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Human and human–yeast chimeric U6 snRNA genes identify structural elements required for expression in yeast

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ABSTRACT

U6 is the most highly conserved spliceosomal snRNA. Previous mutational studies have shown that the majority of essential residues in U6 are located in a region of 35 nucleotides encompassing a conserved hexanucleotide and stem I and stem II of the U4-interaction domain. Although the yeast and human U6 RNAs are 80% identical in this region, the human U6 gene cannot functionally replace the yeast gene *in vivo*. The human gene is not transcribed when placed in the context of yeast flanking sequences. Transcription of the human gene, but not its function, can be stimulated by the introduction of an A block promoter element in the U6 coding region. Using a set of human-yeast chimeras, we show that the 5′ domain and the 3′ terminal region of the human U6 gene can each functionally replace the corresponding yeast domains. However, a combination of both domains in a single molecule is lethal. The basis of the inability of the human U6 snRNA to function in yeast cells is discussed.

INTRODUCTION

The removal of nuclear introns is mediated by the interaction of the precursor mRNA with four snRNPs and additional factors in a complex called the spliceosome (1–4). The assembly of the spliceosome is a stepwise process, which is similar in yeast and mammalian cells (5–7). Both the snRNAs and the protein components have been conserved during evolution. Indeed, microinjection studies in Xenopus oocytes show that fungal and plant snRNAs are capable of assembling with stockpiled snRNP proteins to form snRNP-like particles (8, 9). These hybrid particles are immunoprecipitable by human autoantibodies of the Sm type. The human U1 and U2 snRNAs are not only assembled into snRNPs but the complexes also function in the splicing of SV40 late pre-mRNA (10). Moreover, it has been shown that the human U2 snRNA can complement a lethal deletion of the yeast U2 gene, arguing that the human snRNA is assembled into a functional yeast spliceosome (11, 12); this result is particularly significant because the yeast U2 snRNA is 6 times larger than its human counterpart (13). The difference in size is largely due to non-conserved regions which can be deleted without affecting function (14, 15). Such non-conserved regions also account for the size differences between the yeast and mammalian U1 and U5 snRNAs (16). Most non-conserved regions of both yeast U1 and U5 snRNAs can be deleted without disrupting snRNA function and both yeast snRNAs can adopt an overall secondary structure equivalent to their human counterparts (17–21).

In contrast to the U1, U2 and U5 snRNAs, the U4/U6 snRNAs are similar in size among species, with U6 being the most conserved snRNA (16, 22). The extensive conservation of U6 RNA and the presence of an intron in the U6 gene from *S. pombe* (23) led to the model that U6 is involved in the catalysis of pre-mRNA splicing and that U4 RNA acts as a negative regulator of U6 activity (16, 24). *In vitro* reconstitution experiments and *in vivo* structure/function analyses using numerous U4 and U6 mutants in both yeast and metazoan systems lend support for this model and indicate that two regions of the U6 molecule are particularly sensitive to mutations: a conserved hexanucleotide ACAGAG located in the central domain of the U6 snRNA and residues encompassing stem I and stem II in the interaction domain (25–28). These studies show that single substitutions in the highly conserved hexanucleotide and in stem I specifically block splicing either at the first or the second step of the splicing reaction (26, 27). Interestingly, the positions that exhibit an inhibition of the second step of the splicing reaction correspond to the location of introns in the *S. pombe* and *R. dacryoidum* U6 genes (23, 29), consistent with the suggestion that these introns inserted into regions of U6 close to the active site of the spliceosome. The mutational analyses also indicate that base-pairing in stem II of the interaction domain is required for U4/U6 assembly but is not sufficient for function (28, 30). In contrast, base-pairing in at least certain positions of stem I is not required for splicing: indeed, U4 mutants in this region are fully viable whereas mutations in the U6 side of the helix are lethal (27, 28). Since compensatory mutations in stem I do not restore function, these results suggest that U6 has an important role in addition to base-pairing with U4 snRNA, perhaps in the catalytic event itself.

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Due to the high conservation in size and sequence between the human and the yeast U6 snRNAs, we anticipated that the human U6 snRNA would be functional when transformed into yeast, if it could be properly expressed. *In vivo*, the yeast U6 gene requires, for its transcription by RNA polymerase III, a B block promoter element located 120 bp downstream of the 3′-end of the coding region (31). Since the transcription of the human U6 gene by RNA polymerase III in mammalian cells does not require tRNA gene-like A and B block promoter elements (32−35), we predicted that the human U6 gene would be expressed if placed in the context of the yeast U6 promoter and terminator. This construct was transformed into a yeast strain carrying a disruption of the chromosomal copy of the U6 gene. Surprisingly, these transformants are inviable. We determined that the human U6 gene exhibits a transcriptional defect. Transcription of the human U6 gene can be greatly stimulated by the introduction of an A block RNA polymerase III promoter consensus element in the 5′ region of the coding sequence. However, this modified human gene still fails to complement a deletion of the yeast gene. By constructing different human-yeast U6 snRNA chimeras, we show that the 5′ domain and 3′ region of the human U6 RNA can replace the yeast counterparts whereas a combination of both domains in a chimeric RNA is lethal.

