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Presence in Leukemic Cells of Avian Myeloblastosis Virus-Specific DNA Sequences Absent in Normal Chicken Cells

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^{3}H-labeled 35*S* RNA from purified avian myeloblastosis virus (AMV) was exhaustively hybridized with an excess of normal chicken DNA to remove all viral RNA sequences which are complementary to DNA from uninfected cells. The [^{3}H]RNA which failed to hybridize was isolated by hydroxylapatite column chromatography which separates DNA-RNA hybrids from single-stranded [^{3}H]RNA. The residual RNA hybridized to leukemic chicken DNA but did not rehybridize with normal chicken DNA. This demonstrates conclusively that DNA from AMV-induced leukemic cells contain viral-specific sequences which are absent in DNA from normal cells.

DNA sequences are present in all apparently normal chicken cells which are complementary to the RNA of avian oncornaviruses and probably represent endogenous virus infomation (1, 3, 8, 10, 12). After infection with avian myeloblastosis virus (AMV) the amount of viral-specific DNA sequences is increased in transformed cells, such as leukemic myeloblasts and kidney tumor cells, as well as in nontransformed virus-producing fibroblasts (1-3). Oncornavirus DNA sequences present after infection with AMV are complementary to the entire AMV RNA and do not simply represent an increase in the amount of endogenous viral DNA. Under DNA excess conditions, 70 to 80% of AMV RNA hybridizes to leukemic DNA, whereas only 30 to 40% hybridizes to normal chicken DNA (4, 6, 9). Similar findings have been reported in cells transformed by Rous sarcoma virus (7). The acquisition of new viral DNA after transformation by avian sarcoma viruses was clearly demonstrated in rat cells and mouse cells which, unlike chicken cells, do not normally contain avian oncornavirus-specific DNA (1, 5, 9, 11).

In this study we demonstrate directly by two successive DNA-RNA hybridizations that certain AMV-specific DNA sequences present in AMV-induced leukemic cells are absent in uninfected chicken cells. (i) Viral RNA sequences shared by normal and leukemic cell DNA were removed by hybridization of sonically treated 35*S* AMV RNA with an excess of normal cellular DNA. (ii) The RNA which failed to hybridize with normal cellular DNA was rehybridized with DNA from leukemic cells or from normal cells. In the second hybridization the residual AMV RNA hybridized with DNA from leukemic cells but not with DNA from normal cells.

MATERIALS AND METHODS

AMV strain A, subgroup B of the Bureau of Animal Industry was used. Leukemic myeloblasts from acutely leukemic chicks were kindly provided by J. W. Beard of Life Sciences, Inc., St. Petersburg, Fla. Embryonated gs-negative eggs were kindly supplied by R. L. Luginbuhl of the University of Connecticut and were hatched in our facilities. The purification and isolation of ^{3}H-labeled 35*S* RNA from purified AMV, the extraction and purification of cellular DNA, and the preparation of cellular DNA and viral RNA for liquid hybridization have been described (9).

The hybridization of viral RNA sonicated into 6 to 7*S* fragments with an excess of DNA was carried out at 65 C in 0.4 M phosphate buffer (9). [^{3}H]35*S* AMV RNA (12,000 counts/min) and 30 mg of gs-negative chicken embryo DNA in 7.5 ml of 0.4 M phosphate buffer (pH 6.8) containing 0.2% sarkosyl were denatured in a water-ethylene glycol bath for 3 min and immediately transferred to a water bath maintained at 65 C. The hybridization reaction was carried out to a Cot (concentration of deoxynucleotides in moles per liter times the time in seconds) of 25,000. After hybridization, the mixture was diluted with cold distilled water containing 0.1% sarkosyl to obtain a final phosphate concentration of 0.05 M. The mixture was added to a column of packed hydroxylapatite (four columns of 10-ml bed volume each were used; DNA grade Bio Gel HTP from Bio-Rad) at 60 C, and the column was washed with 30 ml of 0.05 M phosphate, pH 6.8, containing 0.1% sarkosyl. Single-stranded and double-stranded nucleic acids were eluted with 0.17 and 0.40 M phosphate buffer, pH 6.8, respectively (9). Both the single-stranded and the double-stranded fractions were passed through hy-

droxylapatite columns a second time, and the eluates were dialyzed extensively at 4 C against 0.01 M Tris, pH 7.4, containing 0.1% sarkosyl. The nucleic acids were precipitated by ethanol, pelleted, dried, dissolved in TM buffer (0.01 M Tris, pH 7.4, 0.01 M $MgCl_2$), treated with DNase, extracted with phenol, and ethanol precipitated. The precipitate was again pelleted, dried, and dissolved in 0.3 ml of cold 0.01 M phosphate, pH 6.8. The nonhybridized RNA fraction contained approximately 6,000 counts/min and approximately 2,000 counts/min of RNA were recovered from the DNA-RNA hybrid fraction.

