

Primary In Vivo Transcription of Vesicular Stomatitis Virus and Temperature-Sensitive Mutants of Five Vesicular Stomatitis Virus Complementation Groups

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Received for publication 16 July 1973

The process of adsorption and RNA transcription by vesicular stomatitis virus (VSV) in cells has been followed by using highly labeled preparations of VSV virions. The initial transcription process (primary parental transcription) is rapid and takes about 4 min to develop complete transcripts of the input genome. The optimal cell temperature for in vivo transcription is between 36 and 39.5 C, although transcription can be detected at 18 C. No negative effect on primary parental transcription was obtained by the presence of actinomycin D, puromycin, or cycloheximide. Demonstrable primary transcription by selected temperature-sensitive mutants of all five complementation groups of VSV was obtained at either 31 and 34 C (permissive temperatures for virus production) or 39.5 C (nonpermissive temperature for virus development). VSV grown in hamster (BHK 21) or chicken embryo cells were more efficiently adsorbed and transcribed in BHK cells.

Vesicular stomatitis virus (VSV) is a bullet-shaped rhabdovirus which possesses an RNA-dependent RNA polymerase in its virion particle (1-6). The polymerase, during in vitro assays, has been found capable of transcribing the viral RNA repetitively, sequentially, and completely, synthesizing a series of RNA species which are complementary to, but smaller than, the viral RNA and which have distinct and distinguishable 5' terminal nucleotide sequences (1-6, 29, 30). In vivo it has been demonstrated that VSV-specific messenger RNA species are also smaller than the virion RNA as well as complementary to it (17, 24). Both lines of study indicate, therefore, that the function of the virion polymerase is to transcribe the viral RNA into multiple messenger RNA species, whose subsequent intracellular course is to direct the synthesis of viral proteins.

In this investigation we have investigated the intracellular parental transcription of wild-type VSV. The method we have chosen involves infecting cells with ³H-nucleoside labeled virus and thereafter determining the number of particles which adsorb per cell, the fate of the viral RNA, and the time-course of intracellular pa-

rental transcription. We have studied the optimal temperature for primary transcription and the effect, or lack of it, of various drugs upon the process.

We have also examined the transcription of virus in contact-inhibited cells as well as the effect of virus grown in BHK cells or chicken embryo fibroblast cells upon adsorption and transcription in cells of either type.

Temperature-sensitive mutants of VSV (Indiana strain) have been described by several investigators (8, 12-16, 20, 21, 25-27). Of the five known complementation groups, mutants in three (groups I, II, and IV) elicit less RNA synthesis in infected cells growing at high temperatures than at low temperatures, or by comparison to that exhibited by the wild-type virus (12-14, 16, 20, 23, 26-28, 32). This has led to the suggestion that their reduced RNA synthesis is consequent to temperature-sensitive transcriptases, transcriptional and/or replicative steps in cells growing at the nonpermissive temperatures and these mutants have been described therefore as RNA-negative types.

We have followed the parental transcription of selected mutants of all five complementation groups of VSV at both permissive and nonper-

missive temperatures and conclude that mutants of all five groups do synthesize RNA at high temperatures and their genomes are transcribed repetitively and probably completely.

MATERIALS AND METHODS

Preparation of virus stocks. Temperature-sensitive (ts) mutants of VSV (Indiana strain) were grown in confluent monolayers of BHK-21 cells at 34 or 31 C as described previously (1). The following Orsay mutants were investigated: group I-5, group II-52, group III-23, group IV-100, group V-45 (12-13), as well as the wild-type strain. Virus stocks, all containing less than 0.01% PFU revertants (except group II-52, which had 1% revertants), were verified by complementation tests and their inability to grow at 39.5 C in BHK-21 monolayers or chicken embryo fibroblasts as shown previously (12, 13, 15, 25). The wild-type virus grew as well at 39.5 or 31 C in either BHK or chicken cells.

Cells. Primary and secondary chicken embryo fibroblast (CEF) monolayer cultures were prepared from 12-day-old chicken embryos. Some CEF cultures were the gift of R. W. Simpson, Rutgers.

