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The Synthesis of a cDNA Copy Complementary to Two snRNAs and the Localization of Their Genes in the Rat Genome

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Summary
Two small nuclear RNAs-U2 and U1ab- were prepared starting from rat liver nuclei. A cDNA copy was synthesised from both RNAs using random primers prepared from calf thymus DNA. The cDNA synthesised is heterogeneous in size, ranging from full transcripts to a few bases. This cDNA copy was hybridised to restricted total genomic rat DNA blotted on nitrocellulose filters or diazotized paper. This allowed the identification of the genes coding for these snRNAs. We could demonstrate that these genes are not randomly interspersed between unique DNA sequences but possess an ordered structure. Furthermore, these genes are not clustered intimately, but are separated by sequences not present in the final snRNAs.

Small nuclear RNA is a population of well defined RNA molecules, present in the nucleoplasm as well as in the nucleolus (1). These molecules vary in length between 80 and 220 nucleotides, and are transcribed from the middle repetitive fraction of the genome (2).

In the past several reports have dealt with the possible role played by these small, long-lived molecules. Miller et al.(3) demonstrated the presence of some of these RNAs in the nuclear skeleton, whereas Deimel et al (4) and Howard (5) showed their presence in nuclear ribonucleoprotein particles carrying HnRNA sequences, suggesting a role in the processing of HnRNA. The presence of some of these RNAs in polymeric ribonucleoproteins extracted from rat liver was demonstrated (6), and it could be shown that these RNAs bind partially to HnRNA via hydrogen bonds (7).

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Nothing is known about the localization of the genes coding for the snRNA. Using a rather indirect system Eliceiri (8) suggested their clustering, but until now no reports have appeared which present conclusive evidence supporting an organised localization of the genes.
In this report we describe the localization in the genome of the genes coding for two species of snRNA, by using CDNA copies to these snRNAs which were hybridized to restricted genomic DNA.

Material and Methods

Isolations of snRNA. snRNA was isolated from rat liver HnRNP complexes, prepared as described (6). Ribonucleoproteins whith a sedimentation coefficient greater than 80S were pooled and the RNA was isolated by incubation with Proteinase K, phenol extraction and precipitation with ethanol. Total RNA was layered on a 5-20% sucrose gradient 80% formamide in 3mM Tris, 3mM EDTA, 0.5% SDS, pH 7.5 and centrifuged for 36 hours at 25°C (IEC centrifuge, Rotor SB283). Fractions containing RNA smaller than 10S were pooled, precipitated with ethanol and separated on 10% acrylamide 98% formamide gels (9). Individual bands were identified, cut out from the gels and the RNA eluted. The elution product was checked for purity by electrophoresis on 10% acrylamide 98% formamide gels.
Preparation of cDNA. The cDNA was prepared by the Method of Taylor et al. (10) using calf thymus DNA as source for the random primers. The mixture was incubated at 42°C with either actinomycin D or pyrophosphate (11). After 45 minutes incubation, the reaction was terminated by adding EDTA to 50 uM. The cDNA copy was separated from the triphosphates by chromatography on Sephadex G-100, freed from RNA by alkaline digestion, precipitated with ethanol and desalted.
Southern blots. Total genomic DNA was restricted with a ten-fold excess of restriction enzyme and precipitated with ethanol. The DNA was loaded on 0.8 or 1.5% agarose gels in 40mM Tris, 4mM Sodium acetate, 1mM EDTA,pH7.8 and electrophoresed in the same buffer. The DNA was transferred to nitrocellulose paper after treatment of the gels as described by Wahl et al. (12). Hybridization, washing and autoradiography were also as described (12).
Northern blots. Polymeric structures were isolated as described (6) and RNA isolated as described above. The RNA was bound to oligo-dT cellulose and the non-bound fraction, containing all the snRNA, separated on 10% acrylamide 0.8% agarose as described (13). The gel was then treated with periodic acid to destroy the acrylamide net and washed twice in 50mM Sodium phosphate buffer,pH 5.5 in the cold. Diazotized paper was prepared as described by Alwine et al. (14) and washed twice in the same buffer as the gel. Transfer of RNA was allowed for 4 hours at 4°C and then for 14 hours at room temperature. Hybridization and washing conditions were the same as described above (13) for the Southern blots. Diazotized paper was also used for DNA transfers, when the restriction fragments were thought to be small.

