Transcription of a yeast U6 snRNA gene requires a polymerase III promoter element in a novel position

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Vertebrate genes coding for U6 small nuclear RNA are transcribed by RNA polymerase III (pol III), using only upstream promoter elements rather than the A and B block internal control regions typical of most pol III transcription units. We show that expression of the U6 gene from the yeast Saccharomyces cerevisiae has two unexpected features: it requires a B block promoter element, and this element is located in a novel position, 120 bp downstream of the coding region. In tRNA genes, the B block is the primary binding site for transcription factor (TF) IIIC, whose function is to promote the subsequent binding of TFIIIB. Both factors are thus implicated in yeast U6 gene transcription. We present a model of the U6 transcription complex based on the structure of yeast and vertebrate U6 promoters.

[Key Words: U6; snRNA; pol III transcription; B block; TFIIIC]

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U6 small nuclear RNA is one of five snRNAs essential for spliceosome-mediated processing of premessenger RNA (for review, see Guthrie and Patterson 1988). It is the only spliceosomal snRNA synthesized by RNA polymerase III (pol III; Kunkel et al. 1986; Reddy et al. 1987); the others are synthesized by pol II [see Dahlberg and Lund 1988]. Genes transcribed by pol III, including transfer RNA and 5S ribosomal RNA genes, characteristically have predominantly intragenic promoters (for review, see Geiduschek and Tocchini-Valentini 1988). For example, tRNA genes contain two conserved intragenic promoter elements, the A and B blocks. The A block is nearest the 5' end of the gene and specifies the point of transcription initiation, whereas the B block is 30–90 bp farther downstream and is the primary binding site of the pol III transcription factor TFIIIC. Binding of TFIIIC is obligatory for binding of another factor, TFIIIB, resulting in formation of a stable transcription complex recognized by pol III. TFIIIB and TFIIIC are presumed to be generally required for pol III transcription. It was therefore surprising when transcription by pol III of a human 7SK gene and a mouse U6 snRNA gene was shown to be dependent only on sequences upstream of the initiation site [Murphy et al. 1987; Das et al. 1988]. Three conserved upstream elements important for vertebrate U6 gene promoter function have been identified: the distal sequence element (DSE; Bark et al. 1987; Carbon et al. 1987; Das et al. 1988; Kunkel and Pederson 1988), the proximal sequence element (PSE; Carbon et al. 1987; Das et al. 1988; Kunkel and Pederson 1988; Mattaj et al. 1988; Lobo and Hernandez 1989), and a T/A-rich element similar in sequence and location to the pol II TATA box [Mattaj et al. 1988; Lobo and Hernandez 1989]. The DSE and PSE have been shown to be functionally interchangeable with similar sequences upstream of the gene coding for U2 snRNA, which is synthesized by pol II [Bark et al. 1987; Kunkel and Pederson 1988; Mattaj et al. 1988].

Remarkably, it is the TATA-like sequence 29–31 bp upstream of the U6 snRNA start site that directs pol III to the U6 gene [Mattaj et al. 1988; Lobo and Hernandez 1989]. A similar requirement for a TATA-like sequence ~30 bp upstream of a pol III start site has been seen for the human 7SK RNA [Murphy et al. 1987] and c-myc genes [Bentley et al. 1989]. Although the authors of these studies concluded that the TATA-like sequence is a binding site for a specific factor that directs pol III to the promoter, a candidate for this factor has not yet been proposed. This leaves open the question of how a sequence element best known as a binding site for a pol II transcription factor, TFIIID (Sawadogo and Roeder 1985), promotes recognition of the U6 gene by pol III.

We isolated previously the U6 snRNA gene (SNR6) from the yeast Saccharomyces cerevisiae and found that it is transcribed in a homologous cell-free pol III transcription system [Brow and Guthrie 1988]. Here, we report the results of a gross mutational analysis of the SNR6 promoter. Surprisingly, expression of the SNR6 absolutely requires a B block promoter element, situated in a novel position: 120 bp downstream of the coding region. The ability of yeast U6 gene deletion constructs

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with intact B blocks, but not those with disrupted B blocks, to compete with 5S rDNA for a limiting transcription factor strongly suggests that the downstream B block binds TFIIIC in vitro. In tRNA genes, TFIIIC also contacts the A block promoter element, we find a match to the A block consensus sequence at the appropriate position in the yeast U6 coding region. Because the function of TFIIIC in tRNA gene transcription is to promote binding of TFIIIB [Kassavetis et al. 1990], both factors are thus implicated in yeast U6 gene transcription. In light of recent evidence that TFIIIB binds to a region centered 20–30 bp upstream of tRNA and 5S rRNA genes [Braun et al. 1989; Kassavetis et al. 1989], we propose that TFIIIB binds the U6 gene TATA-like element and directs recognition of the U6 gene by pol III.

Results

DNA sequence flanking the yeast U6 SNR6 gene

The isolation and initial characterization of the U6 snRNA gene from the yeast S. cerevisiae is described in Brow and Guthrie [1988]. At that time, the presence of a TATA-like sequence at position −30 and a sequence identical to the 3'-half of the human U6 gene PSE at position −60 were noted. For mouse and human U6 genes, <80 bp of 5'-flanking DNA (encompassing both of these elements) are necessary and sufficient to promote in vitro transcription [Das et al. 1988; Kunkel and Pederson 1988]. In vivo, the DSE, located 220 bp upstream of the start site, is required additionally for efficient expression of the mammalian genes [Bark et al. 1987; Das et al. 1988; Kunkel and Pederson 1988]. A preliminary deletion analysis of SNR6 revealed that a subclone containing 300 bp of 5'-flanking and 30 bp of 3'-flanking DNA (−303/+145) was not expressed in vivo or transcribed in vitro [data not shown]. We therefore undertook a more detailed deletion analysis to locate the essential promoter element missing from this subclone but present in a larger, active subclone (−539/+629, see Fig. 1).

