Nucleotide Sequence Relationships of Avian RNA Tumor Viruses: Measurement of the Deletion in a Transformation-Defective Mutant of Rous Sarcoma Virus

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Stocks of cloned helper-independent Rous sarcoma virus (RSV) spontaneously segregate transformation-defective (td) mutants that appear to have an RNA genome composed of smaller subunits than those of the patent virus. Differential hybridization and competitive hybridization techniques involving reactions between viral RNA and proviral sequences in host cell DNA (under conditions of initial DNA excess) were used to measure the extent of the deletion in a td mutant of Prague strain (Pr) of RSV (Pr RSV-C). Viral 60 to 70S RNA sequences labeled to 1 to 5 × 10⁷ counts per min per μg with ¹²⁵I were characterized with respect to their properties in hybridization reactions and used to reinforce data obtained with [³H]RNA of lower specific activity. By these techniques, about 13% ± 3% of the sequences Pr RSV-C that formed hybrids with DNA from virus-induced sarcomas appeared to be deleted from the genome of td Pr RSV-C. Studies comparing hybridization of RNA from Pr RSV-C and td Pr RSV-C with RSV-related sequences in normal cells, and competition experiments with RNA from the endogenous chicken oncornavirus Rous-associated virus type 0 (RAV-0) provided evidence that the majority, if not all, of the RNA sequences of Pr RSV-C deleted from its transformation-defective mutant are not represented in normal chicken DNA. Competition studies with a leukosis virus, RAV-7, indicated this virus also lacks a genome segment of about the same size as the deletion in the td mutant. Finally, the genome of all three "exogenous" viruses was found to lack a small segment (about 12%) of sequences present in the endogenous provirus of RAV-0.

The C-type RNA tumor viruses of chickens have been cultivated in tissue culture on chicken embryo cells and, over a period of years, separated into specific groups. Two of the readily identifiable general groups are the sarcoma viruses that produce transformation in tissue culture and sarcomas in susceptible animals and the leukosis viruses (often isolated from sarcoma virus stocks) that do not transform chicken embryo cells in tissue culture, but will produce lymphomatosis in susceptible animals. Cloned stocks of helper-independent sarcoma viruses appear spontaneously to give rise to transformation-defective mutants (27), and recent studies of the migration of dissociated RNA from mutant and parent viruses on polyacrylamide gels suggested the deletion of a segment from the constituent subunits of the RNA genome of the mutant (17).

To clarify further the genetic relationships between these various types of viruses and to begin to define genome segments related to their disparate biological properties, we have attempted to measure the nucleotide sequence relationships between the RNA genome of Prague strain (Pr) of Rous sarcoma virus (RSV), subgroup C (Pr RSV-C) and a spontaneous transformation-defective (td) mutant arising from the same strain (td Pr RSV-C). Further comparisons were made with a standard subgroup C leukosis virus, Rous-associated virus (RAV) type 7 (RAV-7), and with RAV-0, an example of the endogenous subgroup E leukosis viruses associated with normal chicken cells (28, 29). The technique used for these studies is based upon Temin's theory of viral replication, which requires a DNA provirus in the genome of host cells (25), and the method involves hybridization reactions between viral RNA and proviral DNA sequences. Conditions for such hybridization studies have been described that are

based upon the use of an excess of reactive DNA (6, 9, 18), and studies employing these conditions have demonstrated the majority, if not all, of the sequences of Pr RSV-C in DNA from virus-induced sarcoma cells (19). A smaller fraction of the sequences of the sarcoma virus was detected in the DNA of normal chicken embryos. Further studies of the same nature revealed the presence of extensive proviral sequences of RAV-0 in the DNA of normal chick cells and demonstrated a high degree of specificity for these hybridization reactions (20).

Recently, we have characterized a method of comparing different viral sequences related to proviral DNA in host cells by measuring the competition of viral RNA from various sources for complementary sequences in the DNA of appropriate types of cells (S. E. Wright and P. E. Neiman, Biochemistry, in press). These initial studies demonstrated that the genomes of Pr RSV-C and RAV-O RNA have extensive homology as well as measurable unshared sequences. Furthermore, a minimum of 85% of Pr RSV-C related sequences in normal DNA appeared to be accounted for by the endogenous provirus of RAV-O.

In the present study, we describe some further improvements in the conditions for hybridization and hybridization competition reactions with oncornavirus nucleic acids. By using these techniques, we measured the extent of the deletion present in the genome of td Pr RSV-C and observed a deletion of similar magnitude in RAV-7. Comparative studies, which included hybridization reactions with endogenous viral sequences in normal cells, provided evidence that most, if not all, of the sequences deleted from the mutant virus are in the segment of the sarcoma virus genome not present in normal chick DNA, that both the transformation-defective mutant and the standard leukosis virus (RAV-7) retain some genome sequences that are not present in normal DNA, and that all three "exogenous" viruses lack a small segment of genome present in the endogenous provirus of RAV-O.

