The genes coding for 4 snRNAs of *Drosophila melanogaster:* localization and determination of gene numbers

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ABSTRACT

Four small nuclear RNAs (snRNAs) have been isolated from Drosophila melanogaster flies. They have been characterized by base analysis, fingerprinting, and injection into Axolotl oocytes. The size of the molecules and the modified base composition suggest that the following correlations can be made: snRNA1 ~ U2-snRNA; snRNA2 ~ U3-snRNA; snRNA3 ~ U4-snRNA; snRNA4 ~ U6-snRNA. The snRNAs injected into Axolotl oocytes move into the nuclei, where they are protected from degradation. The genes coding for these snRNAs have been localized by "in situ" hybridization of 125-I-snRNAs to salivary gland chromosomes. Most of the snRNAs hybridize to different regions of the genome: snRNA1 to the cytological regions 39B and 40AB; snRNA2 to 22A, 82E, and 95C; snRNA3 to 14B, 23D, 34A, 35EF, 39B, and 63A; snRNA4 to 96A. The estimated gene numbers (Southern-blot analysis) are: snRNA1 : 3; snRNA2 : 7; snRNA3 : 7; snRNA4 : 1-3. The gene numbers correspond to the number of sites labeled on the polytene salivary gland chromosomes.

INTRODUCTION

Eucaryotic cells contain a set of small, stable, low molecular weight RNAs not involved in protein synthesis[1]. They are predominantly localized in the cell nucleus, but some have also been found in the cytoplasm[1]. The presence in all eucaryotic cells[2] and the sequence conservation during evolution[3-7] suggest important roles of these RNAs. A great variety of functions have been assigned to these so-called small nuclear RNAs (snRNAs), including mRNA splicing[6,8], translation regulation[9], transport of mRNA to ribosomes[10], transcription regulation[11], chromatin structure[12], DNA replication[13], etc., but basically their functions remain still elusive.

A study of the "in vivo" functions of the snRNAs is confronted with questions on the genetic as well as on the molecular level. Drosophila melanogaster, which has been investigated on

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both levels extensively, is an especially well suited organism for such an approach. We therefore isolated a number of snRNAs from D.melanogaster and characterized them in comparison with snRNAs of other organisms described in the literature. We also studied the number of genes coding for these RNAs and localized their position on D.melanogaster salivary gland chromosomes.

MATERIALS AND METHODS

Isolation of snRNAs, [^{32}P]-labeling: Three to six day old flies of Drosophila melanogaster (Oregon R strain) were collected and stored at -90°C. Three to four gram of flies were ground to a fine dust in liquid nitrogen and subsequently homogenized in a Dounce homogenizer on ice after addition of 30 ml homogenization buffer (10 mM Tris-HCl pH 8; 1 mM $CaCl_2$; 1 mM $MgCl_2$; 350 mM sucrose). The homogenate was filtered through a nylon net (Polymen, PES 400-37 ASTM) and centrifuged at 1200xg for 20 min. at 4°C. The pellet was resuspended in homogenization buffer and again centrifuged. This step was repeated 3 times. Cytoplasmic RNA was extracted from the first supernatant, nuclear RNA from the last pellet. The nuclei were lysed by addition of 15 ml 0.1 mM Na-acetate pH 5; 0.5 M SDS; 10 µg/ml polyvinylsulfate; 500 µg/ml bentonite; 1 mM $MgCl_2$; 0.1 M NaCl. Cytoplasmic and nuclear RNA were phenol-extracted according to Kirby[14], the high molecular weight RNAs precipitated with 3 M K-acetate[15], and the low molecular weight RNAs precipitated from the supernatant by addition of 2.5 vol. ethanol. The PAGE system of Fradin and coworkers[16] was used. A 10% gel was used for the first dimension and run for 18-24 h. The second dimension was run on a 20% gel for 5-6 d. The RNAs were eluted as described by Dudler et al.[17].

Two gram of 3 to 6 day old adults were fed with a solution of 1 mCi monosodium-[^{32}P]-phosphate (New England Nuclear, NEX-063, 1250 mCi/mM, 25 mCi/ml) which contained 1% acetic acid and 20% sucrose for 12 hours. Extraction of the RNA and 2-dim. PAGE was performed as described above.

