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Comparison of an Avian Osteopetrosis Virus with an Avian Lymphomatosis Virus by RNA-DNA Hybridization

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Myeloblastosis-associated virus (MAV)-2(0), a virus which was derived from avian myeloblastosis virus and induced a high incidence of osteopetrosis, was compared with avian lymphomatosis virus 5938, a recent field isolate which induced a high incidence of lymphomatosis. The following information was obtained. (i) MAV-2(0) induced osteopetrosis, nephroblastoma, and a very low incidence of hepatocellular carcinoma. No difference was seen in the oncogenic spectrum of end point and plaque-purified MAV-2(0). (ii) ^{125}I-labeled RNA sequences from MAV-2(0) formed hybrids with DNA extracted from osteopetrotic bone at a rate suggesting five proviral copies per haploid cell genome. The extent of hybridization of MAV-2(0) RNA with DNA from osteopetrotic tissue was more extensive (87%) than was observed in reactions with DNA from uninfected chicken embryos (52%). (iii) Competition of unlabeled viral RNA in hybridization reactions between the radioactive RNA from the two viruses and their respective proviral sequences present in tumor tissues showed that 15 to 20% of the viral sequences detected in these reactions were unshared. In contrast, no differences were detected in competition analyses of RNA sequences from the two viruses detected in DNA of normal chicken cells. (iv) MAV-2(0) 35*S* RNA was indistinguishable in size from avian lymphomatosis virus 5938 35*S* RNA by polyacrylamide gel electrophoresis.

Avian sarcoma viruses have an integrated proviral sequence in the chromosome of the infected and transformed cell (20, 27). Cells infected with avian leukemia and leukosis viruses also contain integrated copies of DNA (14, 21). Using conditions of DNA excess, the RNA sequences present in exogenous chicken viruses are related to, but not identical with, the endogenous sequences present in uninfected cells (13, 14). For example, 18% of the RNA sequences from certain field isolates of avian leukosis virus did not hybridize with the DNA sequences present in the endogenous provirus (14). Since activated endogenous viruses have so far been incapable of inducing neoplasms in susceptible chickens (H. G. Purchase, personal communication), it is possible that the portion of the leukosis virus genome which remains unhybridized to the endogenous virus represents the genetic information for malignant conversion.

The rationale of the present study was to compare the hybridization kinetics of viruses which differ in their oncogenic potential (4). A number of authors have reported the isolation of viruses with only part of the oncogenic potential expressed (R. M. Dougherty, R. H. Conklin, J. P. Whalen, and H. S. DiStefano, Fed. Proc. **27**:681, 1968; 17, 24). We have selected two viruses for comparison, one which induces a high incidence of osteopetrosis (24) and a recent field isolate which induces a high incidence of visceral lymphomatosis (14).

MATERIALS AND METHODS

Viruses and viral culture. Avian lymphomatosis virus (ALV) 5938 is a recent field isolate which was contributed by H. G. Purchase and which has recently been characterized in tissue culture and in susceptible chickens (14, 16). Briefly, this isolate retains both subgroup A and B interference patterns after a single end point dilution, does not transform chicken embryo fibroblasts in culture, but produces a very high incidence of lymphoid leukosis when injected into newly hatched line 15 × 7 chickens. The virus was propagated on chicken embryo cells of the C/BE and C/E phenotype from leukosis-free embryos obtained from Heisdorf and Nelson Farms, Redmond, Wash., by previously described techniques (19).

A serum was obtained from a chicken infected with avian myeloblastosis virus (AMV); the serum, when

injected into susceptible chickens, caused a high incidence of osteopetrosis (24). This virus, identified as myeloblastosis-associated virus (MAV)-2(0), was cloned by an end point technique and was used in most of the present studies without further biological purification. In addition, a plaque-purified derivative of MAV-2(0) was obtained using the technique of Graf (8), and this cloned virus was employed in certain experiments. Approximately 10^5 PFU were injected intravenously into 12-day chicken embryos, and osteopetrosis developed within 1 month after hatching. Birds were sacrificed when incapacitated, autopsies were performed, histological sections were prepared when appropriate, and bone samples were taken for DNA extraction.

