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Separation of DNA Sequences Complementary to the RNA of Avian Myeloblastosis Virus from Chicken DNA by Alkaline Cesium Chloride Density Sedimentation

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Density gradient sedimentation in alkaline cesium chloride of DNA from normal chicken embryos or leukemic myeloblasts fragmented to a size of 13*S* revealed that the DNA sequences complementary to 70*S* avian myeloblastosis virus RNA sedimented in the high guanine plus cytosine region ahead of the main peak of cellular DNA. When the DNA was fragmented into pieces of 6.6*S* there was a broader distribution of the DNA sequences complementary to the viral RNA. This technique could be employed as a step towards the isolation of DNA copies of the entire viral RNA genome from the mass of host cellular DNA.

The putative DNA copy of the entire RNA genome of avian myeloblastosis virus (AMV) is needed to characterize the viral genome, the viral mRNA species, and the viral-specific DNA sequences present in normal or infected cells. The viral-specific DNA synthesized by the virion RNA-dependent DNA polymerase on the endogenous RNA appears to represent a transcript of all the sequences in viral RNA (8), but is less than 4 to 10*S* in size (7, 9, 12), and every sequence in the oncornavirus RNA is not represented at the same frequency in the DNA product (10, 15). The majority of the DNA sequences are apparently copied from a limited fraction (5–10%) of the total viral genome (7, 15). Also, the small RNA species present in virions and contaminating cellular RNA and DNA species may be copied. The use of purified enzyme and viral RNA is an improvement but does not resolve the problem, since the exact composition of the viral genome is unknown.

Another approach to the characterization of the viral genome is to study the nature of the virus-specific DNA sequences in infected cells. As a step toward the isolation of the viral DNA template from cellular DNA, we investigated whether DNA complementary to AMV RNA could be separated from bulk chicken cellular DNA by alkaline cesium chloride density sedimentation. The viral RNA, and consequently the viral DNA, have an average content of 52% guanine plus cytosine (G + C) (13), whereas only 42% of the chicken DNA is G + C. Thus, the viral DNA should have a considerably greater density than chicken DNA and should sediment ahead of the bulk DNA in CsCl density gradients. To obtain a better resolution, the DNA was fractionated under denatured conditions in alkaline cesium chloride; fragments of two different sizes, 13.2*S* and 6.6*S*, were analyzed by this method. We also analyzed DNA from normal chicken cells which contain fewer and qualitatively different oncornavirus-specific DNA sequences (1, 4, M. Shoyab, M. A. Baluda, and R. M. Evans, unpublished data) than virus-producing leukemic cells transformed by AMV.

MATERIALS AND METHODS

The AMV BAI strain A subgroup B was used. ^{3}H-labeled 70*S* AMV RNA was prepared as described earlier (2, 4).

Chicken embryo DNA, chicken myeloblast DNA, and mouse embryo RNA were prepared as described previously (4). Only normal chicken embryos that were not virus producers were used (1).

Chicken embryonic ^{3}H-labeled 28*S* rRNA was isolated from secondary chicken embryo fibroblast cultures, when 10-cm Falcon culture dishes were approximately three-fourths covered. The regular medium was replaced with 10 ml of medium containing ^{3}H-uridine (20 μCi/ml) plus dialyzed calf (5%) and chicken (5%) sera and 10^{-6} M thymidine. After 12 h of incubation the cells were dispersed with trypsin and pelleted by centrifugation at 4 C. The cells were

resuspended in cold hypotonic buffer (0.1 M NH_4Cl, 0.005 M $MgCl_2$, 0.03 M Tris-hydrochloride (pH 7.6), and 0.005 M mercaptoethanol; 25 ml per 1 ml of packed cells), allowed to swell for 15 min, and homogenized at 0 C by 15 strokes of a steel dounce homogenizer with a clearance of 0.002 inches (0.00508 cm). Sucrose was added to the homogenate to a final concentration of 0.25 M. The homogenate was centrifuged at 10,000 rpm for 10 min in a refrigerated Sorvall centrifuge to remove nuclei and mitochondria. The post-mitochondrial supernatant solution was centrifuged at 105,000 $\times$ *g* for 1 h to pellet the ribosomes. The ribosomal pellet was resuspended in extraction buffer (0.1 M NaCl; 0.01 M Tris-hydrochloride (pH 8.5), and 0.001 M EDTA), and the RNA was extracted as described previously (4). The 28*S* rRNA was isolated after neutral sucrose velocity gradient sedimentation (5–20% sucrose in 0.1 M NaCl, 0.01 M Tris-hydrochloride (pH 7.4), 0.001 M EDTA).

The DNA (250 μg/ml) was fragmented either in a Waring blender to a size of 13*S* (5 min in 1-min pulses after intervals of 1 min at 0 C), or to a size of 6.6*S* by sonic disruption (Bronson sonifier at a setting of 4 for 5 min in 1-min pulses after intervals of 1 min at 0 C). The size of the DNA was determined in alkaline sucrose velocity gradients by using M-13 phage DNA (20*S*) as marker. The molecular weight of the DNA fragments was calculated by the method of Studier (14).

