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Restricted Replication of Vesicular Stomatitis Virus in Human Lymphoblastoid Cells

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Replication of vesicular stomatitis virus (VSV) is restricted in one human lymphoblastoid cell line (Raji), but not in another similar cell line (Wil-2), compared with growth in HeLa cells. This restriction is characterized by a low proportion of cells yielding infectious virus and is associated with limited production of 42*S* virion RNA. Primary transcription of 13*S* and 26*S* VSV-specific RNA is not restricted in Raji cells, and the 13*S* RNA produced contains adenylate-rich sequences. This suggests that the block in Raji cells involves some step required for the replication of virion RNA.

Numerous examples of restriction of virus replication in animal cells have been described (3, 4, 7, 11, 13-15). In some cases the nonpermissive state can be attributed to lack of cell surface receptors or inability to uncoat the virion (4, 11), whereas in most the precise molecular basis of the restriction remains unclear. Among primary cells or tissues, lymphocytes are of particular interest since resting lymphocytes are nonpermissive for several RNA and DNA viruses, whereas those activated by antigens or mitogens acquire the capacity to support virus replication (1, 6, 10, 12, 16). The molecular basis for the restriction of virus replication in lymphocytes and the enhancement of virus replication upon activation of the cells is unknown. Because only a small proportion (usually fewer than 10%) of primary lymphocytes can be activated to support virus replication by most agents, we have chosen to study virus replication in human lymphoblastoid cell lines as a possible model system for the nonpermissiveness of primary lymphocytes to virus infection. We have screened several lymphoid cell lines for susceptibility to infection by vesicular stomatitis virus (VSV), poliovirus, and Newcastle disease virus. We have selected both a cell line permissive for VSV replication, Wil-2, derived from the spleen of a donor with no lymphoproliferative disease (8), and a second, nonpermissive or restrictive for VSV replication, the Raji cell line, derived from a patient with Burkitt's lymphoma (3).

VSV, a membrane-maturing virus of the rhabdovirus group, was chosen for study of restriction of virus replication in lymphoid cells because it has a wide host range, its replication is restricted in the Raji cell line, and because some stages of its replicative cycle can be defined and studied independently. The results of the present study indicate that the restriction of VSV replication in Raji cells involves a block in the replication of virion RNA.

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MATERIALS AND METHODS

Cells and virus. HeLa cells (S3 strain) and mouse L cells were grown in Spinner culture in Joklik modified Eagle medium (MEM) containing 5% fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and glutamine (2 mM). Human lymphoblastoid cell lines, Wil-2 and Raji, were maintained in suspension cultures in Dulbecco modified Eagle medium (DME) supplemented with penicillin, streptomycin, glutamine, and 10% fetal calf serum. The stock of VSV, Indiana strain, was prepared in secondary chicken embryo cells as described (6). The titer was 10^9 to 3×10^9 PFU/ml.

Infectious centers assay. The efficiency of VSV infection in various cell lines was determined in an infectious centers assay described elsewhere (6). Briefly, 2×10^6 cells were exposed to VSV at a multiplicity of infection (MOI) of 10 PFU/cell at 37 C. After various periods of time the cultures were treated with 0.02 ml of guinea pig anti-VSV serum, an amount sufficient to neutralize all input virus. The infected cells were washed three times in MEM, and duplicate 0.2-ml samples of 10-fold dilutions were plated in medium containing 1% agar (Ionagar No. 2, Oxoid, Colab Laboratories, Inc., Chicago Heights,

Ill.) above a monolayer of L cells. Plaques were scored 48 h later, after the plates had been stained with neutral red (0.1 mg/ml in phosphate-buffered saline).