Results

Isolation and purity of snRNA. After pooling the RNA from the formamide sucrose gradients, fractions smaller than 10S were separated on 10% acrylamide 98% formamide gels. Parallel slots were stained with toluidine blue and served as markers for the isolation of the different snRNA species. Bands corresponding to a nucleotide number of between 171 and 168 were isolated together. These bands correspond to the U1b and U1a snRNA according to the nomenclature of Busch et al. (1), whereas the band with 196 nucleotides correspond to the U2 snRNA. After desalting the RNAs, 1 /ug of each fraction was applied to an acrylamide-formamide gel and submitted to electrophoresis. As can be seen in Fig.1 U2,U1a-U1b occur as single bands, without cross contamination, thus making these RNAs suitable for reverse transcription.

Reverse transcription. 200 ng of each of the separated snRNAs were hybridized to 80 /ug random primers and reverse transcribed. The cDNA was separated from non-incorporated nucleotides and the RNA hydrolised with NaOH. Under the conditions described above, 20 ng of cDNA was obtained, corresponding to an efficiency of 10% by weight. This yield is the same as with a

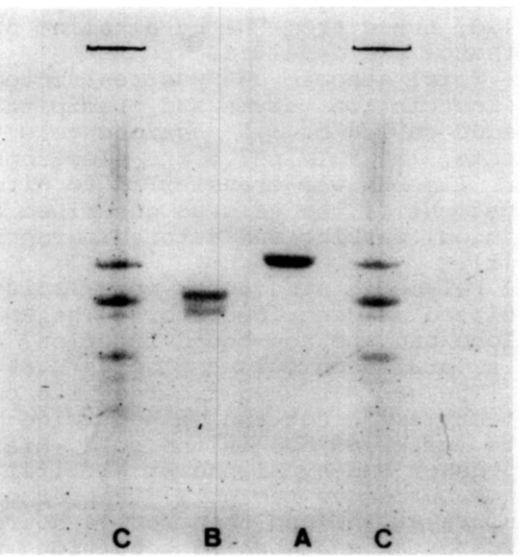

Fig.1.- Formamide acrylamide gel of separated snRNAs.A)U2,B)U1ab,C) total snRNA.

heterogeneous RNA template using oligo-dT as primer, but much less than with ribosomal RNA and calf thymus random primers; in this case about 45% of the template RNA was reverse transcribed. Incubation of the reaction mixture at 37°C (6) or addition of pyrophosphate instead of actinomycin D do not change the percentage being transcribed.

The length of the cDNA was checked after separation on acrylamide-formamide gels. The mean length for both cDNAs was about 120 bases, which is big enough for paper hybridization.

Hybridization of snRNAs and their respective cDNAs. In order to check the validity of the cDNA probe prepared for the hybridization with restricted genomic DNA, the total non poly-A-containing RNA from ribonucleoprotein complexes greater than 80S, which contains practically all the snRNA, was separated on acrylamide-agarose gels and transferred to diazotized paper. Fig. 2 shows the results of the hybridization between U1ab and their cDNA. Only one defined band could be observed at the position where U1ab are localised. After overexposing the X-ray plate, one very faint band was detected, but this band does not comigrate with any of the major bands seen in the gel after staining with toluidine blue and probably represents a degradation product of U1b, as has been recently reported by Lerner et al. (15) Hybridization of the same RNA with U2 cDNA gives also only one defined band, indicating that the cDNA copy is a faithful transcript from the respective snRNA.

