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*HOMOLOGY BETWEEN RNA FROM ROUS SARCOMA VIRUS AND DNA FROM ROUS SARCOMA VIRUS-INFECTED CELLS**

BY HOWARD M. TEMIN

MCARDLE MEMORIAL LABORATORY, UNIVERSITY OF WISCONSIN

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Recently, it has been suggested[1-4] that the provirus of Rous sarcoma virus-infected cells is composed of DNA and that therefore the life cycle of Rous sarcoma virus (RSV) involves transfer of information from the RNA of the genome of the infecting virus to DNA of the provirus and from the DNA of the provirus to RNA of the progeny virus. This hypothesis is based mainly upon the results of experiments with inhibitors of nucleic acid synthesis.

The isolated virions of RSV contain RNA as their sole nucleic acid component.[5-7] The role of RNA as the genetic material of RSV is supported by the recent report of the isolation from Rous sarcoma cells of an infectious nucleic acid which is sensitive to destruction by RNase and not by DNase.[8] In further support of this role of RNA is the observation that stoppage of DNA synthesis in Rous sarcoma cells by FUDR or by amethopterin does not prevent production of new RSV.[3, 9-11]

However, prevention of DNA synthesis in cells soon after they have been exposed to RSV does prevent infection and initiation of virus production.[3, 4, 12, 13] This inhibition, by amethopterin, FUDR, BUDR, or cytosine arabanoside, is specifically prevented by the presence in the medium of the appropriate deoxyribonucleoside. Therefore, although the virion of RSV does not contain DNA and DNA synthesis is not needed for virus production after it has begun, DNA synthesis is needed for the initiation of virus production (establishment of the provirus).

Treatment of virus-producing Rous sarcoma cells with actinomycin D prevents production of infectious RSV[1, 11, 14, 15] and of virus nucleic acid.[3] Since actinomycin D prevents DNA-directed RNA synthesis,[16] this result is in agreement with the hypothesis that the provirus of RSV is DNA.

This hypothesis also most simply explains the other results and leads to the prediction that Rous sarcoma virus-infected cells contain some DNA not found in parallel uninfected cells, and that this new DNA should be homologous with RNA from RSV.

In this paper experiments are presented which confirm this prediction by showing that labeled RNA from RSV has homology with DNA from RSV-infected cells, but not with DNA from parallel uninfected cells. Two different techniques for confirming the presence of homology have been used. The first depends upon the fact that a specific RNA-DNA complex is resistant to degradation by RNase[17, 18] and can be trapped on a nitrocellulose filter.[19, 20] The second depends upon the fact

that a specific RNA-DNA complex is not dissociated at 60°C in 0.3 *M* salt, but is dissociated at 75°C in 0.0015 *M* salt.[21] Both methods have been extensively used by others.

Materials and Methods.—General: The virological and tissue culture techniques used in this work have been previously described in detail.[22] The strain of RSV used was isolated from the standard strain of Bryan.

Isolation of DNA: Secondary cultures containing 1.5×10^6 chick fibroblast cells were made in 100-mm Petri dishes. Half of the cultures were infected with *morph*^{r} RSV at a multiplicity of infection of about 1. All cultures were then overlaid with agar. After incubation for about 8 days, the cells in each culture were transferred to two 100-mm Petri dishes and were grown in fluid medium for 2–4 days. The cells were then trypsinized and frozen in dimethyl sulfoxide.[23]

About 2×10^8 uninfected cells and 2×10^8 parallel RSV-infected cells were thawed, centrifuged out of the dimethyl sulfoxide-containing medium, and suspended in a medium containing 80% glycerol.[24] The cells in glycerol were left in the freezer overnight. They were then disrupted with five strokes of a loose-fitting Dounce homogenizer, and the isolated nuclei were collected by centrifugation. The packed nuclei, in a volume of about 1 ml, were suspended in 6 ml of 0.15 *M* NaCl, 0.02 *M* Na citrate, pH 7 (SSC).[25] One ml of 10% dupanol and 2 ml of 5 *M* NaCl were added, and the solutions were mixed by rocking to avoid shearing. Ten ml of a mixture of 3:1 chloroform:butanol was added, and protein was extracted by rocking for 30 min at room temperature. The mixture was centrifuged, and the aqueous layer was mixed with two volumes of absolute ethanol. The DNA was collected on a glass rod and dissolved in 3.4 ml of SSC/10. Two-tenths milliliter of 10% dupanol and 0.4 ml of 10 × SSC were added, and more protein was removed by three 10-min extractions with equal volumes of SSC-saturated phenol. The DNA was precipitated again with ethanol, collected on a glass rod, dissolved in 2 ml of SSC/100, and treated at 37°C for 30 min with 50 μg of pancreatic RNase (Worthington), which had been placed in a boiling waterbath for 5 min to inactivate DNase. In this low salt concentration any double-stranded RNA would be degraded by the RNase.[26] Three-tenths milliliter of 10 × SSC was added, and the RNase was removed by three more phenol extractions. The phenol was removed by extraction with ether. The DNA was reprecipitated with ethanol, and dissolved in 2 ml of SSC/100. When specified, the DNA was denatured by placing in a boiling waterbath for 5 min.