35*S* AMV RNA exhaustively hybridized with leukemic myeloblast DNA was treated as the AMV RNA hybridized with normal chicken DNA.

RESULTS AND DISCUSSION

The kinetics of hybridization of unfractionated 35*S* AMV RNA with DNA from normal or leukemic cells are shown in Fig. 1. As reported recently (7, 9), AMV RNA hybridized in a biphasic manner with either DNA suggesting the presence of fast and slowly hybridizing viral-specific DNA sequences. At a Cot of 10,000, 78% of the AMV RNA was rendered RNase resistant by leukemic DNA, whereas only 42 to 44% was hybridized with normal DNA. Approximately equal proportions of AMV RNA were hybridized by the fast- and slowly-reacting sequences in leukemic DNA. Only approximately one-third of the AMV-specific DNA in normal cells represents fast-reacting sequences.

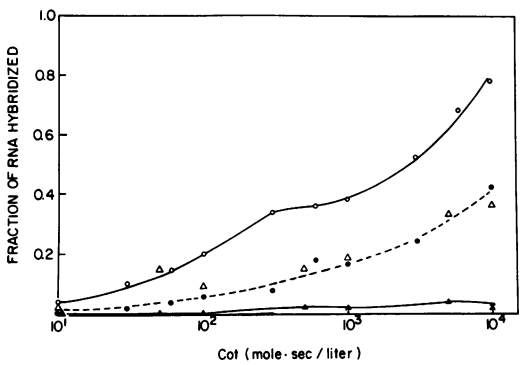

FIG. 1. *Liquid hybridization in excess of leukemic or normal DNA of sonically treated unfractionated 35S AMV RNA and of 35S AMV RNA which failed to hybridize with gs-negative chicken embryo DNA. The reaction mixture contained 4 mg of sonically treated DNA per ml fragmented to a size of 6 to 7S, 1,200 counts per min per ml of unfractionated or fractionated 35S AMV RNA (specific activity 1.1 × 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 silicone-stoppered tubes. After boiling for 3 min in a water-ethylene glycol bath, the tubes were quickly transferred to a water bath at 65 C. 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 (fraction A) and T_1 RNase's to determine the fraction of RNA rendered RNase resistant. Symbols: ○, hybridization of AMV RNA with leukemic DNA; ●, hybridization of AMV RNA with normal DNA; △, hybridization of residual AMV RNA with leukemic DNA; ▲, hybridization of residual AMV RNA with normal DNA.*

35*S* AMV RNA was separated into two fractions by hybridization with an excess of normal chicken DNA to a Cot of 25,000: (i) RNA which hybridized, and (ii) RNA which did not. Each RNA fraction was then rehybridized with a large excess of DNA from leukemic myeloblasts or from normal cells (4, 9). The rehybridization curves depicted in Fig. 1 show that the RNA which had not hybridized with normal DNA again did not hybridize significantly with normal DNA even at a Cot of 10,000. However, this RNA does hybridize with biphasic kinetics to leukemic DNA, suggesting that both fast- and slowly-reacting sequences were still present in the residual RNA. Approximately equal proportions of residual RNA formed hybrids with the fast- and the slowly-hybridizing viral-specific sequences in leukemic DNA. However, only 36% of the residual RNA was rendered RNase resistant by leukemic DNA at a Cot of 10,000, whereas we expected 60 to 70% RNase resistance. The experiment was repeated with similar results. Since sonically treated AMV RNA was incubated about 106 h at 65 C to achieve 25,000 Cot and the processing of the residual RNA consumed a long time, it is possible that the RNA was greatly reduced in size by thermal scission, i.e., large enough to be precipitated by trichloroacetic acid but not of sufficient length to form stable hybrids with complementary DNA. It is also possible that the hybridized RNA was reduced to a very small size by RNase treatment especially if the RNase susceptible regions due to imperfect matching are randomly distributed in the viral RNA. Nevertheless, it is clear that the viral RNA sequences complementary to both normal and leukemic DNAs are removed by the first cycle of hybridization with normal DNA. This experiment provides unequivocal evidence for the addition of new viral DNA sequences after infection of normal chicken cells by AMV.

The AMV RNA sequences which hybridized to gs-negative DNA in the first cycle of hybridization were eluted from the hybrids and rehybridized with normal or leukemic DNA to a Cot of 10,000. The results (Fig. 2C) show that approximately 40% of the eluted RNA reformed hybrids with either leukemic or normal DNA.

