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Orientation-Dependent Transcriptional Activator Upstream of a Human U2 snRNA Gene

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We examined the structure of the promoter for the human U2 snRNA gene, a strong RNA polymerase II transcription unit without an obvious TATA box. A set of 5′ deletions was constructed and assayed for the ability to direct initiation of U2 snRNA after microinjection into *Xenopus* oocytes. Sequences between positions −295 and −218 contain an activator element which stimulates accurate initiation by 20- to 50-fold, although as few as 62 base pairs of 5′ flanking sequence are sufficient to direct the accurate initiation of U2 RNA. When the activator was recloned in the proper orientation at either of two different upstream locations, the use of the normal U2 start site was stimulated. Inversion of the element destroyed the stimulation of accurate U2 initiation, but initiation at aberrant upstream start sites was enhanced by the element in both orientations. A 4-base-pair deletion that destroyed the activity of the element lies within a sequence (region III) which is highly conserved among U2 genes from different organisms. Mutations in the activator also affected the ability of the U2 template to compete with a wild-type U1 gene in coinjection experiments. We propose that the element enhances the efficiency of transcription in part by facilitating the association of a limiting factor with transcription complexes. Human U1 snRNA genes possess a region homologous to U2 region III, and we suggest that upstream activator elements may be a general feature of snRNA promoters.

The small RNAs (U1, U2, U3, U4, U5) found in the nucleus of most eucaryotic cells seem to represent a special class of RNA polymerase II transcripts. Like other RNA polymerase II transcripts, such as mRNA, snRNAs are capped, and their synthesis is inhibited by α-amanitin (8). Unlike most polymerase II transcripts, snRNAs are metabolically stable, are not polyadenylated, and contain an unusual 2,2,7-trimethyl guanosine cap structure instead of the 7-methyl guanosine cap found on mRNA (for a review, see reference 31). Most if not all snRNAs are integral components of small nuclear ribonucleoprotein particles, which have been proposed to play a role in RNA processing events in the cell nucleus (for a review, see reference 12).

The genes encoding U2 and U1, the most abundant of the snRNAs, have been cloned from a number of vertebrates, including rats (38, 41), mice (19, 28), *Xenopus laevis* (3, 16, 20, 46), and humans (18, 26, 39, 45). The genomic organization of the human U2 genes is particularly simple. The 10 to 20 U2 genes are organized in a uniform, tandem array with a 6-kilobase repeat unit. Recombinant bacteriophage containing multiple copies of the repeat unit have been isolated, and the immediate sequence environment of the gene has been determined (39, 45). All of the human U2 genes appear to be clustered at a single site on chromosome 17 (13). The 30 to 50 (15) U1 genes in the human genome share extensive flanking sequence homology at great distances from the snRNA coding region (18) and are clustered at a single site on human chromosome 1 (14, 27). Although recombinant clones containing adjacent U1 genes have not been isolated, current data suggest that the human U1 genes are arranged in a large, possibly tandem, array (L. Bernstein, V. Lindgren, U. Francke, and A. Weiner, submitted for publication).

U1 and U2 are the most abundant of the snRNAs, each present at about 10^6 copies per cell (43). The ability of only 40 to 70 U1 and U2 genes to generate this many transcripts each cell generation suggests that the promoters for these genes must be much more active than the promoters for abundant housekeeping mRNAs. Comparison of the sequences flanking snRNA genes with sequences flanking mRNA genes failed to reveal the existence of elements known to function in initiation of transcription of mRNA (e.g., TATA boxes and CAAT boxes); however, comparison of snRNA genes with each other revealed significant blocks of sequence homology that may function in initiation of transcription of these genes (26, 36, 39). Transcription of human U1 RNA in *Xenopus* oocytes requires sequences around −220 upstream of the cap site, and sequences between positions −105 and −6 upstream of the cap site are important for specifying the site of initiation in a fashion formally analogous to a TATA box (36). Our initial studies of the human U2 gene suggested, as did the work of others (44), that regions upstream of the U2 gene are also important; however, these studies did not include an internal reference gene which would allow quantitative analysis of the sequence requirements. Strangely, a region from positions −40 to −60 upstream of the U2 cap site has a striking homology to the −220 region of U1 (39), while the −220 region of U2 is homologous to a region near −85 of the U1 gene (see Fig. 1), suggesting that important sequence elements might be arranged differently in the two genes.

To obtain a more unified understanding of snRNA promoter structure, we generated a set of resections, mutations, and rearrangements upstream of the human U2 coding region and analyzed the ability of the mutant templates to direct the accumulation of human U2 snRNA after injection into *Xenopus* oocytes. In contrast to human U1 genes, only 62 nucleotides of 5′ flanking sequence are necessary to direct the synthesis of U2, although at low (2 to 5%) efficiency. We found that alteration of a region of homology between human, rat, and *Xenopus* U2 genes (region III), centered around position −220 in the human gene, is detrimental to

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efficient transcription, and impairs the ability of the template to compete with a coinjected U1 gene. This activator element has some properties of a transcriptional enhancer because it stimulates transcription from upstream sites in an orientation-independent fashion; however, it differs from classical enhancers because it stimulates correct U2 initiation only in the natural orientation.

MATERIALS AND METHODS

Enzymes. Restriction endonucleases were purchased from New England BioLabs, Inc., except *Asu*II, which was from Promega Biotec. Calf intestine alkaline phosphatase and polynucleotide kinase were purchased from Boehringer Mannheim Biochemicals. Reverse transcriptase was from Life Sciences, Inc. Large-fragment DNA polymerase (Klenow polymerase), genetically engineered to lack amino-terminal 5′ to 3′ exonuclease activity (10), was used for end polishing and dideoxy sequencing and was generously provided by Cathy Joyce. S1 nuclease was from Sigma Chemical Co.

Construction of DNA templates. All plasmids and phage were derived from a subclone containing the 896-base-pair (bp) *Stu*I-*Acc*I U2 gene fragment of λU2.24 (39). In sequential reactions, the *Stu*I end at position −556 was ligated to the filled-in *Eco*RI site, and the *Acc*I end at position +152 was ligated to the filled-in *Sal*I site of plasmid pML2d (35). This resulted in the generation of *Eco*RI and *Sal*I ends at the 5′ and 3′ ends of the transcription unit, respectively. This subclone is called *dl*-556 (see Fig. 2). We have found that the *Stu*I site used to create this clone is highly refractory to cleavage by *Stu*I unless the DNA is obtained from a *dcm*⁻ host, so in this case the parental plasmid was grown in *Escherichia coli* GM271 (from M. Marinus through the *E. coli* Genetic Stock Center, Yale University, New Haven, Conn.). All other plasmids were grown in strain HB101 or DH1, and M13 derivatives were grown in strain JM103 (17).

The *Eco*RI-*Sal*I fragment was subcloned into phages M13mp8 and M13mp9 (22) and sequenced by the dideoxy-chain termination method (34). Subclones containing portions of the insert were also sequenced, and ambiguities were resolved by electrophoresis on 7 M urea–8% polyacrylamide gels containing 25% formamide. The sequence upstream of position −295 is not considered confirmed because only the coding strand has been sequenced.

Deletions of various portions of the 5′ flanking region of the gene were generated by restriction endonuclease digestion, end polishing when necessary, and religation. For convenience, restriction sites which would generate deletions with an interesting endpoint and reconstruct an *Eco*RI site at the endpoint were used. For example, the 5′ deletions to positions −295 and −62 (*dl*-295 and *dl*-62) were constructed by partial digestion of *dl*-556 with *Ban*II and then by complete digestion with *Eco*RI and 3′ end polishing with Klenow polymerase. In the case of the *Ban*II ends, which have 3′ extensions, the 3′ to 5′ exonuclease activity of the Klenow polymerase converted the extensions to blunt ends containing a 5′ deoxycytidine residue. When this blunt end was ligated to a filled-in *Eco*RI end, an *Eco*RI site was regenerated. In like manner, the 5′ deletion to position −218 (*dl*-218) was made by using the unique *Sph*I site (see Fig. 2A). The −3 deletion was made by subcloning a *Hin*PI fragment extending from position −3 to 92 nucleotides past the 3′ end of the coding region (position +92). To provide a control for the deletion of 3′ flanking sequences, we constructed a clone containing an intact 5′ flank but with a 3′ flanking deletion to +91 by resection with exonuclease *Bal*31. The details of this construction [*dl*-556(3′+91)] will be presented elsewhere (C.-Y. Yuo, M. Ares, and A. Weiner, submitted for publication).

