Sequences More than 500 Base Pairs Upstream of the Human U3 Small Nuclear RNA Gene Stimulate the Synthesis of U3 RNA in Frog Oocytes†

Dick Suh,‡ David Wright,§ and Ram Reddy*,‡

Department of Pharmacology, Baylor College of Medicine, One Baylor Plaza, Houston, Texas 77030, and Department of Genetics, M. D. Anderson Hospital and Tumor Institute, Texas Medical Center, Houston, Texas 77030

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ABSTRACT: Small nuclear RNA (snRNA) genes contain strong promoters capable of initiating transcription once every 4 s. Studies on the human U1 snRNA gene, carried out in other laboratories, showed that sequences within 400 bp of the 5′ flanking region are sufficient for maximal levels of transcription both in vivo and in frog oocytes [reviewed in Dahlberg and Lund (1988)]. We studied the expression of a human U3 snRNA gene by injecting 5′ deletion mutants into frog oocytes. The results show that sequences more than 500 bp upstream of the U3 snRNA gene have a 2–3-fold stimulatory effect on the U3 snRNA synthesis. These results indicate that the human U3 snRNA gene is different from human U1 snRNA gene in containing regulatory elements more than 500 bp upstream. The U3 snRNA gene upstream sequences contain an *Alu*I homologous sequence in the −1200 region; these *Alu*I sequences were transcribed in vitro and in frog oocytes but were not detectable in HeLa cells.

Among the six abundant U snRNAs, U3 snRNA is unique in its localization to the nucleolus [reviewed in Busch et al. (1982) and Reddy and Busch (1988)]. Since part of the U3 snRNA is found associated with preribosomal RNA (Prestayko et al., 1970; Zieve & Penman, 1976), U3 snRNA is thought to be involved in the maturation of rRNAs (Busch et al., 1982; Epstein et al., 1984; Parker & Steitz, 1987). Evidence for this proposal was recently obtained by studies using chemical cross-linking agents which showed that U3 snRNA is hydrogen-bonded to a specific region near the initiation site in the external transcribed spacer region of the preribosomal RNA (Maser & Calvet, 1989; Stroke & Weiner, 1989). Recently, it has been demonstrated that intact U3 snRNPs are required for an early processing step of pre-rRNA (Kass et al., 1990). In the case of yeast, deletion of U3 snRNA genes is lethal (Hughes et al., 1987). These data show that U3 snRNA serves an essential cellular function.

Two studies indicated that nucleolar U3 snRNA synthesis may be independent of nucleoplasmic U1 or U2 snRNA synthesis. Toyocamycin, an adenosine analogue, preferentially inhibits synthesis of rRNA in mammalian cells, blocking the conversion of 45S precursor rRNA molecules to 5.8S, 18S, and 28S RNAs. Toyocamycin, at low concentrations (0.2 μg/mL), completely inhibited the synthesis of U3 snRNA, while the synthesis of U1 and/or U2 snRNA was not affected (Hamelin et al., 1973). The synthesis of U3 snRNA was preferentially stimulated compared to U1 or U2 snRNA synthesis in rats treated with thioacetamide (Ro-Choi et al., 1976). These results indicate that U3 snRNA synthesis can be independent of U1 or U2 snRNA synthesis and the synthesis of nucleolar U3 snRNA may involve one or more factors different from those involved in the synthesis of U1 and U2 snRNAs.

The genes coding for U3 snRNA have been isolated and characterized from different genomes. Analysis of rat U3 snRNA genes indicated features common to and different from other U snRNA genes (Stroke & Weiner, 1985). The U snRNA genes coding for U1, U2, or U4 RNAs, characterized thus far, contain an essential proximal sequence element around the −50 region, an enhancer at the −200 region, and a 3′ formation signal at the +10 region [reviewed in Dahlberg and Lund (1988) and Reddy and Singh (1990)]. These three signals are also present in human and rat U3 snRNA genes (Stroke & Weiner, 1985; Suh et al., 1986; Yuan & Reddy, 1989). Since these signals are common to all trimethylguanosine cap containing snRNA genes, the region of the U3 snRNA gene responsible for selective inhibition or stimulation must be elsewhere. Our aim has been to identify the cis-acting elements which are unique to U3 snRNA gene. One such conserved region apparently unique to U3 snRNA genes is the "U3 box". This sequence is present immediately upstream of the enhancer region in both rat and human U3 snRNA genes (Stroke & Weiner, 1985; Suh et al., 1986; Yuan & Reddy, 1989).

To identify the cis-acting elements present in the human U3 gene, 5′ deletion mutants were constructed, and the efficiency of transcription was assayed in frog oocytes. The results show that sequences more than 500 bp upstream of the U3 snRNA gene have a stimulatory effect on U3 RNA synthesis. In this respect, the human U3 snRNA gene appears to be different from the human U1 snRNA gene, where the cloned U1 snRNA gene, containing only 400 bp of upstream sequences, was optimally expressed in frog oocytes (Murphy et al., 1987) and also in mouse cells after transfection [Schenborn et al., 1985; reviewed in Dahlberg and Lund (1988)].