The amount of DNA was determined by measurement of optical density. The ratio of optical densities at 260 and 280 mμ for the native DNA was about 2. The DNA, either native or denatured, was found at the bottom of the tube after centrifuging on a 3-ml gradient of 5–30% sucrose in 0.15 *M* NaCl[27] at 28,000 rpm for 5 hr in the SW39 rotor of the Spinco Model L ultracentrifuge.

Preparation of labeled virus nucleic acid: To each of four confluent cultures of Rous cells in 100-mm Petri dishes, 50 μc of tritiated uridine, 3 curies/mmole (Volk Chemical Co.), was added in 6 ml of medium. All particles of viral size had been removed from the serum in the medium by centrifugation for 1 hr at 30,000 rpm in the 30 rotor of the Spinco Model L ultracentrifuge. The serum also had been dialyzed against isotonic Tris buffer to remove uridine. After 19 hr, the medium containing the tritiated uridine was removed, and fresh medium, made with centrifuged serum that had not been dialyzed, was added. This medium was harvested 10 and 29 hr later, combined with the 19-hr supernatant, and filtered through a membrane filter (porosity 0.3 μ). The virus was concentrated from the filtrate by centrifuging for 20 min at 35,000 rpm in the SW39 rotor of the Spinco Model L ultracentrifuge on 2 ml of an 11–33% exponential gradient of potassium tartrate in 0.02 *M* Na citrate. The virus-containing band of light-scattering material in the center of the gradient was removed with a pipette and dialyzed for 4 hr against isotonic Tris buffer. The virus was rebanded in a potassium tartrate gradient, and the band of light-scattering material was collected by dripping from the bottom of the tube. The band was diluted with water and the virus was spun down (35,000 rpm for 35 min in the SW39 rotor) in a siliconized centrifuge tube. Control experiments mixing labeled material from uninfected cells with unlabeled virus and then purifying the virus have shown that 85% of the label in this material is in viral RNA.[3]

The labeled virus was suspended in 2 ml of SSC with bentonite. It was shaken with ether for 5 min at room temperature. The upper layer was discarded and the ether removed from the aqueous phase by flushing the tube with nitrogen. The nucleic acid was extracted by shaking at

60°C two times with phenol saturated with SSC. The phenol was removed by shaking with ether. The ether was removed by flushing with nitrogen. In one experiment (Table 3C) the RNA was passed over Sephadex G-200 before use.

The RNA extracted in this way formed a very broad band in sucrose gradient centrifugation, which clearly indicates that the RNA was partially degraded. Assuming the specific activity of the viral RNA was the same as that of cell RNA, the specific activity of the viral RNA would be about 5000 cpm/μg.

Preparation of labeled cell RNA: Confluent cultures of Rous sarcoma cells were labeled for 19 hr with 30 μc tritiated uridine. The cells were harvested 24 hr after the medium containing the tritiated uridine was removed. The RNA was extracted with dupanol and hot phenol as has been described previously.[3] It had a specific activity of about 3000 cpm/μg. It is mainly ribosomal and soluble RNA, although label continues to appear in virus over 24 hr after the removal of label from the supernatant medium.

Labeled bacterial RNA: RNA from CR-34 Thy$^-$, Leu$^-$, Threo$^-$ *E. coli* was kindly supplied by Dr. William Sly, Department of Biochemistry, University of Wisconsin. This RNA was isolated by extraction with phenol at 60°C from a culture of *E. coli* following a two-min pulse with H^3-uridine. It had a specific activity of about 3100 cpm/μg.

