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Topology of Repeated Sequences: Relationship of Nuclear RNA to the Repeated Sequences of the Main and Satellite DNA in Mouse Plasmocytoma Cells

(hybridization)

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ABSTRACT **The topology of repeated sequences in mouse plasmocytoma DNA was studied by high-resolution CsCl density gradient centrifugation and heterogeneous nuclear RNA·DNA hybridization. Satellite region DNA of plasmocytoma cells contains additional components and hybridizes specifically with entire heterogeneous nuclear RNA molecules. A linkage is demonstrated between the A+T-rich satellite sequences and those hybridizing with heterogeneous nuclear RNA. Heavy DNA also hybridizes specifically with heterogeneous nuclear RNA molecules that show sequence similarity to heterogeneous nuclear RNA hybridized to satellite DNA. These results could suggest that part of satellite DNA became heavier after integration of some other DNA species, which could belong to a virus or to immunoglobulin repetitive genes. Dispersed, highly repetitive, short nucleotide sequences could constitute recognition sites for such a process.**

The discovery of repeated sequences in eukaryotic DNA raised the problem of their possible function. The two possibilities that have been considered are either a role in regulation or their occurrence in structural genes (1, 2). Studies of heterogeneous nuclear RNA (HnRNA)·DNA hybridization could provide some general information on the organization and possible function of repeated DNA. HnRNA contains rapidly hybridizing sequences (3–5). Hybridization experiments with DNA from fractionated chromosomes have shown an homology between HnRNA sequences that hybridize rapidly to the DNA of different sets of chromosomes. These rapidly hybridizing sequences represent a part of HnRNA (15–20%) not yet exactly estimated (4).

DNA repeated sequences occur in families with a broad range of repetition frequency, similarity, and length (1). One of these families, the most reiterated one, is present in all chromosomes in the centromeric region (6). In some species (e.g., mice) these families of sequences can be separated on the basis of nucleotide composition by centrifugation to equilibrium in CsCl or Ag^+-Cs_2SO_4 (7–10). They appear as a discrete band (satellite), distinct from the main-band DNA. Although some controversy exists on this point (11, 12), it is generally accepted that this kind of DNA is not normally transcribed into RNA molecules (11) and may play some role in chromosome structure and folding (13–15). The present experiments provide some more information on the distribution of repeated sequences that are complementary to HnRNA and their topological relationship to nontranscribed repetitions in mouse plasmocytoma DNA.

Abbreviation: HnRNA, heterogeneous nuclear RNA.

MATERIALS AND METHODS

Cells. The MPC-11 plasma cell tumor (adapted to culture) provided by Dr. M. D. Scharf was maintained in BALB/c mice by subcutaneous transplantations. Cells released by shaking tumor fragments (16) were used to prepare DNA and rapidly labeled RNA. Cell cultures were also used for labeling experiments.

Fractionation Procedures. HnRNA greater than 45 S was prepared as described (17, 4) by labeling plasmocytoma cells in culture (2×10^7 cells per ml) for 90 min with 0.5 mCi/ml of [^{3}H]uridine. The specific activity of HnRNA was about 50 to 75×10^4 cpm/μg. High-molecular-weight DNA (3 to 4×10^7) was prepared from nuclei that had been cleaned by detergent (4). A combination of the method of Flamm *et al.* and a "density gradient multispeed method" (18, 19) was used to increase resolution of DNA components of slightly different G+C content in preparative CsCl equilibrium density gradients. The final solution of DNA (0.65 *A* units/ml) in CsCl, 10 mM Tris·HCl, 1 mM EDTA (pH 8.2), adjusted to a density of 1.700 g/ml in 15-ml gradients overlayed with paraffin oil, was centrifuged in a Spinco 30 rotor for 40 hr at 28,000 rpm followed by 50 hr at 20,000 rpm to reduce the slope of the gradients. Fractions were collected from the bottom of the tubes by suction with a peristaltic pump, and the DNA was recovered by centrifugation after 5-fold dilution. ^{14}C-Labeled satellite DNA was purified by the technic of Flamm *et al.* (18) using 100–150 μg of DNA per 15-ml gradient. Sonication was used to reduce the size of DNA in some experiments. The DNA was diluted to 50 μg/ml and sonicated (15 sec at 20–21 kHz) under standard geometrical conditions in a MSE Sonicator. Molecular weight was determined by the method of Burgi and Hershey (21), with bacteriophage λ DNA as a marker of size.

