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Sites of RNA polymerase III transcription initiation and Ty3 integration at the U6 gene are positioned by the TATA box

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ABSTRACT **The function of a TATA element in RNA polymerase (EC 2.7.7.6) III transcription of a naturally TATA-containing U6 snRNA gene and a naturally TATA-less tRNA gene was probed by transcription and Ty3 transposition analyses. Deletion of the TATA box from a U6 minigene did not abolish transcription and Ty3 integration but changed the positions of initiation and insertion. Insertion of the U6 TATA box at three positions upstream of the TATA-less *SUP2* $tRNA^{Tyr}$ gene resulted in novel transcription initiation and Ty3 integration patterns that depended upon position of the insertion. Nevertheless, the predominant tRNA gene initiation sites were not affected by insertion of the TATA sequence and remained at a fixed distance from the internal *box A* promoter element. Insertions of the TATA box upstream of a *SUP2 box A* mutant affected the level of transcription and restricted the use of upstream start sites, but they neither enhanced the use of TATA-dependent initiation sites nor restored expression to the level of the wild-type gene. We conclude that (*i*) the U6 TATA box is essential *in vivo* for correct initiation but not for transcription, (*ii*) a TATA box does not compensate for a weak *box A* sequence and so cannot perform equivalently, and (*iii*) the TATA-binding protein, and probably components of transcription factor IIIB, are present on the target at the time of Ty3 integration.**

The *Saccharomyces cerevisiae* retrotransposon Ty3 integrates upstream of class III genes (1, 2). Mutation of either of the essential tRNA gene promoter elements, *box A* or *box B*, eliminates the ability of the gene to direct Ty3 insertion, suggesting that the integration machinery recognizes either promoter elements or components of the transcription complex as the active target. The class of genes transcribed by RNA polymerase (EC 2.7.7.6) III (pol III) encompasses members with diverse promoters, including TATA-containing and TATA-less forms. For tRNA and 5S, which are TATA-less genes, formation of the pol III transcription complex is dependent upon internal promoter elements (reviewed in ref. 3). For tRNA genes, transcription complex formation begins with binding of transcription factor (TF) IIIC to the *box A* and *box B* elements, followed by binding of TFIIIB (4), in a sequence-independent manner, upstream of the initiation site (5, 6). Recently, TATA-binding protein (TBP), the DNA recognition subunit of TFIID (reviewed in ref. 7), has been demonstrated to be essential for the transcription of all class III genes (8–11).

Transcription of some class III genes, such as the vertebrate U6 small nuclear RNA genes, requires a TATA element *in vivo* and *in vitro* and is dependent upon TFIID *in vitro* (12). For vertebrate U6 genes, the essential promoter elements are located exclusively upstream (13–15). For the *S. cerevisiae* U6 gene, *SNR6*, TBP has been shown to be necessary for transcription *in vivo* (9) and *in vitro* (16). However, a yeast strain in which natural TATA-containing sequences upstream of the essential *SNR6* gene were replaced had reduced levels of U6 RNA but was viable despite the inactivity of this gene *in vitro* (17). The interpretation of this result was complicated by the presence of a sequence upstream of the gene with similarity to the TATA consensus. For *in vivo* expression, a *box B* motif centered at position +240 is required (17). In contrast, a gene lacking the downstream *box B* can be transcribed in a purified *in vitro* system (16). *SNR6* also contains a *box A* motif beginning at position +21. In the following study, the novel *SNR6* promoter configuration and the insertion specificity of Ty3 were exploited to investigate the role of the TATA element in pol III transcription and to determine whether transcription factors are bound to the target gene at the time of Ty3 integration.

MATERIALS AND METHODS

Plasmid Constructions. Culturing of bacteria and manipulations of DNA were performed essentially as described in Ausubel *et al.* (18). Test genes were contained on a high-copy, *HIS3*-marked, yeast shuttle vector, pDLC237 (2). Construction of plasmids carrying the wild-type (wt) U6 gene and flanking sequences [base pairs (bp) −539 to +629] (pDLC369), the *sup2+b* gene (pDLC356), and the *sup2G56+b* mutated *box B* derivative (pDLC565) have been described (2). Truncated versions of *SNR6*, lacking the upstream TATA sequences (bp +6 to +629) or downstream *box B* sequences (bp −120 to +125), were excised from plasmid pNH6 or pTaq6 (17), respectively, by cleaving with *Hin*dIII, filling in the 5′ overhanging end, and cleaving with *Eco*RI. These fragments were inserted between the *Eco*RI and *Sma* I sites of pDLC237. An *Eco*RI fragment containing the wt *SNR6* was then inserted into the *Eco*RI site between the 2-μm sequence and the wt or one of the truncated *SNR6* genes, creating target plasmids pDLC583, pDLC563, and pDLC564, respectively.