A plaque-purified MAV-2 was derived from Rous sarcoma virus (RSV[MAV-2]) (generously provided by T. Graf [Max Planck Institut für Virusforschung, Tubingen, Germany]). This virus will be designated MAV-2(TG) to denote that it is a separate isolate. Pr-RSV-C was kindly provided by P. K. Vogt (University of Southern California School of Medicine, Los Angeles, Calif.) and was used because it contains an equal mixture of *a* and *b* 35*S* RNA subunits, in contrast to the Pr-RSV-C strain previously characterized in our laboratory, which contains only the *a* subunit (25). Conditions for the growth of both transforming and nontransforming viruses in C/E cells have been described (22, 23).

Isolation and ^{125}I labeling of viral RNAs. Virus harvested from cultures of ALV 5938 and plaque-purified MAV-2(0) was isolated by buoyant density centrifugation in 20 to 60% sucrose gradients, and viral RNA was extracted by the sodium dodecyl sulfate-phenol method (18). High-molecular-weight 60-70*S* RNA was separated from low-molecular-weight species by sedimentation in preformed 15 to 30% glycerol gradients in 10 mM NaCl-1 mM EDTA-0.2% sodium dodecyl sulfate-10 mM Tris, pH 7.4, at 48,000 rpm for 50 min at 20 C in an SW50.1 rotor. The 60-70*S* peak was located in gradient fractions by assay of absorbance at 260 nm and precipitated by the addition of 2 volumes of cold ethanol. About 2 μg of viral RNA from each virus was labeled with ^{125}I by a modification (15) of the method of Tereba and McCarthy (26) to a specific activity of 10^7 to 3×10^7 counts/min per μg. The size and characteristics of iodinated viral RNA in hybridization reactions have been described (15).

Preparation of DNA. DNA was extracted by the method of Marmur (12) from normal chicken embryos, from bursal lymphomas induced by ALV 5938, and from osteopetrotic tissues induced by MAV-2(0). In the case of osteopetrotic tissue, this bony material was first minced with bone shears and homogenized at low speed in a Virtis homogenizer for 2 min. Remaining extraction procedures were identical to those for DNA from other tissues. DNA was fragmented to an average size of 130,000 daltons by limited depurination followed by alkaline hydrolysis (11).

RNA-DNA hybridization. The conditions, assay, and characterization of hybridization reactions between ^{125}I-labeled viral RNA sequences and a modest excess of proviral DNA have been described in detail (15, 16). Briefly, hybridization reaction mixtures consisting of 0.5 mg of single-stranded cellular DNA fragments and 4×10^{-4} μg of ^{125}I-labeled viral RNA (1,000 to 3,000 counts/min) in 0.75 M NaCl-0.075 M sodium citrate-50% formamide were incubated for varying periods of time at 49 C (the apparent thermal rate optimum for the reaction). The minimum excess of complementary proviral DNA (assuming a proviral molecular weight of 3×10^6) present in cellular DNA with a frequency of one complete copy/haploid genome would be about 20-fold under these conditions. Hybridization was assayed by acquisition of resistance of the ^{125}I-labeled RNA to digestion by pancreatic ribonuclease. The fraction of viral RNA hybridized was corrected for background reactions measured at zero time and with heterologous DNA (5 to 7%). Thermal stability of viral RNA-proviral DNA hybrids formed under these conditions has been measured (15) and demonstrates a T_m of 81 to 83 C in 0.12 M sodium phosphate. Results of hybridization kinetic studies were plotted as a function of C_0t, where C_0 is the concentration of bulk cellular DNA in moles of nucleotides per liter and t is the time in seconds (3). Values of C_0t were not corrected for sodium concentration. The theoretical curves for hybridization kinetics of ^{125}I-labeled MAV-2(0) RNA and proviral DNA present with a frequency of one copy and five copies/haploid genome were generated by computer-assisted computations which have been described in detail (16). These computations are based upon estimates of rate constants of DNA reassociation (K_1) and RNA-DNA hybridization (K_2) corrected for sizes of the reacting polynucleotides, including the effects of progressive thermal scission on the RNA. The initial ratio K_2:K_1 was assumed to be 0.75 as reported by Hutton and Wetmur (9).