Base analysis, [^{125}I]-iodination, and fingerprints: Uniformly [^{32}P]-labeled snRNAs were digested with nuclease P 1 and snake venom phosphodiesterase and chromatographed on 2-dim. thin layer plates according to standard procedures[18]. The "in vitro" proce-

dure described by Prensky[19] was followed in detail for the iodination of the snRNAs (New England Nuclear, Na[^{125}I], NEZ-033L). Nuclease T 1 digestions and separation of the oligonucleotides was done according to Brownlee[20].

Injection of Axolotl oocytes, analysis of RNA accumulated in the nuclei: Ambystoma mexicanum (Axolotl) oocytes of 3-4 mm diameter were injected with 5000 cpm [^{125}I]-snRNA/oocyte and incubated in Barth's solution[21] for 18 h. The nuclei of 5-10 oocytes were then isolated with forceps and the RNA extracted from the nuclei and the cytoplasm as described above. The RNA was loaded on a 1-dim. 10% PAGE and subsequently autoradiographed.

"In situ" hybridization: The hybridization buffer was: 2xSSAE, 50% deionized formamide, E.coli tRNA (1000-4000 fold molar excess), Drosophila melanogaster 5S RNA (30-50 fold molar excess) (1xSSAE: 15 mM Na-acetate pH 5; 150 mM NaCl; 10 mM EDTA). The hybridization was done at 40° C for 15 h with $R_o t$ of 2x, 4x, and 8x$R_o t_{1/2}$, respectively. $R_o t_{1/2}$ was assumed to be 2×10^{-3} Mol·sec·l^{-1} ($R_o t_{1/2}$ for tRNA[17]: 1.3×10^{-3} Mol·sec·l^{-1}). Preparation of the polytene salivary gland chromosomes from giant (gt, 1-0.9) larvae and autoradiography were done as described by Dudler et al.[17].

DNA preparation, restriction enzyme digests, agarose gel electrophoresis, Southern-transfer, and filter hybridization: The protocol of Dudler et al.[17] was followed in detail. Each nitrocellulose filter was incubated with 1×10^6 cpm [^{125}I]-snRNA at 42° C for 26 h. The $R_o t$ values were: $snRNA_1$: 3.1×10^{-3} Mol·sec·l^{-1}; $snRNA_2$: 4.8×10^{-3} Mol·sec·l^{-1}; $snRNA_3$: 1.7×10^{-3} Mol·sec·l^{-1}; $snRNA_4$: 1.1×10^{-3} Mol·sec·l^{-1}.

RESULTS

Isolation and characterization: Preparation of intact snRNAs from antibody precipitated[6] RNPs isolated from adult Drosophila melanogaster is difficult due to the high nuclease content of the flies (K.Schäfer, personal communication). Therefore, the RNAs were extracted by direct phenol-extraction in order to obtain sufficient non-radioactive material for the [^{125}I]-iodination experiments. Quantitative differences can be observed between the ethidium bromide stained gels (not shown) and the autoradiographs of [^{32}P]-labeled RNAs (Fig. 1), but the number and the location of the spots are identical. The RNAs used for "in situ" hybridi-