Radiolabeling of VSV-specific RNA. Cells (3×10^6/ml) were infected at an MOI of 10 PFU/cell. One hour after addition of VSV, actinomycin D (gift of Merck, Sharp & Dohme, Rahway, N.J.) was added to 4 μg/ml, and in some cases cycloheximide (Calbiochem) was also included at a final concentration of 100 μg/ml. Incubation at 37 C was continued for another hour, and ^{3}H-uridine (28 Ci/mmol, New England Nuclear Corp.) was added to a final concentration of 10 μCi/ml. At various times thereafter duplicate 0.1-ml samples of infected cells were transferred into 2 ml of cold Earle salt solution, collected by centrifugation, and resuspended in 1 ml of distilled water, and 1 ml of 10% trichloroacetic acid was added. The precipitate was collected on Whatman GF/A glass fiber filters, dried, and 6 ml of scintillation fluid (2 volumes of toluene, 1 volume of Triton X-100, containing 4 g of PPO per liter, Nuclear Ass.) added. Radioactivity was determined in a Beckman scintillation counter. For determination of total (intra- and extracellular) radioactivity incorporated, samples of infected cell cultures were directly precipitated in 5% trichloroacetic acid and treated as above.

Analysis of VSV-specific RNA on sucrose gradient. For sucrose gradient analysis of VSV-specific RNA, 3×10^7 cells of each cell line (5×10^6 cells/ml) were infected and radiolabeled as above. At 7 h postinfection cells were washed in cold Earle salt solution, collected by centrifugation, and resuspended in 1.5 ml of RSB (0.01 Tris [pH 7.2], 0.01 M NaCl, 0.0015 M $MgCl_2$). The cells were allowed to swell for 15 min at 4 C and were disrupted in a stainless steel Dounce homogenizer; cell disruption was monitored by phase microscopy. Nuclei were removed by centrifugation (1 min at 2,000 rpm at 4 C) and the supernatant cytoplasmic extracts were collected. Sodium dodecyl sulfate (SDS) was added to 0.5%, and cytoplasmic extracts were layered on 37-ml 15 to 30% linear sucrose gradients prepared in NETS buffer (0.1 M NaCl, 0.001 M EDTA, 0.01 M Tris, pH 7.0, 0.1% SDS). The gradients were centrifuged at 20 C for 17 h at 22,000 rpm in a Spinco SW27 rotor. Fractions (approximately 1.3 ml) were collected through a Gilford recording spectrophotometer to monitor optical density (OD), with cellular ribosomal RNA (28S and 18S) serving as internal OD markers in each gradient. Acid-precipitable radioactivity in each fraction was determined by collecting the precipitates on Whatman GF/A glass fiber filters and assaying as above.

Identification of A-rich regions in VSV-specific 13S RNA. Cells (5×10^7) of each line were infected as above and radiolabeled with either ^{3}H-uridine (28 Ci/mmol, New England Nuclear Corp.) at 150 μCi/ml, or ^{3}H-adenosine (10 Ci/mmol, New England Nuclear Corp.) at 150 μCi/ml from 2 to 7 h postinfection, in the presence of 4 μg of actinomycin D per ml. At 7 h postinfection cytoplasmic extracts were prepared and centrifuged on sucrose gradients. Samples of 20 μliters of each fraction were assayed for acid-precipitable radioactivity, and fractions comprising the area of the 13S peak of virus-specific RNA were pooled. A 25-μg amount of carrier yeast RNA was added, the solution was brought to 0.2 M in NaCl, and the RNA was precipitated with 2.5 volumes of 95% ethanol at −70 C overnight. The pellets were collected by centrifugation and resuspended in 1 ml of buffer containing 0.01 M Tris (pH 7.2), 0.2 M NaCl, and 0.01 M EDTA. T1 ribonuclease (50 U) (Calbiochem) and 10 μg of pancreatic ribonuclease (Calbiochem or Worthington) was added, and the digestion was performed for 60 min at 37 C. The reaction was stopped by the addition of 0.5% SDS, 25 μg of carrier yeast RNA was added to each sample, and the RNA was reprecipitated with ethanol as above. The undigested radiolabeled RNA was analyzed by polyacrylamide gel electrophoresis as previously described (2) The electrophoresis was carried out for 18 h at 9 mA/gel at room temperature in 10%, 18-cm cylindrical acrylamide gels containing 0.1% SDS. The gels were crushed in a Savant automatic gel crusher, fractions were collected into scintillation vials, and radioactivity was determined in a Beckman LS200 scintillation counter.