Techniques for recognizing specific hybridization: (a) *Nygaard-Hall technique:*[28] Known quantities of denatured DNA and labeled RNA were suspended in 0.6–1.2 ml of 0.5 *M* NaCl, 0.02 *M* Na citrate, 0.01 *M* Tris, pH 7.2, and 0.001 *M* EDTA in screw-capped vials and incubated at 60°C. After 24 or 48 hr, the mixture was diluted to 10 ml with the same buffer, and 50 μg of RNase was added. The RNase was allowed to act for 15 min at 37°C. The entire mixture was then passed through a membrane filter (27-mm B-6 filter of Schleicher and Schuell Co.) which had previously been soaked overnight in buffer. The filter was then washed with four 10-ml portions of buffer, dried, and counted in a liquid scintillation counter, usually for 200 min.

(b) *Bolton-McCarthy technique:*[21] One mg of denatured DNA in 1 ml of SSC/100 was mixed in a boiling waterbath with 1 ml of 6% agar (Oxoid Agar—Agar #3) and pipetted to a beaker in an ice bath. The DNA-agar was then pushed through a wire screen into 150 ml of 2 × SSC at 60°C. The large granules were allowed to settle, and the fine granules were removed. This process was repeated twice more. The granules were then washed on a Buchner funnel with 500 ml of 2 × SSC at 60°C. They were partially dried, and placed in screw-capped vials. RNA in 2 × SSC was added, and the vials were incubated at 60°C for 16 hr. The agar-DNA was then transferred to a jacketed column and washed at 60°C with ten 5-ml portions of 2 × SSC. This washing took about 90 min. The temperature of the column was then raised to 75°C, and the agar-DNA was washed with five 5-ml portions of 0.01 SSC. To each of the fifteen fractions was added 250 μg RNA and 1 ml cold 60% PCA. The precipitate was collected on a membrane filter, dried, and counted in a liquid scintillation counter.

The amount of DNA in the agar was determined by adding 0.1 gm agar-DNA to 0.9 ml 5 *M* $NaClO_4$, heating at 60°C until all the agar dissolved, and measuring the optical density at 260 mμ. Agar without DNA served as a blank.

Results.—In this work, an attempt was made to show whether or not RSV-infected cells contain a new DNA homologous to viral RNA. To establish this, labeled viral RNA was annealed with DNA from RSV-infected cells and with DNA from parallel uninfected cells and checked for formation of RNA-DNA hybrids. Two different methods of analysis, that of Nygaard and Hall, and that of Bolton and McCarthy, were used.

Hybridization by the Nygaard-Hall technique: Various concentrations of denatured DNA from parallel cultures of RSV-infected and uninfected cells were annealed with 990 cpm of viral RNA in a volume of 0.6 ml. After 24 hr the samples were treated with RNase and filtered. In Figure 1 is plotted the per cent of the added counts which were trapped on the filter.

At every concentration of DNA the amount of RNA retained on the filter was higher when DNA from RSV-infected cells was used for hybridization. In

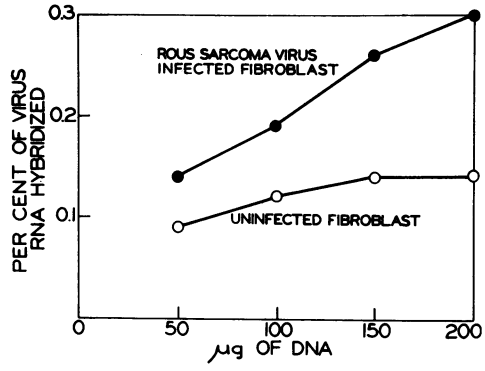

FIG. 1.—Hybridization of viral RNA with DNA from RSV-infected and uninfected cells (Nygaard-Hall technique). The precision of counting in this and subsequent experiments is 0.02% of the cpm added.

addition, when the amount of DNA was increased, an increase in the amount of RNA retained on the filter was observed with DNA from RSV-infected cells, but not with DNA from parallel uninfected cells. The amount of RNA specifically hybridized with the DNA from RSV-infected cells (the amount retained on the filter with the DNA from RSV-infected cells minus the amount retained on the filter with the DNA from uninfected cells) was directly proportional to DNA concentration, about 0.04 per cent per 50 μg.

