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**Mouse DNA sequences complementary to small nuclear RNA U1**

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**ABSTRACT**

A mouse genomic library was screened for sequences complementary to U1 nuclear RNA. Out of the eight clones tested, none contained more than one copy of U1. Six of them were identical and one of those (clone ØU1-XIII) was further analysed. This latter clone contained no other gene for discrete species of small size RNA in the 8 Kb EcoRI fragment encoding U1. A 248 bp BglII fragment from ØU1-XIII encompassing the full length of U1 as well as flanking regions on both sides has been subcloned and sequenced in M13 phage. Although the coding region was 96.5% homologous to rat U1a RNA, there is no direct evidence that this clone is a true gene. 3' and 5' flanking sequences of this as well as other published clones have been searched for homologies and the results of this search are discussed.

**INTRODUCTION**

SnRNAs are a class of discrete small size RNA molecules which are present in high copy number in eucaryotic nuclei (for review, see ref. 1). Although their discovery dates back to 15 years ago (2), they have long remained quite mysterious both with regard to their function and their biosynthesis. Recently, however, several reports have pointed out to the sequence complementarity of U1 RNA with intron-exon junctions and based splicing models on this observation (3-5).

Even more elusive has so far remained their biosynthesis with two basic questions still unresolved. The first question asks whether they are synthesized directly in mature form or as part of a larger precursor. For lack of direct evidence, the fact that most snRNAs are capped or somehow blocked at their 5' termini has been taken as a suggestion that the cap site was indeed the site of transcription initiation as observed for pre-messenger RNA transcribed by RNA-polymerase II. On the other hand, the existence of a very large precursor has been inferred from UV inactivation experiments (6).

The second question asks which type of RNA polymerase is involved in

their synthesis. Even this apparently simple point has not yet been settled unambiguously as all three types of enzyme have in turn been blamed for it. Except for a single report suggesting a role for RNA polymerase I (7), the choice now seems to be restricted to polymerases II (8) and III (9,10), both possibilities not being mutually exclusive.

In vitro transcription studies are obviously needed to answer both these questions. As a first step towards this aim, we have been looking for mouse genomic clones containing U1 RNA sequences.

### MATERIALS AND METHODS

#### Vector and bacterial strains

E. coli DP50 (dap<sup>-</sup>, thy<sup>-</sup>, rec B/C, sup E, sup F, r<sub>k</sub><sup>-</sup>/m<sub>k</sub><sup>-</sup>) was used as the recipient host for recombinant plasmids and for growing phages. E. coli JM103 ( $\Delta$  lac-pro, thi, str A, end A, sbc B15, hsd R4, sup E, F' tra D36, pro AB, lac I<sup>9</sup>,  $\Delta$  M15) was used as the host for M13 mp7 phage. Both phage and bacterial strains were obtained from BRL.

pBR325 (11) was obtained from Dr. P. Ferrer. The Mm 31 plasmid (12) containing a mouse B1 sequence inserted into pBR322 was kindly provided by Dr. G.P Georgiev.

#### Screening of the genomic library

The partial EcoRI mouse genomic DNA library in Charon 4A was obtained from Dr. F. Blattner. Phages were grown on DP50 as described in the excellent cookbook provided with the library and purified by CsCl banding according to Yamamoto *et al.* (13). Screening was carried out according to Woo (14).

#### DNA preparations

Plasmids and replicative form of M13 mp7 phage were prepared by isopycnic centrifugation in CsCl-ethidium bromide as described by Haseltine *et al.* (15).

Phage DNAs were prepared by extracting purified virus three times with one volume of phenol-chloroform-isoamyl alcohol (24:24:1) and then three times with diethylether. Residual ether was then removed under vacuum.

Single-stranded M13 mp7 DNA was prepared for sequencing as described by Schreier and Cortese (16).

All DNA preparations were kept at - 20°C until use.