Hybridizations. RNA·DNA hybridization was carried out for prescribed times at 45° in 0.30 M NaCl–0.030 M Na citrate, 0.1%(w/v) sodium dodecyl sulfate, 30% (v/v) formamide (4). DNA·DNA hybridization was conducted in 0.30 M NaCl–0.030 M Na citrate, 0.1% sodium dodecyl sulfate, 30% formamide. Under these conditions denatured DNA is not retained by the nitrocellulose, although it is still able to hybridize with homologous DNA bound to filters. [^{14}C]DNA was denatured in 15 mM NaCl–1.5 mM Na citrate by bringing the pH of the solution to 13 with 1 M KOH (20). After 10 min, the solution was neutralized with 1 M KH_2PO_4, chilled, diluted

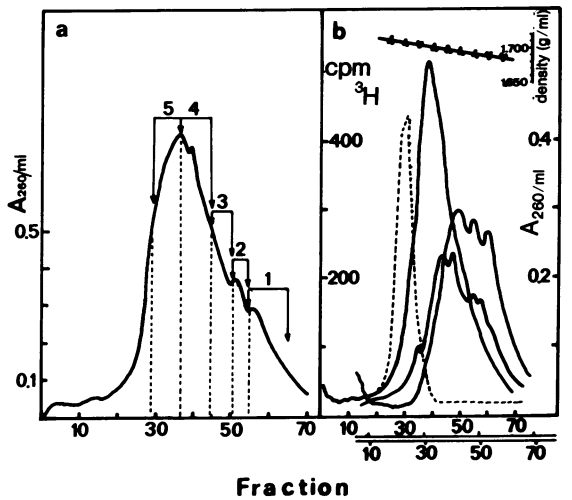

FIG. 1. (*a*) Buoyant density profile of total nuclear mouse DNA from plasmocytoma tumor cells banded in CsCl. (*b*) From right to left, buoyant density of recycled fraction areas 1, 2, and 3. Positions in the figure are relative to the marker (^{3}H-labeled λ DNA). Dashed line, cpm; solid line, absorbance.

with 0.30 M NaCl–0.030 M Na citrate, 30% formamide in the appropriate volume, and distributed to the vials.

RESULTS

Analysis of Mouse DNA by High-Resolution CsCl Density Gradient Centrifugation. Relatively high-molecular-weight nuclear DNA (30 to 40 × 10^6) from plasmocytoma cells was centrifuged in CsCl under the conditions described in *Materials and Methods* to increase resolution of components. The buoyant density profile of the DNA is depicted in Fig. 1*a*, and shows two shoulders in its light part or "satellite region." If fractions from the light portion of the initial gradients (indicated by arrows in Fig. 1*a*) are collected and recycled in new CsCl density gradients under the same centrifugation conditions, resolution of these components is increased (Fig. 1*b*). In this experiment, labeled λ DNA was used as a density marker (density, 1.709 g/ml), and the respective positions of individual fractions relative to the marker are represented in the same figure. Fractions 1, 2, and 3 actually band at different densities, and "satellite-region" DNA is resolved into more than one component with slightly different buoyant densities, from 1.690 to 1.695 g/ml. This pattern of satellite-region DNA, to my knowledge, has not been reported, either with preparative or analytical gradients (10, 11, 18).

