Cross-reaction of snRNA and an Alu I-like sequence from rat with DNAs from different eucaryotic species

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ABSTRACT

Sequence homologies to rat U1-snRNA and U2-snRNA were investigated in DNA from 23 eucaryotic species (3 lower eucaryotes, 4 plants, and 16 animals) using dot hybridization at various stringency conditions. Cross-hybridization among very distantly related species in e.g. plants-insects or mold-vertebrates is not the rule; there are, however, examples for stronger homologies like *Rattus-Dictyostelium*.

Furthermore, DNA from all 23 species was analysed for sequence homologies with the repetitive DNA sequence B1 (an Alu I family equivalent) from rat. We observed a wide range of homologies covering some plants and insects, up to vertebrates. Hybridization at increasing stringency conditions revealed species with higher degree of homology to the rat B1 sequence: maize, chicken, and hamster.

INTRODUCTION

A wide variety of eucaryotic species (from *Dictyostelium* to human) have been shown to contain a group of low molecular weight, stable, partially modified RNAs called small nuclear and cytoplasmic RNA, snRNA or scrRNA (for review see [1]). Currently, a great deal of attention is being focused on them, particularly on the snRNAs designated U1 through U6, 4.5S and 7S. Although they have been described about 15 years ago (2,3,4) and their primary structures have been analysed (5), their function still remains unknown. The recent interest in these molecules can be attributed to two observations: i) snRNA might be involved in processing nuclear RNA (6-9) and ii) sera from patients with several autoimmune diseases react with certain ribonucleoproteins (RNPs) which contain species of snRNA (10,11).

In several laboratories the genes coding for various types of snRNA have been investigated in more detail. Hybridization studies and DNA sequence analysis suggest a considerable degree of conservation (12-17), perhaps reflecting a preserved function of the gene product. We therefore have asked whether such conservation exists for a wide collection of genomes with differing degrees of evolutionary relationship.

Interspersed non-clustered repetitive DNA exists in most, if not all, species. These sequences have been also postulated as regulators of gene expression (18 and references within). One of the most abundant interspersed repeated sequences in human DNA is the so called Alu I-family (18). Its counterpart in rodents has been designated as B1 family (19,20). We and others have found that members of the Alu/B1 family can be found close to the location of various gene sequences (21-25), in one case a B1-sequence was found 600 bp upstream of a rat U1-snrRNA related sequence (26). Using a cloned copy of this rat B1-sequence, we have studied the occurrence of B1-related sequences in the same species as used for the study with snRNAs. The goal of these experiments was to analyse the distribution of these repetitive sequences as compared to the snRNA genes which are represented in the genomes as fewer copies. We present results demonstrating that the rat snRNAs as well as the B1 copy are able to cross-hybridize to a number of the DNAs investigated in distantly related species.

MATERIALS AND METHODS

DNA sources

DNA from three lower eucaryotic species, *Physarum* and *Dictyostelium*, (both Myxomycetes), and *Kluyveromyces lactis* (an Ascomycete) were used in this study, along with DNA from four plants, two dicotyles (*Rhinanthus*, *Sinapis*), and two monocotyles (*Cymbidium*, *Zea*), and from 16 animal species with representatives from *Diptera*, *Echinoidea*, fishes, *Amphibia*, birds and mammals. Abbreviations for all species used are listed in Table 1. DNA from the following cell lines: *Muntiacus muntjak* (ATTC CCL 157); *Potorous tridactylus* (PtK2); *Cricetulus griseus* (CHV79); *Mus musculus* (3T3); *Rattus rattus* (Novikoff hepatoma) (=Hep); *Homo sapiens* (HeLa) and from mouse, rat, *Xenopus* and *Triturus* livers, and human placenta was extracted as published previously (27). Herring sperm DNA was obtained from Boehringer, Mannheim. After isolation, all DNAs were checked on agarose gels for determination of size distribution.

