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# Hybridization of Rous Sarcoma Virus Deoxyribonucleic Acid Polymerase Product and Ribonucleic Acids from Chicken and Rat Cells Infected with Rous Sarcoma Virus[1]

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Rous sarcoma virus (RSV)-specific ribonucleic acid (RNA) in virus-producing chicken cells and non-virus-producing rat cells infected with RSV was studied by hybridization with the endogenous deoxyribonucleic acid (DNA) product of the RSV virion DNA polymerase system. By hybridizing the total DNA product with excess virion RNA, the product DNA was separated into hybridized ("minus") and nonhybridized ("plus") DNA. The "minus" DNA was complementary to at least 20% of the RNA from RSV which remained of high molecular weight after denaturation. A maximum of approximately 65% hybridization was observed between "minus" DNA and RSV RNA or RSV-infected chicken cell RNA. A maximum of about 60% hybridization was observed between "minus" DNA and RSV-infected rat cell RNA. RSV-infected chicken cells contained RSV-specific RNA equivalent to about 6,000 virions per cell. RSV-infected rat cells contained RSV-specific RNA equivalent to approximately 400 virions per cell. Neither cell type contained detectable RNA complementary to virion RNA. The RSV-specific RNA in RSV-infected rat cells did not appear to be qualitatively different from that in RSV-infected chicken cells.

Virions of ribonucleic acid (RNA) tumor viruses contain endogenous deoxyribonucleic acid (DNA) polymerase systems capable of synthesizing DNA using virion RNA as a template (2, 24). Much of the DNA product has a sequence complementary to that of the virion RNA (7, 18). It has been reported that at least 85% of the virion RNA is copied by the Rous sarcoma virus (RSV) endogenous DNA polymerase system (7); however, all portions of the genome may not be copied to an equal extent (25). It has been reported that RNA of RSV (5, 10) or murine leukemia virus (13) could be detected in infected cells, but not in uninfected cells, by hybridization with the DNA product of the appropriate virion DNA polymerase system. Extensive characterization of the DNA product used in the hybridization, or of the hybrids themselves, has not been reported.

The experiments presented in this paper compare virus-specific RNA in chicken and rat cells infected with RSV. R(B77) cells, a line of rat embryo fibroblasts transformed in vitro by the B77 strain of avian sarcoma virus (1, 16), produce no detectable RSV-like particles yet contain the RSV genome (1, 5). In these respects, they resemble most other RSV-transformed mammalian cell lines (12). Previously (5), we showed that R(B77) cells contain some B77 virus-specific RNA detectable by hybridization with labeled B77 virus product DNA. It was not determined whether this RNA was qualitatively or quantitatively different from that observed in B77 virus-infected chicken cells or whether it had a sequence identical or complementary to that of virion RNA. To study these problems, we have performed hybridization experiments with the DNA product of the endogenous B77 virion DNA polymerase system separated into fractions complementary or identical in sequence to the virion RNA. The results of these experiments suggest that R(B77) cells contain less RNA of the same sequence as B77 virus RNA than do chicken cells, but that there is no qualitative difference between the B77 virus-specific RNA in these two

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types of cells. Neither type of cells contained detectable RNA with a sequence complementary to that of B77 virion RNA.

## MATERIALS AND METHODS

General experimental procedures for this laboratory were described previously (20, 21).

**Viruses and cells.** The B77 strain of avian sarcoma virus was grown in C/O chicken embryo cells in culture and concentrated and purified as described previously (4).

High-specific-activity $^{32}P$-labeled B77 virus was prepared as follows. Six secondary cultures of chicken embryo fibroblasts in 60-mm plastic culture dishes (Falcon Plastics, Division of B-D Laboratories, Los Angeles, Calif.) were infected with virus and maintained in Eagle medium with 5% fetal bovine serum. Five days after infection, the medium was changed to phosphate-free Eagle medium for 1 day. A 30-ml amount of phosphate-free Eagle medium containing 10 mCi of $^{32}P$-phosphate (carrier-free, New England Nuclear Corp., Boston, Mass.) was then added to the cultures. One day later, the supernatant medium was removed from the cultures, and virus was purified as described previously (5). RNA was extracted from the $^{32}P$-labeled B77 virus as described below with the addition of 100 $\mu$g of yeast RNA (Worthington Biochemical Corp., Freehold, N.J.) as carrier. The amount of $^{32}P$-labeled B77 virus RNA was estimated from the amount of virus RNA obtained from parallel unlabeled cultures, purified as described below. This estimate and the amount of radioactivity obtained were used to estimate a specific activity of approximately $2 \times 10^6$ counts per min per $\mu$g of viral RNA. Approximately 85% of this RNA preparation was precipitable with trichloroacetic acid. Less than 1% of the trichloroacetic acid-precipitable radioactivity remained after treatment with 0.2 N NaOH at 25 C for 12 hr.

Uninfected and B77 virus-infected chicken embryo fibroblasts were prepared as described previously (4). Rat embryo fibroblasts transformed in vitro by B77 virus [R(B77)] cells and uninfected rat embryo fibroblasts were previously described (5).

**Nucleic acids.** Labeled endogenous B77 virion DNA product was prepared from standard polymerase reactions (24) carried out in a total volume of 1.25 to 6.25 ml containing either thymidine-5′-triphosphate-$^3H$ ($^3H$-TTP, 17.5 Ci/mmole, Schwarz BioResearch, Inc., Orangeburg, N.Y.) or deoxycytidine-5′-triphosphate-$^3H$ ($^3H$-dCTP, 22.6 Ci/mmole, Schwarz BioResearch, Inc.), or both labeled deoxyribonucleoside triphosphates. These reactions were divided into an appropriate number of tubes at about 0.5 ml per tube, incubated for 1 hr at 40 C, and then pooled for nucleic acid extraction. $^{32}P$-labeled DNA was prepared from large-scale polymerase reactions containing 10 $\mu$Ci of $\alpha$-$^{32}P$-TTP (27.2 Ci/mmole, New England Nuclear Corp.) per ml of reaction. One pmole of $^{32}P$ incorporated corresponded to approximately 48,000 counts per min. Nucleic acids were extracted from polymerase reactions by using sodium dodecyl sulfate and diethyl pyrocarbonate as previously described (4).