Results

Construction of the Human U3 Maxigene. The clone hU3BS(−2kb/169) contained human DNA corresponding to 2 kb of upstream sequences and 169 bp corresponding to 1–169 of human U3 snRNA inserted between the *Hind*III and *Eco*RI sites of the Bluescript vector. This plasmid DNA was digested with *Bam*HI and *Xba*I, and a DNA fragment corresponding to 166–217 and also 49 bp of the 3′ flanking region [a *Sau*3AI/*Xba*I fragment from clone hU3(−5/+49)] was in-

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‡ Baylor College of Medicine.

§ Texas Medical Center.

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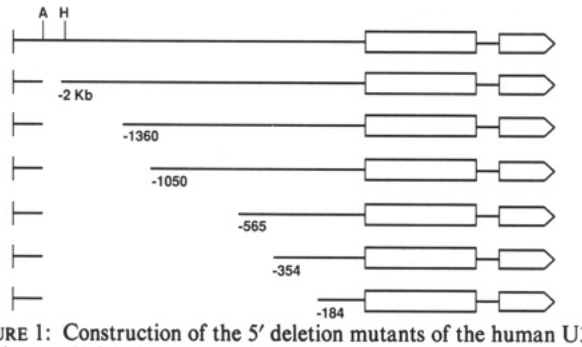

FIGURE 1: Construction of the 5′ deletion mutants of the human U3 maxigene. 5′ deletion mutants were constructed from the hU3-mx-(−2kb) clone, by using the exonuclease III/mung bean nuclease method of Henikoff (1987). The *Apal* (A) *Hin*dIII (H) restriction enzymes were used to create the 3′ and 5′ overhanging termini, respectively. The box indicates the transcribed portion of the U3 RNA gene, and the boxed arrows indicate the direction of transcription. The break in the U3 gene at position 169 is due to the insertion of a polylinker sequence; the inserted sequence is CATGAATTCCTGCAGCC-CGGGGGATCCACTAGTTGTTC. The numbers indicate the base pairs upstream from the U3 initiation site.

serted. This resulted in a 4 bp repeat of the U3 coding sequence and a 34 bp long insert of vector sequence. A schematic representation of the human U3 maxigene is shown in the top line of Figure 1. The resulting maxigene supported a 255-nucleotide-long transcript, which is 38 nt longer than the wild-type human U3 RNA transcript. The 5′ deletion mutants were constructed from this maxigene by using the exonuclease/mung bean nuclease method of Henikoff (1987); the clones designated −1360, −1050, −565, −354, and −184 contain 1360, 1050, 565, 354, and 184 bp, respectively, upstream to the transcription initiation site. The sequence near the 5′ and in the case of every deletion mutant was defined by sequencing the plasmid DNA by the method of Sanger et al. (1977).

Upstream Sequences Have a Stimulatory Effect on the Transcription of the U3 Maxigene in Xenopus Oocytes. To carry out studies on the role(s) of the 5′ flanking regions of the transcription of the U3 RNA, successive regions of the maxigene were deleted. The five deletion mutants utilized in this study are shown in Figure 1. These mutants contained 1360, 1050, 565, 354, or 184 bp of the 5′ flanking sequence and were injected into frog oocytes with ^{32}P-labeled precursor.

The RNA transcripts were fractionated on polyacrylamide/7 M urea gels and subjected to autoradiography (Figure 2A). Lanes 3–7 contain the transcripts from the 5′ deletion mutants, containing successively less of the 5′ flanking sequence. Transcripts of the expected mobility were seen on the autoradiograms, labeled MxU3 for the maxi-U3 snRNA and U6 for the mouse U6 snRNA transcripts. The intensity of the MxU3 transcript obtained from Mx(−2kb) (lane 3) was clearly greater than that for the MxU3 transcript band seen in lanes 4–7, which contained transcripts synthesized from clones containing less of the 5′ flanking region. Since the U6 snRNA gene, used as an internal standard, is transcribed with nearly equal efficiency in all cases (Figure 2A, lanes 3–7), the difference in the levels of MxU3 transcripts is apparently due to the different efficiencies of the deletion mutants. Lane 2 contained the wild-type human U3 snRNA gene used as a control sample. The stimulation by upstream sequences (−565 to −2 kb), observed in the case of human U3 snRNA gene, is interesting in that these results are in contrast to the results obtained by other investigators with the human U1 snRNA gene.