Hybridization of cDNA and restricted genomic DNA. Eco RI restricted rat genomic DNA was separated on agarose gels and hybridized to U2 and U1 ab cDNAs. Two well defined bands could be demonstrated with molecular weights of 2.5 kb and 1.6 kb respectively (Fig.3) After overexposing the autoradiograms it is possible to observe three more faint bands with molecular weights of 2.0,0.9 and 0.6 kb.

The U2 RNA is 196 bases long, indicating that the Eco RI restriction fragments contain some genes or one gene and a large non coding sequence. This conclusion is based on the assumption that Eco RI will not cut in between a DNA sequence which corresponds to U2, as already published by Busch et al. (1) and the fact that cDNA from rat liver is hybridizing exactly at the same position as Novikoff Hepatoma DNA, tumor which snRNAs have been sequenced (see discussion). Furthermore Lerner et al

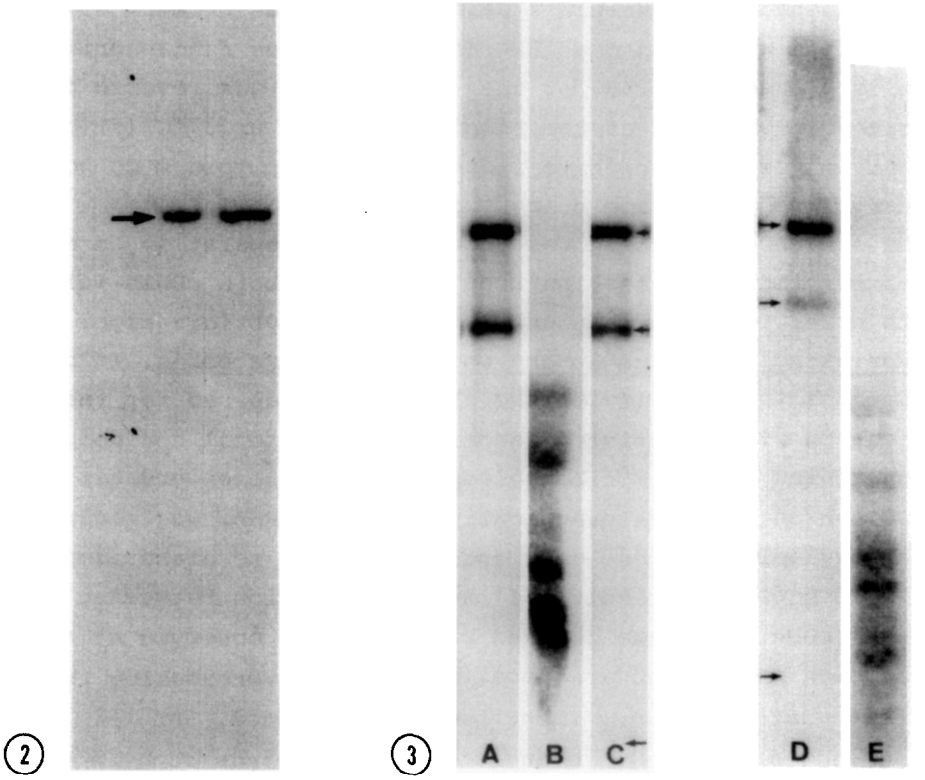

Fig.2.- Autoradiogram of the hybridization between poly A-devoided polymeric RNA blotted on diazotized paper and a cDNA copy synthesized from U1ab snRNA. The arrow indicate the position of U1ab determined in a parallel slot stained with toluidine blue.

Fig.3.- Autoradiogram of the hybridization between cDNA complementary to U2 snRNA and rat DNA restricted with Eco RI (A),AluI (B), Novikoff hepatoma DNA restricted with Eco RI (C). Hybridization between cDNA complementary to U1ab and rat DNA restricted with Eco RI (D) or AluI (E). The arrows (from top to the bottom) indicate the position of 2.5 kb,1.6 kb and 0.4 kb markers.

pointed out a high degree of sequence conservation in the snRNAs during differentiation and evolution (15). By using AluI, an enzyme known to cut the DNA copy of U2 at nucleotide 132 (see discussion) it should be possible to obtain further information about the organization of the genes, e.g. if they are organised in clusters or if the genes are separated by non-coding sequences. AluI restricted DNA hybridizes in very well defined fragments to U2 cDNA, with molecular weights ranging from 1.1 kb to 0.4 kb. This indicates that the genes are probably separated by sequences not present in the final RNA transcript.