As a first step in this analysis, we sequenced more of the 5'- and 3'-flanking DNA. The sequence shown in Figure 2a represents the entirety of SNR6 upstream DNA present in our original genomic clone, and about 500 bp of downstream DNA. The additional 5' sequence data revealed a solo delta element upstream of the conserved TATA-like and PSE-like sequences, spanning base pairs −425 to −91 [Fig. 2a]. Delta elements are the long terminal repeats of the Ty retrotransposon [Cameron et al. 1979] and are responsible for both the initiation of Ty transcription by pol II and the polyadenylation of the transcript [Elder et al. 1983]. Solo and Ty-associated delta elements are often located immediately upstream of tRNA genes [Gafner et al. 1983], and one has also been found upstream of a 5S rRNA gene [Piper et al. 1984]; this association is presumably the result of preferential transposition of Ty to these sites, frequently followed by excision of the transposon via homologous recombination between the two delta elements. The discovery of a solo delta upstream of the U6 gene reinforces the notion that preferential integration of the Ty transposon is a general property of pol III transcription units.

Figure 2b shows an alignment of the Ty-associated delta ADH2-6'L and the SNR6 solo delta, which are 78% identical. The delta TATA box, transcription initiation site, and polyadenylation site are all well conserved, whereas the upstream activating sequence (UAS) is more degenerate. The SNR6 delta is oriented such that, if active, it would be transcribed in the direction away from the U6 gene. Although Ty-linked delta elements similarly positioned upstream of pol II transcription units can greatly increase their expression [for review, see Williamson 1983], this is not true of solo delta elements, which lack a downstream enhancer-like sequence. Thus, the SNR6 solo delta is not necessarily expected to influence synthesis of U6 snRNA.

Another surprising finding came from the 3'-flanking DNA sequence analysis: 120 bp downstream of the U6 snRNA-coding region lies an excellent match to the pol III B block promoter element consensus [Fig. 2a,c]. The seven most highly conserved nucleotides of the 11-base consensus [Galli et al. 1981] are present. The B block is the primary binding site for TFIIIC and is required for transcription of tRNA genes and other [but not all] pol III transcription units. Although no functional B block has ever been found to lie outside of the coding region with which it is associated, our preliminary analysis suggested this downstream B block might be required for yeast U6 snRNA synthesis.

In vitro transcription of SNR6 deletion constructs

To determine the sequences required for U6 snRNA synthesis in S. cerevisiae, 5' and 3' deletions of SNR6 were constructed. The transcriptional activity of these deletion constructs was tested in both an in vitro and an in vivo assay system. The in vitro assay used a whole-cell extract preparation active in tRNA and 5S rRNA gene transcription [Klekamp and Weil 1982]. As reported previously [Brow and Guthrie 1988], the full-length subclone of SNR6 directs synthesis of U6 snRNA in this cell-free system [see Fig. 3, lane 2]. Identification of the transcript is based on its size (112–115 nucleotides), its hybrid selection by a small SNR6 subclone (−120/+125; data not shown), and changes in its length when internally deleted SNR6 templates are used [see below]. A set of four transcripts 200–300 nucleotides in length is also produced, the nature of these transcripts is discussed below. Synthesis of all the transcripts is insensitive to 1 mg/ml α-amanitin [data not shown], which, given the drug sensitivity of the yeast polymerases [Schultz and Hall 1976; Valenzuela et al. 1976], suggests they are products of pol III. Furthermore, when transcription is carried out in the presence of low levels of tagetitoxin, a specific inhibitor of pol III [Steinberg et al. 1990], the yield of all transcripts is significantly reduced [T. Steinberg and R. Burgess, pers. comm.].

Figure 1 shows the structure of the 5'- and 3'-deletion constructs and summarizes their activity in vitro. The supporting data are presented in Figure 3a. Six different
5' ends were tested. End points from −539 to −120 are all approximately equally active in vitro (Fig. 3a, lanes 2–6), indicating that sequences within the solo delta are not important for in vitro transcription. The +6 construct, which substitutes the entire 5' flank as well as 5 bp of coding DNA with vector DNA, is completely inactive (Fig. 3a, lane 7). This is not surprising as it lacks the position of the following sequence elements is indicated (arrows indicate direction of transcription): (solo delta) long terminal repeat of Ty retrotransposon, [P] PSE-like sequence; (A) A/T-rich TATA-like sequence, (SNR6) U6 snRNA gene; (B) B block consensus match; (T) downstream oligo T stretch. The grey bars represent yeast DNA present in the various deletion constructs. A plus sign indicates that in vitro transcription from this template was easily detected or that transcripts containing only this SNR6 allele are viable. A minus sign indicates that transcription was not detected, or strains were inviable (see Fig. 3).