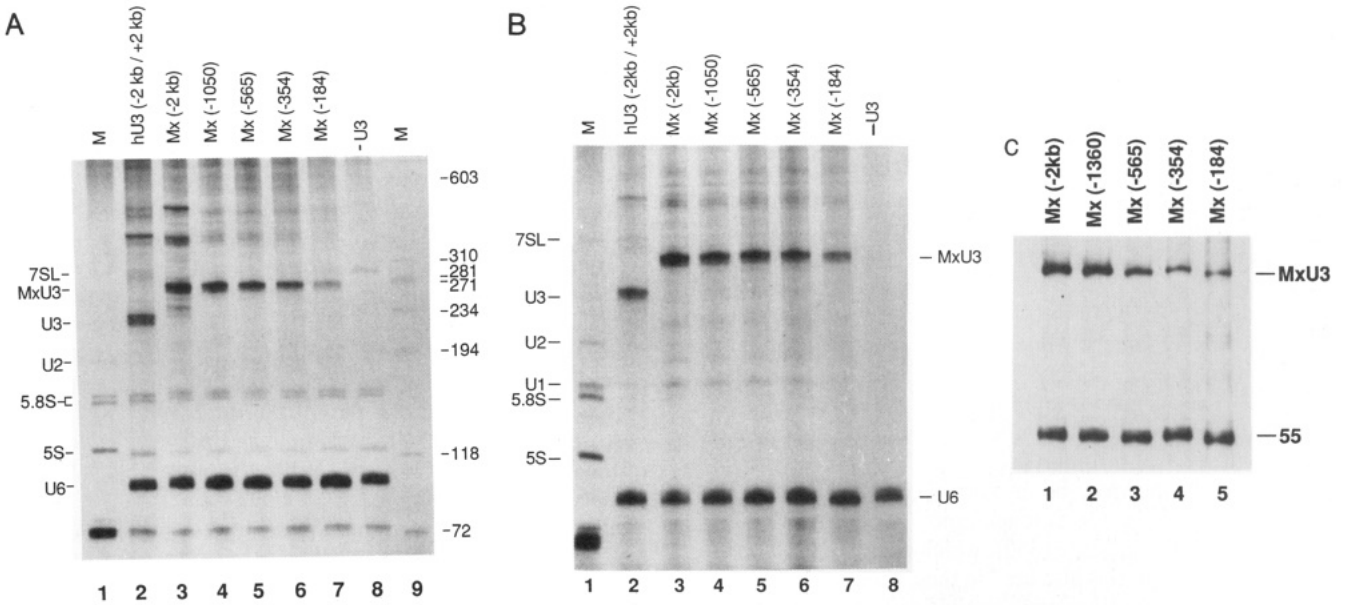

FIGURE 2: (A) Transcription of the 5′ deletion templates in *Xenopus* oocytes. The deletion mutants were injected into *Xenopus* oocytes with [α-^{32}P]GTP, along with a mouse U6 RNA gene (Das et al., 1988) as an internal standard. The RNA transcripts were analyzed on a 5% polyacrylamide/7 M urea gel and subjected to autoradiography. Lane 1, in vivo ^{32}P-labeled HeLa 4–8S RNA; lane 2, hU3(−2kb/+2kb) clone; lane 3, Mx(−2kb); lane 4, mx(−1050); lane 5, Mx(−565); lane 6, Mx(−354); lane 7, Mx(−184); lane 8, no U3 DNA; lane 9, ^{32}P-labeled DNA size markers. Transcripts marked MxU3 and U6 are from the injected DNAs. (B) Hybrid selection of transcripts of samples described in (A). Two cloned DNAs in Bluescript vector were immobilized on nitrocellulose disks: (1) clone hU3(−5/+49) which contains the coding region of U3 and 5 bp on the 5′ side and 49 bp on the 3′ side; (2) a mouse U6 clone [mU6(−23/+149) from Das et al. (1988)]. These filters were used to hybrid-select transcripts from the deletion mutant templates and analyzed on a 5% polyacrylamide/7 M urea gel. Lane 1, in vivo ^{32}P-labeled HeLa 4–8S RNA; lane 2, hU3(−2kb/+2kb) clone; lane 3, Mx(−2kb); lane 4, Mx(−1050); lane 5, Mx(−566); lane 6, Mx(−354); lane 7, mx(−184); lane 8, no U3 DNA. Indicated on the right are the transcripts from the U3 maxigene and the mouse U6 gene. (C) Transcription of the 5′ deletion templates in *Xenopus* oocytes. The deletion mutants were injected into *Xenopus* oocytes with [α-^{32}P]GTP, with a Syrian hamster 5S gene (Hart & Folk, 1982) as an internal standard. The products were analyzed on a 5% polyacrylamide/7M urea gel and subjected to autoradiography. Lane 1, Mx(−2kb); lane 2, Mx(−1360); lane 3, Mx(−565); lane 4, Mx(−354); lane 5, Mx(−184). Unlabeled HeLa nuclear 4–8S RNA was run adjacent to lane 5, stained with methylene blue, and subsequently marked on the autoradiogram. MxU3, the U3 maxigene transcript; 5S, 5S RNA from 5S DNA injected as an internal standard.

