JOURNAL OF VIROLOGY, Jan. 1976, p. 106-113

Vol. 17, No. 1
Printed in U.S.A.

Ribonucleotide Sequence Homology Among Avian Oncornaviruses

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Received for publication 11 August 1975

RNA sequence relatedness among avian RNA tumor virus genomes was analyzed by inhibition of DNA-RNA hybrid formation between ^{3}H-labeled 35*S* viral RNA and an excess of leukemic or normal chicken cell DNA with increasing concentrations of unlabeled 35*S* viral RNA. The avian viruses tested were Rous associated virus (RAV)-0, avian myeloblastosis virus (AMV), RAV-60, RAV-61, and B-77 sarcoma virus. Hybridization of ^{3}H-labeled 35*S* AMV RNA with DNA from normal chicken cells was inhibited by unlabeled 35*S* RAV-0 RNA as efficiently (100%) as by unlabeled AMV RNA. Hybridization between ^{3}H-labeled 35*S* AMV RNA and DNA from leukemic chicken myeloblasts induced by AMV was suppressed 100 and 68% by unlabeled 35*S* RNA from AMV and RAV-0, respectively. Hybridization between ^{3}H-labeled RAV-0 and leukemic chicken myeloblast DNA was inhibited 100 and 67% by unlabeled 35*S* RNA from RAV-0 and AMV, respectively. It appears therefore that the AMV and RAV-0 genomes are 67 to 70% homologous and that AMV hybridizes to RAV-0 like sequences in normal chicken DNA. Hybridization between AMV RNA and leukemic chicken DNA was inhibited 40% by RNA from RAV-60 or RAV-61 and 50% by B-77 RNA. Hybridization between RAV-0 RNA and leukemic chicken DNA was inhibited 80% by RAV-60 or RAV-61 and 70% by B-77 RNA. Hybridization between ^{3}H-labeled 35*S* RNA from RAV-60 or RAV-61 and leukemic chicken myeloblast DNA was reduced equally by RNA from RAV-60, RAV-61, AMV or RAV-0; this suggests that RNA from RAV-60 and RAV-61 hybridizes with virus-specific sequences in leukemic DNA which are shared by AMV, RAV-0, RAV-60, and RAV-61 RNAs. Hybridization between ^{3}H-labeled 35*S* RNA from RAV-61 and normal pheasant DNA was inhibited 100% by homologous viral RNA, 22 to 26% by RNA from AMV or RAV-0, and 30 to 33% by RNA from RAV-60 or B-77. Nearly complete inhibition of hybridization between RAV-0 RNA and leukemic chicken DNA by a mixture of AMV and B-77 35*S* RNAs indicates that the RNA sequences shared by B-77 virus and RAV-0 are different from the sequences shared by AMV and RAV-0. It appears that different avian RNA tumor virus genomes have from 50 to 80% homology in nucleotide sequences and that the degree of hybridization between normal chicken cell DNA and a given viral RNA can be predicted from the homology that exists between the viral RNA tested and RAV-0 RNA.

From previous studies by us and other workers (12, 16, 17, 21) it has become apparent that avian oncornaviruses share RNA sequences with each other but vary in their degree of relatedness. Also, different avian species contain avian oncornavirus-specific DNA sequences that are different (17, 21). In this study we determined the extent of base sequence homology that exists between some avian oncornaviruses belonging to different subgroups. Ribonucleotide sequence relatedness among different viruses was analyzed by competition hybridization between 35*S* viral RNA and an excess of normal or leukemic chicken DNA.

MATERIALS AND METHODS

Viruses. Avian myeloblastosis virus (AMV) BAI strain A, subgroup B, was kindly provided by J. W. Beard. Rous associated virus-0 (RAV-0) and RAV-60, subgroup E, RAV-61, subgroup F, and avian sarcoma virus B-77(w), subgroup C, were kindly provided by H. Hanafusa either as virus-producing infected cells (RAV-0 and RAV-60) or as frozen virus [RAV-61 and B-77(w)]. Viruses were produced in our laboratory as described earlier (17).

Cells and embryonated eggs. The source of embryonated avian eggs and the methods of cell culture have also been described (17).