The activity of the 5'-deletion constructs indicated that failure of our earlier subclone to be transcribed was due to the lack of an essential downstream promoter element. To determine whether this element is the B block-like sequence, five different truncated 3' ends were tested in combination with the functional −120 5' end (Fig. 1): +387, +312, and +246 all contain the putative B block promoter element, although +246 has only an additional 3 bp downstream of it; +235 replaces all but the first 2 bp of the B block with vector DNA (Fig. 2c) and +145 has only 30 bp of yeast DNA downstream of SNR6. In vitro, all 3' deletions containing the B block have approximately equal activity (Fig. 3, lanes 6, 10–12), but those lacking most or all of the B block are completely inactive (lanes 8 and 9).

To confirm that the inactivity of the +145 and +235 deletions is due to lack of a functional B block and not a result of bringing inhibitory sequences into the proximity of the U6 gene, a 2-bp deletion was made in the B block of the −120/+629 construct, removing base pairs +238 and +239. Base pair +238 corresponds to the C residue at position 5 of the B block consensus; mutations at this position are the most detrimental to TFIIIC binding (Baker et al. 1986) and promoter function (Alison et al. 1983; Newman et al. 1983; Fabrizio et al. 1987) yet identified in yeast tRNA genes. The SNR6 B block is palindromic, with the axis of symmetry between positions +238 and +239. Thus, the +239 deletion removes the corresponding position 5 C residue from the reverse orientation, precluding any possible bidirectional activity of the downstream B block. The +238/+239 deletion construct is completely inactive in vitro (Fig. 3, lane 13), verifying that it is the B block itself that is essential for yeast U6 gene transcription. (Template mixing experiments showed that this DNA preparation did not contain inhibitors of U6 gene transcription.)

In addition to the requirement for 5'-flanking sequences and the downstream B block, intragenic sequences were also found to affect the level of yeast U6 synthesis. In vitro transcription of a mutant lacking 22 bp of 3'-coding sequence (Δ+87/+108, kindly provided by R. Bordonne) yielded at least fourfold more RNA than was obtained from the same amount of wild-type template (Fig. 3, cf. lanes 14 and 15). It is not clear whether this is due to the decreased distance between the B block and the site of initiation (see Discussion) or to the removal of inhibitory sequences. With respect to the latter possibility it may be significant that within this deletion lies an 8-bp direct repeat of sequences present in the upstream PSE-like region (cf. positions −55/−48 with +102/+109).

The deletion analysis also provides clues to the identity of the four additional discrete transcripts produced by the full-length SNR6 clone. Their approximate lengths are 235, 260, 275, and 295 nucleotides (Fig. 3a). These transcripts are produced by all the 5'-deletion constructs, including +6/+629 and, therefore, do not require sequences upstream of SNR6 for their synthesis. Indeed, linearization of the template at the BclI site in the middle of the U6 gene abolishes U6 snRNA synthesis but does not affect accumulation of the 235–295 nucleotide products (data not shown). However, the +87/+108 deletion construct does not generate the longest transcript (Fig. 3a, lane 14), whose promoter or coding sequence must therefore overlap with the U6 gene. None of the transcripts is produced by the +238/239 deletion, so their synthesis is also B-block dependent, but because the B block is palindromic, one can not infer from this the orientation of the transcripts. The 3' deletions that maintain the B block (+246, +312, +387) do not produce the 235–295 nucleotide transcripts, but instead generate longer transcripts whose lengths correlate with the extent of the deletion (visible in Fig. 4, indicated by arrows). From these data, we conclude that the four transcripts most likely initiate immediately downstream of the U6 gene (approximately at positions +125, +145, +160, and +185) and terminate.
in the stretch of seven T residues at positions +416 to +422 [Fig. 2a]. In the 3'-deletion constructs containing the B block but lacking the oligo T stretch, the transcripts are expected to continue into vector sequence until encountering an oligo T stretch there. The location of such oligo T stretches in the vector is consistent with the approximate length of the observed transcripts. The +87/+108 deletion apparently destroys upstream sequences required for synthesis of the longest transcript. Whether these transcripts, which have not yet been detected in vivo, represent functional RNAs is unknown.

**Competition of SNR6 deletion constructs with 5S rDNA**

The identification of a conventional pol III promoter element in a U6 snRNA gene is unprecedented and indicates that initiation of transcription of the yeast U6 gene is likely to involve general pol III factors. That vertebrate U6 genes also use general pol III factors was suggested by competition experiments in which the U6 gene was found to compete with 5S rDNA for a limiting transcription factor in vitro [Reddy et al. 1987] and in vivo [Carbon et al. 1987]. Because transcription of 5S rDNA genes requires TFIIIB and TFIIIC (as well as the 5S-specific factor TFIIIA), this suggests that vertebrate U6 genes use at least one of these common pol III factors. However, there is no information concerning the binding site on the U6 gene of the pol III factor titrated out by U6 DNA.

We found that the yeast U6 gene can compete with either a tRNA gene or a 5S rRNA gene in the cell-free transcription system (unpubl.). If TFIIIC is the factor sequestered by the yeast U6 gene, or if TFIIIC binding is required for pre-mRNA processing, we would expect to detect a 5S-specific factor (e.g., TFIIIB), the competitive strength of a given U6 transcription factor. The results of an experiment with 5S rDNA as competitor are shown in Figure 4. In the presence of 75 fmoles of the -120/+235 construct, which lacks most of the B
block, competes no better than vector DNA. The construct containing the entire B block and 3 bp of downstream sequence \((-120/+246)\) is not a strong competitor, reducing 5S rDNA transcription only by about a quarter, to 72% of control. Thus, even though this deletion construct is transcribed in vitro with approximately the same efficiency as the full-length clone, the absence of sequences downstream of the B block must impair its ability to bind a factor limiting for 5S rDNA transcription. The additional sequences important for binding must be <66 bp downstream of the B block, as constructs containing the +312 and +387 end points exhibit full competitive strength. Sequences within the B block are essential for competition, as the A+238/9 internal deletion construct competes no better than vector DNA (data not shown).

