The EMBO Journal vol.7 no.10 pp.3125-3134, 1988

Elements required for transcription initiation of the human U2 snRNA gene coincide with elements required for snRNA 3′ end formation

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Communicated by J.D.Watson

Formation of the human U1 and U2 snRNA 3′ ends requires both a conserved sequence, the 3′ box, located downstream of the snRNA termini and sequences within the snRNA promoter regions. Indeed, replacement of the U1 snRNA promoter by mRNA promoters inhibits U1 3′ end formation. We have now mutated the 5′ flanking region of the human U2 gene and assayed the effects on initiation of transcription and 3′ end formation. The 5′ flanking region of the U2 gene contains two major promoter elements, a previously characterized distal element that enhances the efficiency of transcription and a proximal element, which our analysis localizes between positions −59 and −43 in a segment conserved in vertebrate snRNA genes. The 5′ flanking region does not contain an element required solely for 3′ end formation. However, when enhancer elements from an mRNA-encoding gene are introduced into a U2 promoter lacking its distal element, 3′ end formation is inhibited. Together, these results suggest that the U2 promoter elements themselves are involved in 3′ end formation, presumably by directing the formation of a unique transcription complex which is compatible with 3′ end formation at the 3′ box. Alteration of the composition of this transcription complex results in increased read-through at the 3′ box.

Key words: enhancers/octamer/U2 transcription complex/U2 proximal element/termination

Introduction

In RNA polymerase II transcription units that direct the synthesis of polyadenylated and non-polyadenylated mRNAs, transcription often continues for hundreds of nucleotides beyond the site of 3′ end processing and terminates heterogeneously in A/T rich regions (see Birnstiel *et al.*, 1985 for a review). The mechanism of termination is poorly understood and may vary in different transcription units. Recent observations indicate that termination in the 3′ flanking region of some viral and cellular genes is dependent on the presence of an upstream polyadenylation site (Acheson, 1984; Whitelaw and Proudfoot, 1986; Logan *et al.*, 1987; Connelly and Manley, 1988), which suggests that termination of transcription may be coupled to polyadenylation. However, the requirement for a 3′ processing site may not be general. Indeed, the termination signal of the gastrin gene seems to function without such a site (Baek *et al.*, 1986), and in the sea urchin H2A histone gene, a non-polyadenylated mRNA encoding gene, transcription termination can occur independently of 3′ processing (Birchmeier *et al.*, 1984; Johnson *et al.*, 1986).

The small nuclear RNA (snRNA) genes U1 to U5 represent a peculiar class of polymerase II transcription units. In these genes, the site of RNA 3′ end formation is signaled by a short conserved block of sequence, the 3′ box, located 9–19 nt downstream of the mature 3′ end of the RNAs and conserved among different species (Hernandez, 1985; Yuo *et al.*, 1985; Neuman de Vegvar *et al.*, 1986; Ciliberto *et al.*, 1986; Ach and Weiner, 1987). Several lines of evidence suggest that the 3′ box directs termination of transcription. First, the longest detectable precursors to mature U1 and U2 RNAs contain only a few extra 3′ nucleotides which are removed in the cytoplasm, probably by an exonuclease (Eliceiri and Sayavedra, 1976; Zieve and Penman, 1976; Eliceiri, 1980; Madore *et al.*, 1984; Kleinschmidt and Pederson, 1987; Zieve, 1987). Second, U1 3′ flanking sequences are strongly underrepresented relative to coding sequences in run-on RNAs elongated in isolated nuclei (Kunkel and Pederson, 1985), and the rare RNA molecules containing 3′ flanking sequences are not precursors to mature U1 RNA (Lobo and Marzluff, 1987). Third, 3′ end formation at the U1 3′ box requires an element(s) located in the U1 5′ flanking sequences (Hernandez and Weiner, 1986; Neuman de Vegvar *et al.*, 1986). Indeed, if the U1 promoter is replaced by a mRNA promoter, 3′ end formation is inhibited and most of the RNA extends to a polyadenylation site inserted downstream of the gene (Hernandez and Weiner, 1986; Neuman de Vegvar *et al.*, 1986). The U2 promoter can, however, substitute for the U1 promoter without deleterious effect on U1 3′ end formation (Hernandez and Weiner, 1986). This observation demonstrates that 3′ end formation is coupled to transcription and most likely arises by transcription termination. snRNA genes, then, serve as a model system to study termination.

The promoters of the vertebrate U1 and U2 genes have a similar organization. They contain a proximal element, required both for efficient transcription and to position the start site (Skuzeski *et al.*, 1984; Ares *et al.*, 1985; Ciliberto *et al.*, 1985; Mattaj *et al.*, 1985; Mangin *et al.*, 1986; J.Murphy *et al.*, 1987). In the human U2 gene, the proximal element has been localized between positions −62 and +1 (Ares *et al.*, 1985). Between positions −60 and −43, 10 out of 18 nt are conserved between the human U2 and U1 genes. The U1 and U2 promoters also contain a distal element, which enhances transcription (Skuzeski *et al.*, 1984) relatively independently of orientation and position (Ares *et al.*, 1985; Mattaj *et al.*, 1985; Mangin *et al.*, 1986; J.Murphy *et al.*, 1987). The well-characterized distal element of the U2 gene is located between positions −240 and −198, and contains two adjacent motifs (Ares *et al.*, 1985; Mangin *et al.*, 1986): a GC-rich box, which may bind the transcription factor Sp1 (Kadonaga *et al.*, 1986), and an octamer motif also found in a variety of promoters and enhancers (Harvey *et al.*, 1982; Parslow *et al.*, 1984; Falkner and

Zachau, 1984; Mason *et al.*, 1985). Each of these motifs binds a protein factor *in vitro*, and both sites can be occupied simultaneously (Ares *et al.*, 1987; Janson *et al.*, 1987). A second GC-rich box is located further upstream in the U2 5′ flanking sequence, around position −265.

The location of the 5′ signals required for 3′ end formation of the U1 and U2 snRNA is not known. The 5′ flanking region of the U1 and U2 genes may contain termination elements distinct from the promoter elements. Such elements could conceivably function in different transcription units, in much the same way as the *nut* site in phage λ can promote antitermination when inserted in other transcription units than the early λ genes (de Crombrugghe *et al.*, 1979). Alternatively, it may not be possible to separate the sequences required for 3′ end formation from promoter elements. Here, we describe a mutational analysis of the human U2 promoter which was undertaken both to localize precisely the U2 proximal element and to localize the element(s) involved in 3′ end formation. Our experiments did not uncover elements involved solely in 3′ end formation. Furthermore, deletion or inactivation of promoter elements did not result in strong inhibition of U1 3′ end formation. Strong inhibition was observed, however, when the U2 distal element was replaced by SV40-derived synthetic enhancers. Thus, elements required for 3′ end formation cannot be dissociated from elements required for initiation of transcription, and introduction of foreign transcriptional elements into the U2 promoter, and thus presumably foreign transcription factors into the U2 transcription complex, inhibits 3′ end formation. These observations suggest that the U2 promoter directs the formation of a specialized transcription complex that is distinct from mRNA transcription complexes and that recognizes the 3′ box as a 3′ end formation signal.

