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Reliability of the RNA-DNA Filter Hybridization for the Detection of Oncornavirus-Specific DNA Sequences

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Denatured DNA from leukemic myeloblasts or uninfected chicken embryos, immobilized on nitrocellulose filters, was hybridized to a vast excess of [^{3}H]70*S* RNA from purified avian myeloblastosis virus. The viral RNA was eluted from the RNA-DNA hybrids, purified, and then rehybridized in solution to an excess of either leukemic or normal chicken embryonic DNA. This study revealed that all the slow and the fast hybridizing viral RNA sequences detectable by liquid hybridization in DNA excess had hybridized to the filter bound DNA. Both techniques also gave similar values for the number of 28*S* ribosomal RNA genes contained in a chicken cell genome: 210 by the liquid hybridization procedure and 218 by the filter hybridization technique. Therefore, filter hybridization can accurately detect DNA sequences present in relatively few numbers in the genome of higher organisms.

Early hybridization studies with nucleic acids from prokaryotes have indicated that the filter hybridization technique between immobilized denatured DNA and an excess of RNA was very specific and could detect all the bacterial DNA sequences complementary to an RNA probe (11, 12, 13, 16, 17). However, RNA-DNA hybridization studies are more complicated in higher organisms. Eukaryotes have a large complex genome, at least one thousand times larger than prokaryotes, of which 25 to 40% consists of sequences reiterated 10^1 to 10^6 times (10). These factors, i.e., large size and internal reiteration, have led some to question the ability of filter hybridization to detect DNA sequences that are present as single copies or reiterated only a few times in the genome of eukaryotes.

DNA sequences complementary to the RNA of avian myeloblastosis virus (AMV) have been detected (i) in all apparently normal chicken cells, (ii) in chicken cells infected or transformed either in vivo or in vitro by avian leukemia or sarcoma viruses, and (iii) in rat cells transformed by B-77 avian sarcoma virus (2, 3, 6). In those studies denatured cellular DNA, immobilized on filters, was hybridized to an excess of viral RNA. The technique did not permit us to determine the fraction of the AMV genome contained in the AMV-specific DNA sequences but this question was resolved by hybridizing AMV RNA in a solution containing a vast excess of cellular DNA (14, 19). It was shown that essentially 100% of the AMV genome is copied into DNA in AMV-induced leukemic cells but that in normal chicken cells there was DNA complementary to only 30 to 60% of the AMV-RNA. Two types of virus-specific DNA sequences, fast and slowly hybridizing, were detected in DNA from normal or from leukemic chicken cells. Rat cells transformed by B-77, avian sarcoma virus (1) contained only slowly reacting DNA sequences, whereas normal rat cells did not contain any AMV-specific DNA. By the filter hybridization technique, AMV-RNA detected seven to nine DNA equivalents of the viral 35*S* RNA subunit even in apparently normal gs-negative chicken embryos which had negligible amounts of the fast-reacting viral-specific DNA sequences. Also, 14 DNA equivalents of the AMV 35*S* subunit could be detected per B-77 transformed rat cell which contained only the slowly hybridizing AMV-specific DNA sequences. These results suggest that even the slowly hybridizing viral-specific DNA sequences can be detected by hybridizing excess viral RNA to denatured cellular DNA immobilized on cellulose nitrate filters (6).

To establish unequivocally whether all the virus-specific DNA sequences can be detected by filter hybridization, the following experiments were performed: (i) AMV-RNA in excess was hybridized with cellular DNA immobilized on filters, (ii) the viral RNA in the RNA-DNA

hybrids was eluted from the filters by denaturation and purified, (iii) the eluted RNA was rehybridized in solution with an excess of cellular DNA. The results revealed that the filter hybridization method could detect all the viral-specific DNA sequences hybridizable by liquid hybridization in DNA excess. Both techniques gave similar values for the number of viral DNA equivalents present per cell genome but the filter method was easier to perform and to interpret and was more reliable. Also, determination of the number of 28S ribosomal genes in chicken cells by both techniques produced identical results.

MATERIALS AND METHODS

Virus. Avian myeloblastosis virus, strain A of the Bureau of Animal Industry, subgroup B (Vogt classification) was used.

Embryonated chicken eggs. Fertilized chicken eggs from parents homozygous for the absence of gs-antigen were kindly supplied by R. Luginbuhl, the University of Connecticut, College of Agriculture and Natural Resources, Storrs, Conn. They were incubated and hatched at our facilities. All the chicken embryos were tested for congenital infection and only nonviremic embryos were used.