The qualitative correlation between transcriptional activity and competitive strength of the deletion constructs strongly suggests that a single factor, which binds the downstream B block, is required for both the transcription of the U6 gene and its competition with

![Figure 3. Expression of SNR6 deletion constructs in vitro and in vivo. (a) Lanes 1–13: pUC118 DNA containing no insert (vector) or the indicated SNR6 fragment (see Fig. 1) in the multiple cloning site was incubated in whole-cell extract with NTPs and \([\alpha^32P]GTP\) as described in Methods, and transcripts resolved on a 6% polyacrylamide, 7 M urea gel. The arrow shows the position of U6 snRNA and the bar, the position of the unidentified high molecular weight transcripts (see text). (Lanes 14 and 15) A similar transcription reaction in which the template was pBluescript DNA containing the 1.4-kb EcoRV-HpaI fragment of pEP6 (i.e., SNR6 \(-539/+629\) with some vector DNA) with or without the indicated intragenic deletion. Markers are a HpaII digest of pBR325. (b) S. cerevisiae strain DAB017 [a trpl leu2 ura3 his4 can1 snr6::LEU2 YCP6[URA3/SNR6]] was transformed with pSE358 (CEN4 ARS1 TRP1) alone (vector) or with the indicated SNR6 fragment inserted in the multiple cloning site. After growth to saturation in synthetic medium lacking leucine, tryptophan, and uracil, transformants were patched on synthetic medium lacking leucine and tryptophan, with or without 0.75 mg/ml 5-FOA. Growth in the presence of 5-FOA (Boeke et al. 1987) requires the loss of YCP6, and therefore indicates that the SNR6 deletion construct borne on pSE358 can functionally replace the full-length SNR6 allele on YCP6.

![Figure 4. Competition of SNR6 constructs with 5S rDNA. Mixtures of 200 ng (\(-75\) fmole) of vector DNA or the indicated SNR6 deletion construct and 45 ng (20 fmole) of pB-1 (5S rDNA) were incubated with 1.5 \(\mu\)l of whole-cell extract for 10 min. NTPs were then added and transcription allowed to proceed for 45 min. Products were resolved on a denaturing gel and bands corresponding to 5S rRNA were excised. Yield of transcript was quantitated by liquid scintillation. The yield relative to control (1386 cpm) is indicated below each lane. The rightmost lane shows transcription of full-length SNR6 clone in the absence of 5S rDNA. The arrows indicate the position of presumptive elongated forms of the 235-295 nt transcripts, resulting from deletion of the oligo T stretch (see text) and visible here because of the shorter exposure time.](https://genesdev.cshlp.org)
the 5S rRNA gene. The quantitative difference in transcription activity and competitive strength of the -120/ +246 construct could be explained if the B-block-binding factor is not limiting for U6 gene transcription, so its reduced affinity for the -120/ +246 template, indicated by the competition results, does not significantly alter the yield of U6 transcript. Of course, the most likely candidate for the B-block-binding factor is TFIIIC. To our knowledge, the contribution of sequences downstream of the B block to the strength of TFIIIC binding has not yet been assessed. However, Camier et al. (1985) report that dimethyl sulfate footprinting of highly purified TFIIIC on a yeast tRNA gene reveals contacts of the factor with nucleotides 8 and 9 bp downstream of the B block; disruption of similar contacts may be responsible for the decreased competitive strength of the -120/ +246 construct.

The U6 gene is a surprisingly strong competitor of 5S rDNA transcription in view of its relatively low transcriptional activity (see Fig. 4), which is therefore unlikely to be due to inefficient binding of the factor limiting for 5S rRNA synthesis. Rather, binding of other components of the initiation complex, or perhaps events after initiation (i.e., elongation, termination, or reinitiation) may limit U6 snRNA synthesis in vitro. It is also notable that the observed competition is nonreciprocal. This could be a consequence of the requirement of 5S rDNA to bind TFIIIA before binding the common factors, TFIIIC and TFIIIB, thus allowing the U6 gene time to sequester these factors. Alternatively, the amount of 5S rDNA present might not be sufficient to make the common factors limiting for U6 snRNA synthesis. More extensive competition analyses, including a variation of the order of template addition, will be required to discriminate between these possibilities.

In vivo expression of SNR6 deletion constructs

Previous studies in mammalian systems have shown that sequences required for maximal transcription of the U6 gene in vitro and in vivo differ markedly, with the latter extending much farther upstream in the 5'-flanking DNA. It was therefore important to determine the activity of our SNR6 deletion constructs in yeast cells. In vivo expression was assayed by a plasmid shuffle protocol (Boeke et al. 1987) in which a full-length SNR6 allele on a URA3 plasmid was replaced by each of the various deletion constructs, carried on a TRP1 plasmid. This was done in a haploid strain with a targeted disruption of the single-copy, essential SNR6 locus. We have shown previously that this strain is completely dependent on the SNR6/URA3 plasmid for growth, evidenced by its inability to survive plating on medium containing the drug 5-fluoroorotic acid (5-FOA), which selects for the small fraction of cells that have spontaneously lost the SNR6/URA3 plasmid (Brow and Guthrie 1988). However, this strain does not exhibit 5-FOA lethality if it contains an additional functional copy of the U6 gene, unlinked to the URA3 gene. Expression of U6 snRNA from 5'- and 3'-deleted SNR6 alleles was therefore tested by introduction into this strain on a TRPI plasmid and the subsequent plating of the cells on medium containing 5-FOA. Growth is indicative of a functional allele of SNR6 on the TRPI plasmid.