The amount of hybridization was also proportional to the amount of RNA used. One hundred micrograms of denatured DNA from RSV-infected or parallel uninfected cells was annealed with 990 or 1980 cpm of viral RNA in a volume of 1.1 or 1.2 ml. After annealing, the samples were treated with RNase and filtered. The amount of RNA retained on the filter doubled as the amount of RNA added was doubled (Table 1). At both concentrations of RNA the per cent RNA retained on the filter with the DNA from RSV-infected cells was two to three times the per cent RNA retained on the filter with the DNA from parallel uninfected cells.

TABLE 1

HYBRIDIZATION OF VIRAL RNA WITH DNA FROM RSV-INFECTED AND UNINFECTED CELLS (NYGAARD-HALL TECHNIQUE)

Viral RNA added (cpm)	Per cent viral RNA hybridized with 100 μg DNA from: Uninfected cells	RSV-infected cells
990 (0.2 μg)	0.07	0.18
990 (0.2 μg)	ND	0.27
1980 (0.4 μg)	0.07	0.19
1980 (0.4 μg)	ND	0.19

The third experiment was annealed for 48 hr, the rest for 24 hr.

TABLE 2

HYBRIDIZATION OF NONVIRAL RNA WITH DNA FROM RSV-INFECTED AND UNINFECTED CELLS (NYGAARD-HALL TECHNIQUE)

RNA Type	RNA Amount	Per cent RNA hybridized with 100 μg DNA from: Uninfected cells	RSV-infected cells
Coli	1 μg	0.04	0.05
RSV-infected cell	2 μg	0.08	0.08
RSV-infected cell	2 μg	0.08	0.07

In the experiments presented in Figure 1 and Table 1, a consistent difference was found between the per cent hybridization of viral RNA with DNA from RSV-infected cells and with that from parallel uninfected cells. In order to show further that this difference was specific, 100 μg of DNA from RSV-infected cells and from parallel uninfected cells was annealed with RNA from *E. coli* or from RSV-infected cells. As seen from the data in Table 2, with these RNA's there was the same amount of hybridization with both types of DNA.

Hybridization by the Bolton-McCarthy technique: The data presented above show that annealing viral RNA with DNA from RSV-infected cells leads to formation of a larger amount of RNase-resistant material than annealing the same amount of viral RNA with DNA from uninfected cells. Experiments were also carried out

to see if annealing viral RNA with DNA from RSV-infected cells leads to formation of a larger amount of material stable to dissociation at 60°C in 0.3 *M* salt than does annealing the viral RNA with DNA from uninfected cells. Viral RNA was annealed with DNA, contained in agar granules, from RSV-infected or from parallel uninfected cells. (There was 15–25% more DNA from the uninfected cells in the granules.) The agar-DNA was then washed with ten 5-ml portions of 2 × SSC at 60°C, and five 5-ml portions of 0.01 SSC at 75°C. The amount of radioactivity in each fraction was determined. Fractions 13–15 were considered background, and an average of their cpm was subtracted from fractions 1 to 12. The total amount of radioactivity in fractions 1 to 12 was called 100, and the amount of radioactivity in each fraction was expressed as a per cent of this total.

TABLE 3

HYBRIDIZATION OF VIRAL RNA WITH DNA FROM RSV-INFECTED AND UNINFECTED CELLS (BOLTON-MCCARTHY TECHNIQUE)

Expt.	DNA from	Fraction Number 6	7	8	9	10	11	12
A	Uninfected cells	−0.2*	−0.3	0.8	0.3	1.9	−0.8	0.3
A	RSV-infected cells	1.7	0.8	1.0	0.2	1.2	2.6	0.3
B	Uninfected cells	1.1	0.6	1.0	0.9	1.2	0.6	0.1
B	RSV-infected cells	2.1	0.3	0.3	−0.2	0.3	3.3	0.8
C	Uninfected cells	1.3	0.9	0.7	0.3	0.4	0.1	−0.2
C	RSV-infected cells	1.1	0.6	0.3	0.3	0.4	0.6	0.0

* Per cent of counts found.

In Table 3 are presented data from three such experiments. In each case, the per cent of the counts appearing in fractions 6–12 are presented. The counts in fractions 11 and 12 represent the specifically bound RNA. In all cases, there was a decrease in the amount of RNA in fractions 11 plus 12 from experiments using DNA from uninfected cells compared with the level found in fractions 8, 9, and 10.