RNA was extracted from purified B77 virus (4) and from cells (5) as previously described.

**Nucleic acid hybridization.** Samples of labeled DNA were distributed in screw-cap vials, and 5 $\mu$liters of phenol and 100 $\mu$g of yeast RNA were added. (The yeast RNA was added to all hybridizations to have an excess of noncomplementary RNA.) Appropriate amounts of test RNA were added to each vial, and all vials were adjusted to the same volume (usually 0.4 ml) with 0.03 M sodium citrate, *p*H 7.0, and 0.3 M NaCl. Annealing was at 68 C for 4 hr or longer. The contents of each vial were then analyzed by equilibrium density gradient centrifugation in cesium sulfate gradients. When it was necessary to hydrolyze the RNA present in some preparations of labeled DNA, the DNA preparations were adjusted to 0.2 N NaOH and 0.05 M tris(hydroxymethyl)-aminomethane (Tris) and incubated overnight at room temperature. After this incubation, a small amount of phenol red was added, and the preparations were neutralized with 0.2 N HCl. DNA was sometimes denatured by heating at 100 C for 4 min followed by cooling on ice.

For study of separated DNA fractions, pooled samples from $Cs_2SO_4$ gradients were annealed by the same procedure with no additional treatment. Since no nonspecific hybridization of such samples with uninfected cell RNA was observed under these conditions, it was not necessary to remove $Cs_2SO_4$ prior to annealing.

Hybridization of $^{32}P$-labeled virus RNA to DNA was performed as follows. DNA preparations were treated with NaOH and neutralized as described above. Samples (50-$\mu$liter) containing various amounts of this DNA in Tris-ethylenediaminetetraacetic acid buffer with approximately 1 M $Cs_2SO_4$ were distributed to vials. A 100-$\mu$g amount of yeast RNA, 5 $\mu$liters of phenol, and approximately 2,000 counts per min of $^{32}P$-labeled virus RNA were added to each vial, to a total volume of 75 $\mu$liters. The samples were incubated at 68 C for 24 hr. Trichloroacetic acid-insoluble $^3H$ and $^{32}P$ radioactivity was determined for 25-$\mu$liter portions of each reaction with no treatment and after treatment with a mixture of 40 $\mu$g of ribonuclease A (Worthington Biochemical Corp.) and 40 units of ribonuclease T1 (California Biochemical Corp., Los Angeles, Calif.) per ml for 1 hr at 40 C. Ribonuclease solutions were treated by heating at 100 C for 10 min before use. Results of these hybridization reactions are expressed as a percentage of ribonuclease-resistant $^{32}P$ counts per minute. The data are corrected for a slight loss of $^3H$ counts per minute (usually less than 10%) observed after ribonuclease treatment. Approximately 4% of the $^{32}P$-labeled RNA was resistant to this ribonuclease treatment prior to annealing with DNA.

**Density gradient centrifugation.** B77 virus RNA was fractionated by centrifugation in linear 5 to 20% sucrose gradients in 0.01 M sodium phosphate, *p*H 7.0, and 0.15 M NaCl. A small amount of $^{32}P$-labeled B77 virus RNA was added as a marker, and the RNA preparation was heated at 100 C for 4 min, followed by cooling on ice, prior to centrifugation. Centrifuga-

tion was for 2 hr at 45,000 rev/min in a Spinco SW50.1 rotor.

Annealing mixtures were analyzed by $Cs_2SO_4$ equilibrium density gradient centrifugation as previously described (5). All data were corrected by subtracting a background of 15 to 20 counts per min. As before, the amount of hybridization was defined as the per cent counts per minute banding at densities greater than 1.53 g/ml in such gradients.

## RESULTS

**Separation of B77 virus DNA "plus" and "minus" fractions.** The product of the endogenous B77 virus virion DNA polymerase reaction (referred to below as B77 virus DNA) contains double-stranded DNA (15), presumably consisting of one strand complementary in sequence to the virion RNA and one strand identical in sequence to the virion RNA. It was thought that separation of these strands prior to further hybridization would provide a more sensitive probe for the specific detection of RSV virion-like ("plus") RNA in cells. RSV DNA product complementary to virion RNA would be expected to hybridize with virion RNA to a greater extent than the total DNA product. Such a separation of B77 virus DNA fractions would also make it possible to look in cells for RNA complementary to that of the RSV virion.

The preparation of separated fractions of B77 virus DNA is shown in Fig. 1. Table 1 summarizes the per cent counts per minute banding in those regions of the gradients shown in Fig. 1B, C, and D. Eighty-seven per cent of the DNA in pool b banded near the density of DNA after annealing with B77 virus RNA (Fig. 1B). Approximately 75% of the DNA in pool c, when annealed with B77 virus RNA and centrifuged in a $Cs_2SO_4$ gradient, banded at either an intermediate density or a density characteristic of RNA (Fig. 1C). Approximately 85% of the DNA in pool d, when annealed with B77 virus RNA and centrifuged in a $Cs_2SO_4$ gradient, banded at the density of RNA or an intermediate density (Fig. 1D). Eighty-seven per cent of the DNA in pool d banded at a density typical of DNA after annealing with RNA from uninfected cells. B77 virus DNA fractions for the experiments described below were prepared as shown in Fig. 1A. Pool b will be referred to as "plus" DNA (based on the convention that the sequence of the virion RNA is "plus"). "Plus" DNA was usually subjected to another cycle of annealing with B77 virus RNA and $Cs_2SO_4$ centrifugation before use. Pool d will be referred to as "minus" DNA. Both DNA fractions sedimented with a sedimentation coefficient of less than 5S in alkaline sucrose gradients (J. M. Coffin, Ph.D. thesis, Univ. of Wisconsin, Madison, 1972).