Hybridization probes

U1-snrRNA and U2-snrRNA were prepared from 100S hnRNP particles extracted from purified rat liver nuclei; the isolated RNA was separated in polyacrylamide gels, and the snRNA bands were eluted as reported previously (28). 5 S RNA from *E. coli* was obtained from Boehringer, Mannheim. Radioactive label (γ ³²P-ATP) was introduced by the polynucleotide-kinase reaction (28). The specific activity obtained was ca. 1×10^7 cpm/ μ g RNA. The rat B1 se-

quence, homologous to the mouse B1 genomic sequences (19) and to the Alu-equivalent sequence in hamster (29) was originally located by sequencing a fragment containing a rat U1-snRNA related sequence subcloned in pBR 322. It was subsequently isolated in its entirety by suitable restriction enzymes and was cloned in pUC8. DNA probes for hybridization were labeled by nick translation in the presence of ^{32}P -dATP and ^{32}P -dTTP.

Southern hybridization

For nitrocellulose blots 15 μg of total genomic DNA was digested to completion with EcoRI endonuclease and fractionated by agarose gel electrophoresis. After transfer, the filters were hybridized at 42°C in 5xSSC, 20 mM phosphate buffer pH 6.9, 50% formamide, 1x Denhardt's mix and 100 $\mu\text{g}/\text{ml}$ of single stranded salmon sperm DNA. The filters were washed at 42°C in 2xSSC and then in 0.1xSSC.

Dot hybridization

A solution containing 1 μg DNA was denatured in NaOH (0.25 M), neutralized in 1 M NH_4 -acetate and applied to nitrocellulose filters as published by Kafatos et al. (30). Hybridization and washing steps were performed as described above. High stringency conditions were applied by using 0.1xSSC and 0.1% SDS at the temperature described in the text. Single stranded salmon sperm DNA was not included at any stage in the prehybridization, hybridization or washing steps.

Melting curves

For melting, the filters were washed in 2 ml 0.01xSSC in scintillation vials by incubating them for 10 min at increasing temperature steps. After each step the filters were removed from the vial and the radioactivity in the supernatant was measured after adding 5 ml "Insta-Gel" scintillation fluid to the aqueous phase.

Sequence homology comparison

The DNA sequencing method of Maxam and Gilbert (31) was used to establish the rat B1 sequence (26). Rat B1 and mouse B1 sequences were compared using a computer search program (39).

RESULTS

1. Melting profiles of dot hybrids

To interpret accurately the results obtained in the dot hybridization, control experiments were performed to investigate the role of the length of the tracer and the sequence homology of the reacting nucleic acids on the melting points of the various hybrids.

Fig. 1 Melting profiles of dot hybrids.

- a) *E. coli* DNA (o-o; ●-●), and cloned U1 sequences from *D. melanogaster* (●-●) and mouse DNA (●-●) were bound to filters (1 µg/dot) and hybridized at 42°C with alkali-fragment *E. coli* 5S RNA (100 b o-o; 50 b ●-●), *Drosophila* U1-snRNA (●-●) and rat U1-snRNA (●-●). After hybridization each filter was washed and stepwise melted at the indicated temperatures.
- b) Filterbound cloned U1 sequences from *D. melanogaster* (D.m.) and mouse (M.m.) were hybridized with *D. melanogaster* U1-snRNA. Replica filters were washed, melted and exposed for autoradiography (1d upper, 3d lower exposure).

The effect of the length of the tracer was studied by degrading aliquots of 5S *E. coli* RNA with alkali for different times to obtain populations of RNA with mean lengths of about 100 nucleotides (for RNA degraded for a very short period) to 50 nucleotides (after degradation for 10 minutes). The resulting fragments were labeled with γ - 32 P-ATP and polynucleotide kinase and hybridized to nitrocellulose-fixed *E. coli* DNA. Melting curves were performed and the melting points determined as described in the material and methods. As Fig. 1 shows, shortening of the tracer from 100 nucleotides length to 50 reduces the T_m value by about 5°C. This value corresponds with that obtained by Kafatos et al. (30) using degraded globin mRNA. The high T_m value shown in Fig. 1a is due to the high G+C content of the *E. coli* 5S RNA (64%).