Results

Assay of snRNA initiation and 3′ end formation

To identify the element(s) in the U2 5′ flanking region required for 3′ end formation, we introduced mutations into the U2 promoter region and monitored their effect on initiation of transcription and 3′ end formation. Because the U1 and U2 3′ boxes are interchangeable (Ach and Weiner, 1987), we took advantage of convenient restriction sites previously engineered into both the U1 and U2 genes to construct a hybrid test gene, encoding an artificial RNA that is easily distinguishable from endogenous HeLa cell transcripts. As shown in Figure 1, this test gene contains the U2 promoter with the first 28 bp of the U2 coding region, followed by a 137 bp fragment of the rabbit β-globin gene cloned in the reverse orientation. Downstream of the globin fragment are two signals for 3′ end formation: first, the U1 3′ end formation signal, carried on a fragment containing the last 6 bp of the U1 coding region and the 3′ box, and second, the L3 polyadenylation site from the major late transcription unit of Adenovirus 2 (Ad2). The availability of a polyadenylation site downstream of the U1 3′ end formation signal ensures that stable RNAs with a discrete 3′ end are formed even if 3′ end formation at the U1 site is abolished by a mutation within the promoter region (Hernandez and Weiner, 1986).

To assay for expression and 3′ end formation, the test plasmid was transfected into HeLa cells together with a plasmid containing the human α-globin gene and a plasmid expressing SV40 large T antigen. The human α-globin gene served as an internal control for efficiency of transfection and RNA recovery. Both the test and reference plasmids carried the SV40 origin of replication, and were therefore replicated in the presence of SV40 large T antigen provided *in trans* by the third plasmid. This served to amplify the signal from the transfected genes so that expression from mutated U2 promoters could be easily detected.

RNAs were analyzed by RNase T_1 protection of various single-stranded RNA probes. To determine the efficiency of transcription as well as the site of initiation, we hybridized RNA from transfected cells to a 5′ RNA probe that is protected across 135 nt by correctly initiated transcripts derived from the transfected test genes (probe A, Figure 1). RNAs derived from cryptic promoters within the vector protect the

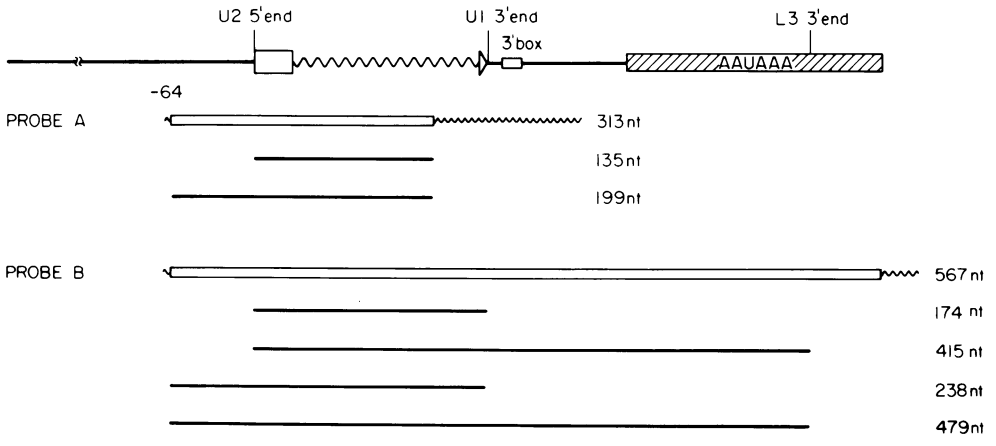

Fig. 1. Top line: Structure of the parent hybrid U2 gene. This hybrid gene consists of the human U2 promoter (thin line), the first 28 bp of the U2 coding region (open box), a fragment derived from the rabbit β-globin gene and inserted in the opposite orientation as in its natural context (wavy line), the last 6 bp of the human U1 gene (arrow head) and 90 bp of U1 3′ flanking sequences (thin line) including the 3′ box, and a fragment derived from Ad2 and carrying the L3 polyadenylation site (stippled box). **Probe A**: riboprobe used for analysis of the 5′ ends of the RNAs. The box represents sequences present in the hybrid construct shown on the top. The wavy lines represent portions of the probes that are not present in the hybrid construct. The portions of the probe protected by correctly initiated RNA (135 nt) and RNA initiated in the vector (199 nt) are indicated. **Probe B**: riboprobe used for analysis of the 3′ ends of the RNAs. The portions of the probe protected by RNA correctly initiated and terminated at the U1 site (174 nt), correctly initiated and polyadenylated (415 nt), initiated in the vector and terminated at the U1 site (238 nt), and initiated in the vector and polyadenylated (479 nt) are indicated.

