Base-Sequence Relationships between Avian Ribonucleic Acid Endogenous and Sarcoma Viruses Assayed by Competitive Ribonucleic Acid–Deoxyribonucleic Acid Hybridization†

Stephen E. Wright*,‡ and Paul E. Neiman

ABSTRACT: Previous studies utilizing RNA–DNA hybridization under conditions of DNA excess demonstrated that the majority of the genomic sequences of an endogenous RNA tumor virus of chickens (RAV-0) and of a helper-independent, highly oncogenic strain of Rous sarcoma virus (RSV) were present in low frequency (1–4 copies/haploid genome) in the DNA of normal chicken cells and of wing-web tumor induced by RSV, respectively. A smaller fraction of the RSV genome was represented in normal chicken DNA. Competitive hybridization studies involving reactions between viral RNA and proviral DNA sequences in host cells were carried out to determine whether the sequences complementary to RSV in normal chicken cells were explained by the presence of the endogenous provirus, RAV-0, and to study the relationship between the RNAs from these two viruses. The results were compared to a theoretical model for competitive hybridization of RNA related to low-frequency genes based on conditions varying from a modest DNA excess to a modest RNA excess. Homologous unlabeled RNA from RAV-0 produced competitive effects in hybridization reactions between labeled RNA and related sequences in host cell DNA which conformed closely to theoretical competition curves, and unlabeled RSV RNA competed in homologous reactions to about 90% of expected values. RNA from a second sarcoma-producing, helper-independent virus of a different origin than RSV, Bratislava sarcoma virus (B77), competed for RSV-related sequences as completely as did homologous RSV RNA. Competition experiments with RNA from different viruses revealed that RSV RNA competed for approximately 80% of sequences complementary to RAV-0 in normal chicken DNA and RAV-0 RNA competed for approximately two-thirds of RSV-related sequences in wing-web tumor DNA. RAV-0 RNA competed for a minimum of 85–90% of the sequences complementary to RSV in normal chicken DNA. It was concluded that the endogenous, nontransforming virus and the sarcoma virus have extensive homology as well as measurable unshared genome segments. Further, almost all of the sequences complementary to RSV in normal chicken DNA were accounted for by the endogenous DNA provirus.

In the past few years numerous reports have strongly supported Temin's hypothesis that RNA tumor viruses replicate (and perhaps modify other cell functions) through a DNA intermediate called a provirus (Temin, 1964). One method for the direct detection of the proviral DNA is hybridization of the viral RNA genome with cellular DNA. Several reports have described RNA–DNA hybridization techniques which detected virus-specific DNA sequences in the genome of host cells (Baluda and Nayak, 1970; Rosenthal *et al.*, 1971; Neiman, 1972, 1973b; Bishop *et al.*, 1973; Varmus *et al.*, 1973). The use of the RNA–DNA hybridization reactions in the presence of an excess of DNA (Gelderman *et al.*, 1971; Melli *et al.*, 1971; Grouse *et al.*, 1972) has allowed quantitative measurement of most, if not all, of the RNA tumor virus genome of a Rous sarcoma virus (RSV[1]) in the DNA of infected cells (RSV-induced wing-web tumor) in low frequency (1–4 copies/cell genome) (Neiman, 1972). A significantly smaller fraction of RSV sequences was found in normal chicken cells. Since production of subgroup E avian leukosis viruses can be induced from normal chicken cells (Weiss *et al.*, 1971), it seemed reasonable that the presence of an endogenous provirus in normal chicken cells might explain the portion of the RSV-related sequences in normal chicken cells. Indeed, further hybridization studies have demonstrated that normal chicken cells do contain a small number of complete, or nearly complete, DNA sequences complementary to RNA from Rous associated virus type 0 (RAV-0) (Neiman, 1973b), an example of such an endogenous chicken oncornavirus (Vogt and Friis, 1971). It remained to be determined whether the RSV-complementary sequences in normal cells were explained by cross reaction with the endogenous provirus of RAV-0. We describe here a technique for the study of sequence relationships between this endogenous virus and RSV, a sarcoma-producing virus, by RNA–DNA competitive hybridization. Because of the low frequency of the viral genome sequences in cellular DNA, the technique used was based on RNA–DNA hybridization in the presence of an initial DNA excess. Our findings indicate that characteristics of competition between identical RNA molecules for low frequency complementary DNA sequences can be predicted with reasonable precision, that the use of this technique demonstrates both extensive homology as well as measurable unshared genome segments within the two types of viruses studied, and that the large majority, if not all, of the RSV-related sequences in normal DNA are those of the endogenous provirus.