Leukemic chicken myeloblasts. Fresh leukemic myeloblasts used for production of ^{3}H-labeled AMV RNA were obtained from the peripheral blood of acutely leukemic chicks which had been infected on hatching day (5). Leukemic myeloblasts used for DNA extraction were kindly provided by J. W. Beard of Life Sciences, Inc., St. Petersburg, Fla., under contract with the Virus Cancer Program of the National Cancer Institute.

Cellular DNA and RNA. The extraction and purification of cellular DNA and RNA have been described (4).

Labeled and cold 35S viral RNAs. The preparation of ^{3}H-labeled 70*S* AMV RNA from purified virions and the isolation of ^{3}H-labeled 35*S* RNA followed published procedures (18). ^{3}H-labeled 35*S* RNAs from other avian viruses were prepared similarly.

Unlabeled 35*S* viral RNAs were also prepared in a similar manner from 2 to 4 liters of 24-h culture media from infected cells. The viruses or viral RNAs were isolated from density or velocity sucrose gradients after spectrophotometric analysis of each fraction.

Preparation of DNA for liquid hybridization. Before use, all DNAs were sonically treated to a size of 6–9S as determined by alkaline sucrose velocity sedimentation (18, 20).

RNA-DNA hybridization and competition by homologous or heterologous viral RNA. The hybridization mixture contained 1.6 mg of sonically treated DNA, 6×10^{-4} μg of ^{3}H-labeled 35*S* viral RNA [600 to 900 counts/min depending upon the specific activity: AMV, 1.9×10^6; RAV-0, 1.1×10^6; RAV-60, 1.2×10^6; RAV-61, 1.6×10^6; and B77(w), 1.1×10^6 counts/min per μg], 0 to 2 μg of unlabeled 35*S* viral RNA, and 0.1% sodium dodecyl sulfate in 0.4 ml of 0.4 M phosphate buffer (pH 6.8). The hybridization mixture was placed in tightly silicone-stoppered tubes, boiled for 3 min in a water-ethylene-glycol bath, quickly transferred to a water bath at 65 C, and incubated for 64 h to reach a C_0t of approximately 15,000 (concentration of nucleotides in moles per liter × time in seconds). The mixture was then diluted with cold water and processed to determine the fraction of ^{3}H-labeled RNA which became RNase-resistant as described previously (18, 20). Viral RNA which hybridized with embryonic mouse DNA under similar conditions (4 to 6%) was subtracted from the experimental values. The values obtained in the presence of yeast RNA but in the absence of cold viral RNA were normalized to 100% and competition is presented as the percentage of maximum hybridization.

Assuming that there are four to six DNA copies of the viral genome per leukemic chicken cell and that the complexity of the viral genome is 3×10^6 daltons (5, 6, 7, 18, 21), the complementary viral DNA sequences would be in 11- to 17-fold excess of ^{3}H-labeled viral RNA in the reaction mixture. The maximum concentration of unlabeled viral RNA used was 2 μg per 0.4 ml, a large excess over ^{3}H-labeled RNA, making it possible to determine the extent of homology that exits between the two viral RNAs.

RESULTS

Base sequence homology between 35S AMV RNA and 35S RNA from RAV-0, RAV-60, RAV-61, or B-77(w). ^{3}H-labeled 35*S* AMV RNA, either with yeast RNA or with increasing concentrations of unlabeled 35*S* AMV RNA or 35*S* RNA from other avian oncornaviruses, was hybridized with DNA from leukemic chicken myeloblasts induced by AMV. In the absence of competing viral RNA, ^{3}H-labeled 35*S* AMV RNA hybridized 71% with leukemic DNA. The data were normalized and plotted as the fraction of AMV RNA remaining hybridized versus increasing concentrations of competing unlabeled RNA (Fig. 1). As expected, maximal inhibition of hybridization was achieved with AMV RNA. At a concentration of 2 μg per 0.4 ml of competing 35*S* AMV RNA 92% inhibition occurred, whereas at the same concentration of 35*S* RAV-0 RNA only 68% competition occurred. Assuming that inhibitions of 92% by homologous RNA and of 68% by RAV-0 are maximal, there appears to be 74% homology between the RNAs of AMV and RAV-0. Since the hybridization of AMV RNA and leukemic DNA was inhibited approximately 60% by RNA from either RAV-60 or RAV-61, these two RNAs appear to have 65% homology with AMV RNA. B-77 35*S* RNA appears to share only 50% of its base sequences with the AMV genome. The competition curves appear to be parallel, suggesting that all the viral-specific sequences in leukemic DNA are present at the same frequency.