MATERIALS AND METHODS

Viruses and cell culture. Recently, cloned Pr RSV-C was cloned again and the immediate progeny was used to produce virus for this study. The deletion mutant td Pr RSV-C was generously supplied by Peter Vogt, as were C/A chicken embryo cell cultures spontaneously releasing RAV-O. Exogenous viruses were 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 (23).

Preparation of viral RNA. Methods for labeling viral RNA with ^{3}H nucleosides and for the subsequent isolation of labeled viral 60 to 70S RNA with a specific activity of 1 to 2 × 10^6 counts per min per μg have been described in detail (19, 20). Unlabeled RNA was extracted by the sodium dodecyl sulfate (SDS)-phenol method (22) from virus isolated by buoyant density centrifugation in 20 to 60% sucrose gradients (as for labeled virus) of virus pellets obtained from 2 to 4 liters of tissue culture fluid, as previously described (S. E. Wright and P. E. Neiman, Biochemistry, in press). High molecular weight viral 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% SDS, 10 mM Tris, pH 7.4, at 48,000 rpm for 50 min at 20 C in an SW 50.1 rotor. The 60 to 70S peak was located by absorbance at 260 nm and precipitated by the addition of 2 volumes of cold ethanol to pooled gradient fractions. Phage T-7 mRNA was a gift from Maxine Linial. All viral RNA was dissolved in water and stored at −176 C until used.

Labeling viral RNA with ^{125}I. To obtain higher specific activity, viral RNA was labeled with ^{125}I by using a previously described technique (4; A. Tereba and B. J. McCarthy, Biochemistry, in press) involving carrier-free ^{125}I as NaI (Amersham Searle) that was fully reduced by treatment with sodium sulfite prior to use as suggested by Prensky et al. (21). Iodination mixtures consisting of 2 μg of viral RNA in 17 μliters of 0.1 mM thallium trichloride, 50 mM sodium acetate, pH 5.0, made about 20 mM in ^{125}I were incubated in sealed glass ampoules at 70 C for 15 min. The ampoule was then opened, the contents were diluted with 0.2 ml of 0.5 M sodium phosphate, pH 7.4, and the vial was resealed and incubated for an additional 45 min at 60 C. Separation of labeled RNA from unbound ^{125}I was accomplished by passing the reaction mixture over a column of Sephadex G-75 equilibrated with 0.12 M sodium phosphate. The specific activity of the RNA appearing in the void volume, assuming 100% recovery, varied from 1 to 5 × 10^7 counts per min per μg. Conversion of the labeled product to acid solubility by pancreatic ribonuclease (20 μg/ml for 30 min at 37 C in 0.12 M sodium phosphate) was greater than 98% and did not differ from the ribonuclease sensitivity of ^{3}H-labeled viral RNA.

Preparation of DNA. High molecular weight DNA was extracted from normal chicken embryos (pools containing usually 10 to 20% of individual embryos positive for viral group-specific antigen), and from 10-day-old sarcomas induced by injection of Pr RSV-C in the wing webs of 1-day-old chicks, by a previously described modification of the method of Marmur (16). The DNA was fragmented to an average molecular weight of 100,000 by the technique of limited depurination followed by alkaline hydrolysis, as previously described in detail (15). The properties of such chicken DNA fragments that are important to nucleic acid homology studies have also been described (19, 20).

RNA-DNA hybridization. We have previously described the conditions, assay methods, and charac-

teristics of "DNA excess" hybridization with avian oncornavirus nucleic acids in detail (19, 20). Briefly, hybridization reaction mixtures with ³H-labeled viral RNA consisting of 1 mg of cellular DNA fragments, 2 to 4 × 10⁻⁴ μg of viral RNA (300 to 600 counts/min) in 0.1 ml of 0.4 M sodium phosphate buffer at pH 7.0, and 0.05% SDS were heated to 98 C for 10 min, chilled in ice, and incubated at 67 C for periods of time up to 96 h. Under such conditions the initial DNA excess, with respect to viral-specific nucleotide sequences, was estimated to range from 40- to 160-fold in different experiments. Individual reaction mixtures were subsequently chilled in ice, diluted with cold 0.12 M phosphate buffer, divided into two portions, and hybridization assayed by aquisition of resistance to pancreatic ribonuclease. For studies with ¹²⁵I-labeled viral RNA by hybridization in phosphate buffer, several modifications were possible because of the greatly increased specific activity of the RNA. Reaction mixtures could be reduced in volume to as low as 15 μliters containing 150 μg of DNA in capillary tubes, or quantities of radioactivity were increased to 6,000 counts/min in a standard reaction mixture with preservation of an adequate DNA excess. Finally, to achieve conditions allowing incubation periods of up to 30 days, some hybridization reactions were carried out in formamide-containing buffers consisting of 0.68 M NaCl, 0.068 M Na citrate, and 45% formamide at 50 C.