Radioautography. Uninfected and infected cells, exposed to actinomycin D and ^{3}H-uridine, were centrifuged onto microscope slides in a cytocentrifuge (Shandon), fixed in methanol for 5 min, dried, and washed (3×10 min) in 5% perchloric acid to remove soluble radioactivity. Dry slides were dipped into a mixture of 2 volumes of NTB2 emulsion (Eastman Kodak Co., Rochester, N.Y.) and 1 volume of distilled water containing a drop of Tween 80, prewarmed to 45 C. The slides were dried for 1 h at room temperature in the dark, and exposed for 1 to 4 weeks in sealed plastic boxes at 4 C. After exposure, slides were developed with D19 Kodak developer (Eastman Kodak Co., Rochester, N.Y.) and stained for 20 min with 0.038% freshly prepared Giemsa stain (Fisher Scientific Co., Fair Lawn, N.J.) in a buffer consisting of 0.0085 M citric acid, 0.023 M sodium phosphate, and 3% absolute methyl alcohol, pH 5.75.

RESULTS

Efficiency of VSV infection in various cell lines. An infectious centers assay was used to determine the proportion of cells undergoing productive infection in a given population. We have shown previously that the data obtained in this assay correlate well with the enumeration of cells associated with mature virus particles by electron microscopy (12). Two human lymphoblastoid cell lines, Wil-2 and Raji, were shown to differ significantly in their ability to support replication of VSV. Table 1 shows that after a 2-h exposure to VSV, when 24% of HeLa cells were productively infected, approximately 8% of Wil-2 cells produced infectious centers. At the same time the proportion of Raji cells that yielded plaques was 10 times lower (0.82%). With longer exposure to the virus (24 h postinfection) 74% of Wil-2 cells became infected

productively (Table 1), and none remained viable at 48 h; in contrast, up to 80% of Raji cells remained viable for several days after initial exposure to VSV (data not shown), and only 15% of them produced infectious centers at 24 h after infection.

^{3}H-uridine incorporation in VSV infected cells. VSV-specific RNA synthesis in infected cells can be divided into two kinds: (i) primary transcription of multiple RNA species, complementary to virion RNA, resulting from the activity of virion-associated RNA polymerase, and (ii) secondary transcription and replication of virion RNA which occurs by a mechanism(s) not understood at present, but which requires virus-specific protein synthesis. Thus, primary transcription, which is the first detectable virus-specific synthetic activity in VSV-infected cells, can be studied in the absence of other viral replicative steps if the cells are treated with an inhibitor of protein synthesis (5, 9). Therefore, incorporation of ^{3}H-uridine by VSV-infected cells was examined in the presence of actinomycin D and cycloheximide, or in the presence of actinomycin D alone (Fig. 1). In VSV-infected HeLa and Wil-2 cells the incorporation of radioactivity was linear for at least 10 h in the presence of actinomycin D alone. In Raji cells, however, the time course of incorporation was different (Fig. 1).

TABLE 1. *Efficiency of VSV infection in continuous human cell lines*

Cell line	Infectious centers (%)[a]	
	2 h[b]	24 h
HeLa	24.30 ± 1.16	92.50[c]
Wil-2	7.92 ± 1.48	74.00
Raji	0.82 ± 0.07	15.00

[a] Cells were exposed to VSV at an MOI of 10 PFU/cell and tested for replication of the virus in an infectious centers assay as described in Materials and Methods. The results are expressed as % infectious centers = no. of plaques per plate × 5 × dilution divided by no. of cells per ml × 100.

[b] Results based on 11 separate experiments.