A construct containing a *Bam*HI decamer linker (5′-CCGGATCCGG-3′) replacing the *Sma*I fragment between positions −270 and −198 was made by religating *Sma*I-digested *dl*-556 DNA in the presence of a 10-fold molar excess of ends of unphosphorylated linker. This construct is called −270/*ins*B10/−198. In a similar way, the *Bam*HI linker was inserted in place of sequences between positions −531 and −61 after complete digestion with *Ban*II and end polishing with Klenow polymerase (−531/*ins*B10/−61). Using an unphosphorylated linker ensured that only a single copy of the linker was inserted.

The relocation and inversion of the 72-bp *Sma*I fragment were achieved by first cloning the fragment into the *Sma*I site of phage M13mp8, in both orientations. Because the *Sma*I site in the vector is bounded on one side by an *Eco*RI site and on the other by a *Bam*HI site, we could then insert the fragment between any pair of *Eco*RI and *Bam*HI sites in whichever orientation we chose. Phage mpSma2 and mpSma4 contain the *Bam*HI site adjacent to positions −198 and −270, respectively, and have an *Eco*RI site at the opposite end. The two constructs containing the *Bam*HI linkers (described above) were digested with *Eco*RI and *Bam*HI and ligated to *Eco*RI-*Bam*HI-digested replicative-form DNA from either mpSma2 or mpSma4. This generated four constructions: p2/−198 and p4/−198 with the *Sma*I fragment (plus linker) inserted at position −198 in the forward and backward orientations, respectively, and p2/−61 and p4/−61 with the *Sma*I fragment (plus linker) inserted at position −61 forward and backward, respectively (Fig. 2B).

The deletion of sequences between positions −223 and −218 (*dl*-223/-218) was accomplished by polishing the 3′ extended ends of *Sph*I-digested *dl*-556 DNA, followed by religating. The insertion of 2 bp at position −211 (*ins*2/−211) was achieved by filling in the unique *Asu*II site and religating (see Fig. 2C). This generated a new *Nru*I site. All constructions were confirmed by restriction endonuclease mapping. In the case of the 4-bp deletion *dl*-223/-218, the *Sma*I fragment containing the mutation was subcloned into phage M13mp8 and sequenced.

Microinjection of oocytes. Oocyte-positive *Xenopus laevis* females were purchased from Nasco and maintained at 20°C. To obtain oocytes, frogs were anesthetized by hypothermia, and partial ovarectomies were performed. After the operation, the frogs were kept in an isolation tank for 1 day before being returned to the colony.

Whole pieces of ovary were maintained in Parafilm-sealed plastic petri dishes at 4°C in a wetting amount of Barth H medium plus penicillin and streptomycin (7). Material stored in this way could be used for up to 1 week. Stage V and VI oocytes were dissected from pieces of the ovary with a watchmaker forceps and a platinum bacterial loop.

We injected the oocyte nuclei with 20 to 40 nl of 88 mM NaCl–10 mM Tris chloride (pH 7.5) containing 10 μCi of [α-^{32}P]rGTP (400 Ci/mmol; Amersham Corp.) and 400 ng of supercoiled DNA per μl, except where otherwise indicated. After incubation at 18 to 20°C for 16 to 28 h, surviving oocytes were homogenized by being drawn up and down with a Gilson Pipetman in 0.4 M LiCl–100 mM Tris chloride (pH 7.5)–25 mM EDTA–1% sodium dodecyl sulfate and 1 mg of proteinase K per ml (20 μl of solution per oocyte). The homogenate was incubated at 42°C for 1 h, extracted once

with water-saturated phenol and once with phenol-chloroform (1:1, vol/vol), and precipitated twice with ethanol. RNA was dissolved in 2 μl of water per oocyte and stored at −20°C.

S1 nuclease mapping. S1 protection experiments were done with end-labeled, strand-separated probes. Probes for 5′ end mapping were labeled at the 5′ ends by phosphatase treatment of the DNA, followed by labeling by kinase with [γ-^{32}P]rATP (3,000 Ci/mmol). Probes were labeled at the 3′ ends by filling in with Klenow polymerase in the presence of [α-^{32}P]dATP (3,000 Ci/mmol). About 10^4 Cerenkov cpm of end-labeled probe was hybridized to total RNA from 0.3 oocyte or from 10^4 HeLa cells. Hybridization was done for 5 h in 30 μl of 80% formamide–0.4 M NaCl–40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] (pH 6.4)–1 mM EDTA at the temperatures indicated in the figure legends. After hybridization, samples were diluted in 200 μl of S1 buffer (30 mM sodium acetate [pH 4.5], 300 mM NaCl, 3 mM $ZnSO_4$, and 10 μg of denatured salmon DNA per ml) and treated with 120 U of S1 nuclease for 1 h at 25°C. Samples were then precipitated twice with ethanol and redissolved for electrophoresis. When the RNA was also radioactively labeled, samples were treated with 5 ng of RNase A (DNase free) in 1 μl just before the addition of electrophoresis sample buffer. Protected DNA fragments were separated by electrophoresis in polyacrylamide gels containing 7 M urea.

Estimation of relative transcription efficiency. To compare different U2 templates quantitatively, we needed to control for the unavoidable variation in amount of DNA injected into the oocyte nucleus. This variation has two causes. First, the sample cannot be reliably delivered to the nucleus, and DNA injected into the cytoplasm of the oocyte is not active in transcription. Second, the volumes injected cannot be controlled accurately. For these reasons we included in our injection samples the *Xenopus borealis* somatic 5S rRNA +20 maxigene as a reference gene (33). After injection and RNA extraction, one oocyte equivalent of RNA from each sample was subjected to electrophoresis on polyacrylamide gels containing 7 M urea. After autoradiography, the 5S maxitranscripts were excised from the gel and counted. We then ran a second gel, normalizing the amount of each sample to include an equivalent amount of 5S maxitranscript. Because this amount of sample represents transcription from an equivalent amount of injected template, we assumed that any differences observed in the efficiency of transcription of the U2 template could be attributed to the mutation in the template and not to variable efficacy of injection. The amount of reference template used was small compared with the test template (1.5 versus 400 ng/μl), and although the 5S maxigene is 500 to 1,000 times more active on a molar basis than the human U2 gene, there was no detectable competition under these conditions (data not shown). To estimate relative transcription from different U2 templates, we scanned autoradiographs of normalized gel samples with a Hoefer GS300 densitometer. Because the processing of U2 from U2+10 is variable in oocytes, the sum of the signals from U2 and U2+10 was taken as an estimate of U2 transcription.

Because we do not know whether individual oocytes have the same relative capacity for transcribing 5S rRNA (an RNA polymerase III product) and human U2 (an RNA polymerase II product), we compared only samples injected on the same day and from the same pool of isolated oocytes. Each sample represented a group of at least eight surviving oocytes which were injected, incubated, pooled, and extracted together.

Primer extension analysis. The 5′ ends of U2 transcripts synthesized in oocytes were analyzed by oligodeoxynucleotide-primed synthesis of reverse transcripts. The oligonucleotide was synthesized and generously provided by Doug Black. The oligonucleotide sequence is 5′-CAGATACTACACTTG-3′ and is complementary to a region in the first loop of the proposed secondary structure of U2 (20). The 3′ end of the primer is 27 bases from the 5′ end of U2 RNA. This region is identical in human and *Xenopus* U2 RNA. The oligonucleotide was labeled at the 5′ end by kinase in the presence of [γ-^{32}P]rATP.