Preparation of virus highly labeled by ^3H -nucleosides. Virus was obtained from one infected T-flask (75 cm², 10 ml of Eagle medium [9], 9×10^6 cells, MOI of 0.1 PFU per cell), by incubation at 31 C (or 37 C for wild-type virus) for 48 h in the presence of 1 mCi of ^3H -uridine (21 Ci/mmol), 1 mCi of ^3H -guanosine (20 Ci/mmol), and 1 mCi of ^3H -adenosine (13 Ci/mmol). Occasionally, 1 mCi of ^3H -cytidine (20 Ci/mmol) was alternately employed in the growth media. After centrifugation, to remove cell debris virus was purified by precipitation with polyethylene glycol (PEG), followed by limited equilibrium gradient centrifugation (3 h at 4 C and 35,000 rpm) in a Spinco SW41 rotor as described previously (1, 6). The supernatant fluid above the visible virus band was removed and the virus was carefully collected by pipette. Under these conditions of virus growth, none of the virus stocks produced detectable amounts of defective virions as determined by two criteria: defective and complete virions would have given a double virus band on this limited equilibrium centrifugation; no defective virion RNA was detected in the final virus preparation when analyzed by polyacrylamide gel electrophoresis (4). To remove sucrose and concentrate virus, the virions were diluted threefold with 0.15 M NaCl, 0.01 M Tris-hydrochloride, pH 7.4, and centrifuged through a 1-ml cushion of 30% (wt/vol) sucrose in the same buffer. The virus pellet was then drained and suspended in 0.3 ml of cold Eagle medium and kept at 4 C. The usual amount of purified virus obtained by this method was about 0.45 mg of virus protein containing 8×10^6 counts per min per mg of protein and having a specific activity of 7×10^6 counts per min per mg of RNA. When the viral RNA was analyzed by polyacrylamide gel electrophoresis (3) approximately 94% of the label was present in VSV viral RNA.

Infection of cells by highly labeled virus. Confluent monolayers of BHK-21 cells in small T flasks (25 cm², 3×10^6 cells) were washed two times with cold Eagle medium (lacking serum) and, after draining,

chilled in an ice bath. A sample of purified virus, diluted in Eagle medium (100 μ liters, 3×10^6 counts/min), was added to the monolayer and incubated at 4 C for 30 min to permit virus adsorption. The flask was then washed successively with 2 volumes of 5 ml of cold (4 C) Eagle medium and then completely filled with prewarmed media and immediately incubated in a circulating water-bath whose temperature was regulated to within 0.1 C of the desired value. Incubation was continued at that temperature for the specified length of time.

Extraction of nucleic acids. RNA was extracted from purified virus as described previously (5). In order to extract nucleic acids from infected cells, the cell monolayer was drained, washed in Eagle medium, removed in 1 ml of 0.14 M NaCl, 0.003 M KCl, 0.01 M sodium phosphate buffer, 0.5 mM EDTA, pH 7.2, and adjusted to 1% sodium dodecyl sulfate (SDS). A 4-ml amount of 0.4 M NaCl, 0.01 M Tris-hydrochloride buffer, pH 7.4, and 0.1% SDS (buffer N) were added, followed by approximately 0.1 ml of diethylpyrocarbonate (as a ribonuclease inhibitor) and 5 ml of phenol-cresol mixture (500 g of redistilled phenol, 70 ml of redistilled m-cresol, 0.5 g of 8-hydroxyquinoline saturated with 200 ml of 0.15 M NaCl, 0.01 M Tris-hydrochloride, pH 7.4). The mixture was sonically treated to fragment DNA, reduce the viscosity of the solution, and aid in the recovery of RNA from the aqueous-phenol interface. Sonic treatment was for 40 s at full power in an M.S.E. ultrasonic disintegrator. After centrifugation, the phenol phase and interface were reextracted with 5 ml of buffer N and the combined aqueous phases were reextracted with 5 ml of phenol-cresol mixture prior to precipitation with 2 volumes of ethanol in a siliconized Corex centrifuge tube (Corning, New York, N.Y.). After storage overnight at -20 C, the nucleic acids were recovered by centrifugation at 10,000 rpm for 30 min in a Sorvall HB4, swinging bucket, rotor, dissolved in 1 ml of 0.4 M NaCl, 0.01 M Tris-hydrochloride, pH 7.4, and reprecipitated with 3 ml of cold ethanol to remove traces of SDS and phenol. After 2 h at -20 C, the nucleic acids were recovered by centrifugation as before, drained, dried, dissolved in 0.2 ml of 0.4 M NaCl, 0.01 M Tris-hydrochloride, pH 7.4, and finally frozen at -20 C until required.