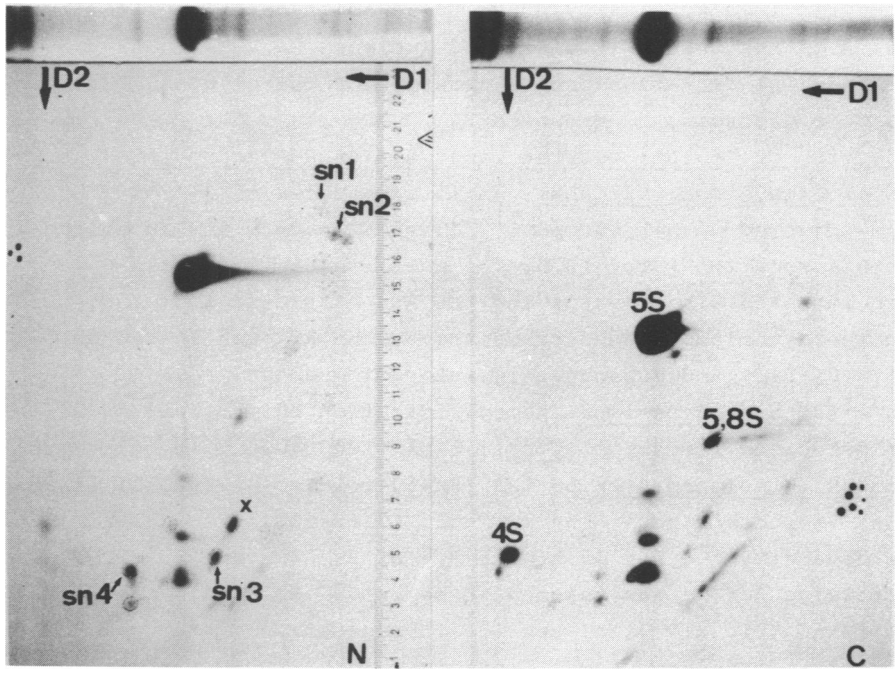

Figure 1: Autoradiographs of two-dimensional PAGE of uniformly [^{32}P]-labeled low molecular weight RNA extracted from nuclei (N) and cytoplasm (C). The upper part of the panel shows the autoradiograph of the first dimension. snRNA$_{1-4}$ are enriched in the RNA fraction isolated from nuclei. Spot X is very weak on ethidium bromide stained gels, and was therefore not considered for [^{125}I]-iodination.

zation and Southern-blot analysis were chosen according to the following criteria: availability of sufficient quantities for iodination, localization predominantly in the nucleus, accumulation in the nucleus after injection into the cytoplasm of Axolotl oocytes, molecular weights similar to U-RNAs characterized in mammalian cells, modified base content similar to mammalian U-RNAs, retention on m-Aminophenylboronate agarose columns[22].

So far three systems of nomenclature have been introduced for small nuclear RNAs[23-25]. Although we have good evidence to correlate the snRNAs studied by us with individual snRNA species described in mammalian cells, we prefer to apply the neutral terms snRNA$_{1-4}$ until the identity is established beyond doubt. The four spots designated as snRNA$_{1-4}$ (Fig. 1) were eluted from ethidium