Hybridization of DNA to Fractions of Different Average G+C Content. In order to perform hybridization experiments, we recycled relatively high amounts of DNA fractions from areas 1, 4, and 5 (gradients of Fig. 1) in new CsCl density gradients. Fraction areas 1, 4, and 5 are, respectively, referred to as satellite-region DNA, main-band light DNA, and main-band heavy DNA. Preliminary tests, performed with fractionated [^{14}C]DNA, have established that fixation and retention to filters were the same for satellite-region and main-band DNA fractions. HnRNA of high specific activity was prepared by labeling plasmocytoma cells for 90 min with [^{3}H]uridine, and hybridization was performed with the entire population of HnRNA (greater than 45 S) molecules. No attempt was made

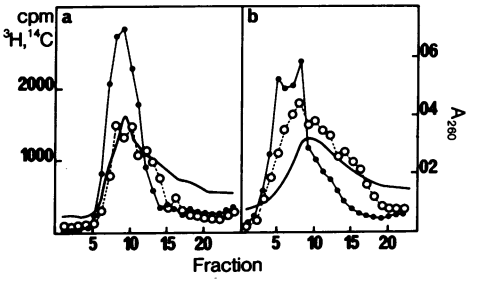

FIG. 2. Hybridization of HnRNA (●) and ^{14}C-labeled satellite DNA (○) to individual fractions of satellite region DNA that had been recentrifuged in CsCl (*a*) before and (*b*) after sonication of this DNA. Solid line, absorbance tracing.

to fractionate the rapidly hybridizing sequences of HnRNA first. However, previous experiments have shown that under the usual conditions of hybridization (20–40 hr) the rapidly hybridizing sequences of these molecules hybridizes predominantly (4). Table 1 shows the result of a hybridization experiment with HnRNA and three fractions of plasmocytoma cell DNA. The amount of RNase-resistant hybrid, tested after digestion of unpaired regions with three RNases (pancreatic and T1 and pancreatic and T2), shows the same hybridization of HnRNA to all the fractions. As satellite DNA is generally accepted not to be transcribed, hybridization in the satellite region of these cells had to be more carefully investigated.

Topological Relationship of Repeated Sequences in Satellite Region DNA. The next experiment was performed in order to look at the relationship between the satellite region DNA sequences that hybridize to HnRNA and the A+T-rich sequences of this fraction. For this purpose the size of DNA was examined by sedimentation velocity by the technic of Burgi and Hershey, with λ DNA as a marker of size (21). We found that the size of total and satellite DNA in our preparations was 40 to 50 × 10^6 daltons. Under suitable conditions of sonication, the size of satellite-region DNA was reduced to 1 × 10^6 daltons. Untreated and sonicated satellite DNA (100 μg) was banded in CsCl density gradients. After analysis of absorbance the collected fractions from each gradient were split, and half the DNA from each individual fraction was loaded onto separate filters after KOH denaturation. HnRNA (1.5 × 10^5 cpm) or sonicated, denatured ^{14}C-labeled satellite DNA (640 cpm/μg, 8600 cpm/vial) was hybridized. The buoyant density of DNA complementary to rapidly hybridizing HnRNA sequences is shown (Fig. 2) in the two different

TABLE 1. *Hybridization of HnRNA to DNA fractions of different average G+C content*

DNA fraction	Measured RNase-resistant hybrid (cpm)		
	Pancreatic + T1	Pancreatic + T2	Blank
MBH	315	320	35
MBL	396	422	34
SAT	311	326	36

HnRNA (70 × 10^5 cpm) was hybridized for 20 hr to 10 μg of DNA per filter. Numbers represent the average cpm of three filters. MBH, main-band heavy DNA; MBL, main-band light DNA; SAT, satellite DNA.

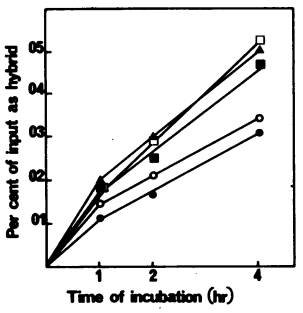

FIG. 3. HnRNA (10^5 total input cpm) was hybridized at 45° in 0.30 M NaCl–0.015 M Na citrate, 30% formamide to filters containing 5 and 10 μg of DNA for 1, 2, and 4 hr. (●) 5 μg and (○) 10 μg of satellite DNA on filters. (▲) 5 μg of main-band light DNA on filters. (■) 5 μg and (□) 10 μg of main-band heavy DNA on filters. The RNase-resistant hybrid was measured. Rates are expressed as a function of the initial input RNA that has been hybridized.

size classes of the native satellite DNA region. Large-molecular-weight satellite-region DNA hybridizes HnRNA as previously found (Fig. 2*a*). Nevertheless, after reduction of DNA size (Fig. 2*b*), a shift in the density of A+T-rich DNA sequences is found (shown by absorbance analysis and DNA·DNA hybridization). This shift is not followed by the radioactivity of HnRNA·DNA hybrids. This experiment suggests that repeated sequences richer in G+C that hybridize to HnRNA are linked to A+T-rich stretches in satellite-region DNA, and this must be in relation to the additional components found in that DNA region, since A+T-rich stretches of satellite DNA are not normally transcribed (11).