We observed that ¹²⁵I-labeled viral RNA remained acid insoluble for periods up to 30 days under such conditions, although specific assay of the size of the RNA throughout the period of the reaction was not carried out. Results of hybridization kinetic studies were plotted as a function of C_ot where C_o is the concentration of cellular DNA in moles of nucleotide per liter and t is the time in seconds (3). No correction was made for sodium concentration. Because a total of 5 to 7% of the RNA from all of the viruses tested acquired ribonuclease resistance in the presence of heterologous mammalian DNA from various sources (e.g., canine, human) at C_ot values less than 100, and because such reactions did not significantly increase at C_ot values between 10² and 10⁴, this level of reaction was subtracted as background from all assays.

Competitive hybridization with viral nucleic acids. Sequence relationships between the different viral RNAs were measured by assaying the competitive effect of unlabeled RNA added to standard hybridization mixtures. Unlabeled viral 60 to 70S RNA could be obtained in sufficient quantity to vary the ratio of reacting viral polynucleotides from an initial modest DNA excess to a modest RNA excess and thus reduce the fraction of radioactive RNA entering hybrids in proportion to the fraction of homologous sequences in the competitor RNA. We have recently described a simple quantitative method for analyzing such competition studies with viral nucleic acids (S. E. Wright and P. E. Neiman, Biochemistry, in press) which was suggested by the work of Straus and Bonner (24) and of Tereba and McCarthy (Biochemistry, in press). Their observations suggested that hybridization kinetics could be

predicted for conditions varying from modest DNA excess to modest RNA excess by solving the general differential equations for simultaneous RNA-DNA hybridization and DNA reassociation for small increments of time with the assistance of a simple computer program. We adapted this method to the conditions required for study of viral nucleic acids and demonstrated that when reactions are assayed at a particular C_ot value, the fraction of RNA entering hybrids in comparison with control hybridization reactions in the absence of competing unlabeled RNA appears to be described by:

$$Y = X/(X + 1) \qquad (1)$$

where Y is the fraction of the control hybridization and X is the viral DNA:RNA ratio in the reaction mixture—at least over the range of conditions used in the experiments described here. This curve, adjusted for the estimated number of proviral copies per genome, was used to compare experimental data in competitive hybridization studies.

RESULTS

Hybridization of ³H-labeled viral RNA with DNA from normal and neoplastic tissue. Figure 1A depicts the kinetics of hybridization of [³H]RNA from Pr RSV-C and from its
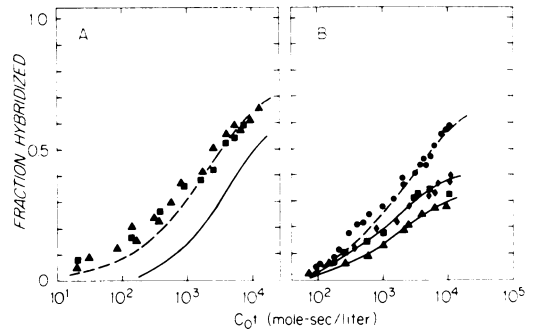

FIG. 1. *Hybridization kinetics of ³H-labeled viral RNA with DNA from normal and neoplastic tissues. Hybridization reactions were carried out with 300 to 600 input counts/min of [³H]RNA from Pr RSV-C (▲), td Pr RSV-C (■), RAV-7 (◆), and RAV-O (●) with specific activities of 1 to 2 × 10⁶ counts per min per μg at 67 C in 0.4 M phosphate buffer as described in the text. (A) Description of the kinetics of reaction of RNA from both the mutant and parent sarcoma viruses with DNA fragments from virus-induced wing web sarcomas. The solid line describes a theoretical kinetic curve for RNA reacting with complementary DNA sequences present with a frequency of one per cell genome, and the dashed line a derivative curve for a frequency of three per cell genome. (B) Description of the kinetics of reaction of RNA from all four viruses with DNA from normal chicken embryos. The dashed line in this case is adjusted to two viral copies per cell. The lower solid lines are not mathematically derived, but simply fit to the data for illustrative purposes.*

transformation-defective mutant with DNA fragments obtained from virus-induced wing web sarcomas. At $C_o t$ values near 10^4, about 66% of the RNA from both viruses formed ribonuclease-resistant hybrids. The data for both viruses cluster closely around a theoretical hybridization kinetic curve estimated for three complete proviral copies per haploid genome. Both the reference and one-copy and three-copy curves were calculated by means of the technique referred to in Materials and Methods and are based on estimates of hybridization rate constants obtained in studies of the kinetics of hybridization of in vitro-prepared RNA complementary to non-reiterated chicken DNA sequences, which we have described in detail (19, 20). The data points for both types of viral RNA show a tendency to describe a biphasic curve with about 40% of the RNA reacting at rates slightly faster than the remainder. This observation is reminiscent of, but less striking than, the evidence for a higher level of reiteration (about 50 to 100 copies) of 30 to 40% of the proviral sequences that we observed previously in studies with a different clone of Pr RSV-C (19) and that has been observed in studies with avian myeloblastosis virus nucleic acids (M. Shoyab et al., J. Virol., in press). Hybridization kinetic studies with normal chick DNA are depicted in Fig. 1B. The data for hybridization with RAV-0 [^{3}H]RNA follows a curve adjusted for about two proviral copies per cell and shows only a minimal suggestion of more amplification of a portion of the genome in accordance with our previous observations (20). Also, as previously reported, a much smaller fraction of Pr RSV-C RNA entered hybrids at $C_o t$ values around 10^4. Because we have shown that the vast majority of RSV-related sequences in normal DNA are indistinguishable (by hybridization) from those of the provirus of RAV-0 (S. E. Wright and P. E. Neiman, Biochemistry, in press), this difference in hybridization is not explained by a decreased reaction rate due to a lower average frequency per cell of a separate set of RSV-related sequences. The simplest interpretation is that this difference reflects the absence of a substantial fraction of the RSV genome from normal chicken cells. The extent of hybridization of [^{3}H]RNA from td Pr RSV-C and RAV-7 falls intermediate between that of the sarcoma virus and the endogenous virus. Because of the relatively low level of radioactivity dictated by the technical limitations of DNA concentration and RNA specific activity, we felt more precise quantitation and interpretation required some improvement in technique.