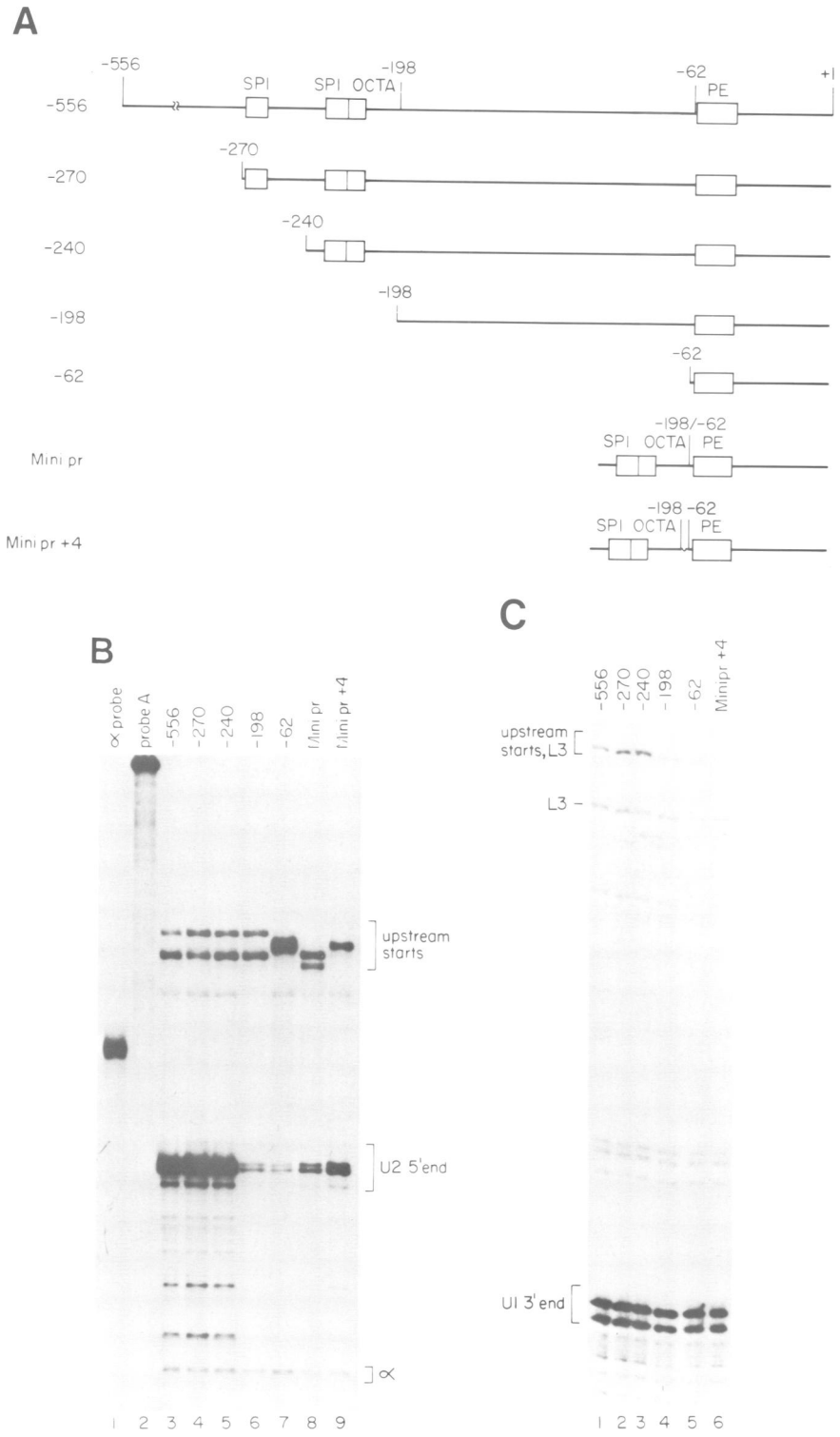

Fig. 2. A. Deletions in the U2 promoter. The lines represent the sequences present in the different mutants. The locations of Sp1 binding site homologies, the octamer motif, and the proximal element as defined in this work are indicated. **B**. Effect of the mutations shown in part A on initiation of transcription. HeLa cells were transfected with a mixture of three plasmids: (i) each of the test constructs indicated on top of the different lanes, (ii) a plasmid carrying the human α-globin gene as an internal reference and (iii) a plasmid encoding the large T antigen of SV40 (see Materials and methods). RNA was hybridized to a mixture of probe A (see Figure 1) and a probe complementary to RNA derived from the human α-globin gene, the hybrids were treated with RNase T_1, and the protected fragments were fractionated on a 6% polyacrylamide sequencing gel. The bands corresponding to RNAs initiated at upstream start sites in the vector (upstream starts), RNA correctly initiated at the U2 cap site (U2 5′ end), and RNA correctly initiated at the α-globin cap site (α) are indicated. RNAs initiated at upstream start sites protect different lengths of probe A, depending on the location of the break of complementarity with the probe. **C**. Effect of the mutations shown in part A on 3′ end formation. HeLa cells were transfected as described above, except that the constructs that gave very low amounts of correctly initiated U2 RNA (−198, −62, and Minipr +4) were modified by insertion of two tandem polyadenylation sites upstream of the U2 promoter (see Materials and methods). RNA was hybridized to probe B (see Figure 1). For the samples −198, −62 and Minipr +4, 20−50 times more total RNA was included in the hybridizations than for the other samples. Bands corresponding to RNA initiated in the vector and polyadenylated (upstream starts, L3), RNA initiated at the U2 cap site and polyadenylated (L3), and RNA initiated at the U2 cap site and terminated at the U1 site (U1 3′ end) are indicated.