† From the Department of Medicine, University of Washington School of Medicine, Seattle, Washington 98195, and the U. S. Public Health Service Hospital, Seattle, Washington 98114. *Received October 15, 1973.* This investigation was supported by Public Health Service Research Grants CA 12895, CA 10895, and CA 05231 from the National Cancer Institute.

‡ Dr. Wright is a fellow of the Leukemia Society of America, Inc. Present address: U. S. Public Service Hospital, Division of Oncology, Seattle, Wash. 98114.

[1] Abbreviations used are: RSV, Rous sarcoma virus; Pr RSV: Prague strain of Rous sarcoma virus subgroup C; RAV-0: endogenous chicken RNA virus: B77: Bratislava 77 avian sarcoma virus; standard buffer: 0.1 M NaCl–0.01 M Tris-HCl–0.001 M EDTA (pH 7.4); C_0t, (moles of DNA nucleotide sec)/liter (Britten and Kohne, 1968).

Experimental Section

Except for the preparation of the unlabeled virus RNA and the competitive RNA–DNA hybridization method, these procedures have been described in detail (Neiman, 1972, 1973b).

Materials. Cells, Embryos, Chickens, and Viruses. Fertile eggs of line 7 chickens were obtained from the USDA Regional Poultry Research Laboratory in East Lansing, Mich. Specific pathogen-free white leghorn chickens as well as chicken embryos for DNA and cells were obtained from Laboratory Associates and Heisdorf and Nelson Farms, Seattle and Redmond, Wash., respectively. Rous-associated virus type 0 (RAV-0), a subgroup E chicken helper-leukosis virus, was selected as an example of an endogenous chicken virus. As mentioned RAV-0 or closely related viruses have been shown to be present in all normal chicken cells tested, and at least a portion of the endogenous chicken virus genome seems to be responsible for partial C-type virus functional expression, such as group specific antigen and chicken helper factor in normal tissues (Dougherty and DiStefano, 1966; Hanafusa *et al.*, 1970; Hayward and Hanafusa, 1973). At present RAV-0 has not been associated with any spontaneous or induced neoplasm. RAV-0 was obtained from chicken embryo cell cultures of the C/A phenotype which spontaneously produced the virus (Vogt and Friis, 1971). For comparison, two helper-independent, subgroup C, highly oncogenic avian sarcoma viruses, Prague stain of Rous sarcoma virus (Pr RSV) and Bratislava 77 avian sarcoma virus (B77), were selected. Both viruses have similar biological properties but represent completely different isolates and a long and separate history in tissue culture (Duff and Vogt, 1969).

Methods. Tissue Culture and Preparation of Virus. Previously described techniques were used for culturing chicken embryo cells (Rubin, 1960). Three 75-cm^3 tissue culture flasks (Falcon plastics) seeded with virus-producing cultures were allowed to grow to confluence for 48 hr. Growth medium was replaced with 5-ml culture medium 199, prepared without unlabeled nucleosides, or with F-10 media (GIBCO), containing 5% fetal calf serum and 100 μCi/ml each of [^{3}H]uridine (22–27 Ci/mmol), [^{3}H]cytidine (27 Ci/mmol), and [^{3}H]-adenosine (8.8 Ci/mmol), Amersham-Searle Corp. The labeling media was replaced every 24 hr and clarified at 12,000g for 10 min, and the labeled virus was sedimented at 110,000g for 30 min. The resulting pellet was suspended, using a water bath sonicator, in 0.2 ml of standard buffer (0.1 M NaCl–0.01 M Tris-HCl–0.001 M EDTA (pH 7.4); Duesberg *et al.* (1968)), layered over a preformed 5-ml 20–60% sucrose (ribonuclease-free) gradient in the same buffer and centrifuged at 110,000g for 4 hr. The labeled virus peak (located by precipitation of small aliquots of gradient fractions) was found at a density of 1.16 g/ml. The banded, labeled fractions were pooled and diluted to 5 ml in cold standard buffer, and the virus was sedimented by centrifugation at 110,000g for 30 min. For the preparation of unlabeled virus a total of 2–3 l. of tissue culture supernatant (F-10, 5% fetal calf serum), from confluent virus-producing chicken embryo cells (collected every 24 hr for RAV-0 and Pr RSV and every 4 hr for B77), was required to obtain a sufficient quantity of RNA for detection by optical density and use in RNA–DNA competition hybridization experiments. The unlabeled virus was clarified, pelleted, and banded in a density gradient as described above for labeled virus. The virus band at 1.16 g/ml was detectable visually.