MATERIALS AND METHODS

Strains and yeast methods

The yeast strain YHM1 (*MAT* a *ura3 his3 lys2 trp1 leu2 snr6::LEU2* YCp50-*SNR6*) has been described previously (27). Media and methods for yeast propagation are as described (36). *S. cerevisiae* strains were transformed by the lithium acetate method (37) and the phenotypes of the mutant constructions were determined using the plasmid shuffle method (38).

Oligonucleotides

HY3′: 5′-AAATATGGAGATCTTCACGAA;
HY5′h: 5′-AGCGAGCACACATGCATGCTTTTCTCC
HY5′y: 5′-CGAACACATGCATGCGAAAAAAAC
Abh: 5′-CGTTCCAATTTTAGCCAATGTGCCACCGAAGCGAGCACAC
Hu-Bcl: 5′-GGCCATGCTGATCATCTCTGTATCG
6−3′m: 5′-AAAAAAAAAATATGGAACGCTTCACGAATTTGCGTGTCATCCTTA
6Δ3′: 5′-CACTCCGATGATAAAAAAAAAACGGTTCATCCTTATGCAGG
H6: 5′-AAAATATGGAACGCTTCAC
6B: 5′-TCATCCTTATGCAGGG
6D: 5′-AAAACGAAATAAATCTCTTTG

Plasmid constructions

A 1.4 Kb EcoRV-HpaI fragment containing the yeast wild type U6 gene (22) was subcloned into the EcoRV site of pBluescript (−) with the upstream sequence closest to the EcoRI site in the polylinker. Oligonucleotide mutagenesis was performed as described (39, 40).

Plasmids pY3′H and pYΔ3′ were obtained by site directed mutagenesis using oligonucleotides 6−3′m and 6Δ3′, respectively. During mutagenesis with oligonucleotide 6−3′m, an insertion of two additional T residues occured in the stretch of ten T residues located at the 3′ end of the U6 coding region. As determined by our study, this has no effect on transcription termination of the U6 genes carried by pY3′H and derived plasmids.

The human U6 'swap' was constructed as follows. An SphI site was introduced in the 5′ flanking region of pY3′H by mutation of nucleotides ACT (positions −7 to −5) to TGC using oligonucleotide HY5′y. A BglII site was created in the 3′ region of the U6 gene carried by plasmid pY3′H by mutating nucleotides CGT (equivalent to positions +102 to +104 in fig.1) to ATC, using oligonucleotide HY3′. The plasmid carrying both of those changes was named pY3′H_{SB}. A 350 base-pair AluI fragment (carried by a BamH1-EcoRI subclone) containing the mouse U6 gene (33) was cloned into the BamH1-EcoRI site of pBluescript (−). After preparation of single-stranded DNA, a SphI site was created in the 5′ coding sequence (positions −7 to −5) of the mouse gene with oligonucleotide HY5′h. This oligonucleotide allows also the replacement of the mouse 5′ adjacent sequences (positions −4 to −1) with the corresponding yeast U6 gene flanking sequences. In addition, a BglII site was introduced at residues corresponding to positions +102 to +104 in the mouse U6 gene using oligonucleotide HY3′. The mouse U6 gene

