HYBRIDIZATION OF RNA FROM ROUS SARCOMA VIRUS WITH CELLULAR AND VIRAL DNA'S

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Abstract.—The RNA of Rous sarcoma virus (RSV) hybridizes with DNA's isolated from all eukaryotes tested (animal and plant) with the possible exception of yeast. Bacterial DNA's show no homology to RSV-RNA. DNA's from the oncogenic viruses, adenovirus type 12 and SV40 show significant homology with RSV-RNA, but those from nononcogenic adenoviruses types 2 and 4 are not homologous.

RSV-RNA hybridized to all of the DNA's examined has the same, or a closely similar, base composition. Moreover, the melting temperatures of all of the hybrids analyzed are very similar, further suggesting that a common base sequence in DNA functions in hybridization with RSV-RNA.

Introduction.—Although considerable evidence exists indicating that oncogenic viruses induce DNA synthesis in contact-inhibited, nongrowing cells,[1–8] including clonally derived differentiated cells,[9, 10] the mechanism by which the viral genome is integrated into the synthetic apparatus of cells is poorly understood. Our interest in this problem prompted us to undertake experiments designed to determine whether cellular DNA's contain base sequences homologous to RSV-RNA. Finding that RSV-RNA hybridizes with DNA's of all eukaryotes tested with the possible exception of yeast, we were led to explore possible base sequence homologies among oncogenic viruses.

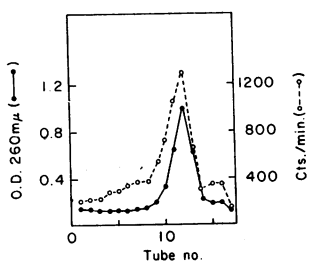
Materials and Methods.—Preparation of highly radioactive RSV-RNA: Secondary fibroblast cultures prepared from 11-day-old chick embryos (avian leukosis virus-free eggs obtained from SPAFAS, Inc., Norwich, Conn.) were infected with a Bryan high-titer strain of Rous sarcoma virus which has been maintained in this laboratory for several years,[10] and incubated at 41°C. The multiplicity of infection was roughly one and foci were observed by the third or fourth day post infection. On the fourth day, the medium was changed to phosphate-free Eagle's minimal essential medium containing 5% dialyzed calf serum, 20 μc of carrier-free $^{32}PO_4$/ml and 0.25 mM each of the four nucleosides. The medium was collected daily for 5 or 6 days. Rous sarcoma virus was precipitated with an equal volume of saturated ammonium sulfate (4°C, pH 7) and purified by sucrose density gradient centrifugation. RNA was extracted by the SDS-phenol method. Usually, 10–20 μg of RNA of specific activity 1 to 4 $\times$ 10^5 cpm/μg were obtained from two 9-cm plates containing about 8 $\times$ 10^6 cells each in 5 ml medium.

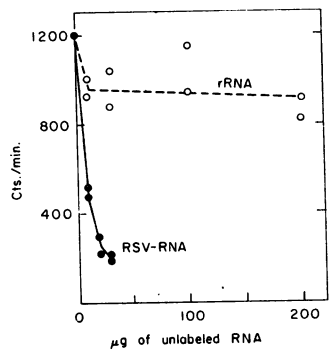
Hybridization technique: Fifty to 100 μg of heat-denatured DNA in 0.1 $\times$ SSC were mixed with 0.5 to 1 μg of ^{32}P-labeled RSV-RNA and 100 μg of chick ribosomal RNA, and the salt concentration was adjusted to equal 2 $\times$ SSC. After incubation at 62°C for 6 to 12 hr, the RNA-DNA mixture was treated with 50 μg of RNase (Worthington Biochemical Corporation, previously heated at 80°C for 10 min to inactivate contaminating DNase) at room temperature for 30 min. In preliminary experiments over the range from 40°C to 75°C we had determined that 62°C was the optimal temperature of incubation. The density of the solution was adjusted to 1.700 gm/ml by adding the appropriate amount of CsCl. After centrifugation at 33,000 rpm for 48 to 60 hr, the fractions were collected and optical densities were determined. Each fraction was diluted with 10 ml

of 2 × SSC and incubated at 60°C for 1 hr to reduce background. Finally, the salt concentration was increased to 4 × SSC, RNA-DNA hybrids were trapped on Millipore filters and their radioactivities were determined in a toluene scintillator. In some experiments, DNA was prehybridized with unlabeled chick ribosomal RNA and fractionated by CsCl centrifugation. DNA filters were then prepared as described by Brown and Weber,[11] and used for hybridization with labeled RNA.