Alkaline CsCl density gradients were run at 35,000 rpm for 65 h in the Beckman SW50 rotor at 22 C. DNA (100–120 μg) was used in each tube in 4.2 ml of CsCl in 0.3 N sodium hydroxide. The final density of the solution was 1.7 g/ml. The neutral CsCl density gradient sedimentation was performed similarly except that the CsCl and the DNA were dissolved in 0.1 $\times$ SSC (SSC equals 0.15 M sodium chloride and 0.015 M sodium citrate). Fractions were collected from the bottom of the tubes, neutralized with Tris-hydrochloride (1 N HCl-0.2 M Tris base), and dialyzed extensively against 0.1 $\times$ SSC (4 liters per 20 ml) with five changes at intervals of 12 h. The density of the cesium chloride fractions was determined from the refractive index.

The dialyzed DNA was denatured by boiling in 0.1 $\times$ SSC for 10 min and rapid cooling in ice water, 20 $\times$ SSC was added to make the DNA solution 6 $\times$ SSC, and the DNA was trapped on nitrocellulose filters by gravity filtration. The method of hybridizing denatured cellular DNA on filters to an excess of ^{3}H-labeled 70*S* AMV RNA or 28*S* ^{3}H-labeled rRNA has been described (1, 4). Depending upon the fraction of the gradient analyzed, the DNA concentration per filter varied from 5 to 19 μg.

RESULTS AND DISCUSSION

Normal chicken embryonic DNA fragmented into pieces of 13*S*, equivalent to a mol wt of 0.95 $\times$ 10^6 (about one-third of a 36*S* RNA molecule), showed, after neutral cesium chloride density sedimentation, a relatively narrow density distribution with a median density of 1.73 g/ml. This density is a little higher than that previously reported for native double-stranded chicken DNA (1.701 g/ml) (11) because the DNA used here had been treated with 0.3 N KOH to remove contaminating RNA before neutralization and dialysis against 0.1 $\times$ SSC at 4 C. This denatured DNA takes a long time to reassociate completely at the proper salt concentration and temperature and is therefore partially single-stranded and partially double-stranded. After alkaline cesium chloride density gradient sedimentation, the DNA had a median density of 1.76 g/ml and a broader density distribution (Fig. 1), indicating a better resolution of the DNA fragments that differ in G + C content.

The distribution of DNA sequences complementary to AMV RNA in different fractions of an alkaline CsCl density gradient is shown in Fig. 1 for DNA from normal chicken embryos and in Fig. 2A for DNA from leukemic chicken myeloblasts. Both figures show that the fractions with the higher densities (1.79–1.80 g/ml) hybridized more with 70*S* AMV RNA than the fractions with lower densities. The degree of complementarity to AMV RNA decreased rapidly with a decrease in density of the DNA and reached a plateau at densities of 1.76 g/ml and lower. The small heterogeneous (4-10*S*) RNA molecules which are associated with the 70*S* viral RNA may be coded for by the cellular genome and may explain why the virus-specific DNA is more broadly distributed over the cellular DNA peak than the DNA complementary to 28*S* rRNA (Fig. 3). DNAs from normal and leukemic cells show qualitatively similar profiles, although the leukemic DNA contained more viral-specific DNA sequences per 100 μg of DNA.

If the size of the leukemic DNA fragments is reduced to 6.6*S* (fragmented by sonic disruption) which corresponds to a mol wt of 168,000, the distribution of the viral-specific DNA sequences with respect to density is different from that obtained with 13*S* fragments. As presented in Fig. 2B, the viral-specific DNA sequences are still present at a higher concentration in DNA with the highest density, but they are more broadly distributed over the entire gradient.

These findings provide additional support for the specificity of the hybridization reaction between viral RNA and complementary DNA and also provide a method for the partial isolation of viral-specific DNA sequences from cellular DNA. Our hybridization technique does not permit the determination of the fraction of the viral genome present in the virus-specific DNA, but in separate experiments it was dem-