Preparation of RNA. RNA was extracted from the radioactive virus pellet by the sodium dodecyl sulfate–phenol method (Robinson *et al.*, 1965) and precipitated with ethanol at −20° for 16 hr. The RNA precipitate (nonvisible) was recovered quantitatively (without carrier) by sedimentation at 12,000g for 20 min. The RNA was dissolved in 0.2 ml of low ionic strength buffer (0.01 M Tris-HCl–0.001 M EDTA (pH 7.4)) to prevent aggregation which tended to occur with RNA preparations greater than 1 μg, and layered over a preformed 5-ml 5–20% sucrose gradient in the same buffer. The 60–70S viral genome was separated from low molecular weight nucleic acids by sedimentation at 110,000g for 135 min at 4°. The fractions containing 60–70S RNA were pooled, the absorbance at 260 nm was measured, and the solution was diluted to 4.5 ml with standard buffer and precipitated overnight with 2 volumes of cold ethanol. Undenatured high molecular weight viral RNA obtained by this method migrated as a sharp band in the 60–70S region when analyzed by a mixed agarose–acrylamide gel technique (Neiman and Henry, 1971). No attempt was made to assay or remove any small molecular weight (4 S or 5 S) RNA which may have been hydrogen bonded to the 60–70S viral genome (Erikson and Erikson, 1972; Faras *et al.*, 1973). The specific activity of the viral RNAs was estimated from the ultraviolet absorbance to be 1.5×10^6 cpm/μg. The RNA was dissolved in 0.4 M sodium phosphate–0.05% sodium dodecyl sulfate (pH 7.0) and stored at −176°.

The unlabeled viral RNA was prepared by the same method as the labeled RNA, except that a modified procedure was necessary to prevent the large quantity of RNA from forming rapidly sedimenting aggregates. Following extraction and precipitation the viral RNA was heated to 40° for 10 min in 0.4 ml of 0.2% sodium dodecyl sulfate–0.01 M Tris (pH 7.4)–0.01 M NaCl–0.001 M EDTA. The dissolved RNA was then layered on a 5-ml 15–30% glycerol gradient in the same buffer and sedimented at 48,000 rpm for 50 min at 20° in a SW 50.1 rotor (P. Duesberg, personal communication). The 60–70S RNA fraction, located by optical density, was treated in the same manner as the labeled RNA. Whole-cell *Escherichia coli* RNA was prepared by a hot phenol–*m*-cresol–sodium dodecyl sulfate procedure (Torelli *et al.*, 1968).

Preparation of DNA. Since DNA from all normal chicken sources tested (line 7 embryos, "leukosis-free" white leghorn chickens) showed no difference with regards to hybridization kinetics with RAV-0 RNA (P. E. Neiman, 1973b, and unpublished observations), line 7 embryos or "leukosis-free" 10-day-old chicken viscera were used as the source for normal chicken DNA. Tumor DNA was obtained from 10-day wing-web sarcomas induced by injection of Pr RSV into 1-day-old chicks. The technique used for DNA extraction was a modification (Hiatt, 1962) of the method of Marmur (1961). DNA was fragmented to an average molecular weight of 100,000 by limited depurination followed by alkaline hydrolysis (McConaughy and McCarthy, 1967; Neiman, 1973b). DNA was dissolved in 0.01 × standard saline citrate (0.00015 M sodium citrate–0.0015 M NaCl) if to be used in a few days or 0.4 M sodium phosphate–0.05% sodium dodecyl sulfate (pH 7.0), if to be stored. The properties (reassociation kinetics, thermal stability, and size characterics) of chicken DNA prepared in such a manner have been previously described in detail (Neiman, 1972).