Leukemic myeloblasts. Leukemic myeloblasts were obtained from the blood of acutely leukemic chicks which had been infected on hatching day (5); many were kindly provided by J. W. Beard of Life Sciences Incorporated, St. Petersburg, Fla.

Cellular DNA and RNA. Cellular DNA was extracted, purified, denatured, and trapped on 0.45-μm nitrocellulose Millipore filters as previously described (6). Extraction and purification of total cellular RNA has also been described (6).

^{3}H-labeled 70S AMV-RNA. The preparation and purification of ^{3}H-labeled AMV and of 70S RNA from purified AMV virions has been described (5, 6).

Virus production tests. Virus production tests were carried out as described earlier (2).

^{3}H-labeled 28 and 18S chicken rRNA. The method for purification of ^{3}H-labeled chicken rRNA has been described (18). The 28 or 18S rRNA was further purified by passing it through a column of poly(U) sepharose to remove any poly(A) containing mRNA (21).

Preparation of DNA for liquid hybridization. The DNA for liquid hybridization was processed as described earlier (19). All DNA was sonically treated to 6.4S as determined by alkaline-sucrose velocity sedimentation.

DNA-RNA hybridization on cellulose nitrate filters. The hybridization of ^{3}H-labeled 70S AMV-RNA with DNA immobilized on nitrocellulose filters was done as described earlier except that 100 filters and 10 ml of ^{3}H-labeled 70S AMV RNA were placed in a single hybridizing vial (2, 6). Also, after RNAse treatment, the filters were treated with 0.15 M sodium iodoacetate solution in 2× SSC (1× SSC = 0.15 M sodium chloride, 0.015 M sodium citrate) for 40 min at 54 C to inactivate RNAse that might remain bound to the filters (20). Subsequently, the filters were washed with warm 2× SSC and 4× SSC (4).

Elution of [^{3}H]RNA. After the final washing, the filters carrying RNA-DNA hybrids were blotted on 3M paper, transferred to a flask containing 50 ml of 0.01× SSC, and boiled for 10 min at 100 C. After boiling, the fluid from each filter was drained by holding the filter against the side of the flask. The filters were then transferred to another flask containing 25 ml of 0.01× SSC, boiled for 10 min at 100 C, and removed as before. The contents of both flasks were pooled (80 to 90% of the hybridized AMV RNA was recovered). Tris-hydrochloride (1 M, pH 7.4) and $MgCl_2$ (1 M) were added at a final concentration of 10^{-2} M and the eluted RNA was treated with DNAse (Worthington Chemical Co.; further purified by glycerol gradient, 10 μg/ml for 30 min at 37 C). After addition of sodium chloride and EDTA (final concentrations of 0.15 and 0.003 M, respectively), the RNA was extracted twice with buffer-saturated phenol plus 1% SDS, precipitated with ethanol at −20 C, pelleted, and redissolved in 0.01 M phosphate buffer, pH 6.8.

Hybridization of [^{3}H]70S RNA, [^{3}H]28S or 18S chick rRNA and rehybridization of eluted [^{3}H]viral RNA in DNA excess. The hybridization of ^{3}H-labeled RNA in liquid was carried out as described earlier (19). All the hybridization reactions were performed in 0.4 M phosphate buffer (pH 6.8) at 65 C.

RESULTS

Liquid hybridization of 70S AMV RNA with an excess of DNA from leukemic myeloblasts or from normal chicken embryos. Figure 1 shows the kinetics of hybridization of 70S AMV RNA with DNA from leukemic or from normal, presumably gs-antigen negative, chicken cells. The leukemic and normal DNAs used contained 19.2 and 9.4, respectively, DNA equivalents of the 35S AMV-RNA subunit per cell genome as determined by the filter hybridization technique in viral RNA excess (6). The data represent the fraction of RNA rendered RNAse resistant with increasing Cot (Cot = concentration of deoxyribonucleotides in mole second per liter). As reported recently (19), the hybridization curves showed at least two components representing fast and slowly hybridizing virus-specific DNA sequences in normal and leukemic chicken cells. Of the AMV RNA, 76 to 82% was rendered RNAse resistant with leukemic myeloblast DNA but only 46% was made RNAse resistant with normal chicken embryo DNA. Approximately equal proportions of the AMV RNA were hybridized by the fast reacting and by the slowly reacting sequences in leukemic DNA. Only approximately one-third of the virus-specific DNA in normal DNA consisted of fast reacting sequences.