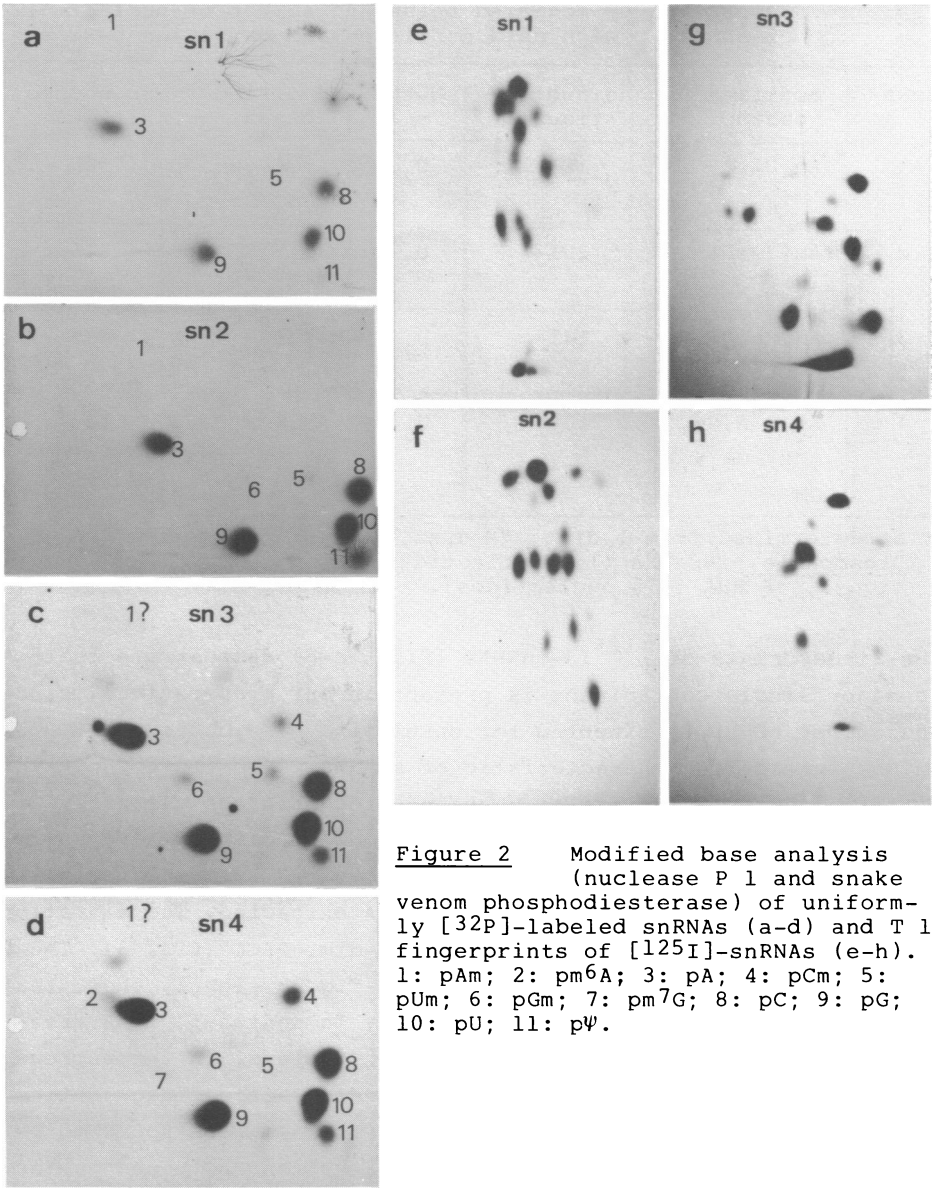

Figure 2 Modified base analysis (nuclease P 1 and snake venom phosphodiesterase) of uniformly [^{32}P]-labeled snRNAs (a-d) and T 1 fingerprints of [^{125}I]-snRNAs (e-h). 1: pAm; 2: pm^{6}A; 3: pA; 4: pCm; 5: pUm; 6: pGm; 7: pm^{7}G; 8: pC; 9: pG; 10: pU; 11: pΨ.

bromide stained 2-dim. PAGE and iodinated "in vitro" with [^{125}I]. Uniformly [^{32}P]-labeled snRNAs were isolated by the same procedure from flies fed with [^{32}P] for 12 hours.

It is shown by base analysis (Fig. 2a-d; Table 1) that snRNA$_{1-4}$ contain many modified nucleotides characteristic for snRNAs[26].

Table 1 Comparison of modified bases and number of nucleotides of $snRNA_{1-4}$ with data published for mammalian U-RNAs[26].

snRNA	modified bases	no.nucleotides*	U-RNA	modified bases	no.nucleotides
1	Am,Um,Ψ	187±	U_2	Am,Gm,Um, Ψ,$m_3^{2,2,7}G$	189
2	Am(?),Um Ψ,Gm	201±	U_3	Am,Ψ, $m_3^{2,2,7}G$	216
3	Am,Cm,Um Ψ,Gm	141±	U_4	Am,m^6A,Ψ,Cm Gm,$m_3^{2,2,7}G$	139
4	Am,m^6A,Gm, m^2G, Um,Ψ, Cm	108±	U_6	Am,m^6A,Gm, m^2G,Ψ,Cm	107

* as determined from 1-dim., 7M urea, 10% PAGE. Markers: Drosophila 5.8S RNA (158 nucleotides), 5S RNA (121 nucleotides), 4S RNA (75 nucleotides).

The fingerprints of [^{125}I]-snRNAs (Fig. 2e-h) demonstrate that no major single contaminant is present in our preparations, since the number of spots expected for snRNA size were obtained.