Competitive hybridization analysis. The sequence relationships between 60-70*S* RNAs from MAV-2(0) and ALV 5938 were measured by determining the competitive effect of unlabeled RNA in standard hybridization reactions. A quantitative method of evaluating such competition studies has been described in detail (16, 28). Competition was assayed as the fraction of radioactive RNA detected in hybrids in comparison with reactions carried out in the absence of competitor. These assays were carried out at values of C_0t where computer-assisted calculations of theoretical hybridization kinetic curves for decreasing ratios of viral DNA to RNA in reaction mixtures predicted a proviral DNA:viral RNA ratio of 1:1 should reduce hybridization by 50% (in comparison with control reactions lacking unlabeled competitor). Under such conditions the effect of increasing quantities of unlabeled homologous competitor is given by $y = x/x + 1$, where y is the fraction of control uncompeted hybridization and x is the viral DNA:RNA ratio. This equation was used to generate theoretical competition curves for comparison with experimental data.

Polyacrylamide gel electrophoresis. Roller culture bottles of MAV-2(0)-, ALV 5938-, and Pr-RSV-C-infected cells were grown (23), labeled with [^{32}P]orthophosphate or [^{3}H]uridine, harvested, and phenol extracted as previously described (5, 25). Supernatant fluids were removed every 2 h, the labeled virus was

pelleted, and RNA was extracted using water-saturated phenol containing 8-hydroxyquinoline. High-molecular-weight RNA was separated from low-molecular-weight species by sedimentation in preformed 5 to 20% sucrose gradients in 0.1 M NaCl-5 mM Tris (pH 7.5)-1% sodium dodecyl sulfate-0.1 mM EDTA for 45 min at 45,000 rpm at 20 C in an SW50.1 rotor. Gradients were fractionated from the bottom by dripping, the high-molecular-weight RNA was located by counting a sample of each fraction, and the peak was precipitated by the addition of 2 volumes of cold ethanol. Gels containing 2.1% polyacrylamide and 0.6% agarose were used to analyze the size of the 35*S* RNA. The preparation of gels and conditions of electrophoresis have been described (7). Gels were fractionated into 1-mm slices, and each slice was dissolved in 0.4 ml of a toluene-protosol-water (10:9:1) mixture and counted in a Beckman liquid scintillation counter using computer-assisted overlap corrections for double label.

RESULTS

Neoplasms induced by MAV-2(0). MAV-2(0) induced osteopetrosis, nephroblastoma, and hepatocellular carcinoma of the tubular type (Table 1). The oncogenic spectrum of MAV-2(0) cloned by an end point technique (24) and passaged by serum transfer did not differ from plaque-purified virus (Table 1). Thus, plaque purification did not eliminate nephroblastoma from the oncogenic spectrum of this virus. In nine chickens infected with plaque-purified MAV-2(0), nephroblastoma and osteopetrosis were present in the same animal. To demonstrate that the high incidence of osteopetrosis was not simply the result of plaque purification of an MAV, a randomly selected plaque of MAV-2 [MAV-2(TG)] was injected into chicks to determine its oncogenic spectrum. This virus induced visceral lymphomatosis as well as osteopetrosis and nephroblastoma (Table 1).

