Point mutations 5' to the tRNA selenocysteine TATA box alter RNA polymerase III transcription by affecting the binding of TBP

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ABSTRACT

The selenocysteine tRNA^Sec^ gene possesses two external promoter elements, one of which is constituted by a strong TATA box. Point mutant analysis performed in this study led to the conclusion that the functional TATA promoter actually encompasses the sequence -34 GGGTATAAAAGG -23. Individual changes at T-31 do not affect transcription much. Position T-29 is less permissive to mutation since transversion to a G, for example, is less well tolerated than at T-31. Interestingly, a double point mutation, converting GG(-33/-32) to TT, causes abrogation of transcription in vivo and severe reduction of transcription in vitro with human TBP. Therefore, data obtained underscore the fact that, in the Xenopus tRNA^Sec^, these two Gs are an integral part of the TATA promoter. Gel retardation experiments indicate that the GG to TT substitution, which led human TBP to lose its ability to support efficient transcription in vitro, correlates with the appearance of an altered pattern of retarded complexes. Altogether, the data presented in this report support a model in which TBP interacts directly with the TATA element of the tRNA^Sec^ gene, in contrast to the type of interaction proposed for classical TATA-less tRNA genes.

INTRODUCTION

Studies in a number of laboratories have shown that the TATA binding protein (TBP) is a general transcription factor serving all three classes of eukaryotic RNA polymerases (reviewed in 1–3). The protein is bipartite. The 180 amino acids constituting the C-terminal core domain exhibit 80% sequence similarity between all species. In contrast, the N-terminal domain of TBPs from these organisms differ significantly in length and sequence (1–3).

Promoters of genes transcribed by RNA polymerases I, II and III (Pol I, Pol II, Pol III) possess different structural organizations. With regard to the Pol III system, three different types of promoters are found (reviewed in 4–6). The Pol III TATA-less promoters include the internal control region of the 5S genes and the A and B boxes of tRNA and Ad2 VAI genes. TFIIIA binds to the internal control region of 5S genes and allows the subsequent binding of TFIIIC and TFIIIA. In contrast, TFIIIC can bind directly to the A and B boxes of the tRNA and VAI genes and recruits TFIIIB. TFIIIB serves the purpose of positioning Pol III for transcription. Both in yeast and mammals, this multisubunit transcription factor contains TBP as an assembly factor (7–12).

The second type of Pol III promoters is exemplified by the most studied, TATA-containing U6 snRNA gene. In vertebrates, transcription is initiated from upstream promoters only (13, reviewed in 4–6,14). In vitro transcription of the yeast and mammalian U6 genes require TBP (15–17) which was shown to be able to bind the human U6 TATA element in vitro (15). Yeast and mouse U6 in vitro transcription in reconstituted systems requires the addition of TFIIIB (16–18), much as the TATA-less Pol III genes. In the case of the vertebrate U6 genes, it is likely that TFIIIB is involved in such a manner. The last, more recently discovered type of Pol III promoters is contained in the selenocysteine tRNA (tRNA^Sec^) gene which displays a situation intermediate between the all internal and the all external gene promoters described above. In the tRNA^Sec^, the existence of external promoters was established (19) and were shown later to consist in a proximal sequence element and a TATA box functionally interchangeable with their U6 counterparts (20). However, although the internal A box is naturally debilitated, the occurrence within the coding region of a perfect match to the B box element consensus exerts a stimulatory effect on in vivo transcription (20).

In the work presented here, we report the unexpected finding that the TATA element required for Pol III transcription of the Xenopus tRNA^Sec^ gene not only consists of the TATAAAA motif itself but also extends in the 5' and 3' direction. With respect to the...
5' end, evidence is shown that the presence of a GG dinucleotide, immediately adjacent to the TATA motif, is essential for efficient transcription both in vivo and in vitro. We also show that the down effect on transcription caused by the double point mutation of the GG dinucleotide correlates well with an altered interaction between human recombinant TBP and its mutated DNA target.