A normalized amount (see above) of RNA from injected oocytes was mixed with uninjected oocyte RNA so that the total amount of RNA was the same in all the reactions and each sample contained transcripts derived from the same amount of injected template. An excess of kinase-labeled primer was mixed with the RNA in 16 μl of 125 mM Tris chloride (pH 8.3)–12.5 mM $MgCl_2$–17.5 mM KCl, heated to 65°C for 5 min, incubated at 37°C for 10 min, and allowed to cool to 25°C slowly (2.5 h). Samples were chilled and made 5 mM in dithiothreitol and 0.5 mM in each deoxynucleoside triphosphate. Reverse transcriptase (1 μl) was added at 17 U per 20-μl reaction, and samples were incubated sequentially at 25°C for 10 min, at 37°C for 30 min, and at 42°C for 30 min. Reactions were terminated by the addition of 2.2 μl of 3 N KOH and incubation at 65°C for 30 min. Samples were neutralized with 4.4 μl of 4 M sodium acetate (pH 5.2). Carrier tRNA (1 μg) was added, and the samples were precipitated with ethanol, suspended in 20 μg of proteinase K per ml–25 mM EDTA (pH 8.0), and incubated for 10 min at 42°C before the addition of electrophoresis sample buffer and gel electrophoresis.

RESULTS

DNA sequence of a transcriptionally active human U2 gene. Various plasmid subclones of λU2.24 (39), as well as DNA from λU2.24 itself, direct the synthesis of the human U2 gene after injection into *Xenopus* oocytes. A plasmid clone containing 556 bp of the 5′ flanking sequence, the 188-bp U2 coding region, and 152 bp of the 3′ flanking sequence was as active as any of the larger subclones or λU2.24 itself (data not shown). Portions of the 896-bp U2 gene fragment were subcloned into phage M13mp8 and M13mp9 and sequenced by the dideoxy-chain termination technique (Fig. 1A). Computer-assisted comparison (30) of this sequence with 5′ flanking sequences of other snRNA genes identified new regions of statistically significant sequence homology. In addition to previously identified regions I and II (39), we noted two new homologies, called regions III and IV (Fig. 1B and 2A). Region III is found 220 to 270 bp upstream of the *Xenopus*, rat, and human U2 genes. Region IV is found just downstream of region III in human and rat U2 genes. A region III consensus sequence is shown in Fig. 1B. A computer search for region III in the human U1 gene HSD4 (17) revealed a significant homology much closer to the U1 cap site (Fig. 1C). In addition to these sequence homologies, we noted six copies of variations on the pentanucleotide 5′-TGAAA-3′ in the 85 bp between positions −235 and −150. Sequence features are interesting, and these were striking, but their importance cannot be judged without information about their function. We used the sequence data to locate useful and interesting sites for targeted in vitro mutagenesis. We then constructed a number of derivatives of the human U2 gene (Fig. 2; see above for details of the constructions).

Human U2 is accurately initiated in frog oocytes. To distin-

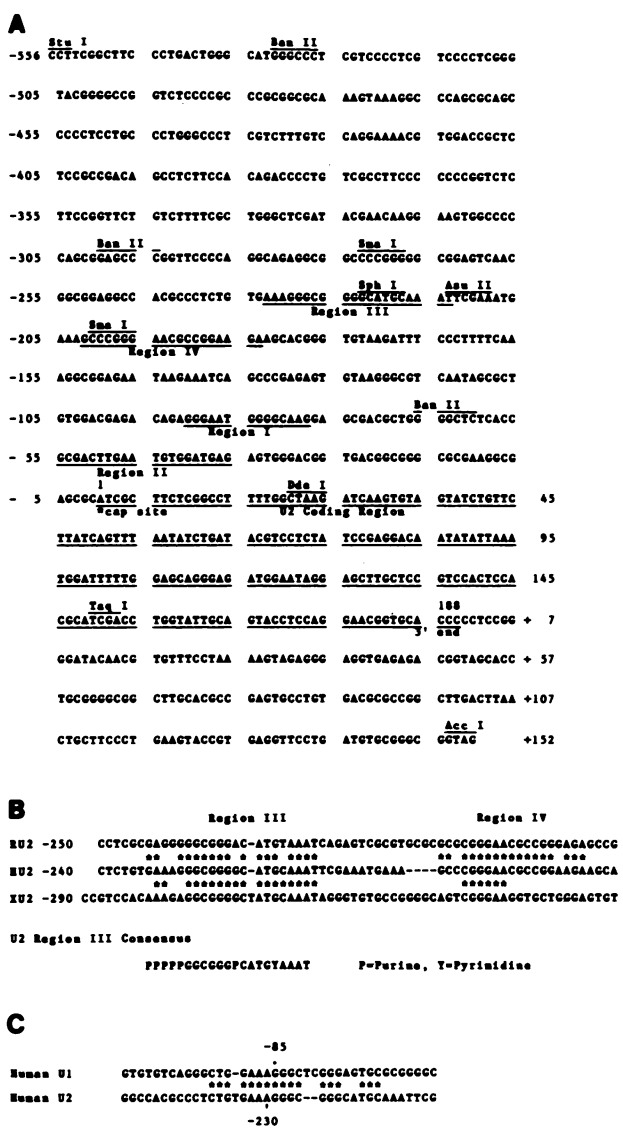

FIG. 1. Sequence and sequence comparisons of the human U2 gene. (A) DNA sequence of a functional human U2 gene. The U2 coding region is underlined, as are regions of 5′ flanking homology with other snRNA genes. Regions I and II are found far upstream in human U1 genes and closer to the cap site in human U2 genes (39). Region III is found in human, *X. laevis*, and rat U2 genes (see part B). Region IV is found in human and rat U2 genes. The U2 cap site is indicated (position 1), and restriction sites discussed in the text are overlined and labeled. (B) Comparison of the region III and region IV homologies of U2 snRNA genes from different species. RU2 is from rats (38), HU2 is from humans (this work), and XU2 is from *X. laevis* (20). The coding strand is shown. Asterisks indicate matched base pairs. Dashes have been used to maximize the match. The number to the left indicates the position relative to the cap site of the first base pair in each of the sequences. A U2 region III consensus is shown. (C) Region III homology in a human U1 gene. The sequence used in the computer search for U2 region III-like homologies in U1 genes was that of HSD4; however, this region is identical in all human U1 genes sequenced thus far (15, 18). The region III of U1 is closer to the cap site in U1 genes than it is in U2 genes. This is the only significant region III homology found by the computer.

guish the human U2 RNA from the endogenous *Xenopus* U2 RNA present in oocytes, we determined hybridization conditions for S1 nuclease mapping which would discriminate between human and *Xenopus* U2 sequences. At sufficiently stringent hybridization conditions, *Xenopus* U2 RNA would not be expected to hybridize with a human U2 probe, because the sequences differ by about 10% (20, 39). RNA from uninjected oocytes or oocytes injected with *dl*-556 or *dl*-62 was then subjected to S1 nuclease mapping (Fig. 3). The probe was a kinase labeled, strand-separated *Taq*I fragment extending from position 151 in the human U2 coding region to −211 upstream. Total RNA from HeLa cells protected a 151-base fragment of the probe at both the low (nondiscriminatory) and the high (discriminatory) stringency conditions of hybridization (Fig. 3A, lanes B and C). In contrast, RNA from uninjected oocytes protected the