Kinetics of hybridization of viral RNA with DNA from normal and virus-infected tissue. The hybridization kinetics of ^{125}I-labeled ALV RNA reacting with DNA from virus-induced bursal lymphomas and with normal chicken DNA have been previously reported (14, 16) and are therefore not shown in this report. With lymphoma DNA the kinetics of hybridization of ALV RNA suggested two or three copies/haploid genome, reaching 73% hybridization at the highest values of C_0t measured (5×10^4 to 8×10^4). Figure 1 shows the results of a kinetic study of the hybridization of ^{125}I-labeled 60-70*S* RNA sequences from MAV-2(0) with DNA fragments from osteopetrotic tissue induced by this virus and from normal chicken embryos. These data from reactions involving DNA from virus-infected tissue conform closely to a theoretical curve for five proviral copies/haploid genome, with hybridization exceeding 85% above C_0t values of 7×10^4. The rate (and extent) of this hybridization reaction thus exceeds that previously observed for reactions between RNA from ALV 5938 and DNA from virus-induced lymphomas (and for RSV RNA reacting with sarcoma DNA) (15). The hybridization of MAV-2(0) RNA with normal chick DNA was considerably less extensive and reached values of about 50%. This latter result was indistinguishable from those previously observed with RNA from a variety of exogenous chicken oncornaviruses, including ALV 5938, reacting with normal chicken DNA (14, 16).

Competition in reactions between viral RNA and DNA from virus-infected tissue. Figure 2 depicts competition by homologous unlabeled MAV-2(0) RNA and ALV RNA in the reaction between ^{125}I-labeled MAV-2(0) RNA and DNA from osteopetrotic tissue. As shown, homologous competitor reduced hybridization of labeled RNA completely, and the data conform closely to the theoretical curve for identical RNA competing for proviral sequences pres-

TABLE 1. *Oncogenic spectrum of three clones of MAV-2*[a]

Virus	Oncogenic spectrum[b]			
	Osteopetrosis	Nephroblastoma	Visceral lymphomatosis	Hepatocellular carcinoma
MAV-2(0)[c]	76/96 (79.2)	15/96 (15.6)	0/96 (<1.0)	1/96 (1.0)
MAV-2(0)[d]	16/23 (69.6)	9/23 (39.1)	0/23 (<4.3)	1/23 (4.3)
MAV-2(TG)[e]	2/9 (22.2)	4/9 (44.4)	3/9 (33.3)	0/9 (<12)

[a] All chicks were injected as 12-day embryos by the intravenous route with approximately 10^5 PFU.

[b] Expressed as the number positive over the number tested. Numbers in parentheses indicate percentage of chickens developing tumors.

[c] Cloned by end point dilution (24).

[d] Subclone of MAV-2(0) derived by plaque purification (8).

[e] Clone picked at random from plaque assay of RSV (MAV-2).

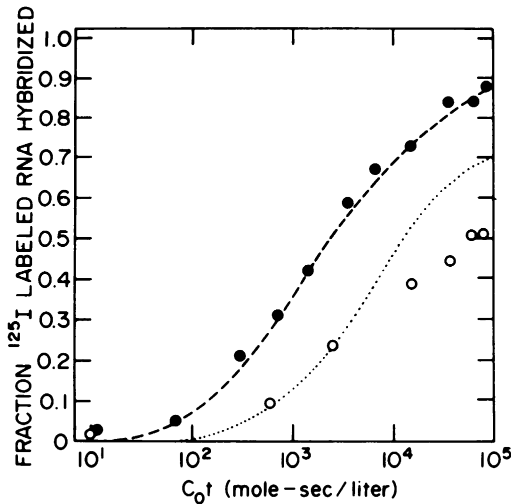

FIG. 1. *Hybridization reaction kinetics. Hybridization reactions were carried out between DNA fragments from osteopetrotic tissues (●) or from normal chicken embryos (O) and ^{125}I-labeled RNA from MAV-2(0). The dotted line represents the computer-assisted calculation of hybridization reactions with proviral sequences present in DNA with a reiteration frequency of one/haploid genome, and the dashed line represents a similarly derived theoretical curve for five copies/haploid genome.*

ent in the reaction at an average frequency of about five copies/haploid genome. In contrast, unlabeled 60-70*S* RNA sequences from ALV 5938 failed to compete completely, since a plateau appeared about 20% above that for complete competition by homologous RNA. Thus a minimum of 20% of the RNA sequences of MAV-2(0) detected in the DNA of osteopetrotic tissue are not present in the RNA of ALV 5938.