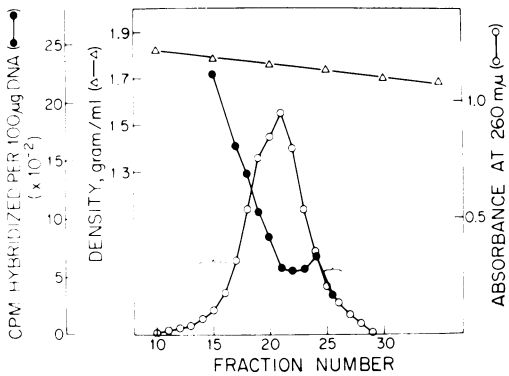

FIG. 1. *Hybridization of ^{3}H-labeled 70S AMV RNA with normal chicken embryo DNA fractionated by alkaline cesium chloride sedimentation. DNA (100–120 μg) fragmented into pieces of 13S was suspended in 4.2 ml of CsCl (final density 1.7 g/ml) plus 0.3 N sodium hydroxide in 5.2-ml polyallomer centrifuge tubes. The remaining volume of the tubes was filled with mineral oil. After centrifugation at 35,000 rpm for 65 h in the SW50 rotor at 22 C, 10 drop-fractions were collected from the bottom of the tubes. Samples (25 μliters) were taken for determination of the refractive index, the remaining material was diluted to 0.4 ml with 0.1 × SSC, and the absorbance at 260 nm was measured in a Zeiss spectrophotometer. Each fraction was neutralized and dialyzed extensively against 0.1 × SSC, boiled for 10 min, and made 6 × SSC, and the DNA was trapped on a nitrocellulose filter. Each filter was hybridized in 0.5 ml of hybridization mixture containing 0.5 × 10^6 counts/min of 70S ^{3}H AMV RNA (sp act: 10^6 counts per min per μg), 3 mg of unfractionated mouse RNA, and 0.05% sodium dodecyl sulfate in 4 × SSC (4). ^{3}H 70S RNA bound to mouse DNA filters (20–30 counts/min) was deducted as background. (—), Pooled fractions were placed on the same filter for hybridization with viral RNA.*

onstrated by hybridization of 70S viral RNA to a large excess of leukemic DNA that the viral-specific DNA in leukemic cells represents more than 60% of the viral genome. The results also indicate that the viral DNA consists of long nucleotide sequences, since its fragmentation into approximately 17 pieces yields segments of varied G + C content, i.e., of varied base sequences. Similar conclusions were drawn from the sharp melting curves and the high melting temperature of the hybrids formed between viral RNA and complementary DNA sequences (4; Shoyab et al., unpublished data) and from the average base composition of the RNA eluted from the hybrids (3). If the DNA sequences complementary to viral RNA had represented short, randomly located sequences, opposite results would have been obtained; long fragments of DNA would have given a broad distribution of the viral-specific DNA sequences and short fragments would have shown a narrow distribution corresponding to the G + C content of the short virus-specific segment.

As a control, ^{3}H-labeled 28S chicken rRNA was used as a hybridizing probe with 13S fragments of normal chicken DNA fractionated

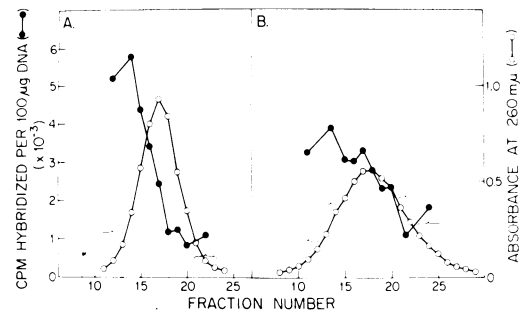

FIG. 2. *Hybridization of ^{3}H-labeled 70S AMV RNA with leukemic myeloblast DNA fractionated by alkaline cesium chloride density sedimentation. A, Leukemic DNA fragmented into pieces of 13S in a Waring blender. The procedure was the same as that described in Fig. 1 except that the hybridized count per minute represents the average of two filters prepared from two similar gradients. B, Leukemic DNA fragmented into pieces of 6.6S by sonic disruption. The procedure was the same as that described in Fig. 2A.*

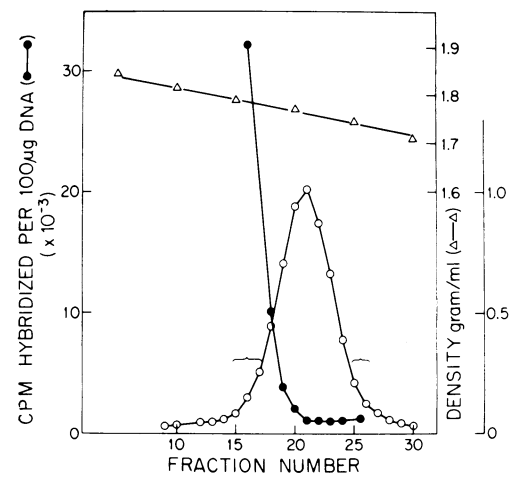

FIG. 3. *Hybridization of ^{3}H-labeled 28S chicken rRNA with normal chicken embryo DNA fractionated by alkaline cesium chloride sedimentation. The DNA was fragmented into pieces of 13S in a Waring blender and processed as described in Fig. 1. The hybridization was carried out with 28S chicken rRNA (10^6 counts per min per ml, sp act: 5 × 10^5 counts per min per μg) in the presence of 3 mg of 4S Escherichia coli RNA per ml. ^{3}H RNA hybridized to E. coli DNA filters (25–28 counts/min) was subtracted as background.*

by alkaline CsCl density gradient sedimentation. As shown in Fig. 3, the DNA sequences complementary to rRNA are also located in the G + C rich fractions. About 80% of the total rRNA is hybridized by the two DNA fractions that are richest in G + C. This is in agreement with the known high G + C content of rRNA (6). Similar results have been obtained in studies with rRNA from *Xenopus laevis* which contains 65 to 67% of G + C (5, 6).

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