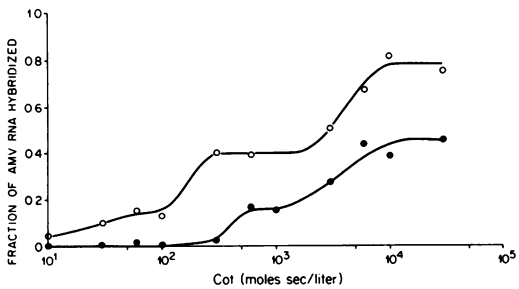

FIG. 1. *Hybridization kinetics of 70S AMV RNA with DNA from leukemic myeloblasts or normal Connecticut chicken embryos. The hybridization mixture contained 4 mg of cellular DNA per ml sheared to a fragment size of 6.4S, 1,000 counts per min per ml of sonically treated ^{3}H-labeled 70S AMV RNA (size 8 to 10S, specific activity 10^6 counts per min per μg) and 0.1% SDS in 0.4 M phosphate buffer, pH 6.8. The hybridization was carried out at 65 C in tightly silicone-stoppered tubes. After boiling for 3 min in a water-ethylene glycol bath, the mixture was quickly transferred to a bath at 65 C. To obtain the lower Cot values in a relatively longer time, lower salt concentrations were used and appropriate correction was made (22). Samples of 0.25 ml were taken at different time intervals and diluted with cold water in an ice-water bath. One-half of each sample was then treated with pancreatic and T_1 ribonucleases to determine the fraction of viral RNA rendered ribonuclease resistant as described earlier (19). A background of 2% obtained at Cot 0 was deducted from all experimental values. Symbols: O, leukemic DNA; ●, normal DNA.*

Liquid rehybridization in DNA excess of AMV RNA eluted from RNA-DNA hybrids formed with leukemic DNA on filters. 70S AMV RNA was first hybridized with leukemic DNA immobilized on nitrocellulose filters in a biphasic system (6). The hybridized RNA was then eluted by denaturation of the RNA-DNA hybrids and rehybridized in liquid with an excess of normal or leukemic chicken DNA. The kinetics of hybridization (Fig. 2) establish clearly that viral RNA eluted from leukemic DNA filters shows kinetics of liquid hybridization with leukemic DNA that are identical with those of AMV-RNA not selected by prehybridization (Fig. 1). The fast-hybridizing sequences in the eluted viral RNA and in the original viral RNA had a similar $Cot^h_{1/2}$, i.e., 110 and 100 mol s per liter, respectively. The slowly hybridizing sequences in the two RNAs had also a similar $Cot^h_{1/2}$, i.e., 2,400 and 2,500 mol s per liter, respectively.

The RNA eluted from leukemic DNA filters and rehybridized with an excess of normal chicken DNA also shows kinetics of hybridization that are similar to those of AMV-RNA not prehybridized. The fast sequences in the eluted RNA and in the original viral RNA hybridized with a $Cot^h_{1/2}$ of approximately 100 and 360, respectively; the slow sequences hybridized with a $Cot^h_{1/2}$ of approximately 6,000 and 5,000, respectively. Only 32% of the eluted viral RNA became RNAse resistant, whereas 46% of AMV-RNA not prehybridized was hybridized to normal DNA. This difference in hybridizability may reflect a difference in homology between AMV and the endogenous viral DNA. Also, these results demonstrate again that the viral-specific DNA sequences in leukemic DNA are different from those in normal chicken embryonic DNA.

Number of rRNA genes determined by filter hybridization or by liquid hybridization. To test the reliability of the filter hybridization technique with a well characterized, homogenous cellular RNA species, we determined the reiteration of ribosomal genes by liquid hybridization in excess of chicken DNA and by filter hybridization in excess of ribosomal RNA. The kinetics of hybridization of 28 and 18S rRNA with an excess of DNA (Fig. 3) were similar; the curves were monophasic and 75 to 86% of the ribosomal RNA was rendered RNAse resistant by chicken DNA. Normalization of the data, after correcting for RNA which hybridized at Cot 0 and for RNA which did not hybridize at Cot infinity (8), showed that the hybridization was a second order reaction with a $Cot^h_{1/2}$ of approximately 43 for both rRNA species. From the pertinent data of Table 1, the reiteration frequency of DNA complementary to 28 or 18S rRNA was calculated to be approximately 210 copies per cell (14).