RNA–DNA Hybridization. Hybridization reaction mixtures consisting of 1 mg of DNA fragments and approximately 2×10^{-4} μg of labeled RNA (300 cpm) with varying amounts of unlabeled RNA in 0.1 ml of 0.4 M sodium phosphate buffer (pH 7.0)–0.05% sodium dodecyl sulfate were denatured 10 min at 100°, chilled in ice, and incubated for 96 hr (cellular

C_0t of 10^4) at 67°. Hybridization was assayed by acquisition of ribonuclease resistance as previously described in detail (Melli *et al.*, 1971; Neiman, 1972, 1973b). Thermal stability studies of viral RNA–cellular DNA hybrids formed under these conditions, which indicated little if any mismatching, have been reported previously (Neiman, 1972, 1973b). All hybridization studies were corrected for nonspecific reactions which have several contributing components. First there was about 1–2% ribonuclease resistance in the radioactive RNA preparations. Additional low-level reaction (3–4%) was noted with *E. coli* DNA. Slightly higher reaction levels were noted with DNA from a variety of eukaryotes, as has been frequently reported (Harel *et al.*, 1970; Yoshikawa-Fukada and Ebert, 1969). These reactions occurred at C_0t values below 100 and did not increase. Since such interactions bear no certain relationship to viral genes, and could not be "competed out" under the conditions used in these studies, they were eliminated as background from the calculations. Reactions of 8% for RAV-0 RNA and 5% for Pr RSV RNA with canine spleen DNA were representative.

If the frequency of viral sequences per diploid cell genome is one, and the size of the viral genome is 10^7 daltons (Bonar and Beard, 1959; Green, 1970), and of the haploid genome of avian cells is 6×10^{11} daltons (Davidson and McIndoe, 1949), then the minimum complementary viral DNA: RNA ratio (D_2: R, eq 2) would approximate 40:1 in a standard reaction mixture in the absence of unlabeled competitor RNA. If the DNA provirus is represented by an allelic gene locus, as is particularly likely for the heiritable endogenous provirus of RAV-0, then the minimum frequency would be one per haploid genome and the minimum DNA excess doubled. Unlabeled homologous RNA was added to the standard reaction mixture in sufficient quantities to produce a modest RNA excess and thereby reduce the radioactivity of the resultant hybrid to a major degree. Different viral RNAs were then compared in a similar fashion.

Results

Theoretical Competition Model. The specific activity of viral RNA and concentration of DNA limited the DNA excess to a modest level. Analytical equations for mathematical prediction of hybridization under conditions varying from moderate DNA excess to moderate RNA excess are not available (Bishop, 1972b). Straus and Bonner (1972) have suggested the numerical solution of the general expressions for DNA reassociation and RNA–DNA hybridization in free solution for small increments of time to generate theoretical hybridization kinetic curves under such conditions. Second-order equations (eq 1–3) have been formulated (Straus and

$$dD_1/dt = -k_1D_1D_2 \quad (1)$$

$$dD_2/dt = -k_1D_1D_2 - k_2D_2R \quad (2)$$

$$dR/dt = -k_2D_2R \quad (3)$$

Bonner, 1972) where D_1 and D_2 are the concentrations of the single strands of unreacted DNA (D_2 being the strand complementary to the RNA), R is the concentration of reactive, unhybridized RNA, and k_1 and k_2 are rate constants for the renaturation of DNA and complementary RNA–DNA hybridization, respectively. For the purpose of providing a theoretical framework for comparison with experimental data, we applied this model to the conditions of viral nucleic

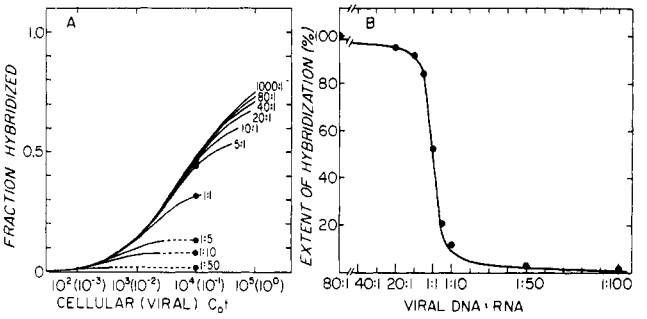

FIGURE 1: (A) Hybridization kinetic curves were obtained by solving the three general equations for DNA reassociation and RNA–DNA hydridization (eq 1–3 in the text) for the indicated ratios of input DNA and RNA at the standard C_0t ((mol sec)/l.) values using the rate constants described in the text. A dashed line represents a flat extension used when the theoretically achievable maximum fraction hybridized was obtained before reaching a cellular C_0t of 10^4. (B) The normalized per cent of hybridization at cellular C_0t of 10^4 derived from indicated points in A (●) is charted at various viral DNA:RNA ratios and compared to the theoretical curve derived from eq 4 in the text.