Since HnRNA has been found to contain sequences that hybridize to repeated DNA, hybridization studies with this material are difficult to interpret. A series of experiments was performed in order to better understand hybridization to DNA fractions of different G+C content.

Comparison of the Reiterated Sequences of Satellite-Region and Main-Band DNA Complementary to HnRNA. (*i*) The frequency of reiteration of the sequences in the different DNA

TABLE 2. *Hybridization and cross-hybridization of HnRNA to DNA fractions of different average G+C content*

Preparative hybridization		Cross-hybridization	
DNA fraction	Eluted hybrid (cpm)	DNA fraction	RNase-resistant hybrid (cpm)
		SAT	2602
SAT	35,000	Blank	30
		MBL	3529
		SAT	2309
MBL	48,000	Blank	39
		MBL	5081
		SAT	2112
SAT	29,500	Blank	25
		MBH	2709
		SAT	2574
MBH	40,000	Blank	51
		MBH	4522

For abbreviations, see Table 1.

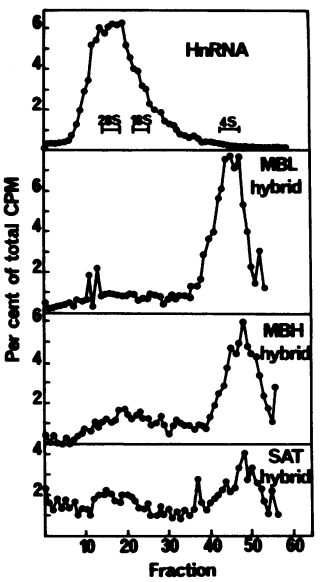

FIG. 4. Electrophoresis in 2% acrylamide–0.5% agarose gels of HnRNA and RNase-resistant hybridized HnRNA recovered from main-band heavy (MBH), main-band light (MBL), and satellite (SAT) DNA. 28S and 18S ribosomal and 4S RNA markers were run on parallel gels.

fractions was studied in an initial experiment. When RNA is hybridized to a great excess of DNA, the rate of hybrid formation, expressed as the fraction of the initial input RNA that has been hybridized, should only be dependent upon the frequency of homologous binding sites in DNA. Neither the concentration nor the specific activity of the RNA should influence the measurement. We have used the term "specific hybridization rate" to describe this measurement (4). The data in Fig. 3 show that the specific hybridization rate of HnRNA is approximately the same for the three DNA fractions tested. This result indicated a similarity in hybridizing sequences with respect to the repetition frequency. Satellite-region DNA hybridizes perhaps at a slower rate to HnRNA than does main-band DNA, but this could be due to the fact that the largest part of this DNA (highly reiterated A+T-rich sequences of satellite) does not hybridize to HnRNA (11).

(*ii*) The degree of homology between the sequences of HnRNA that hybridize to satellite-region and main-band DNA fractions was investigated in the cross-hybridization experiment described in Table 2. HnRNA (3.10^6 cpm) was hybridized for 40 hr on filters containing 10 μg of main-band heavy, main-band light, and satellite DNA. The hybridized RNA was recovered after RNase treatment from each DNA fraction and hybridized to the homologous and heterologous DNAs. There is a selection of rapidly hybridizing sequences in the first round of hybridization because the percentage of the hybridized input increased from 1 to 10% when hybridized sequences are rehybridized. The result of the cross-hybridization shows that the sequences hybridized to the satellite DNA region are similar to the reiterated sequences of the main-band DNA. Such a similarity is difficult to understand for DNA sequences that differ in base composition from 34% to 42% G+C.