With one striking exception, the in vivo results closely parallel the in vitro data (Fig. 1). Plasmids containing the deletion alleles with end points from -539 to -120 all rescue the strain with a chromosomal SNR6 disruption after plating onto 5-FOA-containing medium, indicating that these constructs are expressed (Fig. 3b, patches 2–6). As also expected, all the 3' deletions that are transcribed in vitro, rescue in vivo, and those that are not transcribed, including the 2-bp deletion in the B block, do not rescue (Fig. 3b, patches 8–13). Thus the downstream B block is essential in vivo as well as in vitro.

The exceptional clone is the +6/+629 construct, which produces no U6 snRNA in vitro, but is active in vivo (Fig. 3, patch 7). This is despite the fact that the conserved PSE-like and TATA-like elements have been replaced by vector DNA. When RNA was isolated from strains containing the active deletion constructs, and the amount of U6 snRNA determined by Northern analysis, the only strain with a significantly altered level of SNR6 expression was the one carrying the +6/+629 deletion allele (Fig. 5). It produced half as much U6 snRNA as the strain containing the next most extensive 5' deletion (cf. lanes 8 and 9). Furthermore, the RNA produced by this construct appeared to be slightly shorter than wild-type yeast U6 snRNA. Primer extension of U6 snRNA synthesized in vivo from the +6/+629 deletion allele confirmed that it is one nucleotide shorter than wild-type U6 and has vector sequence at its 5' end (Fig. 6a).

How is this truncated gene expressed in the absence of the conserved upstream elements? An alignment of wild-type and +6/+629 SNR6 upstream sequences reveals a fortuitous 5 of 8 match between the vector DNA in the +6/+629 construct and the SNR6 TATA-like sequence, with identical spacing from the transcription initiation site mapped by primer extension (Fig. 6b). Furthermore, immediately flanking the coding sequence is another 5 of 8 match. In the position of the 3'-PSE-like box on the 1 of 11 match is found, although a 7 of 11 match is present in the +6/+629 construct 5 bp closer to the TATA-like element, as shown in Figure 6b. Thus, although we cannot yet rule out dispensability of these upstream conserved elements, expression of the +6/+629 construct in vivo is most likely due to fortuitous sequence identity between the vector and SNR6 5'-flanking DNA.

The inability of the +6/+629 construct to be transcribed in vitro indicates clearly that the U6 promoter has been damaged significantly. Why then is in vivo expression of U6 snRNA from this template down at most only twofold? One possibility is that an upstream binding factor is present in limiting concentration in the cell-free system, but not in vivo. This would render the cell-free system more sensitive to mutations...
that decrease the affinity of upstream promoter elements for the putative factor. Alternatively, there may exist in the cell a compensatory regulatory mechanism to counteract the decreased U6 snRNA synthesis resulting from replacement of the wild-type SNR6 allele with the +6/+629 construct. At present, however, there is no evidence for such feedback regulation of U6 snRNA synthesis in any organism.

Although we have not yet determined the growth rate or temperature sensitivity of strains producing U6 snRNA from the +6/+629 construct, their viability indicates that changes in the 5' end of the RNA due to the presence of vector sequence are not grossly deleterious to function. Figure 6c compares the proposed structure of the 5'-terminal stem of +6/+629 U6 snRNA with wild type and the consensus structure for all U6 snRNAs. Although +6/+629 U6 snRNA can form the conserved CG pair and retains the CAY sequence at the base of the stem, it has an adenosine rather than the otherwise universally conserved guanosine residue at the 5' end of the stem, and lacks pairing at the first two positions in the stem. More extensive phenotypic analysis will be required to determine whether these changes from the consensus structure, or the twofold decrease in U6 snRNA level, have a detectable effect on U6 function.

Discussion

The S. cerevisiae U6 snRNA gene promoter

We demonstrated that the S. cerevisiae U6 snRNA gene has an essential promoter element positioned 120–130 bp downstream of its 3' end. The DNA sequence of this element matches closely that of the consensus pol III B block internal control element, known to be the primary binding site of TFIIIC in tRNA genes. Furthermore, deletion in the U6 downstream element of 2 bp corresponding to residues key to binding of TFIIIC by the tRNA B block destroys U6 promoter function in vitro and in vivo. It is, therefore, very likely that the role of the yeast U6 gene downstream B block is to bind TFIIIC. The results of in vitro transcription competition experiments assessing the effect of SNR6 and its 3'-deletion alleles on 5S rRNA gene transcription (Fig. 4) are consistent with binding of TFIIIC by the downstream B block. Thus, the yeast U6 gene promoter has two unexpected features: 1) It uses a conventional pol III promoter element not seen in vertebrate U6 genes, and 2) this essential promoter element is downstream of the gene, a position unprecedented in any pol III transcription unit.