[c] HeLa cells were assayed at 7 h because at later times cell viability decreased.

In the presence of actinomycin D and cycloheximide the time course of uridine incorporation was very similar for all three cell lines. Table 2 shows the ratios of total trichloroacetic acid-insoluble radioactivity incorporated by each cell line at 16 h postinfection in the presence of actinomycin D to that incorporated in the presence of actinomycin D and cycloheximide. The amounts of radioactivity incorporated by uninfected control cultures (Table 2) show that there is essentially no difference in uptake or incorporation of ^{3}H-uridine among the different cell lines. Inhibition of protein synthesis reduced the incorporation of ^{3}H-uridine into VSV-specific RNA by HeLa and Wil-2 cells, presumably by preventing secondary transcription and replication, but had no effect on incorporation of radioactivity by VSV-infected Raji cells.

Analysis of VSV-specific RNAs by velocity sedimentation on sucrose gradients. Figure 2 shows a sucrose gradient analysis of VSV-specific RNA produced in the three cell lines

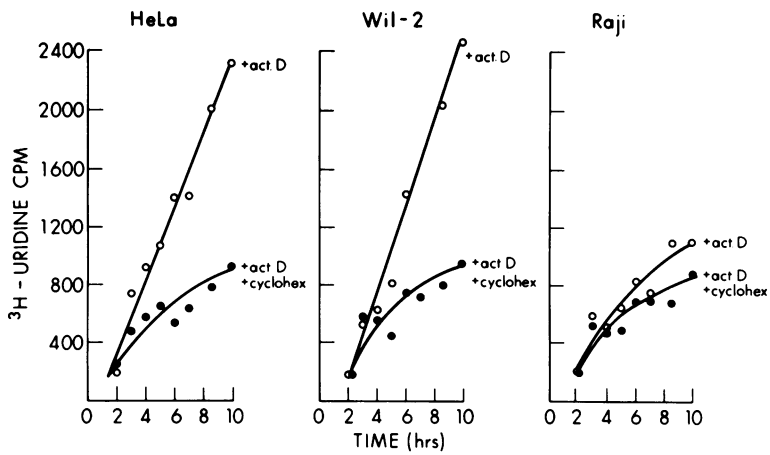

FIG. 1. *Incorporation of ^{3}H-uridine by VSV-infected cells. Virus at MOI of 10 PFU/cell was added at time 0; actinomycin D and cycloheximide were added at 1 h, and ^{3}H-uridine at 2 h postinfection (for concentrations, see Materials and Methods). At indicated times the radioactivity incorporated was determined as described.*

incubated with ^{3}H-uridine between 2 and 7 h postinfection in the presence of actinomycin D. The relative proportions of 26S and 13S VSV RNA are virtually identical in all three cell lines. In contrast, the relative amounts of 42S virion RNA are different in each cell line. Raji cells produce the lowest relative amount of virion RNA, consistent with their restricted capacity to produce infectious virus. Table 3 shows the efficiency of lymphoblastoid cell lines in the production of each class of VSV-specific RNA relative to HeLa cells. The accumulation of 42S virion RNA is consistent with the number of infectious centers obtained in each cell line (cf. Table 1).

The presence of considerable amounts of 13S and 26S VSV RNA in Raji cells and the incorporation of ^{3}H-uridine into VSV-specific RNA in the presence of actinomycin D and cycloheximide demonstrate that early stages of infection, i.e., adsorption, penetration, uncoating, and primary transcription, are not restricted.