#### Enzymes

Restriction enzymes were from Biolabs except EcoRI which was prepared in our laboratory and BglIII and HpaII which were kindly provided by Dr. G.

Roizes. All were used under the conditions specified by Biolabs. T4 DNA ligase was also from Biolabs. T4 polynucleotide kinase was from P.L. Biochemicals.

#### Subcloning procedures

100 ng of pBR325 DNA linearized by EcoRI was ligated to an equimolar amount of shotgun EcoRI fragments from  $\phi$ U1-XIII using 10 units of T4 DNA ligase (Biolabs) in 25  $\mu$ l of 50mM Tris-HCl pH7.6, 10mM MgCl<sub>2</sub>, 20mM DTT, 50  $\mu$ g/ml nuclease free bovine serum albumin (BRL), 1mM ATP. After overnight incubation at 16°C, recombinant plasmids were transfected into competent DP50 cells according to Mandel and Higa (17). Southern blots of quickly extracted plasmid DNA (18) of several individual clones were probed with (<sup>32</sup>P) U1 RNA (see below). All positives plasmids had an 8 Kb insert.

One of the above plasmids was purified and 20 ng were digested with BglII and ligated to 100 ng of Bam HI cleaved M13 mp7 replicative form DNA as described above. Recombinant clones were selected as white colonies on indicator plate according to Messing *et al.* (19). Minicultures (1 ml) of individual clones were centrifuged and the supernatants directly analysed by probing Southern blots with (<sup>32</sup>P) U1 RNA (see below).

#### Preparation of (<sup>32</sup>P) U1 snRNA probe

Crude nuclear extracts of sonicated nuclei from 10 g HeLa cells were prepared as described (20). Nuclear RNAs were phenol-extracted and centrifuged for 24 hrs at 4°C through a 15-30% w/v sucrose gradient in 100 mM Na acetate pH 5.0, 100 mM NaCl, 1 mM EDTA in a Beckman SW27 rotor. The upper third of the gradient containing the snRNAs was collected and ethanol precipitated. snRNAs were resolved by electrophoresis on 12.5%-8M urea acrylamide gels in Tris-borate buffer (21) and the U1 band visualized by ethidium bromide fluorescence, was eluted as described (22).

The cap structure of U1 RNA should have prevented its 5' terminal labeling. Enough endonuclease activity was present however to generate 5' OH ends suitable for labeling. Indeed, U1 was significantly degraded after labeling as shown by gel electrophoresis (not shown).

5' terminal labeling was done as described (23). 100  $\mu$ Ci (<sup>32</sup>P)- $\gamma$ -ATP dried under vacuum were resuspended in 20  $\mu$ l H<sub>2</sub>O. 1  $\mu$ g of snRNA, 3  $\mu$ l of 10X kinase buffer (10 X = 100mM Tris-HCl pH 7.5, 100mM MgCl<sub>2</sub>, 100mM dithiothreitol) and 1  $\mu$ l T4 polynucleotide kinase (5 units) were added. The volume was adjusted to 30  $\mu$ l and the mixture was incubated for 1 hour at 37°C. Incorporated radioactivity was assayed by trichloroacetic acid precipitation. Incorporation was usually about 5 to 7.10<sup>6</sup> dpm/ $\mu$ g snRNA.

### Nick-translation of DNA

Nick-translation of DNA was performed with Amersham nick-translation kit and yielded a specific activity of  $150 \cdot 10^6$  dpm/ $\mu$ g.

### RNA selection by hybridization.

Total ( $^{32}$ P) radioactive nuclear RNA was prepared as described (24).

20  $\mu$ g of recombinant pBR325 plasmid carrying the 8 Kb EcoRI fragment of  $\phi$ U1-XIII was denatured and bound to nitrocellulose filters followed by hybridization and elution of RNA (25).