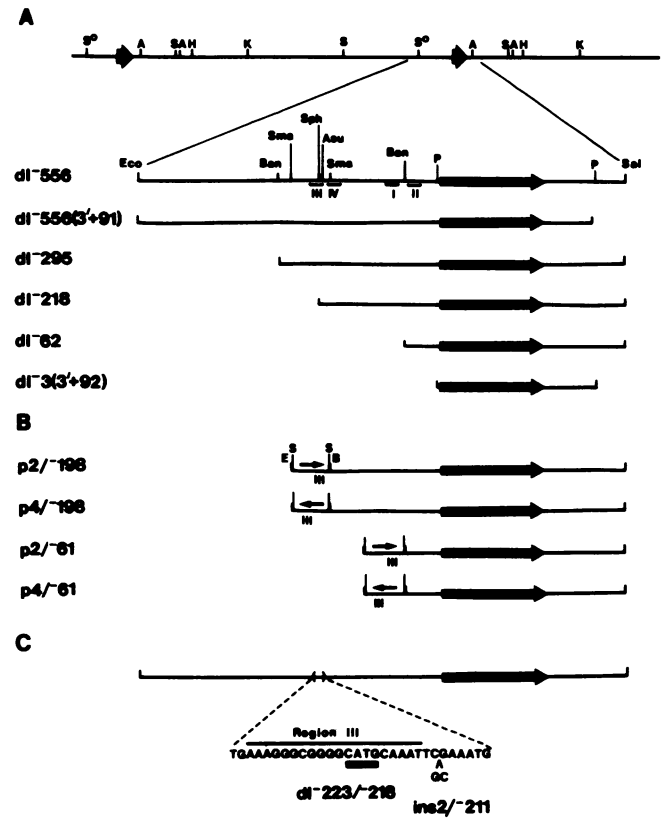

FIG. 2. Derivatives of the human U2 gene tested for transcriptional ability. (The construction of these derivatives is described in the text.) (A) 5′ resection mutants. The top shows the insert from λU2.24 and the location of the *Stu*I-*Acc*I fragment used to generate *dl*-556. A, *Acc*I; S, *Stu*I; S°, a *Stu*I site resistant to cleavage in *dcm*$^+$ methylated DNA; H, *Hin*dIII; K, *Kpn*I. The *Acc*I site distal to the 3′ end of the coding region is absent from about half the U2 family members in the human genome. The bottom shows location of restriction sites and regions I to IV on the map of *dl*-556. Ban, *Ban*II; P, *Hin*PI. DNA sequences remaining in the other derivatives are shown. (B) Inversion and relocation of the *Sma*I fragment. A *Bam*HI linker was used to reclone the *Sma*I fragment containing region III in either orientation at its natural position at −198 or just upstream of −61. B, *Bam*HI; E, *Eco*RI; S, *Sma*I. (C) Two discrete mutations of region III. The sequence spanning region III is shown. The mutation in *dl*-223/-218 removed the sequence 5′-CATG-3′ from region III. The mutation in *ins*2/−211 inserted 5′-GC-3′ between −211 and −212, just downstream of region III.

probe efficiently only after hybridization under low-stringency conditions (Fig. 3A, cf. lanes D and E).

RNA from oocytes injected with the wild-type U2 template *dl*-556 protected fragments the same size as HeLa cell U2 RNA after high-stringency hybridization (Fig. 3A, lane G; cf. with lane C), indicating that human U2 RNA is correctly initiated after injection of the human gene into *Xenopus* oocytes. In addition to correctly initiated human U2 RNA, we also detected a major start at about −180, as well as an array of perhaps 20 aberrantly initiated transcripts with 5′ ends mapping from position −45 to beyond −211 (lane G). Our assay overestimated the abundance of aberrant upstream starts, because these transcripts are longer, have greater guanine-plus-cytosine content, and thus hybridize to the probe more efficiently than does RNA with a normal 5′ end under high-stringency conditions. Consequently, the hybridization conditions required to discriminate between human and *Xenopus* U2 did not allow us to determine quantitatively the ratio of aberrant upstream starts to normal starts.

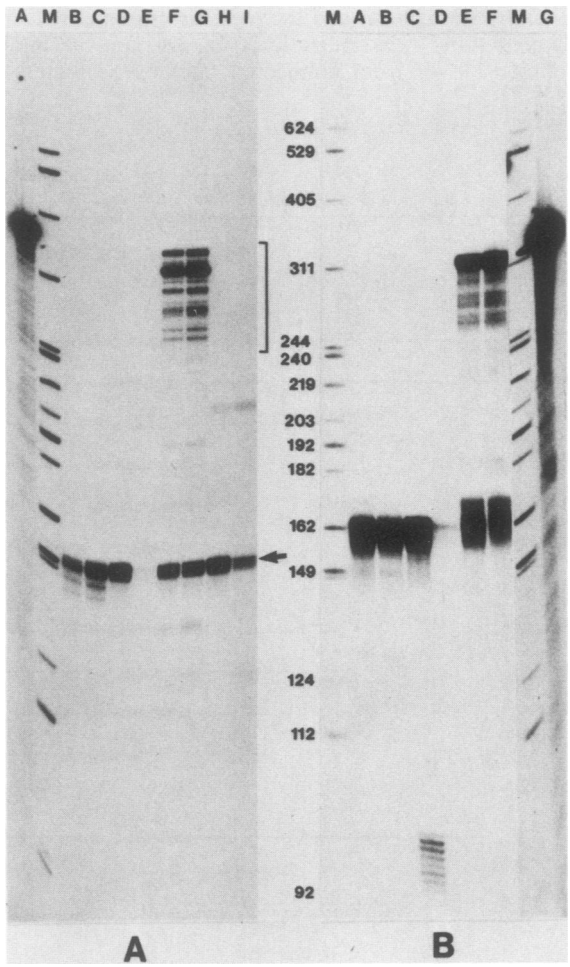

FIG. 3. S1 nuclease mapping of human U2 RNA synthesized in injected oocytes. (A) 5′ end mapping. RNA from 10^4 HeLa cells (lanes B and C), uninjected oocytes (lanes D and E), and oocytes injected with human U2 template *dl*-556 (lanes F and G) or *dl*-62 (lanes H and I) was hybridized to the template strand of the 5′ end-labeled *Taq*I fragment at either 37°C (lanes B, D, F, and H) or 45°C (lanes C, E, G, and I), digested with S1 nuclease, and separated on a 7 M urea–6% polyacrylamide gel. Untreated probe (lane A) and filled-in *Hpa*II fragments of pBR322 (lane M) are shown as size markers. The arrow indicates protected fragments corresponding to correctly initiated U2. The array of upstream starts is bracketed. (B) 3′ end mapping. RNA from 10^4 HeLa cells (lanes A and B), uninjected oocytes (lanes C and D), or oocytes injected with the U2 template *dl*-556 (lanes E and F) was hybridized to the template strand of the 3′ end-labeled *Dde*I-*Sal*I fragment at either 43°C (lanes A, C, and E) or 50°C (lanes B, D, and F), digested with S1 nuclease, and separated on a 7 M urea–6% polyacrylamide gel. Untreated probe (lane G) and filled-in *Hpa*II fragments of pBR322 (lane M) are shown as size markers.

RNA from oocytes injected with *dl*-62 DNA also protected the probe under stringent hybridization conditions (Fig. 3A, lane I). Although apparently less RNA was present (cf. lanes G and I), 62 bp of 5′ flanking sequence is sufficient to direct accurate initiation of human U2 RNA in oocytes. An additional protected fragment of about 213 bases (lane I) corresponded to the extent of homology between the probe and the template and presumably represented transcripts entering the U2 coding region from upstream vector sequences.

Most human U2 transcripts synthesized in oocytes are longer at the 3′ end than mature human U2. To determine whether the human U2 transcripts synthesized in oocytes have the same 3′ end as mature human U2 RNA, we used a 3′-end-labeled, strand-separated probe extending from the *Dde*I site at position 22 in the U2 coding region to position +152 in the 3′ flanking sequence, at the junction between the insert and the vector. Again, we determined stringent hybridization conditions which would discriminate human U2 RNA from endogenous *Xenopus* U2 RNA. As expected, HeLa cell RNA protected a 165-base fragment of the probe after hybridization at either high- or low-stringency conditions (Fig. 3B, lanes A and B). RNA from uninjected oocytes, however, protected the probe efficiently only after hybridization at low- (lane C) and not at high-stringency conditions (lane D).

