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U6 small nuclear RNA is transcribed by RNA polymerase III

(cloned human U6 gene/"TATA box"/intragenic promoter/α-amanitin/La antigen)

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ABSTRACT **A DNA fragment homologous to U6 small nuclear RNA was isolated from a human genomic library and sequenced. The immediate 5'-flanking region of the U6 DNA clone had significant homology with a potential mouse U6 gene, including a "TATA box" at a position 26–29 nucleotides upstream from the transcription start site. Although this sequence element is characteristic of RNA polymerase II promoters, the U6 gene also contained a polymerase III "box A" intragenic control region and a typical run of five thymines at the 3' terminus (noncoding strand). The human U6 DNA clone was accurately transcribed in a HeLa cell S100 extract lacking polymerase II activity. U6 RNA transcription in the S100 extract was resistant to α-amanitin at 1 μg/ml but was completely inhibited at 200 μg/ml. A comparison of fingerprints of the *in vitro* transcript and of U6 RNA synthesized *in vivo* revealed sequence congruence. U6 RNA synthesis in isolated HeLa cell nuclei also displayed low sensitivity to α-amanitin, in contrast to U1 and U2 RNA transcription, which was inhibited >90% at 1 μg/ml. In addition, U6 RNA synthesized in isolated nuclei was efficiently immunoprecipitated by an antibody against the La antigen, a protein known to bind most other RNA polymerase III transcripts. These results establish that, in contrast to the polymerase II-directed transcription of mammalian genes for U1–U5 small nuclear RNAs, human U6 RNA is transcribed by RNA polymerase III.**

The U series of small nuclear RNAs (snRNAs) consists at present of eight species, U1–U8 (1–3), of which at least three, U1, U2, and U7, are cofactors for messenger RNA processing reactions (2, 4–7). Several lines of evidence indicate that U1–U5 snRNAs are transcribed by RNA polymerase II (8–16). However, U6 snRNA has features that distinguish it from other U snRNAs. It does not contain a 2,2,7-trimethylguanosine cap structure at its 5' end (17, 18), and it lacks "domain A," a single-stranded region containing the sequence RA(U)$_n$GR (R = purine nucleoside; $n > 3$) flanked by hairpin structures, which is present in U1, U2, U4, and U5 snRNAs (19).

These facts prompted us to investigate the possibility that the transcription of U6 snRNA differs from that of the other snRNAs. In this paper we report the isolation and sequencing of a human U6 snRNA gene and describe experiments demonstrating that U6 snRNA is transcribed by RNA polymerase III. (Brief accounts of this work were presented at the UCLA Symposium on Transcriptional Control Mechanisms, April 6–13, 1986, Keystone, CO, and the Cold Spring Harbor RNA Processing Meeting, May 14–18, 1986.)

MATERIALS AND METHODS

Cloning and Sequencing of a Human U6 Gene. A partial *Hae* III/*Alu* I library of human fetal liver DNA in λ phage Charon 4A (20) was screened with a ^{125}I-labeled U6 RNA probe (21, 22) using a modified *in situ* plaque hybridization protocol (23). One of several positive clones was plaque-purified and subsequently shown by restriction mapping to contain a 12-kilobase-pair (kbp) insert. A 3.7-kbp *Eco*RI fragment containing U6-hybridizing sequences was subcloned into pBR322 for further restriction mapping. An 800-base-pair (bp) DNA fragment containing U6 homologous sequences was excised using *Ava* I and inserted into the *Sma* I site of M13mp8 replicative form DNA (M13/U6) (24). These parent phage DNAs, containing either strand, and additional subclones carrying smaller inserts (25) were used as templates for dideoxy sequencing (26, 27).