Sequence homology between RAV-0 RNA and RNA from AMV, RAV-60, RAV-61 or B-77(w). The maximal level of hybridization obtained between ^{3}H-labeled RAV-0 RNA and DNA from leukemic cells induced by AMV was 66% in the presence of yeast RNA. This hybridization was inhibited 96% by homologous unlabeled RNA at a concentration of 2 μg per 0.4 ml (Fig. 2). RAV-60 and RAV-61 RNA inhibited hybrid formation approximately 80%, suggesting a homology of 83% between RAV-0 and the genome of these two viruses. Under similar conditions, B-77(w) RNA inhibited 71% of the hybridization between RAV-0 RNA and leukemic DNA, whereas AMV RNA suppressed it by 67%. The concentration of homologous RNA needed for 50% competition was the same for RAV-0 or AMV RNA, i.e., approximately 10^{-2} μg per 0.4 ml (Fig. 1 and 2). AMV and RAV-0 appear to be 67 to 71% homologous (Fig. 1 and 2), whereas AMV and B-77 seem to have only

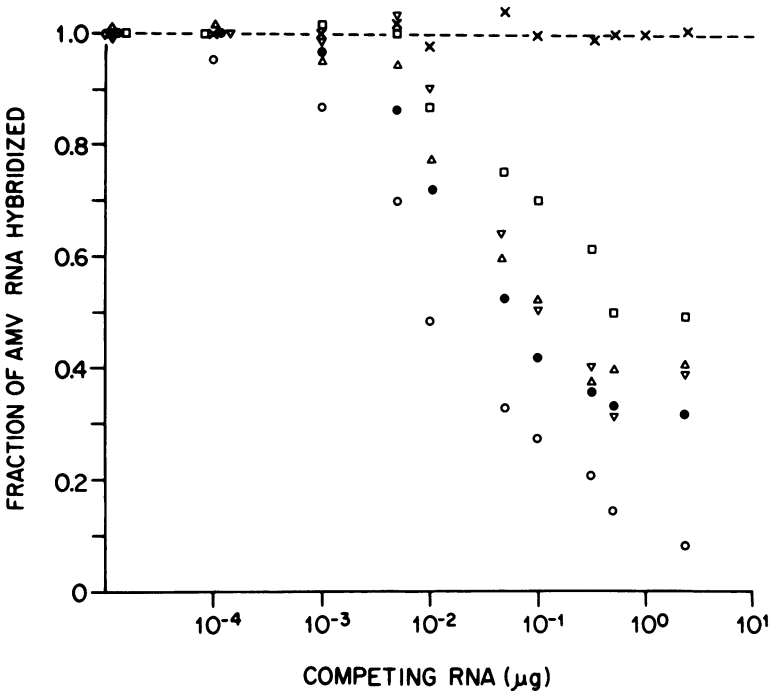

FIG. 1. *Hybridization of 35S ^{3}H-labeled AMV RNA with an excess of leukemic chicken myeloblast DNA in the presence of unlabeled homologous or heterologous viral RNA. The procedure is described in Materials and Methods. Symbols: ○, AMV 35S RNA; ●, RAV-0 35S RNA; △, RAV-60 35S RNA; ▽, RAV-61 35S RNA; □, B-77(w) 35S RNA; ×, yeast RNA.*

