, double-stranded oligonucleotides were preincubated with XOS150 for 15 min at 22°C prior to addition of plasmid templates. Reactions were terminated by adding 125 μl of 20 mM EDTA (pH 8.0)-0.5% sodium dodecyl sulfate (SDS) containing 5 μg of polyuridylic acid (Boehringer Mannheim). Labeled transcripts were recovered following phenol extraction and ethanol precipitation, dissolved in the formamide loading solution (90% formamide, 10 mM EDTA [pH 8.0], 0.05% bromophenol blue, 0.05% xylene cyanol), heated for 2 min at 75°C, and electrophoresed in 10% polyacrylamide gels containing 8 M urea. Primer extension analyses were carried out according to a previously described method (6) with an oligonucleotide primer (5′-CTCTGTCGCTAGATAGCTACAGGTTTG-3′) and MMLV reverse transcriptase (New England Biolabs).

RESULTS

In vitro tRNA$^{[Ser]Sec}$ gene transcription. X. laevis oocytes, which have been used extensively in *in vivo* transcription of the tRNA$^{[Ser]Sec}$ gene (2,4,7), were employed to prepare a cell-free system to analyze *in vitro* transcription of this gene. An S150 extract of oocytes was prepared and found to be self-sufficient in producing Pol III-dependent transcripts (Fig. 1). Figure 1A shows the promoter organization of the 5S, tRNA^{Met}, satellite I and tRNA$^{[Ser]Sec}$ genes. *In vitro* transcription of each of these genes is shown in Fig. 1B, lanes 1-4, respectively. Transcription of the tRNA^{Met}, satellite I RNA and tRNA$^{[Ser]Sec}$ genes results in multiple transcripts of different sizes (lanes 2, 3 and 4). *In vivo* transcription of these same genes, on the other hand, yielded transcripts of a single size (7). The observed heterogeneity regarding the sizes of transcripts generated *in vitro* most likely originated from inefficient processing of precursor transcripts of these RNAs. The tsp of the tRNA$^{[Ser]Sec}$ gene used *in vitro* was shown to be the same as that used *in vivo* by primer extension studies (see below and also Fig. 2B). Hence, the longer transcript observed *in vitro* was not due to the presence of a leader sequence generated from an alternative tsp.

Promoter element requirements in vitro. The elements controlling tRNA$^{[Ser]Sec}$ gene transcription *in vivo* were defined previously through microinjection assays employing *Xenopus* oocytes (2-5). These elements included a TATA box, a PSE (2-5) and an AE (3,5). The internal A and B boxes were not required for basal level expression (4). To determine the role of these elements in *in vitro* transcription and to better characterize the regions governing transcription, the wild type and corresponding promoter mutant genes (see Fig. 2A) were transcribed in *Xenopus* oocyte extracts. Transcripts were analyzed by direct labeling (Fig. 2B) and by primer extension (Fig. 2C). As shown in lane 2 of Fig. 2B, deletion of the AE did not affect the level of transcription *in vitro*. Mutation of the PSE reduced its expression *in vitro* (lane 3) but not as completely as observed *in vivo* (2-4). Mutation of the TATA box, however, completely