The recovery of labeled RNA from infected cells was monitored as follows. Samples of cells, the aqueous phases, ethanol supernatant fluids, and final RNA preparation were mixed with 5% (wt/vol) trichloroacetic acid in the presence or absence of 10^5 counts/min of ^3H viral RNA to act as an internal standard for ^3H quenching. The acid-insoluble counts per minute were corrected for quenching and the label present in each phase of the operation was computed. It was determined that in this procedure for nucleic acid extraction, better than 85% of the label present in the initial cell suspension was recovered in the final RNA preparation.

Cellulose column chromatography (11). Cellulose (CF 11, Whatman brand), equilibrated in TSES buffer (0.15 M NaCl, 0.05 M Tris-hydrochloride, 0.001 M EDTA, pH 7.2, 0.1% SDS) was treated with 0.1% diethyl pyrocarbonate, and packed into a 45-cm column in a 10-ml burette. The column was washed

with 100 ml of TSES solution followed by 100 ml of a mixture of 35% ethanol: 65% TSES buffer (vol/vol). An RNA sample was applied to the column in the same solution and eluted with 20 ml of this solution, then 20 ml of 15% ethanol: 85% TSES buffer (vol/vol), and finally with 20 ml of TSES buffer alone. Eluant fractions (2 ml each) were precipitated by trichloroacetic acid and the acid-insoluble radioactivity was determined. Approximately 80% of the label applied to the column was recovered in the various eluants (see 11).

Preparation of ^{32}P -labeled VSV *in vitro* transcription product RNA. Transcription product representative of the entire genome of VSV was prepared by using ^3H -uridine labeled VSV and ^{32}P - α -UTP to label the product RNA species as described previously (6, 29). After incubation at 31 C for 8 h, the reaction mixture was treated with sodium deoxycholate (1 mg/ml), adjusted to 1 M NaCl, and passed through a column of 4% agarose to remove the virion ^3H -RNA-ribonucleoprotein complexes from free ^{32}P product RNA and other reaction ingredients (6). Fractions containing ^{32}P product RNA were pooled, extracted by phenol-cresol, precipitated by ethanol, and the residual-labeled triphosphates removed by chromatography through Sephadex G-50 (5). The fractions containing ^{32}P -RNA were again pooled, the RNA was precipitated by ethanol and finally dissolved in 0.5 ml of 0.4 M NaCl, 0.01 M Tris-hydrochloride, pH 7.4, and frozen at -20 C until use.

RNA annealing, ribonuclease digestion. Annealing of RNA samples is dependent on RNA concentration, temperature, salt, and time of annealing. Since we annealed RNA samples in which the RNA molar concentrations were low, the time-course of annealing was monitored to obtain as much annealing as possible. This was achieved by taking samples of infected cell RNA (after 20 min of incubation at 38 C: 1,500 ^3H counts per min per sample of 100 μliters) and incubating the RNA at 60 C in sealed tubes for various time periods. The samples were each expelled into 0.55 ml of 0.4 M NaCl, 0.01 M Tris-hydrochloride, pH 7.4, and 0.3-ml volumes precipitated by trichloroacetic acid before or after digestion with pancreatic ribonuclease A (20 μg per ml, 37 C, 30 min). It was found that between 24 and 36 h of annealing were sufficient to attain maximum annealing (approximately 55% of the ^3H label in this case). Consequently, as a standard procedure, infected cell RNA samples (containing at least 1,500 ^3H counts per min per 100- μliter volume-unless stated otherwise) were annealed for 2 days at 60 C. Since the annealing was so long, it was critical that the RNA preparations were free from cell nucleases. For this reason, diethyl procarbonate treatment was included in the extraction procedure, and the acid-insoluble counts always compared with total counts initially added. Without the diethyl procarbonate treatment it was found that there was some loss of acid-insoluble label during the annealing time course for several RNA samples. This was not the case for RNA extracted by the foregoing procedure.

Since RNA annealing is concentration dependent for both species which are being annealed, with RNA samples isolated from infected cells prior to 20 min postinfection or containing less than 1,500 ^3H counts

per min per 100 μliters , there was no guarantee that in these cases maximum annealing was attained and so the values presented for those samples represent minimum values (see text).

Nuclease digestion of unannealed RNA samples was performed in a similar manner to that described above.