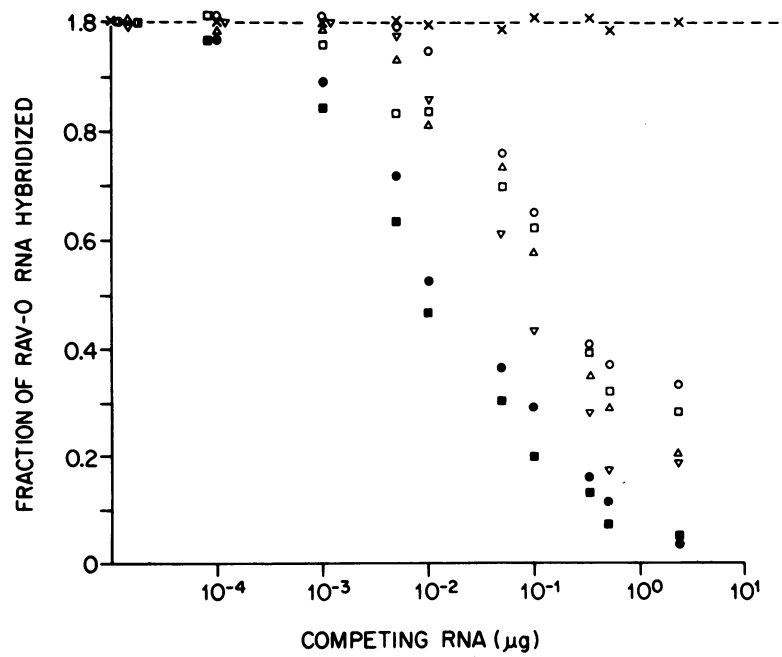

FIG. 2. *Hybridization of 35S ^{3}H-labeled RAV-0 RNA with an excess of leukemic chicken myeloblast DNA in the presence of unlabeled homologous or heterologous viral RNA. Symbols: ○, AMV 35S RNA; ●, RAV-0 35S RNA; △, RAV-60 35S RNA; ▽, RAV-61 35S RNA; □, B-77(w) 35S RNA; ■, B-77(w) + AMV 35S RNAs; ×, yeast RNA.*

52% homology and RAV-0 and B-77 67%. To determine whether the regions of homology between AMV and RAV-0 and between B-77 and RAV-0 are the same or different, RNA from AMV and B-77 were tested together at the same concentration for their inhibition of hybridization between RAV-0 and leukemic DNA. It was found that together B-77 and AMV RNAs suppressed almost completely hybridization between RAV-0 RNA and leukemic chicken DNA (Fig. 2). The inhibition curve with both RNAs was even displaced to the left of the curve with homologous RNA. This might be due to the higher concentration of competing sequences with the mixed RNAs since AMV RNA and B-77 RNA individually have already approximately 70% homology with RAV-0 RNA. These results show that some of the RNA sequences shared by AMV and RAV-0 are different from the sequences shared by B-77 and RAV-0.

Hybridization between 35S RAV-60 RNA and leukemic chicken DNA in the presence of competing viral RNAs. In the presence of yeast RNA at a C_0t of 15,000, RAV-60 35*S* RNA hybridized approximately 40% with leukemic chicken DNA. All the RAV-60 RNA sequences that hybridized with leukemic chicken DNA were competed 100% by either homologous RNA, AMV RNA, or RAV-0 RNA (Fig. 3). This suggests that RAV-60 RNA hybridized only to DNA sequences homologous with RAV-0 and AMV in leukemic chicken cells. Also, since RAV-61 RNA inhibited hybridization between RAV-60 and leukemic DNA by 87%, some RAV-60 sequences shared with RAV-0 and AMV are not present in RAV-61.

Hybridization between 35S RAV-61 RNA with leukemic chicken myeloblast DNA or normal pheasant DNA in the presence of competing viral RNAs. At a C_0t of 15,000, 30% of 35*S* RAV-61 RNA was made RNase resistant by DNA from leukemic chicken myeloblasts. Competition curves between ^{3}H-labeled 35*S* RAV-61 RNA and an excess of unlabeled 35*S* RNA from RAV-61, AMV, RAV-0, or RAV-60 are shown in Fig. 4. The competition curve for the homologous RNAs was the same as that found for RAV-0, RAV-60, or AMV RNA. The extensive inhibition (94%) of hybrid formation between 35*S* RAV-61 RNA and leukemic chicken DNA by 35*S* RNA from RAV-0, RAV-60, or AMV suggests that RAV-61, like RAV-60, hybridized to RAV-0- and AMV-specific sequences.