U6 minigene (U6mg) constructs, TATA-containing *SUP2* genes, and promoter mutants were created by site-directed oligonucleotide mutagenesis. Minigenes were deleted for positions +3 to +12 of the wt gene. The TG at positions −3, −2 in the wt gene was changed to GT in U6mg and U6mgΔTATA, to create a *Spe* I site. U6mgΔ5′, subcloned from pNH6 (+6/629) by cleavage at the *Eco*RI site (between positions −15 and −14 in the nontemplate strand) and ligation downstream of pDLC237 vector sequence, differs upstream of that site from U6Δ5′, which was subcloned downstream of a copy of wt *SNR6*. Thirteen base pairs, including the A at +1, were deleted from U6Δ5′ to make the transcribed sequence of U6mgΔ5′ the same as the other minigene constructs. The *box B* C56G mutation was introduced into *sup2+bTATA* constructs by replacing the *Bst*EII/*Bam*HI fragment containing the downstream half of the gene and 3′

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Abbreviations: pol, RNA polymerase; TF, transcription factor; TBP, TATA-binding protein; wt, wild-type; U6mg, U6 minigene.

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flanking sequences with the corresponding fragment from pDLC565 (*sup2G56+b*).

Recovery of Ty3 Insertions into Target Plasmids. Yeast strain yDLC221 (*MATa ura3-52 his3-Δ200 ade2-101 lys2-1 leu1-12 can1-100 gal3 Ty3::GTy3-1*) contains an integrated, galactose-inducible Ty*3* element that complements transposition of marked Ty*3* elements (1). The yDLC221 strain was transformed with pDLC348, which contains a galactose-inducible, Neo^R-marked Ty*3* element, and with the integration target plasmid. Transposition was induced in synthetic medium containing galactose, and plasmids containing insertions were recovered as described (2). Insertion sites were mapped by restriction enzyme and dideoxynucleotide sequence analyses.

Primer Extension Analysis of RNA Species. Gene expression was quantitated by reverse primer extension. Oligonucleotides complementary to nucleotides (nt) +49 to +67 of *SNR6* transcripts and nt −4 to +12 (relative to the U6mg major start site) of the U6mg and U6mgΔ5′ transcripts were radiolabeled with [γ-^{32}P]ATP and used to prime extension reactions. Pre-tRNAs were quantitated by primer extension using an end-labeled, *sup2+b*-specific oligonucleotide (19).

RESULTS

Transcription and Target Analysis of U6 Constructs. The discrete promoter elements of the U6 gene suggested that it was a system in which we might deduce the involvement of specific pol III transcription factors in Ty*3* targeting. To investigate the role of *SNR6* promoter elements in Ty*3* integration, the targeting efficiencies of a wt allele of *SNR6* and mutant alleles with unrelated 5′ (U6Δ5′) and 3′ (U6Δ*box B*) flanking sequences were determined. These alleles were each placed on target plasmids, approximately 1000, 530, or 650 bp, respectively, downstream of the wt *SNR6* (Fig. 1). The U6Δ5′ gene lacking the conserved TATA element was used as a target at a frequency similar to that of the wt gene (Fig. 1), and insertion occurred at the positions, −5/−1 and −6/−2 (gene distal/proximal base pairs of the 5-bp target