A double reciprocal plot (7) of the amount of 28 or 18S ribosomal RNA hybridized by the

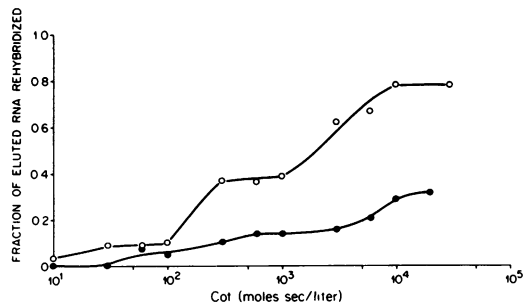

FIG. 2. *Hybridization kinetics of viral RNA, eluted from leukemic DNA filters, with an excess of leukemic or normal chicken DNA. The AMV-RNA was hybridized to leukemic DNA immobilized on filters and eluted as described in the text. The conditions of liquid hybridization in DNA excess were identical to those of Fig. 1. Symbols: O, leukemic DNA; ●, normal DNA.*

filter method to 100 μg of chicken DNA as a function of increasing concentration of rRNA is shown in Fig. 4. The amount of RNA hybridized at infinite RNA concentration was obtained from the line most closely approximating the experimental data determined with a computer program for linear regression. Depending upon whether all the experimental points or only those obtained with the four highest RNA concentrations were used in the program, the saturation values ranged from 12,900 to 14,200 counts per min per 100 μg of DNA for 28S rRNA and from 8,100 to 9,600 counts per min per 100 μg of DNA for 18S rRNA. These saturation values correspond to a redundancy per diploid cell of 184 to 202 for 28S rRNA genes and 292 to 342 for 18S rRNA genes.

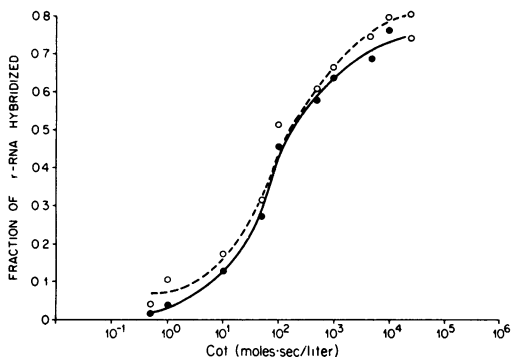

FIG. 3. *Liquid hybridization kinetics of 28 or 18S chicken rRNA with an excess of chicken DNA. The experimental conditions were the same as those described in Fig. 1 except that 3,000 counts per min per ml of ^{3}H-labeled 28 or 18S rRNA (specific activity 6.0 × 10^5 counts per min per μg) were used instead of viral [^{3}H]RNA. Symbols: O, 18S rRNA; ●, 28S rRNA.*

DISCUSSION

These findings demonstrate that both the fast and the slowly hybridizing virus-specific DNA sequences present in chicken cells can be detected and quantitated by the filter hybridization technique under our experimental conditions which have been described previously (2). Comparison of Fig. 1 and 2 shows that the entire viral genome can be hybridized to denatured leukemic DNA immobilized on filters. If this were not the case, e.g., if filter hybridization could detect only the repetitive viral sequences (15), the eluted RNA should have rehybridized completely by a Cot of 300 and the slowly reacting components should be absent in Fig. 2. In addition, the maximum amount of RNA hybridized by leukemic or normal DNA was the same with either the nonhybridized or the eluted viral RNA.

The effectiveness of the filter hybridization was further substantiated by comparing the number of 28S chicken ribosomal genes determined by both methods of hybridization, i.e., approximately 210 and 193 copies per chicken cell, respectively. The number of 18S rRNA genes was estimated to be approximately 317 copies per cell genome by filter hybridization. This is higher than the number of copies of the 28S rRNA genes and may have resulted from the degradation of some of the 28S rRNA molecules during isolation, thereby contaminating the 18S RNA species and raising the apparent number of 18S rRNA genes.