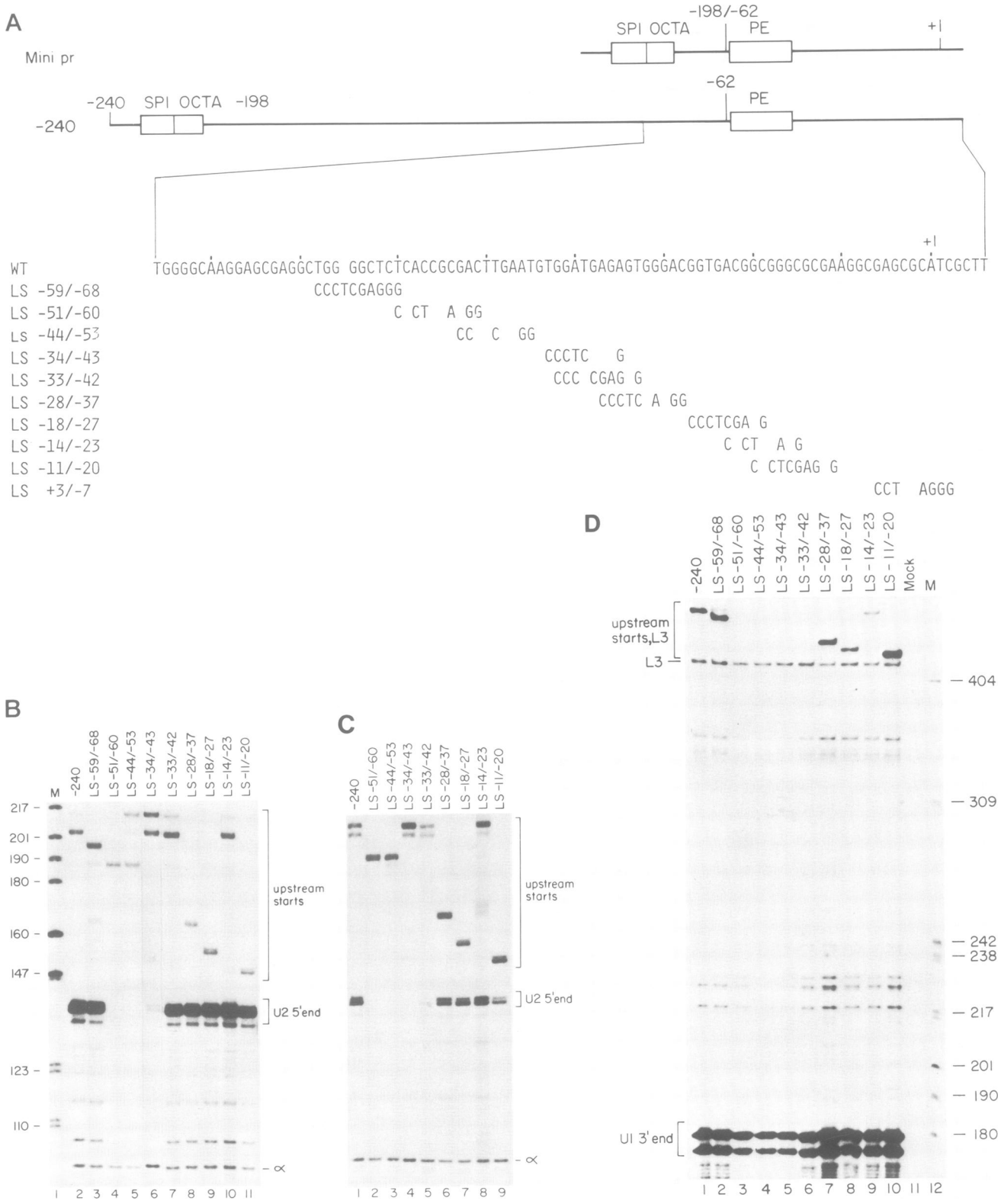

Fig. 3. A. Linker scanning mutations in the proximal region of the U2 promoter. At the top of the figure, the structures of the Minipr and −240 constructs are indicated. The changes caused by each of the linker scanning mutations are shown below the sequence of the U2 proximal region. All linker scanning mutations were introduced into both constructs, except LS−59/−68, which substituted sequences absent in the Minipr construct. **B**. Effect of the linker scanning mutations introduced into the −240 constructs on initiation of transcription. HeLa cells were transfected with the different mutants indicated on top of the lanes and RNAs analyzed as described in the legend of Figure 2B. The protected fragments corresponding to RNA initiated upstream in the vector (bands labeled 'upstream starts') have different sizes; this is because these RNAs contain the different linker scanning mutations, which introduce breaks of complementarity with the probe. **C**. Effect of the linker scanning mutations introduced into the Minipr construct on initiation of transcription. **D**. Effect of the linker scanning mutations introduced into the −240 construct on 3′ end formation. HeLa cells were transfected with the constructs described in Figure 3A, except that the constructs that gave very low amounts of correctly initiated RNA (LS−51/−60, LS−44/−53, LS−34/−43, and LS−33/−42) were modified by insertion of two tandem polyadenylation sites upstream of the U2 promoter (see Materials and methods). The bands corresponding to RNA initiated in the vector (bands labeled 'upstream starts') have different sizes for the same reason indicated above. **Lane 11**: probe B was hybridized to RNA derived from mock-transfected cells.

whole 5′ region of the probe (199 nt) unless there are RNase T_1 sensitive mismatches with the probe due to mutations within the first 64 bp of the promoter. The bands corresponding to these RNAs are labeled 'upstream starts' in the Figures. A second α-globin probe generating a 98 nt protected fragment served to assay expression from the reference plasmid.

We examined where the transcripts derived from the U2 promoter ended by using a probe that maps the 5′ and 3′ ends of the RNA simultaneously (probe B, lower part of Figure 1). This probe extends from upstream of the normal start site of U2 transcription to downstream of the L3 polyadenylation site, allowing us to distinguish among (i) RNAs initiated at the U2 start site of transcription and terminated at the U1 3′ end formation site (174 nt), (ii) RNAs correctly initiated and extending to the polyadenylation site (415 nt), (iii) RNAs initiated at cryptic promoters in the vector and terminated at the U1 3′ end formation site (238 nt), and (iv) RNAs initiated at cryptic promoters and extending to the polyadenylation site (479 nt). In our preliminary experiments, we found that when the U2 promoter was weakened by mutation, RNAs initiating at cryptic promoters within the vector and extending to the polyadenylation site were the predominant RNA species hybridizing to the probe and generated a strong signal at the top of the gel, which obscured other important bands (not shown). Therefore, for 3′ end formation analysis, we inserted two tandem polyadenylation sites upstream of the U2 promoter in the constructs where initiation of transcription at the U2 cap site was severely reduced. This did not affect the efficiency of transcription initiation at the U2 cap site (not shown), but diminished considerably the amount of stable RNA reading through the U2 hybrid gene. (For example, compare bands labeled 'upstream starts, L3' in lane 2 of Figure 3D, where no upstream polyadenylation site was inserted, to those in lane 3, where an upstream polyadenylation site was inserted.)

Analysis of the distal region of the U2 promoter

We tested the role of the distal region of the U2 promoter in snRNA initiation and 3′ end formation by 5′ to 3′ deletion of sequences between positions −556 and −62 upstream of the cap site (Figure 2A). Figure 2B shows the effects of these deletions on U2 initiation. The deleted promoters directed initiation of transcription at a position close to or coinciding with the normal U2 start site (Figure 2B, compare lanes 4−7 to lane 3, bands labeled 'U2 5′ end'). Deletion of the most distal GC-rich box (deletion −240, lane 5) had no effect on initiation of transcription. In comparison to the α-globin signal (labeled α in the Figure), the efficiency of transcription initiation was reduced ~20-fold by deletion of the distal element (deletion −198, lane 6), and ~50-fold by a further deletion to position −62 (deletion −62, lane 7). Twenty or 40% of the wild-type levels of transcription could be restored by inserting the distal element next to the proximal element, depending on the exact spacing and/or the sequence between the two elements (Minipr and Minipr+4 constructs, lanes 8 and 9).

We used probe B (see Figure 1) to monitor the effects of the deletions on 3′ end formation, and the results are shown in Figure 2C. For the mutants that were severely debilitated for U2 transcription, we increased the amount of total RNA in the hybridizations so as to obtain a clearly detectable signal. For ease of interpretation, we normalized all of the 3′ end analyses to obtain equal intensity bands corresponding to polyadenylated RNA initiated at the U2 cap site (bands labeled 'L3' in the Figure). Thus, inhibition of 3′ end formation at the U1 site can easily be visualized as a decrease in the intensity of the bands corresponding to RNA initiated at the U2 cap site and ending at the U1 site (labeled 'U1 3′ end' in the Figure).

As evidenced by the constant intensity of the 'U1 3′ end' bands in Figure 2C, the different deletions had little or no effect on the efficiency of 3′ end formation at the U1 site (lanes 1−6). This result differs from the large increase in polyadenylated RNA we observed previously upon deletion of the U2 enhancer, using a 3′ end-labeled probe that mapped the 3′ ends independently of the 5′ ends (Hernandez and Weiner, 1986). These polyadenylated RNAs were most likely initiated in the vector, since in these experiments, we had not inserted a polyadenylation site upstream of the U2 promoter (see above). Here, we obtained a faint band corresponding to polyadenylated RNAs initiated in the vector (labeled 'upstream starts, L3' in the figure). Interestingly, we never detected a band corresponding to RNAs initiated upstream in the vector and ending at the U1 3′ end formation site, even when polyadenylated RNAs initiated upstream in the vector were clearly visible (see for example Figure 3D, lanes 1 and 2). This shows that passage of the polymerase through the U2 promoter region is not sufficient for 3′ end formation at the U1 site.

Analysis of the proximal region of the U2 promoter

We next analyzed the proximal region of the U2 promoter. We introduced linker scanning mutations between positions −68 and +3 that substituted 5−10 bp at a time without changing the spacing of the different promoter elements (Figure 3A). The only exception was the linker scanning mutation LS−59/−68, which inserted one additional base pair in the U2 promoter region. The mutations were introduced into both the −240 and Minipr constructs shown in Figure 2A, except for mutations LS−59/−68, which substituted sequences that are absent in the Minipr construct (Figure 3A).