In saturation experiments, DNA filters were prepared by the method of Gillespie and Spiegelman.[12] DNA filters were incubated in 4 × SSC containing 0.2 to 5 μg/ml of ^{32}P RSV-RNA and 100 μg of chick ribosomal RNA at 62°C for 12 hr with shaking. Hybrids thus formed were treated with RNase at 50 μg/ml for 30 min at 37°C in 2 × SSC. Each side of each filter was washed five times with 10 ml of 4 × SSC. The background was about 0.05 to 0.1% of input RNA, which is about 10 times higher than that observed with ribosomal RNA's incubated and treated as described above. The addition of 0.1% SDS (purified twice by crystallization with ethanol) which has been reported to be effective in decreasing background of RNA synthesized *in vitro*,[13] did not work under the conditions just described. Washed filters were dried and counted. After counting, filters were washed with chloroform and the DNA was hydrolyzed in 2 ml of 0.2 *N* HCl for 10 min. in a boiling water bath The optical density at 260 mμ was read to determine the amount of DNA on a filter. During hybridization, release of DNA from filters was significant as observed by the increase of counts and optical density of blank filters when incubated with DNA filters. Therefore, only one kind of DNA filter was incubated in each vial and blank filters for background were incubated in separate vials. The amount of RNA hybridized was corrected to the value of 100 μg of DNA using the amount of recovered DNA after hydrolysis of each DNA filter.

Results.—Hybridization of RSV-RNA with chick DNA: RSV-RNA was found to hybridize with DNA isolated from uninfected chick fibroblasts (Fig. 1). The specificity of this hybridization was studied by competition with RSV-RNA and chick ribosomal RNA. Competition of 10 to 20 per cent was observed with 200 μg of ribosomal RNA and over 80% with 30 μg of RSV-RNA (Fig. 2). Harel *et al.*[14, 15] and Wilson and Bauer[16] reported that RSV and AMV-RNA's hybridized





(*Left*) FIG. 1.—CsCl fractionation of hybrids between DNA from uninfected chick embryo fibroblasts and ^{32}P RSV-RNA. The DNA was extracted using the SDS-phenol method from secondary cultures of fibroblasts prepared from 11-day-old embryos and was purified on a methylated albumin Kieselguhr column. 100 μg of DNA and 1.5 μg of ^{32}P RSV-RNA (1×10^5 cpm/μg) were used.

(*Right*) FIG. 2.—Competition of RSV-RNA with ribosomal RNA from chick embryo fibroblasts (uninfected). Filters containing about 100 μg of chick embryo DNA were incubated under hybridization conditions described in *Materials and Methods* with 0.4 μg of ^{32}P RSV-RNA (2×10^5 cpm/μg) in the presence of various amounts of unlabeled RSV-RNA or chick embryo ribosomal RNA.

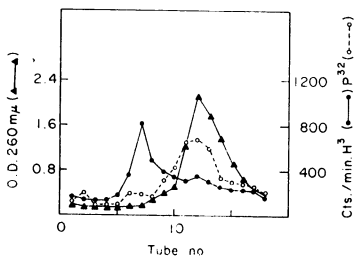


FIG. 3.—Hybridization of chick embryo fibroblast DNA with ribosomal RNA and RSV-RNA. 200 μg of DNA sheared as described by Brown and Weber,[11] prehybridized with 100 μg of ribosomal RNA was fractionated by CsCl centrifugation. DNA filters prepared as described in *Materials and Methods* were cut in half. One half was hybridized with 3 μg of ^{3}H-labeled 18S normal chick embryo ribosomal RNA (1.5×10^{5} cpm/μg) and the other half was incubated with 1 μg of ^{32}P RSV-RNA (4×10^{5} cpm/μg).

with DNA from infected and uninfected chick and rat cells. Both Harel *et al.* and Wilson and Bauer reported saturation values almost equivalent to those found by others[17, 18] in hybridizing mammalian cellular DNA and ribosomal RNA. Moreover, Harel *et al.* observed 20 per cent competition using cellular RNA and the figure reported by Wilson and Bauer was 80 per cent. We interpret the findings of these earlier saturation and competition experiments as indicating contamination of their RSV-RNA preparations by cellular RNA.