acid hybridization used here. Calculations were made by using estimates of rate constants ($k_1 = 100$, $k_2 = 35.7$) obtained from previously reported hybridization kinetic experiments (Neiman, 1972), with data similar to that shown in Figure 2. The estimate for k_1 was derived from the rate constant for the reassociation of the bulk of the cellular DNA fragments and k_2 from the rate of hybridization of RNA complementary to nonreiterated chicken DNA sequences prepared *in vitro*. At the level of one provirus copy per diploid cell genome the estimated concentrations of viral nucleic acids in standard reaction mixtures, in terms of moles of nucleotide per liter, are D_1 and $D_2 = 2.5 \times 10^{-7}$, each, and $R = 6.75 \times 10^{-9}$. These estimates were adjusted upward in the various experiments for the estimated average viral gene frequency. The equations were solved for successive small increments of time producing decreases in D_2 less than or equal to 10%. This procedure necessitated the use of a simple computer program. Theoretical curves were obtained in this manner which indicated expected kinetics of hybridization for various RNA inputs. These are displayed in Figure 1A. The curve for a DNA: RNA ratio of 1000:1 closely approximated a curve calculated using analytical equations for an infinite DNA excess (not shown) (Bishop, 1972b). Assumptions contained in these estimates and derivative calculations included (a) all, or nearly all, of the complementary viral RNA will eventually react in the presence of an adequate DNA excess, (b) the rate constant for proviral DNA reassociation (k_1) does not differ significantly from that of the rest of the cellular DNA, (c) the rate constant for hybridization of viral RNA with proviral DNA (k_2) does not differ significantly from that estimated for the reaction of complementary RNA prepared *in vitro* and, (d) the rate of dissociation of duplexes under the conditions used in these experiments is negligibly small. It should be stressed that all of these assumptions are subject to some error, the extent of which remains to be precisely determined. Nevertheless the potential utility of such calculations in analyzing hybridization reactions between nucleic acids from animal cells made reasonable an attempt to determine how well such a technique might be predictive in the present study.

If the data points for extent of hybridization indicated in Figure 1A at cellular C_0t of 10^4 for varying viral DNA: RNA ratios are normalized and charted against that ratio, they are

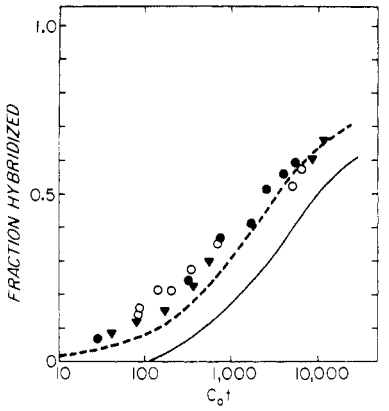

FIGURE 2: Hybridization kinetics with tumor DNA. The fraction of ribonuclease resistant hybrids of [^{3}H]RNA from the cloned standard stock of Pr RSV (▼), a second clone of the same standard stock of Pr RSV (●), and RAV-0 (○) with Pr RSV wing-web tumor DNA is charted at various C_0t values. The kinetics of hybridization were corrected for background ribonuclease resistance and nonspecific interaction of viral RNA with *E. coli* DNA (4%). The solid line represents a "one copy curve" derived from previous RNA–DNA hybridization kinetics for RNA complementary to nonreiterated sequences in wing-web tumor DNA, as described in the text. The dashed line represents the same curve adjusted to a frequency of three viral genomes per cell.

found to follow a curve (Figure 1B) which is described by the equation

$$Y = X/(X + 1) \tag{4}$$

where Y is the extent of hybridization and X is the viral DNA:RNA ratio. This curve, adjusted for the estimated proviral copy frequency per cell, was used as a model with which experimental data from the following competition hybridization studies were compared.