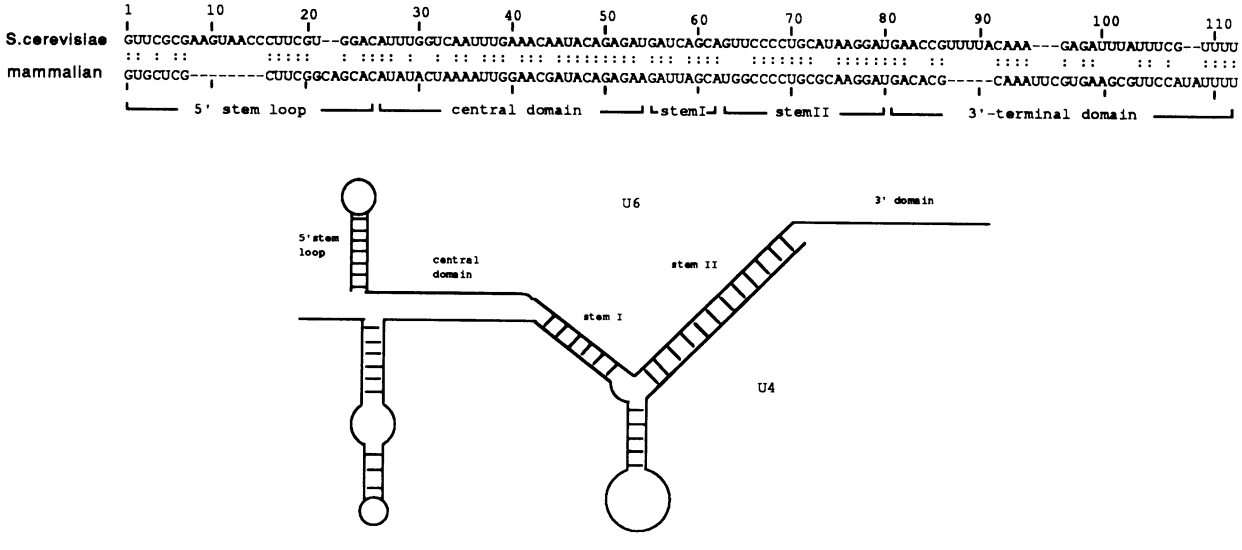

Figure 1. Comparison of the *S. cerevisiae* (22) and human (34) U6 snRNA sequences. The nucleotide position numbers refer to the yeast sequence. Gaps have been introduced to allow maximum sequence identity (22). A schematic drawing of U4/U6 RNA secondary structure is also presented.

carrying the SphI and BglII sites was named pM-U6SB. Plasmid pHu was constructed by replacing the small SphI-BglII fragment of pY3′H_{SB} with the corresponding fragment from pM-U6SB.

Plasmid pAHu was obtained by site-directed mutagenesis using single stranded DNA isolated from plasmid pHu and oligonucleotide Abh. This oligonucleotide allows the introduction of an A block consensus sequence in the 5′ coding region of the mamalian U6 gene (see fig.4A). Plasmid pAHu-1 was constructed by mutations of the A (position 54 in Fig. 1) and the T (position 58) of pAHu to T and C, respectively, using oligonucleotide Hu-Bcl. These changes create a BclI site in the U6 coding sequence. Plasmid pAHu-2 was constructed by replacing the small BclI-KpnI fragment of pAHu-1 with the corresponding BclI-KpnI fragment from plasmid pY3′H. Plasmid pAHu-Y was created by substituting the small BclI-KpnI fragment of pAHu-1 with the large BclI-KpnI fragment from plasmid pYU6 carrying the wild type yeast U6 gene.

The different DNA restriction fragments were isolated on low melting agarose gel (FMC Biochemicals) and ligation performed according to (41). All constructions were made in a Bluescript(−) (Stratagene) background and the mutant genes were subcloned into pSE358 (CEN, ARS, TRP1, AmpR) which is a derivative of pUN10 (42). The coding region sequences of all constructs were determined by dideoxynucleotide sequencing using Sequenase (U.S. Biochemicals).

RNA isolation, Northern blot analysis and primer extension

Total yeast RNA was prepared using the guanidium thiocyanate method (43). Electrophoresis, transfer and hybridization conditions were performed as described previously (44). Primer extension analysis of total RNA was performed using oligonucleotide-primed dideoxynucleotide sequencing (45).

RESULTS

The human 3′-terminal domain can replace the yeast 3′ region

As a first step in designing the expression of the human U6 gene in yeast, we engineered an interspecies chimera carrying the body of the yeast U6 RNA (positions 1 to 80 in fig.1) and the 3′ domain of the human U6 gene (positions 81 to 112). A yeast U6 snRNA mutant deleted from positions 87 to 108 was also constructed to determine the functional requirement of this 3′ domain. A schematic drawing of both constructs (pY3′H and pYΔ3′, respectively) is shown in fig.2A. The U6 mutants were subcloned into the vector pSE358 and their phenotype was determined by streaking cells onto plates containing 5-fluoroorotic acid (5-FOA) (38). Since 5-FOA selects for cells having lost the URA3 plasmid, the phenotype of cells on this media will be due to the U6 mutant genes. A deletion of the yeast U6 snRNA sequences encompassing nucleotides 87 to 108 is lethal, whereas the replacement of this domain with the corresponding human sequence allows yeast to grow in the absence of a wild type gene at all temperatures tested. The 3′ substitution mutant strain may have a slight cold sensitive phenotype based on a smaller size of colonies after several days of growth at 18°C.

Northern analyses of cells carrying the pY3′H plasmid as the sole copy of the U6 gene indicate that the chimeric RNA is found at a level similar to that found for the wild type yeast U6 snRNA (fig.2B, compare lanes 2 and 3). In contrast, the amount of the 3′ deletion mutant is decreased by approximately 5-fold compared to wild type U6 snRNA in a heterozygous strain (Fig.2B, lane 4). This is unlikely to be due to a transcriptional defect since in vitro transcription of the 3′ deletion mutant gene using a homologous transcription extract yields fourfold more RNA than the wild type yeast U6 gene (31).

