The human U1 snRNA promoter and enhancer do not direct synthesis of messenger RNA

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ABSTRACT

We examined the ability of the 5' flanking region sequences of a human U1 RNA gene to direct synthesis of functional mRNA. When fused to chloramphenicol acetyltransferase (CAT) coding region sequences, the upstream sequences of the U1 gene were able to stimulate the synthesis of functional CAT mRNA in 293 cells but not in HeLa cells. Most of the polyadenylated CAT mRNA in 293 cells originated from cryptic promoters in the upstream U1 sequences, but nearly all of the CAT-specific RNA originating at position +1 (relative to the U1 gene promoter) was non-polyadenylated; this confirmed that the bona-fide U1 gene promoter was unable to direct efficient synthesis of poly-A⁺ mRNA. Our results demonstrate that the snRNA gene promoter and enhancer elements, although very efficient in transcription of snRNAs, are unable to direct transcription of polyadenylated mRNAs. However, other sequences in the 5' flanking region of the human U1 gene can activate transcription of functional mRNA, with 5' ends upstream of the normal transcription start site.

INTRODUCTION

SnRNA genes are transcribed by RNA polymerase II (for a review, see 1). Because of the very efficient transcription of these genes in vivo (one initiation per gene every 3-4 seconds) it is tempting to use their promoters to drive the synthesis of mRNAs. However, several features distinguish snRNA promoters from those normally used to make mRNAs (1). Upstream of the coding region are a proximal promoter element (centered around position -50, relative to the U1 cap site) that fixes the point of transcription initiation, and a distal promoter element which acts as an enhancer (upstream of position -200) (2-10). Downstream of the coding region is a highly conserved sequence, the snRNA 3' end signal, which is required for 3' end formation (11-14) and which functions only when an snRNA promoter region

is used to initiate transcription (13,14). Transcripts initiated at an snRNA promoter are not polyadenylated, even if they lack the snRNA coding region and contain normal cleavage and polyadenylation signals (13); instead, their 3' ends are located about 20 nucleotides upstream of the snRNA 3' end signal.

The studies reported here were undertaken to investigate whether an snRNA enhancer and promoter could direct the synthesis of a functional mRNA in tissue culture cells. We determined that the human U1 RNA 5' flanking region was able to direct the synthesis of functional chloramphenicol acetyltransferase (CAT) after transfection of U1-CAT chimeric genes into human 293 cells. Analysis of the RNA in these cells demonstrated the existence of two populations of CAT-specific RNA. One class, with a cap site at position +1 (relative to the U1 promoter) was non-polyadenylated, and thus unlikely to be functional; the other class, with 5' ends mapping within the upstream flanking region, was polyadenylated. We conclude that the functional mRNAs were probably generated from cryptic promoters in the U1 5' flanking region rather than from the U1 RNA promoter itself.

MATERIALS AND METHODS

Construction of recombinant DNAs.

The pSV2cat, pA10cat and pcat3M DNAs (Fig. 1) were kindly provided by G. Khoury, National Institutes of Health, Bethesda, MD (15). The pHU1-1D plasmid carrying the human U1 RNA gene, HU1-1, has been described previously (16).

Sequences from the 5' flanking region of the HU1 gene were inserted into the BglII site of the pcat3M vector, immediately upstream of the CAT gene coding region. The pcat3M/-393 template was prepared by ligation of a BamHI-BglII fragment of pHU1-1D DNA, extending from position -393 to position -6, into the BglII site of pcat3M. XhoI sites at the endpoints of a set of Bal31 generated deletion mutants of pHU1-1D (8) were converted to BglII sites. The resulting BglII fragments, extending from positions -280, -231 or -203 to position -6, were inserted into the BglII site of the pcat3M vector so that position -6 of the HU1 gene was always proximal to the CAT coding region sequences (Fig. 1).