### Sequencing

Sequencing by the chain terminators method was as described by Sanger *et al.* (26). We used the SP16 primer which was provided by Dr. O. Hagenbüchle. Reaction products were fractionated on two thin 40 cm 6% acrylamide-bisacrylamide (SERVA) (19:1) - 8 M urea (Schwarz Mann ultrapure) gels as described by Sanger *et al.* (21). The first gel was run for 1.5 hour at 1400 V in order to resolve the first 150 nucleotides. The second one was run for 6 hrs to give a resolution from nucleotide 100 to nucleotide 350. Gels were fixed 5 minutes in 10% acetic acid - 20% ethanol and then dried on a BioRad gel dryer with heating. They were autoradiographed for 16 hours at room temperature.

### Electrophoresis in agarose gels and southern blotting

Agarose (Sigma - type II) gels were run in TBE (90mM Tris base, 90mM boric acid, 2mM EDTA, pH 8.3) at 10 Volt/cm in the presence of 0.5  $\mu$ g/ml ethidium bromide. Transfer of DNA to cellulose nitrate sheets (Schleicher & Schüell BA85) was done according to the procedure of Southern (27) as modified by Smith and Summers (28). Hybridization was carried out in 6 x SSC (1 x SSC = 0.15M NaCl - 0.015M Na citrate) containing 50% v/v formamide, 0.5% SDS, 10mM Na Phosphate pH 6.5 at 42°C. Filters were then washed three times for 10 min. in 2 x SSC, 0.5% SDS at room temperature, twice for 10 min. in 50% formamide, 2 x SSC, 0.5% SDS and finally rinsed rapidly twice with 0.2 x SSC.

## RESULTS

### Considerations on U1 RNA sequences

Determination of the complete nucleotide sequence of U1 from various species (29) has revealed extensive homologies between man and rat which differ at only two out of 165 nucleotides. These sequences are fully compatible with the T1 fingerprints of rat U1 (30) and mouse U1a (31). In this latter species, Lerner *et al.* (31) have observed another subspecies,

U1b, which can be distinguished from U1a on the basis of its migration as well as of its fingerprint. Although fingerprints of both species are largely similar, they differ at the level of three oligonucleotides. Of special interest is oligonucleotide 15(30, 31) because it corresponds to an HpaII site (Figure 1) which can be predicted from the U1 sequences of man, rat and chicken (29). Indeed, oligonucleotide 15 no longer exists in U1b fingerprints and is replaced by oligonucleotide b (31) whose sequence clearly shows the disappearance of HpaII site (Figure 1)

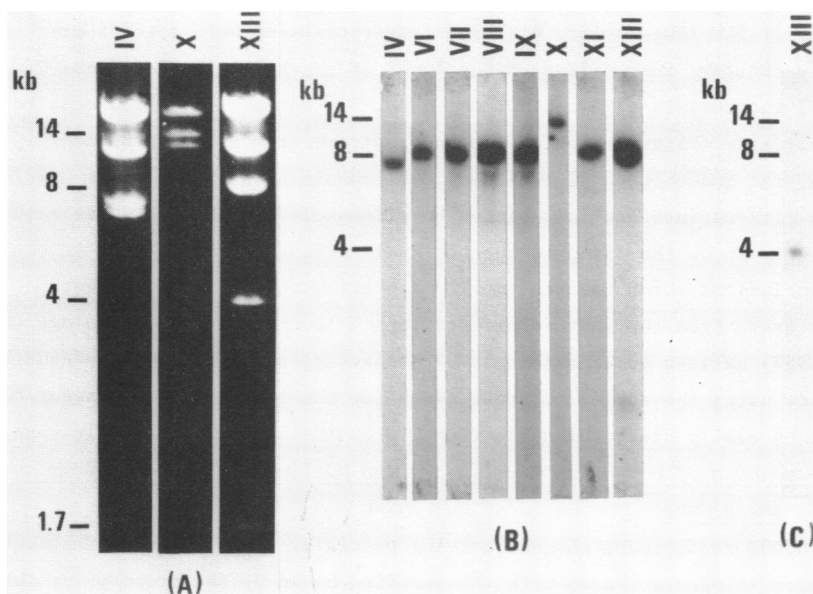
Two conclusions which have an important bearing on the following work can be drawn from the above considerations : 1) the degree of homology (at least 95%) between human and mouse U1 RNA sequences is more than adequate to allow using the former to probe genes for the latter ; 2) U1b sequences should be easily distinguishable from U1a on the basis of HpaII cleavage.