***In Vitro* Transcription in a HeLa Cell S100 Extract.** HeLa cells were propagated in suspension culture as described (28). S100 extracts were prepared from exponentially growing cells (29) and stored in aliquots at −80°C. Transcription reactions were carried out in a final volume of 20 μl containing 50 μg of plasmid DNA per ml, 0.5 mM ATP, 0.5 mM UTP, 0.5 mM CTP, 25 μM unlabeled GTP, 10 μCi of [α-^{32}P]GTP (1 Ci = 37 GBq), 20 mM Hepes (pH 7.9), 75 mM KCl, 2.2 mM $MgCl_2$, 0.25 mM dithiothreitol, and 10 μl of S100 extract. Samples were incubated at 30°C for 1 hr, and nucleic acids were isolated by phenol/chloroform extraction and ethanol precipitation. Some RNA samples were selected by hybridization to 10–20 μg of M13/U6 DNA immobilized on nitrocellulose (30). RNAs were fractionated on 10% polyacrylamide/8.3 M urea gels as described (31).

The U6 plasmid DNA used for the *in vitro* transcription experiments was constructed by cloning the entire polylinker + 800-bp U6 DNA insert from M13mp8/U6 replicative form DNA into the pGEM1 vector (Promega Biotec, Madison, WI). As positive controls we used pBR322 plasmid DNA templates containing a *Xenopus laevis* tRNA^{Met} gene (pXltmet1) (32) and a *Xenopus borealis* somatic 5S RNA gene (pXbs 201) (33), kindly provided by K. Vrana and D. Brown (Carnegie Institution of Washington, Baltimore).

Transcription in Isolated Nuclei. Isolation of nuclei from HeLa cells, transcription reactions, slot hybridizations, and snRNA–ribonucleoprotein complex (snRNP) antibody immunoprecipitations were carried out as described (16). Hybrid-selection of RNA was on M13 clones of human U1 DNA (16), U2 DNA (34), U4 DNA (30), and the presently described M13/U6 DNA clone.

RESULTS

Cloning of a Human Genomic DNA Sequence Homologous to U6 RNA. A human genomic DNA library (20) was screened with human placental U6 RNA that had been labeled with ^{125}I and purified by preparative gel electrophoresis. One of several positive λ phage plaques was investigated further. A 3.7-kbp *Eco*RI DNA fragment that contained a U6 RNA-

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Abbreviations: snRNA, small nuclear RNA; snRNP, snRNA–ribonucleoprotein complex; kbp, kilobase pair(s); bp, base pair(s).

complementary sequence was then cloned into pBR322. Further subcloning of an 800-bp *Ava* I fragment (Fig. 1*a*) into M13mp8 generated the U6 DNA clone used for the experiments described in this paper.

The sequence of the clone's U6-homologous region and flanking DNA is shown in Fig. 1*b*. Comparison of the human DNA sequence homologous to U6 RNA with sequences of rat (17) and mouse (37) U6 RNAs, known to be identical (35), revealed perfect correspondence. (Human U6 RNA has not been sequenced, but it is known to have the same, or very similar, sequence as rodent U6 RNA based on the respective RNase T1 fingerprints, ref. 38; see also Fig. 2*b*.) This clone therefore appears to be an authentic gene for human U6 RNA, as opposed to a U6 pseudogene (21, 39).

It is of interest that the 5′ flanking regions of the human and mouse (36) U6 DNA clones have a high degree of sequence homology (Fig. 1*c*). In particular, there is a TATA sequence located 26–29 nucleotides upstream from the presumed U6 RNA transcription start site that is also found at position −28 to −31 in the mouse genomic clone. Although the presence of a TATA sequence in this position is characteristic of a RNA polymerase II promoter element, the results that follow show that human U6 RNA is in fact transcribed by RNA polymerase III.