Nearest neighbor analysis of $\alpha$-$^{32}$P-thymidine

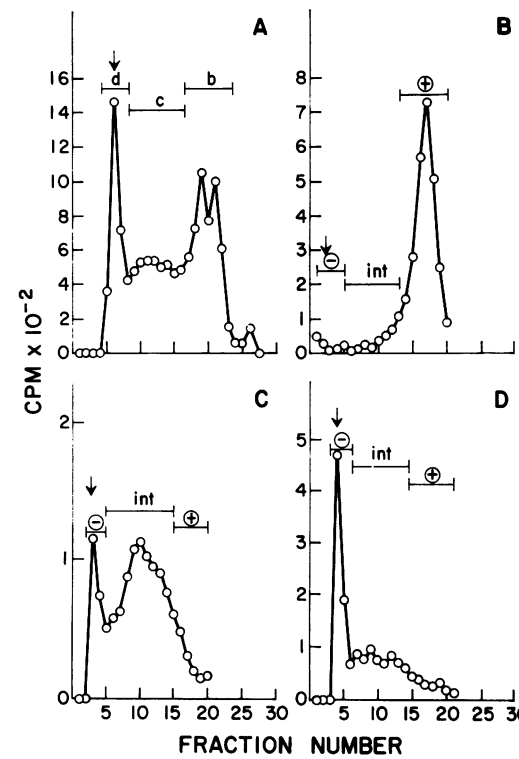


FIG. 1. *Preparation and analysis of separated fractions of B77 virus DNA. A, An endogenous DNA polymerase reaction containing both $^3$H-TTP and $^3$H-dCTP was carried out with B77 virus, and the total nucleic acids were extracted after 1 hr of incubation. This nucleic acid preparation was heated at 100 C for 4 min and cooled on ice. A 5-μg amount of B77 virus RNA and 50 μg of uninfected rat cell RNA were added, and the mixture was annealed at 68 C for 12 hr. The annealing mixture was then centrifuged in a $Cs_2SO_4$ equilibrium density gradient, and samples of each fraction were counted (○). Fractions were pooled as indicated by the horizontal bars. B, Pool b was heated at 100 C for 4 min, cooled on ice, and annealed with 2.5 μg of B77 virus RNA and 50 μg of uninfected rat cell RNA. Annealing was at 68 C for 8 hr. The annealing mixture was analyzed in a $Cs_2SO_4$ equilibrium density gradient, and the radioactivity of samples of each fraction was determined (○). C, A sample of pool c was subjected to the same treatment as described in B above. D, Pool d was treated with 0.2 N NaOH for 16 hr at 25 C to degrade the associated RNA and neutralized with HCl. A 1.25-μg amount of B77 virus RNA and 50 μg of uninfected chicken cell RNA were added, and the mixture was annealed at 68 C for 4 hr. The annealing mixture was analyzed on a $Cs_2SO_4$ equilibrium density gradient, and radioactivity was determined for all fractions (○). The density gradient was similar in all gradients. The location of an RNA precipitate band in all gradients is marked with an arrow. Data from these gradients are summarized in Table 1. The horizontal bars in B, C, and D mark the regions of the gradients considered as "plus," "intermediate," and "minus" for the calculation of recoveries of these fractions.*

TABLE 1. *Recoveries of different fractions of $^3$H-labeled B77 virus DNA*[a]

| Starting fraction | Per cent counts/min[b] recovered as | | |
|---|---|---|---|
| | "Minus"[c] | "Intermediate"[c] | "Plus"[c] |
| Total product (Fig. 1A) | 26 | 34 | 40 |
| Pool b (Fig. 1B) | 4 | 9 | 87 |
| Pool c (Fig. 1C) | 17 | 59 | 25 |
| Pool d (Fig. 1D) | 47 | 39 | 14 |
| Pool d (not shown)[d] | 1 | 13 | 87 |

[a] Data were taken from the experiment described in Fig. 1.

[b] Data are expressed as per cent total radioactivity recovered from each gradient.

[c] These fractions are indicated by horizontal bars in Fig. 1B, C, and D.

[d] Data from a gradient containing an annealing mixture run in parallel to that shown in Fig. 1A, except that no B77 virus RNA was added.

TABLE 2. *Hybridization of B77 virus "minus" DNA to fractions of B77 virus RNA*[a]

| RNA fraction[b] | Counts/min in density > 1.53 g/ml | Total counts/min | Per cent counts/min in density > 1.53 g/ml |
|---|---|---|---|
| None | 280 | 2,500 | 11 |
| Complete | 1,700 | 2,700 | 63 |
| 20 to 50*S* | 1,800 | 2,900 | 61 |
| 3 to 7*S* | 580 | 2,300 | 25 |

[a] A preparation of $^3$H-labeled B77 virus "minus" DNA was annealed with the indicated RNA species under standard conditions at 68 C for 4 hr and analyzed in $Cs_2SO_4$ equilibrium density gradients.

[b] A preparation of B77 virus RNA was heated at 100 C for 4 min, cooled on ice, and centrifuged in a 5 to 20% linear sucrose gradient for 1.25 hr at 48,000 rev/min in an SW50.1 rotor. The indicated fractions were pooled, and equal amounts (approximately 5 μg) were used in the hybridizations.

monophosphate (TMP)-labeled B77 virus product DNA showed that approximately 80% of the TMP was not next to TMP, and therefore that the product DNA was not copied from poly(A) regions of the virus RNA (J. M. Coffin, Ph.D. thesis).

**Relationship of B77 virus DNA fractions to B77 virus RNA.** RSV virions contain both high- and low-molecular-weight RNA (17). The high-molecular-weight RNA is assumed to contain the RSV genome. The low-molecular-weight RNA contains transfer RNA (9). To determine whether a significant portion of B77 virus "minus" DNA was complementary to the genome RNA, the experiment shown in Table 2 was carried out. The same amount of hybridization was observed with the complete B77 virus RNA and the denatured high-molecular-weight fraction of B77 virus RNA after removal of associated low-molecular-weight RNA (8). This result indicates that primarily the high-molecular-weight RNA of B77 virus was copied in the endogenous virus DNA polymerase reaction. Mostly genome RNA should, therefore, be detected in hybridizations between B77 virus "minus" DNA and cell RNA. It is uncertain whether the low-molecular-weight RNA which hybridized to B77 virus "minus" RNA was virion transfer RNA or breakdown products of the high-molecular-weight RNA.

To draw conclusions from hybridization experiments of the type discussed here, it is necessary to know how much of the B77 virus genome was represented by the B77 virus product DNA. Duesberg and Canaani (7) have reported that at least 85% of RSV RNA would hybridize to the product of the RSV endogenous DNA polymerase system. However, these authors found it necessary to use very large amounts of DNA to achieve this much hybridization. In contrast, Varmus et al. (25) have reported that the double-stranded product of the RSV endogenous DNA polymerase product reanneals at a rate characteristic of a complexity of only about 20% or less of the size of the virion RNA. The experiments of Fig. 2 were performed to determine how much of the B77 virus RNA was represented by the "plus" and "minus" fractions of the endogenous DNA product.