Hybridization of ^{125}I-labeled viral RNA in phosphate and formamide-containing buffers. The marked increase in the specific activity of ^{125}I-labeled viral RNA offered the possibility of overcoming some of the limitations we encountered, providing the presence of the iodine on the 5 position of 1 to 5% of the cytosine residues in the RNA did not adversely affect the formation of hybrids with proviral DNA. In standard phosphate buffer at 67 C, the rate of hybridization of ^{125}I-labeled RAV-0 RNA with normal chicken DNA was almost identical to that of RAV-0 [^{3}H]RNA, as shown in Fig. 2A. Furthermore, assay of the thermal stability of the iodinated RAV-0 RNA-normal chick DNA hybrids by thermal chromatography on hydroxylapatite columns (Fig. 3B) demonstrated a melting temperature of 84 C, which is not significantly different from that previously observed for viral [^{3}H]RNA-proviral DNA hybrids (19, 20). The use of formamide-containing buffers was then introduced in an attempt to establish conditions necessary for significantly increasing the maximum $C_o t$ value to which reaction mixtures could be incubated. Figure 3A depicts a curve demonstrating that the optimal temperature for hybridization in the formamide-containing buffer was about 50 C, which produced a rate of hybrid formation only slightly lower than that achieved in the standard phosphate buffer at 67 C and allowed reactions to be carried out to $C_o t$ values near 10^5 without the viral RNA becoming acid soluble or the cessation of hybrid formation due to thermal degradation. This advantage is further illustrated in Fig. 2B where RAV-0 [^{125}I]RNA is shown to form hybrids with normal chick DNA to the extent of about 74% at a $C_o t$ value of about 10^5 and to nearly 80% when reacted with DNA from Pr RSV-C induced sarcomas. This higher percentage in the latter case reflects a slightly increased rate of hybridization, indicating that an average of at least one more copy of sequences complementary to most of the RAV-0 genome was present in the DNA of the tumor cells.

Thus, we could extend the observation of differential hybridization with normal chick DNA described in Fig. 1B to obtain more precise quantitation. Table 1 shows the results of hybridization of ^{125}I-labeled RNA from RAV-0, RAV-7, td Pr RSV-C, and Pr RSV-C with normal chick DNA fragments in formamide-containing buffer to $C_o t$ values of about 10^5. Inspection indicates that about 50% of the radioactive RNA from Pr RSV-C entered ribonuclease-resistant duplexes under these condi-
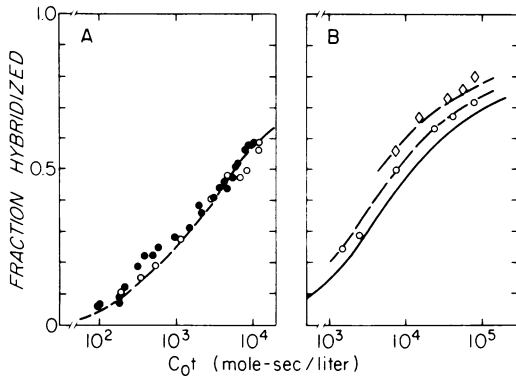
FIG. 2. Hybridization of ^{125}I-labeled viral RNA. (A) Comparison of the kinetics of the hybridization reaction of RNA from RAV-O labeled with ^3H (●, same data as Fig. 1B) and ^{125}I (○) with normal chicken embryo DNA. In the case of [^{125}I]RNA, reactions were carried out in 0.05 ml of the standard phosphate buffer at 67 C containing 0.5 mg of DNA fragments and 1,500 counts/min of labeled RNA with a specific activity of 1.2×10^7 counts per min per μg. (B) Illustration of the reaction of the same ^{125}I-labeled RAV-O RNA with normal chick DNA (○) and DNA from Pr RSV-C-induced sarcomas (◆) under the same conditions except that formamide-containing buffer was used (see text) and reactions were carried out at 50 C. The solid line represents theoretical "one-copy kinetics" under these conditions, and the middle and upper derivative dashed curves represent the expected rate of reactions with DNA sequences present with a frequency of two and three copies per cell genome, respectively.
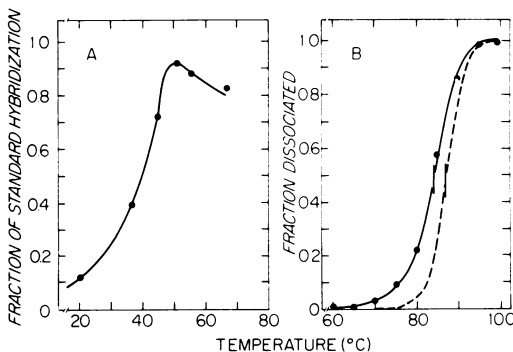