The human U3 maxigene used in this study contained 38 extra nucleotides near the 3′ end of the U3 RNA (Figure 1). To verify that the U3 maxigene initiates and terminates accurately, the maxi-U3 RNA was fingerprinted after complete T1 RNase digestion. Every oligonucleotide expected from the wild-type U3 RNA was found. In addition, five oligonucleotides from the inserted polylinker sequence were also found (results not shown). Therefore, the maxigene supports the synthesis of maxi-U3 RNA similar to the unmodified U3 gene.

As is most evident in lanes 2 and 3 of Figure 2A, other bands were seen which did not correspond to the transcripts of expected size, and these were not endogenous *Xenopus* RNA transcripts. It seemed likely that these unexpected transcripts were being synthesized from the upstream regions of the U3 snRNA gene. This raised the possibility that the intensity of the band seen for MxU3 in lane 3 might contain not only MxU3 but also transcripts unrelated to U3 snRNA. If, for example, these unexpected transcripts were synthesized by sequences upstream of position −585, the observed stimulation of Mx(−2kb) might be an artifact of comigrating transcripts unrelated to U3 snRNA.

To rule out this possibility, the total RNA transcripts analyzed in Figure 2A were hybrid-selected by using cloned U3 DNA containing only the transcribed portion and a U6 snRNA gene. The hybrid-selected RNAs were eluted and analyzed on a polyacrylamide/7 M urea gel. As shown in Figure 2B, lane 3, which corresponds to the MxU3(−2kb) clone, showed the greatest intensity of the MxU3 band. Again, the mouse U6 RNA transcripts, which were used as an internal standard, were expressed at approximately the same levels (Figure 2B, lanes 2–8). This hybrid-selection experiment shows that the different levels of intensities of the MxU3 transcripts seen in lanes 3–7 are indeed the result of different levels of MxU3 transcription, and not due to some aberrant transcripts which may be comigrating with the MxU3 transcript.

Figure 2C shows the results of a similar experiment as in Figure 2A, except that the 5S gene was used as an internal standard. It has been shown that the U6 snRNA gene, which is transcribed by pol III, shares several factors with the RNA polymerase II genes [reviewed in Dahlberg and Lund (1988) and Reddy and Singh (1990)]. To rule out the possibility that the U6 gene may somehow interfere with the transcription of the U3 snRNA gene, 5S RNA gene was used as an internal standard. The 5S gene is an extensively characterized gene that has been shown to be synthesized by RNA polymerase III. No known transcription factor for RNA polymerase II genes has been shown to be involved in the synthesis of 5S RNA [reviewed in Geiduschek and Tocchini-Valentini (1988)]. In addition, Mx(−1360), which contains 1360 bp of the upstream region, was also used in these series of injections. Nevertheless, the findings were similar to the results shown in Figure 2A. The MxU3 band from the Mx(−2kb) clone (Figure 2C, lane 2) had the highest intensity, and Mx(−1360) gave a similar level of transcription. Mx(−565) (lane 4) showed a severalfold lower level of transcription.

Quantitation of U3 snRNA Transcripts from Deletion Mutants. In order to get a quantitative assessment of the stimulatory effect of the upstream regions on the level of MxU3 transcription, the autoradiograms were quantitated by a scanning densitometer. The results, as shown in Figure 3, indicate that the clone containing 2 kb of the 5′ flanking region appeared to transcribe the MxU3 RNA at 2–3-fold more compared to the clone containing 565 bp of the 5′ flanking region (compare lanes −2kb and −565, Figure 2A,C). Quantitation of the other deletion mutants showed a slight (less than 1.5 times) stimulatory effect of Mx(−1025) compared to Mx(−565). Comparison of the levels of transcription between Mx(−565) and Mx(−354) was not significant. As is consistent with the findings for the other U snRNA genes [reviewed in Dahlberg and Lund (1988)], Mx(−184) showed a significant decrease in the level of transcription compared to the Mx(−565) clone.

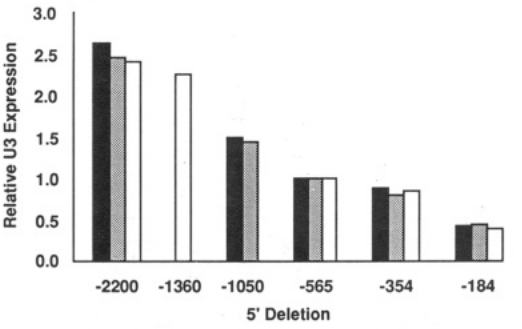

FIGURE 3: Quantitation of transcription of the U3 maxigene deletion mutants. As described in Figure 2, different U3 snRNA gene constructs were transcribed in *Xenopus* oocytes. The RNAs were isolated and fractionated on polyacrylamide gels. A scanning densitometer was used to quantitate the ratio of U3 gene expression to a control. The abscissa shows the deletion mutants used (i.e., −565 represents the U3 snRNA gene mutant with 565 bp upstream of the cap site). The synthesis from the −565 mutant is taken as 1, and the increase or decrease in relation to this mutant is shown on the ordinate. The ratios are plotted as a function of the U3 deletion mutant from which they are obtained. The black bars are from quantitation of U3 versus a mouse U6 snRNA gene control from total transcripts. The dotted bars are from the quantitation of U3 versus a mouse U6 snRNA gene from hybrid-selected samples. The white bars are from the quantitation of U3 versus a Syrian hamster 5S gene from total transcripts.