Figure 3 clearly shows that the DNA containing sequences for ribosomal RNA is different from that which is homologous to RSV-RNA. Sheared chick embryo DNA was first hybridized with chick embryo ribosomal RNA and fractionated by CsCl centrifugation. DNA homologous to ribosomal RNA sedimented faster than the rest. DNA filters prepared as described were used for the second hybridization with ^{32}P-labeled RSV-RNA and ^{3}H-labeled 18S ribosomal RNA. ^{3}H-radioactivity was detected in the heavier fraction while ^{32}P-radioactivity was in the main DNA peak.

Homology between RSV-RNA and DNA from various cell types: The ability of RSV-RNA to hybridize with DNA isolated from other cell types was examined using CsCl centrifugation. It should be emphasized that these hybridizations were done in the presence of chick ribosomal RNA. The DNA's extracted from HeLa cells and peas hybridize with RSV-RNA to about the same extent as clone 2 (a Schmidt-Ruppin transformed hamster cell line provided by Dr. P. Sarma) and Rous sarcoma virus-infected chick myotubes (Fig. 4*A–D*). No hybridization was detected with *M. lysodeikticus* DNA (Fig. 4*E*). As we reported previously, similar centrifugation patterns were obtained for Rous sarcoma virus hybrids with DNA's from L cells, calf thymus, turkey, goose, *R. pipiens*, salmon sperm, and *Euglena*, but not *E. coli* or yeast DNA.[19]

Homology between RSV-RNA and viral DNA: Adenovirus types 2, 4, and 12 were chosen because they reveal marked differences in oncogenicity within a group of related viruses. The first two are weakly or nononcogenic while the last is highly oncogenic. RSV-RNA hybridizes much more extensively with adenovirus 12 DNA than with that of adenoviruses 2 and 4 (Fig. 5). The densities of DNA from adenoviruses 2, 4, 12, and KB cells are 1.716, 1.717, 1.708, and 1.699, respectively.[23] As the optical density peak was found at density of 1.710 in Figure 5*C*, the radioactivity detected represents a hybrid between RSV-RNA and adenovirus 12 DNA, not of RSV-RNA and contaminating cellular DNA. Another oncogenic virus, SV40, also has DNA base sequences homologous to RSV-RNA (Fig. 5*D*). The density of SV40 DNA is 1.700 and is indistinguishable

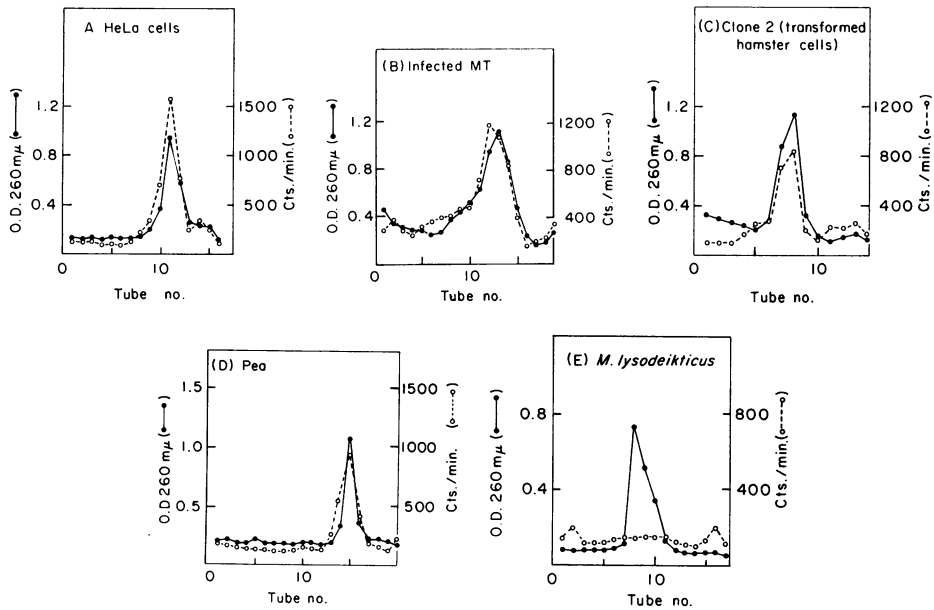


FIG. 4.—CsCl fractionation of hybrids between ^{32}P RSV-RNA and DNA's of various cell types. HeLa and clone 2 cells were grown in Eagle's minimum essential medium containing 5% calf serum. DNA was extracted by SDS-phenol method followed by RNase-pronase treatment. Chick embryo myotubes infected with Rous sarcoma virus were obtained by centrifuging cells ($80 \times g$, 30 sec) from mixed fibroblast and muscle cultures 48 hr post infection which had been layered over fetal calf serum. The lower fraction was about threefold enriched in myotubes but still contained myoblasts and fibroblasts. DNA's of pea and *M. lysodeikticus* were provided by Dr. J. Sinclair. About 60 to 100 μg of DNA were hybridized with 1.5 μg of ^{32}P RSV-RNA ($1–4 \times 10^5$ cpm/μg) and 100 μg of unlabeled chick ribosomal RNA.