A maximum of about 25% of $^{32}$P-labeled B77 virus RNA was resistant to ribonuclease after it was annealed with either complete B77 virus DNA or B77 virus "minus" DNA. A maximum of only about 10% of the B77 virus RNA was resistant to ribonuclease after annealing with "plus" DNA. Less than 5% of the B77 virus RNA was resistant to ribonuclease after annealing with no added DNA. The specificity of the hybridization observed in this experiment is demonstrated by the fact that 0.12 μg of B77 virus RNA in the annealing mixture reduced the hybridization of $^{32}$P-labeled B77 virus RNA to complete DNA by more than 50%. These results indicate that at least 20% of the RSV RNA is represented by the complete B77 virus DNA and by the "minus" DNA.

In another experiment, it was shown that annealing of B77 virus "plus" DNA with B77 virus "minus" DNA led to formation of double-stranded molecules and prevented the "minus" DNA from hybridizing with B77 virus RNA (J. M. Coffin, Ph.D. thesis). The "plus" B77

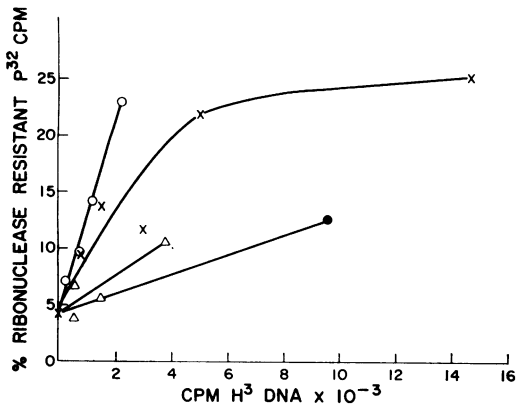


FIG. 2. *Hybridization of B77 virus RNA to B77 virus DNA fractions. Dilutions of total, "plus," and "minus" DNA product were annealed with approximately 500 counts/min of $^{32}$P-labeled B77 virus RNA as described in the text. Annealing was for 24 hr at 68 C. At the end of this time, trichloroacetic acid-insoluble radioactivity was determined for all fractions with and without ribonuclease treatment in about 0.5 M $Cs^+$. The specific activity of the B77 virus RNA was approximately 2 × 10$^6$ counts per min per μg, and the specific activities of the B77 virus DNAs were approximately 1.5 × 10$^6$ counts per min per μg. Symbols: complete (unfractionated) B77 virus DNA (×); B77 virus "minus" DNA (○); B77 virus "plus" DNA (△). One annealing mixture contained $^{32}$P-labeled B77 virus RNA, complete B77 virus DNA, 0.12 μg of unlabeled B77 virus RNA, and $^{32}$P-RNA (●).*

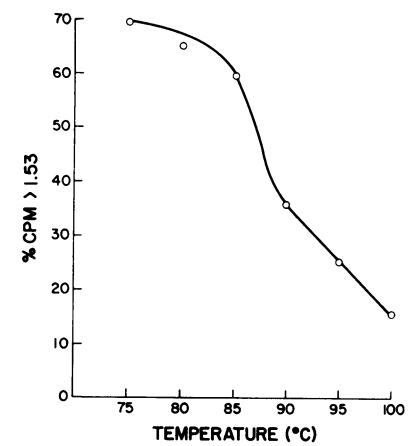


FIG. 3. *Thermal denaturation of the hybrid between B77 virus "minus" DNA and B77 virus RNA. $^3$H-labeled B77 virus DNA-B77 virus RNA hybrid was prepared as described in the legend of Fig. 1. Samples were diluted with Tris-EDTA buffer to approximately 0.3 M $Cs_2SO_4$ and heated at the indicated temperatures for 10 min. All samples were cooled on ice and analyzed in $Cs_2SO_4$ equilibrium density gradients. The per cent counts per minute banding at greater than 1.53 g/ml was determined for all gradients (○). In a similar experiment, no difference was observed in the per cent counts per minute banding at greater than 1.53 g/ml between untreated B77 virus RNA-"minus" DNA hybrids and hybrid heated to 75 C.*

virus DNA was complementary to a minimum of 80% of the B77 virus "minus" DNA.

**Properties of B77 virus DNA-B77 virus RNA hybrids.** The hybrid of the B77 virus RNA and B77 virus "minus" DNA was denatured over a broad temperature range (approximately 20 C) with a $T_m$ of about 87 C (Fig. 3).

Similar B77 virus "minus" DNA-B77 virus RNA hybrids were treated with 10 μg of ribonuclease A per ml in approximately 1 M $Cs_2SO_4$ at 40 C for 30 min and were analyzed by $Cs_2SO_4$ equilibrium density gradient centrifugation. Most of the ribonuclease-treated hybrid banded in a single peak at about 1.55 g/ml, a density characteristic of hybrids with approximately equal amounts of RNA and DNA, instead of at about 1.65 g/ml (data not shown). Thus, the banding of B77 virus "minus" DNA-B77 virus RNA at the density of RNA in $Cs_2SO_4$ gradients appears to be due to the presence of regions of single-stranded RNA in the hybrid molecules. A similar result has been reported by Duesberg and Canani (7). However, the extent of the difference in density between untreated and ribonuclease-treated hybrids probably reflects not the relative amounts of RNA and DNA in the hybrids, but coprecipitation of the hybrids with RNA in the gradients (5). For this reason, all $Cs_2SO_4$ gradients described here contained at least 50 μg of cell RNA.

**Use of separated fractions of RSV product DNA to detect RSV-specific RNA in RSV-infected chicken and rat cells.** The results of hybridization between B77 virus "minus" DNA and B77 virus RNA, B77 virus-infected chicken cell RNA, and R(B77) cell RNA are shown in Fig. 4. Most of the DNA banded at the density of DNA after annealing with uninfected rat cell RNA (Fig. 4A). A significant fraction of the DNA banded at the density of RNA after annealing with B77 virus RNA (Fig. 4B), B77 virus-infected chicken cell RNA (Fig. 4C), or R(B77) cell RNA (Fig. 4D). These results show that B77 virus "minus" DNA hybridized not only with B77 virus RNA but also with RNA from B77 virus-infected chicken and rat cells.