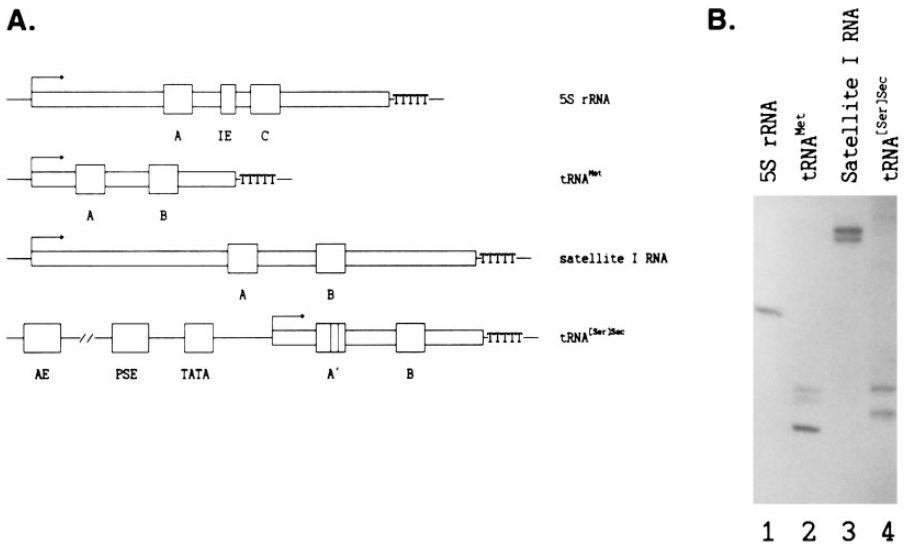

FIG. 1. Transcription of various Pol III transcribed genes in *Xenopus* oocyte extracts. (A) Promoter organization of genes encoding 5S rRNA, $tRNA^{Met}$, satellite I RNA and $tRNA^{[Ser]Sec}$. The arrows show the tsp and the regions downstream of the arrows to the poly T tract depict the coding sequences. Boxes with letters under them represent promoter elements (the A, IE, C boxes of 5S RNA genes, the A and B boxes in $tRNA^{Met}$ and satellite I RNA genes and the upstream AE, PSE and TATA box elements, the two vertical lines within the A′ box represent the two extra base pairs [T14/C15] in this region that deviate from all other eukaryotic canonical A boxes and B box of the $tRNA^{[Ser]Sec}$ gene). (B) Plasmids containing genes for 5S RNA (lane 1), $tRNA^{Met}$ (lane 2), satellite I RNA (lane 3), and $tRNA^{[Ser]Sec}$ (lane 4) were incubated in the extract, transcripts recovered and analyzed as described in Materials and Methods.

abolished $tRNA^{[Ser]Sec}$ gene transcription *in vitro.* Thus, this element is indispensable to the expression of the gene both *in vitro* (lane 4) and *in vivo* (2-4). Mutation of the A and B boxes resulted in transcripts that were inefficiently processed (see lanes 5 and 6 and ref 4). The level of transcription was not impaired by mutation in the internal boxes as shown by primer extension analysis that was performed on each of the transcripts shown in Fig. 2B to determine the respective tsp (see legend to Fig. 2). The transcripts derived from the A or B box mutants manifested the same tsp and level of transcription (compare lanes 6 and 7 to 2, respectively). This observation confirmed that the longer transcripts generated from the A and B box mutants were inefficiently processed. The transcripts generated from the mutant PSE and the T14/C15 deletion also had the same tsp as wild type. As expected, no transcript from the TATA box mutant could be detected by primer extension (see lane 4).

To determine whether the factors binding to the AE, the PSE and the TATA box are required for *in vitro* $tRNA^{[Ser]Sec}$ transcription, double-stranded oligonucleotides containing one of these promoter elements were independently added to the transcription reactions. The TATA box and PSE oligonucleotides inhibited $tRNA^{[Ser]Sec}$ transcription in a dose-dependent manner, while the AE oligonucleotide did not decrease the level of transcription in similar amounts (Fig. 3). These results together with those from the transcription assay with the AE-deleted template (lane 2 of Fig. 2B) suggest that Staf, the AE-binding transcription factor (8), does not have a significant role in *in vitro* transcription of the $tRNA^{[Ser]Sec}$ gene.

Pol III specificity of $tRNA^{[Ser]Sec}$ gene transcription. Since $tRNA^{[Ser]Sec}$ transcription *in vitro* tolerated mutations or deletions in AE, PSE, A box and B box but not in the TATA box, we could determine if the region spanning the TATA box to the tsp is sufficient for recognition by Pol III. G-less transcription cassettes were constructed (9) in which the upstream regions