Radioautography of VSV-infected cells. Since the level of ^{3}H-uridine incorporation into VSV-specific RNA in infected Raji cells was considerably higher than would be expected on the basis of the number of infectious centers, the question arose whether all cells of this line could produce some species of VSV-specific RNA but only a few could complete the infectious cycle, or alternatively, whether only a small proportion of Raji cells was able to undergo infection at all, and was responsible for the total incorporation of radioactivity observed. To study this directly, infected cells of each cell line were incubated with ^{3}H-uridine and the proportion of radiolabeled cells was determined by radioautography. Table 4 shows that while the fraction of labeled Raji cells appears somewhat lower than Wil-2 or HeLa cells (40% versus 64 and 82%, respectively), it is threefold higher than the proportion of virus producing cells found in this cell line (a maximum of 15%). In radioautographs of VSV-infected Raji cells generally fewer grains per cell

TABLE 2. *Total ^{3}H-uridine incorporation in VSV-infected cells after 16 h of infection*

Cell line	CPM[a]			VSV + AcD/ VSV + AcD + Cyclohex.
	Uninfected control	VSV + AcD	VSV + AcD + Cyclohex.	
HeLa	20,220	9,297	4,418	2.26 ± 0.46
Wil-2	18,559	8,644	5,123	1.90 ± 0.14
Raji	17,578	5,972	5,863	0.95 ± 0.11

[a] Acid-precipitable radioactivity; results shown here are averages based on six separate experiments.

TABLE 3. *Production of VSV-specific RNAs by lymphoblastoid cell lines*[a]

RNA	HeLa	Wil-2	Raji
13S	100	119.3	116.9
26S	100	124.3	108.0
42S	100	56.7	25.6

[a] Based on data shown in Fig. 2. The areas under each peak were integrated; the values for VSV-specific RNA produced in HeLa cells were taken as 100%, and the values for Wil-2 and Raji cell lines expressed as the percentage of HeLa cell values.

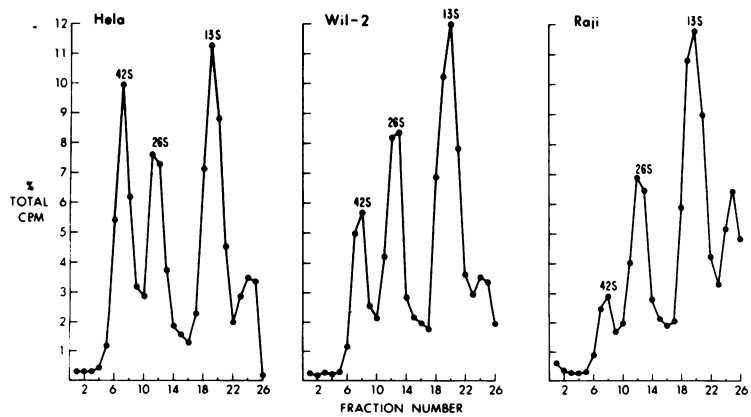

FIG. 2. *Sucrose gradient analysis of VSV-specific RNA produced in HeLa, Wil-2, and Raji cells. Cells were infected and labeled with ^{3}H-uridine, and cytoplasmic extracts were prepared and analyzed on 15 to 30% sucrose gradients as described in Materials and Methods. Total radioactivity per gradient was: 47,368 counts/min (HeLa), 40,754 counts/min (Wil-2), and 27,052 counts/min (Raji).*

TABLE 4. *Frequency of cells incorporating ^{3}H-uridine into VSV-specific RNA*[a]

Cell line	Total no. cells	No. labeled cells	Labeled cells (%)
HeLa	1,041	853	82
Wil-2	1,256	802	64
Raji	1,206	484	40

[a] Cells of each line were infected in the presence of actinomycin D, labeled with ^{3}H-uridine between 20 and 23 h postinfection, and subjected to radioautography as described. Only intact cells were counted and those containing more than 10 grains in the cytoplasm were scored as positive.

were observed than in corresponding samples of Wil-2 or HeLa cells, as would be expected if each cell was limited in its capacity to produce virus, and the number of grains in Raji cells often approached background values. Since only cells containing at least 10 grains in the cytoplasm were scored as positive, the value shown in Table 4 for Raji cells is a minimal estimate, and the actual proportion of radiolabeled cells of this line may be higher than 40%.