The hybridization of Eco RI restricted DNA to U1ab cDNA gives a similar pattern to the one seen with U2 cDNA (Fig.3). Hybridization of AluI restricted DNA to U1ab cDNA allows the recognition of several bands in the low molecular weight region of the gel, similar but not identical to those fragments observed with U2 cDNA. This also indicates a non homogeneous structure of the U1ab genes, as is the case for U2. AluI does not cut in the U1ab DNA.
Hybridization of U1ab cDNA to SalI restricted DNA-SalI is also no cutting in the U1ab DNA- allows to identify two major bands with molecular weight greater than 4 kb, which indicates also a regular organization of the genes in the rat genome.

Discussion.
Our results demonstrate that it is possible to synthesise a complementary DNA copy to isolated snRNA species in vitro. The existence of such a copy will facilitate the analysis of the genes coding for snRNAs, and make possible the elucidation of the functions of these well defined RNA molecules. The use of random primers for the reverse transcription allows the synthesis of a rather heterogeneous cDNA, with a mean molecular weight of about 50.000 daltons. We notice that it is not necessary to separate the large transcripts from the small ones to obtain reproducible results in the filter hybridization. It has been demonstrated, that snRNA genes are more than 1000 times repeated in the genome of different species (2) but there was until now no direct evidence about the organization ot these genes in the animal genome. Indirect evidence was resented by Eliceiri (8) about the possible clustering of these genes. Furthermore nothing is known about the role played by these snRNAs in the cell nucleus. Different reports point out a possible role in transcription (6), during processing of HnRNA (7,15) or in the formation of the nuclear skeleton (3). Knowledge about the structure of the genes could help in understanding more about the function of snRNA.
The results presented here demonstrate that the genes coding for U2 and U1ab are not interspersed between unique genes of different length, as could be expected if these genes have a function as regulators in the transcription of the genomic DNA. In this case, a diffuse, non-defined hybridization pattern

should be found. Using the known sequences for U2 and U1b from Novikoff hepatoma cells (18,19) -the snRNA sequences are very stable during differentiation, and our cDNA hybridizes exactly in the same fragments to rat and Novikoff DNA, as shown in the results- we constructed a computer programme designed to analyse the fragments produced by different restriction enzymes. Eco RI is suggested not to cut in to U2 DNA. It shows nevertheless two major well defined bands, indicating a non-homogeneous structure of the U2 genes. Thus we should mention that snRNAs are transcribed as precursors, being processed to the final snRNA in the nucleoplasm (20). Digestion of the genomic DNA with AluI, allows the identification of several fragments with molecular weight varying between 1.1 and 0.4 kb, indicating heterogeneity between the genes or sets of genes.

Similar results were obtained for U1ab. We were able to find well defined fragments with Eco RI,SalI, Hind III (results not shown), and AluI, none of the enzymes cutting in the final snRNA sequence, indicating a similar structure for these RNAs to U2 RNA.

The fact that restriction with Eco RI allows the recognition of similar fragments hybridizing with U2 and U1ab, suggests a related localization of both snRNAs in the genome. Nevertheless, the great fragment after Eco RI digestion is 2.5 kb long, containing therefore nor more than 10 to 12 snRNA genes or perhaps much less. Furthermore, AluI, an enzyme not cutting in the sequence of U1ab, shows several bands after hybridization with both U2 and U1ab, but it is not possible to identify as identical both patterns. Taken together all these results, a structure could be suggested in which the genes for each of U2 and U1ab are not intimately clustered and are probably also not interspersed with one another, but are more probably in groups separated by sequences not present in the final RNA transcripts. An analysis of the organization of the genes should be more exact after cloning them in bacterial plasmids. Such experiments are underway in our laboratory.

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