Figure 2 also depicts the data for the reciprocal competition experiment—that is, the competitive effect of unlabeled MAV-2(0) RNA in the reaction between ^{125}I-labeled 60-70*S* ALV RNA sequences and DNA from ALV-induced bursal lymphomas. The observation that ALV proviral sequences appear to be present in lymphoma DNA at about one-half of the frequency estimated in the osteopetrosis system affected the calculation of viral DNA:RNA ratios and thus the shape of the theoretical competition curves shown in the figure. Again, homologous ALV RNA competed completely in this reaction, and the data conformed reasonably well to the theoretical curve, as was true in more extensive previously reported experiments (13, 14). In simultaneous reactions MAV-2(0) RNA failed to compete completely, giving a competition plateau about 15% above the level for complete competition. This presumably reflects the fraction of ALV 5938 RNA sequences detected by this method in the DNA of infected tissue which is not present in the RNA of MAV-2(0).

Competition for ALV sequences in normal chicken embryo DNA. Figure 3 depicts competition in the reaction between ALV 5938 ^{125}I-labeled RNA and normal chicken embryo DNA. In striking contrast to the results obtained with DNA from virus-induced tumors, unlabeled RNA from MAV-2(0) competed completely in this reaction (as did homologous ALV RNA). These results are identical with those obtained

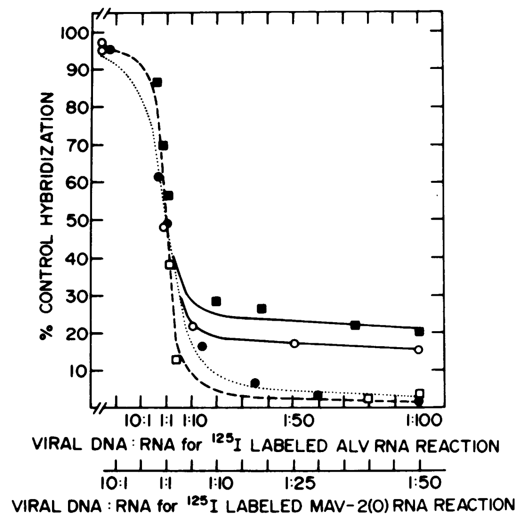

FIG. 2. *Competitive hybridization with DNA from virus-infected tissues. Unlabeled 60-70S RNA (0 to 1 μg) from MAV-2(0) (●) or ALV 5938 (■) was added to standard reaction mixtures for hybridization of ^{125}I-labeled 60-70S RNA sequences from MAV-2(0) with DNA from osteopetrotic tissues. For an estimated average of five copies/haploid genome, 0.02 μg of unlabeled homologous RNA was expected to produce a viral DNA:RNA ratio in the reaction mixture of 1:1. The theoretical curve for competition by homologous RNA is given by the dotted line for reactions incubated to C_0t values of 1.7×10^4. Reactions lacking unlabeled competitor demonstrated 74% hybridization at this C_0t value, which was normalized to 100% on the ordinate. In reactions between ^{125}I-labeled RNA from ALV and DNA from ALV-induced lymphomas, the same quantities of unlabeled MAV-2(0) RNA (O) and ALV 5938 RNA (□) were added. The lower estimate of proviral sequence frequency suggested that about one-half as much added RNA, 0.01 μg, would produce a 1:1 DNA-RNA ratio in the reaction mixture. This is reflected in the different abscissas for reactions with ALV 5938 and MAV-2(0) RNA. The theoretical competition curve in the reaction with labeled ALV RNA is given by the dashed line.*