Identification of A-rich regions in 13S VSV RNA. The preceding experiments demonstrated that the production of 13*S* VSV RNA is not blocked during restricted infection which takes place in Raji cells. In spite of similar sedimentation properties, some differences may exist between 13*S* RNAs produced in different cell lines. One of the characteristic features of 13*S* VSV RNA produced during infection of HeLa cells is the presence of an adenylate-rich sequence (2). This property was used to compare 13*S* RNA made in VSV-infected Wil-2 and Raji cells. ^{3}H-uridine- or ^{3}H-adenosine-labeled VSV-specific RNA sedimenting at approximately 13*S* (cf. Fig. 2) was digested with pancreatic and T1 ribonuclease, and nuclease-resistant acid-insoluble material was subjected to polyacrylamide gel electrophoresis (Fig. 3). Since the radioactivity in uridine-labeled material was rendered completely acid soluble by pancreatic and T1 ribonuclease, removal of any possible contamination by double-stranded RNA by gel filtration (2) was unnecessary. Adenosine-labeled, ribonuclease-resistant material derived from VSV-specific 13*S* RNA produced in both Wil-2 and Raji cells migrated more slowly than the bromophenol blue dye marker (approximately 4*S*) on 10% polyacrylamide gels and was heterogeneous. Minor differences in the electrophoretic patterns of adenylate-rich segments of 13*S* VSV RNA from the two cell lines were observed (Fig. 3), but the significance of this difference is not clear. The results demonstrate, however, that in both cases A-rich oligo-nucleotides are present, as has been reported previously for permissive HeLa cells (2).

DISCUSSION

Cells of the Raji lymphoblastoid line restrict the growth of VSV. Results of experiments described here indicate that the block probably does not involve early stages of infection and seems to be associated with limited production of 42*S* virion RNA. Interferon is not responsible for this restriction because Raji cells do not produce interferon under normal growth conditions, as reported previously (17) and confirmed in this laboratory. In addition, Raji cells support certain virus-specific synthetic activities, e.g., transcription, which are inhibited by interferon (9). Both the results of infectious centers assays and electron microscope enumeration of Raji cells associated with mature virus particles at 24 h after infection (12) indicate that the maximal proportion of cells able to support VSV replication in this line is approximately 15%, as compared with 74 to 100% in permissive cell lines (Wil-2 or HeLa). Early stages of infection—adsorption, penetration, uncoating—seem unlikely as the primary block because at least 40% of Raji cells support VSV-specific RNA synthesis (Table 4) and because considerable amounts of 13*S* and 26*S* VSV specific RNAs were found in these cells after infection (Fig. 2). However, the relative amount of 42*S* virion RNA was depressed in these cells, in agreement with the infectious centers assay. Although selective degradation of 42*S* virion RNA does not seem to be a likely primary cause of restriction in Raji cells, further experiments are required to exclude this possibility. When the activity of input virion-associated RNA polymerase was examined by following the incorporation of ^{3}H-uridine into VSV-specific RNA in the presence of cycloheximide, essentially no differences were found between restrictive and permissive cells (Fig. 1). In addition, incorporation of ^{3}H-uridine into VSV-specific RNA did not change significantly in Raji cells in the presence or absence of cycloheximide; 13*S* VSV RNA can be easily identified in both restrictive and permissive cells. This suggests that mainly input virion-associated RNA polymerase may be active in these cells, and that further replication may occur to only very limited extent. When this species of virus-specific RNA was examined for the presence of adenylate-rich sequences, no major differences