When RNA from RSV-infected cells was annealed with DNA from RSV-infected and uninfected cells, a result similar to that shown in Table 3 was found (Table 4). In both experiments, there was an increase in the amount of RNA in fractions 11

TABLE 4

HYBRIDIZATION OF RSV-INFECTED CELL RNA WITH DNA FROM RSV-INFECTED AND UNINFECTED CELLS (BOLTON-MCCARTHY TECHNIQUE)

Expt.	DNA from	Fraction Number 6	7	8	9	10	11	12
A	Uninfected cells	0.7*	0.5	0.6	0.4	0.6	0.2	0.1
B	Uninfected cells	0.6	0.7	0.6	0.2	0.5	0.4	0.0
A	RSV-infected cells	0.3	0.2	0.2	0.2	0.2	3.8	2.5
B	RSV-infected cells	1.0	0.1	0.3	0.2	0.2	0.9	0.0

* Per cent of counts found.

plus 12 compared with the level found in fractions 8, 9, and 10 from experiments using DNA from RSV-infected cells but not when using DNA from uninfected cells. This result is different from that with the Nygaard-Hall technique.

However, the difference between the hybridization with DNA from RSV-infected cells and from parallel uninfected cells is specific. If the two types of DNA are annealed with RNA from bacteria, no hybridization is found (Table 5).

Discussion.—In this work, an attempt was made to find a DNA, homologous to viral RNA, present in RSV-infected cells which is not present in uninfected cells.

TABLE 5

HYBRIDIZATION OF RNA FROM *E. coli* WITH DNA FROM RSV-INFECTED AND UNINFECTED CELLS (BOLTON-MCCARTHY TECHNIQUE)

	Fraction Number						
DNA from	6	7	8	9	10	11	12
Uninfected cells	1.3*	1.0	0.8	0.5	0.6	0.01	0.02
RSV-infected cells	1.1	0.6	0.5	0.3	0.5	0.02	−0.05

* Per cent of counts found.

Since this new DNA would be expected to be only a very minute fraction of the total cellular DNA, great care was taken to carry out all operations in parallel on equivalent samples of material from RSV-infected and uninfected cells.

The results using two independent methods of analysis show a consistent difference between hybridization of viral RNA with DNA from RSV-infected cells and with DNA from parallel uninfected cells. This result is most simply explained by the hypothesis that the DNA of RSV-infected cells has a region of new DNA, formed at infection, homologous to viral RNA.

Under these conditions this new DNA does not hybridize with nonhomologous RNA. The increased hybridization of RNA from RSV-infected cells to the DNA from RSV-infected cells seen with the Bolton-McCarthy technique was probably due to viral RNA found in these virus-producing cells.

The failure to find this increased binding with the Nygaard-Hall technique is probably due to a difference between the two techniques. In the Nygaard-Hall technique, the DNA is not prevented from aggregating or renaturing. Therefore, the hybridization with RNA stops. In the Bolton-McCarthy technique, the DNA is trapped in agar so that it cannot aggregate or renature. Therefore, in Nygaard-Hall technique, but not in the Bolton-McCarthy technique, the presence of nonspecific binding due to ribosomal RNA could prevent the occurrence of specific binding by viral RNA before the DNA aggregated or renatured.

Therefore, it seems that the simplest hypothesis to explain these data is that the provirus of Rous sarcoma is a region of DNA homologous with viral RNA stably integrated into the molecules of cellular DNA in the nucleus. It would then be similar to the prophage of lambda.[20, 29, 30] This similarity could then be the basis for the previously observed similarities between the behavior of RSV and temperate phages.[22, 31–37]

The existence of a DNA provirus for RSV, an RNA virus, would suggest that the formation of a DNA provirus may be a necessary step in viral carcinogenesis. The hypothesis of information transfer from RNA to DNA would suggest that this mechanism should be looked for in other cases of somatic information storage, e.g., differentiation, antibody synthesis, and memory.

Summary.—Chick embryo cells infected with Rous sarcoma virus, an RNA virus, have been shown to contain some new DNA homologous to the viral RNA. This new DNA was not homologous to unrelated RNA. The techniques of hybridization of Nygaard and Hall and of Bolton and McCarthy were both used to demonstrate the presence of this new DNA.

Abbreviations: BUDR, 5-bromodeoxyuridine; cpm, counts per minute; EDTA, disodium ethylene diamine tetraacetate; FUDR, 5-fluorodeoxyuridine; ND, not determined; PCA, perchloric acid; RSV, Rous sarcoma virus; SSC, 0.15 *M* NaCl, 0.02 *M* Na citrate, pH 7.

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