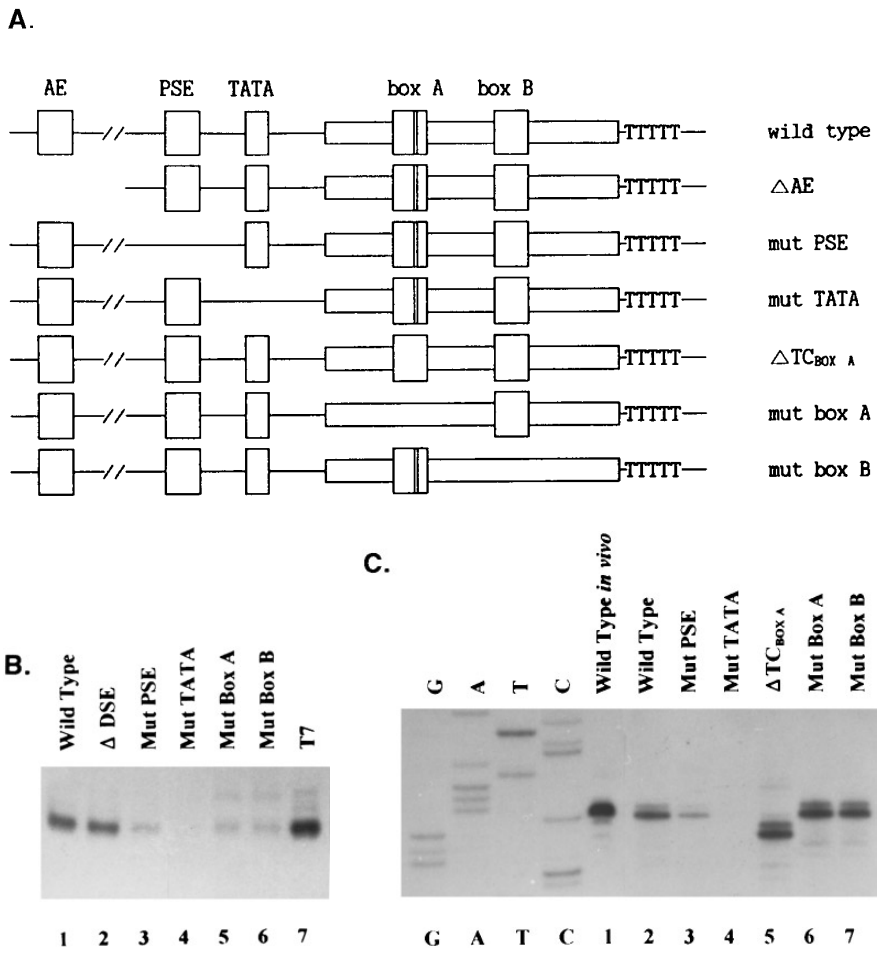

FIG. 2. Transcription of the tRNA$^{[Ser]Sec}$ gene harboring a deletion of the AE or mutations in the A box, B box, TATA box, or PSE in *Xenopus* oocyte extracts. (A) Wild type or mutant templates of the tRNA$^{[Ser]Sec}$ gene. The symbols depicting coding regions and controlling elements are described in the legend to Figure 1.(B) Analysis of labeled transcripts by electrophoresis and autoradiography. Lanes show transcripts generated from the following genes: 1, wild type; 2, AE-deleted mutant; 3, PSE mutant; 4, TATA mutant; 5, box A mutant; 6, box B mutant; and 7, T7 RNA polymerase-derived control transcript. The AE-deletion and mutations in the other elements were described previously (4). (C) Primer extension analysis of the tRNA$^{[Ser]Sec}$ transcripts from wild type or mutant templates. Products of the sequencing reaction for the wild type tRNA$^{[Ser]Sec}$ gene template (lanes G, A, T, and C) were laid in parallel with the primer extension products (lanes 1–7) for locating the 5′ end of the transcripts. Primer extension from the *in vivo* transcript were included (lane 1). Analyzed transcripts were from the following templates: wild type (lane 2), PSE mutant (lane 3), TATA mutant (lane 4), T14/C15 deletion mutant (lane 5), box A mutant (lane 6) and box B mutant (lane 7).

of the tRNA$^{[Ser]Sec}$ gene were linked with a short tetranucleotide (5′-ACTT-3′) repeat sequence ending with a poly T tract (see left hand portion of Fig. 4). The poly T tract dictates Pol III termination. The construct with the intact PSE-TATA module produced an RNase T1-resistant transcript at the α-amanitin concentration expected of Pol III transcription (Fig. 4, PTG$^-$ panel). The construct lacking the PSE showed a similar pattern of transcription manifesting Pol III specificity although the transcription level was slightly diminished (Fig. 4, TG$^-$). The TATA box was also found to be indispensable for transcription in this assay (Fig. 4, PG$^-$) as was observed *in vivo* (4). Polymerase specificity of these transcription templates was also

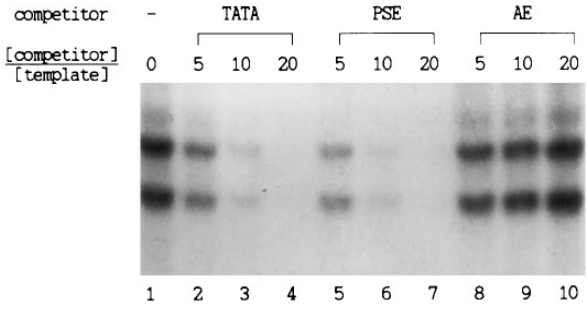

FIG. 3. Inhibition of tRNA$^{[Ser]Sec}$ transcription by oligonucleotides containing the TATA box or the PSE sequence. Double-stranded oligonucleotides encoding the TATA box (lanes 2–4), the PSE (lanes 5–7) or the AE (lanes 8–10) were included in the transcription reactions at 5 fold (lanes 2, 5 and 8), 10 fold (lanes 3, 6 and 9) or 20 fold (lanes 4, 7 and 10) molar excess amounts.

confirmed by checking sensitivities of the transcription reactions toward targetitoxin, a specific inhibitor of Pol III transcription. Targetitoxin completely blocked transcription from the PTG$^-$ or TG$^-$ template, but did not affect AdMLP transcription (data not shown). These data suggest that the region between the TATA box and the tsp is sufficient for Pol III to recognize the tRNA$^{[Ser]Sec}$ gene promoter.

