A U6 snRNA gene with an internal promoter is juxtaposed to an snRNP protein sequence within an intron of a human G protein gene

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ABSTRACT

A complex locus on human chromosome 1 brings together sequences homologous to a G protein and two components of the RNA processing machinery of eukaryotic cells. Specifically, the seventh intron of the human G3α gene contains a fusion of a partial snRNP E protein pseudogene to a variant U6 snRNA gene. The novel U6 sequence contains nine point mutations and a one nucleotide deletion relative to the major U6 gene from humans. Unlike all other vertebrate U6 genes characterized to date, the variant U6 gene is efficiently transcribed by RNA polymerase III even in the absence of all natural flanking sequences. The union of elements from the signal transduction pathway and the RNA processing machinery suggests the possibility of functional interplay.

INTRODUCTION

Small nuclear ribonucleoprotein (snRNP) complexes containing snRNAs U1–U13 are integral components of the RNA processing machinery of eukaryotic cells (1–3). The spliceosomal snRNPs (those containing snRNAs U1, U2, U5, and U4/U6) have each been shown to be required for efficient excision of introns from primary gene transcripts (4). The protein components of the spliceosomal snRNPs have been studied extensively in the past few years, and it is now clear that the U1, U2, U5, and U4/U6 snRNPs each have a distinctive complement of snRNP proteins. However, all of these snRNPs do share a subset of common snRNP proteins, including a ‘core’ of small proteins that have been designated D, E, F, and G (5).

The U4/U6 snRNP is unique among this group of snRNPs that are required for mRNA splicing in that it contains two base-paired snRNAs (6,7). Additionally, the U6 component of the U4/U6 snRNP is atypical in that it has a unique cap structure, lacks the binding site for the common snRNP proteins, and is synthesized by RNA polymerase III rather than RNA polymerase II (8–10). Surprisingly, analysis of the regulation of transcription of the major U6 genes in mammals has demonstrated that sequence elements upstream of U6 genes are both necessary and sufficient for correct initiation of transcription (11,12). Thus, the internal promoter elements common to many other polymerase III transcripts do not appear to play an important role in the expression of the most abundant U6 RNAs found in mammalian cells.

Further interest in U6 has been stimulated by studies of U6 genes in yeast. Sequence analysis reveals that U6 is the most conserved spliceosomal snRNA through evolution, suggesting that it must have a particularly crucial role in eukaryotic cells (4). Particularly interesting is the finding that the U6 gene from the fission yeast, Schizosaccharomyces pombe, contains an intron (13). This finding has led to the speculation that U6 might be directly involved in catalysis during splicing. The discovery of a transcriptionally-active variant U6 gene within a G protein intron raises the possibility that U6 snRNAs might have additional roles in gene regulation.

MATERIALS AND METHODS

Cloning, sequencing, and mapping of LH87
Genomic clone LH87 was isolated from a human genomic library as described previously (14). The human genomic library was purchased from Clontech Laboratories, Inc. (Palo Alto, CA) and was generated from a partial Mbol digest of human leukocyte DNA. Clone LH87 and several other independent isolates were subcloned into plasmid and M13 vectors for restriction and sequence analysis. In situ hybridization data were collected and analyzed as described (16).

In Vitro transcription
U6 transcription reactions were carried out in HeLa cell S100 extracts prepared according to Weil et al., (17). Transcription reactions were assembled as described by Kunkel et al. (8), with minor modifications. Specific reaction conditions are given in the figure legends. Uncut plasmid DNA (20 µg/ml unless otherwise noted) was used as template. Reactions were incubated at 30°C for 90 min. Transcription products were purified by phenol extraction and either analyzed directly by electrophoresis
and autoradiography or further purified by hybridization selection as described (18).

In Vitro mutagenesis

A pair of oligonucleotides (5'-ctcgtgatgtGTTGCTTGTTCGTAG-3' and 5'-tcggtgatgtAAAAATATGGGAACGGTTTC-3') were used as primers for amplification of a DNA fragment corresponding precisely to the 87U6 gene. The amplified DNA fragment was cut with EcoRI and cloned into pBluescribe- and pUC18 to generate 87U6 clones lacking human flanking sequences.

RESULTS

Genome structure

While screening a human genomic DNA library for sequences homologous to one of the common snRNP proteins, the 11,000 dalton E protein (15), we isolated a group of clones that hybridized only to probes from the 3' end of the E protein cDNA. Sequencing studies on this group of genomic clones revealed the presence of a putative U6 snRNA pseudogene immediately upstream of DNA sequences coding for the 3' terminal 213 nucleotides of the E protein mRNA. Further sequence analysis indicated that this unusual combination of sequences was located in an intron of the human gene for a signal transduction G protein, G33α (Fig. 1A).