Figure 1. Structure of recombinant DNAs containing the CAT gene. The CAT gene coding region (heavy arrow), SV40 DNA (solid line; 72 base pair repeat or enhancer region designated by the box), and pBR322 DNA (dashed line) are indicated. The structures of the templates (not to scale) are from reference 15. Chimeric pcat3M plasmids, containing regions of the HUI gene upstream from position -6, are also shown; the solid circles and squares represent the HUI gene enhancer and TATA box, respectively (7,8). The small arrow (below the top template) indicates the primer (used in Figure 5) whose 5' end is 253 nucleotides within the CAT coding region.

Fragments from the 5' flanking region of the HUI gene were ligated into the pA10cat vector (which lacks a transcriptional enhancer) at BglII and BamHI sites, upstream or downstream of the CAT sequences, respectively. The chimeric genes were named to reflect the number of HUI gene base pairs present in the fragment.

SVHSplac vector DNA was obtained from M. Green (17). The maxi-U1 Bi33 gene with 5' flanking region sequences extending to positions -393, -280, -231 and -203 (Fig. 2) was inserted into the BamHI site of this vector.

Transfection of cells.

293 and HeLa cells (18) were maintained in Dulbecco modified

Figure 2. Ability of the HU1-1 5' flanking region to stimulate CAT activity was assayed in transfected cells. CAT enzyme activity in extracts of 293 or HeLa cells that had been transfected with pcat3M, pcat3M/-393 and pSV2cat DNAs (cf. Figure 1). The autoradiogram shows the chromatogram which separated [^{14}C]-chloramphenicol (CM) from its acetylated forms (AcCM).

Eagle medium supplemented with 10% fetal calf serum.

Transfections were performed using 6 μg of supercoiled DNA per 60 mm dish of cells. The co-precipitate of calcium phosphate and DNA was washed-off the cells after 24 hours, the cells were refed with fresh medium and harvested 44 to 48 hours after initial exposure to DNA (19,20).

CAT assays.

For CAT assays, each plate of cells was washed several times with 40 mM Tris HCl (pH 7.4), 1 mM EDTA and 150 mM NaCl and incubated 5 min in 1 ml the same buffer. The cells were scraped off the plates, pelleted, resuspended in 0.25 M Tris HCl (pH 7.8) and disrupted either by sonication or by 3 freeze-thaw cycles accompanied by vigorous mixing. The cell debris was pelleted and the supernatant was saved and stored at -70°C . Enzyme activities were assayed in a final volume of 150 μl , which was made from 70 μl of 1 M Tris HCl (pH 7.8), up to 55 μl of cell extract, 0.25 μCi [^{14}C]chloramphenicol (45 mCi/mmol; Amersham),

and 20 μ l of 4 mM acetyl coenzyme A (21). The amount of extract protein added (22) was constant for each assay except for the blank control which contained 55 μ l of 0.25 M Tris HCl (pH 7.8) and the enzyme control which contained 0.01 U of purified CAT enzyme (Pharmacia). The CAT enzyme assay was linear for at least 60 min of incubation, the time used in the experiments presented here.

RNA analyses.

Nucleic acids were isolated from cells by lysis in urea (23) and extraction with phenol/chloroform; the nucleic acids were precipitated with ethanol, and resuspended in 10 mM Tris Cl (pH 8), 1 mM EDTA prior to use.

For analysis by northern blot hybridization, nucleic acids were electrophoresed through a 12% polyacrylamide gel (30:0.8) containing 7 M urea. The RNAs in the U1 RNA region of the gel were transferred to a Gene Screen Plus membrane (New England Nuclear Corp.) by electroblotting with an LKB Transphor unit. The membrane was prehybridized in 0.9 M NaCl, 1% SDS, 10 mM Tris (pH 7.5) and 1 mM EDTA and hybridization was in the same solution containing 10^4 - 10^5 cpm/ml [32 P]-labeled probe. The probe was RNA complementary to the coding region of the HU1 gene, synthesized with SP6 RNA polymerase (Promega, Madison, WI) (24).