The effects of these mutations on initiation of transcription were analyzed as described above, and the results obtained with the −240 and Minipr constructs are shown in Figures 3B and C, respectively. LS−59/−68, which substituted eight base pairs between position −59 and −68, had no effect on the efficiency of initiation (Figure 3B, lane 3). Similarly, modifications in the region extending between positions −37 and −15 did not alter the efficiency nor the accuracy of transcription initiation (Figure 3B, lanes 8, 9 and 10, and Figure 3C, lanes 6, 7 and 8). In sharp contrast, three linker scanning mutations (LS−51/−60, LS−44/−53 and LS−34/−43) that introduced mutations between positions −60 and −35 reduced transcription to barely detectable levels (Figure 3B, lanes 4, 5 and 6; Figure 3C, lanes 2, 3 and 4). This essential region contains a block of sequence that is conserved in mammalian snRNA genes (see Discussion). LS−33/−42 and LS−11/−20 had an intermediate effect. LS−33/−42 had a severe and LS−11/−20 a weak inhibitory effect when inserted in the Minipr construct (Figure 3C, lanes 5 and 9), but both mutations had little or no effect in the −240 construct (Figure 3B, lanes 7 and 11). These differences suggest that these two mutations destroy elements that are redundant in the −240 promoter.

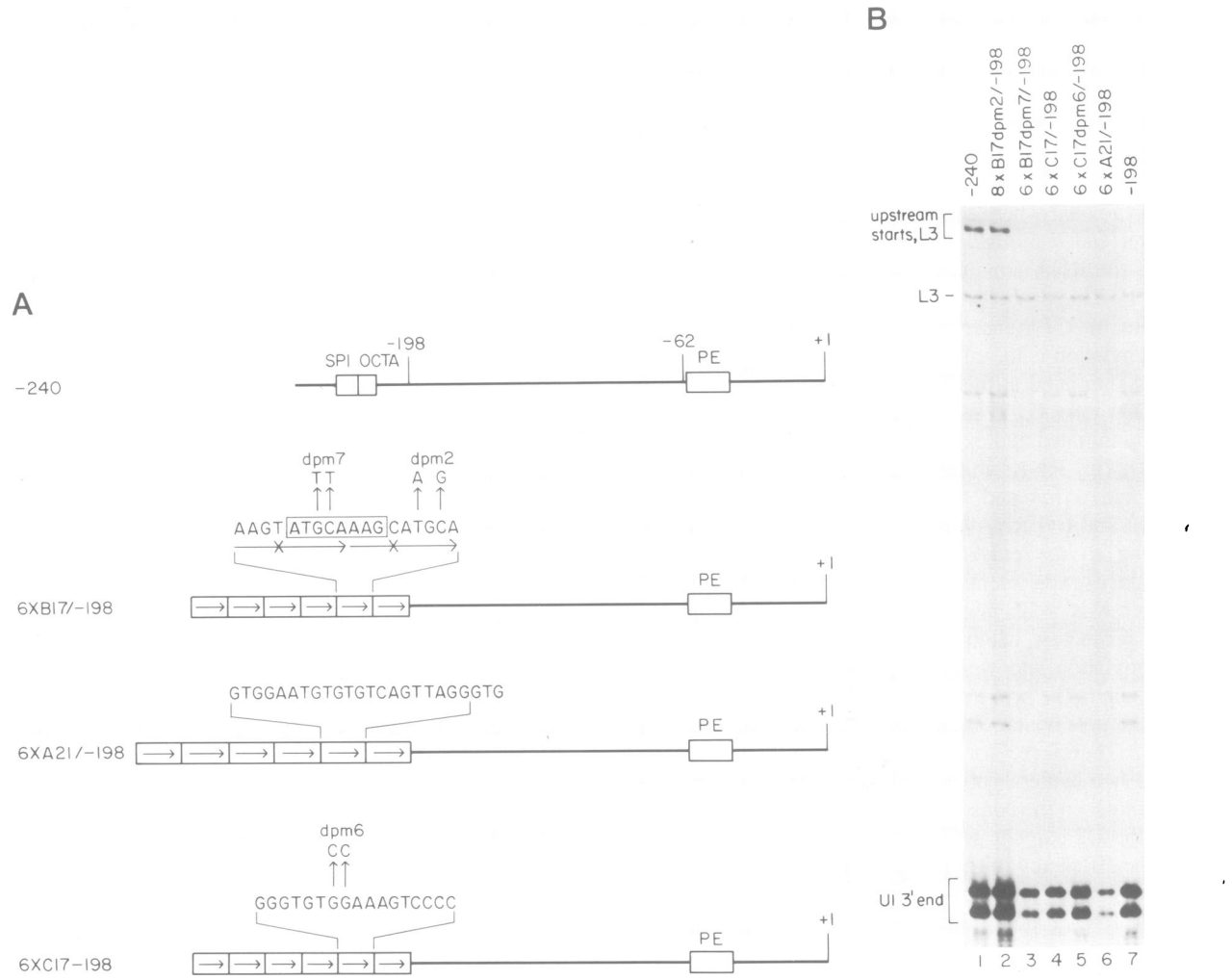

Fig. 4. Replacement of the U2 distal element by SV40-derived synthetic enhancers. The top line shows the −240 construct. In the different replacement constructs, six tandem copies of the SV40 A, B (eight tandem copies for the 8 × B17*dpm*2 element), and C elements were inserted at position −198 upstream of the start site of transcription. The sequence of each repeat and the double point mutations present in 8 × B17*dpm*2, 6 × B17*dpm*7 and 6 × C17*dpm*6 are indicated. In the B element sequence, the Sph motifs are underlined and the octamer motif is boxed. **B.** Effects of the U2 distal element replacements on 3′ end formation. In the constructs 6 × B17*dpm*7/−198, 6 × C17/−198, 6 × C17*dpm*6/−198, 6 × A21/−198 and −198, two tandem polyadenylation sites were inserted upstream of the U2 promoter region.

The last linker scanning mutation shown in Figure 3A, LS+3/−7, modified sequences at and around the start site of transcription. Consequently, the break of complementarity between the probe and RNA derived from this promoter mutant occurred downstream of the 5′ end of the RNA. Initiation of transcription from this mutant was therefore anlayzed by primer extension, which showed that RNAs derived from the LS+3/−7 promoter initiated a few nucleotides upstream of the normal start site of transcription and were much less abundant than RNA derived from the parental construct (not shown). Because the RNA derived from the LS+3/−7 construct is slightly different in sequence from RNA derived from the parental construct, this reduction could be due to post-transcriptional effects such as differences in RNA stability, rather than to transcriptional effects.

The effects of the linker scanning mutations on 3′ end formation were examined using probe B (Figure 1). As shown in Figure 3D with the −240 constructions, none of these mutations had a strong effect on 3′ end formation (LS+3/−7 is not shown). Densitometric analyses indicated that the linker scanning mutations LS−44/−53 and LS−34/−43, which strongly reduced initiation of transcription, inhibited 3′ end formation at the U1 site <2-fold when introduced into the −240 construct. This effect was even less pronounced when the mutations were inserted in the Minipr construct (not shown), and was weak compared to the inhibitory effect observed in hybrid promoter constructs (see below). Thus, none of the substitutions in the proximal region of the U2 promoter had a strong inhibitory effect on 3′ end formation.