RESULTS

Optimal temperature for primary parental transcription of VSV. Monolayers of BHK-21 cells infected with ^3H -labeled wild-type VSV were incubated at different temperatures and the cellular nucleic acids were extracted at either 0, 20, or 60 min postinitiation of incubation. The percentage of ^3H -ribonuclease resistance of the RNA was determined before or after annealing and the results are plotted in Fig. 1. Even at 18 C, the lowest temperature examined, it was found that there was an appreciable increase in ribonuclease resistance of the input ^3H -RNA before annealing and much more after annealing for either the 20- or 60-min sample. For all the other temperatures (28 to 41 C) there was a significant increase in ^3H -ribonuclease resistance prior to annealing and this increase principally occurred during the first 20 min for samples incubated between 36.5 and 41 C. After annealing, there was a considerable increase in ^3H -ribonuclease resistance (to 100% for the 20-min samples of the 38 and 39.5 C incubations). However, RNA extracted from infected cells incubated at 41 C did not show as great an increase in ribonuclease resistance (either before or after annealing) as those incubated at 36.5 to 39.5 C, possibly reflecting a poorer capacity of the cells to support life functions at that temperature.

It should be noted that each time point for each temperature represented a single monolayer of approximately 3×10^6 BHK cells, so that it was conceivable that some monolayers were not exactly equivalent to others. However, in this and for other experiments reported below, the monolayers used in an experiment were all prepared at the same time, and used within 12 h of attaining confluency.

Despite these qualifications, it is clear from the results presented in Fig. 1 that the labeled RNA became ribonuclease resistant (before or after annealing) as a function of the incubation temperature and time. These results could be interpreted to indicate that viral RNA was transcribed intracellularly into complementary RNA species.

Is the ^3H label recovered from infected cells VSV RNA? The recovery of ^3H -label from infected cells and annealing to give a ribonuclease-resistant product, was no guarantee that the label was VSV RNA. For instance, although

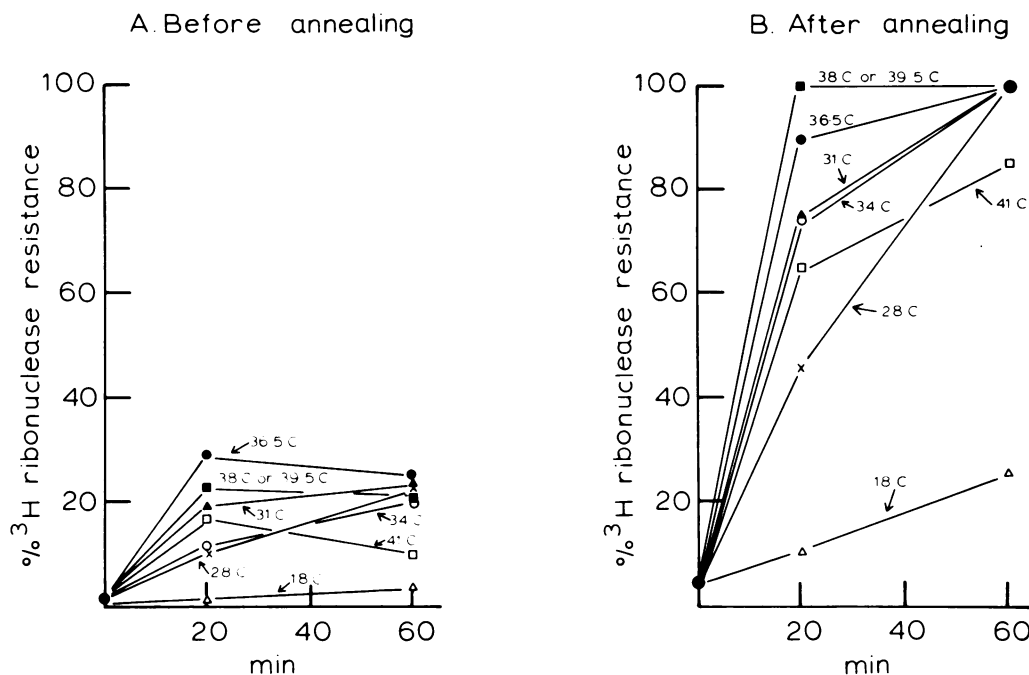


FIG. 1. Effect of temperature upon the *in vivo* transcription of VSV. A preparation of ^3H -labeled, purified VSV (2.6×10^6 counts per min per 100 μl of Eagle medium) was added to prewashed cold monolayers of BHK-21 cells and allowed to adsorb for 30 min at 4 C. One monolayer (the 0 time sample) was washed to remove excess virus and the cells recovered and extracted for nucleic acids (containing 1.7×10^4 ^3H counts/min). Other infected monolayers were washed with cold media then prewarmed media followed by incubation in circulating waterbaths for 20 or 60 min. The cells were washed and extracted for nucleic acids. The mean quantity of label (\pm standard deviation) recovered for all the time points was $13,000 \pm 4,500$ ^3H counts/min and the monolayers contained 3×10^6 cells per flask. The ^3H percentage of ribonuclease resistance before and after annealing was determined.