Expression of the human U6 gene in yeast

To construct a total swap of the yeast U6 gene with the human counterpart, SphI and BglII restriction sites were introduced by site directed mutagenesis in the 5′ flanking region and 3′ terminal domain, respectively, of the U6 gene carried by the pY3′H plasmid (for more details, see Materials and Methods). The resulting plasmid was named pY3′H_{SB}. The same sites were also introduced in the 5′ flanking sequence and 3′ domain of a mouse U6 gene carried by a 350 base-pair AluI fragment (kindly provided by R. Reddy; sequence as in 33). We have previously shown that introduction of an SphI site at positions −7 to −5 in the 5′ flanking region of the yeast wild type gene has no effect on *in vivo* function of the U6 gene (27). The 110 bp SphI-BglII fragment carrying the mouse U6 gene was cloned into the large

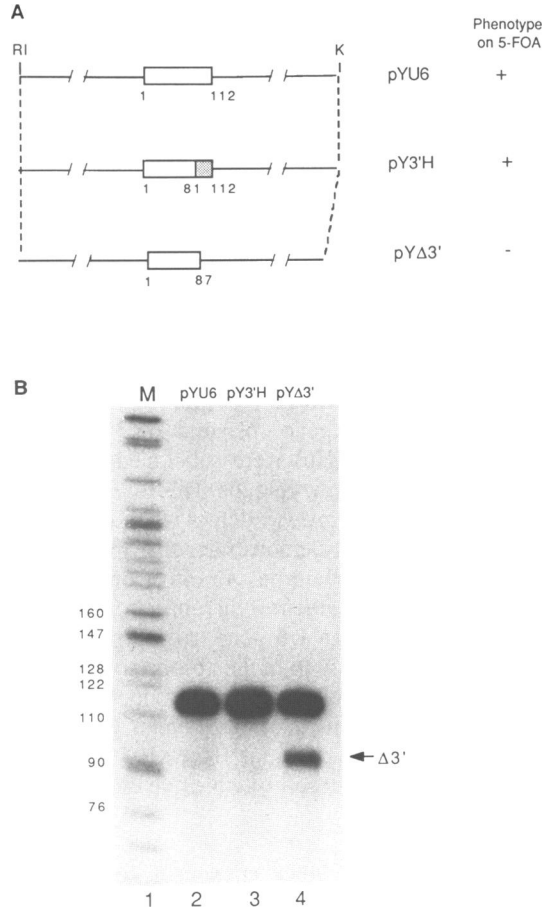

Figure 2. Expression of U6 mutants. (A) The schematic of the constructs and the ability (+) or inability (−) of the mutant strains to grow on medium containing 5-FOA are indicated. The white rectangles denote the yeast coding sequences and the stippled area represents the human sequence. The numbers refer to the sequence shown in fig.1. Restriction sites noted are EcoRI (RI), KpnI (K). (B) Northern blot analysis on total RNA isolated either from an homozygous strain carrying only the pY3′H plasmid (lane 3) or from a heterozygous strain carrying both a yeast wild type U6 gene and the pYΔ3′ plasmid (lane 4). Lane 2 represents total RNA prepared from a wild type strain carrying the pYU6 plasmid and the vector pSE358. Hybridization was performed with oligonucleotide 6B complementary to residues 67−82 of yeast U6 snRNA. M: labeled HpaII pBR325 fragments.