LH87 is one of the group of 23 independent isolates containing this gene arrangement. All have been restriction mapped, and four of the 23 clones have been analyzed at the DNA sequence level. Since the organization of the U6, E protein and G protein sequences is the same in all clones, further discussion will be limited to the prototypic clone of this group, LH87.

The E protein homologous region of LH87 includes the last 64 nucleotides (nts) of protein coding sequence, and contains eleven mismatches relative to the parental sequence (Fig. 1C). The U6 sequences found immediately upstream of the E protein pseudogene sequence (87U6) have 91% homology to an expressed human U6 gene, pGem/U6 (8). Half of the ten differences between the U6 sequences are clustered in the middle of the U6 coding region, which is thought to be important for interaction of U6 with U4 snRNA (19).

There is some limited similarity between the 5' flanking sequence of 87U6 and the human U6 gene isolated by Kunkel et al., (8) including the presence of a 12 nt purine-rich sequence between 150 and 200 nts upstream of the transcription start site in both U6 genes (Fig. 1C, asterisks). However, none of the upstream transcriptional control elements (the octamer motif, proximal sequence element (PSE) and TATA box) found in the major vertebrate U6 genes, pGem/U6 (8). Half of the ten differences between the U6 sequences are clustered in the middle of the U6 coding region, which is thought to be important for interaction of U6 with U4 snRNA (19).

The E protein gene begins at nucleotide position -404 and ends at position -616 (Fig. 1B). This exon and the immediate flanking intron regions are identical to that reported by Itoh et al. (20), (Fig. 1B). The possibility that the LH87 group of clones represents an unusual cloning artifact peculiar to a single genomic library has been excluded by Southern blot analysis of one of the original G33α clones isolated by Itoh et al. (20). These studies confirmed the presence of E protein and U6 sequences in a 622 nt EcoRI fragment of clone βHG13-81 (data not shown).

A portion of the remaining 87U6 5' upstream sequence that we have analyzed extends beyond the limits of the published genomic sequence for G33α, but does match that of human cDNAs for G33α (21 -23). This similarity defines the beginning of exon 9, which is separated from exon 8 by a 95 nucleotide intron (Fig. 1B). Exon 9 contains a single mismatch in the 3' untranslated region compared to the cDNA sequence reported by Codina et al. (21), and maintains homology to the longer G33α cDNA sequenced by Didsbury and Snyderman (22) throughout the region we have sequenced.

Mapping

The unique functional E protein gene is located on chromosome 1 at 1q25-q43, (16) and previous efforts at mapping G proteins have also localized the human G33α gene to an undetermined locus on chromosome 1 (24). Although it seems likely that an RNA intermediate was involved in the dispersion of the E pseudogene sequence in the human genome, the sequence data do not exclude the possibility that the LH87 locus overlaps that of the functional E protein gene.

To evaluate the hypothesis that the E protein sequence in LH87 represented an alternative exon of the E protein gene and to clarify the relationship of the G33α sequence in LH87 to previously characterized G protein genes, we used in situ hybridization to map the location of the LH87 sequence in the human genome.

An 880 nucleotide Sphi-SphiI fragment from exon 9 of the G33α gene (Fig. 1A) was used to make a nick-translated probe for in situ hybridization. The results of the in situ hybridization are shown in Figure 2A and 2B. The LH87 probe mapped to a region near the centromere of chromosome one, at 1p13—21 (Fig. 2B). As depicted in Figure 2B, this places LH87 on the opposite arm of chromosome 1 from the expressed E protein gene, and rules out the possibility that the E protein homologous sequences are a functional part of the major E protein.
Figure 1. A. Sequence organization of genomic clone LH87. The solid line represents genomic DNA. Stippled boxes represent exons for the G3α protein, the cross-hatched box represents the E protein pseudogene, and the open box represents the 87U6 gene. The portion of genomic DNA representing the G3α protein exon 7 is not present in clone LH87. The shaded boxes containing arrows represent Alu repeats. Restriction enzymes are: A = Acc I; E = Eco RI; He = Hinc II; Sp = Sma I; Sph I; Xb = Xba I. B. Sequence comparison of clone LH87 with G3α protein nucleotide sequences. Humgip3α8 is the sequence presented by Itoh et al. (20), for the human G3α protein exon 8 and immediate flanking sequences. Humgtpbp is sequence of a human cDNA for a G3α protein (23). Humgkas is cDNA sequence for a human Gk protein (21). Humgiab is a cDNA sequence for a Gα protein from human lymphocytes (37). Nucleotide number 1 is located at the start of the U6 gene while nucleotide -1 is the first nucleotide upstream of the U6 gene. A (-) indicates identity with LH87 while a (.) indicates a deletion. Lower case letters indicate intron sequences. The amino acid sequence of the carboxyl-terminus of the G3α protein is shown above the nucleotide sequence. C. Sequence comparison of clone LH87 with U6 and E protein sequences. Human (8) and mouse (12) U6 genes have been aligned above the U6 gene from clone LH87, while the E protein cDNA sequence is aligned below the sequence of LH87. Direct repeats flanking the U6/E protein pseudogene region and other sequence features mentioned in the text are indicated.
transcription unit. These data also provide the first regional localization of the G3α gene on the human genome.