To insure that these hybrids were not the result of hybridization between poly(A) and poly(dT) (3, 14), nearest neighbor analysis of $^{32}$P-TMP-labeled B77 virus "minus" DNA in hybrids with B77 virus RNA and RNA from B77 virus-infected rat and chicken cells was performed. Approximately 80% of the TMP was not next to

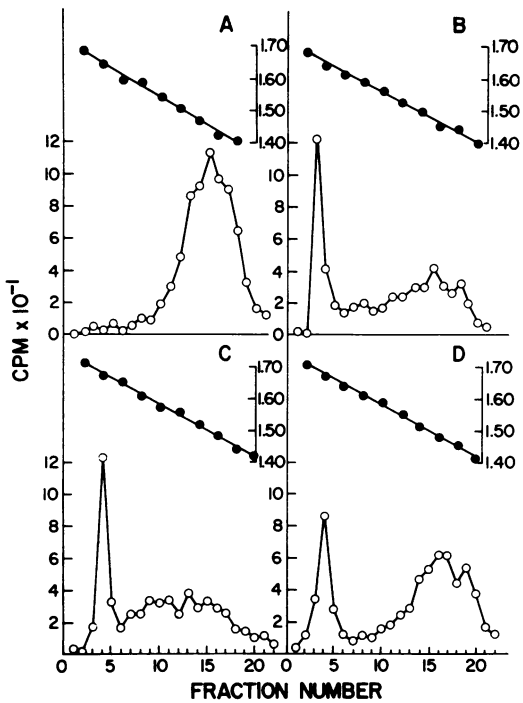


FIG. 4. *Hybridization of B77 virus "minus" DNA with B77 virus RNA and cell RNA. Samples of "minus" DNA were annealed under standard conditions with the following RNA. A, 50 μg of uninfected rat cell RNA; B, 50 μg of uninfected rat cell RNA plus 0.31 μg of B77 virus RNA; C, 55 μg of B77 virus-infected chicken cell RNA; D, 160 μg of R(B77) cell RNA. Annealing was at 68 C for 7 hr. All annealing mixtures were analyzed in $Cs_2SO_4$ equilibrium density gradients. Twenty-drop fractions were collected, and the density was determined for alternate fractions (●). Trichloroacetic acid-insoluble radioactivity was determined for each fraction (○).*

TMP (J. M. Coffin, Ph.D. thesis). Therefore, the hybridization was not between poly(A) and poly(dT).

To determine whether hybrids between B77 virus "minus" DNA product and B77 virus-infected chicken cell RNA or R(B77) cell RNA were denatured over a temperature range similar to that of B77 virus RNA-"minus" DNA hybrids, the experiment described in Table 3 was carried out. The behavior of both hybrids with cellular RNA was similar in this experiment. Both were almost completely denatured by heating to 90 C, and approximately one-half of each was denatured by heating to 80 C. In contrast, less than 10% of the hybrid between B77 virus "minus" DNA product and B77 virus RNA was denatured by heating to 80 C. The $T_m$ of the hybrids between B77 virus "minus" DNA product and B77 virus-infected chicken cell or R(B77) cell RNA was somewhat less than the $T_m$ of the hybrid between B77 virus "minus" DNA and B77 virus RNA.

TABLE 3. *Thermal denaturation of hybrids between B77 virus "minus" DNA and B77 virus-infected cell RNA*[a]

| RNA species in hybrid | Per cent counts/min > 1.53 g/ml after heating at | | |
|---|---|---|---|
| | 25 C | 80 C | 90 C |
| B77 virus-infected chicken cell | 84 | 53 | 4 |
| R(B77) cell | 84 | 45 | 11 |
| B77 virus[b] | 70 | 67 | 37 |

[a] Hybrids between $^3$H-labeled B77 virus "minus" DNA and cell RNA were prepared as described in the legend of Fig. 4. Pooled hybrid fractions were heated and analyzed in $Cs_2SO_4$ gradients under conditions identical to those described in the legend of Fig. 3.

[b] Data from Fig. 3.

The above results indicate that hybrids were formed between RNA extracted from both B77 virus-infected chicken cells and B77 virus-infected rat cells. In a large number of experiments (for example, Fig. 4A), no significant hybridization has been observed between B77 virus "minus" DNA and uninfected chicken cell RNA or uninfected rat cell RNA, although amounts of up to 200 μg of these RNA species per hybridization have been used. This lack of hybridization is in agreement with results previously reported by others (10, 13). Thus, the B77 virus "minus" DNA used here did not appear to contain DNA complementary to RNA from normal, uninfected cells.

Data from experiments like that shown in Fig. 4 are replotted in Fig. 5 as per cent counts per minute banding at densities greater than 1.53 g/ml versus amount of test RNA used in the hybridizations. B77 virus RNA and B77 virus-infected chicken cell RNA both hybridized a maximum of about 60% of the B77 virus "minus" DNA. R(B77) cell RNA hybridized a maximum of about 45% of the B77 virus "minus" DNA.

There are several possible reasons for the failure of the B77 virus "minus" DNA to band entirely at densities of greater than 1.53 g/ml in gradients where maximum hybridization was observed. It was possible that the B77 virus "minus" DNA used in this experiment contained fragments of DNA too small to form stable hybrids. It was also possible that some of the DNA was hybridized to molecules of RNA too small to