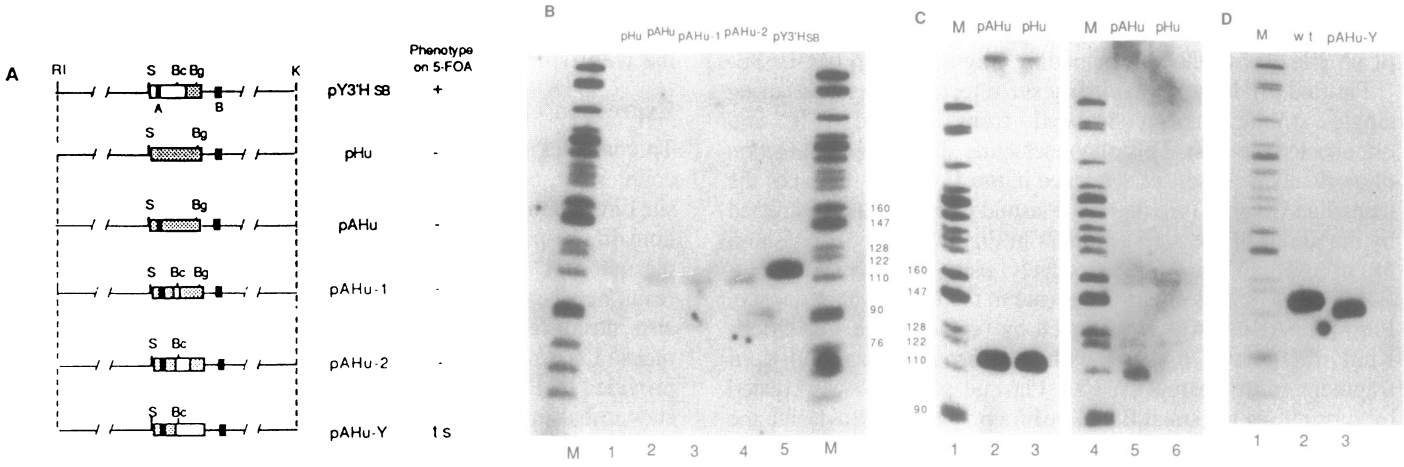

Figure 3. Expression of human U6 and human-yeast chimeras. (A) Schematic drawing of the constructs used (for construction details, see text and Materials and Methods). The white and stippled rectangles indicate yeast and human sequences, respectively. The black boxes represent the A and B block promoter elements. Restriction sites noted are SphI (S), BclI (Bc), BglII (Bg), EcoRI (RI), KpnI (K). The viability (+) or non-viability (−) of the mutant strains on medium containing 5-FOA is indicated at right. ts=temperature sensitive. (B) Northern analysis of total RNA isolated from heterozygous strains carrying a wild type U6 gene and the mutant gene indicated above each lane. Hybridization was done using a mixture of ^{32}P-labeled oligonucleotide HY3′ complementary to the human U6 RNA 3′ domain containing a BglII site and H6 complementary to the wild type human 3′ domain in pAHu-2. Equivalent amounts (10 mg) of RNA were loaded in each lane as determined by the intensity of tRNA bands on an ethidium bromide-stained gel and by the intensity of wild type U6 RNA after hybridization of a blot with oligonucleotide 6D (data not shown). (C) Northern analysis of RNA isolated from heterozygous strains carrying plasmids pAHu and pHu. The blots were probed with oligonucleotide 6B complementary to yeast U6 RNA (left panel) and oligonucleotide HY3′ complementary to human U6 RNA (right panel). (D) Northern blot of total RNA isolated from a wild type strain (lane 2) and from a homozygous 5-FOA resistant strain carrying the pAHu-Y plasmid (lane 3). Hybridization was done using oligonucleotide 6B.

SphI-BglII fragment isolated from the pY3′H_{SB} construct to yield plasmid pHu (Fig.3A). Since the mouse U6 and the human U6 snRNAs are identical in sequence (33, 34), the construct pHu carries a human coding sequence in the context of the yeast flanking sequences. The 1.4 Kb EcoRI-KpnI fragment containing the chimeric yeast-human gene (plasmid pY3′H_{SB}) and the human U6 gene (plasmid pHu) were subcloned into the yeast vector pSE358. Yeast cells carrying the pY3′H_{SB} plasmid as the sole source of U6 have no growth defect when compared to a wild type strain. This result demonstrates that the introduction of a BglII site in the 3′ domain has no effect on the ability of the chimeric gene to complement a disruption of the yeast U6 gene. In contrast, the human U6 gene carried by the plasmid pHu is unable to support growth in the absence of the wild type U6 gene. This defect is not due to the introduction of a BglII site in the 3′ terminal domain of the human gene since the restoration of nucleotides at positions 102 to 104 to human wild type sequences does not restore growth of yeast cells (data not shown).

The introduction of an A block consensus sequence in the human gene stimulates its transcription

To determine the defect responsible for the lethality of the human U6 gene, we performed Northern analysis on heterozygous cells carrying both the wild type U6 gene and the plasmid pHu. As shown in fig.3B (lane 1) and 3C (lane 6), no human RNA can be found when the blots are hybridized with a human specific probe. The lack of human U6 RNA in the heterozygous cells can be explained either by RNA instability or by a transcriptional defect (or both). The second possibility would be intriguing since the human U6 gene is presumably under control of the yeast U6 promoter and terminator. Moreover, as described in the Introduction, it has been reported that no internal coding

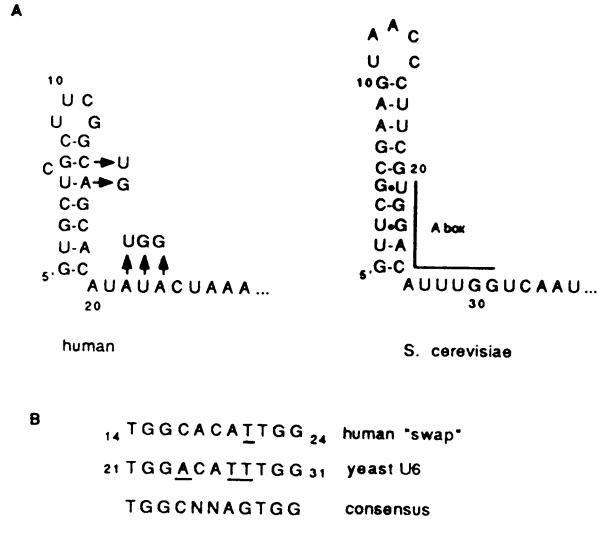

Figure 4. (A) Secondary structure of the 5′ stem loops from human and *S. cerevisiae*. The mutations introduced in the 5′ region of the human U6 gene to obtain an A box motif are indicated. (B) Comparison of the A block sequences present in the human U6 gene 'swap' (construct pAHu) and the yeast wild type U6 gene (construct pYU6) with the tRNA gene consensus sequence (31, 47). The nucleotides which differ from the consensus sequence are underlined.