The B block is the dominant element determining promoter strength in tRNA genes, but tDNA transcription also requires an A block element, which directs initiation at a site about 20 bp upstream of its 5' border. Binding of the A block by TFIIIC is probably a key step in the placement of TFIIIB upstream of tRNA genes [Kassavetis et al. 1990, and references therein]. We therefore looked for a match to the 11-bp A block consensus in SNR6. Beginning at position +21 of the S. cerevisiae U6 gene is a sequence that is identical to the A block consensus at 6 of the 9 nondegenerate positions [Fig. 7a]. Furthermore, two of the three deviations from the consensus are at sites where mutations have been found to be phenotypically neutral; only the G → T transversion at the eighth position [Fig. 7a] is known to be a promoter down mutation [Allison et al. 1983]. Therefore, it seems likely that this sequence is acting as an A block promoter element, although further mutational analysis will be required to confirm this.

The downstream location of the yeast U6 gene B block...
Figure 6. Analysis of the in vivo transcript from the +6/+629 SNR6 construct. (a) Primer extension of total RNA from strains containing the +6/+629 and -120/+246 SNR6 deletion constructs. Total RNA (15 μg of each) from the preparations used in lanes 9 and 10 of Fig. 5 were subjected to primer extension (in the presence of 2', 3'-dideoxynucleoside triphosphates) with oligonucleotide 6B. Products were displayed on a sequencing gel. The short exposure (top) shows that U6 snRNA from the +6/+629-bearing strain is one nucleotide shorter. The long exposure shows that the G at position 5 in the -120/+246 transcript (arrow) is changed to a C in the +6/+629 transcript, as expected given the substitution of SNR6 coding sequence for vector DNA. (b) Alignment of sequences upstream of the wild-type and +6/+629 alleles of the S. cerevisiae SNR6. The wavy arrows indicate the 5' end of the respective in vivo transcripts, as determined by primer extension. The dashes mark gaps introduced to optimize alignment. (c) Proposed secondary structure of the +6/+629 U6 snRNA 5'-terminal stem. The 5'-terminus of the transcript of the +6/+629 allele of SNR6 is shown in a secondary structure that most closely conforms to that of wild-type S. cerevisiae U6 snRNA (Brow and Guthrie 1988) and the consensus structure for all U6 snRNAs (Roiha et al. 1989). N indicates any nucleotide, Y, a pyrimidine, and a dash base pairing.

promoter element indicates there is not a fixed spatial relationship between the presumptive TFIIIC binding site and the sites of transcription initiation and termination. An obligatory ordering and/or proximity of these sites might have been expected given the evidence that the La protein, which is implicated in termination of transcription by mammalian pol III (Gottlieb and Steitz 1989) and which also may be present in yeast (Brow 1987), appears to be tightly associated with TFIIIC (Gottesfeld et al. 1984; Gottlieb and Steitz 1987). Thus, it has been proposed that, in an active transcription complex, the upstream side of TFIIIC binds TFIIIB and is involved in capturing pol III, whereas the downstream side binds La and facilitates termination (Gottlieb and Steitz 1989). This simple geometry is not possible for the yeast U6 gene, as the initiation and termination sites are both upstream of the B block. The proposed juxtaposition of factors and sites could be achieved, however, by extensive looping and folding of the DNA (some degree of which must be required in any case to allow binding of the putative A block by TFIIIC; Fig. 7b). If this in fact occurs, there may be a minimum allowable distance between the termination site and the downstream B block, the extent of which is determined by steric constraints to DNA folding.

The distance between the SNR6 transcription initiation site and its downstream B block (240 bp) is greater than in any other natural pol III transcription unit of which we are aware. However, artificial tRNA gene constructs with similar or even greater spacing (but in which the B block remains intragenic) retain some in vitro promoter activity (Dingermann et al. 1983; Fabrizio et al. 1987). Cannon et al. (1986) studied the effect of progressively increasing the 5'-end to B block spacing by inserting DNA between the A and B block of the adenovirus VARNA1 gene, which has a tRNA-like pol
III promoter. They found that in vitro transcription efficiency drops off sharply with 5’-end/B block separations greater than about 100 bp. Furthermore, with spacings that are greater than ~130 bp, alternative initiation sites closer to the B block are used, suggesting the large A-to-B block distance promotes the recognition of “cryptic” A blocks. A similar phenomenon may be responsible for the production of the 235–295 nucleotide transcripts from SNR6 clones in the whole-cell extract. For example, the longest of these appears to initiate approximately at position +125, just downstream of the U6 gene. Nineteen base pairs downstream of this position is a 9 of 11 match to the putative SNR6 A block (Fig. 7a). It should be noted, however, that the effect of the +87/+108 deletion on formation of this transcript (Fig. 3a) indicates that sequences upstream of the presump-
tive start site are also important for its initiation. The importance of sequences upstream of the U6 gene itself are considered below.

Implications for U6 genes in other organisms

The SNR6 promoter structure described above is remarkably similar to that of a tRNA gene, suggesting that the S. cerevisiae U6 gene is a “missing link” between classical tRNA-like pol III transcription units and the U6 genes of vertebrates, which have exclusively up-
stream promoters (Fig. 7b). It would be of interest to de-
termine when the shift from tRNA-like to upstream U6 gene promoters occurred in phylogeny. Toward this end we re-examined the published U6 gene sequences and discovered a 10 of 11 match to the B block consensus in the U6 gene from the fission yeast, Schizosaccharomyces pombe (Tani and Ohshima 1989). Remarkably, this potential B block promoter element is located in the intron (at positions +68 to +78), adjacent to and up-
stream of the UACUAAC sequence at the site of branch formation. The “extragenic” location of the B block in both yeasts may be a reflection of incompatibility of B block sequences with function of the highly conserved U6 snRNA. The S. pombe U6 gene also has an A block-
like sequence beginning at position +17. Within the class Ascomycetes, S. pombe is only distantly related to S. cerevisiae (Sogin et al. 1989). tRNA-like U6 gene pro-
motors may therefore be a general feature of this class, which encompasses most of the yeasts as well as fila-
mentous fungi.