Sequence of the 5′ Flanking Region of the Human U3 Gene. Since the 5′ flanking sequences of the U3 gene showed a stimulatory effect on transcription, we further characterized this region; 1536 base pairs of the human U3 snRNA gene 5′ flanking sequence were sequenced (Figure 4), by using the dideoxy chain termination method. Analysis of this sequence showed stretches of DNA that were homologous to human *Alu*I sequences. Since *Alu*I sequences are members of the highly repetitive sequences (approximately 300 000 copies per haploid genome), the presence of an *Alu*I sequence near the U3 gene was not surprising. The interesting finding was that these *Alu* sequences, or the region very near them, were affecting the rate of transcription from the U3 snRNA gene.

Synthesis of an Alu Transcript by the Upstream Sequences of the Human U3 RNA Gene. When the human U3 snRNA gene containing 2 kb of the 5′ flanking sequences was transcribed in vitro, two prominent RNA transcripts and a third band with less intensity were consistently observed (Figure 5, lanes 4 and 5). The two prominent bands, designated T450 and T600, were fingerprinted for identification. Interestingly, T450 and T600 RNAs were structurally related to each other but not related to human U3 RNA (data not shown).

T450 RNA Is Transcribed Also in Frog Oocytes. To study whether the T450 and T600 transcripts observed during in vitro transcription are also observed in vivo, the U3 DNA was injected into frog oocytes, and the synthesized RNAs were fractionated on polyacrylamide gels. An autoradiograph of the RNA transcripts synthesized in frog oocytes is shown in Figure 5 (lanes 2 and 3). The results indicate that the T450 RNA observed in in vitro studies is also synthesized in the frog oocytes (compare lanes 2 and 3 with lanes 4 and 5 of Figure 5). However, the T600 RNA was not observed in the frog

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                (-1536) TGATCTTGGCTCACTG CAGCCTCTGCCTCCCGGGTT -1501
CAAGCAATTCTCCTGCCTCA GCCTCCCCAGTAGCTGGGAT TACAGGCGCAGGCCACCACG -1441
CCCGGCCAGTTTTTGTATTT TTAGTAGAGACGAGGTTTCA CCACGTTGGCCAGGATGGTC -1381
TTGATCTCCTGACCTGGTGA TCCTACCCGCCTCGGCCTCC CAAAGTGCTAGGATGACAGG -1321
CGTGAGCCACCGTGCCCGGT CCAGTTGTGTTTTTATGCAC TGGCAAGGAACAATTCAAAA -1261
ATGTAATTAAGAAAACCGCT GGGCGTGGTGGCTCACGCCT GTAGTCCCAGCACTTTGAGC -1201
GGCCGAGGCGGGTGGATCCC TTGATCCCAGGAGTTCAAGA CCATCCTGGGCAATGTGGTG -1141
AAACCCCCTCCCTACAAAAA ATACAAAAAATTAGCGGCAG TGATGGCATGTGCCTACGAT -1081
CCCAGCTACTCAGGAGGCTG ACGTGGGAGGATCACCCGAG CCTGGGGGTCAAGGCTGCAG -1021
TGAGCTGTCACATCATGCAT CATTGCACTCCAGCCTGAAA AAGGAGTGAAATTCTGCAAC  -961
ATGTTACAACGTGAATGAAC CTTGAAAACGTCATTCTAAG TGAAATAAGCCAGATACAAA  -901
AGGACAATATTGCATGTTTC CACTTATAGAGATACCTACA AGAATCAAATTCATAGAGAC  -841
GGAAAGTAGAATAGTGGTTA AAGGGGTCTGGGCGGAGGGA GGAAAGGGAAGTTTGTTTTA  -781
CGGGTAGAGTTTCAGTTTGG GATGTCGAAAAAGTTCTGGA GATAAATAATGGTGATGGTT  -721
ACATGCCAATGGCTACACGA ATGTACTTAATGCCACTGAA TTGTATATGTGAAAAATGGT  -661
TAAAATGGTAAATTTTGTAT CTATTTAATACCATCCCCCC CTAAAAAAAAAATTGTTTTT  -601
AAGAGTCAAGATCTCACTCT GTCTCCCAGGCTGGGGTGCA GTGGGGTAACTGATCAGAGC  -541
TCACTGCAGCTTTGAACTCA GCCAGCTTCCCTGACTCAAA CGATCAATCCGCTTCAGCCT  -481
CCCGAGTAGCTGGGACTACA GACGGTGCCATCACGCCCAG CTCATTGTTGATTCCCGCCC  -421
CCTTGGTAGAGACGGGATTC CGCTATATTGCCTGGGCTGG TGTCGAACTCATAGAACAAA  -361
GGATCCTCCCTCCTGGGCCT GGGCGTGGGCTCGCAAAACG CTGGGATTCCCGGATTACAG  -301
GCGGGCGCACCACACCAGGA GCAAACACTTCCGGTTTTAA AAATTCAGTTTGTGATTGGC  -241
TGTCATTCAGTATTATGCTA ATTAAGCATGCCCGGTTTTA AACCTCTTAAAACAACTTTT  -181
AAAATTACCTTTCCACCTAA AACGTTAAAATTTGTCAAGT GATAATATTCGACAAGCTGT  -121
TATTGCCAAACTATTTTCCT ATTTGTTTCCTAATGGCATC GGAACTAGCGAAAGTTTCTC   -61
GCCATCAGTTAAAAGTTTGC GGCAGATGTAGACCTAGCAG AGGTGTGCGAGGAGGCCGTT    -1
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FIGURE 4: Sequence of the upstream region of the U3 RNA gene. The noncoding strand of the upstream region of the human U3 RNA gene is shown. The nucleotide sequence of both strands was obtained by the dideoxy chain termination method (Sanger et al., 1977). The numbers are relative to the initiation site of the U3 RNA (−1 is the first nucleotide upstream of the initiation site). Putative pol III termination signals (the T stretches) are underlined.