We then examined the effect of the sequence homology on the T_m value of DNA-RNA hybrids in dot hybridization. At first, *Drosophila* U1-snRNA (53% G+C) extracted from Kc cells, with a mean length 60 to 70 nucleotides after

mild alkali treatment, was hybridized to a cloned *Drosophila* U1 gene sequence (13) forming 100% homologous hybrids. In a melting curve, the T_m was found at 57°C (Figure 1a) thus corresponding with the data of beta-globin mRNA (51% G+C) dot hybrids (30). For comparison of the optical signal intensity the points of the curve were exposed onto a film (Figure 1b). Then, in order to determine the effect of mismatching RNA-DNA hybrids on the T_m labeled rat U1-snRNA was hybridized to a clone containing a rodent U1 gene sequence with 94.6% homology to the published rat U1-snRNA (5). Here, the T_m in the plot was found at 55°C (Figure 1a). The 5.4% mismatching derives from point mutations, thus making the difference in the T_m values smaller than calculated. Finally, we hybridized *D. melanogaster* U1-snRNA with the rodent U1 clone mentioned above (70.6% homology). The resulting hybridization was extremely weak, even after long exposure, so that no significant signals were observed on the autoradiograms (Figure 1b) and no accurate T_m value could be calculated.

Therefore, we concluded that using our hybridization conditions only homologies well above 70% would be observed.

2. Homologies of U1-snRNA sequences

Following the data from Figure 1 we performed dot hybridization experiments with DNA from 23 species and in vitro labeled snRNA degraded to a mean length of 50 nucleotides. This resulted in clear signals for all mammalian, *Chironomus thummi* and *Dictyostelium* DNA whereas of the four plant species only *Sinapis* gave a strong hybridization signal (Table 1, Figure 2). From the positive DNAs, serial filters with new dots were prepared and washed at increasing temperature after hybridization. When the filters were washed at 50°C and at 55°C, some of the hybridization signals became weaker at the higher temperature. At 55°C, the T_m , the strongest hybridization is seen only with DNA from rat, while somewhat weaker signals are produced by DNA from *Dictyostelium*, *Chironomus*, human and sea urchin (Figure 3). Above 65°C, no signals but a weak remainder in the rat DNA can be detected in the autoradiograms.

An obvious difference is observed for the various DNAs from mammals. At all temperatures tested rat DNA displays a stronger signal than e.g. human. The close homology of both U1 snRNA sequences has been demonstrated (5, 12) and therefore the distinct difference in signal intensity suggests a high degree of divergence in the pseudogene sequences, particularly since at least 10^5 homologous gene copies are necessary to produce a faint hybridization dot (data not shown) under the hybridization conditions we are using.

Table 1

Hybridization of genomic DNA to specific rat sequences at 42°C after washing at 42°C in 0.1 x SSC.

DNA from species			Hybridization with rat sequences		
			U1	U2	B1
Escherichia coli (Bacterium)	Eco	-	-	-	o
Kluyveromyces lactis (Yeast)	Kla	-	-	-	-
Physarum polycephalum (Mold)	Phy	-	-	-	-
Dictyostelium discoideum (Mold)	Ddi	+	+	+	-
Sinapis alba (Mustard)	Sal	+	+	+	+
Rhinanthus spec. (Family Scrophulariaceae)	Rhi	+	-	-	-
Cymbidium spec. (Orchid)	Cym	-	-	-	-
Zea mays (Maize)	Zma	-	+	+	+
Drosophila melanogaster (Fruitfly)	Dme	-	+	+	+
D. virilis (Fruitfly)	Dvi	-	+	+	+
Chironomus tentans (Midge)	Cte	-	-	-	-
C. thummi (Midge)	Cth	+	-	-	-
Paracentrotus lividus (Mediterranean sea urchin)	Pli	+	+	+	+
Lytechinus variegatus (Atlantic sea urchin)	Lva	+	+	+	o
Clupea harengus (Herring)	Cha	+	+	+	+
Xenopus laevis (Frog)	Xle	+	o	o	+
Triturus spec. (Newt)	Tri	-	o	o	o
Gallus gallus (Chicken)	Gga	+	+	+	+
Potorous tridactylus (Rat kangaroo)	Ptr	+	+	+	+
Muntjac muntjak (Indian muntjak)	Mmj	+	+	+	+
Cricetulus griseus (Chinese hamster)	Cgr	+	+	+	+
Mus musculus (Mouse)	Mmu	+	+	+	+
Rattus rattus (Rat)	Rra	+	+	+	+
Homo sapiens	Hsa	+	+	+	+

Genomic DNA from listed species (a three letter abbreviation code for each scientific name is indicated) shows hybridization signals (+) or fails to hybridize (-) to the following rat sequences:

U1-snRNA, U2-snRNA, B1, (o= not investigated).