Oocytes injected with the wild-type human U2 gene contained transcripts with three major classes of 3′ end (Fig. 3B, lane F): transcripts with the mature U2 3′ ends (cf. lane F with lane B), transcripts with 6 to 10 extra nucleotides at the 3′ end, and transcripts which extend into the 3′ flanking sequences and protect the full length of the probe. These data indicate that formation of the 3′ end of human U2 RNA is inefficient in *Xenopus* oocytes, but we cannot tell whether U2 RNAs with 3′ extensions represent normal U2 precursors or aberrant byproducts of 3′ end formation. However, U2 transcripts with 6 to 10 extra 3′ nucleotides (which we call U2+10), have been identified in HeLa cells by pulse-labeling (1, 47), in rat cells by S1 nuclease mapping (37, 38), and in oocytes after injection of a human U2 gene (44). Recently, by using the Ya antiserum specific for U2 snRNPs (24), one of us was able to immunoprecipitate from HeLa cells an RNA which comigrates with the human U2+10 seen in oocytes (M. Ares, unpublished data). In addition, we found that U2+10 can be processed to mature U2 RNA upon reinjection into oocytes (Yuo et al., submitted for publication). We agree with the suggestion (1, 47) that the longer transcript is a natural precursor of U2 snRNA which is processed during small nuclear ribonucleoprotein assembly or maturation. Perhaps because of gross sequence differences between human U2+10 and a potential *Xenopus* U2 precursor (20) the oocyte may process the human transcript inefficiently.

Efficient U2 transcription requires activator sequences far upstream of the cap site. The S1 nuclease mapping data indicated that as few as 62 bp of 5′ flanking sequence were

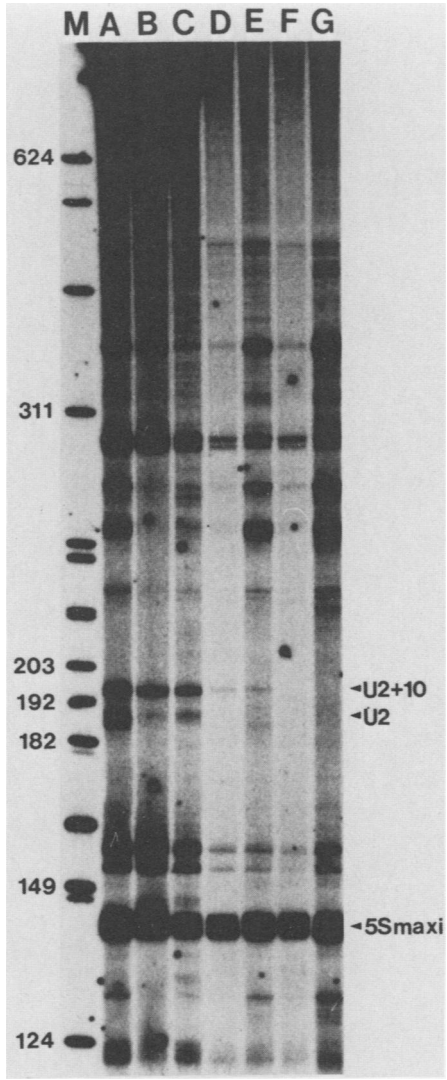

FIG. 4. Relative expression of human U2 templates containing 5′ flanking sequence deletions. Labeled RNA synthesized in oocytes from a normalized amount (see text) of injected template DNA from *dl*-556 (lane A), *dl*-556(3′+91) (lane B), *dl*-295 (lane C), *dl*-218 (lane D), *dl*-62 (lane E), *dl*-3(3′+92) (lane F), and pUC8 (lane G) was separated on a 7 M urea–6% polyacrylamide gel. Filled-in *Hpa*II fragments are shown as size markers (lane M). Arrows indicate the positions of migration of U2+10, U2, and 5S maxigene reference transcripts. Maps of these constructs are shown in Fig. 2A.

sufficient for a low level of accurate U2 initiation, but we could not be confident that the reduced S1 signal reflected the efficiency of transcription rather than the efficiency with which the template was delivered to the oocyte nucleus (see above for a more detailed explanation). To control for variation in template delivery, we coinjected each U2 construction with a fixed amount of a *X. borealis* 5S RNA +20 maxigene (33) which produces several transcripts of about 140 bases. By normalizing the amount of 5S maxitranscript in each sample, we were able to compare the level of U2 transcription derived from the same amount of the different mutant U2 templates.

After injection and incubation of each U2 template, together with [α-^{32}P]rGTP and a constant amount of reference 5S maxigene, we extracted total labeled RNA and resolved it on a denaturing polyacrylamide gel (Fig. 4). As expected from the S1 nuclease mapping experiment, transcripts corresponding to U2 and U2+10 were observed after injection of the wild-type U2 gene (*dl*-556, lane A). These transcripts are derived from the U2 gene because they can be hybrid selected by single-stranded DNA from an M13 clone containing only the U2 coding sequence in the appropriate orientation (data not shown). Because both U2 and U2+10 resulted from initiation at the U2 cap site (Fig. 3), we took the sum of the two transcripts to reflect the efficiency of transcription. We do not know the basis for variability in the amounts of mature U2 compared with U2+10; however, the variation is not reproducibly correlated with any particular promoter mutation and so is not due to changes in the promoter. Deletion of template sequences to position −295 (*dl*-295, lane C) did not reproducibly reduce the U2 and U2+10 syntheses, but deletion to −218 reduced the accumulation of U2 and U2+10 by 20- to 50-fold (cf. lanes C and D). Deletion to −62 did not further reduce the level of accurate U2 initiation (lane E), but deletion to −3 abolished detectable U2 synthesis (lane F). Because the −3 deletion also lacks sequences past +92 in the 3′ flank, a construct containing 556 bp of 5′ flanking sequence (as in wild-type *dl*-556) but lacking 3′ flanking sequences past +91 was tested as a control. Deletion to +91 on the 3′ side of the gene did not affect efficient U2 transcription in oocytes (lane B). As expected, injection of vector pUC8 (40) in the same amount as the U2 templates did not result in synthesis of U2 or U2+10 (lane G). We conclude that sequences between positions −295 and −218 contain an element capable of activating transcription of human U2 templates in oocytes. Moreover, as few as 62 bp but more than 3 bp of 5′ flanking sequence are required for accurate initiation of human U2 transcripts in oocytes.

The activator enhances accurate U2 initiation in an orientation-dependent fashion. To investigate the gross organizational requirements for upstream activator function, we constructed several rearrangements of the U2 gene. Basically, the 72-bp *Sma*I fragment located between positions −198 and −270 was first subcloned and then recloned in both orientations, either at its normal distance upstream of the gene at −198 or upstream of position −61 (Fig. 2B). These four constructs, together with a number of controls, were injected into oocytes along with [α-^{32}P]rGTP and the reference 5S maxigene. Labeled RNA was extracted and analyzed directly by electrophoresis and autoradiography (Fig. 5). Recloning the *Sma*I fragment in its normal position and orientation resulted in deletion of sequences upstream of −270 and insertion of 10 bp at position −198, but these changes did not affect the efficiency of transcription (p2/−198, lane B) relative to the wild-type U2 gene (*dl*-556, lane A). Inversion of the *Sma*I fragment at this position (p4/−198, lane C) reduced the efficiency of U2 transcription to the same low level (2 to 5%) as deletion of all sequences upstream of position −62 (*dl*-62, lane F). Recloning the *Sma*I fragment just upstream of −61 in its natural orientation resulted in a 10-fold stimulation of accurate U2 initiation relative to *dl*-62 (p2/−61, lane D; cf. to lane F), but recloning the *Sma*I fragment at this position in the opposite orientation apparently reduced accurate transcription (p4/−61, lane E; cf. to lane F). These results indicate that the transcriptional activator can stimulate initiation at the normal U2 cap site only in its natural orientation. In addition, activation of correct U2 initiation by the element is at least partially independent of position, because relocation of the *Sma*I fragment to a position nearer to the U2 cap site still stimulates initiation.