sequences are necessary for the transcription of the human U6 gene. However, a recent study (31) reports the existence, in the 5′ portion of the yeast U6 gene, of an RNA polymerase III A block consensus sequence (positions 21−31, see Fig. 4). Examination of the human U6 coding region reveals no similar sequence. To test the hypothesis that the defect of the human gene is transcriptional, we introduced an A block consensus

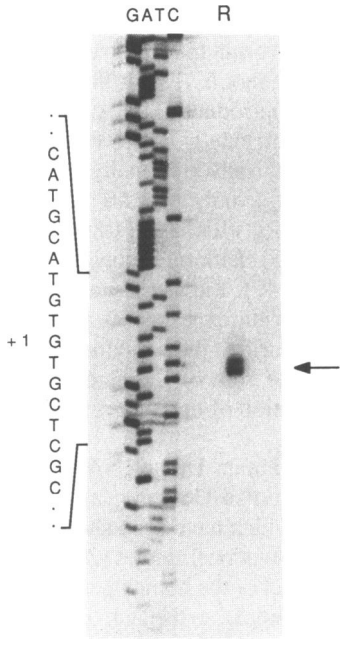

Figure 5. Primer extension analysis of the 5′ end of the temperature sensitive human-yeast U6 chimera. Total RNA isolated from homozygous strain carrying only the pAHu-Y plasmid was subjected to primer extension with ^{32}P-labeled oligonucleotide 6D complementary to residues 92−112 of yeast U6 snRNA. The DNA sequence of the coding region of the U6 chimera was performed with non-labeled oligonucleotide 6D and ^{35}S-dATP and was loaded on the gel beside the RNA primer extension reaction (lane R). The start site of the chimeric RNA, indicated by an arrow, corresponds to residue G at position +1 in the human 5′ domain (Fig.4A). The sequence shown corresponds to the RNA coding sequence.

sequence in the human U6 gene by mutating five positions in its 5′ region as shown in fig.4A. When the resulting plasmid, named pAHu (see fig.3A) is transformed into yeast, no complementation is observed. However, Northern analyses performed on RNA isolated from heterozygous strains carrying both yeast wild type gene and the pAHu plasmid indicate that human U6 RNA can be detected (fig.3B, lane 2; fig.3C, lane 5). When the same RNA samples are fractionated on a parallel gel, transfered to a nylon membrane and the blot probed with oligonucleotide 6B complementary to yeast U6 RNA, equivalent levels of wild type transcripts are found in each lane (fig. 3C, left panel, lanes 2 and 3). This result shows that the absence of human U6 transcript in lane pHu and its presence in lane pAHu are not due to differences in the amounts of RNA loaded in each lane. We can not rule out the possibility that the human U6 gene carried by the pHu plasmid produces unstable RNA, and that the mutations creating an A block in the 5′ stem loop allow an increased stability of the human RNA. This hypothesis is unlikely, however, since the secondary structure of the 5′ stem loop of the human gene carried by plasmid pHu is expected to be, at least, as stable as the 5′ stem loop structure of the RNA carried by plasmid pAHu (Fig.4A). These results suggest that the presence of an A block in the human U6 coding sequence is required for transcription of the gene by the yeast transcription machinery, in the context of yeast 5′ and 3′ adjacent sequences. Similar conclusions on the transcription of the human U6 gene have been obtained when plasmid pHu and pAHu are used in a yeast *in vitro* transcription system (M. Kaiser and D. Brow, personal communication).

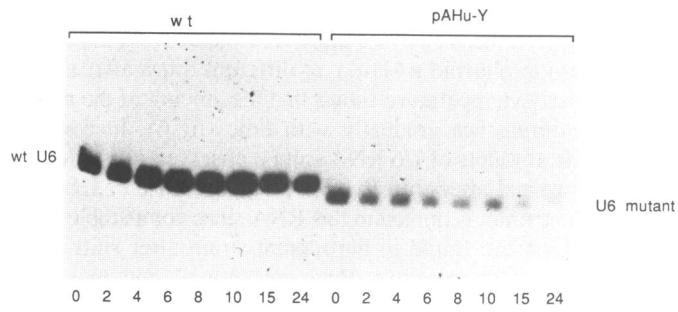

Figure 6. Effect of temperature shift on U6 snRNA level. Total RNA was prepared from strains carrying either the plasmid pAHu-Y or the wild type U6 gene at various times after shift at 37°C. 10 μg of RNA was then fractionated on 6% polyacrylamide/urea gel and transfered to nylon membrane. Hybridization was performed with oligonucleotide 6B. The strains and the time points (in hours) are indicated at the top and below the panels, respectively.