Fig. 2 Hybridization of rat U1-snRNA to genomic DNA. 1 μ g of genomic DNA from each species indicated were filterbound, hybridized to labeled U1-snRNA (50% formamide, 42°C) and washed at the same temperature in 0.1xSSC-0.1% SDS. The discrepancy in the signal between HeLa and human placenta (Hsa) DNA was insubstantial in other experiments and was not observed for U2 (Figure 4) and B1 hybrids.

Because of their different genomic sizes the rat DNA dots represent 4×10^5 haploid genomes, the Dictyostelium dots, however, 8×10^7 ; a 200 fold excess by which the clear signal in this lower eucaryote can be explained given just several related sequences per genome.

3. Hybridization pattern of U1-snRNA in genomic sequences

To examine in greater detail the genomic distribution of U1-snRNA genes, DNA from rat, mouse, hamster, rat kangaroo and muntjak was digested with EcoRI

Fig. 3 Increasing stringency for U1-snRNA hybrids. All positive species from Fig. 2 were hybridized and then washed in 0.1 x SSC at increasing temperatures (45°, 55°, 65°C).

Fig. 4 Hybridization of rat U2-snRNA to genomic DNA. Filterbound genomic DNA from indicated species was hybridized to rat U2-snRNA (50% formamide, 42°C).

restriction endonuclease, transferred to nitrocellulose filters after gel electrophoresis, and the blots were hybridized to U1 RNA sequences. The restriction/hybridization patterns of these mammalian DNAs vary considerably, showing the following major bands: mouse (Ltk⁻ cells) 1.65 kb, 7.0 kb; hamster (CHV79 cells) 7.0 kb; muntjak 8.1 kb, 10 kb; rat kangaroo 8.0 kb (17). This indicates that regions in the environment of U1-snRNA coding and related sequences vary even among closely related species.

4. Homologies of U2-snRNA sequences

In a similar approach as used for establishing sequence homologies with rat U1-snRNA, rat U2-snRNA was hybridized to genomic DNA from 23 species. Among the lower eucaryotes, only *Dictyostelium* showed a significant hybridization signal; among the plants, *Sinapis* and *Zea* were positive. In the order of Diptera, we found both *Drosophila* species to hybridize whereas both *Chironomus* species were negative. All other animals gave clear hybridization signals, whereas a control hybridization with *E. coli* DNA was negative (Figure 4, Table 1). When the washing temperature was raised (40-61°C) a reduction in hybridization efficiency of various DNAs with rat U2 sequences was observed (Figure 5). At 55°C, (T_m), strong signals from rat, mouse, and fruit fly were noted, while at 61°C, 4°C below complete melting, only the rat DNA displayed an efficient hybridization signal.

Here a weaker hybridization is observed with DNA from hepatoma cells than with rat liver DNA. Since these are derived from the same species both cell types should be comparable. Novikoff hepatoma cells, however, are known for completely aneuploidic chromosomal sets.

5. Homologies of Alu 1-like sequences

We have observed Alu 1-related sequences in cloned rat DNA fragments which show homology to U1- and U2-snRNA (26); Alu 1 sequences have also been

Fig. 7 Hybridization of rat B1 sequences at increasing stringency. DNA from species observed to hybridize at low stringency (40°C, 0.1 x SSC) was filterbound, hybridized to a cloned rat B1 sequence and washed in 0.1xSSC at increasing temperatures (40°, 49°, 55°, 61°).

DISCUSSION

Current studies on genes coding for various small nuclear snRNAs have disclosed that their primary structure is similar in many of the species investigated up till the present. Taking this evidence into account a model had been proposed suggesting a common ancestor gene for all the known snRNA sequences (33). Evidence has also been presented for a comparable secondary structure in several snRNAs from different mammals, suggesting that these genes may be related in their origin and perhaps in their function (40). These parallels in the assumed function of U1 and U2 snRNA in processing steps (6-9) have focused attention on these molecules.