One interesting characteristic of snRNAs is their tendency to migrate back into the nuclei when injected into the cytoplasm[27]. Therefore, purified [^{125}I]-snRNAs were injected into the cytoplasm of Axolotl oocytes. After incubation for 18 h the nuclei and cytoplasm were separated and the RNA extracted. The migration was studied by analysis of the RNA on 1-dim. PAGE (Fig. 3). The results of the experiments presented in Fig. 3 clearly show, that all four snRNAs migrate into the nuclei. In contrast, tRNA treated identically remained to 96% in the cytoplasm. In the nucleus of the oocytes the $snRNA_{1-4}$ seemed protected against degradation, while the snRNAs that remained in the cytoplasm were found to be partially degraded. The degraded material of $snRNA_1$ found in the nucleus (Fig. 3c) might be due to transport of degraded $snRNA_1$ into the nucleus.

Localization of the genes: The elegant method of "in situ" hybridization allows the localization of genes for which no mutants are known. The only prerequisite is a pure primary gene product or the gene and flanking sequences. After "in vitro" [^{125}I]-iodi-

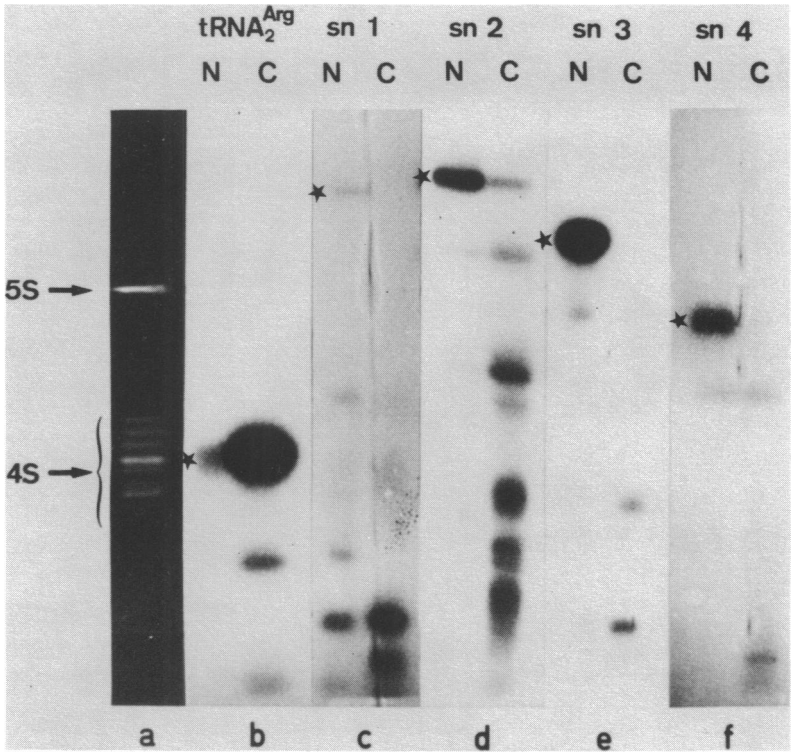

Figure 3 RNA migration in Axolotl oocytes. The oocytes were injected previously with [^{125}I]-snRNA into the cytoplasm. 10% PAGE of RNAs isolated from nuclei (N) or cytoplasm (C). Lane a: total, unlabeled low molecular weight RNA, stained with ethidium bromide; lane b: [^{125}I]-tRNA-Arg; lanes c-f: [^{125}I]-snRNA$_{1-4}$. All snRNAs accumulate in the nucleus, tRNA-Arg does not. snRNA2-3 are protected in the nucleus, snRNA$_1$ is partially degraded. All snRNAs are degraded in the cytoplasm. ★= size of the injected RNA.

nation the four snRNAs were hybridized "in situ" to polytene salivary gland chromosomes of the Drosophila melanogaster mutant giant. The following cytological regions were labeled (Fig. 4a-j): snRNA$_1$: 39B and 40AB; snRNA$_2$: 22A, 82E, and 95C; snRNA$_3$: 14B, 23D, 34A, 35EF, 39B, and 63A; snRNA$_4$: 96A. With the exception of region 39B (labeled by snRNA$_1$ and snRNA$_3$) all regions are labeled by one snRNA only. It follows from this result that the genes coding for snRNA$_{1-4}$ are not clustered in the Drosophila melanogaster genome.