Screening of mouse genomic library and preliminary characterization of the clones

70,000 recombinant phages carrying partially digested EcoRI fragments in Charon 4A were screened with the purified human U1 RNA probe using the procedure of Woo (14). Out of this population which is supposed to represent about one fifth of the genome, 15 clones hybridized with U1. Eight of them were randomly selected for further characterization by restriction digestions followed by probing southern blots with ( $^{32}\text{P}$ ) U1 RNA. Figure 2B shows the results of Eco RI digestion of all eight clones : U1 RNA hybridized to an 8Kb band for six out of these eight clones, to a 7.7 Kb band for clone  $\emptyset\text{U1-IV}$  and to a 14 Kb band for  $\emptyset\text{U1-X}$  (Figure 2B). Ethidium bromide fluorescence shows the content of EcoRI fragments of each clone (Figure 2A). Again six were identical (only the pattern of  $\emptyset\text{U1-XIII}$  is shown) with three fragments of respectively 1.75, 4 and 8 Kb.  $\emptyset\text{U1-IV}$  clone contained two fragments of 7.7 and 7 Kb while  $\emptyset\text{U1-X}$  contained a single 14 Kb insert.

Man, rat or chicken U1 sequence (29)	$\begin{array}{c} 70 \quad \text{HpaII} \\   \quad \text{---} \\ \text{GCA}_{\text{m}} \text{CUCGGGAU} \end{array}$
Oligonucleotide 15 from rat U1 (30) and mouse U1a (31)	$\text{CA}_{\text{m}} \text{CUCGG}$
Oligonucleotide b from mouse U1b(31)	$\text{CA}_{\text{m}} \text{CUUUG}$

Figure 1 : U1 RNA sequences surrounding the HpaII site.  
Sequences are taken from the references in parentheses.

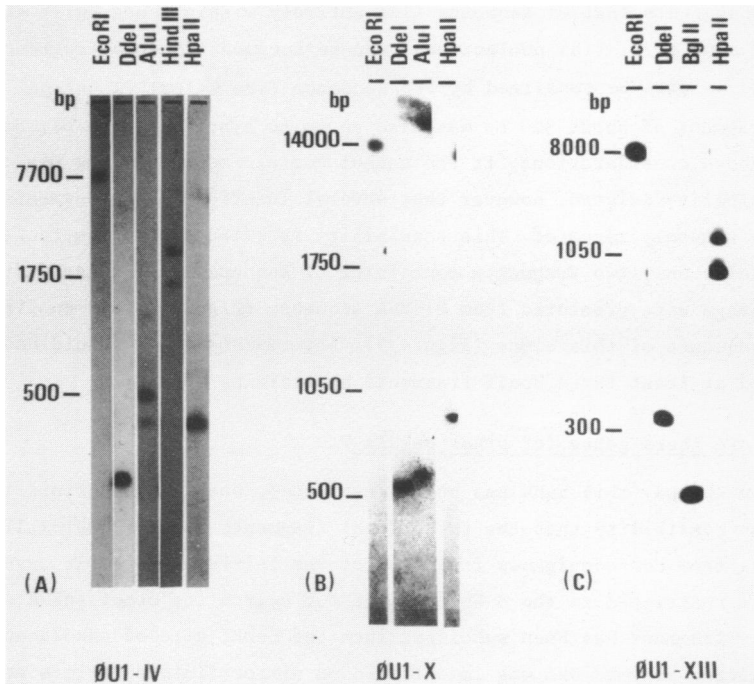


**FIGURE 2** : A) Electrophoregram of EcoRI digests of several ØU1 clones. DNA of each clone (0.5 microgram for ØU1-IV and ØU1-XIII, 0.1 microgram for ØU1-X) was digested by EcoRI, electrophoresed on 1% agarose gel and stained by ethidium bromide as described under Materials and Methods. Fragment sizes were derived from a calibration curve made with various reference markers (not shown).