TABLE 1. *Pertinent quantitative data*

Determination	Amt
DNA per chicken cell genome	2.4×10^{-6} μg
Number of genomes per 100 μg of chicken DNA	4.17×10^{7}
Specific acitivity of 28S or 18S rRNA	6×10^{5} counts per min per μg
Mass of 28S rRNA molecule	2.82×10^{-12} μg
Mass of 18S rRNA molecule	1.12×10^{-12} μg
DNA per *E. coli* genome	4.48×10^{-9} μg
$Cot^{h}_{\frac{1}{2}}$ of *E. coli* c-RNA hybridized to homologous DNA	22 mol s/liter
$Cot^{h}_{\frac{1}{2}}$ of 28 or 18S rRNA	42.7 mol s/liter
Liquid hybridization	
Cellular DNA	4 mg/ml
Viral DNA[a] (complementary to viral RNA)	0.142 μg/ml
Viral RNA	1.6×10^{-3} μg/ml
Filter hybridization	
Viral RNA	1 μg/ml
Cellular DNA/filter	50 μg
Viral DNA[a]	1.775×10^{-3} μg/ml

[a] Viral DNA concentration estimated assuming there are 19 identical DNA equivalents of the viral RNA subunit (molecular weight 2.7×10^6) per leukemic cell.

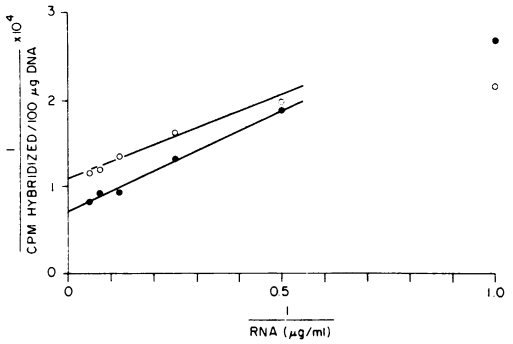

FIG. 4. *Hybridization of 28 or 18S ribosomal RNA with chicken DNA immobilized on filters. The reciprocal of counts per minute hybridized per 100 μg of DNA were plotted versus the reciprocal of concentration of input ^{3}H-labeled 28 or 18S rRNA by using the five highest rRNA concentrations. Hybridization was performed for 10 h at 70 C with three experimental DNA filters for each RNA concentration used. Three E. coli DNA filters were used to determine the nonspecific binding of ribosomal RNA (10 to 30 counts/min), and were deducted as background from the experimental chicken filters. The hybridization mixture contained 2.5 mg of E. coli soluble RNA per ml, varying concentrations of 28 or 18S ^{3}H-labeled r-RNA (specific activity 6.0 × 10^5 counts per min per μg) and 0.05% SDS in 4× SSC. The average amount of DNA per filter was 9 μg. Symbols: O, 18S rRNA; ●, 28S rRNA.*

A simple consideration of the kinetic parameters involved in the two methods of hybridization reveals that filter hybridization should detect the slowly reacting sequences. The rate of the hybridization reaction is directly proportional to the RNA concentration and to the concentration of its complementary DNA. Based on the presence of 19 DNA equivalents of the AMV 35*S* subunit per leukemic cell, the product of the two reactants is estimated to be 2.27×10^{-4} $(\mu g/ml)^2$ in the liquid hybridization mixture and 1.78×10^{-3} $(\mu g/ml)^2$ in the filter hybridization mixture (Table 1). Therefore, the rate of hybridization to DNA on filters will be approximately eight times faster than the rate of hybridization to DNA in suspension. Also, from the reactant concentrations and the duration of the reaction, the effective Cot (defined as total Cot multiplied by the fraction of virus-specific DNA in cellular DNA) can be estimated to be the same under the conditions of filter hybridization and liquid hybridization. Assuming that there are 19 DNA equivalents of 35*S* subunit per cell, in our liquid hybridization in DNA excess the effective Cot is approximately 0.5 mol s per liter when the total reaction Cot is 10,000 mol s per liter. Under our filter hybridization conditions in viral RNA excess (approximately 1 $\mu g/ml^1$), the effective Cot was 0.5 after 10 h of incubation. This includes correction for the salt concentrations and for the fact that only one DNA strand takes part in the hybridization (22). Although the reactants are not freely diffusible in the filter method, this is compensated by the fact that the effective concentration of the reacting DNA species does not decrease because the reassociation of DNA is prevented by immobilization of the denatured DNA. In addition, the filter hybridization method is experimentally uncomplicated and easily quantitated, requires a small amount of DNA and takes place in a relatively short time interval, and therefore RNA degradation is minimized. However, hybridization of RNA with an excess of DNA must be used to determine the fraction of the viral genome which is represented in the DNA and to study qualitative differences between viral-specific sequences in different DNAs. The limitations and advantages of both procedures have been reviewed recently (9).

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