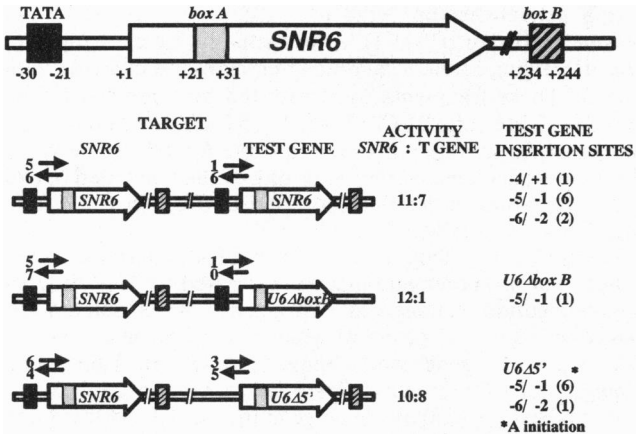

FIG. 1. Target efficiency of wt and mutant U6 genes. Wide open arrow, U6-coding region; narrow open box, flanking sequences; solid box, TATA sequence; shaded box, *box A*; hatched box, *box B*. Solid arrows above each gene indicate the direction of transcription of integrated Ty*3* elements. The target efficiency of the upstream *SNR6*, relative to the downstream test gene, is indicated in the first column. The sites of Ty*3* insertions given in the second column are indicated as the gene distal/proximal base-pair positions of the 5-bp target duplication generated by Ty*3* integration. Not all insertions analyzed were into the tandem target and not all insertions into the tandem target were sequenced. The number of insertions observed at each position is shown in parentheses. Transcription of U6Δ5′ was shown by primer extension analysis to initiate with an A and is indicated by * (unpublished data; ref. 17).

duplication), used in the wt target. When the construct containing the U6Δ*box B* allele was tested as a target for Ty*3* integration, 1 of 13 insertions occurred at position −5/−1 of the mutant gene (Fig. 1). These data suggested that the TATA element is not essential for efficient targeting and that *box B* and, by inference, TFIIIC are important, but not essential, for targeting.

The complete dispensability of sequences upstream of *SNR6* for Ty*3* integration suggested that requirements for integration and transcription might be separable. To examine transcription of *SNR6* with modified promoter elements in the presence of the endogenous gene, a minigene (20) was constructed by deleting bp +3 to +14 and designated U6mg. Primer extension analysis showed that the major transcript produced from U6mg was 12 nt shorter than the transcript produced from the wt gene and was initiated at the same position (Fig. 2). Minor start sites were observed upstream at positions −4, −8, and −10, each contributing 2–5% of U6mg transcripts. Primer extension analysis using an oligonucleotide specific for U6mg transcripts did not detect initiation at position −12, which would have coincided with products of the wt gene, for the minigene or its derivatives described below (unpublished data).

To investigate the role of 5′ flanking sequence in expression of the U6 gene, the transcription of an U6mg allele with a Ty*3* insertion, U6mg::Ty*3*(−4/+1), and of an allele in which vector sequences replaced natural sequences upstream of the major U6mg initiation site, U6mgΔ5′, was tested. Transcription was reduced and initiation occurred upstream of the natural U6mg for both of these constructs (Fig. 2). Therefore, although the majority of U6mg transcripts was initiated at the same distance from upstream sequence in the minigene as for the wt gene, in each U6mg with a modified 5′ flanking sequence, the distribution of initiation sites shifted upstream so that the distance between the start site and positions downstream of the deletion approached that of the wt gene. This result suggested that both upstream and downstream sequences were involved in positioning the initiation site. To test whether the TATA box affected choice of the initiation site, the 10 bp that spanned positions −30 to −21 (5′-TATAAATAAA-3′) was deleted from U6mg. This construct was designated U6mgΔTATA. Initiation sites were at +1, −3, −4, −8, −10, and −19 (approximately 17%, 6%, 39%, 18%, 18%, and 2% of U6mgΔTATA transcription, respectively) (Fig. 2). Thus, deletion of the TATA-containing sequence enhanced the use of previously minor upstream sites.

To determine whether pol II was responsible for the U6 transcripts with altered initiation sites, bp position 5 of the *box B* consensus sequence (3) was changed from C to G in U6mg and U6mgΔTATA. The analogous mutation in the *SUP4* tRNA^{Tyr} gene (nt +56 in the mature tRNA numbering convention) disrupts TFIIIC binding (21) and eliminates tRNA expression *in vivo* (22) and *in vitro* (23). The C56G mutation eliminated use of the *SUP2* tRNA^{Tyr} gene as a target for Ty*3* transposition (2). Neither *box B* mutant allele, U6mgG56 nor U6mgΔTATAG56, produced detectable transcripts (Fig. 2). Thus, transcripts detected by primer extension were products of pol III activity.