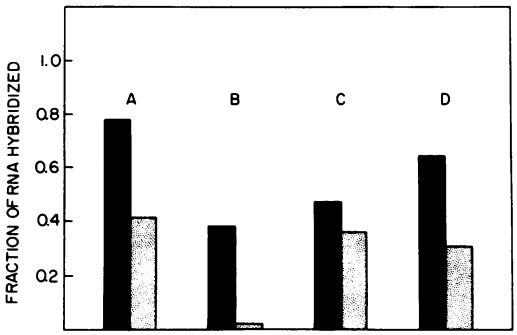

FIG. 2. *Rehybridization with normal or leukemic DNA of various AMV RNA fractions. The reaction mixtures and hybridization procedures were similar to those in Fig. 1. The following RNA fractions were used: A, unfractionated 35S AMV RNA; B, AMV RNA remaining single-stranded after hybridization with normal DNA; C, AMV RNA eluted from hybrids formed with normal DNA; D, AMV RNA eluted from hybrids formed with leukemic DNA. Symbols: ■, leukemic DNA; ⊠, normal DNA.*

Again, possibly due to the small size of the RNA, as discussed in the previous paragraph, the level of hybridization obtained was less than the expected 60 to 70%. Similar levels of rehybridization with leukemic or normal DNA were achieved with 35*S* AMV RNA eluted from gs-negative DNA trapped on nitrocellulose filters after a first hybridization in RNA excess (unpublished data).

A control experiment was carried out with a first cycle of hybridization between 35*S* AMV RNA and leukemic DNA. The nonhybridized fraction was negligible. After melting, the RNA isolated from the hybrids was rehybridized with normal and leukemic DNA (Fig. 2D); 64 and 30% of the RNA rehybridized with leukemic and normal DNA, respectively.

The present study supports our earlier findings (9) and provides conclusive evidence for the addition of new AMV-specific DNA sequences in leukemic cells transformed with AMV. These results add compelling evidence for the existence of a DNA intermediate in the replication of the viral RNA and suggest that new viral genetic information may be involved in oncogenesis.

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LITERATURE CITED

1. Baluda, M. A. 1972. Widespread presence, in chickens, of DNA complementary to the RNA genome of avian leukosis viruses. Proc. Nat. Acad. Sci. U.S.A. **69**:576-580.
2. Baluda, M. A., and W. N. Drohan. 1972. Distribution of deoxyribonucleic acid complementary to the ribonucleic acid of avian myeloblastosis virus in tissues of normal and tumor-bearing chickens. J. Virol. **10**:1002-1009.
3. Baluda, M. A., and D. P. Nayak. 1970. DNA complementary to viral RNA in leukemic cells induced by avian myeloblastosis virus. Proc. Nat. Acad. Sci. U.S.A. **66**:329-336.
4. Bishop, J. O. 1972. Molecular hybridization of ribonucleic acid with a large excess of deoxyribonucleic acid. Biochem. J. **126**:171-185.
5. Harel, L., J. Harel, and G. Frezouls. 1972. DNA copies of RNA in rat cells transformed by Rous sarcoma virus (RSV). Biochem. Biophys. Res. Commun. **48**:796-801.
6. Melli, M., C. Whitfield, K. V. Rao, M. Richardson, and J. O. Bishop. 1971. DNA-RNA hybridization in vast DNA excess. Nature N. Biol. **231**:8-12.
7. Neiman, P. E. 1972. Rous sarcoma virus nucleotide sequences in cellular DNA: measurement by RNA-DNA hybridization. Science **178**:750-753.
8. Rosenthal, P. M., H. L. Robinson, W. S. Robinson, T. Hanafusa, and H. Hanafusa. 1971. DNA in uninfected and virus-infected cells complementary to avian tumor virus RNA. Proc. Nat. Acad. Sci. U.S.A. **68**:2336-2340.
9. Shoyab, M., M. A. Baluda, and R. Evans. 1974. Acquisition of new DNA sequences after infection of chicken cells with avian myeloblastosis virus. J. Virol. **13**:331-339.
10. Varmus, H. E., R. A. Weiss, R. R. Friis, W. Levinson, and J. M. Bishop. 1972. Detection of avian tumor virus-specific nucleotide sequences in avian cell DNAs. Proc. Nat. Acad. Sci. U.S.A. **69**:20-24.
11. Varmus, H. E., J. M. Bishop, and P. K. Vogt. 1973. Appearance of virus-specific DNA in mammalian cells following transformation by Rous sarcoma virus. J. Mol. Biol. **74**:613-626.
12. Weiss, R. A., R. R. Friis, E. Katz, and P. K. Vogt. 1971. Induction of avian tumor viruses in normal cells by physical and chemical carcinogens. Virology **46**:920-938.