B) Localization of U1 sequences in EcoRI fragments from several ØU1 clones. 0.5 microgram of DNA from each clone was digested by EcoRI, electrophoresed on 1% agarose gel, transferred to nitrocellulose and hybridized with ( $^{32}$ P) U1 RNA probe as described under Materials and Methods. Fragment sizes were derived as in A).

C) Localization of B1 sequence in EcoRI fragments of clone ØU1-XIII. The experiment was exactly as in B) except that the blot was probed with nick-translated Mm31 plasmid carrying the B1 sequence as described under Materials and Methods.

The group of six clones was further analysed by digestion with ten other restriction enzymes (Bam HI, PstI, AccI, DdeI, HaeIII, AluI, HpaII, HindIII, BglII and AtuCI). Undistinguishable patterns (not shown) were obtained in all cases suggesting they are extremely similar or even identical, as might have occurred if they were derived from a single initial clone (see Discussion). Of special interest was the cleavage by HpaII which was predicted to cut within U1a sequence (Figure 1) and which indeed yields two fragments hybridizing with U1. Clone ØU1-XIII randomly selected as representative of this group of potential U1a genes was therefore used for a more thorough investigation (see below).



**FIGURE 3** : Localisation of U1 sequences on different restriction fragments from clones ØU1-IV, ØU1-X and ØU1-XIII. The experiment was carried exactly as in Figure 2B except that gels were 1.2% in agarose.

Neither clone ØU1-IV nor ØU1-X has an HpaII site (Figure 3A and B) : they cannot therefore carry true U1a genes but at best pseudogenes. Alternatively, they might be genes or pseudogenes for U1b. Additional sites for HindIII (and as a consequence for AluI whose recognition sequence is included in that of HindIII) are found in ØU1-IV but not in ØU1-X. Neither of these two clones have been further investigated yet.

*Detailed characterization of clone ØU1-XIII.*

We have asked several specific questions pertaining to this particular clone.

1) How many copies of U1 gene?

Among the many restriction digests of ØU1-XIII which have been studied, a few especially informative ones are presented in Figure 3C. After cleavage with BglII, only one band at about 250 bp contains sequences hybridizing to

U1. This suggests that U1 sequence lies entirely within these BglIII sites. Only one copy of U1 (165 nucleotides) can be accommodated in a fragment of such size as will be confirmed by its sequence (see below). A unique DdeI fragment of about 320 bp was also shown to hybridize with U1. Based on the above considerations, it too cannot contain more than one U1a copy. The possibility existed, however that several identical DdeI fragments could be tandemly repeated. This possibility is ruled out by the fact that HpaII yields only two fragments containing U1 sequences as expected from the cleavage site predicted from U1 RNA sequence (Figure 1) and confirmed by DNA sequence of this clone (Figure 5). Any tandem repeat should have generated at least three HpaII fragments hybridizing with U1.

### 2) Are there genes for other snRNAs ?

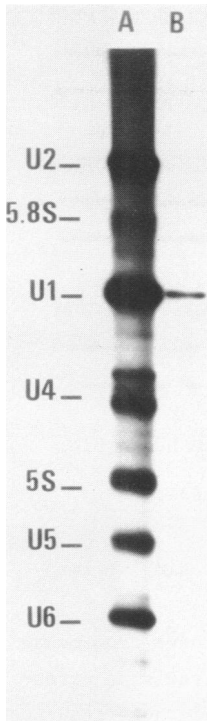
From the way this bank has been constructed, one cannot definitely exclude the possibility that the three EcoRI fragments found in ØU1-XIII might originate from non-contiguous fragments of the initial genome. We have therefore restricted to the 8 Kb fragment our search for other snRNA sequences. This fragment has been subcloned into the EcoRI site of pBR325 and the resulting plasmid DNA was immobilized on nitrocellulose filters and hybridized to total (<sup>32</sup>P)RNA from long-term labeled HeLa cells. The results shown in Figure 4 clearly show that U1 has been successfully recovered by this procedure but that no sequence coding for another discrete RNA species is found within the 8 Kb of this fragment.