At a C_0t of 15,000, 39% of 35*S* RAV-61 RNA hybridized with normal pheasant DNA. Unlabeled homologous RNA suppressed this hybridization by 94% at a concentration of 2 μg per 0.4 ml and by 50% at a concentration of approximately 10^{-2} μg per 0.4 ml (Fig. 5). However,

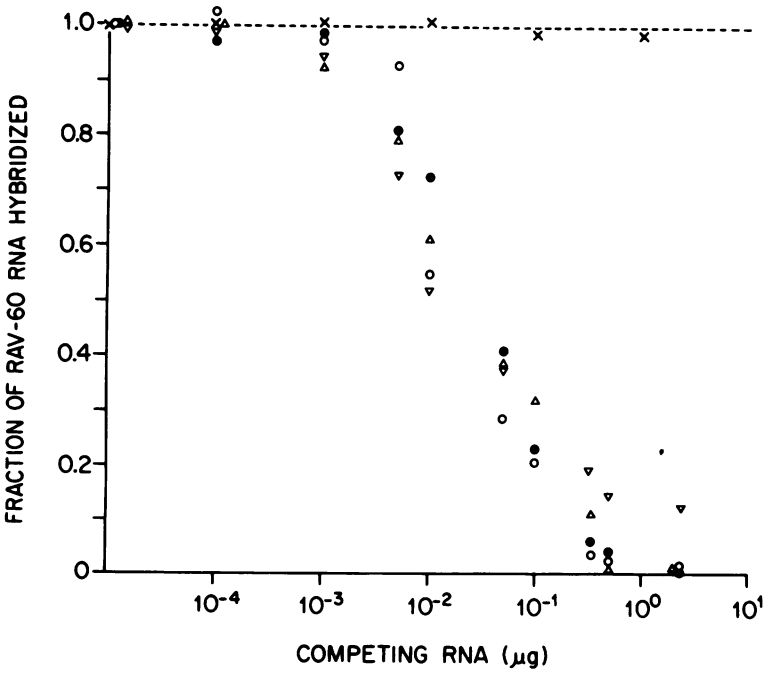

FIG. 3. *Hybridization of 35S ^{3}H-labeled RAV-60 RNA with an excess of leukemic chicken myeloblast DNA in the presence of homologous or heterologous viral RNA. Symbols: ○, AMV 35S RNA; ●, RAV-0 35S RNA; △, RAV-60 35S RNA; ▽, RAV-61 35S RNA; ×, yeast RNA.*

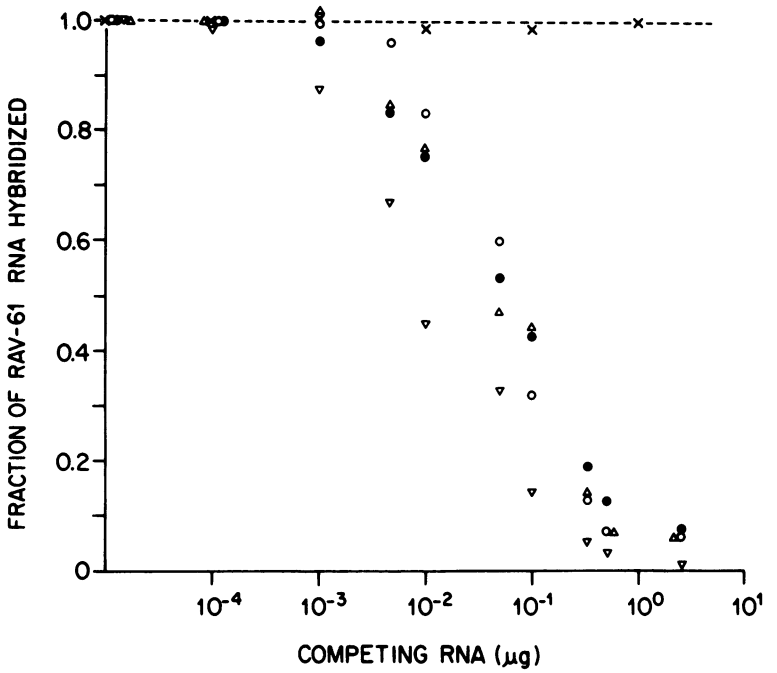

FIG. 4. *Hybridization of ^{3}H-labeled 35S RAV-61 RNA with an excess of leukemic chicken myeloblast DNA in the presence of homologous or heterologous viral RNA. Symbols: ○, AMV 35S RNA; ●, RAV-0 35S RNA; △, RAV-60 35S RNA; ▽, RAV-61 35S RNA; ×, yeast RNA.*