**MATERIALS AND METHODS**

**Mutagenesis and constructs**

Construction of the mutated *X. laevis* tRNA<sup>sec</sup> gene was done by site-directed mutagenesis with the *in vitro* kit from Amersham. Construct tRNA<sup>sec</sup> -100, +99 contains a 199 bp fragment encompassing the *X. laevis* tRNA<sup>sec</sup> gene from position -100 to +99 (21), amplified by PCR from the tRNA<sup>sec</sup> -318, +103 construct (20). It was cloned between the *EcoRI* and *SmaI* sites of pBS<sup>+</sup> (Stratagene).

**Oocyte microinjections**

*X. laevis* oocyte nuclei were microinjected with 20 nl of DNA as performed as in (27). In vitro produced ** performed as described in (26). The experiment with a tRNA<sup>5</sup>TBP (hTBP) was from Promega. Transcription reactions in vitro were carried out near homogeneity as described in (23). Human recombinant TATA binding protein (TBP) and human recombinant Sec<sup>5</sup> tRNA<sup>sec</sup> was purified in vitro transcription assays.

**RESULTS**

*In vivo* functional importance of purine nucleotides flanking the *Xenopus* tRNA<sup>sec</sup> TATA box

Previous experiments indicated that the *Xenopus* tRNA<sup>sec</sup> TATA box is not limited to the TATAAAA sequence only but that sequences surrounding this element might also be important for promoter activity (20). Therefore, in order to delineate precisely those sequences that influence transcription, several point mutations were created (Table 1) and assayed by injection into *Xenopus* oocyte nuclei, under competitive conditions. Competition experiments ensure that mutations which provoke only a mild effect in the single injection assay show a much more discernible phenotype when coinjected along with a competitor.

*Xenopus* tRNA<sup>sec</sup> gene. Competition presumably reflects a decrease in the ability of the mutants to compete for transcription factors which are needed to form a stable transcription complex. The competitor here is a *Xenopus* tRNA<sup>sec</sup> maxigene carrying

**Table 1.** Point mutations introduced into the TATA promoter of the *Xenopus* tRNA<sup>sec</sup> gene and relative transcription of the TATA mutants

<table>
<thead>
<tr>
<th>Mutation</th>
<th>wt</th>
<th>tRNA&lt;sup&gt;sec&lt;/sup&gt;</th>
<th>yTBP</th>
<th>hTBP</th>
</tr>
</thead>
<tbody>
<tr>
<td>CGGAGGCGTATACAAAAAGTTGGTTAGATCTGGGAAATGGGAGTAC-3'</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>AGATCTG</td>
<td>31/-25</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>G &gt; C</td>
<td>37/-36</td>
<td>AG &gt; CT</td>
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</tr>
<tr>
<td>10</td>
<td>G &gt; T</td>
<td>35</td>
<td>G &gt; C</td>
<td>100</td>
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<td>30</td>
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</tbody>
</table>

hTBP and yTBP refer to human and yeast TBP, respectively. Values are in percentage of the wt level. They indicate the residual transcription level or, in the cases of the G to A or G to T mutations at -32, values above the wt level. They arise from two independent *Xenopus* injections and from three independent *in vitro* transcription experiments. Oocyte stands for transcriptional values obtained upon *Xenopus* oocyte injections. Quantitative data were derived as explained in Materials and Methods.

**Electrophoretic mobility shift assay**

The sequences of the 37-mer double-stranded oligodeoxynucleotides used in Figure 4 were as follows:

wt, top strand (−47/−11): 5'-TAAATGGTGGGAGGGTTATAAAAGGAAATTGGGAGTAC-3'.

GG -33/-32 to TT, top strand (−47/−11): 5'-TAATGGTGAGGAGGTTTTATAAAAGGAAATTGGGAGTAC-3'.

TATAAAA (−31/−25) to AGATCTG, top strand (−47/−11): 5'-TAATGGTGGGAGGGAGATCTGGGAAATGGGAGTAC-3'.

The single-stranded DNAs were 5' end labeled and then annealed according to (28). The binding reaction and gel shift conditions were essentially as in (23) using 4 mM MgCl<sub>2</sub> in the gel, 50000 cpm (Cerenkov) of DNA duplex (2500 cpm/fmol) and 20 ng hTBP.