Hybridization Kinetics. In order to obtain estimates of proviral DNA concentrations for the planned competition experiments some hybridization kinetic experiments not previously performed were carried out. Figure 2 depicts the hybridization kinetics of RAV-0 and Pr RSV [^{3}H]RNA with DNA from Pr RSV-induced sarcomas. The data indicate

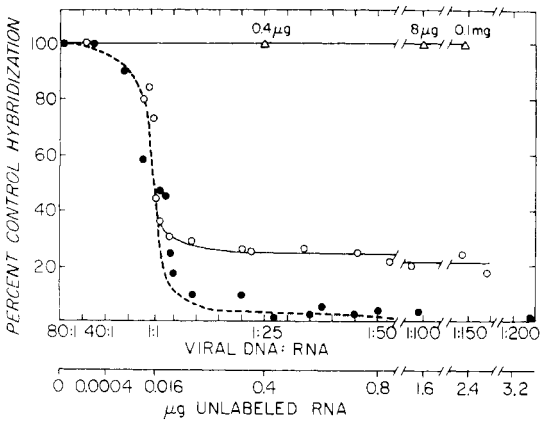

FIGURE 3: Competition of unlabeled RNA from RAV-0 (●) or Pr RSV (○—○) or whole-cell *E. coli* RNA (△—△) in the hybridization reaction between RAV-0 [^{3}H]RNA and normal chicken DNA. The corrected (see text), "uncompeted" hybridization at C_0t value of 10^4 (55–60%) was normalized to 100%. Dashed line represents the theoretical competition curve (Figure 1B) adjusted for a frequency of 2 viral genomes/cell as indicated on the two abscissas. Thus a DNA:RNA ratio of 1 corresponds to the presence of 0.016 μg of each viral nucleic acid in the reaction mixture.

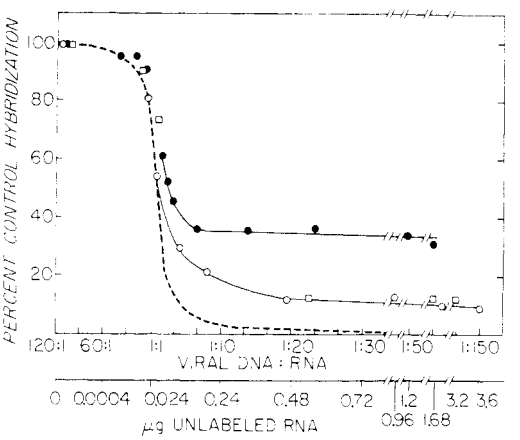

FIGURE 4: Competition of unlabeled RNA from RAV-0 (●—●) or Pr RSV (○—○) or B77 (□) in the hybridization of Pr RSV[^{3}H]RNA with DNA from Pr RSV induced wing-web tumors. Extent of hybridization is determined as described in Figure 3. Dashed line represents a theoretical competition curve adjusted for a frequency of 3 viral genomes/cell. Thus a DNA:RNA ratio of 1 corresponds to 0.024 μg of viral RNA in the reaction mixture.

approximately 60–65% hybrid formation at cellular C_0t values of 10^4 for both viruses, and seem to follow a curve estimated for a proviral DNA sequence frequency of 3 per cell genome. This is about 1–2 copies more than was estimated for RAV-0 sequences in normal chicken cell DNA (Neiman, 1973b). A biphasic contour is observed with a slightly more rapid rate of hybridization for 30–40% of the viral RNA. This "two-phase" kinetic curve is less striking than previously observed with RNA from a different stock of Pr RSV (Neiman, 1972). The hybridization kinetics of the RNA from these cloned stocks of Pr RSV with normal DNA did not differ from previous published results, reaching approximately 30% hybrid formation at a cellular C_0t value of 10^4 (Neiman, 1972).

Competitive Hybridization. A series of competition experiments were then conducted, and the resultant data were plotted against the theroetical curves discussed in previous sections. Figure 3 depicts the competitive effect of unlabeled RNA in the reaction between labeled RAV-0 RNA and normal chicken DNA. Homologous RAV-0 RNA competed close to the theoretical curve adjusted for a proviral frequency of 2 copies/cell. An unrelated RNA (*E. coli*) failed to reduce the radioactivity of the viral nucleic acid hybrids even in massive quantities. Pr RSV RNA competed for approximately 80% of the RAV-0 related sequences in normal DNA. Figure 4 describes competition experiments with RSV related sequences in sarcoma DNA. Homologous RSV RNA competed to approximately 90% of the theoretical value for complete competition. RNA from B77 competed almost as completely as that from Pr RSV. In contrast RAV-0 RNA competed to only 65% of the value for homologous RNA. The data cluster closely around a theoretical competition curve adjusted for about 3 copies of proviral sequences per tumor cell and are, thus, in close agreement with the hybridization kinetics (Figure 2). Competition with labeled Pr RSV for related sequences in normal chicken DNA is illustrated in Figure 5. This experiment was designed to determine the relationship of the endogenous provirus to RSV related sequences in normal chicken DNA. The ordinate was not normalized since the fraction of the RSV genome in normal cells has not been precisely determined. As can be seen in the figure RAV-0 RNA competed to the extent of at least 85–90% with