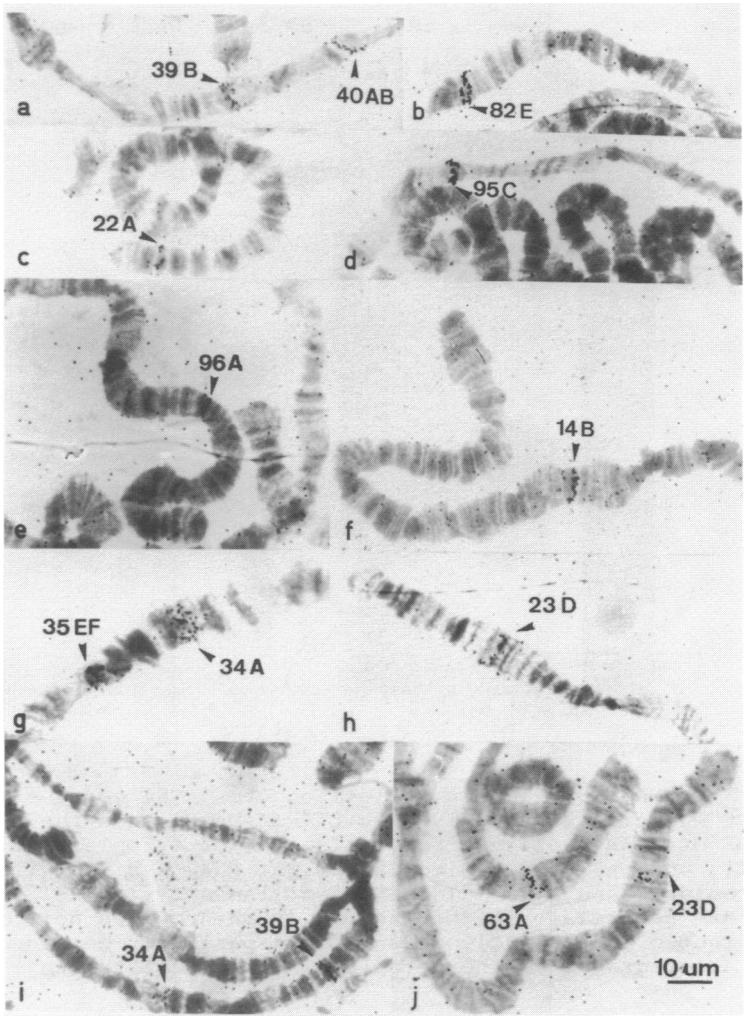

Figure 4 "In situ" hybridization of [^{125}I]-snRNA$_{1-4}$ to polytene salivary gland chromosomes of the D.melanogaster mutant giant. a: snRNA$_1$; b-d: snRNA$_2$; e: snRNA$_4$; f-j: snRNA$_3$.

Number of genes: Estimation of gene numbers from "in situ" hybridization data is difficult. Nevertheless, we compared grain numbers on the labeled loci with grain counts of tRNA hybridizations to loci where gene numbers are known from extensive cloning of these regions[28,29]. From these data (not shown) it can be concluded that each locus contains only one to