from the DNA of BSC-1 cells by CsCl centrifugation. However, not only was the virus purified twice by centrifugation in CsCl of density 1.32, but also the findings of preliminary saturation experiments suggest that the saturation value in hybrids between RSV-RNA and SV40 DNA is significantly higher than in hybrids between RSV-RNA and mammalian cellular DNA. These facts would appear to rule out the possibility that the hybrid observed in Figure 5*D* is caused by contaminating cellular DNA in the SV40 DNA preparation.

Saturation experiments: A typical saturation curve is shown in Figure 6. The amounts of RSV-RNA required to saturate DNA from various sources are indicated in Table 1.

The molecular weights of adenovirus 2-DNA and adenovirus 12-DNA are reported to be 2.4×10^7 and 2.3×10^7, respectively.[23] Therefore, the saturation figure 0.005% obtained for adenovirus 2 DNA corresponds to a DNA segment of molecular weight 1200 (4 nucleotides) and 0.06% for adenovirus 12 DNA corresponds to a DNA segment of molecular weight 13,000 (about 45 nucleotides). Niyogi and Thomas[24] reported that 11 nucleotide pairs are the minimum number for significant hybridization. Therefore, the saturation value for adenovirus 2 DNA would represent a nonspecific hybridization, although it is close to that for mammalian DNA in terms of the amount of DNA hybridized.

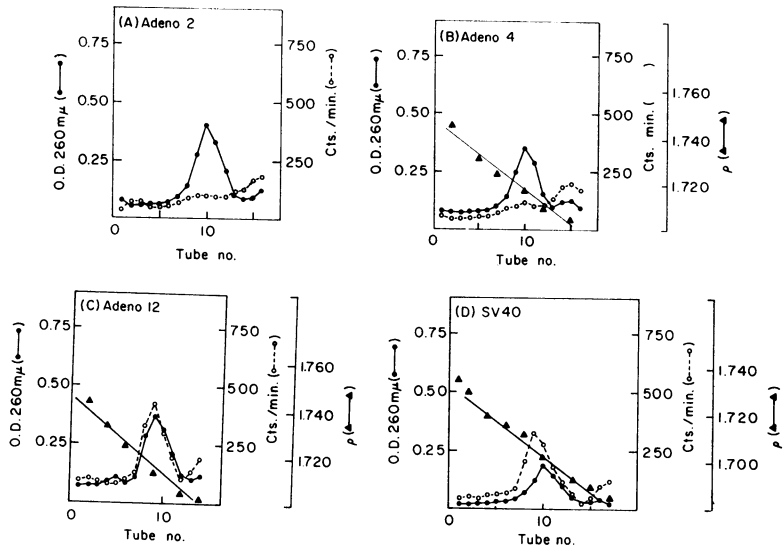


FIG. 5.—CsCl fractionation of hybrids between ^{32}P RSV-RNA and viral DNA's. DNA's of adenovirus types 2, 4, and 12 (obtained from ATCC) were extracted from purified virus stock according to the methods described by Green and Piña.[20, 21] SV40 (provided by Drs. H. Green and B. Pollack) was purified from infected BSC-1 cell stock according to the methods reported by Black *et al.*[22] DNA was extracted by the SDS-phenol method. About 4 to 30 μg of DNA, 1 μg of ^{32}P RSV-RNA ($0.8–2 \times 10^5$ cpm/μg) and 100 μg of chick ribosomal RNA were used for hybridization.