U6 RNA Transcription *in Vitro* Possesses α-Amanitin Sensitivity Characteristic of RNA Polymerase III. When the cloned human U6 DNA was used as template in a HeLa cell S100 extract previously demonstrated to be nonpermissive for polymerase II transcription (29, 40), a RNA species having an electrophoretic mobility corresponding to U6 was synthesized (Fig. 2*a*, lane 6). This putative U6 RNA product was selected by hybridization to recombinant M13/U6 DNA (Fig. 2*a*, lanes 9 and 10). The transcripts produced in the HeLa S100 extract from *Xenopus* tRNA and 5S rRNA genes are shown in Fig. 2*a*, lanes 1 and 2, respectively. (Note the presence, in all reactions, of slower-migrating RNAs, lanes 1–3, 6, 7. These behaved as polymerase III transcripts, based on α-amanitin sensitivity, and were produced in reactions that contain a plasmid vector without any insert, but they were not observed when template DNA is omitted.) Transcription of the human U6 DNA was unaffected by a low concentration of α-amanitin (1 μg/ml) but was completely blocked at 200 μg/ml (Fig. 2*a*, lanes 7 and 8), the latter inhibitor concentration being that required to shut off transcription of 5S rRNA, a known polymerase III product (lanes 2–4). The comparable results for hybrid-selected U6 RNA (lanes 9–11) showed that the aforementioned α-amanitin sensitivity was that of bona fide U6 RNA. These results establish that the human U6 DNA clone is a template for accurate transcription by RNA polymerase III.

To further establish the authenticity of the U6 gene transcribed in the S100 extract, we compared fingerprints of this transcript with U6 RNA labeled with ^{32}P *in vivo*. The RNase T1 oligonucleotides of the *in vivo* U6 RNA (Fig. 2*b*) and the RNA transcribed from the cloned U6 DNA template *in vitro* (Fig. 2*c*) had the same composition as deduced from secondary analyses (see Fig. 2 legend). Pancreatic RNase fingerprints and secondary analyses of the two RNAs also indicated congruent sequences (data not shown). The fingerprint spots from the U6 RNA transcribed *in vitro* (Fig. 2*c*) that are displaced relative to their corresponding spots from *in vivo* U6 (Fig. 2*b*) are consistent with a lack of ribose 2′-*O*-methylation in the S100 extract—i.e., oligonucleotides 7, 11, 13, 14, 18a, 18b—or heterogeneity in the number of 3′-terminal uracil residues—i.e., oligonucleotides 19a–e (see Fig. 2 legend for additional details).

HeLa Cell U6 RNA Is Transcribed by RNA Polymerase III. To investigate the species of RNA polymerase responsible for U6 gene transcription in the cell, we examined the effects of α-amanitin on labeling of U6 RNA in isolated nuclei (16) and intact cells. [^{32}P]RNA synthesized in isolated nuclei was hybridized to nitrocellulose-immobilized M13 recombinant DNAs carrying inserts homologous to U1, U2, U4, or U6 snRNA. Whereas transcription of U1 and U2 RNAs was strongly inhibited in the presence of 1 μg of α-amanitin per ml, U6 synthesis was relatively unaffected (Fig. 3).

To further characterize the U6 RNA labeled in isolated nuclei it was preparatively hybridized to the M13/U6 DNA clone, eluted, and analyzed by electrophoresis. As shown in Fig. 4, lanes 5 and 6, the U6 DNA-selected RNA contained a set of two or three bands that migrated at the position of a U6 RNA marker. The extremely specific nature of the U6 DNA hybridization is illustrated by the results in lanes 9 and 10 of Fig. 4. Despite the prolonged autoradiographic exposure, the only low molecular weight RNA selected was U6.

The effects of α-amanitin on labeling of U6 RNA relative to other species are also illustrated in Fig. 4. It can be seen that U6 RNA transcription was resistant to α-amanitin at 1 μg/ml (lanes 6 and 10) but was completely inhibited at 200 μg/ml (lanes 7 and 11). The synthesis of 5S rRNA (lanes 1–3) showed a similar sensitivity to α-amanitin as U6 (lanes 4–6), again in support of the conclusion that the latter is transcribed by RNA polymerase III. Note also, at the tops of lanes 1–3, the sensitivity of larger, heterogeneous nuclear RNA to α-amanitin at only 1 μg/ml, indicating that these are products of RNA polymerase II, as expected (43).