94% of the label applied to the cells was in VSV viral genomes (see Materials and Methods), it was possible that a small amount of non-VSV RNA was preferentially adsorbed by the cells or that the label which entered the cells was randomized by processes of degradation and then incorporated into cellular RNA. Note that no method was used to remove cell DNA from the extracted nucleic acids, so that if label entering the cells ended up in cellular RNA, we would have been measuring cellular RNA-DNA hybridization.

In order to determine if the label recovered from infected cells was VSV viral RNA, the following experiment was conducted. A sample of infected cell RNA, extracted 60 min post-initiation of incubation at 38 C, was diluted and annealed with or without purified ^{32}P -labeled VSV product RNA for 4 h at 60 C and the ^3H -ribonuclease resistance was determined before or after annealing (Table 1). The ^3H -ribonuclease resistance of the infected cell RNA without additional ^{32}P -RNA, was 11 and 17%,

respectively. In the presence of VSV ^{32}P transcription product RNA, the ^3H -ribonuclease resistance increased to 100% with a concomitant increase in the ^{32}P -ribonuclease resistance. It was concluded, therefore, that at least 83% (and probably all) of the ^3H -RNA recovered from infected cells was in fact VSV viral RNA.

Effect of various multiplicities of viral adsorption upon the primary transcription of VSV. It was calculated from the specific activity of the VSV viral RNA (7×10^6 counts per min per mg of RNA) that 1 count/min of ^3H was equivalent to 2×10^6 RNA molecules (or viral particles) on the assumption that the gram molecular weight of VSV RNA is about 4×10^6 g (4) and that there is one RNA molecule per virion. In most of the experiments reported here about 3×10^6 ^3H counts/min of virus inoculum were applied to BHK-21 monolayers containing 3×10^6 cells. Of these 6×10^{10} virus particles, an average of 3×10^8 were adsorbed (1.5×10^4 ^3H counts/min: multiplicity of adsorption of 1,000). In different experiments the multiplicity

TABLE 1. Annealing of nucleic acids extracted from ³H-VSV infected cells to ³²P-VSV transcription product RNA^a

Determination	Annealing conditions	Ribonuclease resistance (%)	
		³ H	³² P
(a) ³ H-VSV infected cell nucleic acids	(i) Unannealed	11	
	(ii) Annealed 48 h	100	
	(iii) Diluted, annealed 4 h	17	
(b) VSV ³² P transcription product RNA	(i) Unannealed		5
	(ii) Self annealed 4 h		5
(c) ³ H-VSV infected cell nucleic acid plus VSV ³² P transcription product RNA	As (a) iii	100	8

^a A sample of ³H-labeled purified VSV was added to a washed cold monolayer of BHK-21 cells and allowed to adsorb for 30 min at 4 C. After washing to remove excess virus, the monolayer was incubated at 38 C for 60 min and the infected cell nucleic acids extracted (see Materials and Methods). The ³H ribonuclease resistance was determined before (line [a] i), or after annealing for 48 h (line [a] ii), or after a ten-fold dilution and annealing for 4 h (line [a] iii). VSV complementary RNA labeled by ³²P was prepared and the ³²P ribonuclease resistance was determined before (line [b] i) or after (line [b] ii) self annealing. A mixture of diluted ³H-VSV infected cell nucleic acids plus VSV ³²P complementary RNA was annealed for 4 h and the ribonuclease resistance of both labels was determined.

of adsorption varied between 2,000 and 500 per cell. The reason for these individual variations is not known.