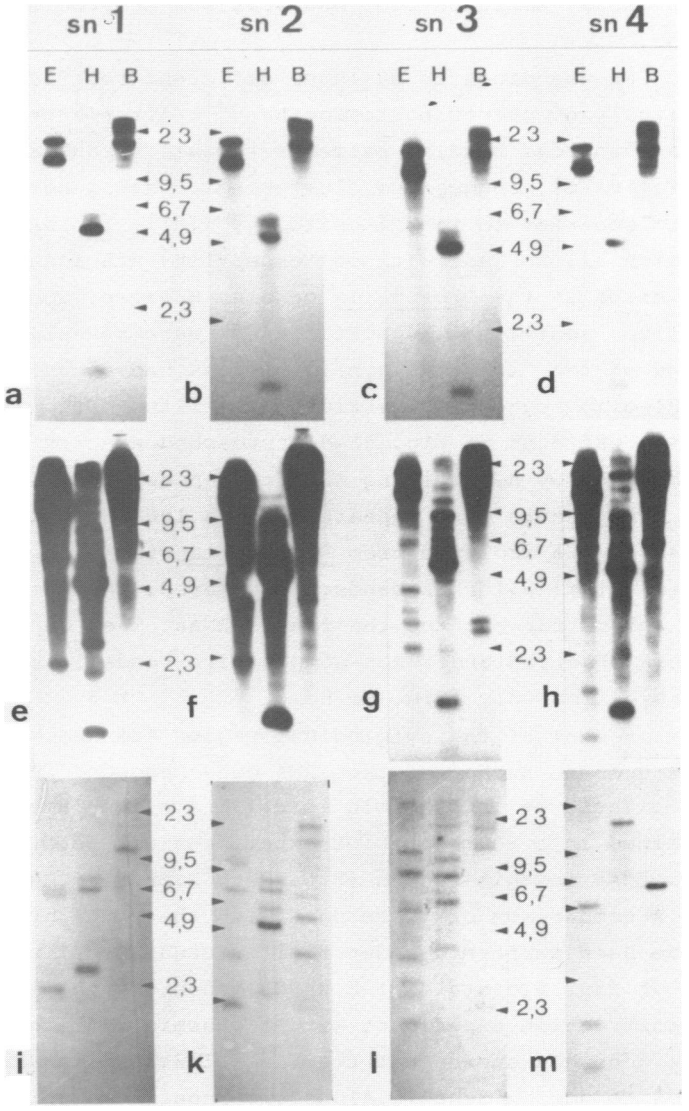

Figure 5 Autoradiograms of the hybridization of [^{125}I]-snRNA$_{1-4}$ to Southern-transfer filters. E = Eco RI digestion; H = Hind III digestion; B = Bam HI digestion. a-d: 12 h exposure time; e-h: 4 d exposure time; i-m: 4 d exposure time, hybridization with 100-fold molar excess of cold rRNA isolated from *Drosophila* ribosomes. rDNA containing bands are labeled in a-d. Additional, snRNA specific bands are revealed upon prolonged exposure time (e-h). Only snRNA specific bands remain after addition of cold rRNA competitor. MW markers in kb.

four snRNA genes. Southern-blot analysis was chosen to test these conclusions.

Genomic DNA was digested by three different restriction enzymes and analyzed according to Southern[30]. After short exposure times identical banding patterns for all 4 snRNAs were obtained (Fig. 5a-d), suggesting clustering of all genes. This would be in contrast to the "in situ" hybridization data, according to which all snRNAs (with one exception) are located at different sites in the Drosophila genome. However, upon prolonged exposure time, additional, specific bands were revealed (Fig. 5e-h). Some of the "in situ" hybridizations had shown labeling of the nucleoli, suggesting hybridization with ribosomal DNA. In fact, the patterns of Fig. 5a-d correspond exactly to the pattern obtained by hybridizing rRNA to genomic DNA[31,32]. Therefore, the experiments were repeated with a 100-fold excess of cold ribosomal RNA isolated from Drosophila ribosomes. As a result (Fig. 5i-m) all rDNA bands disappeared. The remaining bands are unique for each of the four snRNAs. They correspond to the bands observed under non-competitive conditions after long exposure time only (Fig. 5e-h).

A large segment of the cytological region 42A has been cloned and the nature and number of the tRNA genes encoded in this region is known[29]. Thus, it is possible to calibrate the intensity of bands obtained in a Southern blot experiment with e.g. [^{125}I]-$tRNA_2^{Arg}$ encoded in this region (R.Dudler, W.Brunner, unpublished results). A comparison of these data with the snRNA hybridizations (which have been performed under similar conditions) suggest that each band of Fig. 5 contains 1-2 snRNA genes. Furthermore, a genomic reconstruction experiment with a plasmid containing one U_2 Drosophila gene has shown that the Eco R I 2.3 kb band in Fig. 5i corresponds to one U_2 gene (C.Alonso, personal communication). From these results, the following numbers of genes were estimated: $snRNA_1$: 3; $snRNA_2$: 7; $snRNA_3$: 7; $snRNA_4$: 1-3.