To verify that the U6 RNA labeled by [^{32}P]GTP in isolated nuclei was indeed a transcription product, hybrid-selected, gel-purified U6 RNA was excised and digested with RNase T2. Two-dimensional thin-layer chromatography (not shown)

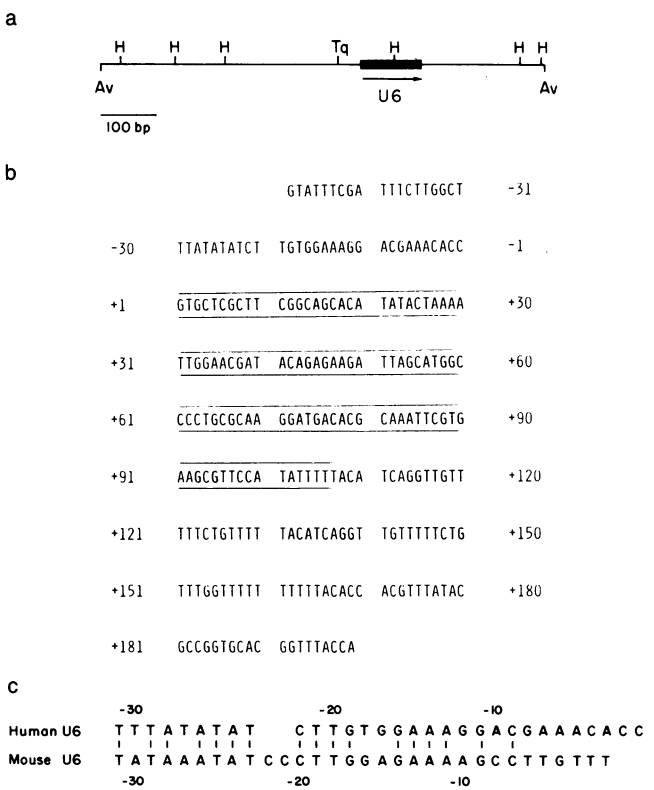

FIG. 1. Human U6 RNA gene locus. (*a*) Restriction map of the 800-bp *Ava* I fragment of human DNA showing the position and transcription orientation of the U6 gene. Av, *Ava* I; H, *Hae* III; Tq, *Taq* I. (*b*) DNA sequence of U6-homologous and flanking regions. Sequencing was carried out by using the dideoxy method on recombinant M13 templates. Nucleotides enclosed by the lines correspond to the sequence of rodent U6 RNA (35). (*c*) Comparison of the 5′ flanking region between potential U6 genes. The mouse U6 DNA-sequence is from Ohshima *et al.* (36). The sequences have been aligned for maximum homology.