The activator enhances aberrant upstream initiation in an orientation-independent fashion. To determine whether the transcriptional activator affects the use of start sites other than the normal U2 cap site, we performed primer extension assays on RNA from oocytes injected with the templates described above. Although direct analysis of labeled RNA is a good way to detect synthesis of transcripts initiating at the U2 cap site, it is not suitable for analyzing the lower abundance RNAs which initiate upstream of the normal cap site. Because the oligonucleotide primer is in vast excess and the hybridization reaction goes essentially to completion, primer extension allows us to determine quantitatively the relative amounts of RNAs initiating at the various upstream start sites. However, the primer is complementary to a highly conserved sequence near the 5′ end of U2 RNA, and so the endogenous *Xenopus* U2 RNA present in oocytes will generate precisely the same extension product as correctly initiated human U2. For this reason we have used the primer extension assay to measure upstream starts only.

When the same normalized RNA samples that were analyzed directly in Fig. 5 were used as templates for primer extension, we easily detected transcripts initiating upstream (Fig. 6). All samples contained large amounts of the 42-base extension product derived from both endogenous *Xenopus* U2 and correctly initiated human U2 transcripts. Densitometric quantitation of the 42-base product showed that an active wild-type gene in this experiment (*dl*-556 or p2/−198) produced an amount of accurately initiated human U2 equivalent to what was already contained in the oocyte (data not shown), suggesting that the upstream starts represent less than 10% of the human U2 transcripts present. As expected from S1 nuclease mapping (Fig. 3A, lane G), injection of the wild-type human U2 gene resulted in an array of upstream starts between positions −60 and −250 with a major start at about −180 (Fig. 6, lane A). Deletion to −218 (lane B) abolished the −180 start and dramatically reduced initiation at other upstream start sites as well. The excellent correlation between the S1 and primer extension assays (cf. Fig. 3 and Fig. 6) argues that these are upstream starts and not simply strong stops for reverse transcriptase. Recloning the *Sma*I fragment in its natural position and orientation (p2/−198, Fig. 6, lane C) restored virtually the same array of minor upstream starts seen in the wild type (lane A), but the major start at position −180 was now shifted to about −190 (lane C), a distance corresponding to the 10-bp linker sequence inserted into the template at position −198 during the recloning. This suggests that the *Sma*I fragment not only increases initiation from an array of potential start sites specified by local sequences, but also directs strong initiation about 20 bp downstream of position −199 regardless of the local sequence context. The ability to direct this initiation is dependent on sequences upstream of −218 (lane B).

When the *Sma*I fragment was recloned at its natural position, but in the opposite orientation (p4/−198, Fig. 6, lane D), we observed a dramatic stimulation of the minor upstream starts seen in the wild type (lane A); however, accurate U2 initiation was not stimulated (cf. Fig. 5, lanes B and C, with Fig. 6, lanes C and D). This suggests that the *Sma*I fragment stimulates the array of aberrant upstream starts in either orientation but stimulates accurate initiation at the normal cap site only in the natural orientation.

Oocytes injected with a U2 template containing the *Sma*I fragment in the natural orientation but closer to the gene at position −61 (p2/−61, Fig. 6, lane E) produced major transcripts initiating at positions −50 to −53, as well minor transcripts which were not detected after injection of the −62 deletion alone (*dl*-62, lane G). The major transcripts starting at positions −50 to −53 initiated at about the same distance downstream of the *Sma*I fragment (20 bp) as did the −180 start in the wild-type gene (lane A) and the −190 start in the reconstructed gene with the linker (lane C). This reinforces our conclusion that the *Sma*I fragment directs initiation at a fixed distance downstream of itself.

Oocytes injected with a U2 template containing the *Sma*I fragment in the opposite orientation at position −61 (p4/−61, Fig. 6, lane F) produced major starts between positions −56 and −53. This construction also stimulated a variety of minor starts not seen in the −62 deletion (cf. lane F with lane G) but to a lesser extent than did inversion of the *Sma*I fragment at its natural position (lane D). Closer comparison

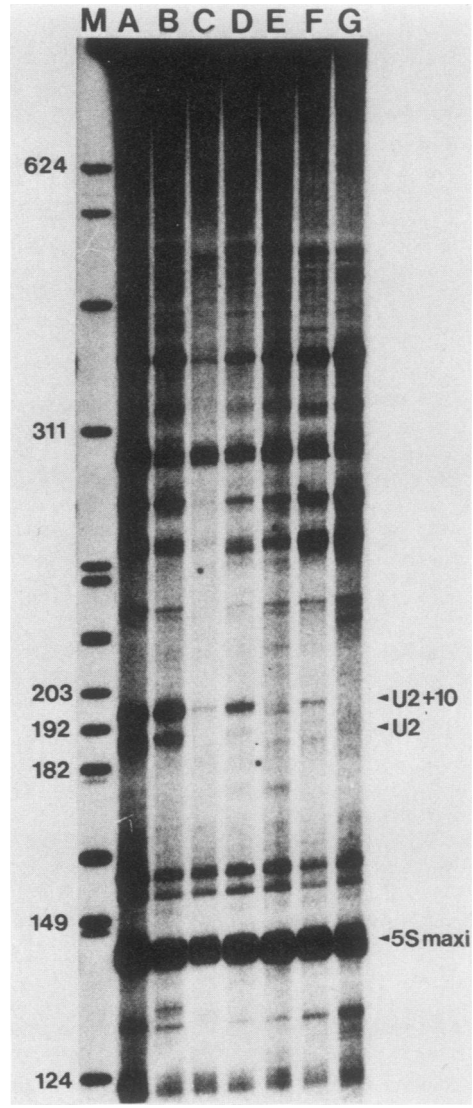

FIG. 5. Relative expression of correctly initiated human U2 from the 5′ flanking sequence rearrangement mutants. Labeled RNA synthesized in oocytes from a normalized amount (see text) of injected template DNA from *dl*-556 (lane A), p2/−198 (lane B), p4/−198 (lane C), p2/−61 (lane D), p4/−61 (lane E), *dl*-62 (lane F), and pUC8 (lane G) was separated on a 7 M urea–6% polyacrylamide gel. Filled-in *Hpa*II fragments of pBR322 are shown as size markers (lane M). Arrows indicate the positions of migration of U2 + 10, U2, and 5S maxigene reference transcripts. Maps of these constructs are shown in Fig. 2A and 2B.

of transcripts from the inverted constructs reveals that p4/−198 produced strong starts around −195, a distance from the *Sma*I fragment equivalent to that of the −53 to −56 starts of p4/−61 (cf. lane D with lane F). This indicates that in the inverted constructs as well as in the naturally oriented constructs, sequences in the *Sma*I fragment direct initiation a fixed distance downstream. Although we do not have direct evidence, we are attracted by the possibility that transcription may proceed bidirectionally from a site within the element.