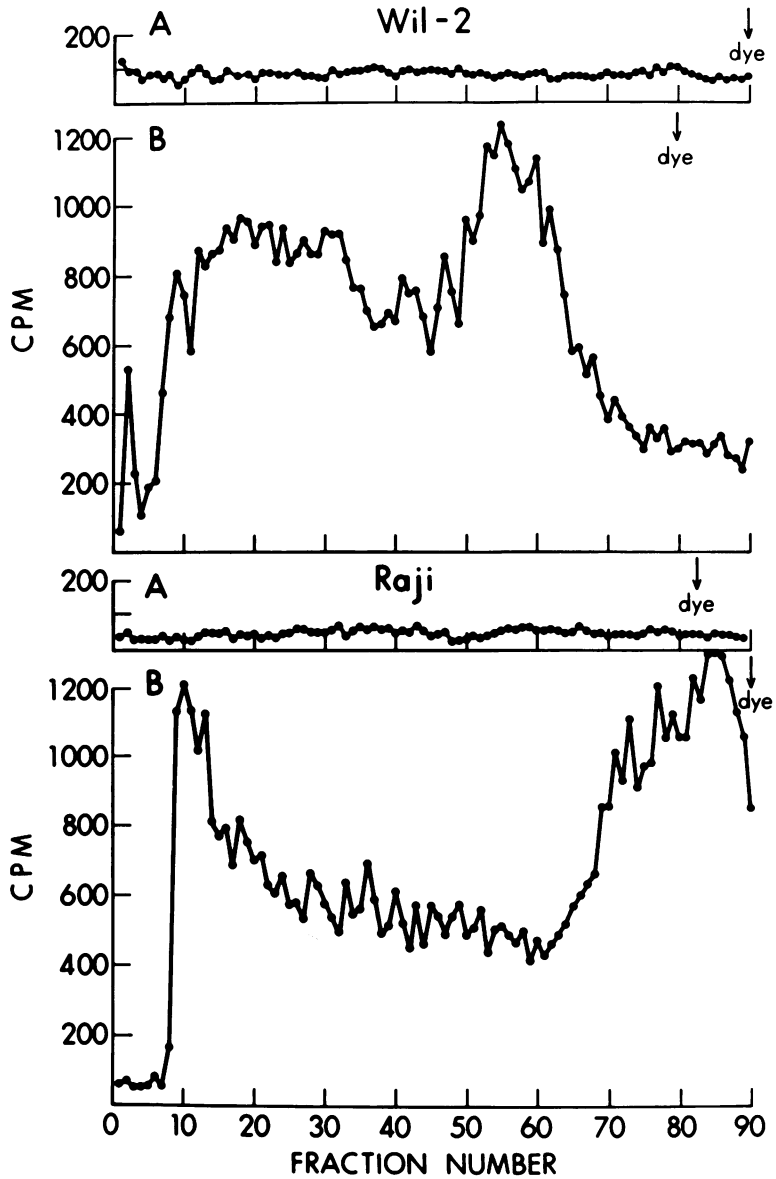

FIG. 3. *Polyacrylamide gel analysis of ribonuclease-resistant fraction of 13S VSV RNA isolated from Wil-2 and Raji cells. (A) ^{3}H-uridine labeled, (B) ^{3}H-adenosine-labeled 13S RNA after digestion with pancreatic and T_1 ribonuclease was subjected to electrophoresis on 10% polyacrylamide gels as described.*

were detected (Fig. 3). Experiments concerning VSV-specific protein synthesis in restrictive and permissive cells should determine whether 13S VSV RNA made in Raji cells is functional in translation. We are also examining other possible differences between restrictive and permissive cells.

Primary lymphocytes do not support replication of VSV, unless activated by antigens or mitogens (1, 6, 10, 12, 16). The nature of this restriction of virus replication is not known. The restriction of VSV replication observed in Raji cells may be similar in nature to the block in primary lymphocytes. VSV, as well as some other viruses, can remain latent or inactive in cultures of lymphocytes for several days under conditions in which free virus would be inactivated, and start to replicate upon stimulation of

the cells by mitogens like PHA or Con A (M. Nowakowski, unpublished data, and 1, 16). This suggests that unstimulated lymphocytes have receptors for VSV and that virus can attach or at least become associated with the cells in such a way as to be protected. The block in primary lymphocytes probably involves some stage of infection occurring after adsorption and penetration, and possibly uncoating.

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