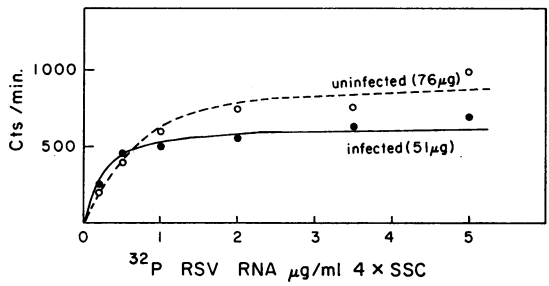
Properties of RSV-RNA hybridizing with cellular and viral DNA's: The base composition of RSV-RNA which is homologous to DNA from both cellular and viral sources was analyzed by two-dimensional paper chromatography (Table 2). Although small differences are observed, in general the base composition of hybridized RSV-RNA is characterized by high G and A and low U, compared to the base composition of the whole RSV-RNA molecule.[25]

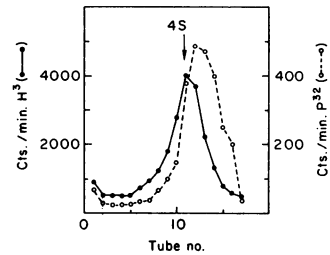
This finding, that the RSV-RNA hybridizing with several DNA's is closely similar in all hybrids, is further supported by melting experiments. The amount of RNA released from hybrids between RSV-RNA and HeLa cell DNA at increasing temperatures is shown in Table 3. The *Tm* of this combination is 74°C (±1°). Comparable *Tm*'s of hybrids between RSV-RNA and other DNA's

TABLE 1. *Saturation values of RSV-RNA hybridized with various DNA's.*

Sources of DNA	Amount of RNA saturating 100 μg of DNA (μg)
Spafas chick fibroblasts	0.010 ± 0.001
RSV-infected chick fibroblasts (72 hr p.i.)	0.010 ± 0.001
Calf thymus	0.004 ± 0.001
Salmon sperm	0.008 ± 0.001
Adenovirus 2	0.005 ± 0.002
Adenovirus 12	0.06 ± 0.01

All figures are the average of 2 or 4 determinations using different preparations of labeled RNA and DNA. Filters contained various amounts of DNA, and saturation value was standardized to per 100 μg of DNA. In *single* experiments values for hamster liver and clone 2 cells both were 0.003.





(*Left*) FIG. 6.—Saturation curve of ^{32}P RSV-RNA hybridized with chick fibroblast DNA. Filters containing DNA from uninfected chick fibroblasts contained an average of 76 μg of DNA and those of Rous sarcoma virus-infected cells (72 hr post infection) contained an average of 51 μg of DNA. Two DNA filters were incubated with various amounts of ^{32}P RSV-RNA (1.2×10^5 cpm/μg) and 100 μg of chick ribosomal RNA per ml in a vial. Each point represents the average of counts from two filters.

(*Right*) FIG. 7.—Sucrose density gradient centrifugation of RSV-RNA hybridized with calf thymus DNA. Hybrids between ^{32}P RSV-RNA and calf thymus DNA were fractionated by CsCl centrifugation. A pattern comparable to Fig. 4*A* was obtained and radioactive fractions were pooled. After adding ^3H-labeled chick sRNA as carrier, the hybrid was precipitated with ethanol and dissolved in 0.1 × SSC. The solution was heated at 98°C for 2 min and then rapidly chilled in an ice bath. The salt concentration was increased to 4 × SSC and the solution was filtered through two Millipore filters. The filtrate was centrifuged on a sucrose gradient of 5 to 20% at 36,000 rpm for 20 hr at 8°C.

are: with chick fibroblasts, 73°C; salmon sperm, 75°C; calf thymus, 74°C; Chinese hamster cells, 72°C; adenovirus 12, 72°C.

The molecular size of RSV-RNA which hybridizes with calf thymus DNA was studied by sucrose density gradient centrifugation using the sRNA of chick cells as a marker. It is about 3*S*, as compared with the 4*S* peak of sRNA (Fig. 7).

Discussion.—The results presented herein clearly demonstrate that RSV-RNA hybridizes not only with DNA from RSV-infected cells, but also with DNA's from normal cells from a variety of animal, and even plant, sources. We wish to emphasize that we find no significant difference in hybridization with DNA from Rous sarcoma virus-infected and uninfected chick fibroblasts.

Temin[26] reported the existence of DNA complementary to RSV-RNA in both Rous sarcoma virus-infected and uninfected chick cells. Although the difference

TABLE 2. *Base composition of RSV-RNA hybridized with various DNA's in the presence of chick embryo ribosomal RNA.*

Source of DNA	A	U	G	C
Spafas chick fibroblasts	30.7	11.9	38.6	18.8
Calf thymus	30.1	10.2	41.0	18.7
Salmon sperm	29.6	11.0	40.6	18.8
Adenovirus 12	30.3	10.7	39.8	19.2
64S RSV-RNA[25]	25.1	22.4	28.3	24.2
3-4S Heat degraded RSV-RNA	21.7	19.6	33.1	25.6