DISCUSSION

A comparison of the size and the modified base composition of the four snRNAs isolated from D.melanogaster nuclei with data published for mammalian U-RNAs[26] (Table 1) suggest that the

following correlations can be made: $snRNA_1 \sim U_2$; $snRNA_2 \sim U_3$; $snRNA_3 \sim U_4$; $snRNA_4 \sim U_6$. Ireland et al.[33], comparing snRNAs isolated from Drosophila K_c cells with mammalian RNAs, found the same striking similarity in molecular size. After microinjection of total [^{32}P]-labeled RNA from HeLa cells into the cytoplasm of Xenopus laevis oocytes, snRNAs migrate into the nucleus[27]. Transfer RNA and 7S RNA remain in the cytoplasm, while 5S RNA migrates into the nucleolus. Migration into the nucleus is also oberved with $snRNA_{1-4}$ (Fig. 3). In the nucleus the snRNAs seem to be protected from degradation, whereas in the cytoplasm degradation takes place. This is an additional confirmation of the conclusion that the four RNAs studied belong to the class of snRNAs described by DeRobertis et al.[27].

A plasmid containing a U_1 gene of Drosophila melanogaster has been isolated and sequenced[34]. But, to our knowledge, no data on the localization and the number of U_1 genes are available so far. The organization and the number of genes coding for $snRNA_{1-4}$ in the D.melanogaster genome are similar in Dictyostelium[7] and in chicken[35], while the mammalian genome contains between hundred and two thousand dispersed genes and pseudogenes coding for the U-RNAs[36-42]. With one exception, all snRNAs label different regions on the salivary gland chromosomes (Fig. 4). Although the exact number of genes cannot be determined by Southern-blot analysis, it is clear that the numbers are small in the Drosophila genome. Since the number of sites labeled on the chromosomes correspond to the number of estimated genes, it can be concluded that each site contains 1-2 genes coding for a snRNA. Cross hybridization due to internal homologies[26] does not seem to be a problem. However, it has to be emphasized that the hybridization approach does not allow any conclusions about the function of these genes. In fact, the existence of pseudogenes, as described for the human[36,37,41] and the mouse[42] genome, cannot be excluded.

An intriguing observation is that all four snRNAs hybridized not only to their specific geneloci but also to rDNA without the addition of cold rRNA as competitor. This finding is interpreted as being due to a contamination of the snRNAs by random rRNA breadkown products on the basis of the following argu-

ments. First, the label on the rDNA bands disappears completely after addition of cold rRNA as a competitor (Fig. 5i-m). Second, hybridization of the snRNA probes to Southern-blots of rDNA containing plasmids[31] label the 28S α+ β_1, 28S β_2, and the 18S DNA containing bands (results not shown). From this it follows, that the contamination is rather due to a random degradation of ribosomal RNA than to a homology of the snRNAs to a specific part of the rDNA. Third, considering the R_ot-values used in our experiments, only a minor contamination hybridizing with repetitive complementary sequences in the genome can produce this striking effect. Thus, due to the vast excess of rRNA in the cell, it is not astonishing that this result is obtained. It has to be acknowledged, however, that homologies between snRNAs and rRNA have been described for U_3-RNA[26,43], and it cannot be completely excluded by our data, that the phenomenon is a consequence of extended base homologies.

Some of the loci hybridizing with snRNAs are covered by Minute deletions[44]. However, these deletions all include dozens of bands, thus rendering the correlation almost meaningless. The mutant shrunken (shr, 2-2.3), manifesting itself by smallness of body and darkness of colour[45], has been localized in the cytological region $22A_3$-$22B_1$, one site labeled by $snRNA_2$ (Fig. 5c). It will be interesting to see, whether a change in the $snRNA_2$ content can be found in this mutant.

The data on the localization and numbers of genes coding for $snRNA_{1-4}$ presented in this paper provide the necessary information for a combined genetic and molecular approach for a detailed study of this interesting class of RNAs.

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