Replacement of the U2 distal element by enhancer elements from an mRNA-encoding gene

The deletions and substitutions described above had little or no effect on 3′ end formation at the U1 site, although some of them strongly affected initiation of transcription. We then asked whether replacing a U2 promoter element with regulatory elements from an mRNA-encoding gene would affect 3′ end formation. We replaced the U2 distal element which contains the GC-rich and octamer motifs with several

Table I. Sequence around position −50 in the human U1 (Manser and Gesteland, 1982), U2 (Ares *et al.*, 1985), U6 (Kunkel and Pederson, 1985) and 7SK (Murphy *et al.*, 1986) genes and in the mouse U6 gene (Reddy *et al.*, 1987)

Human U2	−63	C T C	T C A C C G C G A C T T G A A T G T * * * * * * * * * * * * *	G G A T G A G A	−35
Human U6	−68	T G C	T T A C C G T A A C T T G A A A G T * * * * * * * * * * * * *	A T T T C G A T	−39
Mouse U6	−70	A A C	T C A C C C T A A C T G T A A A G T * * * * * * * * * * * * *	A A T T G T G T	−42
Human 7SK	−68	A C T	T G A C C − T A A G T G T A A A G T * * * * * * * * * * * * *	T G A G A C T T	−41
Human U1	−64	A A G	T G A C C G T G T G T G T A A A G A * * * * * * * * * * * * *	G T G A G G C G	−36
Consensus			T N A C C (G/C) (T/A/G) (A/C/G) (T/G) A A A G T		

wild-type and mutant synthetic enhancers. As depicted in Figure 4A, these synthetic enhancers consist of six (or in one case eight) tandem repeats of the genetically defined A, B and C elements of the SV40 enhancer (Herr and Clarke, 1986), and have been shown to stimulate transcription when positioned >2 kb downstream of the human β-globin promoter (Ondek *et al.*, 1987).

The B element of the SV40 enhancer is composed of an imperfect repeat of a 9 bp sequence, the Sph motif (underlined in Figure 4A) (Zenke *et al.*, 1986); the junction of the two Sph motifs (Sph motif II on the left and Sph motif I on the right) forms an octamer motif (boxed in Figure 4A) nearly identical to the octamer motif found in the U2 distal element. The synthetic B element constructs contain a reiterated 17 bp sequence (B17) that upon multimerization recreates the 18 bp Sph II and I sequence. We used two mutant versions of the B17 synthetic enhancer. In 8 × B17-*dpm*2, each of eight reiterated B17 elements contains the double point mutation *dpm*2 (Herr and Gluzman, 1985) that modifies the Sph I motif and destroys enhancer function (Ondek *et al.*, 1987) (see Figure 4A). In 6 × B17*dpm*7, the double point mutation *dpm*7 modifies the octamer motif, but leaves enhancer function intact in HeLa cells (W.Herr, personal communication; see Figure 4A for the location of the point mutations). When the 8 × B17*dpm*2 and 6 × B17*dpm*7 synthetic enhancers were inserted at position −198 upstream of the U2 start site of transcription, replacing the U2 distal element, the pattern of activation was opposite to that observed with the β-globin promoter. The 8 × B17*dpm*2 element, inactive with the β-globin promoter, activated transcription from the U2 promoter to wild-type levels, whereas 6 × B17*dpm*7, very active with the β-globin promoter, barely stimulated U2 transcription. (The effects on initiation of transcription will be described elsewhere; M.Tanaka, W.Herr and N.Hernandez, unpublished results.)

We then examined 3′ end formation with probe B (Figure 1). Surprisingly, although insertion of 6 × B17*dpm*7 (with the octamer motif destroyed) into the U2 promoter did not change the start site of transcription, it inhibited 3′ end formation at the U1 site >4.5-fold (Figure 4B, lane 3). In contrast, 8 × B17*dpm*2 (in which the octamer is intact) slightly stimulated 3′ end formation at the U1 site (Figure 4B, lane 2). Thus, an enhancer element containing a motif similar to a motif found in the U2 distal element stimulated transcription and 3′ end formation, whereas an enhancer containing motifs that are foreign to the U2 promoter did not stimulate transcription and strongly inhibited 3′ end formation at the U1 site.

The U2 distal element was also replaced by the other SV40-derived synthetic enhancers 6 × A21 and 6 × C17. These enhancers, like the 6 × B17*dpm*7 construct, failed to stimulate transcription (not shown). In addition, 6 × A21 had the strongest (>5-fold) inhibitory effect on 3′ end formation we observed in this work (Figure 4B, lane 6). 6 × C17 inhibited 3′ end formation weakly, but the effect was reproducible (Figure 4B, lane 4). Interestingly, a mutant version of the 6 × C17 construct, 6 × C17*dpm*6, which is inactive when tested downstream of the human β-globin gene (Ondek *et al.*, 1987), did not inhibit 3′ end formation (Figure 4B, lane 5). Thus, only elements able to enhance transcription from an mRNA promoter inhibited 3′ end formation at the U1 site.

Discussion

Structure of the U2 promoter

We have analyzed in detail the structure of the human U2 promoter. This analysis shows that the U2 promoter is essentially bipartite, consisting of a distal element characterized previously (Ares *et al.*, 1985; Mangin *et al.*, 1986), which serves to enhance transcription, and of a proximal element whose 5′ border is located between positions −59 and −54 and whose 3′ border is located at position −43. As shown in Table I, a weak sequence similarity can be found (10 out of 18 base pair match) at the equivalent location in the human U1 promoter; this region of the U1 promoter is partially contained in the U1 proximal element as defined by deletion analysis (J.Murphy *et al.*, 1987). Although the proximal elements of the *Xenopus* U1 and U2 genes have been localized to a similar position relative to the cap site (Ciliberto *et al.*, 1985; Mattaj *et al.*, 1985), they have little or no sequence similarity with the human U1 and U2 proximal elements. Thus, the proximal elements of snRNA genes seem to have diverged considerably in different species. Interestingly, however, a good match to the human U2 proximal element can be found in the human and mouse U6 genes (14 and 12 out of 18 base pair match, respectively, see Table I), as well as in the human 7SK gene (10 out of 18 base pair match, see Table I) (Kunkel *et al.*, 1986; Kunkel and Pederson, 1988; Reddy *et al.*, 1987; Das *et al.*, 1988; S.Murphy *et al.*, 1986, 1987). These genes are transcribed by RNA polymerase III, and these sequence similarities in each case are located in a region that is required for efficient transcription (S.Murphy *et al.*, 1987; Kunkel and Pederson, 1988; Das *et al.*, 1988). The proximal element is thus of considerable interest, since it may

represent a binding site for transcription factors shared by RNA polymerase II and RNA polymerase III.