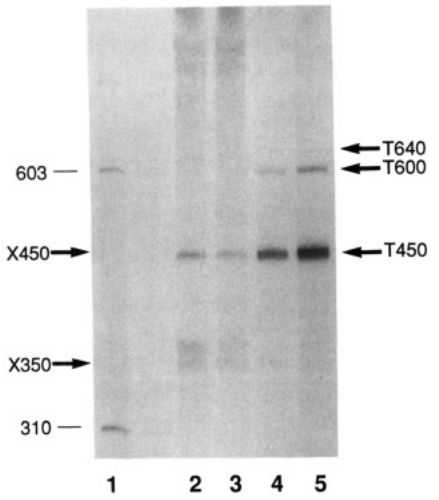

FIGURE 5: Synthesis of an *Alu* transcript by the upstream sequences of the human U3 RNA gene. The U3 maxigenes were transcribed in *Xenopus* or in vitro in the presence of [α-^{32}P]GTP. The transcripts were isolated and analyzed on 5% polyacrylamide/7 M urea gels. Lane 1, ^{32}P-labeled ϕX174 DNA, digested with *Hae*III; lane 2, Mx(−1360) transcribed in *Xenopus*; lane 3, Mx(−2kb) transcribed in *Xenopus*; lane 4, Mx(−2kb) transcribed in vitro; lane 5, Mx(−1360) transcribed in vitro. The in vitro transcriptions were done by using HeLa Weil extracts. The arrows indicate the *Alu* transcripts detected. X450, ~450-nt-long transcript from *Xenopus*; X350, ~350-nt-long transcript from *Xenopus*; T450, ~450-nt-long transcript synthesized in vitro; T600, ~600-nt-long transcript synthesized in vitro; T640, ~640-nt-long transcript synthesized in vitro.

oocytes. Instead, a broad diffuse RNA band, labeled X350, was observed (Figure 5, lanes 2 and 3). This RNA was found by fingerprinting to be structurally related to human U3 RNA