For primer extension experiments, nucleic acids were centrifuged through a CsCl cushion and the RNA in the pellet (total RNA) was ethanol precipitated and resuspended in 10 mM Tris HCl pH 7.6, 1 mM EDTA. The polyadenylated RNAs (poly-A⁺) were prepared from the total RNA fraction using oligo-(dT) chromatography (25). A 5' end labeled fragment was derived by exonuclease III digestion of the 102 nucleotide-long PvuIII-EcoRI fragment within the CAT coding region (26). This primer and the RNA samples were resuspended in 30 μ l of 33 mM Tris HCl pH 8.3, 10 mM MgCl₂, 40 mM KCl, 1 mM dithiothreitol, 40 μ g/ml actinomycin D and 1 mM of each deoxynucleoside triphosphate. The reaction was initiated by addition of 5 units of AMV reverse transcriptase (Promega) and incubated at 37°C for 1 hr. The reaction was stopped with 20 mM EDTA and 0.1 M NaOH (final concentrations). After an additional 30 min incubation at 37°C, the nucleic acids were precipitated, resuspended and electro-

TABLE 1: Effect of U1 promoter deletions on CAT activity in 293 cell extracts

<u>Plasmid DNAs</u>	<u>Relative CAT Enzyme Activity</u>
pcat3M/-393	100%
/-280	52%
/-231	25%
/-203	19%
pcat3(Vector)	8%

CAT enzyme activities were assayed in extracts from cells transfected with plasmid DNAs containing different extents of the 5' flanking region of the HU1 gene. The HU1 sequences, extending from positions -6 to positions -203, -231, -280 or -393, were inserted immediately upstream of CAT coding region sequences in pcat3M (see Figure 1).

phoresed for 19 hr at 350V through a 6% polyacrylamide gel (30:0.8) containing 7 M urea.

RESULTS

Sequences in the HU1 5' flanking region stimulate expression of a chloramphenicol acetyltransferase gene.

We introduced the 5' flanking region of the U1 RNA gene into pcat3M, a vector with no eukaryotic enhancer or promoter, to test the ability of the U1 flanking region to drive expression of an mRNA gene. This plasmid, which we call pcat3M/-393, contains the 5' flanking region sequence of the HU1 gene from positions -393 to -6, inserted immediately upstream of the CAT coding region sequences in the pcat3M vector (Figure 1). Human 293 cells were transfected with the pcat3M/-393 and pcat3M DNAs or with pSV2cat DNA (a control in which the CAT gene was driven by an SV40 early promoter and enhancer). As shown in Figure 2, substantial CAT enzyme activity was observed in extracts of 293 cells transfected with either pcat3M/-393 recombinant DNA or pSV2cat DNA. Cells transfected with the pcat3M DNA, lacking an eukaryotic enhancer or promoter, expressed only a low background level of enzyme activity. Very little CAT activity was observed in extracts of HeLa cells transfected with pcat3M/-393 DNA (Figure 2).

The U1 gene enhancer does not activate CAT gene expression.

Some of the sequences within the HU1 5' flanking region that were responsible for the stimulation of CAT activity in 293 cells

Figure 3. Synthesis of maxi-U1 RNA in 293 cells transfected with deletion mutants of the maxi-U1 (Bi33) clone. Cells were transfected with recombinant DNAs constructed from Ψ SVHSplac DNA (vector) and deletion mutants of a human maxi-U1 RNA gene, Bi33, with 5' flanking sequences extending to positions -393, -280, -231 or -203. The autoradiogram of the northern blot shows the amounts of maxi-U1 RNA (198 nt) and endogenous, wild-type U1 RNA (165 nt) that accumulated in these cells. A map of the maxi-U1 clones is presented below the autoradiogram.

were mapped by transfecting cells with a set of chimeric CAT genes containing different portions of the HUI gene 5' flanking region, but no SV40 promoter or enhancer (pcat3M). As shown in Table 1, deletion of progressively larger regions of the U1 5' flanking sequences resulted in a gradual decrease in CAT activity, but deletion of the U1 enhancer (located in the region between positions -231 and -203) (1,7,27) did not cause an abrupt drop in CAT activity. In contrast, the snRNA enhancer greatly affected synthesis of maxi-U1 RNA in 293 cells (Figure 3) or HeLa cells (data not shown) as has previously been demonstrated for this and other cloned snRNA genes (2-10,28) in a variety of cells. The relatively small effect of deletion of this element on CAT expression indicates that the snRNA enhancer does not significantly influence CAT mRNA synthesis from pcat3M/U1 chimeric genes.