The next experiment was designed to look at the size of perfectly hybridized sequences of HnRNA, to compare the size of the hybrids from fractions with different G+C con-

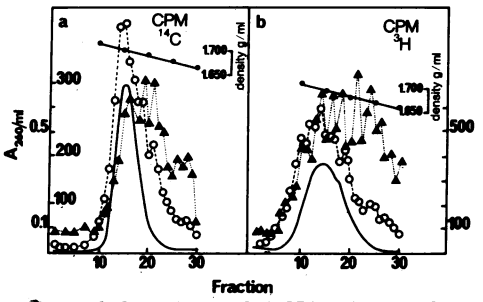

FIG. 5. Recycled main-band DNA of two different size classes, molecular weights 40 × 10^6 (*a*) and 10^6 (*b*), was banded in CsCl gradients and fractionated. Each fraction was hybridized with [^{3}H]HnRNA (○) and satellite [^{14}C]DNA (▲). Solid line, absorbance.

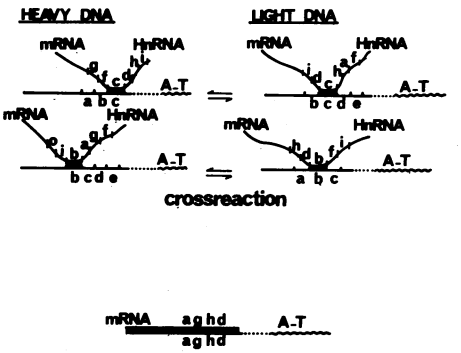

FIG. 6. (*Top*) Specific binding of HnRNA to dispersed reiterated sites. (*Bottom*) Specific binding of entire HnRNA molecules. See text for details.

tents, and to see what part of the entire HnRNA molecule they represent.

(*iii*) In order to rid HnRNA preparations of residual DNA fragments, the greater than 45S fraction of HnRNA was heated for 5 min at 100° and passed through a nitrocellulose filter. Under such conditions the size of the molecules is generally reduced to 28–18 S. HnRNA (5 × 10^6 cpm) of that size was then hybridized on filters to 10 μg of main-band heavy, main-band light, and satellite DNA. After RNase digestion, washing, and iodoacetate treatment of the filters to eliminate any residual RNases, the hybridized RNA was eluted and its size was examined by acrylamide gel electrophoresis.

Nonhybridized HnRNA molecules show (Fig. 4) a broad profile of sizes, with a mean value of about 28 S; very few molecules are found in the region smaller than 18 S. Eluted hybrids from all DNA fractions show two interesting features. (*a*) There is a rather homogeneous size distribution of sequences found in the region of 5–4 S that is common to the hybrids recovered from all these DNA fractions. (*b*) In addition, RNA of 18–28 S is recovered from hybrids formed with DNA derived from the satellite DNA region and to a lesser extent from the heavy DNA region. We describe such hybridization as site-specific for the entire HnRNA molecule.

The results of these hybridization experiments show that: (*1*) On the basis of size redundancy and sequence similarity, repeated sequences of HnRNA represent a small and rather homogeneous fraction of the large HnRNA molecules. The occurrence of such sequences in a broad spectrum of DNA molecules with a different G+C content could be explained if we taken into account the large size of DNA relative to the size of the RNA hybrid. Under such conditions, a small homogeneous sequence could be present in DNA molecules with a different average buoyant density. (*2*) Specificity of hybridization in satellite DNA region and heavy DNA must be related to the appearance of an additional component in satellite region shown from first and second CsCl gradients of plasmocytoma cell DNA and might also be related to the crossreaction between these molecules and those hybridized to heavy DNA.