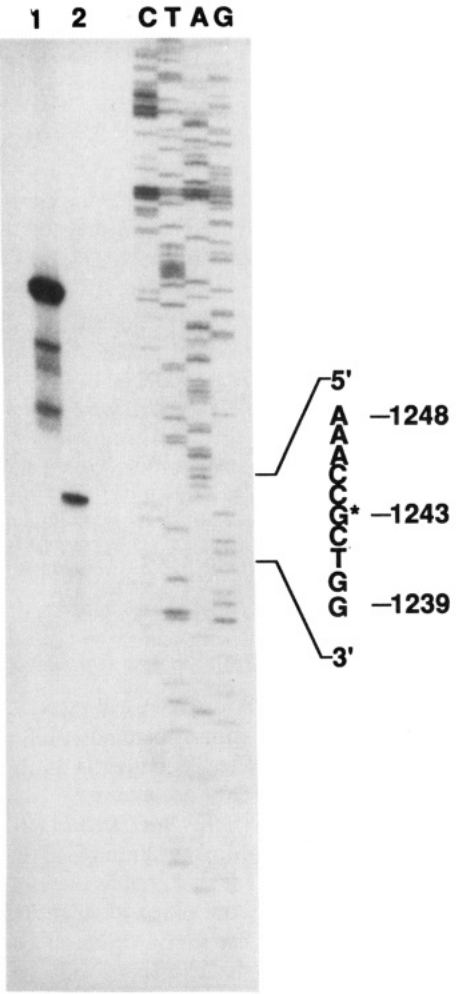

FIGURE 6: Primer extension analysis of the upstream *Alu* transcript initiation site. With an oligonucleotide complementary to the *Alu* transcript, a cDNA strand was synthesized by using reverse transcriptase. The reaction products were electrophoresed alongside sequence ladders of the corresponding DNA region. The sequence reactions are labeled C, T, A, and G. Lane 1 contains the reaction product for the HeLa U1 RNA that was used as a control. Lane 2 contains the reaction product for the *Alu* transcript. The sequence of the DNA near the region of initiation is indicated to the right of the gel. The initiation nucleotide G is marked with an asterisk. The numbers indicate the position relative to the initiation site of the U3 RNA; 5′ and 3′ indicate the orientation of the DNA.

(results not shown) and may represent some aberrant transcription initiation of human U3 RNA genes in frog oocytes.

T450 and T600 RNAs Are Synthesized by RNA Polymerase III. The sensitivity of the T450 and T600 RNAs to α-amanitin was used to evaluate the type of RNA polymerase involved in the synthesis of these RNAs. There was no inhibition at 1 μg/mL concentration, and the concentration of α-amanitin had to exceed 200 μg/mL to inhibit the synthesis of the T450 and T600 in vitro (data not shown). Therefore, it is concluded that RNA polymerase III is responsible for their synthesis.

Primer Extension Analysis To Determine the Initiation Point of the Alu Transcript. *Alu* transcript was synthesized in vitro and isolated on a polyacrylamide/7 M urea gel, and the 5′ terminus of the T450 *Alu* transcript was determined by primer extension analysis. By use of an oligonucleotide complementary to positions −1143 to −1159, the labeled primer was extended with reverse transcriptase. As a control reaction, HeLa 4–8S RNA was used with an oligonucleotide specific for the U1 RNA at position 123. The products were run on a sequencing gel, alongside the sequencing reaction products

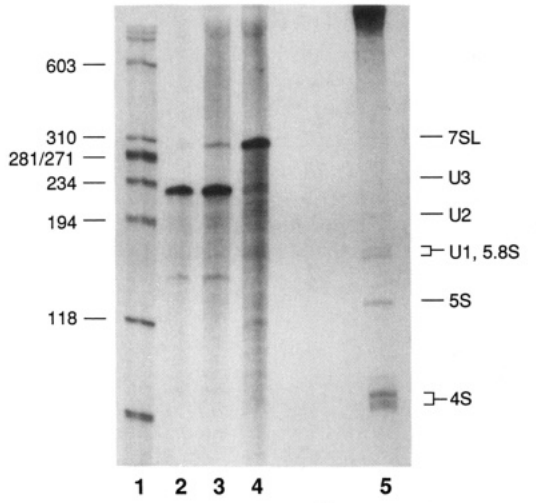

FIGURE 7: Hybrid selection of in vivo ^{32}P-labeled HeLa cell RNA. HeLa cells were ^{32}P labeled in vivo, and its RNAs were isolated. DNA clones were immobilized ont nitrocellulose filters and used to hybrid-select the ^{32}P-labeled RNA. Lane 1, ϕX174 DNA, digested with *Hae*III as a molecular weight marker; lane 2, clone hU3-(−5/+49), lane 3, clone Mx(−2kb); lane 4, clone containing *Alu*I sequences [pDL 41-48 from Ullu and Weiner (1985)]; lane 5, in vivo ^{32}P-labeled HeLa total RNA.

of the DNA. This result (Figure 6) places the initiation nucleotide at position −1243. The T600 and T640 RNAs also initiated at position −1243 (data not shown).

T450 and/or T600 RNAs Are Not Detectable in HeLa Cells. Since the T-450 RNA was synthesized in in vitro transcription systems as well as in *Xenopus* oocytes, the following experiment was carried out to see whether these RNAs are present in HeLa cells. The HeLa cells were uniformly labeled with [^{32}P]orthophosphate in tissue culture, and the labeled RNAs were hybridized to DNA clones. As shown in Figure 7, when the RNAs were hybridized to clone hU3-5/+49 (lane 2), only the U3 RNA was hybrid-selected. In lane 3, clone Mx(−2kb) hybrid-selected the U3 RNA; in addition, the 7SL RNA (which has high homology to *Alu* sequences) was also hybrid-selected. The T450 or T600 RNA transcripts were not detectable by this method. In lane 4, a plasmid containing the *Alu*I sequence hybrid-selected the 7SL RNA; again, the T450 or T600 RNAs were not detectable. These results indicate that these RNAs are not made in vivo. Alternately, if these transcripts are made, they may have a short half-life.