Our analysis also shows that the U2 promoter contains other elements which contribute to a lesser extent to the efficiency of initiation of transcription in our assay. Thus, directly 3′ to the proximal element is an element uncovered by the mutation LS−33/−42. Examination of the sequence in this region reveals weak similarities to the coreA and coreC motifs (Weiher *et al.*, 1983; Ondek *et al.*, 1988) as well as to the GT-I and GT-II motifs (Zenke *et al.*, 1986) of the SV40 enhancer. LS−33/−42, which does not change any of the base pairs on the conserved segment shown in Table I, reduces transcription significantly when introduced into the Minipr construct, but has little or no effect in the −240 construct. This may indicate that the region between position −198 and −62, which is deleted in the Minipr construct, contains element(s) that can compensate for loss of function in the element mutated by LS−33/−42. That the region between positions −198 and −62 contributes to promoter efficiency is also suggested by the reduced levels of transcription derived from the Minipr construct as compared to the wild-type promoter, although this difference could also be due to spacing effects.

The linker scanning mutations LS−11/−20 and LS+3/−7 reduce the efficiency of transcription, the first one only in the Minipr context, the second one in both the Minipr and the −240 constructs. LS−11/−20, like LS−33/−42, may be uncovering an element required only in the context of the Minipr for efficient transcription. This element may correspond to the A element found in the human U1 promoter (J.Murphy *et al.*, 1987).

Sequences involved in 3′ end formation

It has previously been shown that formation of the 3′ end of the human U1 gene requires sequences located in the U1 and U2 promoter regions (Hernandez and Weiner, 1986; Neuman de Vegvar *et al.*, 1986). This observation suggests that the U1 and U2 promoter regions contain one or several binding site(s) for factors involved in 3′ end formation. To identify these sites, we have monitored the effects of the different promoter mutations on 3′ end formation. Deletion of all 5′ flanking sequences up to position −62, just upstream of the proximal element, had no effect on the efficiency of correct 3′ end formation. Similarly, most of the linker scanning mutations in the region between −62 and the cap site had no effect on 3′ end formation; the only exceptions were linker scanning mutations within the proximal element, which inhibited 3′ end formation <2-fold. These results indicate that the U2 promoter region does not contain an element involved solely in 3′ end formation, suggesting that the elements required for 3′ end formation coincide with elements required for initiation of transcription.

It may seem surprising, then, that none of the mutations that reduced initiation of transcription strongly affected 3′ end formation. This suggests that the residual transcription derived from a mutated U2 promoter is qualitatively the same as that derived from a wild-type promoter. Thus, perhaps the reduction in transcription efficiency results from infrequent formation of the transcription complex and not from a change in the composition of this complex. Simply destroying transcriptional elements could not then, reveal which elements are essential for 3′ end formation.

The importance of initiation elements for 3′ end formation is particularly well illustrated by the experiment in which we replaced the U2 enhancer by synthetic enhancers derived from SV40. Each synthetic enhancer that could activate transcription from mRNA promoters inhibited 3′ end formation at the U1 site, and some of these replacements (6 × A21 and 6 × B17/*dpm*7) had the strongest inhibitory effect observed among the different constructs analyzed here. In contrast, synthetic enhancers carrying point mutations that inactivated their ability to enhance transcription from mRNA promoters did not inhibit U1 3′ end formation. Thus, the weak inhibitory effect of 6 × C17 was abolished by the double point mutation *dpm*6, which also suppressed the enhancer activity of the C element for mRNA promoters (Ondek *et al.*, 1987). And 6 × B17/*dpm*7, which is an active enhancer for mRNA transcription, inhibited 3′ end formation strongly whereas 8 × B17/*dpm*2, which is inactive as an enhancer for mRNA transcription, had no deleterious effect. In fact, 8 × B17/*dpm*2 even activates transcription from the U2 promoter (unpublished results), probably because it contains intact octamer motifs similar to the octamer found in the U2 distal element. Thus, introduction of foreign transcription signals into the U2 promoter, and probably foreign transcription factors into the U2 transcription complex, inhibits 3′ end formation. However, introduction of transcription signals such as the octamer motif that belong to the natural U2 promoter has no deleterious effect.

Together, these results suggest that proteins involved in forming the U2 transcription complex are also involved in 3′ end formation. This idea is reinforced by the observation that passage of the polymerase through the whole U2 promoter region is not sufficient to promote 3′ end formation at the U1 site. Indeed, the readthrough transcripts initiated at cryptic promoters within the vector all extend to the polyadenylation site inserted downstream of the U1 3′ box. Therefore, 3′ end formation at the U1 site not only requires U1 or U2 promoter sequences, it requires that the transcription complex be formed at a U1 or U2 promoter.

The effect of transcription complex composition on 3′ end formation may not be unique to snRNA genes. Premature termination downstream of the c-*myc* promoter is abolished when the c-*myc* promoter is replaced by the major late promoter of Ad2 (Bentley and Groudine, 1988). Although this effect may be due to differences in the structure of the RNA or to changes in the distance separating promoter and termination site (Bentley and Groudine, 1988), it is tempting to speculate that in this case termination is also dependent on the composition of the transcription complex, and therefore on promoter sequences. It will be of interest to determine whether other premature termination sites, such as the one reported in the target sequence (TAR) of the HIV *trans*-activator *tat* (Kao *et al.*, 1987), are recognized with different efficiencies when placed downstream of different promoters.

How could the transcription factors binding to the U2 promoter influence an event that can happen at least 506 bp (Hernandez and Weiner, 1986) downstream of the cap site? We have discussed several possibilities previously, in particular the possibility that the U1 and U2 snRNA promoters are recognized by a variant form of RNA polymerase II, perhaps lacking an elongation factor (Hernandez and Weiner, 1986). All the results presented here are consistent with such a model, which is attractive since studies

in prokaryotic systems have shown that several forms of RNA polymerase with different susceptibilities to termination signals exist. Thus, in phage λ, the N and Q proteins modify the RNA polymerase at *nut* and *qut* sites to a terminator-resistant form (Grayhack *et al.*, 1985; Barik *et al.*, 1987 and references therein). Sequences far upstream of the termination site can also influence the efficiency of rho independent termination by *E.coli* RNA polymerase in the absence of any additional protein factor, suggesting that these sequences promote a conformational change of the enzyme to a configuration resistant to termination (Wieland and Chamberlin, 1988). Our data are also consistent, however, with other models such as looping of the DNA template to bring promoter sequences and 3′ box in close proximity.

Materials and methods

Promoter mutant constructions

Parent construct. The parent construct is derived from a pUC13 plasmid carrying a U2 gene marked at position +28 by insertion of an *Xba*I restriction site (Mangin *et al.*, 1986). The U2 sequences downstream of the *Xba*I site were deleted and replaced by a *Taq*I−*Bam*HI fragment derived from the second exon of the rabbit β-globin gene, but with a 50 bp deletion around the internal *Mst*II site and inserted in the opposite orientation as in the natural context. This fragment is followed by a *Sal*I−*Xba*I fragment from the construct −47/−6, *Sal*I series (Hernandez, 1985) carrying the last 6 bp of the human U1 coding region and 90 bp of U1 3′ flanking sequence, a 215 bp fragment containing the L3 polyadenylation site of Ad2 (see Hernandez and Weiner, 1986), and a *Bam*HI−*Hin*dIII 178 bp fragment derived from the plasmid pAO (Zenke *et al.*, 1986) and containing the SV40 origin of replication and the three 21 bp repeats.