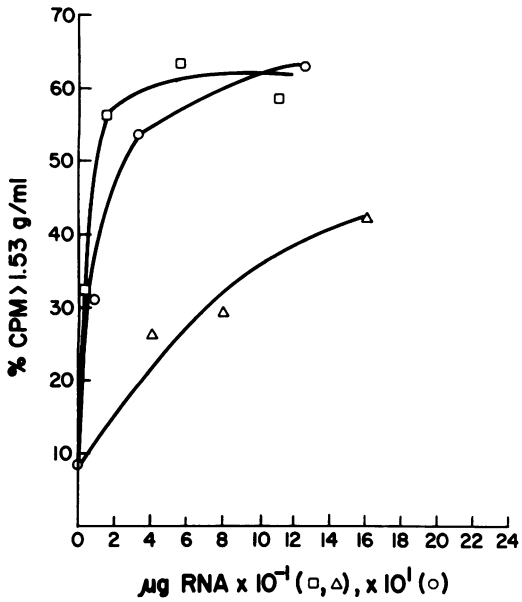


FIG. 5. *Hybridization of B77 virus "minus" DNA with B77 virus RNA and cellular RNA. Mixtures containing $^3$H-labeled B77 virus "minus" DNA product and the indicated amounts of B77 virus RNA* (○), *B77 virus-infected chicken cell RNA* (□), *or R(B77) cell RNA* (△) *were prepared. After annealing, all mixtures were analyzed in $Cs_2SO_4$ equilibrium density gradients, some of which are shown in Fig. 4. The per cent counts per minute banding at greater than 1.53 g/ml was determined for each gradient.*

increase the density to greater than 1.53 g/ml. A third possibility was that some DNA-RNA hybrids formed during annealing were degraded during subsequent operations. Such degradation could have been due either to spontaneous denaturation of the hybrids or to nuclease activity. Hybrids isolated from $Cs_2SO_4$ gradients and analyzed on further gradients without being treated under denaturing conditions also did not band entirely at densities greater than 1.53 g/ml (Table 3). This result suggests that part of the reason hybrids between B77 virus RNA and B77 virus "minus" DNA did not band entirely at densities greater than 1.53 g/ml was degradation of hybrids formed during annealing.

B77 virus RNA hybridized "minus" DNA about 100 times as efficiently, per μg of RNA, as did B77 virus-infected chicken cell RNA. Assuming that a B77 virus virion contains $10^7$ daltons ($1.7 \times 10^{-11}$ μg) of RNA (6) and that a cell contains $10^{-5}$ μg of RNA, then the B77 virus-infected chicken cells contained B77 virus "plus" RNA equivalent to about 6,000 virions per cell. (This argument applies only to the 20% or more of the viral RNA which was detected by hybridization with the B77 virus "minus" DNA used in these experiments.)

R(B77) cell RNA hybridized "minus" DNA about 16-fold less efficiently, per μg of RNA, than B77 virus-infected chicken cell RNA. If the B77 virus RNA species detected in the B77 virus-infected chicken cells and in the R(B77) cells were qualitatively similar, a similar argument shows that R(B77) cells contained B77 virus "plus" RNA equivalent to about 400 virions per cell.

The hybridization of B77 virus "minus" DNA to R(B77) cell RNA reached a maximum of only 45%. To determine whether it was possible to obtain a greater amount of hybridization than that observed in the experiment described in Fig. 5 by increasing the concentration of R(B77) cell RNA present in the annealing mixture, the experiment of Table 4 was performed. The extent of hybridization under these conditions, when measured as per cent DNA hybridized, should have been independent of the DNA concentration. It was found that 59% hybridization of B77 virus "minus" DNA was obtained with the larger amount of R(B77) cell RNA, and 46% hybridization was obtained with the lesser amount of R(B77) cell RNA. These results suggest that the R(B77) cell RNA which hybridized to B77 virus "minus" DNA was not qualitatively different from B77 virion or B77 virus-infected chicken cell RNA which hybridized to B77 virus "minus" DNA.

This conclusion is supported by the results of an experiment with the DNA which did not hybridize to R(B77) cell RNA. No difference in ability to hybridize with R(B77) cell or B77 virus-infected chicken cell RNA was found between the B77 virus "minus" DNA and the preparation

TABLE 4. *Hybridization of B77 virus "minus" DNA with large amounts of R(B77) cell RNA*[a]

| RNA | Per cent counts/min > 1.53 g/ml |
|---|---|
| 50 μg of uninfected rat cell | 7.5 |
| 50 μg of uninfected rat cell + 1 μg of B77 virus | 69 |
| 190 μg of R(B77) cell | 46 |
| 790 μg of R(B77) cell[b] | 59 |

[a] Annealing was for 6 hr at 68 C with 3,000 counts/min of "minus" DNA. All gradients were analyzed by $Cs_2SO_4$ equilibrium density gradient centrifugation.

[b] This reaction contained 12,000 counts/min of B77 virus "minus" DNA. Only one-fourth of the mixture was analyzed in a $Cs_2SO_4$ gradient.

which did not hybridize with R(B77) cell RNA (J. M. Coffin, Ph.D. thesis).

**Absence of B77 virus "minus" RNA in cells infected with B77 virus.** RNA tumor viruses most likely replicate through a DNA intermediate (19, 23). For this reason, there is no requirement that cells infected with an RNA tumor virus contain RNA complementary to the virion RNA. It was possible, however, that the messenger RNA for RSV was complementary to the virion RNA. It was also possible that R(B77) cells could contain B77 virus "minus" RNA, due to some error of transcription, even if "minus" RNA was absent from B77 virus-infected chicken cells. These hypotheses were tested by the use of B77 virus "plus" DNA (Fig. 6).

In all gradients with B77 virus "plus" DNA alone, no hybridization significantly above background was observed. The hybridization of B77 virus "plus" DNA to B77 virus RNA (Fig. 6B) indicates that this "plus" DNA preparation contained less than 2% contamination with "minus" DNA. From this data it can be calculated that 40 μg of B77 virus-infected chicken cell RNA hybridized 55% of B77 virus "minus" DNA (Fig. 6E) and that there was less than 1/200 as much B77 virus "minus" RNA in B77 virus-infected chicken and rat cells as there was "plus" RNA in B77 virus-infected chicken cells. Also, no significant hybridization of B77 virus "plus" DNA product with cellular RNA was observed in similar experiments when the RNA was heated at 100 C prior to annealing (data not shown). Thus, it seems most likely that the messenger RNA of the B77 virus genome in B77 virus-infected cells is "plus" RNA, identical in sequence to the virion RNA, at least for the 20% or more of the B77 virus RNA measured here.

## DISCUSSION

The experiments described in this paper were undertaken to determine whether RSV-infected mammalian cells contained RSV-specific RNA and to compare such RNA with the RSV-specific RNA present in RSV-infected chicken cells. For these purposes, hybridization reactions were performed between the DNA product of the B77 virus virion endogenous DNA polymerase system and virus or cell RNA.