**Transcription of the 87U6 gene in vitro**

To determine if the variant U6 gene found in LH87 could be expressed despite the lack of homology to known U6 regulatory sequences, a construct containing 87U6 plus 1,4 kilobases (kb) of 5' and 1 kb of 3' flanking sequences (p87Hc) was incubated in a HeLa cell S100 extract capable of supporting transcription by RNA polymerase III (17). As shown in lane 3 of Figure 3, the LH87 construct directed the synthesis of a labeled product that co-migrated with the U6 RNA produced from another human U6 gene, pGem/U6 (lane 1; ref. 8). The RNA synthesized from both the consensus and variant U6 templates specifically hybridized to single stranded DNA containing sequence complementary to human U6 RNA, but failed to hybridize to single stranded DNA complementary to U2 snRNA. Vector sequence alone did not yield any products that are recovered by hybrid selection (lanes 5 and 6). These results suggest that the variant U6 gene from LH87 can be efficiently and accurately transcribed in vitro.

To further investigate the regulation of transcription of the variant 87U6 gene, increasing amounts of α-amanitin were added to the in vitro transcription reactions. As shown in Figure 3B (lane 3), the transcription of 87U6 was not affected by α-amanitin at a concentration of 1 μg/ml, but transcription was abolished when the concentration of α-amanitin was increased to 200 μg/ml (lane 4). These results are consistent with those obtained for the major U6 genes from human and mouse, and suggest that the variant 87U6 gene was being transcribed by RNA polymerase III in the HeLa extract.

**87U6 Transcription in the absence of 5' flanking sequence**

The results shown in Figure 3 suggest that the efficiency of transcription of 87U6 by RNA polymerase III in vitro was similar to that of pGem/U6, even though 87U6 lacks all known U6 transcriptional regulatory sequences. These findings establish that the transcription of these two U6 genes relies upon different sequence elements, even though they are transcribed by the same polymerase. Since many polymerase III genes utilize internal promoters rather than elements in flanking sequences, we used the polymerase chain reaction (PCR) to produce plasmids that contained the entire coding region of 87U6 but lacked any human 5' or 3' flanking sequences. As shown in lane 5 of Figure 4, the minimal 87U6 constructs continued to be suitable templates for transcription in vitro, yielding approximately as much U6-sized product as the complete pGem/U6 with all known 5'
regulatory elements (lane 6). Similar results were obtained with minimal 87U6 constructs cloned in both directions in two different vectors (pUC 18 and pBS-; data not shown). Some of the 87U6 minimal PCR constructs also yielded a higher molecular weight product as shown in lane 5. This larger band was not selected on filters containing U6 DNA, and has not been characterized further.

Transcription of both human U6 genes was considerably less than that obtained with two constructs containing a murine U6 gene, mU6 -89/+84 and mU6 -79/+84 (ref. 12; lanes 1 and 2, Figure 4). Both of the functional mouse U6 constructs contained sufficient 5' flanking sequence to support transcription by polymerase III in our HeLa cell extracts. However, in agreement with the results of Das et al. (12), no U6 RNA was synthesized from two mouse U6 constructs that contain lesser amounts of 5' flanking sequences, mU6 -23/+84 and mU6 6/+84 (lanes 3 and 4). Thus, the transcription of the minimal 87U6 construct cannot be attributed simply to the inability of our extracts (or incubation conditions) to discriminate between active and inactive U6 templates.

The low molecular weight band that is detectable in lanes 3 and 4 was also present in every other lane, including the vector only control (lane 7). Since this labeled species was also found when template was omitted from the incubation (not shown), it probably represents end-labeling of an RNA present in the extract by the α-32P-GTP.