FIG. 3. Properties of RAV-O [^{125}I]RNA-normal chicken DNA hybrids. (A) Temperature optimum. Reaction mixtures containing RAV-O [^{125}I]RNA and normal chicken DNA fragments were incubated in formamide-containing buffer at various temperatures to a $C_o t$ value of 10^4. The extent of hybridization was assayed and plotted as the fraction of the value (58%) obtained in standard phosphate buffer at 67 C. (B) Thermal stability. Reaction mixtures incubated to $C_o t$ value of 10^4 were diluted in 0.12 M phosphate treated with ribonuclease, extracted with phenol, and

TABLE 1. Hybridization of [^{125}I]RNA from the various chicken oncornaviruses with normal chicken DNA[a]

Source of RNA	$C_o t$	Input radio-activity[b] (counts/min)	Extent of hybridiz-ation[c]	Normal-ized
RAV-0	10^5	1,500	74	1.0
RAV-7	10^5	1,400	62	0.84
td Pr RSV-C	10^5	2,350	55	0.75
Pr RSV-C	10^5	2,550	50	0.67

[a] The conditions for hybridization in formamide-containing buffers were as detailed in the text and Fig. 2B.

[b] ^{125}I-labeled RNA from RAV-0 had a specific activity (in counts per min per μg) of 10^7; from RAV-7, 1.1×10^7; from td Pr RSV-C, 4.5×10^7; and from Pr RSV-C, 5.6×10^7.

[c] Each assay was performed in quadruplicate. Percentages are means with a range of ± 2%.

tions, which was about 67% of the extent of hybridization of RAV-0. If this 33% difference in reaction was a reflection of a segment of RSV genome not contained in normal cells (as seems reasonable from our previous observations, S. E. Wright and P. E. Neiman, Biochemistry, in press), then a major deletion from this segment should result in an increase in the extent of hybridization (conversely, a deletion in the portion of the RSV genome contained in normal chick DNA should produce a decrease in the fraction of RNA entering hybrids). Interestingly, the extent of hybridization of ^{125}I RNA from td Pr RSV-C was 55% in this experiment or some 75% of the reaction of RAV-0 RNA. RNA from RAV-7 demonstrated even more extensive hybridization amounting to 87% of the reaction of endogenous viral RNA, suggestive of a slightly smaller percentage of "exogenous" genome than was present in the td mutant sarcoma virus. Nevertheless, because differential hybridization could conceivably be affected by factors other than sequence sharing, it was important to compare these viral RNAs by another technique. Therefore, these data were

passed over a small column of Dowex-50 according to the method of Gelderman et al. (6). The hybrids and the reassociated cellular DNA were then applied to hydroxylapatite columns (in a ratio of 1 mg of input DNA to 1 ml of column volume) at 60 C in 0.12 M phosphate buffer. Thermal chromatography was carried out by raising the temperature of the column in increments and monitoring the dissociated DNA (---) by optical density at 260 nm and acid-precipitable [^{125}I]RNA (●) in the effluent buffer.

compared with more direct measurements of sequence relationships obtained by competitive hybridization discussed in the following section.

Competitive hybridization studies of sequence relationships between the different viral RNAs. The study of genetic relationships between the four viruses was extended by detailed competition experiments depicted in Fig. 4. In each case, the extent of hybridization obtained at C_ot values of 10^4 in the absence of competitor unlabeled RNA was normalized to 100% to facilitate comparisons. The addition of unlabeled homologous RNA in the hybridiza-

FIG. 4. *Competitive hybridization studies. These experiments were carried out in standard phosphate buffer at 67 C with reaction mixtures incubated to C_ot values of 10^4. (A) Competition in the reaction between labeled RNA from RAV-O and normal chicken cell DNA. Reaction mixtures included either 600 counts/min of [^{3}H]RNA or 1,500 counts/min of [^{125}I]-RNA with a specific activity of 10^7 counts per min per μg. Hybridization in the absence of unlabeled competitor was about 60%, which was normalized on the ordinate. Unlabeled RNA from RAV-O (●), RAV-7 (◆), td Pr RSV-C, (■), Pr RSV-C (▲, △ for competition with [^{125}I]RNA) and T-7 phage mRNA (×) was added in increasing quantities and the resultant extent of hybridization plotted in comparison with the theoretical curve for competition by homologous RNA (----) derived as described in the text. (B) Competition in the reaction between labeled RNA from td Pr RSV-C and sarcoma DNA. Conditions were as for the other experiments except that, in experiments with iodinated RNA, 2,000 counts/min of [^{125}I]RNA with a specific activity of 4.5×10^7 counts per min per μg were used. Symbols for the unlabeled competitor RNAs are as for (A) with the solid symbols representing competition with [^{3}H]RNA and the open symbols (RAV-O, ○; td Pr RSV-C, □) for competition with [^{125}I]RNA. Extent of control hybridization in the absence of unlabeled competitor was 64%. (C) Competition in the reaction between labeled RNA from Pr RSV-C and sarcoma DNA. Symbols for unlabeled competitor RNA are as above; the significance of solid and open symbols and the extent of control hybridization (64%) is as for the other figures. Competition with ^{125}I-labeled Pr RSV-C RNA was carried out with 3,000 counts/min of [^{125}I]RNA with a specific activity of 5.6×10^7 counts per min per μg in the reaction mixture.*