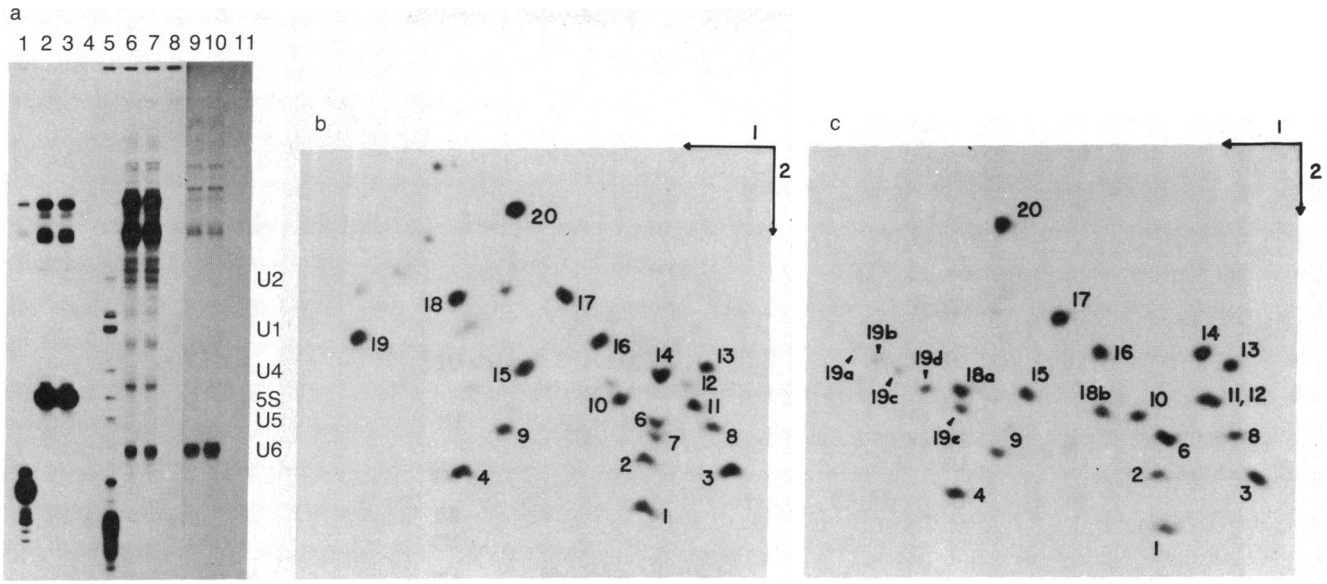

FIG. 2. U6 RNA is accurately transcribed in a HeLa S100 extract. (*a*) Transcripts synthesized during incubation in an S100 extract (29) were labeled with [α-^{32}P]GTP, isolated by phenol extraction and ethanol precipitation, and separated by electrophoresis on a 10% polyacrylamide/8.3 M urea gel. Lane 1, *X. laevis* methionyl tRNA gene (pXltmet1). Lanes 2–4, *X. borealis* 5S RNA gene (pXbs201); 0, 1, and 200 μg of α-amanitin per ml, respectively, was included in the transcription reaction mixtures. Lane 5, HeLa nuclear RNA markers labeled *in vitro* using [^{32}P]pCp and T4 RNA ligase. The migration positions of major small RNAs are indicated at the right of lane 11. Lanes 6–8, human U6 RNA gene; 0, 1, and 200 μg of α-amanitin per ml, respectively, was included in the transcription reaction mixtures. Lanes 9–11, RNAs transcribed from the human U6 template as shown in lanes 6–8 were further purified by hybrid-selection with M13/U6 DNA (30). The lanes shown are from the same gel as lanes 1–8 but result from a 12-fold longer autoradiographic exposure. (*b*) Ribonuclease T1 fingerprint of U6 RNA from HeLa cells. U6 RNA was isolated from HeLa cells labeled with [^{32}P]orthophosphate and purified by gel electrophoresis, subjected to ribonuclease T1 digestion, and separated by electrophoresis on cellulose acetate strips in the first dimension and homochromatography on polyethyleneimine cellulose plates in the second dimension (41, 42). The numbering of the oligonucleotide spots is according to Epstein *et al.* (17) based on identification of their composition by secondary analysis. They are consistent with the following sequence, with asterisks to the right of the nucleotides denoting probable sites of nucleotide methylation modifications: 1, G; 2, AG; 3, CG; 4, UG; 6, AAG; 7, A*AG; 8, CAG; 9, AUG; 10, CUCG; 11, CAA*G; 12, AACG; 13, AC*ACG; 14, C*CC*C*UG; 15, CUUCG; 16, AUACA*G; 17, CAAAUUCG; 18, AUUA*G*CAUG; 19, UUCCAUAUUUUU$_{OH}$; 20, CACAUAUACUAAAAUUG. (*c*) Ribonuclease T1 fingerprint of U6 RNA transcribed *in vitro*. RNAs transcribed in four separate reactions with each of the α-^{32}P-labeled nucleoside triphosphates were purified by gel electrophoresis, mixed, digested with ribonuclease T1 and fingerprinted as in *b* above. Some spots are displaced from their positions in *b*, probably due to a lack of methylation and to 3′-terminal heterogeneity. These are 11, CAAG; 13, ACACG; 14, CCCCUG; 18a, AUUAG; 18b, CAUG; 19c, UUCCAUAUUU$_{OH}$; 19d, UUCCAUAUU$_{OH}$; 19e, UUCCAUAU$_{OH}$. Oligonucleotides 19a and 19b could not be determined by secondary analysis but their positions relative to 19c, 19d, and 19e are consistent with 19a, UUCCAUAUUUUU$_{OH}$; 19b, UUCCAUAUUUU$_{OH}$. Oligonucleotide 7 in *b* becomes identical to oligonucleotide 6 in *c* by the lack of the *in vivo* modification. A detailed analysis of ribonuclease T1 and pancreatic ribonuclease fingerprint data of *in vivo* and *in vitro* transcribed U6 RNA is available from the authors upon request.