The corrected (relative to 5S RNA maxil) transcriptional values were quantitated with a Fuji Bioimage Analyzer BAS 2000.
a wild-type promoter but an insertion into the coding sequence enables it to be distinguished from the trnA\textsuperscript{Sec} gene in which the mutations are performed (20).

Mutants were tested in three groups. The values of their template activities are given in Table 1. The first group introduced mutations 3' to the core TATAAAA motif, ranging from positions -14 to -25. Figure 1A shows that mutations at positions -18 to -21 (lanes 3 and 2) do not lead to altered transcription levels. Changing GA to TC (-23/-22) reduces the transcription level to 90% of the control value (compare lanes 5 and 4). In contrast, an overlapping mutant substituting GGA to TTC (-24/-22) exerts a more pronounced effect since the transcription level is 30% of the wt value (lane 6). The effects of point mutations at position A-25 vary upon the identity of the replacing base. An A to G change did not affect the transcription level much, whereas an A to C mutation led to a 30% transcription level (Figure 1A, lane 6). The effects of point mutations at position A-25 vary upon the identity of the replacing base. An A to G change did not affect the transcription level much, whereas an A to C mutation led to a 30% transcription level (Figure 1A, lane 6). In contrast, a transition at G-32 is much less tolerated than transversions since the G to A change generates a template whose activity is only 20% of the wt level (compare lanes 17 and 13 in Figure 1A). Surprisingly, the GG to TT double mutant at -33/-32 is almost lethal (Figure 1A, lane 11) since it only yields 5% of transcriptional activity. When looking at the moderate effects furnished by the individual G to T mutations at -33 or -32, this finding was unexpected. Clearly, the combined presence of the single point mutants G-33 to T-33 and G-32 to T-32 to form the double point mutant GG to TT exacerbates in a non additive but rather cooperative fashion the modest down effects provoked by the single point mutations. The last group of mutants are dedicated to two positions in the core TATAAAA motif. T-29 or T-31 changed individually to G or C did not alter the transcription level significantly (Figure 1B, lanes 1—4). The most pronounced effect is caused by a T to G mutation at position -29 which, however, still displays 75% of transcriptional activity.

Figure 1. Transcriptional activities assayed by Xenopus oocyte injection of Xenopus trnA\textsuperscript{Sec} templates carrying point mutations in the TATA element. (A) Effects of point mutations flanking the TATA sequence. The point mutations tested are shown above the lanes and in Table 1. A wt control was introduced wherever the injections were done in separate experiments. Positions of 5S maxi, trnA\textsuperscript{Sec} maxi and tRNA\textsuperscript{Sec} transcripts are indicated. (B) Effects of mutagenizing positions T-31 and T-29 of the TATA box. The nature of the mutation is indicated above the lanes and also in Table 1. Positions of the transcripts are as in (A). Quantitative analysis for (A) and (B) is given in Table 1.
It appears that the sequence we consider as representing the functional TATA box of the Xenopus tRNA^Sec^ gene is longer than the TATA motif itself and actually encompasses positions -34 GGGTATAAAAAGG -23 with a possible extension to A -22. Lastly, it is remarkable to note the strong down effect of the mutation which changes GG to TT at -33/-32.

Recombinant TBP relieves the inability of Xenopus egg extracts to support TATA dependent and TATA less transcription of Xenopus tRNA^Sec^.

Surprisingly, while a Xenopus egg extract can support transcription of a classical tRNA gene (29), it does not so for the Xenopus tRNA^Sec^ gene (Figure 2A, lanes 1 and 5). Transcription, however, is restored upon, and stimulated by the sole addition of recombinant yeast or human TBP (Figure 2A, compare lanes 2 -4 with lane 1 and lane 6 with lane 5). Three products, named I, II and III appear upon transcription. A template which harbors a clustered point mutation in the TATA box (-31/-25 in Table 1), abolishing transcription in injected oocytes (20), compromises transcription which generates bands II and III but does not affect band I (Figure 2A, lane 8). Another template, carrying a mutation in the PSE which severely inhibits transcription in oocytes (20), is not deleterious in vitro (Figure 2A, lane 7). This shows that under the in vitro conditions employed, transcription of the products contained in bands II and III requires TBP in a TATA box-dependent manner but is not dependent upon the integrity of the PSE sequence.