To determine whether the sequence of cell RNA detected by hybridization with B77 virus DNA was similar or complementary to B77 virus RNA, DNA fractions capable of detecting only one or the other of the two RNA species were prepared. Preparation of these fractions was accomplished by annealing the total B77 virus DNA with B77 virion RNA and separating hybridized ("minus") from nonhybridized ("plus") DNA in $Cs_2SO_4$ equilibrium density gradients. Separation of the B77 virus DNA by hybridization to B77 virus RNA yielded two fractions: "minus" DNA, complementary to at least 20%

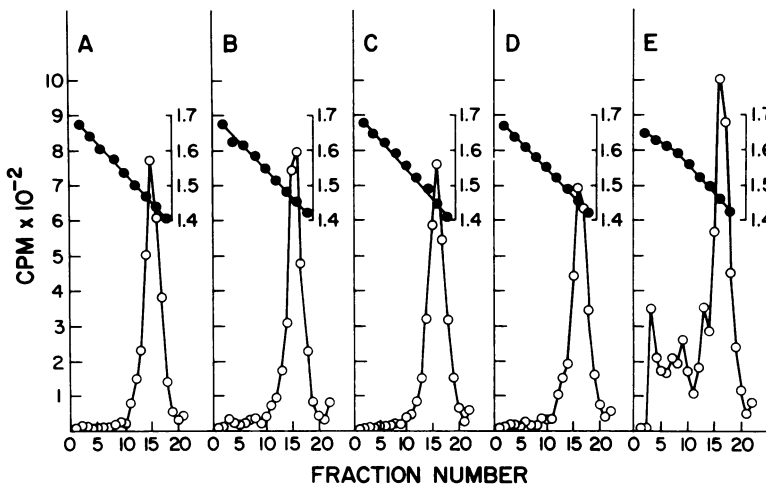


FIG. 6. *Failure of B77 virus "plus" DNA to hybridize with B77 virus RNA or B77 virus-infected chicken or rat cell RNA. $^3$H-labeled B77 virus "plus" DNA was annealed with 2.5 μg of B77 virus RNA and centrifuged again in a $Cs_2SO_4$ gradient. Samples of 3,000 counts/min of the nonhybridized DNA from this gradient were annealed with each of the following RNA species: A, 120 μg of uninfected chicken cell RNA; B, 120 μg of uninfected chicken cell RNA plus 5.5 μg of B77 virus RNA; C, 225 μg of R(B77) cell RNA; D, 240 μg of B77 virus-infected chicken cell RNA; E, 40 μg of B77 virus-infected chicken cell RNA and 3,000 counts/min of B77 virus "minus" DNA. Annealing was at 68 C for 4 hr. All annealing mixtures were analyzed by $Cs_2SO_4$ equilibrium density gradient centrifugation. Fifteen-drop fractions were collected, and density in g/ml (●) and trichloroacetic acid-insoluble radioactivity (○) were determined.*

of the viral RNA, and "plus" DNA, complementary to at least 80% of the "minus" DNA, and therefore identical to at least 16% of the virus RNA (J. M. Coffin, Ph.D. thesis).

Both "plus" and "minus" B77 virus DNA were heteropolymers and were small, with sedimentation constants of less than 5S in sucrose gradients. This small size is consistent with previously reported sedimentation values in alkaline sucrose gradients for the complete DNA product of the RSV endogenous DNA polymerase (15). B77 virus "minus" DNA was mostly complementary to viral RNA which was of high molecular weight after heating.

B77 virus "minus" DNA proved to be a sensitive probe for the detection of B77 virus "plus" RNA in cells However, the interpretation of hybridization experiments using this "minus" DNA is somewhat limited since as much as 80% of the B77 virus genome RNA may not be detected by hybridization with B77 virus "minus" DNA.

As expected, B77 virus "minus" DNA hybridized with B77 virus RNA, B77 virus-infected chicken cell RNA, and R(B77) RNA to a greater extent than did unfractionated B77 virus DNA. In addition, more consistent results were obtained with the "minus" DNA than with the unfractionated DNA. The relative efficiencies of hybridization of B77 virion RNA, B77 virus-infected chicken cell RNA, and R(B77) cell RNA were similar whether complete B77 virus DNA or B77 virus "minus" DNA was used.

Surprisingly, hybrids between B77 virus "minus" DNA and B77 virus-infected chicken cell RNA or R(B77) cell RNA melted at a somewhat lower temperature than hybrids between B77 virus "minus" DNA and B77 virus RNA. The reasons for this difference are unknown. It might reflect a problem in the specificity of the cell hybrids.

Neither B77 virus "plus" nor "minus" RNA complementary to the DNA used here have been observed in uninfected chicken or rat cells. B77 virus RNA complementary to "minus" DNA equivalent to as few as 30 virions per cell could have been detected.

B77 virus-infected chicken cell RNA hybridized both "minus" and total B77 virus DNA approximately 1% as efficiently, per μg of RNA, as B77 virus RNA. However, it cannot be concluded from this result that 1% of the cell RNA is viral, since the portion of the virus RNA detected may not be representative of the entire virus RNA in cells. The RNA that was detected was equivalent to about 6,000 virions per cell. RNA from R(B77) cells hybridized both "minus" and complete B77 virus DNA much less efficiently than B77 virus-infected chicken cell RNA. Also, a lower maximum hybridization of "minus" and complete B77 virus DNA to R(B77) cell RNA was observed. The failure to obtain the same maximum hybridization of B77 virus "minus" DNA with R(B77) cell RNA as with B77 virus-infected chicken cell RNA appears to be the result of a quantitative difference between the two RNA species.

It is likely that this lower amount of B77 virus-specific RNA in R(B77) cells is related to a lower rate of transcription from the DNA provirus, although a much greater rate of breakdown cannot be excluded. If transcription and breakdown of B77 virus RNA occurred at the same rate in R(B77) and B77 virus-infected chicken cells, there should have been more of this RNA in R(B77) cells, since more of this RNA would have been released as virions from B77 virus-infected chicken cells. The failure of R(B77) cells to produce B77 virus may be related to the low amount of B77 virus RNA in these cells.