revealed ^{32}P radioactivity corresponding to all four nucleoside 3′-monophosphates, thus ruling out end-labeling of preexisting U6 RNA by [^{32}P]GTP in isolated nuclei.

The labeling of U6 RNA in growing HeLa cells during a short pulse with [^{3}H]uridine was also found to be relatively insensitive to an α-amanitin concentration that substantially reduced the transcription of U1. Cells were incubated for 30 min in the presence or absence of α-amanitin at 70 μg/ml and then were labeled with [^{3}H]uridine for 90 min. Whereas U1 and U2 RNA synthesis was inhibited 70% and 89%, respectively, the labeling of U6 RNA was reduced by only 37% (data not shown; see also refs. 15 and 44).

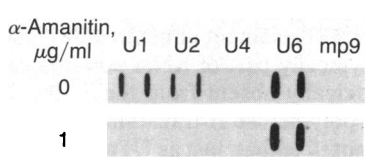

FIG. 3. Transcription in isolated nuclei. HeLa nuclei were incubated in the presence of [α-^{32}P]GTP with or without 1 μg of α-amanitin per ml. Total RNA was isolated and hybridized to recombinant M13 DNAs immobilized as slots on duplicate nitrocellulose strips. After washing, the strips were treated with RNases A and T1 (16) and exposed to preflashed x-ray film. All DNAs were loaded as duplicate samples. M13/U1 (16) and U2 (34) DNAs are subclones of human genes. U4 corresponds to a human U4 RNA pseudogene subcloned into M13 (30). The M13/U6 DNA is described in the text. Control M13 DNA containing no insert of human DNA was immobilized in the slots designated mp9.

RNA polymerase III transcripts are known to be transiently associated with a protein recognized by anti-La antibodies (45, 46). Most of the small RNAs synthesized in high abundance in isolated HeLa cell nuclei were quantitatively immunoprecipitated by a human autoantibody with La specificity (Fig. 5), in confirmation of previous results (45). When La antibody-selected RNA labeled in isolated nuclei was further purified by hybridization-selection to U6 DNA, it was found that U6 RNA was efficiently bound by the anti-La antibody (Fig. 5, lanes 5 and 6). In contrast, a monoclonal Sm antibody did not immunoprecipitate U6 RNA or any other discrete small RNA species detectable on the gel (lanes 3 and 7). The existence of newly transcribed U6 RNA in a RNP complex with the La antigen is consistent with its synthesis by RNA polymerase III.

DISCUSSION

The fact that U6 small nuclear RNA has a blocked 5′ end (17) and, like U1, U2, U4, and U5 snRNAs, is precipitated from nuclear extracts by Sm antibody (48) has made it reasonable to suppose that U6 is closely related to the other U snRNAs