Discussion

The main finding of this study is that the regions upstream of −565 of the human U3 gene have a stimulatory effect on U3 RNA synthesis. The results from studies on U1 and U2 RNA genes [reviewed in Dahlberg and Lund (1988)] showed that sequences within 400 bp upstream of the initiation site are sufficient to transcribe these RNAs at maximal levels. Although Murphy et al. (1987) have shown that the human U1 snRNA gene requires the region −335 to −395 to compete with other U snRNA genes, this region did not affect the level of transcription. The upstream sequences contain an *Alu*I transcription unit, which can be transcribed in vitro.

Alu sequences have been found in the 5′ flanking elements of a number of genes (Duncan et al., 1979; DiSegni et al., 1981; McKinnon et al., 1986; Oliviero & Monaci, 1988; Hudson et al., 1990; Parmentier et al., 1989; Salbaum et al., 1988) and in some cases were shown to enhance the transcription of the neighboring pol II transcription units. There are at least two mechanisms by which *Alu* sequences seem to exert their enhancing effects. In one mechanism, the *Alu* sequences appear to act somewhat as traditional enhancers. That is, through the binding of transacting factors, the *Alu* sequences increase transcription from nearby pol II promoters. This would be the case for the haptoglobin gene and possibly in the case of the human U3 snRNA gene (Duncan et al., 1979). In addition, this is consistent with the accumulating evidence showing that there are a number of factors and sequences interchangeably recognized by pol II and pol III (Nakataki et al., 1990; Lobo et al., 1990; Bark et al., 1987; Kunkel & Pederson, 1988; Lobo & Hernandez, 1989).

The second mechanism of *Alu* enhancement, which was recently proposed for the ϵ-globin gene, involves transcriptional interference (Wu et al., 1990; Allan & Paul, 1984). In contrast to the above described enhancer roles for *Alu* sequences, it has also been reported that an *Alu* sequence from an African green monkey can act as a negative regulatory element (Saffer & Thurston, 1989).

The mechanism by which the upstream sequences are stimulating transcription of U3 RNA is not known. It appears likely that the *Alu* transcription unit present in front of the human U3 gene is enhancing transcription, acting as a traditional enhancer similar to that observed in some of the above studies [for example, see Oliviero and Monaci (1988)]. There are several questions that remain to be investigated. It is not known which particular DNA sequence motif is responsible for the stimulation. Finer deletons of the region between −1360 and −1050 should provide the answer to this question. It will also be interesting to know whether the stimulation is transferable to other genes, e.g., other U snRNA genes and mRNA genes.

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Phased Adenine Tracts in Double-Stranded RNA Do Not Induce Sequence-Directed Bending†

Yuh-Hwa Wang, Michael T. Howard, and Jack D. Griffith*
Lineberger Cancer Research Center, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27514
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ABSTRACT: Tracts of four to six adenines phased with the DNA helix produce a sequence-directed bending of the helix axis. Here, using gel electrophoresis and electron microscopy (EM), we have asked whether a similar motif will induce bending in a duplex RNA helix. Single-stranded RNAs were transcribed either from short synthetic DNA templates or from *Crithidia fasciculata* kinetoplast bent DNA, and the complementary single-stranded RNAs were annealed to produce duplex RNA molecules containing blocks of four to six adenines. Electrophoresis on polyacrylamide gels revealed no retardation of the RNAs containing phased blocks of adenines relative to duplex RNAs lacking such blocks. Examination by EM showed most of the molecules to be straight or only slightly bent. Thus, in contrast to DNA duplexes, phased adenine tracts do not induce sequence-directed bending in double-stranded RNA. Analysis of the distribution of molecule shapes for the highly bent *C. fasciculata* DNA showed that the adenine blocks do not act cooperatively to induce DNA bending and that the molecules must equilibrate between a spectrum of bent shapes.

The folding of natural RNA creates molecules of great structural complexity. As seen in ribozymes and ribosomal RNA, folded RNA contains segments of perfectly and imperfectly paired duplexes joined by regions containing nonpaired bulges of varying size. The duplex regions themselves may contain unusual sequence arrangements, which in duplex DNA can create bends, left-handed helices, cruciforms, and slipped structures (Wells, 1988). A major challenge in understanding RNA folding is to understand the structure of each of these elements alone. In some cases, lessons can be taken from what is known for duplex DNA; as described below this appears to be true for bulges. In this paper we have investigated the possibility of "RNA bending" by phased blocks of oligo(rA).

In duplex DNA, bulges of one to five bases have been shown to create stiff kinks that can cause significant electrophoretic retardations of the bulge-containing DNA (Bhattacharyya & Lilley, 1989; Hsieh & Griffith, 1989; Rice & Crothers, 1989). On the basis of this finding, the laboratories of Lilley and Draper (Bhattacharyya et al., 1990; Tang & Draper, 1990) showed that bulges introduce kinks into the RNA helix, the magnitude depending on the number and types of bases in the

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* To whom correspondence should be addressed.

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