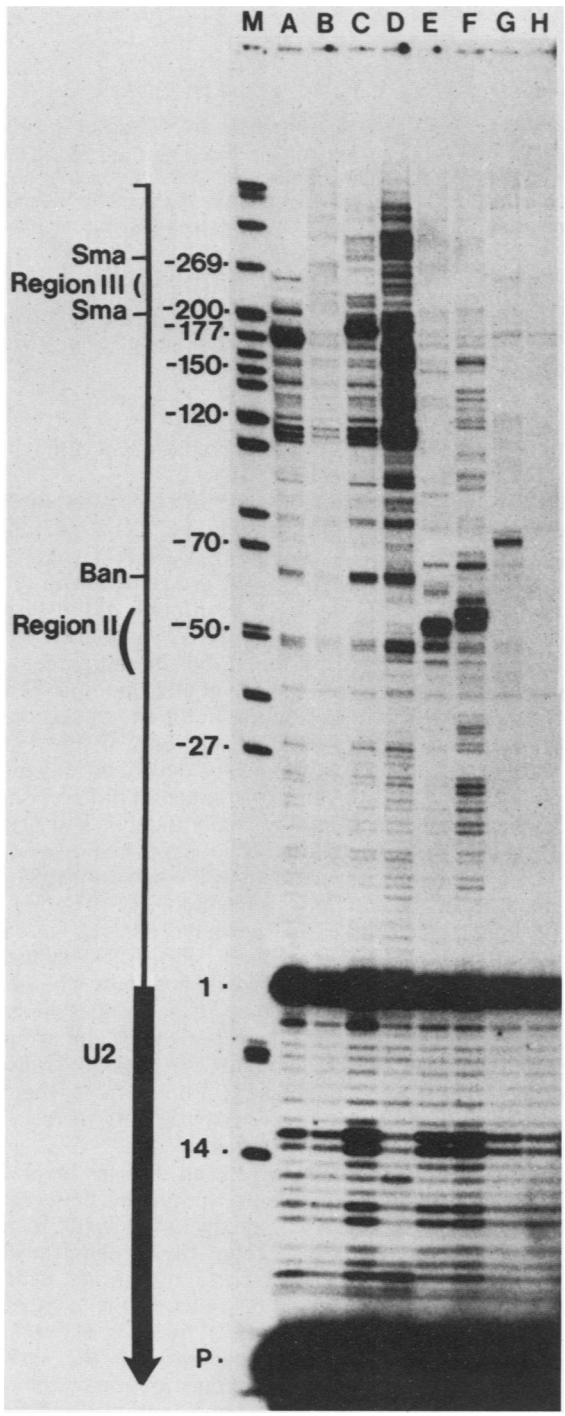

FIG. 6. Relative expression of upstream initiated U2 transcripts from the 5′ flanking sequence rearrangement mutants. The 5′ ends of RNA synthesized in oocytes from a normalized amount (see text) of injected DNA from *dl*-556 (lane A), *dl*-218 (lane B), p2/−198 (lane C), p4/−198 (lane D), p2/−61 (lane E), p4k/−61 (lane F), *dl*-62 (lane G), and pUC8 (lane H) were mapped by oligonucleotide primer extension. All samples were made up to the same total amount of RNA by addition of RNA from uninjected oocytes. Extension products were separated on a 7 M urea–8% polyacrylamide gel. Filled-in *Hpa*II fragments of pBR322 are shown as size markers (lane M), but are numbered by the position they represent relative to the U2 cap site. The diagram to the left of the gel shows features of the wild-type human U2 gene to the scale of the gel. Maps of these constructs are shown in Fig. 2A and 2B.

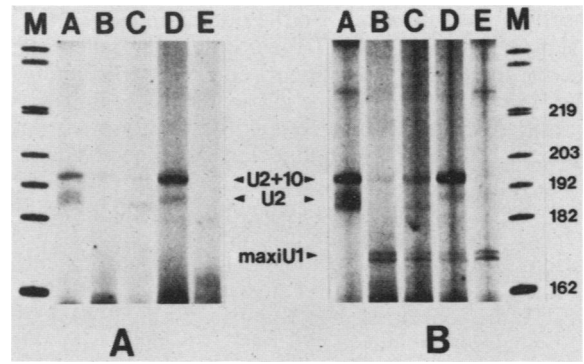

FIG. 7. Transcriptional activity and competitive ability of discrete mutations in and near region III. (A) Transcriptional activity. Labeled RNA synthesized in oocytes from a normalized amount of injected DNA from *dl*-556 (lane A), *dl*-218 (lane B), *dl*-223/-218 (lane C), *ins*2/−211 (lane D), and pUC8 (lane E), along with *Hpa*II fragments of pBR322 (lane M). (B) Competitive ability. Labeled RNA synthesized in oocytes from a normalized amount of a 7:1 mixture of the test template plus the U1 maxigene. Increased synthesis of the U1 maxitranscript reflected reduced competitive ability of the coinjected U2 template. Shown are *dl*-556 plus U1 maxigene (lane A), *dl*-218 plus U1 maxigene (lane B), *dl*-223/-218 plus U1 maxigene (lane C), *ins*2/−211 plus U1 maxigene (lane D), and pUC8 plus U1 maxigene (lane E), along with *Hpa*II fragments of pBR322 (lane M) separated on a 7 M urea–6% acrylamide gel. Arrows indicate position of migration of U2+10, U2, and U1 maxitranscripts.

A discrete mutation in region III abolishes transcriptional activation and impairs competitive ability. From the human U2 gene constructs described above, we deduced that sequences between positions −270 and −218 are important for transcriptional activation and that the *Sma*I fragment between positions −198 and −270 spans the activator. The *Sma*I fragment contains a sequence homology found in U2 genes of rats and *X. laevis* (region III, Fig. 1B). We made two discrete mutations in this region, a deletion of 4 bp between −223 and −218 (*dl*-223/-218) and an insertion of 2 bp at −211 (*ins*2/−211, Fig. 2C). We tested these mutants for the ability to direct U2 transcription by injecting them into oocytes together with the reference 5S maxigene and [α-^{32}P]rGTP. Labeled RNA was isolated, normalized to the 5S maxitranscript as before, and compared with RNA from *dl*-556 (Fig. 7A, lane A) and *dl*-218 (lane B). Mutant *dl*-223/-218 (lane C) was no more efficient in directing accurate initiation of U2 than was *dl*-218 (lane B), demonstrating that deletion of just 4 bp in the center of region III, 220 bp upstream of the U2 cap site, is enough to destroy

transcriptional activation. On the other hand, insertion of 2 bp at position −211 (lane D) had little or no effect on the ability of the template to support efficient transcription.

We also found by coinjection experiments that the upstream element can confer competitive ability in *cis* upon a functional U2 transcription unit (Fig. 7B). In each case, the same amount of U2 template was coinjected with a human U1 maxigene. (This maxigene generates transcripts of about 180 bases resulting from the insertion of an *Eco*RI linker at position 27 in the coding region [N. Hernandez and A. Weiner, unpublished data]. Transcripts from wild-type human U1 are obscured by *Xenopus* 5.8S rRNA.) When wild-type U2 template *dl*-556 was coinjected with the U1 template at a 7:1 ratio, little U1 maxitranscript was detectable (Fig. 7B, lane A). Deletion to −218 of the U2 template resulted in a 20-fold increase in the synthesis of U1 maxitranscript (lane B), the same level observed when the vector alone, instead of a U2 template, was coinjected with the U1 maxigene (lane E). This indicates that sequences upstream of position −218 in U2 are involved in mediating the competition.

For other relatively large deletions tested, we found a one-to-one correspondence between templates having a functional transcriptional activator and those behaving as competitive templates (*dl*-295 competes; *dl*-62 and *dl*-270/-198 do not; data not shown), suggesting that the sequences involved in transcriptional activation lie near the sequences involved in the competition effect. The much smaller 4-bp deletion in mutant *dl*-223/-218 significantly reduced, but did not completely eliminate, the competitive ability of the U2 template (lane C), although transcriptional activation was destroyed (Fig. 7A, lane C). Insertion of 2 bp at position −211 also generated a template with reduced competitive ability (Fig. 7B, lane D), although surprisingly this template was efficiently transcribed (Fig. 7A, lane D).

The competitive effect cannot be mediated by the upstream element alone. We were interested in determining whether the activator element by itself could display competitive ability. A transcriptional activator upstream of the rRNA promoter of *X. laevis* competes away transcription even when not linked to an active promoter (32). This is not the case for the human U2 transcriptional activator. Coinjection of subclones of the U2 gene 5′ flanking sequence with the U1 maxigene failed to reduce maxigene transcription (Fig. 8.) Injection of a subclone containing the 72-bp *Sma*I fragment (lane B) or the 231-bp *Ban*II fragment extending from positions −65 to −296 (lane C) was no more effective at competing away U1 maxigene transcription than was the vector alone (lane A), although the complete wild-type U2 gene can compete (lane D). We conclude that the competition effect mediated by the upstream sequences may require ongoing transcription or sequences downstream of −61 to stabilize the association of the template with a limiting transcription factor.