To test directly whether the isolated U1 enhancer was capable

Figure 4. Inability of the human U1 gene enhancer to stimulate CAT activity in transfected 293 cells. Extracts were made from 293 cells transfected with pA10cat recombinant DNAs with the 40 bp enhancer-containing fragment inserted upstream or downstream of the CAT gene, in the BglII or BamHI sites respectively (Fig. 1). These sites had been converted to XhoI sites by the addition of linkers, and the fragment was in the same (s) or opposite (op) orientation relative to the direction of transcription as in the HU1-1 gene.

of activating transcription of mRNA genes driven by an mRNA promoter, a 40 bp fragment of the HU1 gene, containing the functional U1 enhancer (from position -230 to -190) (7), was ligated into pA10cat DNA; this latter template contains the CAT gene downstream of the SV40 early promoter, but it lacks an enhancer. Regardless of whether the U1 enhancer fragment was inserted upstream or downstream of the promoter, in either orientation, it was unable to stimulate CAT activity above background levels (Figure 4).

When larger fragments of the U1 5' flanking region were placed in the CAT vector lacking an enhancer, some stimulation of CAT activity was observed (data not shown). However, the magnitude of the stimulation of CAT activity depended on both

Figure 5. Primer extension analysis of CAT-specific RNAs from 293 cells transfected with pSV2cat, pcat3M/-393 and pcat3M DNAs. Total cellular RNA (60 μ g; lanes 1-4) or poly-A⁺-selected RNA (6 μ g; lanes 5 and 6) was hybridized to a 5'-end labeled primer prepared from the PvuII-EcoRI fragment of the CAT gene and transcribed by reverse transcriptase. The size markers are 5'-end labeled MspI restriction fragments of pBR322 DNA. Total RNA from non-transfected 293 cells was used for the control (lane 4). The 5' end of the primer is 253 nucleotides downstream of position +1 of the U1 promoter in pcat3M/-393 DNA. The arrow denotes the (primarily non-polyadenylated) transcript with a 5' end at position +1 of the U1 promoter and the arrowheads indicate polyA⁺ RNAs with 5' ends within the U1 5' flanking region.

the position and orientation of the U1 5' flanking region fragments indicating that this region might have cryptic promoters which could direct transcription through the CAT gene sequences.

Most of the poly-A⁺ CAT mRNA is initiated in the upstream sequences of the HUI promoter.

The 5' ends of CAT RNAs which were transcribed from pSV2cat, pcat3M/-393 and pcat3M templates were mapped by primer extension using a DNA priming fragment from within the CAT gene. As shown in lane 1 of Figure 5, transfection with pSV2cat DNA resulted in the accumulation of two species of CAT RNA. The 5' end of the major RNA (which makes a reverse transcript ~310 nucleotides long) mapped to the major start site of the SV40 promoter. Both the major and the minor pSV2cat RNAs were polyadenylated (lane 5).

Transfection with the chimeric pcat3M/-393 DNA resulted in accumulation of at least five different CAT-specific RNAs (lane 2). The 5' end of the predominant RNA (denoted by an arrow, making a cDNA ~250 nucleotides long) mapped to position +1 relative to the U1 promoter, but only a small fraction of this RNA (and of another one which is about 10 nucleotides longer) was polyadenylated (lane 6). Several polyadenylated CAT-specific RNAs did accumulate in high amounts in cells transfected with pcat3M/-393 DNA (lane 6). These RNAs had 5' ends mapping to positions around -180, -80 and -70 of the upstream U1 sequences (denoted by arrowheads). Presumably, these RNAs arose from mRNA promoter-like sequences in the HUI 5' flanking region. We were unable to detect any CAT-specific RNA in the total RNA isolated from HeLa cells transfected with pcat3M/-393 DNA (data not shown).