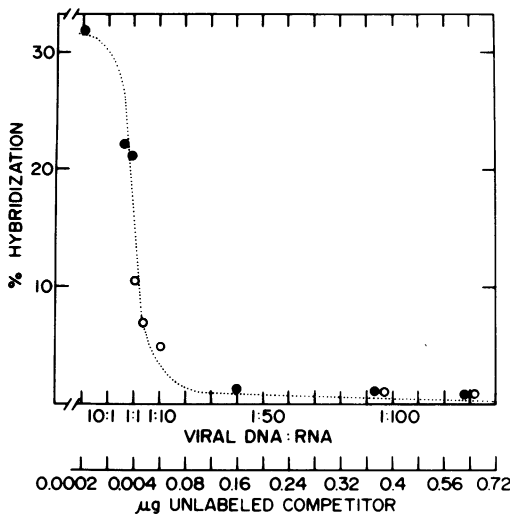

FIG. 3. *Competition studies with normal chicken embryo DNA. Various quantities of unlabeled RNA from ALV 5938 (●) and MAV-2(0) (○) were added to standard reactions between ^{125}I-labeled RNA from ALV and normal chicken embryo DNA. Reaction mixtures were handled as described in Materials and Methods and in the legend to Fig. 2. The theoretical competition curve based on an estimate of 1 proviral copy/haploid genome is given by the dotted line.*

with other exogenous chicken leukosis-sarcoma virus in previous studies (14, 16) including ALV 5951, RSV, B77, and Rous-associate virus-7. Thus, we cannot identify by this method any sequences of ALV 5938 detected in normal chicken DNA which are not shared with MAV-2(0) and a number of other biologically different exogenous viruses of the chicken leukosis-sarcoma complex.

Size of MAV-2(0) and ALV 5938 RNA. The 5% increment in the fraction of divergent RNA sequences detected in MAV-2(0) is at the limit of significance in the competitive hybridization system but is consistent with a genome (and therefore an RNA subunit) which is slightly larger than that of ALV. This disparity in hybridization competition between MAV-2(0) and ALV 5938 (Fig. 2) prompted the investigation of the genome sizes of the two viruses. In a preliminary analysis using polyacrylamide gel electrophoresis, MAV-2(0) 35*S* RNA appeared to be 0.5 to 1 fraction larger than that of ALV 5938 35*S* RNA (Fig. 4). These data are consistent with the finding that MAV-2(0) 35*S* RNA was smaller than Pr-RSV-C *a* RNA but slightly larger than Pr-RSV-C *b* RNA (Fig. 5). Pr-RSV-C demonstrates two peaks of 35*S* RNA by gel electrophoresis (Fig. 5); the larger is the *a* size class attributable to the nondefective Pr-RSV-C genome, and the smaller is the *b* size class attributable to the presence of a spontaneous transformation-defective mutant. Since *b* RNA is 12% smaller than *a* RNA (6) and corresponds to three fractions in our gels (Fig. 5), a size difference of 0.5 to 1 fraction (Fig. 4) suggests that MAV-2(0) 35*S* RNA is approximately 2.5 to 5% larger than ALV 5938 35*S* RNA.

However, reversal of the labels used in Fig. 4, such that ALV 5938 RNA was labeled with [^{3}H]uridine and MAV-2(0) RNA was labeled with [^{32}P]orthophosphate, followed by electrophoresis in polyacrylamide gels, did not confirm that MAV-2(0) 35*S* RNA was larger than ALV 5938 35*S* RNA.