Because Ty*3* integration does not show sequence dependence at the insertion site (1, 2, 24), the presence or absence of the TATA element should only affect the position of integration indirectly through the action of proteins such as TBP. We reasoned that if integration occurred at the same positions in *SNR6* and U6mg relative to transcription initiation, but these positions were redistributed together with initiation upon deletion of the TATA box, then positioning of the integration complex must be affected by transcription factors bound upstream of the start site. Three independent Ty*3* insertions targeted to U6mg were collected and mapped

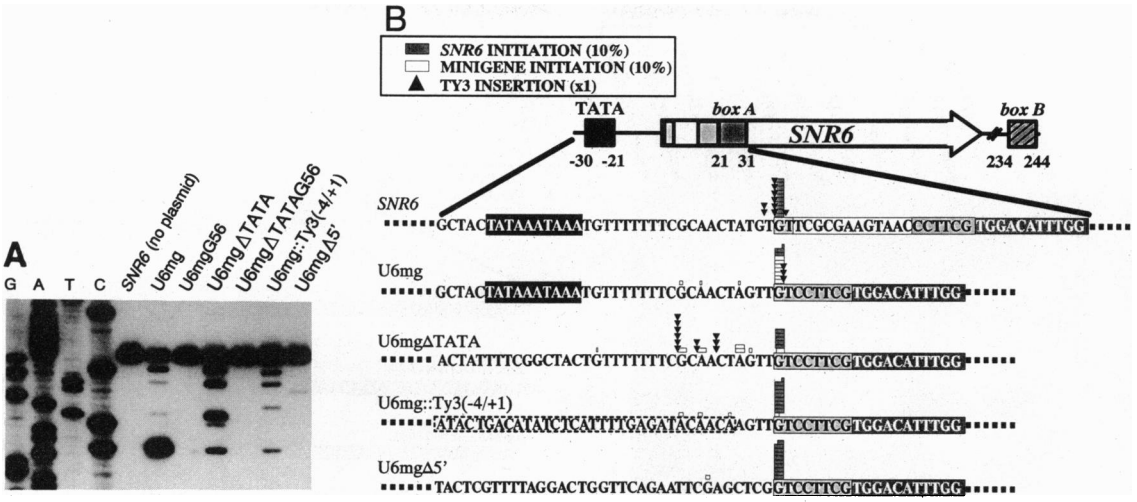

FIG. 2. Expression of U6mg constructs. (*A*) U6 expression was examined by reverse transcription of total RNA isolated from cells containing the indicated construct on a high-copy plasmid using a ^{32}P-labeled, U6-specific oligonucleotide. Primer extension products were separated by electrophoresis through a denaturing, 10% polyacrylamide/bisacrylamide (19:1) gel and visualized by autoradiography. The dideoxy sequence ladder, shown in the left four lanes, was generated by extension of the U6 oligonucleotide primer annealed to a U6mg plasmid DNA template. Products are shown of reverse transcription of 1 μg of RNA from each transformant. (*B*) Data are summarized for transcription and Ty*3* insertions for the U6mg plasmid constructs. Insertion sites for *SNR6* have been described (2). The dashed box indicates Ty*3* sequence. The pattern of transcription initiation is indicated by blocks above the start sites. Each full block represents 10% of total extension products: shaded blocks represent expression from the endogenous *SNR6* gene; open blocks represent transcription from U6mg plasmid constructs. Each Ty*3* insertion is presented as an arrowhead above the sequence indicating the U6 gene-proximal nick sites inferred from the 5-bp target duplication to occur on the opposite strand. Sequence of the integrated Ty*3* element begins with the sixth bp upstream. The sequence of U6Δ5′ differs from that of U6mgΔ5′ and for the upstream region is (−35) 5′-AGTCGTTGGGTACCGAGCTCGAATTCGAGCTCGGT-3′ (−1).

to position −4/+1, relative to the major initiation site of the minigene (Fig. 2*B*). The pattern of Ty*3* insertions upstream of U6mgΔTATA compared to U6mg shifted upstream together with the pattern of transcription initiation. All 11 Ty*3* insertions into this target gene occurred upstream of the insertions into U6mg: 3 at −11/−7, 2 at −13/−9, and 6 at −15/−11 (Fig. 2*B*). This redistribution of insertion sites with the deletion of TATA argued that transcription factors bound at the time of transcription initiation are present prior to Ty*3* integration.