DISCUSSION

External vs. internal U6 promoters

The variant U6 gene in LH87 is unique among all previously characterized vertebrate U6 genes in that its transcription by RNA polymerase III in vitro is not dependent upon flanking DNA sequences. In fact, transcription of previously characterized mammalian U6 genes requires only the initiating G nucleotide of the U6 coding sequence and the PSE and TATA elements found in the immediate 5' flanking sequence (11,12,25,26). Elimination of the remainder of the U6 coding sequence does not influence the efficiency of transcription by RNA polymerase III in vitro. The 87U6 gene represents the opposite extreme. Following the paradigm of the 5S RNA and tRNA-type RNA polymerase III transcription units, all required promoter elements reside within the 87U6 coding sequence. Thus, one or more of the ten mutations that distinguish 87U6 from the major human U6 gene is responsible for converting an entirely external RNA polymerase III promoter into an entirely internal promoter.

Although we have not yet mapped the internal sequences responsible for the function of 87U6, it seems unlikely that this feature of 87U6 arose by chance during divergence of this gene copy from a parental U6 gene that required flanking sequences for expression. It is more likely that some (or all) of the sequence differences between 87U6 and the wild type U6 were necessary for the function of an ancestral, internally controlled U6 gene. In this regard, it is interesting that 3 of the 10 sequence differences between 87U6 and the consensus human U6 reside within the previously noted homology to the A box of tRNA genes. While two of these changes are neutral, one of these sequence changes (G to A at position 57) does improve the homology to the A box consensus (Fig. 5, top).

In contrast to the typical tRNA-like polymerase III transcription unit, the coding region of the 87U6 gene does not contain a recognizable homology to the tRNA B box. Studies of tRNA gene function suggest that this element is not absolutely required for RNA polymerase III function (27), and the 87U6 coding region may be an example of a polymerase III transcription unit that lacks a functional B box.

However, it may be significant that there is a good match to the B box consensus within the adjacent E protein sequence (Fig. 5, bottom and Fig. 1C). The B box-like sequence embedded in the E protein sequence begins 70nts downstream of the U6-E protein junction. We have not directly tested the influence of the E protein segment on transcription of 87U6. It is clear that the E sequences are not absolutely required for in vitro transcription of the 87U6 gene, since constructs lacking these sequences yield as much U6 sized product as the wild type human U6 gene. However, as evidenced by the influence of a downstream B box on the transcription of a U6 gene in S. cerevisiae (28), the external location of the B box sequences does not exclude the possibility of interaction with other RNA polymerase III promoter elements.

Thus, while only the internal A box might be required to obtain basal expression of 87U6, the B box homology in the 3' flanking region might influence the rate of expression of this gene in vivo. Selective pressure based upon an influence of the E sequences on 87U6 function would also provide a plausible explanation for why the 87U6 and E protein sequences at this locus have diverged only minimally from the parent sequences.

Speculations on the origin and function of the unusual sequence arrangement in LH87

The additional factor that must be considered in discussions of the evolution of this locus is the location of the 87U6 gene at this locus is...
RNA and E protein in human cells. With this possibility in mind, it is interesting that most cells seem to contain a molar excess of U6 over U4, and that metabolically distinguishable subpopulations of U6 RNA have been described in human cells (32,33). The possibility that U6 RNAs not found in complete U4/U6 snRNPs might have additional roles in cellular regulation has not been addressed experimentally.

It is also possible that the variant 87U6 gene product could be involved in the splicing of the G3αx transcript. Two of the ten differences that distinguish between 87U6 and the consensus human U6 sequences occur at positions that have been absolutely conserved during evolution (4,34). The consensus C at position 11 is a U in 87U6, and the conserved U at position 64 is a C in 87U6 (thereby conserving the run of four C’s at this location). However, neither of these changes occurs at sites that have been demonstrated experimentally to be important for viability, assembly with U4, or spliceosome formation. In fact, mutational analysis in yeast has established that substitution of the conserved C at the 5’ end of U6 does not compromise viability (34,35).

The phenotype produced by mutation of the conserved U in the U4 interaction domain has not been reported, but none of the changes in 87U6 in this region would be predicted to abolish the proposed base pairing interactions between U4 and U6 (4,19). Thus, there is no reason to believe that 87U6 RNA could not be assembled into a functional U4/U6 snRNP.

Discussion of possible roles for the 87U6 product in cellular regulation makes the assumption that the 87U6 gene is expressed in vivo. The evidence presented here demonstrates that the 87U6 gene contains a functional promoter. Since the G3αx gene is actively expressed in a variety of tissues, the 87U6 gene should be accessible to the transcriptional machinery and is likely to be expressed in human cells in vivo. While fingerprinting of the major U6 from HeLa cells suggests that the 87U6 product is not a predominant product of these cells (8), there is no evidence that excludes the possibility that other forms of U6 are expressed at low levels in HeLa cells or at high levels in other human cells. Assessment of the relative contribution of each of the 200 or more U6-related sequences in the human genome (36) to the total pool of human U6 RNA will require further studies of U6 gene structure and function.

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REFERENCES