RNA from the sarcoma virus for complementary DNA sequences. This observation indicates that the vast majority if not all of the sequences in normal chicken DNA complementary to RSV are explained by the endogenous provirus of RAV-0.

Discussion

The close fit of the experimental data to the theoretical curve for competition by RAV-0 RNA in homologous competition reactions suggests that the techniques used for predicting DNA–RNA hybridization at different DNA:RNA ratios, as described in the Results section and illustrated in Figure 1, may be useful for analysis of competition experiments with RNA transcribed from low frequency genes in eukaryotic systems. It must be emphasized, however, that such an observation does not prove that the assumptions and estimates from which the curves were calculated (particularly the hybridization rate constant, k_2) are precisely correct. For example, the fact that we cannot be certain that the rate constant for the reassociation of the proviral DNA is the same as that of the bulk of the cellular DNA and the fact that we do not know whether hybridization, assayed by ribonuclease resistance, would eventually proceed to completion if cellular C_0t values of 10^6 or greater were practically obtainable mean the copy number estimates and derivative calculations are subject to modest degrees of error as has been previously discussed (Straus and Bonner, 1972; Neiman, 1972, 1973b). In addition the estimate of the viral RNA hybridization rate constant (k_2) is subject to error because it was based on the hybridization of *in vitro* prepared complementary RNA which may well differ in base composition and secondary structure from viral RNA. Further it has recently been shown that k_2 is proportional to the square root of the size of the reacting RNA (Hutton and Wetmur, 1973). Since hybridization reactions with low frequency sequences in animal cell DNA require long periods of time, and in this study elevated temperature, progressive thermal scission in the RNA may well produce an apparent k_2 which is falsely low. Thus, no conclusion can be drawn with respect to the precision of the estimate of k_2 used. Nevertheless the experimental determination of the amount of homologous RNA necessary to produce 50% competition in studies with both RAV-0 and Pr RSV gave estimates of provirus reiteration frequency which are quite close to those suggested by analysis of the kinetics of hybridization of RNA from the two viruses (which were based on comparisons with complementary RNA) and indicates a very low number of viral genome per cell. A similar mathematical analysis of the hybridization of ribosomal nucleic acid has been recently reported (Tereba, 1973; Tereba and McCarthy, 1973). Bishop (1972a) and Bishop *et al.* (1972) have also demonstrated the competitive effect of m- and rRNA under similar conditions.

The reason for the 10% or so of RSV RNA radioactivity remaining at DNA:RNA ratios where it would be predicted that the unlabeled homologous RNA would be present in sufficient excess to produce complete competition is not immediately apparent. The possibility of the presence of a small fraction of labeled RNA in the RSV preparation complementary to high frequency sequences (>200 copies/cell) in the tumor DNA cannot be excluded. Because of the low amount of radioactivity used in these studies (to obtain a modest initial DNA excess), differences in the 5–10% range approach the limit of significance. These considerations raise the question of the effect of amplification of a portion of the

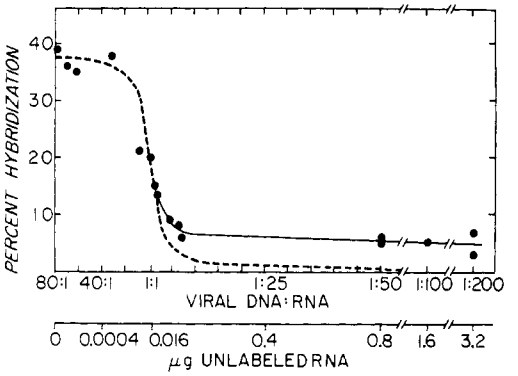

FIGURE 5: Competition of unlabeled RNA from RAV-0 (●—●) in the hybridization of Pr RSV [^{3}H]RNA with normal chicken DNA. Per cent of hybridization is the actual fraction hybridized (not normalized) after background correction (see text). Dashed line represents a curve adjusted to a frequency of about 2 partial viral genomes/cell.