Effects of a TATA Box on tRNA Expression. Although TBP is known to position pol II transcription complexes at TATA-containing promoters by sequence-specific interaction with the DNA (reviewed in ref. 7), less is known about how TBP is recruited to and makes contact with TATA-less promoters. Therefore, we asked whether the U6 TATA box could influence selection of initiation sites and potentially Ty*3* integration sites at a naturally TATA-less gene. Using site-directed oligonucleotide mutagenesis, 10 bp containing the U6 TATA box was inserted at three locations upstream of the *SUP2* tRNA^{Tyr} gene, so that it occupied positions −11/−20, −21/−30, and −31/−40 relative to the major *SUP2* transcription initiation site. The *SUP2* allele used in this analysis, *sup2+b*, contained a 6-bp insertion within the 14-bp intron so that pre-tRNA could be monitored specifically (19). Pre-tRNA is rapidly processed to its mature form (25) and pre-tRNA levels should therefore reflect the rate of transcription. The predominant *sup2+b* start sites were +1 (10 bp upstream of the coding region for the mature tRNA) and −2, which represented 83% and 17% of transcription, respectively (Fig. 3). Insertion of the TATA box, to occupy position −21/−30, corresponding to the location of the TATA box relative to the start site of the U6 gene, increased transcription initiation at the −2 site to nearly 40% of total transcription. Introduction of the TATA box at −11/−20 did not significantly alter the ratio of initiation at the +1 and −2 positions; however, new initiation sites downstream at +4 and +5 were observed, which represented approximately 5% and 3% of total transcription, respectively (Fig. 3). When the TATA box was placed at −31/−40 a slight decrease of initiation at position −2 was observed together with a new initiation site at position −10. Approximately 7% of pre-tRNAs were initiated at this novel site. The primer extension analyses of RNA produced from the three *sup2+bTATA* constructs indicated that the U6 TATA box could alter selection of the initiation site of a naturally TATA-less pol III-transcribed gene. As reported previously (19), Ty*3* insertions beginning at position −17 of the *SUP2* gene in either orientation increased usage of the −2 initiation site, but no novel initiation sites were detected as were found in this study for the TATA element insertions. Nevertheless, the major initiation site was not shifted either by the displacement of sequence caused by insertion of 10 bp or by the heterologous TATA element. This showed that the downstream promoter effects dominated start-site selection and suggested that the natural upstream sequence did not function analogously to the U6 TATA box.

The effect of the TATA box on Ty*3* integration was examined by mapping Ty*3* insertions at each *sup2+bTATA* mutant (Fig. 3*B*). Previous work showed that Ty*3* integrates from positions −4/+1 to −7/−3 but predominantly at −5/−1 (10 of 14 insertions) (2). Twenty-six Ty*3* insertions targeted to the *sup2+bTATA−21/−30* were recovered and found to be at positions −2/+3 (1), −5/−1 (9), −6/−2 (2), and −7/−3 (14). The predominant insertion site was shifted 2 bp upstream relative to that of the wt gene. Twenty-four Ty*3* insertions targeted to the *sup2+bTATA−11/−20* allele were mapped and found to be at positions −3/+2 (1), −5/−1 (22), and −7/−3 (1). Thus, position −5/−1 was even more favored over upstream insertion sites in this TATA-containing gene than in the wt gene. The insertion at −3/+2 is also further downstream than insertion sites observed previously for *SUP2*. This pattern of 18 Ty*3* insertions at the *sup2+bTATA−31/−40* gene was similar to the pattern at the wt gene. The distribution of Ty*3* insertion sites upstream of each TATA-containing tRNA gene was therefore congruent with the respective patterns of transcription initiation and