DISCUSSION

The AE of the tRNA$^{[Ser]Sec}$ gene, which stimulates transcription of this gene *in vivo* (3,5), did not enhance its expression *in vitro.* The AE may bind a protein factor which relieves the inhibitory effects of nucleosome assembly on transcription *in vivo.* This type of anti-repression process may be bypassed in the cell-free system since naked templates were employed. In the oligonucleotide competition assays described in this study, excess amounts of the PSE

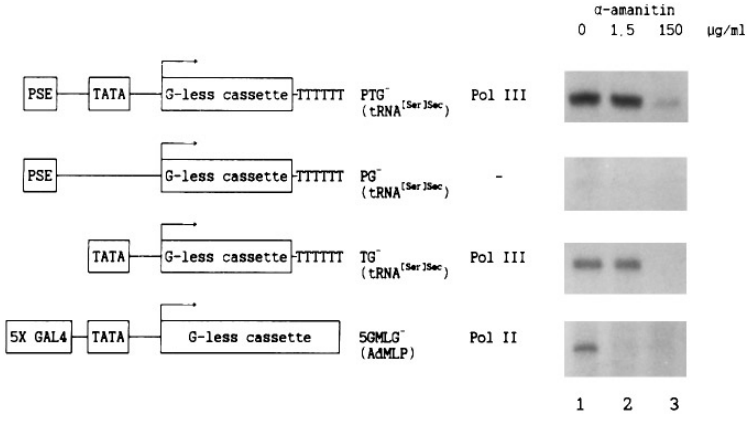

FIG. 4. RNA polymerase-specificity of transcription from the tRNA$^{[Ser]Sec}$ gene promoter. G-less transcription templates were constructed in which the upstream wild type or mutant promoter elements of the tRNA$^{[Ser]Sec}$ gene were linked to a 28 bp ACTT tetranucleotide repeat. A T stretch followed the 3′ end of the repeat sequence which served as a Pol III termination sequence. PTG$^-$ designates the construct encoding wild type PSE and TATA box (−73/−1), PG$^-$ that encoding wild type PSE and the mutant TATA box (−73/−1) and TG$^-$ that encoding wild type TATA box only (−33/−1). An AdMLP/G-less template (designated 5GMLG$^-$) was also employed as a representative of class II genes. The 5× GAL4 indicates five consecutive binding sites for the GAL4 transcriptional activator. The G-less templates were transcribed in the presence of 0 (lane 1), 5 (lane 2), or 150 μg/ml (lane 3) α-amanitin.

oligonucleotides inhibited $tRNA^{[Ser]Sec}$ transcription. However, multiple point mutations in the PSE or deletion of the PSE did not completely prevent transcription of the gene. Recently, it has been reported that a human factor that binds to the PSE sequence (10,11,12) interacts with TATA-binding protein (TBP; 10) or contains TBP as an integral subunit (11). In the present study, it may be that the PSE oligonucleotide added to extracts sequestered the endogenous TBP which was already associated with the PSE binding factor (designated PTF in ref 12), and thus inhibited $tRNA^{[Ser]Sec}$ transcription. Although interaction between the PSE and PTF might not be absolutely required for transcription of the $tRNA^{[Ser]Sec}$ gene *in vitro,* PTF might somehow stabilize binding of TBP to the $tRNA^{[Ser]Sec}$ TATA box and thereby increased the level of transcription.

The upstream −33/−1 region of the $tRNA^{[Ser]Sec}$ gene successfully mediated Pol III transcription *in vitro.* The $tRNA^{[Ser]Sec}$ gene TATA box *per se* cannot constitute the determinant for Pol III specificity since its sequence and position are in perfect conservation with Pol II TATA motifs. Other studies have shown that TATA elements alone directed bidirectional transcription by Pol II and Pol III in nuclear extracts from *Drosophila* embryos (13) and *Acanthamoeba* (14). A TATA box likely manifests a tendency to support both Pol II and Pol III transcriptions, at least *in vitro.* Nearby sequences or transcription factors that interact with them may help commit the TATA box to serve as a basal promoter of one type of RNA polymerase. The PSE may contribute to determining Pol III specificity by facilitating recruitment of a Pol III-specific TBP-containing complex *in vivo.*

The *in vitro Xenopus* oocyte transcription assay described in the present study has permitted us to focus on the role of the region spanning the TATA box to the tsp in $tRNA^{[Ser]Sec}$ transcription *in vitro* by Pol III. These studies have also shown differential requirements of the AE and the PSE in $tRNA^{[Ser]Sec}$ transcription *in vivo* and *in vitro* and thereby provided clues for unique roles of these elements in transcription of the $tRNA^{[Ser]Sec}$ gene *in vivo.*

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