### 3) Are there known repetitive sequences in the vicinity of U1 sequence ?

The mouse genome is interspersed with 40-80,000 copies of a 130 bp sequence, called B1 (32), which are the equivalent of human Alu family (33). An EcoRI digest of clone ØU1-XIII was probed with Mm31 plasmid containing this sequence. As shown in Figure 2C, there is no such sequence within the 8 Kb fragment but rather in the 4 Kb fragment. Knowing that U1 sequence is roughly in the central part of the 8 Kb fragment (unpublished data), we can therefore exclude the presence of a B1 sequence in the close vicinity of U1.

By the same token, we have looked for the presence in ØU1-XIII of satellite DNA sequences (34) as well as of sequences from the middle interspersed family I characteristic of rodents (35). These latter sequences amount to 1% of the genome and are stretches of about 3 Kb, part of which can be isolated from EcoRI genomic digests as a conspicuous discrete fragment of 1,350 bp. None of the three fragments of ØU1-XIII was found to contain any of these two types of sequences (results not shown).





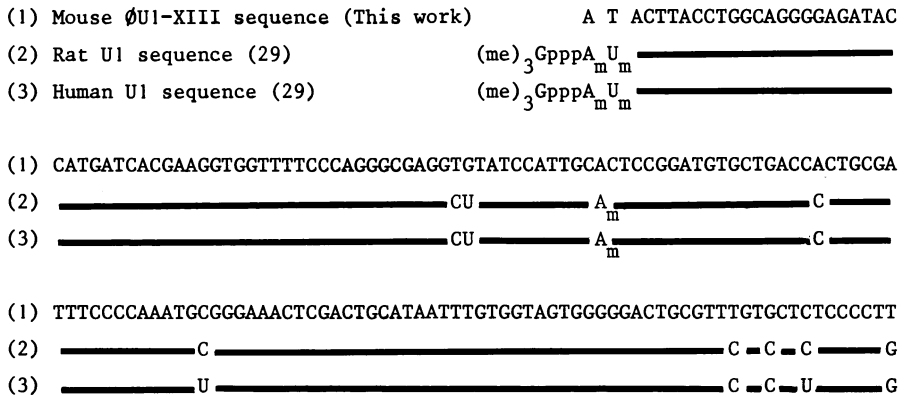
**FIGURE 4 :** Hybrid selection of ( $^{32}\text{P}$ ) in vivo labeled total HeLa nuclear RNA with the 8Kb EcoRI fragment of  $\phi\text{U1-XIII}$  subcloned into pBR325 plasmid. RNA selection by hybridization followed by electrophoresis on 12.5% acrylamide-urea gels and autoradiography were as described under Materials and Methods. Track (A) : total ( $^{32}\text{P}$ ) in vivo labeled HeLa nuclear RNA ; Track (B) : hybrid selected material.

#### 4) Nucleotide sequence of the BglII fragment

The unique BglII fragment hybridizing to U1 RNA was excised from the plasmid carrying the 8Kb EcoRI fragment and in turn recloned into the Bam HI site of phage M13 mp7. The sequence was obtained by the chain terminator method.

The coding sequence for U1 is easily recognized and has been aligned to the U1a RNA sequences of rat and man in Figure 5. The present gene exhibits respectively 96.5% and 95% homology with rat and man U1a. Should this sequence be transcribed into RNA, its fingerprint would differ from that of Lerner *et al.* (31) at the level of oligonucleotide 19. It is also interesting to note that all six differences lie outside of the few discrete positions where variability between species is observed at the RNA level (29). The 5' terminal sequence which are supposed to interact with splicing sites on pre-messenger RNA are completely conserved.