tion reactions between labeled RNA from RAV-0, *td* Pr RSV-C, and Pr RSV-C and cellular DNA fragments containing their respective proviruses produced a reduction in the fraction of RNA entering hybrids that conformed closely to the theoretical curve adjusted to two proviral copies per cell in the case of RAV-0 sequences in normal cells and to three proviral copies per cell in the case of sarcoma virus sequences in wing web tumor DNA. The amount of RNA required to produce the competitive effects observed at the various DNA: RNA ratios is displayed on the lower abcissa. The determination of the apparent 1:1 ratio of viral DNA and RNA in the reaction mixture allowed us to make these sequence frequency determinations based on an estimated viral genome size of 10^7 daltons (2) and a haploid chicken genome size of 6×10^{11} daltons (5). It should be noted that homologous competitor RNA produced a maximum of 93% of the expected competitive effect in reactions between [^{3}H]RNA from Pr RSV-C and sarcoma cell DNA. In contrast, the competition of homologous RNA was virtually as predicted in reactions between RAV-0 RNA and the endogenous provirus in normal chicken DNA. The significance of the 7% ribonuclease-resistant radioactivity above background associated with the labeled Pr RSV-C RNA, which persists despite an apparent 100-fold excess of unlabeled homologous competitor, remains to be determined. In any case, for purposes of tabulating the extent of homology of the other viral genomes with Pr RSV-C, we have used the experimental homologous competition data rather than the theoretical curve for complete competition. Finally, the comparative studies included the repetition of some experiments with ^{125}I-labeled RNA to increase the quantity of radioactivity and enhance the accuracy of the determination.

Figure 4A deals with competition in the reaction between labeled RNA from RAV-0 and normal chick DNA. The data indicate that the unlabeled RNA from all three "exogenous" viruses failed to compete as completely as homologous unlabeled RAV-0 RNA, suggesting that approximately 12% of the sequences of the endogenous provirus of RAV-0 are missing from the RNA genome of the other three viruses. This is a smaller difference than the 18 to 20% suggested by our previous experiment with Pr RSV-C RNA alone as competitor, which was performed with much smaller amounts of radioactivity, (S. E. Wright and P. E. Neiman, Biochemistry, in press).

Figure 4B demonstrates that unlabeled Pr

RSV-C RNA competed as effectively as homologous RNA for *td* Pr RSV-C-related sequences in wing web sarcoma DNA (as would be expected). RNA from RAV-0, however, failed to compete as extensively as homologous *td* Pr RSV-C viral RNA, indicating the existence of proviral DNA sequences in sarcomas related to the transformation defective mutant that are not present in the genome of the endogenous virus. Figure 4C indicates that competition by unlabeled RAV-0 RNA was even less extensive in the reaction between labeled RNA from Pr RSV-C and its proviral sequences in tumor DNA, suggesting the presence of an additional segment of genome in the sarcoma virus (unrelated to RAV-0) that is missing from *td* Pr RSV-C. The direct test of this proposition is also demonstrated in Fig. 4C where unlabeled *td* Pr RSV-C RNA is seen to compete incompletely in this reaction (although to a greater extent than did RAV-0 RNA), indicating at least a 13% deletion in the RNA of the transformation defective mutant. The apparent quantitative sequence relationships between the genomes of the viruses studied in this fashion are summarized in Table 2. Because the pertinent hybridization competition studies in this table are in reasonable agreement with the data in Table 1, which is based on the extent of hybridization of all four viruses with normal chick DNA, and because of our previous direct demonstration that nearly all of the Pr RSV-C-related sequences (and, presumably nearly all the sequences of *td* Pr RSV-C) in normal chicken cells were accounted for by the endogenous provirus of RAV-0, it is possible to graphically display these findings in

terms of the relationship of the viruses to each other and to their representation in normal chicken DNA. This is schematically depicted in Fig. 5.