Defective domains of the human U6 RNA

An explanation for the functional defect of the human U6 gene carried by plasmid pAHu is that the RNA is unable to associate with the yeast U4 snRNA due to the reduced sequence complementarity in stem I and stem II of the interaction domain. Indeed, by examining the sequence of the human and yeast U6 snRNAs in this region, we observe that they differ at one position in stem I (residue 58) and five positions in stem II (residues 63−65 and 73−74) (fig.1). These substitutions are expected to result in the loss of six hydrogen bonds between U4 and U6. To test whether these differences in the interaction domain are responsible for the defect of the human U6 RNA, we restored the yeast sequences either in stem I or in both stems. The details for construction of plasmid pAHu-1, carrying yeast stem I sequences and plasmid pAHu-2, carrying yeast stem I and stem II sequences are described in Materials and Methods. A schematic representation of both plasmids is shown in Fig. 3A. Both plasmids are unable to support yeast growth suggesting that the defect of the human U6 gene in yeast can not simply be explained by the differences in stem I and stem II of the interaction domain. Northern analyses shown in fig.3B indicate that the steady state level of the mutant RNAs found in the respective heterozygous strains are equivalent but are significantly lower than the RNA level found in a heterozygous strain carrying the viable pY3′H_{SB} plasmid. This observation may reflect less efficient assembly of the human and chimeric RNAs into snRNPs particles.

To define the cause of the defect of the human U6 gene in yeast, we constructed an additional hybrid carrying the 5′ stem-loop and the central domain of the human U6 gene (positions 1 to 53 in fig.1) and the yeast U6 gene sequences encompassing stem I, stem II and the 3′ terminal domain (positions 54 to 112). This construct was named pAHu-Y (fig.3A). When transformed into yeast, this plasmid can support yeast growth at 18°C and 30°, but not at 37°C. Northern analyses performed on RNA isolated from homozygous cells carrying only plasmid pAHu-Y grown at permissive temperature shows that steady state level of the mutant RNA is twofold lower than its wild type counterpart (Fig.3D, lane 3). The RNA has the expected size of about 106 nucleotides, suggesting that the hybrid RNA possesses the correct 5′ and 3′ ends. As determined by primer extension analysis (fig.5), the 5′ end of this human-yeast chimera abuts precisely the G corresponding to the transcriptional initiation site. The doublet observed in lane R may represent a primer extension

artefact and is also observed for a wild type U6 snRNA (data not shown). Northern analyses on RNA isolated from the mutant strain carrying plasmid pAHu-Y at different times after shift at non-permissive temperature shows that the amount of the mutant U6 RNA diminishes gradually with time (fig.6). In contrast, comparable amounts of U6 RNA can be observed in a wild type strain before and after shift to 37°C. The decrease of U6 RNA in the mutant strain is limited to this RNA since comparable levels of U5 snRNA are found in the mutant strain after shift to the non-permissive temperature (data not shown) and may be a consequence of the inability of the mutant RNA to form a stable snRNP.

DISCUSSION

We have analysed essential domains of the yeast U6 snRNA by the use of interspecies chimeras and by a total swap of the yeast U6 gene with the human counterpart. Our data provide several new insights on structural elements required for expression of the yeast U6 gene.

Unlike most genes (5S rRNA, tRNA) transcribed by RNA polymerase III, it has been shown that the mammalian U6 gene does not require intragenic promotor elements, since the entire coding region of the U6 gene can be removed without any effect on the efficiency of transcription both *in vivo* and *in vitro* (33, 35). Transcription of vertebrate U6 genes requires a combination of upstream promoter and control elements (PSE, DSE and TATA-like box) similar to those involved in polymerase II transcribed genes (46). A different picture has emerged in yeast, since it has been shown that a B block promoter element, located 120 bp downstream of the 3′ end of the U6 gene is essential for transcription by RNA polymerase III (31). These authors also pointed out that a sequence resembling an A block consensus was located in the 5′ region of the U6 gene and might be involved in transcription of the yeast U6 gene *in vivo*. Here, we present evidence that this A block promoter element plays an important role for efficient transcription of the U6 gene *in vivo*. First, we showed that a human U6 gene, placed in the context of yeast promoter and terminator, is not stably expressed in yeast; subsequent introduction of an A block consensus sequence in the U6 coding region stimulated RNA formation to detectable levels. It is interesting to note that a chimeric RNA carrying the 5′ stem loop of *S. pombe* and the body of yeast U6 is functionally expressed in yeast (27), although the replacement of the yeast 5′ stem loop in this chimera (named U6−5′Sp) disrupts the A block sequence TGGACACTGG (positions +21 to +31). The successful expression of the U6−5′Sp RNA can be explained by the presence in the central domain of the *S. cerevisisae* U6 gene of a second A block-like motif with the sequence TGGT-CAATTTG (positions +29 to +39 in fig.1). This sequence possesses 8 nucleotides in common with the tRNA gene A block consensus sequence (47) and is thus likely to replace the motif located in the U6 wild type 5′ region for transcription of the chimeric U6−5′Sp gene. Notably, the U6−5′Sp RNA is found at 5 fold lower levels than wild type U6 in a heterozygous strain (27). This difference in the RNA level could reflect competition between both genes for limited transcription factors binding to the A block element.