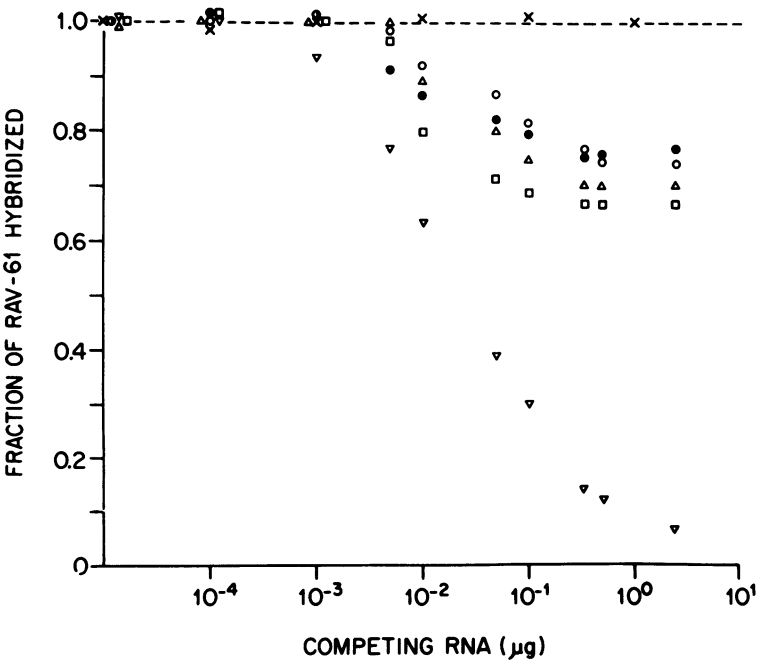

FIG. 5. *Hybridization of ^{3}H-labeled 35S RAV-61 RNA with an excess of normal pheasant DNA in the presence of homologous or heterologous viral RNA. Symbols: ○, AMV 35S RNA; ●, RAV-0 35S RNA; △, RAV-60 35S RNA; ▽, RAV-61 35S RNA; □, B-77(w) 35S RNA; ×, yeast RNA.*

there was only 22 and 26% inhibition by 35S RNA from RAV-0 and AMV, respectively, whereas there was 30 and 33% inhibition by RAV-60 and B-77, respectively (Fig. 5). These results suggest that the pheasant DNA sequences (probably endogenous pheasant virogenes) which hybridized with RAV-61 RNA have only 20 to 30% homology with RNA from RAV-0, RAV-60, B-77, or AMV. These findings are consistent with our previous results on hybridization between these viral RNAs and pheasant DNA (17).

Inhibition by 35S RAV-0 RNA of hybridization between 35S AMV RNA and normal chicken DNA. AMV RNA, 35S or 70S, hybridizes with normal chicken DNA 50 to 65% as much as with DNA from leukemic cells transformed by AMV (18–20). We had postulated that hybridization between AMV RNA and normal chicken DNA might be due to homology between AMV RNA and the endogenous virus genome. If that is the case, RAV-0 RNA should completely inhibit hybridization between AMV RNA and normal chicken DNA. As shown in Fig. 6, 35S RNA from RAV-0 inhibited by 98% hybrid formation between normal chicken DNA and 35S AMV RNA. Also, both competition curves in Fig. 6 representing homologous and heterologous viral RNAs follow the same kinetics, supporting our earlier conclusion that all the viral-specific sequences in chicken DNA are present at the same concentration.

DISCUSSION

Base sequence homologies among RNA tumor virus genomes can be estimated by competition of DNA driven RNA-DNA hybridization as was previously done for the determination of relatedness among RNAs of cellular and viral origin (8, 9, 11, 16, 23, 25). Competition RNA-DNA hybridization analysis as performed in this study can also be used to estimate the analytical complexity of the complementary viral DNA sequences by the method derived by Bishop et al. (8, 9). This procedure requires determination of the concentration of homologous RNA which gives 50% competiton. At that level of competition, the ratio of reacting complementary DNA to RNA is 2. One also needs to know the complexity of the cellular genome and the frequency of reiteration of the RNA sequences in DNA. Viral-specific DNA sequences are reiterated four to six times per diploid leukemic myeloblast genome (17), and the complexity of the chicken genome is 1.09×10^{12} daltons (20). Since there is 50% competition by homologous viral RNA at a concentration of approximately 10^{-2} μg/0.4 ml, the complexity of complementary viral DNA is estimated at approximately 3×10^{6} daltons. This estimate is in agreement with the complexity of avian oncornavirus genome determined by other methods (5–7).