Examination of the flanking sequences of clone XIII reveals the presence of an A-rich tract abutted to the 3' end of the coding region (Figure 6). This is itself followed by a 14 bp sequence, a direct repeat of which (except



**FIGURE 5** : Comparison of  $\emptyset$ U1-XIII coding sequence with rat and human U1 snRNA sequences. Sequences are taken from the references in parentheses.

for two differences) immediately precedes the 5' end of the coding region (Figure 7).

Comparisons of 5' flanking sequences (Figure 7) of U1 clones from mouse (this work), chicken (8), drosophila (36) and man (37, 38) reveals no extensive homologies but enough limited homologies to show that all these sequences are not completely unrelated. Probably the most significant homology concerns the few nucleotide immediately adjacent to the 5' end of U1 - i.e. overlapping the direct repeat - which appear to be closely related in drosophila, chicken and mouse clones. Interestingly enough, this region is completely different from that of true human U1 genes (37, 38). Further upstream, however, some limited homologies can be found between pairs of clones including the



**FIGURE 6** : Comparison of the 3' flanking regions of U1 sequences in mouse  $\emptyset$ U1-XIII, chicken and human clones. Sequences are taken from the references in parentheses. Dotted lines represent the coding sequence. Directly repeated sequences are underlined. Overhead dots show the two differences with the 5' flanking sequence of Figure 7. Homologous residues between the three clones are joined by solid or dotted lines.



report by Alonso *et al.* (45) suggesting that U1 and U2 hybridize to the same rat genomic fragments. No B1 sequence was found in the EcoRI fragments which contained U1 in a roughly central position thereby excluding that they could be in close vicinity.

In the coding region, the sequence of the BglII fragment from clone ØU1-XIII differs from that of rat (29) at six positions. All six differences lie outside of the few discrete positions where variability between species has been tolerated by evolution. Such a finding is certainly not in favor of clone ØU1-XIII bearing a true U1 gene. One should keep in mind, however, that RNA sequences overlook minor species. Indeed, Roop *et al.* (8) have observed some minor sequence heterogeneity in U1 cDNA clones. Wise and Weiner (46) also reported minor differences in U3 RNA from *Dictyostelium*. Even more divergence is observed for clone ØU1-IV and ØU1-X although they have not been analysed in sufficient detail to warrant further elaboration.

Examination of the flanking sequences of clone XIII reveals the presence of an A-rich tract abutted to the 3' end of the coding region (Figure 6). This is itself followed by a 14 bp sequence, a nearly perfect direct repeat of which immediately precedes the 5' end of U1 (Figure 7). This configuration is highly reminiscent of that observed in human U1-101 clone described by Van Arsdell *et al.* (47) and which was an obvious pseudogene. These authors believe that such sequence arrangements are produced by integration of cDNA copies of U1 RNA at staggered cuts in the genomic DNA which would generate the direct repeats observed here. However, a further expectation which follows from this model is that integration should occur at random and therefore that flanking sequences outside the repeat should be completely unrelated in different clones. Flanking sequences on both sides have been compared in different species. From this comparison, it appears that despite the lack of extensive homologous regions common to either side of all clones, there are enough limited homologies to show that all these sequences are not completely unrelated. Moreover, these homologies significantly overlap the directly repeated sequences. This situation therefore argues against a random insertion mechanism. In any case, there is at present no strong evidence in support of the above described mouse U1 sequence being a true gene nor is there compelling evidence to the contrary. Only *in vitro* and *in vivo* transcription experiments could give a clear answer. In this context, it should be noted that the only U1 gene (human) to have so far met these criteria (37) is the one whose structure departs most strongly from the features observed in either our clone or the chicken clone (8).

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