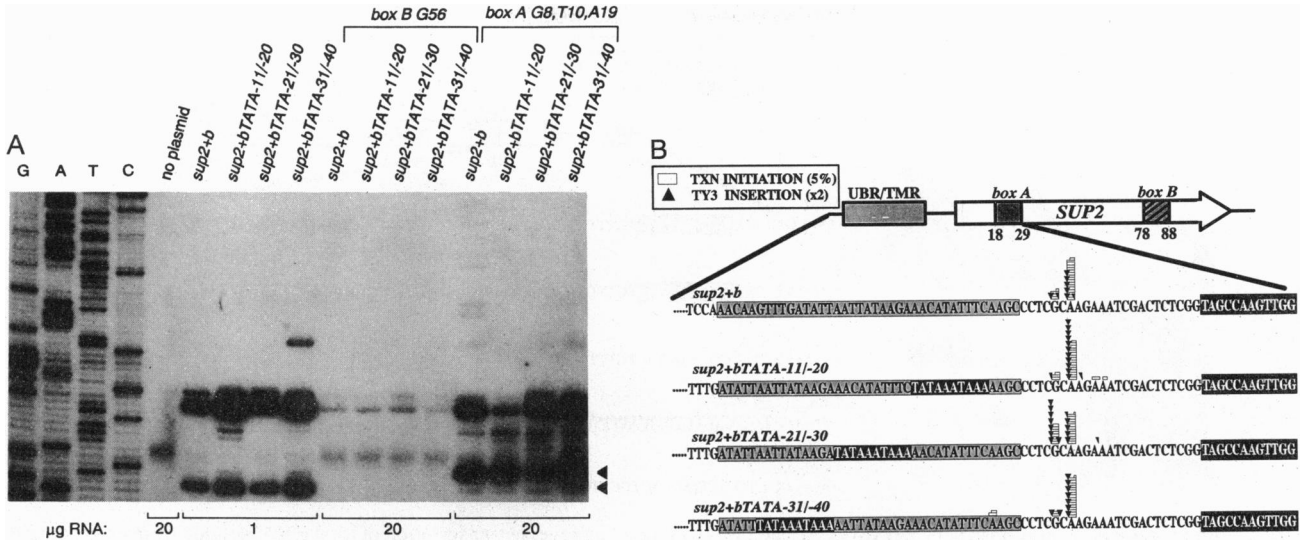

FIG. 3. Expression of TATA-containing tRNA genes. (*A*) Transcription was analyzed by reverse transcription of RNA isolated from cells transformed with high-copy plasmids containing the indicated tRNA gene construct. Extension reactions were primed with ^{32}P-labeled oligonucleotides complementary to plasmid-derived pre-tRNAs. The left four lanes contain a DNA sequence ladder generated using *sup2+b* DNA and the *sup2+b*-specific oligonucleotide. Primer extension products were separated by electrophoresis through a denaturing 10% polyacrylamide/bisacrylamide (19:1) gel and were visualized by autoradiography. Products are shown in the indicated lanes of reverse transcription of 1 µg of RNA each from transformants containing the *sup2+b* construct and the three TATA box-containing derivatives with wt internal promoter elements and 20 µg of RNA each from transformants containing mutant constructs with an altered *box B* (G56) or *box A* (G8, T10, A19). Extension products representing the 5′ end processed pre-tRNA are denoted by the solid arrowheads. (*B*) Data are summarized from the primer extension analysis and from Ty*3* insertions for the *sup2+b* gene and the three TATA box-containing derivatives. Insertion sites for the wt gene have been described (2). Each full open block represents 5% of transcription initiation. Ty*3* insertions are presented as arrowheads above each sequence indicating the tRNA gene-proximal integration nick site. Each full arrowhead represents two insertions.

presumably reflects constraints imposed by the transcription complex.

Our results showed that in *SUP2* the natural promoter prevailed over a heterologous TATA box for start-site selection. In transcription of tRNA genes, the *box A* element directs initiation a fixed distance upstream (2, 26, 27). *SNR6* contains a sequence from bp +21 to +31 that matches the *box A* consensus at only six of nine positions. We postulated that if TBP bound to the tRNA promoter in the same way as it did to the U6 promoter, then decreasing the similarity to the consensus sequence of the *box A* in the tRNA gene might enhance TATA box-specific initiation. To test this prediction, the *box A* sequences of the *sup2+b* gene and the three TATA box-containing derivatives were mutated and the effects on transcription initiation were examined by primer extension analysis. The *box A* consensus sequence, defined as tRNA positions +8 to +19, is TRGCNNAGY(N)GG (R = G or A; N = G, A, T, or C; Y = T or C) (3). The T8, A14, G18, and G19 positions are invariant. Position +17 is absent from some genes and is represented as (N). An altered *box A* sequence was generated with mutations T8G, G10T, and G19A. Primer extension analysis showed that transcription from this mutant was decreased >20-fold from that of *sup2+b* (Fig. 3*A*). In addition, the 5′ end processing site was shifted 2 nt upstream (Fig. 3*A*). A similar change was observed for the tRNA species produced from a G10T, A14G, G15T *box A* mutant that did not show decreased transcription (unpublished data). Transcription from the *sup2+bTATA−11/−20 box A* mutant was reduced relative to the *sup2+b box A* mutant, but the *sup2+bTATA−21/−30* and the *sup2+bTATA−31/−40 box A* mutants showed equal or greater expression than the TATA-less mutant. The transcription pattern of the *sup2+b box A* mutant showed several minor initiation sites upstream of the wt start site that were not present in the TATA-containing derivatives. Thus, as observed for U6mg, the TATA sequence restricted initiation at upstream sites. However, the TATA box was unable to restore transcription to the level of the wt gene by directing initiation to sites specified by its position.