5′ deletions. The different 5′ deletions were constructed using natural restriction sites in the U2 promoter. The deletion −247 was obtained from M.Ares (clone NE, Ares *et al.*, 1987). In the deletions that strongly reduced promoter activity, we inserted two tandem early polyadenylation sites from SV40 carried on a duplicated *Bcl*I−*Bam*HI 235 bp fragment (obtained from M.Tanaka) just upstream of the U2 5′ flanking sequence. We determined that insertion of this fragment had no effect on initiation of transcription (not shown), but reduced considerably the amounts of RNAs reading through the U2 promoter sequences from cryptic initiation sites within the vector. These modified constructs were used for 3′ end formation analyses.

Linker scanning mutations. The linker scanning mutations were constructed as described by McKnight and Kingsbury (1982). The marked construct −531/*ins*B10-61 (Ares *et al.*, 1985; Mangin *et al.*, 1986), which contains unique *Bam*HI and *Xba*I sites at positions −61 and +28, respectively, was cleaved with *Bam*HI and treated with *Bal*31 to generate 5′ to 3′ deletions. The resected plasmids were then fitted with *Xho*I linkers, circularized and used to transform DH5. The endpoints of the deletions were determined by double-strand sequencing (Chen and Seeburg, 1985). *Xho*I−*Xba*I fragments were then isolated from some of the deleted plasmids. Similarly, the marked U2 dl-556 construct (Mangin *et al.*, 1986), which contains unique *Eco*RI and *Xba*I sites at positions −556 and +28, respectively, was cleaved at the *Xba*I site and treated with *Bal*31 to generate 3′ to 5′ deletions. The resected plasmids were treated as above. *Eco*RI−*Xho*I fragments were isolated from deletions matching the 5′ to 3′ deletions. Matching fragments from 5′ to 3′ and 3′ to 5′ deletions were then used to reconstruct mutant U2 promoters containing *Xho*I linker substitutions at different positions. For 3′ end formation analyses, tandem polyadenylation sites from SV40 (see above) were inserted upstream of the U2 5′ flanking sequences in the constructs LS−51/−60, LS−44/−53, LS−34/−43 and LS−33/−42.

Replacement of the distal element. The U2 fragment extending from position −247 to −198 and containing the U2 distal element was replaced by *Hin*dIII−*Pst*I fragments (obtained from T.Baumruker and W.Herr) carrying six tandem copies of the SV40 A, B*dpm*2, B*dpm*7 (W.Herr, unpublished), C and C*dpm*6 elements (oligonucleotides A21, MB17, C17 and MC17; Ondek *et al.* 1987). For 3′ end formation analyses, tandem SV40 polyadenylation sites were inserted upstream of the U2 5′ flanking sequence in the constructs 6 × B17*dpm*7/−198, 6 × A21/−198, 6 × C17/−198 and 6 × C17*dpm*6/−198.

HeLa cell transfections

HeLa cells were transfected by the calcium phosphate co-precipitation method. 10 mm dishes were transfected with a mixture of four plasmids: (i) the test plasmid (3 or 13 μg); (ii) the plasmid πSVHPα2 (0.5 μg), which contains the human α-globin gene and an SV40 origin of replication (Treisman *et al.*, 1983); (iii) the plasmid pSVEori- (4 μg), a derivative of pSVE (see Hernandez, 1985) which contains the entire early region of SV40 including the transcriptional enhancer but in which the SV40 origin of replication has been destroyed and (iv) pUC 118 carrier DNA to adjust the total amount of DNA to 20 μg. pSVEori- encodes the SV40 large T antigen, which allows replication of the test plasmid and the πSVHPα2 internal reference plasmid. In this system, efficient expression from the U2 promoter was dependent on inclusion of pSVEori- in the transfections and on the SV40 origin of replication in the test plasmid (not shown). With 13 μg of test plasmid, the cells were saturated for U2 transcription, whereas with 3 μg of test plasmid the assay was in the linear range of response. Each mutant was tested under these two conditions. The results were qualitatively the same, but the different mutations had a more pronounced effect on initiation of transcription at saturating levels of DNA, which were the conditions used in the results shown in Figures 2−4.

RNA preparation

For preparation of cytoplasmic RNA, HeLa cells were rinsed twice with PBS, scraped from the plates with a rubber policeman, collected in 1.3 ml of PBS and tansferred into a microcentrifuge tube. The cells were centrifuged for 5 s and resuspended in 200 μl of ice cold buffer containing 150 mM NaCl, 10 mM Tris−HCl pH 8. 10% NP40 was then added to a final concentration of 0.65% to lyse the cells. The nuclei were pelleted by a 1 min centrifugation in the cold room, and the supernatant was removed to a fresh tube containing 20 μl of 10% SDS and 3 μl of proteinase K, 20 mg/ml. The mixture was incubated at 37°C for 30 min or longer, extracted with phenol, and the nucleic acids were precipitated with ethanol. The RNA preparations were then treated with DNase in the presence of RNasin, and reprecipitated.

RNA analysis

RNase protection mapping was performed as described (Zinn *et al.*, 1983), except that the hybrids were treated with RNase T_1 (2 μg/ml) only. After RNase T_1 treatment, we fractionated half of each sample on a gel. The intensity of the 'α' band in 5′ end analyses and of the 'L3' band in 3′ end analyses was monitored visually, and a second gel fractionation with the RNAs normalized to the intensity of these bands was performed with the remaining material. Quantification of the results was performed by densitometric analysis of the autoradiograms; samples were normalized relative to the 'α' signal for initiation of transcription, and relative to the 'L3' signal for 3′ end formation.

Initiation of transcription was also examined by primer extension, with a mixture of two primers (not shown). One primer hybridized to RNA derived from the reference α-globin gene, and the second primer hybridized to the β-globin portion of the hybrid U2 gene. After hybridization, the primers were extended with AMV reverse transcriptase, and the resulting cDNAs were fractionated on a sequencing gel. This analysis confirmed that the doublet obtained with probe A and labeled 'U2 5′ end' in the Figures is diagnostic of RNA correctly initiated at the U2 cap site.

Acknowledgements

We thank A. Weiner in whose laboratory this work was initiated. We thank M.Ares for the plasmid NE and several *Bal*31 deletions of the U2 promoter; T.Baumruker and W.Herr for fragments containing artificial enhancers; M.Tanaka for a tandem duplication of the SV40 early polyadenylation site; D.Green, J.Duffy and M.Ockler for artwork and photography. We are grateful to M.Gilman, W.Herr, S.Lobo and M.Tanaka for helpful suggestions and discussions during the course of this work, and G.Das, M.Gilman and W.Herr for comments on the manuscript. We also thank J.D.Watson for support and improvements on the manuscript. This work was supported by funds from the Robertson Research Fund (Cold Spring Harbor Laboratory) and by a grant (RO1 GM38810-01) to N.H. from the National Institutes of Health.

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Received on June 8, 1988