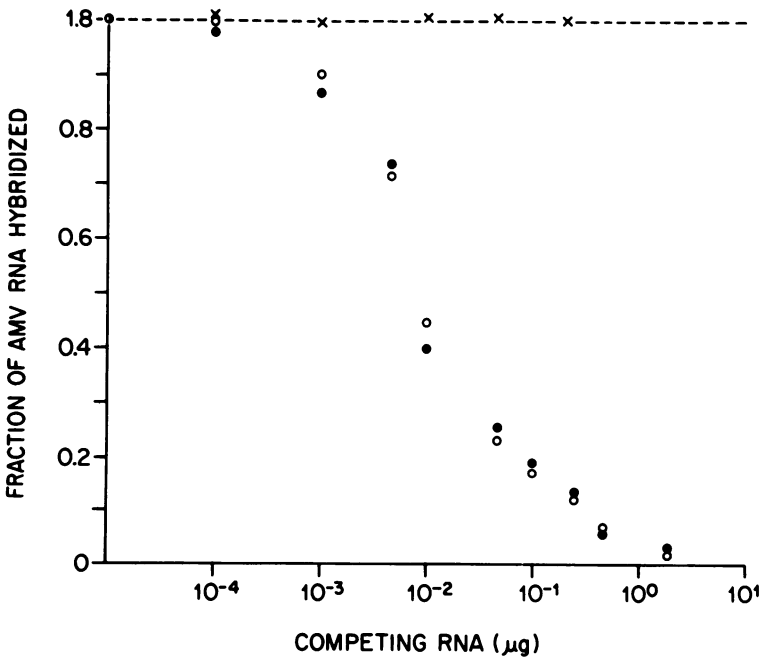

FIG. 6. *Inhibition of hybrid formation between ^{3}H-labeled 35S AMV RNA and normal K-137 chicken DNA by 35S RAV-0 RNA. Symbols:* O, *AMV 35S RNA;* ●, *RAV-0 35S RNA.*

Our findings indicate that there are three types of nucleotide sequences in avian RNA tumor virus genomes: (i) those unique to a given virus, (ii) those present in all viruses, and (iii) those which are shared by a few closely related viruses. Probably the sequences common to all avian tumor viruses were conserved during the evolution of these viruses from a common primordial ancestor because they provide essential gene products. The sequences which are unique to a given avian RNA tumor virus might have arisen either by fast divergence or by recombination of the viral nucleic acid with other viruses or host genetic material. The AMV and RAV-0 genomes appear to be 65 to 70% homologous, and the complexity of both viral RNAs appears to be the same. Also, the portion of 35*S* AMV RNA which hybridizes with normal chicken DNA appears to be homologous with RAV-0 RNA. This study points out that RAV-0 RNA rather than exogenous viral RNA should be used as probe to investigate transcription of viral genes in uninfected chicken cells because the endogneous viral genome appears to be only 65 to 80% homologous with other avian oncornavirus genomes.

AMV and B-77, which differ extensively in their biological properties, have only 50% homology (1, 2, 10, 22). Interestingly, these two viruses have also only 50% homology with RAV-0, but the regions homologous to RAV-0 are different and together represent approximately the entire RAV-0 genome.

RAV-0 and RAV-60, which belong to subgroup E and presumably have similar envelope structural components (24), show the most extensive homology (82%) among the viral RNAs tested. RAV-61 contains a substantial portion of its genome which hybridizes with pheasant DNA and is not homologous to any of the viral RNAs tested. This supports our previous claim that RAV-61 may have arisen due to recombination between an endogenous pheasant virus and a chicken leukosis virus (17).

ACKNOWLEDGMENTS

The excellent technical assistance of Elizabeth Shen, Julia Tang, and Gigi Hsu is gratefully appreciated. We thank H. Hanafusa and J. W. Beard for generous supplies of viruses and cells.

This work was supported by Public Health Service grant no. CA-10197 from the National Cancer Institute.

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