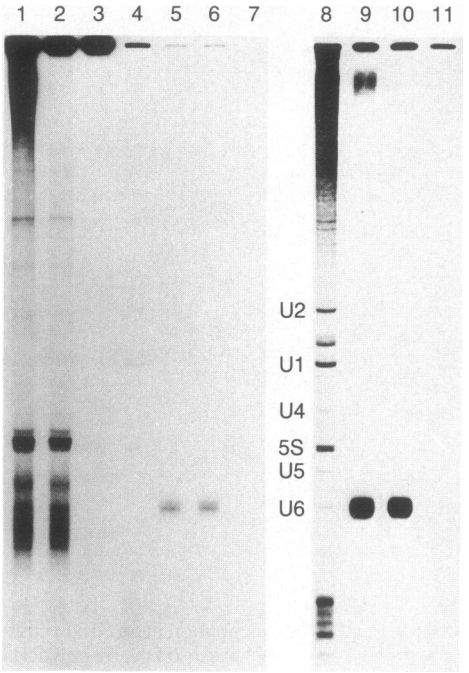

FIG. 4. Transcription of U6 RNA in isolated nuclei is sensitive to α-amanitin at 200 μg per ml. Total RNA synthesized in isolated nuclei in the presence of [α-^{32}P]GTP was isolated and electrophoresed on a 10% polyacrylamide/8.3 M urea gel before (lanes 1–3) or after selection of U6 RNA by hybridization to M13/U6 DNA (lanes 5–7 and 9–11). The gel was fluorographed to visualize [^{3}H]RNA markers in lanes 4 and 8. Lanes 1–3, total RNA synthesized in the presence of 0, 1, or 200 μg of α-amanitin per ml, respectively. Lane 4, HeLa nuclear RNA markers labeled *in vivo* with [^{3}H]uridine. Lanes 5–7, RNA selected by hybridization to M13/U6 DNA after synthesis in the presence of 0, 1, or 200 μg of α-amanitin per ml, respectively. Lanes 8–11, same as lanes 4–7 after a longer autoradiographic exposure.

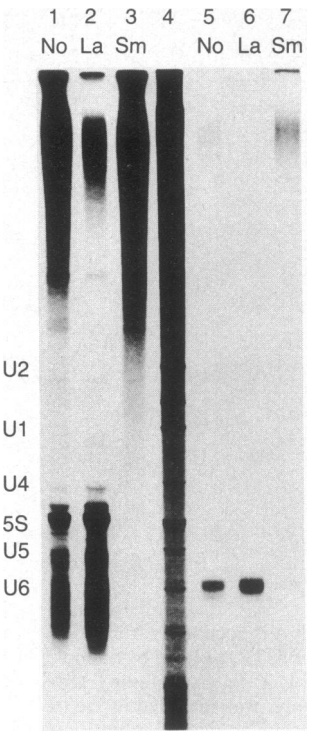

FIG. 5. U6 RNA transcribed in isolated nuclei is complexed with the La antigen. After incubation with [α-^{32}P]GTP under standard transcription conditions (16), nuclei were lysed by sonication and immunoprecipitated with La antibody or a monoclonal Sm antibody (47). Total RNA was isolated, a small portion was saved for gel analysis, and the remainder was selected by hybridization to M13/U6 DNA. RNA was fractionated by electrophoresis followed by fluorography to allow visualization of [^{3}H]RNA markers. Lanes 1–3, total RNA isolated from equal aliquots of nuclei following no antibody selection (lane 1) or following immunoprecipitations with La antibody (lane 2) or monoclonal Sm antibody (lane 3). Lane 4, HeLa nuclear RNA markers. Lanes 5–7, RNA selected by hybridization to M13/U6 DNA isolated from identical portions of nuclei following no antibody selection (lane 5), reaction with La antibody (lane 6), or reaction with monoclonal Sm antibody (lane 7).

and is synthesized by the same RNA polymerase—namely, polymerase II (8–16). The results of the present study establish that, to the contrary, U6 RNA is transcribed by RNA polymerase III. The evidence includes the transcription of a cloned human U6 gene in a HeLa cell S100 extract system that is nonpermissive for polymerase II activity, the α-amanitin sensitivity of U6 RNA transcription in the S100 system, isolated HeLa nuclei, and intact cells, and the quantitative association of newly synthesized U6 RNA with a protein, the La antigen, that has previously been shown to bind other RNA polymerase III transcripts.