DISCUSSION

The U1 gene 5' flanking region sequences drives transcription of the CAT gene in transfected 293 cells, but the transcripts either are non-polyadenylated or have 5' ends which do not correspond to position +1 relative to the U1 promoter start site. The lack of significant levels of polyadenylated RNA with 5' ends at positions +1 is in keeping with our previous discovery that most transcripts made from snRNA promoters cannot be cleaved and polyadenylated (13). Moreover, such transcripts are likely to encounter sequences within the CAT gene that resemble an snRNA 3' end signal well enough (29) to bring about 3' end formation

before the entire mRNA coding region has been transcribed (13). If functional CAT mRNA must be polyadenylated, synthesis of the mRNA responsible for the CAT activity would have to be driven by sequences in the U1 gene flanking region that act like cryptic mRNA promoters.

Thus it is likely that the ability of this region to make mRNA in transfected 293 cells reflects the activity of the cryptic mRNA promoters rather than that of bona-fide snRNA promoter. The 5' flanking regions of snRNA genes contain binding sites for several factors that activate transcription by RNA polymerase II (30-32). Some of these sites may well be responsible for the effects of the U1 gene 5' flanking region on mRNA transcription.

The U1 enhancer normally increases the level of transcription of U1 RNA from the U1 promoter by more than an order of magnitude. The specificity of this enhancer for snRNA gene promoters was demonstrated by its ability to activate expression of an enhancer-less snRNA gene (7 and Figure 3) but not the enhancer-less mRNA gene in pA10cat DNA (Figure 4). Moreover, deletion of the U1 enhancer from the pcat3M vectors had relatively little effect on the level of CAT activity, indicating that the U1 enhancer did not activate the mRNA-like promoters in the HU1 5' flanking sequences (Table 1).

Although the U1 enhancer is unable to affect functional transcription of an mRNA gene (the CAT gene), the converse appears to be true: an mRNA enhancer from SV40 can activate transcription from an enhancer-less snRNA promoter (5). We note that the early SV40 promoter, unlike the promoters for several other genes, is able to compete (albeit weakly) with the HU1 gene for transcription factors in X. laevis oocytes (7).

U1 RNAs with 5' ends corresponding to upstream mRNA start sites have not been detected in 293 cells, HeLa cells or X. laevis oocytes (8,33; J. Murphy, E.T.S and J.E.D, unpublished). Thus, the absence of significant amounts of CAT or CAT mRNA in transfected HeLa cells is not surprising. It is unclear why the upstream cryptic mRNA promoters are used in transfected 293 cells; the presence of the Ela gene product in 293 cells cannot be solely responsible since transfection of HeLa cells with

Ela-producing plasmids (34) does not lead to activation of these cryptic promoters (E.T.S., unpublished). Furthermore, given that these promoters do function in 293 cells, it is unclear why they are not used to make U1 RNAs with extra nucleotides at their 5' ends. Perhaps transcription from these promoters is normally suppressed by other sequences, such as the coding region or the 3' end signal, which are absent from the U1/CAT constructions used here. Also, U1 transcripts starting at mRNA promoters might be difficult to detect since their 3' ends would not respond to the U1 3' end signal (13,14), causing heterogeneity in size and possible instability.

These studies show that expression of chimeric U1/CAT genes is cell specific: moreover, the levels of CAT activity observed in 293 cell extracts do not respond as expected to the presence or absence of elements needed for U1 RNA synthesis. Thus we conclude that the level of CAT activity cannot be used as a measure of U1 promoter strength in cells transfected with chimeric U1/CAT genes and that snRNA gene enhancer and promoter elements are specific for the synthesis of snRNAs.

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