No significant B77 virus "minus" RNA, either single- or double-stranded was detected in either B77 virus-infected chicken cells or in R(B77) cells by hybridization with B77 virus "plus" DNA. "Minus" RNA equivalent to approximately 30 virions per cell would have been detected by this procedure. This result suggests that the messenger for that portion of the B77 virus genome represented by "plus" DNA was not "minus" RNA.

Since it is likely that hybridization of virus DNA with cell RNA will be widely used to determine the relationship of viruses to natural tumors, the following points are worth emphasizing. Viral DNA used as a probe in such experiments must be isolated from hybrids with high-molecular-weight virion RNA. It is not sufficient to demonstrate that a population of virus DNA has some molecules capable of hybridizing to high-molecular-weight virion RNA. The hybridized DNA must also be shown to be a heteropolymer. Furthermore, even positive results cannot imply viral etiology. DNA hybridizable to viral RNA and DNA has been found in normal uninfected cells (2a, 11). Such DNA may be that postulated by the oncogene or protovirus theories (23).

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## LITERATURE CITED


1. Altaner, C., and H. M. Temin. 1970. Carcinogenesis by RNA sarcoma viruses. XII. A quantitative study of infection of rat cells *in vitro* by avian sarcoma virus. Virology **40**:113–134.
2. Baltimore, D. 1970. RNA-dependent DNA polymerase in virions of RNA tumor viruses. Nature (London) **226**: 1209–1211.

2a. Baluda, M. A. 1972. Widespread presence in chickens of DNA homologous to the RNA genome of avian leukosis viruses. Proc. Nat. Acad. Sci. U.S.A. **69**:576–580.

3. Baluda, M. A., and D. P. Nayak. 1970. DNA complementary to viral RNA in leukemic cells induced by avian myeloblastosis virus. Proc. Nat. Acad. Sci. U.S.A. **66**:329–336.
4. Coffin, J. M., and H. M. Temin. 1971. Comparison of Rous sarcoma virus-specific deoxyribonucleic acid polymerases in virions of Rous sarcoma virus and in Rous sarcoma virus-infected chicken cells. J. Virol. **7**:625–634.
5. Coffin, J. M., and H. M. Temin. 1971. Ribonuclease-sensitive deoxyribonucleic acid polymerase activity in uninfected rat cells and rat cells infected with Rous sarcoma virus. J. Virol. **8**:630–642.
6. Duesberg, P. H. 1970. On the structure of RNA tumor viruses. Curr. Top. Microbiol. Immunol. **51**:79–104.
7. Duesberg, P. H., and E. Canaani. 1970. Complementarity between Rous sarcoma virus (RSV) RNA and the *in vitro*-synthesized DNA of the virus-associated DNA polymerase. Virology **42**:783–788.
8. Erikson, E., and R. L. Erikson. 1971. Association of 4*S* ribonucleic acid with oncornavirus ribonucleic acids. J. Virol. **8**:254–256.
9. Erikson, R. L. 1969. Studies on the RNA from avian myeloblastosis virus. Virology **37**:124–131.
10. Garapin, A. C., J. Leong, L. Fanshier, W. E. Levinson, and J. M. Bishop. 1971. Identification of virus-specific RNA in cells infected with Rous sarcoma virus. Biochem. Biophys. Res. Commun. **42**:919–925.
11. Gelb, L. D., S. A. Aaronson, and M. A. Martin. 1971. Heterogeneity of murine leukemia virus *in vitro* DNA: detection of viral DNA in mammalian cells. Science **172**: 1353–1355.
12. Gelderblom, H., H. Bauer, and H. Frank. 1970. Investigations on virus production in RSV mammalian tumors. J. Gen. Virol. **7**:33–45.
13. Green, M., H. Rokutanda, and M. Rokutanda. 1971. Virus specific RNA in cells transformed by RNA tumor viruses. Nature N. Biol. **230**:229–232.
14. Harel, L., J. Harel, F. Lacour, and J. Huppert. 1966. Homologie entre genome du virus de la myeloblastose aviare (AMV) et genome cellulaire. C. R. H. Acad. Sci. Paris **263**:616–619.
15. Mizutani, S., D. Boettiger, and H. M. Temin. 1970. DNA-dependent DNA polymerase and a DNA endonuclease in virions of Rous sarcoma virus. Nature (London) **228**: 424–427.
16. Moore, E. G., and H. M. Temin. 1971. No correlation between conversion by RNA sarcoma viruses and increased agglutinability of cells. Nature N. Biol. **231**:117–118.
17. Robinson, W. S., A. Pitkanen, and H. Rubin. 1965. The nucleic acid of the Bryan strain of Rous sarcoma virus: purification of the virus and isolation of the nucleic acid. Proc. Nat. Acad. Sci. U.S.A. **54**:137–144.
18. Spiegelman, S., A. Burny, M. R. Das, J. Keydar, J. Scholm, M. Travnicek, and K. Watson. 1970. Characterization of the products of RNA-directed DNA polymerases in oncogenic RNA viruses. Nature (London) **227**:563–567.
19. Temin, H. M. 1964. Nature of the provirus of Rous sarcoma. Nat. Cancer Inst. Monogr. **17**:557–570.
20. Temin, H. M. 1967. Studies on carcinogenesis by avian sarcoma viruses. V. Requirement for new DNA synthesis and for cell division. J. Cell. Physiol. **69**:53–63.
21. Temin, H. M. 1968. Studies on carcinogenesis by avian sarcoma viruses. VIII. Glycolysis and cell multiplication. Int. J. Cancer **3**:273–282.
22. Temin, H. M. 1971. The role of the DNA provirus in carcinogenesis by RNA Tumor viruses, p. 176–187. *In* L. G Sylvestri (ed.), Proc. 2nd Lepetit Colloq., North-Holland, Amsterdam.
23. Temin, H. M. 1971. Mechanism of cell transformation by RNA tumor viruses. Annu. Rev. Microbiol. **25**:609–648.
24. Temin, H. M., and S. Mizutani. 1970. RNA-dependent DNA polymerase in virions of Rous sarcous virus. Nature (London) **226**:1211–1213.
25. Varmus, H. E., W. E. Levinson, and J. M. Bishop. 1971. Extent of transcription by the RNA-dependent DNA polymerase of Rous sarcoma virus. Nature N. Biol. **233**: 19–21.