The DNA sequence of the cloned human U6 gene raises interesting points. As shown in Fig. 6, from positions 46 to 59 of the U6 RNA-congruent sequence there is substantial homology with the intragenic control region of *Xenopus* 5S rRNA genes (50) and the box A consensus sequence of eukaryotic tRNA genes (51). The relative distance of the box A sequence from the 5′ end of human U6 RNA places this gene in the so-called class 2 of polymerase III genes (51), constituting the only other member at present besides 5S genes. Moreover, the 3′ end of the U6 gene is terminated by a run of thymines, again a feature of polymerase III genes that typically end with a run of four or more thymine residues (52). Interestingly, the 3′ flanking region of the U6 gene contains four additional clusters of five or more thymines (Fig. 1*b*) that may be similar to "backup" termination sites common to 5S genes (52).

By analogy with other well-characterized RNA polymerase III transcripts (51), the box A homology region of U6 RNA (nucleotides 48–59) is probably part of an intragenic control region for U6 RNA transcription. At the same time, the close homology between 5′ sequences flanking human and mouse U6 genes (Fig. 1*c*) points to the possible transcriptional importance of these upstream sequences as well. Variable results have been obtained on the role of 5′ flanking sequences in the control of polymerase III transcription. Upstream sequences do not markedly affect the efficiency of *in vitro* synthesis of several well-studied polymerase III genes (53–55). However, in other cases 5′ flanking sequences drastically modulate the level of *in vitro* transcription (ref. 56 and references cited therein; also refs. 57, 58).

An intriguing sequence in the human U6 gene's 5′ flanking region is the TATA element at nucleotides −26 to −29. This sequence is typically part of RNA polymerase II promoters but is paradoxically absent from the 5′ flanking regions of genes for the polymerase II-transcribed snRNAs U1 and U2 (59, 60). It is possible that, just as U1 and U2 genes apparently

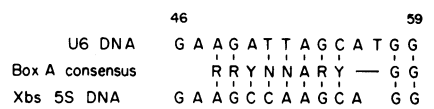

FIG. 6. U6 DNA contains a sequence homologous to the internal control region of eukaryotic tRNA genes and a *Xenopus* 5S RNA gene. The box A consensus sequence of eukaryotic tRNA genes is from ref. 51. R denotes a purine; Y, a pyrimidine; and N, an unspecified base. The 5S DNA sequence shown is that of a *X. borealis* somatic-type 5S rRNA gene (49).

represent a special class of polymerase II-transcribed RNAs, the U6 gene is distinct from the previously characterized 5S and tRNA polymerase III transcription units.

A true gene for human U6 RNA had not been isolated prior to the present study. U6 pseudogene-like sequences have been reported (21, 39), none of which shares the immediate 5′ flanking homology that we are presently noting between human and mouse U6 genes. The number of transcriptionally active U6 genes in any species or cell is unknown, but the total number of U6-homologous genomic loci has been estimated to be about 200 (21).

Transcripts of RNA polymerase III invariably have either triphosphate or monophosphate 5′ ends. The present finding that U6 RNA is transcribed by polymerase III renders its blocked 5′ terminus (17) particularly intriguing. The synthesis of conventional 7-methylguanosine caps on polymerase II transcripts is either a pre- or cotranscriptional initiation event (61). Whether the unusual 5′ structure on U6 RNA is added like polymerase II caps or reflects an entirely different reaction is of interest, as is the chemical identification of this end group.

A yeast snRNA gene has been identified that resembles a composite of U4- and U6-like RNA sequences (62). In mammals, U4 and U6 RNAs are encoded by distinct genes but nevertheless associate in heterodimers (18, 63–65). This suggests that U4 and U6 RNAs function together and raises the possibility that during evolution mammalian U4 and U6 genes have become unlinked from some ancestral module (62). The bimolecular association of these two small RNAs, transcribed by RNA polymerase II and III, respectively, raises interesting questions regarding the temporal and spatial coordination of their RNA processing, RNP assembly, and dimerization in cells.

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