DISCUSSION

The two viruses used in this study differ widely in the types of neoplasms induced. ALV 5938 induced lymphoid leukosis in virtually all injected chicks (16), whereas MAV-2(0) induced osteopetrosis in approximately 80% of the embryos injected (24; Table 1). It is interesting that plaque purification eliminated neither nephroblastoma nor hepatocellular carcinoma from the oncogenic spectrum of MAV-2(0). Two points regarding this finding are relevant. (i) Hepatocellular carcinoma of the tubular type was induced by both end point and plaque-purified MAV-2(0). This rare neoplasm has previously been associated only with the MC29 strain of avian myelocytomatosis (1, 10; E. A. Hillman, Ph.D. thesis, Duke University, Durham, N.C., 1972). However, the appearance of this neoplasm may somehow be associated with the ability to induce neoplasms involving epithelial cells such as nephroblastomas. (ii) Application of the most appropriate cloning procedure yet developed for avian leukosis viruses (8) did not result in the appearance of a monopotent virus. It is not known whether the selection of a different clone of subgroup B virus from AMV would result in a monopotent virus, but the nonselective search for such a clone would be difficult, since such a search would require the injection of a large number of chickens and long observation periods. For example, the in vivo characterization of a plaque-purified isolate [MAV-2(TG)] resulted in a high incidence of lymphoid leukosis, as well as nephroblastoma and osteopetrosis (Table 1). This clone was selected without regard to oncogenicity, whereas MAV-2(0) has been selected on the basis of high osteopetrosis induction. We are currently investigating the oncogenic spectrum of MAV-2(0) in the 15 × 7 cross of chickens to determine whether lymphoid leukosis can be induced in this extremely susceptible population (H. G.

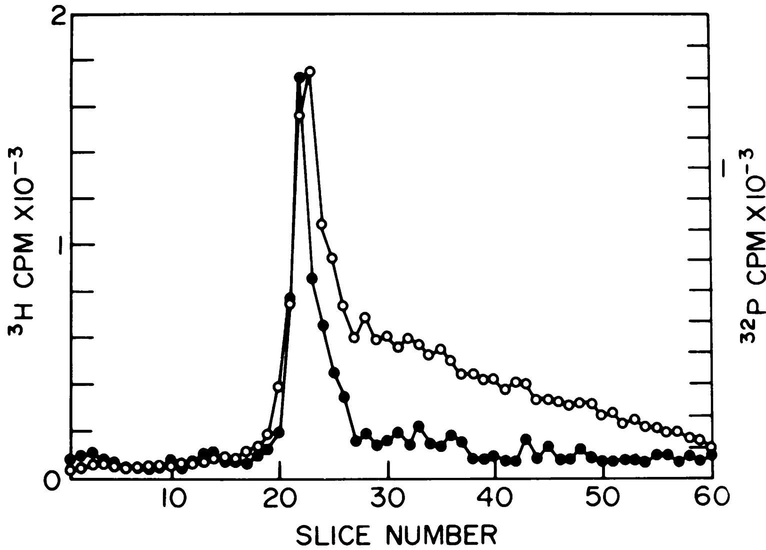

FIG. 4. *Size of MAV-2(0) and ALV 5938 35S RNA. Comparison of the size of [^{3}H]uridine-labeled MAV-2(0) 35S RNA (●) and ^{32}P-labeled ALV 5938 35S RNA (O). Heat-denatured RNA samples were co-electrophoresed for 3 h, sliced into 1-mm fractions, and counted. Electrophoresis was from left to right.*