DISCUSSION

A number of recent reports have described the use of ^{125}I-labeled RNA in hybridization reactions (7, 21; A. Tereba and B. J. McCarthy, Biochemistry, in press). In the studies reported here, viral RNA labeled with ^{125}I appeared to form hybrids with chicken DNA that have similar properties to those formed with ^{3}H-labeled RNA. The markedly enhanced specific activity obtained by this method provides a solution for some of the shortcomings of this hybridization technique for studies of RNA tumor virus nucleic acids. For example, it is no longer necessary to use such large amounts of DNA to obtain the required excess of proviral sequences. More importantly, for this study we were able to use sufficient quantities of radioactivity to more accurately quantitate the results of hybridization kinetics and competition analyses with the different viruses. As has been described (8, 14) the use of formamide-containing buffers allows the hybrids to form at lower temperature, decreases the rate of thermal degradation of the reacting molecules, and thus allows the study of more extensive reactions.

There remain a number of problems with this technique of hybridization which affect such things as the precision with which we can estimate rate constants for hybridization and obtain derivative calculations such as proviral copy numbers. For example, the theoretical hybridization kinetic curves for single-copy sequences in cellular DNA were derived from
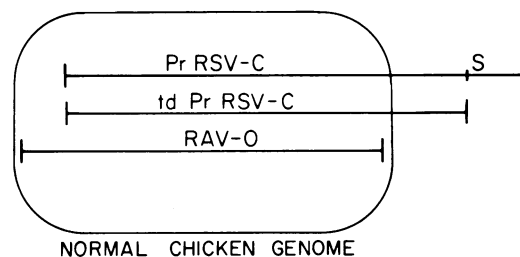
TABLE 2. *Sequence relationships between chicken oncornaviruses by competitive hybridization*[a]

Source of labeled RNA	Competing RNA			
	RAV-0	RAV-7	*td* Pr RSV-C	Pr RSV-C
RAV-0	1.0	0.88	0.88	0.88
td Pr RSV-C	0.87	—	1.0	1.0
Pr RSV-C[b]	0.72	0.87	0.87	1.0

[a] The figures are the apparent extent of homology in terms of the fraction of labeled viral RNA sequences (which formed hybrids under these experimental conditions) that are present in the unlabeled competitor. The data are obtained from the apparent competition "plateaus" shown in Fig. 4.

[b] Experimentally determined competition by homologous RNA was used as the basis for calculations rather than the theoretical curve, where a small discrepancy was observed in the case of Pr RSV-C nucleic acids, as shown in Fig. 4A and elaborated in the text.

FIG. 5. *Schematic relationship of the genome of chicken oncornaviruses to the normal chicken genome. The letter S represents at least some genes required for in vitro transformation and sarcomagenesis. The solid lines represent apparently homologous genome segments (subject to the limit of sensitivity of the hybridization technique) but do not have reference to structural characteristics. For example, we do not have evidence that the unshared portions of RAV-O and Pr RSV-C are at the ends of molecules.*

hybridization kinetic data from previous studies with in vitro-prepared complementary RNA that may differ from viral RNA in molar base ratio and secondary structure. The inhibitory effect of the latter upon the rate of hybridization of rRNA was recently demonstrated (24). The fact that the present studies were carried out near the optimal temperature for hybrid formation (Fig. 3A) suggests that any effect of secondary structure on the hybridization of viral RNA should have been minimized.

Another problem relates to the fact that the calculations of copy number estimates are based on the assumption that all of the viral RNA will eventually form hybrids with DNA from appropriate host cells if it were practical to carry out reactions to C_ot values in excess of 10^6. Although the extended hybridization shown in Fig. 2B is supportive of that concept, it is possible that we are slightly underestimating the number of proviral copy numbers in host DNA by this technique, as has been previously discussed (19, 20). Furthermore, the conditions for all of these studies permit thermal degradation of the viral RNA to an extent that was not determined. The recently reported effect of RNA size on hybridization rate constants (11) suggests that if thermal scission was extensive, the apparent rate of hybridization of viral RNA might be artificially depressed. Nevertheless, the competition studies with homologous RNA shown in Fig. 4 provide further evidence that the reiteration frequency of most of the proviral sequences is quite low.

The principal purpose of this study was to obtain a measure of the genetic relationship of cloned, helper-independent RSV to a transformation-defective deletion mutant that spontaneously segregated from stocks of the sarcoma virus. It must be noted before proceeding that the conclusions drawn from this study are stated as though the very extensive, but incomplete hybridization of the sarcoma virus RNA with proviral sequences in the DNA of virus-induced tumors (and RAV-0 RNA with normal DNA) is representative of the entire genome. Although studies demonstrating the transmission of Pr RSV with purified cellular DNA (10) provide strong support for this proposition, rigorous interpretation is limited to the 60 to 64% of the viral sequences that were detected in hybrids in the control reactions for the competition study. The competition experiment described in Fig. 4 demonstrates that the mutant lacks about 13% of the sequences present in the provirus of RSV. However, as noted, competition by homologous unlabeled RNA from the sarcoma virus fell about 7% short of the theoret-

ical value, a problem not observed in homologous RNA competition reactions of RAV-0 RNA and td Pr RSV-C RNA with their respective proviral sequences in cellular DNA. Therefore, it is possible that the small fraction of RNA sequences in the sarcoma virus preparation that were not "competed out" (under the conditions of our experiments) are also missing in RNA of td Pr RSV-C. Because these radioactive sequences behave differently from the rest of the RNA from Pr RSV-C, we have not included this small fraction in the calculations of sequence relatedness between the viral genomes (and thus introduced another possible source of error in precision).