The SNR6 upstream TATA-like element, which is highly conserved in sequence and location (~29 to ~31) among U6 genes (Fig. 7b), and the A block may collabo-
rate in specification of the U6 snRNA 5’ end. The TATA-like sequence of vertebrate U6 genes has been shown to be the sole determinant specifying their recogni-
tion by pol III rather than pol II (Mattaj et al. 1988; Lobo and Hernandez 1989). Kassavetis et al. (1989) and Braun et al. (1989) have shown that the analogous region of tRNA and 5S rRNA genes is bound by TFIIIB in a TFIIIC-dependent fashion. Although many tRNA and 5S rRNA genes do not exhibit a strong upstream sequence dependence, suggesting that TFIIIB can bind in a se-
quence-independent manner, TATA-like sequences are often present ~30 bp upstream of tRNA and 5S rRNA genes, and, in some cases, are absolutely required for promoter function (Larson et al. 1983; Selker et al. 1986; Garcia et al. 1987). Furthermore, movement of the TATA-like sequence with respect to the coding region of a Neurospora 5S rRNA gene can result in a corre-
sponding shift in the transcriptional start site (Tyler 1987).

A simple explanation of these observations is that

**Figure 7.** Model for the interaction of pol III trans-
scription factors with yeast and human U6 genes (a) The putative S. cerevisiae U6 gene A block sequence is shown below the consensus tRNA gene A block promoter element (Galli et al. 1981). Also shown is a downstream sequence with a 9 of 11 match to the putative yeast U6 A block, which may direct syn-
thesis of the 295 nucleotide downstream transcript (see text). Positions with respect to the U6 snRNA start site are indicated. (b) This highly schematic figure shows a model of transcription complex assembly on a typical tRNA gene and the yeast and human U6 genes. Genes (striped bars) with their asso-
ciated promoter elements (black bars) are drawn approximately to scale ([A] A block; [B] B block; [T/A] TATA-like sequence; [PSE] proximal sequence ele-
ment; [3’PSE] portion of human PSE conserved in yeast; [DSE] distal sequence element). The open bar in the tRNA gene indicates that the TATA-like ele-
ment is present in some, but not all, tRNA genes. Transcription factors (grey ellipses) are shown in contact with their putative binding sites. Tanaka et al. [1988] have proposed that Oct-1 binds the U2 snRNA gene DSE; the U6 DSE may bind this or another factor. No PSE-binding factor has yet been identified. See Discussion for a detailed description.
TFIIB binds all pol III transcription units 20–30 bp upstream of the start site, preferentially at a TATA-like sequence, but with little regard to sequence when a strong A block [acting at a distance via TFIIC] is present. Our data, suggesting an essential role for TFIIC in SNR6 transcription, support the notion that TFIIB binds the yeast U6 gene. How might TFIIB binding be accomplished in vertebrate U6 genes, which lack a B block and so are not expected to bind TFIIC directly? One can imagine a situation analogous to that which occurs with 5S rRNA genes, which also lack a B block. 5S rDNA binds a gene-specific transcription factor, TFIIIA, via an intragenic promoter element unrelated to the A and B blocks. Binding of TFIIIA is necessary and sufficient for association of TFIIC with the 5S rRNA gene, which in turn is necessary for the upstream binding of TFIIB [Braun et al. 1989, and references therein]. Thus, TFIIC effectively replaces the B block sequence, acting as an adaptor between 5S rDNA intragenic sequences and TFIIC. It is plausible that as yet unidentified proteins that bind sequence elements upstream of vertebrate U6 snRNA genes [e.g., the PSE; Fig. 7b] perform the same function. Alternatively, U6 genespecific upstream binding proteins could interact directly with TFIIB, as proposed by Kassavetis et al. [1990].

It is already known that transcription of the mouse U6 gene in vitro requires at least one factor not used by tRNA or 5S rRNA genes [Reddy 1988]. This is unlikely to be a DSE-binding protein as the DSE has been shown previously to be dispensable in vitro [Das et al. 1988; Kunkel and Pederson 1988]. However, a DSE-binding protein [suggested to be Oct-1 in U2 snRNA genes; Tanaka et al. 1989] may facilitate binding of a U6 PSE-binding factor in vivo [Fig. 7b]. Given the far-downstream location of the SNR6 B block, upstream binding proteins may also be required for efficient assembly of the yeast U6 gene transcription complex. In this regard, it will be interesting to determine the effect on yeast U6 synthesis of substitutions in the SNR6 upstream sequences identical to the 3'-half of the human PSE.

Very recently, Moenne et al. [1990] have reported in vitro transcription of our −120/+125 subclone of SNR6 with partially purified TFIIB and pol III. They find that transcription of this clone, which lacks the downstream B block, does not require TFIIC. Because we determined that this subclone will not complement the SNR6 disruption and is not transcribed in the whole-cell extract, the TFIIC independence observed in the purified system is unlikely to be of physiological relevance. However, the work of Moenne et al. provides further evidence in support of the involvement of TFIIB in U6 gene transcription.