DISCUSSION

We undertook this investigation to determine whether the TATA and downstream promoter elements of *SNR6* function separately or coordinately to target Ty*3* and to determine whether pol III transcription factors are bound to the target gene at the time of Ty*3* insertion. The results showed that efficient Ty*3* transposition requires a functional *box B* sequence but that neither transcription nor transposition requires the conserved TATA element. Nevertheless, deletion of the TATA box significantly affected the positions of transcription initiation and integration. These data argue that the yeast U6 gene is a member of the class of genes, including the *Xenopus* tRNA$^{(Ser)Sec}$ gene (28), that are TATA-containing but depend on downstream elements for accurate and efficient transcription. In this study, the correspondence between the effect of a TATA sequence on Ty*3* insertion sites and transcription start sites indicated that the Ty*3* integration complex binds predominantly at target genes that are associated with proteins of the initiation complex.

Our results demonstrated that the TATA and downstream element(s) make distinct contributions to placement of the initiation complex at *SNR6*. The *SNR6* start site occurred 30 bp downstream of the first T of TATA, even in the U6mg construct where the initiation site was 12 bp closer to *box A*. Similarly, Ty*3* insertions at U6mgΔTATA, but not at U6mg, occurred at the same distance from *box A* as insertions at the wt target. Thus, in the absence of TATA, the initiation complex is positioned by downstream promoter elements.

Transcription of the *SUP2* tRNA^{Tyr} gene containing upstream TATA insertions demonstrated that, in the tRNA gene context, downstream promoter elements act independently of a TATA element to position the initiation complex. Nevertheless, the use of some TATA-dependent initiation sites and repression or enhancement of normal transcription of these constructs suggested that the initiation complex composed of TFIIIB and TBP and potentially TFIIIC could respond in a sequence-specific manner to changes upstream

of the initiation site. This argues, albeit indirectly, that in at least a subset of transcription complexes formed over naturally TATA-less genes, TBP is available to contact the DNA and to exploit a TATA sequence if present. Supporting this notion, even when an identifiable TATA element is lacking, the region centered around 22 bp upstream of the initiation site has been found to affect the level of tRNA gene expression (29). In addition, TBP interacts with a variety of sequences, including some that match the consensus poorly (30, 31). Our observations and those of others are consistent with a model in which TBP is delivered by different mechanisms to naturally TATA-less and TATA-containing genes but may contact the DNA in a similar way.

Some property of the tRNA gene, not shared by the U6 gene, must constrain the influence of the heterologous TATA element. It neither shifted major initiation sites nor compensated for a debilitated *box A*. The proximity of *box A* and *box B* in *SUP2* relative to the distance in *SNR6* is potentially such a property. In tRNA genes, TFIIIC binding is optimal when *box A* to *box B* spacing is 30–60 bp (27). The distance between these promoter elements affects interaction of TFIIIC with the *box A* element (27, 32). In *SNR6*, the separation of a nonconsensus *box A* motif from a *box B* motif by >200 bp may result in a greater influence of the TATA box over the position of initiation. Our results and those of others suggested that TFIIIC also contributes differently to transcription of the U6 gene than to that of the tRNA gene. For example, conditions can be achieved *in vitro* where U6 expression is not dependent on TFIIIC. In our study removal of the U6 *box B* sequence did not completely eliminate Ty*3* integration, as appeared to be the case for a *box B* C56G mutation in the *SUP2* tRNA (2).

The specificity of Ty*3* integration makes it a unique and useful tool to evaluate pol III initiation complex formation *in vivo*. Further definition of the precise interactions between the integration machinery and the transcription complex will facilitate understanding of transcription initiation and yield insights into the process of retroviral target selection.

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