For the sake of clarity in further experiments, it appeared necessary to assign identities to the three bands that occur upon transcription. Only band III should represent the mature tRNA^Sec^ since it migrates at the same level as a tRNA^Sec^ arising from injected oocytes (lane 9). We will next consider the origin of the other two products. The experiment shown in Figure 2B, lane 2 takes advantage of a template lacking its genuine run of Ts acting as a Pol III terminator. In vivo, termination of transcription takes place at the T stretch present downstream in the vector and the extra length is then properly trimmed to produce the mature tRNA^Sec^ (20). Under in vitro conditions, the situation is different. Band III is still present but bands I and II disappeared (Figure 2B, lane 2). Two larger products (bands IV and V) arose which very likely originate from unprocessed tRNAs whose transcription terminated at the downstream Ts of the vector. Therefore, bands I and II showed in lane 1 (and in Figure 2A) likely represent unprocessed tRNAs with 3’ termini ending at the authentic run of Ts occurring immediately downstream of the tRNA^Sec^ sequence.

Mapping of the 5’ ends of the products was investigated by primer extension. Figure 2C shows that under conditions employed in Figure 2A or B, two products (I and II) appeared with the wt tRNA^Sec^ (lane 2) while the TATA-less tRNA^Sec^ mutant led to band I (lane 3). We will focus our attention on

Figure 2. In vitro transcription of the Xenopus tRNA^Sec^ gene in Xenopus egg extracts supplemented with yeast or human TBP. (A) Transcription in vitro supported by yeast or human TBP generates three products. yTBP and hTBP and the yeast and human TBP, respectively. -PSE and -TATA represent PSE-less and TATA-less Xenopus tRNA^Sec^ mutant promoters, respectively. Lanes 2 -4 contained 9, 12 and 18 ng of yTBP, respectively. The experiments in lanes 6 -8 contained 10 ng of hTBP each. The origin of bands I, II and III refer to the three types of transcripts obtained and are mentioned in Results. In lane 9 is shown the Xenopus tRNA^Sec^ obtained after Xenopus oocyte injection and used as a size marker. (B) In vitro transcription of a Xenopus tRNA^Sec^ template lacking its genuine Pol III terminator (shown above lane 2 and described in Materials and Methods). In each lane, transcription used 15 ng of yTBP. Bands IV and V are described in Results. (C) Primer extension performed to map the 5’ ends of the products contained in bands I and II. -TATA refers to the same TATA-lacking template as in (A). T7 tRNA^Sec^ is transcribed in vitro under the control of a T7 promoter as mentioned in the text and in (27). Bands I and II are as in (A) and (B) and discussed in Results. The amount of hTBP used in this experiment is given in Materials and Methods.

Figure 3. Analysis of transcription in vitro from mutations in the TATA motif in the presence of yeast TBP (A) or human TBP (B). Reactions were carried out as described in Materials and Methods. Templates used are shown above the lanes and in Table 1. -TATAAAA is the TATA-less tRNA^Sec^ mutant promoter as in Figure 2. Quantitative analysis is given in Table 1.
these two bands, the faster migrating product in lanes 2 and 3 not being considered since it is shorter at the 5' end than the full-length tRNA^Sec produced under the control of a T7 promoter (lane 4). Comparison of lanes 2 and 4 indicates that band II corresponds to tRNA^Sec molecules initiated at the authentic 5' end, while comparison of lanes 2 and 3 with lane 4 reveals that band I contains molecules initiated upstream of the normal start site of transcription. Thus, bands I in Figure 2A, B and C contain the same RNA products and band II in Figure 2C corresponds to band III in Figure 2A and B. The absence, in Figure 2C, of the equivalent to band II observed in Figure 2A and B, again argues that this band contains a tRNA possessing a 3' extension but a mature 5' end.