proviral DNA sequences on competition analyses of the kind described here. Host cell DNA sequences complementary to a fraction of some C-type viral RNAs such as that from RD-114 (Neiman, 1973a), avian myeloblastosis virus (Shoyab *et al.*, 1974), and some clones of RSV (Neiman, 1972) appear to be present in a frequency of 50–100 copies. It should require, therefore, concentrations of unlabeled RNA 25–50-fold higher than those used in the present study to produce complete competition in studies with nucleic acids from these viruses. Host cell sequences related to the particular clone of RSV selected for this study, as well as to RAV-0, did not appear to demonstrate this peculiar partial amplification in sufficient degree to affect the competition curves (with the exception of the small fraction of RSV RNA discussed above). The significance of this apparent variation in the frequency of virus related DNA sequences remains to be determined.

Bishop *et al.* (1972) have recently presented a simple equation for the estimation of the molecular weight of reacting polynucleotides in competitive RNA–DNA hybridization experiments performed under conditions similar to those of the present study. For this calculation the complexity of the DNA, the amount of RNA needed to produce a DNA:RNA ratio of 1 (50% competition), and a reiteration frequency estimate for the complementary sequences in cellular DNA are required. With a range in the estimate of the copy number of proviral sequences of from 1–4/cell, the molecular weight of these two viruses, calculated by this method, ranges from 0.3 to 1 $\times$ 10^7. This estimate is in agreement with, but no better than, previously published estimates by other methods (Bonar and Beard, 1959; Green, 1970).

The principal conclusion of this study is that the sarcomagenic virus shares about two-thirds of its sequences with the endogenous virus, RAV-0, and about one-third of its sequences are unique to RSV. Conversely about four-fifths of the endogenous provirus is shared with the transforming virus and about one-fifth appears to be unique to RAV-0. This interpretation (and the normalization of the ordinate of Figures 3 and 4) presupposes that the extensive, but partial, hybridization achieved at a cellular C_0t of 10^4 is representative of the entire genome, an assumption for which there is considerable biological evidence (Hill and Hillova, 1972a,b; Weiss *et al.*, 1971). The unshared sequences of Pr RSV do not seem to be present in the normal chicken cell since these studies demonstrate that almost all of the Pr RSV sequences in normal chicken DNA are shared with RAV-0.

It would be helpful, in the comparative study of RNA tumor viruses, to assign unshared functions to unshared genome sequences. One obstacle to this interpretation, in the present study, is the question of simple genetic divergence. This process needs to be considered if for no other reason than the difference in the history of the two viruses, with RSV having a long history in tissue culture while RAV-0 was obtained directly from its natural source. The competition experiment with Pr RSV and B77, a comparable sarcoma virus of completely separate origin and different history of tissue culture, suggests, however, that simple sequence divergence, unrelated to function (and detectable by this hybridization technique), may not be very extensive in the chicken oncornavirus system. Admittedly this finding is not conclusive. For example, it says nothing about the evolution of endogenous viruses separate from that of sarcoma viruses. Nevertheless it is provocative to speculate that the capacity for sarcomagenesis and *in vitro* transformation may lie in the segment of the RSV genome unshared with RAV-0 and exogenous to the normal chicken cell. Study of nontransforming deletion mutants derived from stocks of Pr RSV now in progress should help clarify this observation. We also observed sequences in the endogenous provirus not present in the genome of RSV. Subject to the same hazards, speculation as to the function of the RAV-0 unique segment may include sequences for envelope antigens distinguishing subgroup C and E viruses, but these may not be sufficient to explain this large difference. The possible role of these sequences in the genetic regulation of endogenous viruses will be the subject of further study.

Acknowledgments

The authors thank Drs. Peter Vogt and Robert Friis for supplying viruses and cultures of chicken embryo cells and for advice regarding materials for these experiments, Dr. Allan Tereba for helpful discussions of the theoretical calculations for competitive hybridization, Dr. Vincent Gallucci for help in deriving eq 4, and Miss Christine McMillin and Mr. Don MacDonnell for excellent technical assistance.

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