We have now looked for the degree of homology in U1, U2 and Alu I-related sequences using genomic DNA from 23 different species ranging from lower eucaryotes and plants to vertebrates, in dot hybridization assays.

From the control experiments described above we estimate that homologies weaker than 70% would not be detected as hybridization signals using the washing conditions as specified. A point to be taken into consideration in the analysis of our results is that multiple copies of divergent snRNA-related genes exist; probably only few copies are colinear with the snRNA since till now only very few of the cloned sequences reported were absolutely homologous with the known snRNA sequences. This implies that the dot hybridization reactions are recognizing preferentially the abundant copies whereas the "bona fide" genes are probably underrated in autoradiograms. Furthermore, the calculation of mismatches in percent gives no direct information on the stability of the hybrids because the size of the clusters of homology also affects the degree of stability.

Interestingly, variation in the degree of homology for the three sequences investigated differs considerably. Rat U1 sequences hybridize to a different set of non-mammalian species than do U2 sequences, and differ in their hybridization efficiencies under stringent conditions. For U1, we have observed hybridization signals with as varied species as the human, sea urchin, midge and mold. U2 sequences, on the other hand, were found, apart from in all vertebrates, in plants like maize and mustard and in *Drosophila* but not in *Chironomus*. However, the fact that the snRNA genes from many lower eucaryotes did not hybridize with rat snRNAs indicates that these sequences are not as conserved as others, e.g. the rRNA sequences.

Nevertheless, it should be kept in mind, that testing the sequence homology of snRNAs in different species as observed in our investigation does not present a probe for similar or different function of the studied molecules. If U1 and U2 play a role in splicing processes, only few bases may be involved in the base pairing with hnRNA (13). This pairing could be sufficient to maintain the function whereas the rest of the sequence could differ more or less from the tracer used in the hybridization experiments.

Another point to be taken into consideration is the differential hybridization signals of DNAs extracted from cells or cell lines derived from the same tissue, when hybridized to snRNA. It should be borne in mind, that these cellular lines differ considerably in their DNA content as well as in their chromosomal composition. This is evident in the case of the Novikoff hepatoma cells, which show a heterogeneous DNA content per cell, when this is determined by cytofluorometry. The same is valid for 3T3 cells, an aneuploid population which differs strongly from the normal diploid cells of the mouse. These differen-

ces in genomic composition may therefore be responsible for the different hybridization signals observed.

Alu/B1-like sequences have been observed in the vicinity of most snRNA genes and pseudogenes (15,21,23,25,26,37). It has been speculated that transposition may have generated and distributed a large number of those sequences throughout the genome (34,35). Recently, as an indication of the mobility of these sequences, small polydisperse circular DNA isolated from monkey kidney cells was reported to display sequence homology to Alu DNA probes (36).

We have now observed a homology of the rat B1 sequence to all vertebrate DNAs investigated, whereas among plants and insects the hybridization is quite disperse. In this context, using the Alu-part found in the 7S cytoplasmic RNA Ullu et al. (41) have recently presented data demonstrating a similar homology to that found in our work. Nevertheless, the fact that several related species do not react when hybridized to the B1 sequence indicates that, at least in non vertebrates, these sequences are not widely distributed. Therefore, this distribution cannot be correlated with an evolutionary event. Since DNA from related species like *Drosophila-Chironomus* did

In summary, our results reveal that U2 and U1 snRNA as well as Alu-related sequences are present in essentially all vertebrate species investigated suggesting an evolutionary conservation. This mechanism will less likely account for the positive hybridization signals in non-related species (several plants and invertebrates) where the sequence investigated would have been generated by other, yet unknown, mechanisms. Homology in certain DNA sequences may have arisen by analogous events, i.e. by selecting for sequences specified by certain needs. It remains to be elucidated what the true function of the snRNAs as well as the Alu 1 sequences is and if these sequences play some definite role in controlling the gene expression as suggested recently.

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