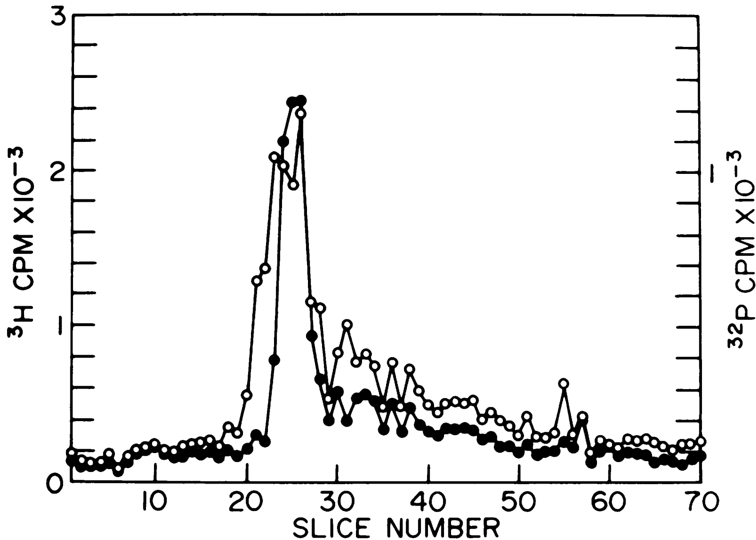

FIG. 5. *Size of MAV-2(0) and Pr-RSV-C 35S RNA. Comparison of the size of MAV-2(0) [^{3}H]uridine-labeled 35S RNA (●) and ^{32}P-labeled Pr-RSV-C 35S RNA (O). Heat-denatured RNA samples were co-electrophoresed for 3 h, sliced into 1-mm fractions, and counted. Electrophoresis was from left to right.*

Purchase, personal communication).

Hybridization reactions between MAV-2(0) RNA and DNA from infected tissues were more extensive than those observed with RNA from other viruses previously studied. The most likely explanation derives from the more rapid rate of reaction of MAV-2(0), suggesting a proviral reiteration frequency 2 to 2.5 times higher than that previously observed with nucleic acids from ALV or RSV, and about 5 times that of proviral sequences in normal chick DNA related to the endogenous virus RAV-0 (13–16). This apparent increase in sequence frequency improves the proviral DNA excess obtained in reaction mixtures, and the more rapid reaction rate decreases the detrimental effect of thermal scission in the RNA, which are the most significant limiting factors in the extent of hybridization which can be achieved. The observation of an increase in extent of hybridization achieved

associated with increasing proviral copy numbers per cell genome is therefore entirely consistent with the predictions of the computer-assisted estimates of hybridization reaction kinetics and supports the proposition that these hybridization reactions are representative of the entire viral genome. Nevertheless, rigorous interpretation of these studies is limited by the partial hybridization reactions achieved.

The reciprocal competitive hybridization studies between the ALV 5938 and MAV-2(0) systems indicate that the viruses differ in a minimum of 15 to 20% of the RNA sequences detected in hybridization reactions with DNA from infected cells. The ability of MAV-2(0) RNA to compete completely and symmetrically with RNA from ALV 5938 for proviral sequences in normal chicken embryo DNA indicates that the approximately 15% of RNA sequences in the lymphoma virus divergent from those of the osteopetrosis virus are confined to the portions of the viral RNA which are not detected in the genome of normal chicken cells by this technique. Therefore, both ALV and MAV-2(0) insert new sequences into the DNA of cells which they infect and "transform," and these virus-specific sequences are grossly divergent between the two viruses. The possibility of an RNA subunit and genome size for MAV-2(0) slightly larger than that of ALV (and intermediate in size between the *a* and *b* RNA subunits of RSV and its transformation-defective mutant) is suggested in data from hybridization competition studies. A similar size difference has been found between avian sarcoma viruses and recombinant viruses derived by crossing-over with avian leukosis viruses (2). However, analysis of the size difference of ALV 5938 and MAV-2(0) viral 35*S* RNA molecules by polyacrylamide gel electrophoresis indicates that a reproducible difference was not observed.

Although it is provocative to wonder whether the sequence differences detected between ALV 5938 and MAV-2(0) may be related to their differing oncogenic potential, such a speculation must be entertained with caution. The absence of a convenient method for detection of transformation-defective mutants arising from these "leukosis" viruses analogous to the transformation-defective segregants in stocks of RSV hampers attempts at characterization of transforming functions in the genome of viruses which are non-focus forming in embryo fibroblast cultures.

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