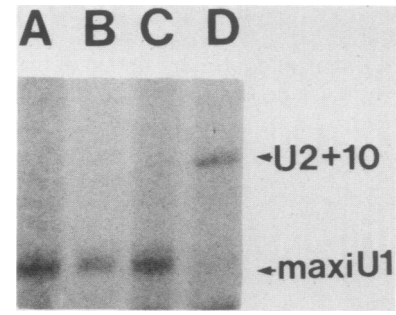

FIG. 8. Competitive ability of subcloned portions of the human U2 gene flanking sequences. Labeled RNA synthesized in oocytes from a normalized amount of a 7:1 mixture of the test DNA plus the U1 maxigene. Shown are the mp8 vector plus U1 maxigene (lane A), mpSma4 (containing the 72-bp *Sma*I fragment from −269 to −199) plus U1 maxigene (lane B), mpBan2 (containing the 231-bp *Ban*II fragment from −65 to −294) plus U1 maxigene (lane C), and mp*dl*-556 (containing the entire 896-bp U2 gene in mp8) plus U1 maxigene (lane D). Arrows indicate the positions of migration of U2+10 and the U1 maxitranscripts.

DISCUSSION

Upstream sequences including region III enhance U2 transcription. Efficient transcription of human U2 RNA in *Xenopus* oocytes required the presence of template sequences far upstream (Fig. 4). The most telling mutation (*dl*-223/-218) removed just 4 bp in the middle of region III but reduced transcription to the same low level (2 to 5% of wild type) as deletion of all sequences upstream of position −62 (Fig. 4 and 7). Our confidence in the importance of region III was strengthened by the existence of a very good region III homology (16 of 17 bp match) at a comparable position in the *Xenopus* and rat U2 genes (Fig. 1B).

Although region III has a number of elements thought to be important in enhancing transcription (2, 21, 29, 42; Fig. 1; 5′-GGGCGG-3′; Z-DNA potential: 5′-GCATGCA-3′; enhancer core: 5′-GTGAAAG-3′), we are reluctant to draw a direct analogy between the U2 transcriptional activator and previously described enhancers for RNA polymerase II. The U2 activator, as defined by the 72-bp *Sma*I fragment that spans region III, stimulated the use of aberrant upstream U2 starts in either orientation (Fig. 6), but it stimulated correct initiation at the normal U2 start site only in the correct orientation (Fig. 5). Like previously described enhancers, the ability of the U2 activator to stimulate both correct and aberrant initiations is at least partially independent of position (Fig. 5), because activation is not abolished by insertion of 10 bp immediately downstream of the activator (p2/−198) or relocation of the activator to position −61 (p2/−61).

It is important to note that we have not yet tested the activity of the element in the absence of U2 sequences downstream of position −61, so we do not know whether the element depends on the presence of sequences including region II for its function. It will also be important to determine whether the U2 activator can activate transcription from other RNA polymerase II promoters or whether it is specific for the U2 promoter. Experiments to test these possibilities are currently in progress.

Activator sequences coincide with an element involved in template competition. Competition experiments between various U2 constructs and a U1 maxigene allow us to make some preliminary suggestions about the mechanism of U2 transcriptional activation. Discrete mutations near sequences important for activation reduced the competitive ability of the U2 template (Fig. 7). A competition effect involving activator sequences suggests that the activator facilitates the binding of a specific transcription factor which is limiting in the oocyte. This cannot entirely explain the competition effect, however, because similar competitive abilities were displayed by two different mutants, one of which is transcriptionally active (*ins*2/−211) and one of which was not (*dl*-223/-218; Fig. 7). Recent experiments with *Xenopus* 5S RNA and histone genes have suggested that competition phenomena in oocytes may be due to differential

abilities of competing templates to assemble transcriptionally active chromatin (4). This result means that the behavior of any mutant U2 template could in principle reflect either an inability to assemble active chromatin or an inability to initiate accurate transcription once properly assembled. If efficient transcription is a two-step process first involving assembly of stable transcription complexes and then transcription initiation, it might be expected that subtle mutations could affect assembly of complexes without affecting the activity of the complex once assembled or have little effect on assembly of complexes but reduce the activity of properly assembled complexes. This may explain why the *ins*2/−211 and *dl*-223/-218 templates behaved differently. In spite of this, the coincidence of template sequences required for efficient transcription and competition hints that the U2 activator might function at least in part by enhancing the assembly of transcriptionally active chromatin.

We also found that the U2 activator by itself could not compete with the U1 maxigene (Fig. 8), indicating that the competitive effect resulting from upstream sequences was facilitated or stabilized by ongoing transcription or by association of sequences downstream of −61.

Region II may be indispensable for U2 and U1 transcription. We showed that only 62 bp 5′ to the U2 start site are sufficient to direct accurate initiation of human U2 RNA in *Xenopus* oocytes. In contrast, synthesis of human U1 in the same oocyte system is not detected when sequences between positions −231 and −203 are deleted from the template (36). In addition, in vitro transcription of human U1 templates requires sequences in the −220 region (26), whereas in vitro transcription of human U2 templates does not (44). Interestingly, sequences between positions −62 and −3 of the human U2 gene include a block of 17 of 20 bp that is also found between positions −231 and −203 in the human U1 gene; this homology has been called region II (39). On the one hand, it may be that region II is absolutely required for both U2 and U1 transcription but that it can be present at different distances from the start site of transcription in different genes: close in U2, far in U1. On the other hand, the far upstream sequences of the U1 gene may represent an element similar to the transcriptional activator upstream of U2 but without which human U1 synthesis cannot be detected in oocytes. Moreover, region II in U2 genes partially overlaps a sequence that has been suggested to function as the equivalent of a TATA box in specifying the precise initiation site for transcription (36). If region II and the proposed TATA box equivalent actually represent two distinct promoter elements, they are packed with surprising efficiency in human U2 genes and are widely spaced in human U1 genes. Many discrete mutations have to be tested before the relative importance and functional equivalence of these sequences can be assessed.

Our conclusions regarding the organization of the U2 snRNA promoter must be tempered by the limitations of the heterologous oocyte assay system. For example, a number of transcriptional enhancer elements display strong tissue or species specificity (5, 37), and some of the initiation factors and promoter elements for RNA polymerase I transcription are known to be species specific (6, 11, 23, 25). Nonetheless, microinjection of *Xenopus* oocytes has proved useful in the analysis of other heterologous genes transcribed by RNA polymerase II (9, 21), including human snRNA genes (36, 44). The oocyte is convenient for our purposes because the assay is rapid, and most of the transcripts derived from the wild-type U2 template are correctly initiated in the oocyte. Although many upstream start sites can be detected by S1 nuclease protection (Fig. 3) and by primer extension (Fig. 6), quantitation of the 42-base extension product from the gel in Fig. 6 suggests that these aberrant starts constitute less than 10% of the human U2 transcripts initiated in oocytes. We do not know whether these aberrant upstream starts are due to the use of heterologous transcription machinery, to overloading of the machinery, or to aberrant chromatin assembly. These uncertainties will not necessarily disappear when we use a homologous in vivo or in vitro assay system, and they need not interfere with our major conclusions.

The synthesis of U2 snRNA makes strong demands on the cellular transcription machinery. This specialized RNA polymerase II promoter must be accurate enough to specify the precise 5′ end of a structural RNA and yet powerful enough to produce about 10^6 transcripts per cell generation by using at most 10 to 20 genes. This corresponds to an average minimum initiation rate of one transcript per gene every 2 s. On a per-gene basis, in fact, the output of the U2 genes is comparable to that of the rRNA genes transcribed by RNA polymerase I. The extraordinary power of the snRNA promoters suggests that they must be different from the typical RNA polymerase II promoter for mRNA. The structure of U2 and U1 snRNA promoters is quite different from that of mRNA promoters, and we expect that snRNA genes will require specialized sequence elements and transcription factors to meet the demands for snRNA synthesis.

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