About 200 μg of DNA (50 μg for adeno 12) were hybridized with ^{32}P RSV-RNA in the presence of 300 μg of chick embryo ribosomal RNA. The hybrids were fractionated by CsCl centrifugation and the radioactive peaks which are comparable to those in Figs. 1 and 4 were pooled. Hybridized RSV-RNA was precipitated with trichloroacetic acid, 2 mg of yeast RNA being added as carrier, and hydrolyzed with 0.3 *N* KOH for 18 hr at 37°C. The resulting nucleotides were separated by two-dimensional paper chromatography. Figures are the average of 3 analyses, except for adeno 12 DNA for which 2 analyses were made.

TABLE 3. *Release of ^{32}P RSV-RNA from hybrids with HeLa cell DNA at increasing temperatures.*

Temperature (°C)	Cpm	Counts as percentage of total released
50	50	0.3
60	60	0.3
65	240	1.3
70	4840	25.4
75	9684	50.9
80	3520	18.5
85	637	3.3
90	20	0.1
100	10	0.1

Three HeLa cell DNA filters were incubated with 3 μg of ^{32}PRSV-RNA (6×10^5 cpm/μg) and 100 μg of chick ribosomal RNA in 1 ml 4 × SSC at 62°C for 8 hr. After incubation, filters were washed with 4 × SSC and treated with 50 μg/ml of RNase at 37°C for 30 min. Filters were incubated with 0.5 mg/ml of pronase (pretreated at 37°C for 2 hr) at 37°C for 15 min, washed with 4 × SSC, dried, and counted. Filters were washed with $CHCl_3$, dried, and incubated in 2 ml of 4 × SSC for 10 min at increasing temperature from 30°C to 100°C. At each point, the solution containing released RNA was removed and the RNA precipitated with an equal volume of 10% trichloroacetic acid at 4°C, using 30 μg bovine serum albumin as carrier. The precipitate was trapped on a Millipore filter, air-dried, and counted. At the final point, 100°C, the DNA filters were incubated for 10 min in 5.0 ml of 0.2 *N* HCl, after which the optical density at 260 mμ was determined to be 1.320.

between infected and uninfected cells was very small because of the low radioactivity of the RNA used, the finding is often cited as evidence in favor of the existence of a DNA provirus. However, the finding of viral particles[27] and of actively replicating RSV-RNA[28] in nonproducing (NP) cell cultures makes it unnecessary to postulate a proviral DNA. Moreover, the provirus hypothesis requires a new kind of enzyme—an RNA-dependent DNA polymerase—to synthesize proviral DNA copied from RSV-RNA as a template. Furthermore, a proviral DNA or a RSV-RNA-dependent RNA polymerase is also required to make progeny RSV-RNA. Although the possibility that one proviral DNA molecule (about 2×10^7 daltons as double-stranded DNA) may exist in a Rous sarcoma virus-infected chick fibroblast nucleus (in which the DNA content is 2.4×10^{-12} gm)[29] remains, there is considerable doubt whether it can be demonstrated by these methods.

We have already remarked that the molecular size of the RSV-RNA hybridizing with the several DNA's is 3*S* and that its base composition is characterized by high G and A compared to intact RSV-RNA. The question may be raised concerning the meaning of the 3*S* value. It is true that, as we have shown, RSV-RNA is degraded under conditions of hybridization to 3 to 4*S* fragments.[19] However, the base composition of the 3*S* moiety in the hybrids under discussion clearly differs from that observed under conditions of degradation (Table 2).

These findings are, on the whole, consistent with those reported by others. Kubinski and Rose[30] reported that the DNA's of oncogenic adenoviruses and SV40 have more dA:dT-rich sequences than those of nononcogenic adenoviruses. Moreover, DNA's from oncogenic viruses are found to be integrated in the host cell genome after infection.[13, 31, 32] In addition, RNA's synthesized *in vitro* with DNA's of SV40[31, 33] and polyoma[31, 34] as templates hybridize with the DNA's of normal cells to an extent which cannot be excluded simply as a nonspecific one.

What is the significance of these common DNA sequences with which RSV-

RNA interacts? Our recent experiments indicate that the cellular DNA hybridizing with RSV-RNA may be part of a membrane-associated, lipoprotein-nucleic acid complex, i.e., it may somehow be associated with the nuclear membrane.[35] The characterization of DNA isolated from membrane-associated chromatin, especially in relation to cellular DNA synthesis, is in progress.

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