The above results establish that in vitro transcription of tRNA^Sec needs TBP but this requirement reflects the formation of two distinct transcription complexes. On the one hand, we observed one type of transcription which is TBP but not TATA box-dependent, yielding band I carrying 5' and 3' unprocessed ends. Given the fact the TATA-less template harbors a B box similarity, this transcription must be dependent only on the B box. In contrast, the use of TATA box mutants attests that bands II and III arise from a template requiring TBP in a TATA box-dependent manner, the extra length of the product in band II being due to an unprocessed 3' end. Interestingly, we can conclude from the above experiments that, under the conditions employed, the TATA box selects the transcription initiation site which coincides with the 5' end of the mature tRNA^Sec.

A double point mutation 5' to the TATA box alters transcription and TBP binding in vitro

The TATA box mutants which have been assayed by injection into Xenopus oocytes were also tested in vitro with Xenopus egg extracts supplemented with recombinant yeast and human TBP (Figure 3A and B, respectively). The transcription pattern is identical to that observed in Figure 2A with the appearance of the three bands. Quantitative data for band III are given in Table 1. In the downstream portion of the TATA sequence, the most pronounced down effect, both with yeast and human TBP, is caused by the combined mutations leading to GGA to TTC (~24/~22), while the double GA to TC mutation induces only a slight decrease in the transcription level. The most dramatic effects observed by using either human or yeast TBP are produced by two distinct single point mutations. One of them resides 3' to the TATA sequence and corresponds to an A→C to C change. The transcription levels are 10% and 35% of the control level with human and yeast TBP, respectively. This position, however, can accommodate a transition much more effectively. The other strong down mutation occurs at position T→C. A transition is tolerated (about 50% of wt level with either TBP) but not a transversion since the T to G change affects transcription much more dramatically with human than with yeast TBP (10% and 45% of the control value, respectively). Either mutation at T→C leads to roughly 50% of transcription using human TBP. With yeast TBP, a T to C transition leaves the transcription level unaffected, while a transversion to a G diminishes it to leave 75% of the wt value. Interesting are the effects arising as a consequence of point mutations introduced into the G-rich cluster 5' to the TATA sequence. Changing G→32 to a C does not affect transcription at all with either of the proteins. With yeast TBP, a G to A or T mutation unexpectedly raises the transcription level to 140 and 170% of the control value, respectively. Using human TBP, a G to A change inhibits transcription moderately (85% of wt level), while the G to T mutation does not modify significantly the transcription level (105%). The effects of the same point mutants introduced at G→33 are different since, in all cases tested, the transcription level is only mildly altered, irrespective of whether human or yeast TBP is employed. With human TBP, the transcription level of the double GG (~33/~32) to TT point mutant drops to 30% of the wt value, what is consistent with the result of the in vivo assay. In contrast, this double mutant does not markedly affects transcription with yeast TBP.

We next asked whether the observed drop in transcription activity originating from certain point mutations reflects impairment or abolition of TBP binding to its DNA target. To test this hypothesis, an electrophoretic mobility shift assay was performed. As shown in Figure 4, a complex was obtained with human TBP and the wt TATA box sequence (lane 2) but not with a DNA derived from the −31/−25 mutant which disrupts the alternation of the A/T motif (lane 4). With the −33/−32 GG to TT mutant which prematurely arrests the long purine string preceding the T/A motif the shifted complex observed in lane 2 is replaced in lane 6 by an array of bands which must contain TBP−DNA complexes since they are retarded relative to free DNA. From this, we conclude that there exists a correlation between the drop in the transcription level and alteration of TBP binding to the TATA box mutant.

DISCUSSION

The tRNA^Sec is the only tRNA gene known so far to possess external promoter elements, one of which being constituted by a TATA element (19,20). The mutational analysis we have performed indicates that the Xenopus tRNA^Sec TATA motif occupies positions −34 GGCTATAAAAGG −23. Therefore, the functional TATA box of this RNA polymerase III-dependent gene is not confined to a mere T/A rich sequence. It is also remarkable that the tRNA^Sec TATA is a close match to the Adenovirus ML Promoter TATA box GGCTATAAAAGG in

![Figure 4. Effects of the double GG to TT point mutation at −33/−32 on TBP binding. The double-stranded DNAs used are shown above the lanes and correspond to point mutations depicted in Table 1. Experimental details are provided in Materials and Methods. hTBP was omitted in lanes 1, 3 and 5. F denotes the migration of the free DNA.](image-url)
which position −32 is occupied by a C and, as found for the latter, it is a strong promotor (30).