Presence of A+T-Rich Satellite-Like Sequences in Main-Band DNA. Recycled main-band DNA (fractions 4 and 5) was used to perform the same type of experiment as for satellite-region DNA. Fig. 5 represents such an experiment, where fractions of main-band DNA, banded in CsCl without reduction of size (40 × 10^6) or after sonication (10^6 daltons), were used to study hybridization patterns of HnRNA and satellite DNA sequences. Each DNA fraction was loaded onto a nitrocellulose membrane, half of which was used for hybridization with HnRNA and half for hybridization with satellite [^{14}C]-DNA. Comparison of the patterns of hybridization in high-molecular-weight and sonicated DNA shows an increase in hybridization of satellite-like sequences in the light part of the gradient of sonicated DNA. Therefore, A+T-rich sequences must be present in main-band DNA, even when this band has been recycled by centrifugation and satellite region removed. There is not a substantial increase in the density of HnRNA hybridizing sequences after reduction of DNA size, and satellite [^{14}C]DNA hybridization coincides with the hybridization of HnRNA in the heavy part of the gradient. As satellite [^{14}C]-DNA used in these experiments could contain some G+C-rich sequences that hybridize to HnRNA, this result cannot distinguish between this possibility and a closer linkage of A+T-rich sequences with HnRNA in main-band DNA.

DISCUSSION

Since it has been shown that HnRNA hybridizes to the repeated DNA (4), studies of hybridization with these molecules are difficult to interpret. Previous experiments have shown an abnormal distribution of sequences complementary to HnRNA among chromosomes. Comparison of HnRNA sequences hybridizing to the DNA of different sets of chromosomes has shown sequence similarity (4). (*i*) It could be that this sequence similarity is due to the presence of internal "signals" in HnRNA molecules that are the same or very similar among different sets of chromosomes. (*ii*) However, it has been shown that some of the HnRNA hybrids were very large and could represent entire HnRNA molecules (22). Dispersion of these molecules could be due to DNA transfers by the mechanism of reverse transcriptase, as is postulated for oncogenic RNA viruses. This could be particularly interesting for cancer cells where such viruses cannot be isolated.

Coexistence of the two possibilities, *i* and *ii*, cannot be excluded, some of the molecules being bound to DNA in the proper place and so hybridized throughout their length and some not. This is explained in Fig. 6 in relation to the present experiments: a, b, c, d, e, f. . . . n are short reiterated sites not tandemly repeated in DNA and present in some part of the HnRNA molecules. Hybridization on these sites could account for specific binding not occurring on the entire HnRNA molecules and could explain crossreactions. If these highly repetitive sequences are contiguous to structural repetitive genes, the chance to bind entirely the proper molecule in-

creases as the number of copies of a particular combination a, g, h, d increases. Then specific hybridization could occur.

The present work is performed in plasmocytoma cells. The MPC_{11} cell line synthesizes large amounts of an immunoglobulin light chain (16) and contains a leukemia-type RNA virus (23) that has been seen by electron microscopy (G. Pagoulatos, unpublished observation). The possibility exists that DNA transfers concerning immunoglobulin genes or DNA provirus could proceed by a common mechanism (24, 25). In this context, topological studies of DNA repeated sequences complementary to HnRNA in these particular cells are very relevant.

The present experiments indicate that additional components exist in these cells in satellite region. We do not know whether this is a general feature of mouse DNA that has never been found before (10, 11, 18), or whether it is specific for BALB/c mouse DNA (26) or plasmocytoma cell DNA. If we take into account that satellite DNA is not normally transcribed, hybridization of HnRNA in that region could be due to the presence of these components of very close buoyant densities. However, it is difficult, in the light of information from the present experiments, to understand the exact topological relationship of the repeated sequences in the satellite region of these cells. This will only become possible after purification of the components of that region. It could be that the additional component is independent of satellite DNA. However, the linkage we have demonstrated in the satellite region between A+T-rich sequences, which are not apparently transcribed, and the sequences hybridizing HnRNA could suggest that this component constitutes part of the satellite that became heavier after integration of some other DNA species. The presence of specific hybridization in heavy and satellite region DNA and the crossreaction between HnRNA sequences hybridized in these two DNA regions are consistent with this explanation. Dispersed, highly repetitive, short nucleotide sequences (see Fig. 6) could constitute recognition sites for such translocation processes.

Many recent reports relate satellites to oncogenic virus infection (27–29). Hybridization studies with RNA from antibody-producing cells show changes in the percentage of hybridization to satellite-region DNA during the immune response (30). However, the relation of DNA in these components with virus or immunoglobulin specific genes deserves further experimentation.

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