Although A and B block promoter motifs are also found in the U6 gene from *S. pombe* (23, 31), it is not known if those elements are essential for transcription. Recently, it has been shown that the U6 gene from *S. pombe* can be transcribed by human pol III *in vivo* and *in vitro* (48) suggesting that the the human pol III machinery can transcribe efficiently and accurately in the presence of these motifs. This is in agreement with a report (49) showing that the introduction of A and B block motifs into a Xenopus U6 gene carrying a TATA element and an enhancer motif allows efficient transcription by RNA polymerase III. However, a gene carrying only A and B blocks with no additional control elements is not efficiently transcribed, indicating an important role of the 5′ flanking sequences for transcription by RNA polymerase III (49). Further mutational studies combined with *in vitro* transcription experiments as well as protein-DNA binding studies using purified transcription factors will be required to define precisely the role of the A block and the upstream sequences in transcription of the *S. cerevisiae* U6 gene by RNA polymerase III.

The failure of the human U6 snRNA to function in yeast is surprising since the human U6 gene carried by plasmid pAHu contains all residues which have been shown to be important for function by previous mutational studies (26−28). One explanation for the functional defect of the human gene may be that the RNA is unstable due, for example, to the lack of a correct cap structure. It has been shown recently that the human U6 RNA possesses a γ-monomethyl phosphate cap (50). The capping determinants of the human U6 snRNA consist of a phylogenetically conserved 5′ stem-loop and a AUAUAC sequence following this structure (51). If the capping machinery in yeast (if indeed it exists) does not recognize the same elements, the human U6 RNA would be unstable. However, this explanation is unlikely since the human-yeast chimera carrying the 5′ stem loop and central domain of human RNA (plasmid pAHu-Y) is efficiently expressed at normal yeast growth temperature.

The defect of the human U6 snRNA and the chimeric human-yeast RNA also can not be attributed solely to the inability to base pair with the yeast U2 snRNA. Cross-linking studies in HeLa nuclear extracts suggest that a portion of the 3′ end of U6 snRNA may base pair with the 5′ region of U2 snRNA (52). Base pairing between U2 and U6 is conserved phylogenetically and may occur over 11 consecutive base-pairs in *S. cerevisiae*. It has been shown recently that base-pairing between U2 and U6 is required for efficient splicing of mammalian pre-mRNA *in vivo* (53, 54). In contrast, such an interaction between both snRNAs seems dispensable in yeast or must be more limited than that hypothesized. Indeed, by examining the structure of the human U6 snRNA and the yeast U2 snRNA, an interaction between both snRNAs would occur only over 4 consecutive base-pairs. If the disruption of interaction between the 3′ region of human U6 and the 5′ region of U2 were responsible for the inviability of cells carrying the complete human U6 gene, one would expect that the chimera with the human 3′ domain (plasmid pY3′H) would also be lethal. As shown in this study, this is not the case. The defect of the human U6 RNA is also not only a consequence of differences in the 5′ stem-loop or in the 3′ terminal domain, since we show here that the human 5′ and 3′ domains can individually replace the corresponding regions of yeast U6 without destroying the function of the U6 snRNA. These results are consistent with previous reports demonstrating that the structure of the 5′ stem-loop of U6 RNA is relatively flexible and that point mutations in the 3′ domain of yeast U6 snRNA are not deleterious to function (27, 31, 55).

An alternative explanation for the lack of function of the human U6 RNA in yeast is that it is not efficiently incorporated into a stable snRNP. In this regard, it has previously been shown that

the human U6 RNA is unable to complement the yeast U6 RNA in a *in vitro* reconstitution system and that the human U6 RNA does not associate with the yeast U4 snRNA to form a U4/U6 snRNP (55). Due to the very low amount of human snRNA present in the heterozygous strain, we were not able to determine by glycerol gradient centrifugation of snRNPs species (44), whether or not this snRNA associates with yeast U4 *in vivo*. But, our results clearly indicate that the inability of human U6 RNA to function in a complementation assay is not simply due to reduced complementarity in the U4/U6 interaction domain, since the restoration of the yeast sequences in both stem I and stem II in the human U6 RNA does not restore function. Taken together, these observations suggest that other element(s), such as species-specific proteins, may play an important role in the efficient formation of U6 and U4/U6 snRNPs. The use of the mutant strains described in this study combined with genetic methods such as complementation could help in the identification of such proteins.

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