The extragenic location of the yeast U6 gene B block has an important practical ramification: it is expected to greatly facilitate genetic analysis of the TFIIC–DNA interaction. Previous genetic studies on B block function have been hampered by the fact that mutation of the promoter element also results in changes in the RNA product. The in vivo phenotype therefore could not be used as a measure of promoter function, as it is also influenced by RNA stability and function. In contrast, mutations in the yeast U6 B block do not alter the coding sequence, thus interpretation of their phenotype is much more straightforward, as illustrated by our deletion analysis. Selection of mutations in TFIIC that allow its recognition of mutated U6 gene B block elements should be possible and is a promising strategy for identifying specific protein–DNA contacts.

Methods

Plasmid constructions

The yeast 5S rDNA clone [pB-1] was described previously [Brow 1987]. Yeast U6 gene-containing plasmids are named according to the location of the ends of the yeast DNA insert [with the end upstream of SNR6 first], and have the suffix “/6”. Yeast centromere-containing shuttle vectors have the prefix “pUC”. All constructs derive from pVH6, which consists of the 1357-bp EcoRV–HpaI fragment of pEP6 [Brow and Guthrie 1988] inserted in the Hinfl site of pUC118, with the EcoRV end closest to the EcoRI site in the poly linker. All the first 188 bp of the pVH6 insert correspond to the EcoRV–BamHI fragment of pBR322, derived from the original genomic clone. The remaining 1169 bp are yeast genomic DNA and are shown in Figure 2A.

Four of the SNR6 5'-deletion constructs [p-539H6, p-424H6, p-317H6, and p-225H6] were generated by exonuclease III [BRL] digestion of KpnI–BamHI-cut pVH6, followed by S1 nuclease [BRL] digestion, filling-in with the Klenow fragment of DNA polymerase I [U.S. Biochemicals], and ligation with T4 DNA ligase [U.S. Biochemicals], essentially as described by Henikoff [1984]. All retain the EcoRI site of pUC118. pCH6 [−120/+629] was constructed from p-225H6 by digestion with EcoRI and Clal, followed by filling-in with Klenow and religation. pNH6 [+/6/+629] was constructed by religation of pVH6 digested with SmaI and NruI. For the 3'-deletion constructs pCRC, pCD6, pCS6, and pCM6, the 770-bp EcoRI–HindIII fragment of pCH6 was digested further with Rsal [+145], Ddel [+246], Sbal [+312], or Msal [+387], respectively, and then ligated with EcoRI–HindIII-cut pUC118, filling-in sticky ends with the Klenow fragment of DNA polymerase I as necessary. pCC6 [−120/+235] was constructed by religation of pCH6 that had been digested with Csp45I [Promega], PstI, and S1 nuclease; pCH6ΔAB (Δ+238/+239) was constructed by the same procedure, except the PstI digestion was not done. All deletion end points were determined by dideoxynucleotide sequencing with Sequenase [USB]. The +87/+108 internal deletion of SNR6 was made by oligo-directed mutagenesis of pRB6.1 [which consists of the EcoRV–HpaI fragment of pEP6 inserted in the EcoRV site of pBluescript−], with the upstream sequences closest to the EcoRI site in the poly linker and was kindly provided by Remy Bordonne. Yeast shuttle vectors containing the various alleles of SNR6 were made by isolating the EcoRI–SphI fragment from each deletion construct and inserting it into EcoRI–SphI-digested pSE358 [related to pUN10, Elledge and Davis 1988].

In vitro transcription

The S. cerevisiae whole-cell extract was prepared according to the procedure of Klekamp and Weil [1982]. Unless specified otherwise, the 22.5-μl transcription reaction contained 40 mM HEPES [pH 7.9], 100 mM NaCl, 7 mM MgCl2, 3 mM dithio-
threitol, 200 ng of DNA and 2 μl of whole-cell extract. After incubation at 20°C for 15 min, 2.5 μl of NTP mix [0.6 mM each ATP, CTP, and UTP, and 25 μM (8 μCi) [α-32P]GTP in transcription buffer was added, and incubation continued for 45–60 min. Transcription was terminated and transcripts purified as described previously [Brow 1987].

In vivo expression

*S. cerevisiae* strains were transformed by the lithium acetate method [Ito et al. 1983] with DNA prepared by the boiling method [Holmes and Quigley 1981] from 0.3 ml of a saturated culture of *Escherichia coli*. Total RNA was extracted from cells in the presence of guanidinium thiocyanate as described by Wise et al. [1983] and electrophoresed on a 6% polyacrylamide, 7 M urea gel. The gel was stained for 30 min with 0.5 μg/ml ethidium bromide in 20 mM NaH2PO4 (pH 6.5) and photographed. RNA was transferred to a nylon membrane and the blot was hybridized with 4 x 106 cpm (Cerenkov) of 5'-α2P-labeled oligonucleotide 6B (5'-TCATCCTTATGCAGGG-3') in 20 ml as described [Brow and Guthrie 1988] except incubation with probe was for 2 days and exposure was for 6 days without intensifying screen.

Primer extension analysis

RNA was subjected to oligonucleotide-primed dideoxynucleotide sequencing according to procedure 2 of Geliebter et al. [1986] with the following modifications. Approximately 7 x 106 cpm (0.7 pmole) of oligo 6B (see above) was annealed to total RNA for 30 min at 45°C, and reverse transcription (without actinomycin D) was also carried out at 45°C for 30 min. The gel (6% polyacrylamide, 7 M urea) was exposed to Kodak XAR-5 film for 5 days without (short), or 8 days with, a lightening Plus (DuPont) intensifying screen.

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Note added in proof

Sequence data described in this paper have been submitted to the EMBL/GenBank Data Libraries.

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