A second important observation is that the data provide significant evidence that the majority, if not all, of the deletion in the transformation-defective mutant occurred in the section of the RSV genome not represented in normal chick DNA. This proposition is based on our previous study of the nearly complete competitive effect of unlabeled RAV-0 RNA in the reaction between Pr RSV-C RNA and normal chick cell DNA, demonstrating that the vast majority of the RSV related DNA sequences in normal chicken cells are accounted for by the endogenous provirus of RAV-0 (S. E. Wright and P. E. Neiman, Biochemistry, in press). Thus, a comparison of Fig. 4B and 4C, indicating that RAV-0 RNA competes more extensively with RNA from the transformation-defective mutant than with Pr RSV-C RNA for complementary sequences in sarcoma DNA, strongly suggests that the genome deletion in td Pr RSV-C RNA is not present in the genome of RAV-0 and thus is absent from normal cells. Furthermore, the extent of the exogenous deletion calculated in this fashion (15 to 16%) is in fair agreement with the apparent size of the total missing segment. However, as noted in our previous competition study (S. E. Wright and P. E. Neiman, Biochemistry, in press), the possibility remains that a maximum of 10 to 15% of the RSV-related DNA sequences of normal chickens might not be shared with RAV-0. This would amount to 7 or 8% of the entire Pr RSV-C genome. Were all of such putative endogenous sequences to participate in the deletion, then only about half of the missing information in td Pr RSV-C would be from the "exogenous" portion of the genome.

The data in Table 1 (and Fig. 4C) appear to rule against that possibility. They are most consistent with the interpretation that about 33% of the genome of Pr RSV-C was not present in normal cells, whereas about 25% of the sequences in the transformation-defective mu-

tant are apparently exogenous. A 10% deletion of the Pr RSV-C genome in the exogenous portion would produce this effect. Thus, combining the results of the several methods of calculation, we estimated a deletion of 13% ± 3%, in this particular mutant, of the portion of the RSV genome that is not represented in normal cells.

If, as seems apparent, at least part of the genome required for transformation by RSV is carried in this large missing segment, one could conclude that at least some of the "oncogene(s)" of this particular RNA tumor virus is not endogenous to normal chicken cells, contrary perhaps to the prediction of the viral oncogene hypothesis (26). There are severe restrictions on such a conclusion, however, because the exogenous viruses used in this study were isolated long ago, have an extended history in tissue culture and, therefore, have an uncertain relationship to viruses causing spontaneous malignancy. Furthermore, at least some transformation-defective mutants of RSV have been shown to retain their capacity to produce lymphoid leukosis in susceptible chickens (1).

In this connection, the data available here suggest that both RAV-7 and td Pr RSV-C retain a significant fraction of genome that was not detected in normal cells. Furthermore, all three exogenous agents are lacking at least 12% of the sequences present in the endogenous provirus of RAV-0, a virus which has a demonstrably different biology than the other agents including, perhaps significantly, the fact that it has not yet been observed to produce lymphomas even in susceptible chickens. It is not possible, however, on the basis of these observations, to sort out the role of such genetic differences, if any, in the specification of envelope antigens and other structural proteins that differ between subgroup E and C viruses, in the regulation of subviral gene expression or viral replication, or in lymphomagenesis. For example, RAV-7 was only partially characterized in this study, largely because of the impracticality at present of obtaining DNA containing a complete provirus. Two statements can be made, however. First, there is a segment of genome missing from RAV-7 in comparison with Pr RSV-C of about the same size as the deletion in the RNA of td Pr RSV-C. Second, as mentioned, this leukosis virus lacks a small segment of the genome of RAV-0, but additional endogenous sequences unrelated to RAV-0 have not been excluded from RAV-7 by the current study. The question of the relationship of leukosis virus to endogenous virus in chickens is being explored further in studies involving re-cent field isolates from lymphoid leukosis.

Finally, it should be noted that the genetic differences described here are presumably minimum differences. Despite the high thermal stability of the viral RNA-cellular DNA hybrids formed under the conditions used in this study, single noncomplementary bases could be present in such structures to the extent of 1% or so of the total number of nucleotide pairs without significantly affecting our measurement of the melting temperature (13). Thus, a fair number of amino acid substitutions could conceivably be present in protein gene products from the different viruses that are coded for by genome segments that appear homologous by the hybridization technique. Resolution of genetic differences beyond that detected by competitive hybridization will require nucleotide-sequencing techniques. An approach to such an analysis by using both hybridization techniques and "fingerprinting" was recently reported (12) that demonstrated the presence of a few oligonucleotides in RNA from sarcoma viruses not present in the genome of their transformation-defective mutants (and no additional sequences in the mutant RNA) compatible with the results of the present study. On the other hand, it would not be surprising if some of the differences detected in RNA by the fingerprinting technique between strains and subgroups of biologically similar viruses were not as striking in competitive hybridization studies.

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