The novel finding arising from this work concerns the critical importance of positions −33/−32 to both in vivo and in vitro transcription. A severe down mutation is provoked in vivo and to a lesser extent in vitro by the double point mutation, while separate mutations at −33 and −32 do not generate such a drastic phenotype. It has already been published by others that a G−34 to A transition reduces yeast TBP binding in vitro (31). However, the work reported here constitutes, to our knowledge, the first detailed study stressing the requirement of bases upstream of the TATA motif for transcription in vivo and in vitro. Transcription in vivo is moderately, if not affected, by substitutions to C or G at T−31 or T−29. Apparently, transcription in vitro is more sensitive to these point mutations and the strongest deleterious effect is observed when G replaces T−29, especially with human TBP. Transcription in vitro is neither PSE nor activator dependent under our conditions. One can thus assume that in vivo, despite the occurrence of a mutated TATA box, the transcription complex is stabilized owing to the involvement of PSE-binding factors and activators, explaining the better in vivo versus in vitro transcription level. By comparing our data with those described in (32) by Wobbe and Struhl, it appears that the effects of their mutational analysis are more dramatic than ours: any single point mutation reduced activity, with an almost complete loss of transcription when T−31 or T−29 were replaced by G.

In the 3′ portion of the TATA box, positions −25 to −23 appear critical. The A−25 to C mutation exerts a significant effect both in vivo and in vitro, while transition to G is more tolerated. This correlates well with the converse experiment by Wobbe and Struhl with the Pol II system (32): they replaced the terminal G by A in the TATAAAG sequence and observed an increase in the transcription level. Our data are also in line with methylation interference experiments which underlined the contribution of A−25 to TBP binding in vitro (33,34).

Is this mutational analysis interpretable in the light of the recently published crystal structures of yeast and Arabidopsis TBP/TATA box complexes (35,36)? It appeared that the effects of some but not all of the mutations can find an explanation at the structural level. In addition, the quantitative effects of the mutations are not immediately discernible from inspection of the 3D models. The deleterious transcriptional effect induced by the presence of C−25 instead of A is certainly due to the presence of G−25 on the other strand: an exocyclic amino group is not tolerated in the Van der Waals interface provided by the wide shallow minor groove in order for the phenylalanine to pack against the base (35,36). However, we are unable to explain why G−25 is less detrimental than C−25. Similarly, we cannot provide any explanation as to why T−31 is more tolerant to mutation than T−29 and especially more tolerant to a transition than a transversion. In any case, according to both models, introduction by mutagenesis of the exocyclic 2-amino of guanines into the minor groove should be inhibitory. It is also difficult to interpret the strong detrimental effect of the double point mutation at −33/−32 on a structural basis since none of the amino acids of the TBPs employed appear to interact in the vicinity of these nucleotides (35,36). Therefore, we are left with the possibilities that these mutations either influence the bending of the DNA by relaxing the compression provided by the G/C rich sequences 5′ to the TATA box or the binding specificity of TBP. It has effectively been proposed that G/C rich flanking sequences are important to promote a particular DNA structure or to confine TBP to the TATA box (33). The altered pattern observed in the gel shift assay could reflect either possibility. It is also possible that a subtle interaction takes place between some residues of the long N-terminal domain of the human TBP and G−33/G−32. These contacts, if they exist, could not have been detected in the published 3D models since the yeast TBP used for the crystallographic studies was truncated so as to leave the C-terminal domain only (35) and Arabidopsis TBP possesses a short N-terminal domain (36).

Finally, whereas the central role of TBP in Pol III transcription is now well established (7−12), little is known on its role in transcription complex assembly. The TATA binding protein was found to be required for TFIIB assembly upstream of the tRNA genes (7,8) but its direct interaction with DNA within the transcription complex has not been demonstrated. The fact that down mutations affected both transcription and DNA−TBP complex formation support a model in which TBP interacts directly with the TATA element of the RNA Sec gene.

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