An experiment was designed to research the effect of widely different multiplicities of viral adsorption upon the primary parental transcription of VSV in order to determine if, under conditions of low or high multiplicities, there was a noticeable effect upon the efficiency of virus transcription. In question was the ability of cells to support the initial phases of growth of large numbers of viruses. As a criterion for transcription efficiency, the percentage of ³H RNA ribonuclease resistance was determined before or after annealing nucleic acids isolated from cells 20 min after initiation of incubation at 38 C. A preparation of ³H-labeled VSV was used at different concentrations to infect BHK cell monolayers. The virus preparation was found to possess a particle to PFU ratio of 400:1. From the cell counts and amount of ³H label extracted from the infected cells, the multiplicities of adsorption examined were: 5, 50, 440, 800, 1,500, 3,000, 6,000, 12,000, and 20,000 particles per cell. The respective percentage of ³H ribonuclease resistance postannealing were 75, 61, 54, 94, 74, 61, 75, 32, and 31% whilst before annealing they were 18 ± 8%. To obtain the lower or very high multiplicities, the experimental protocol was changed. For instance, for the low values a fraction of the amount of virus normally used to infect one 25-cm² monolayer, was applied to three large T-flasks (3 × 10⁷ cells), whereas for the higher values, normal (or greater) amounts of virus were applied to 3 × 10⁴ cells in individual wells of a microtest plate (Linbro Chem. Co., New Haven, Conn.) and the

infected cell nucleic acids extracted after addition of uninfected cells of the usual proportions.

Apparently, for up to 6,000 adsorbed virions per cell, irrespective of the multiplicity of adsorption, the initial phases of virus growth were not appreciably different.

Effect of cell age upon transcription of the VSV viral genome. Duplicate monolayers of BHK-21 cells were prepared from serial twofold dilutions of a cell inoculum. The cells were grown until all four dilutions had attained confluency and then one of each pair was infected at the same time with the same amount of ³H-labeled virus. After 20 min of incubation at 38 C, the infected cell nucleic acids were extracted and the ³H-ribonuclease resistance determined before or after annealing. The duplicate uninfected cell monolayers were resuspended and cell counts were obtained. Values for the cell counts of the four serially prepared monolayers were 6 × 10⁶, 4.5 × 10⁶, 4.7 × 10⁶, and 3.7 × 10⁶ and the respective ³H ribonuclease resistances postannealing were 43, 52, 45, and 48%. No significant difference in the total label recovered from the four samples was observed (±20%).

It was concluded, therefore, that cells which had just, or had for some time attained confluency, were equally efficient at supporting the primary transcription of VSV—at least to the extent of three generation equivalents beyond the time of confluency and contact inhibition.

Number of adsorbed virus which are transcribed. In the experiments described in Fig. 1, about one-fifth of the ³H-RNA recovered in the extracted nucleic acids for the 20-min samples of the 38 and 39.5 C incubations was

ribonuclease resistant prior to annealing. After annealing, 100% of the label became ribonuclease resistant. In order to determine how long it took a complete transcript of the VSV genome to be made, it was necessary to determine how many of the adsorbed genomes were transcribed. The results of Fig. 1 can be interpreted to indicate that by 20 min a minimum of 20% of the input genomes were transcribed (presumably more or less completely) or as much as 100% (presumably mostly partially) or somewhere between these two values. This interpretation rests on the assumption that the presence of ribonuclease resistance indicates that that proportion of the total RNA was transcribed.

An alternate means was investigated for determining how many genomes were involved in RNA transcription. The method involved cellulose chromatography to separate single-stranded from partially or completely double-stranded RNA molecules. Monolayers of BHK-21 cells were infected with ^3H -labeled wild-type virus and incubated at 38 C. Nucleic acids were extracted at 0, 20, and 60 min of incubation and the percentage of ^3H -ribonuclease resistance was determined without prior annealing. The value obtained for the 0-time sample was 4%, whereas those of the 20- and 60-min samples were 14%. Samples of infected cell nucleic acids were chromatographed on cellulose (11) and the distribution of acid-insoluble label was determined after elution with buffers (A, B, and C) which elute DNA and transfer RNA (A), single-stranded RNA (B) or double- and multistranded RNA species (C) (Fig. 2). The percentage of the total recovered label eluted by solution C increased from 2% for the zero time sample to 11 and 15%, respectively, for the 20- and 60-min samples.

It was clear from these results that not only did the amount of ribonuclease-resistant RNA increase in the extracted nucleic acids as a function of the incubation time, but also that the majority of the recovered labeled RNA species was single stranded. This latter conclusion was corroborated when it was determined that the RNA eluted by solution B for the 20- and 60-min samples (Fig. 2) possessed negligible ribonuclease resistances (0.6 and 2%, respectively). Since the initial ribonuclease resistance of the extracted nucleic acids agreed well with the number of genomes recovered in the solution C eluants (which for the 20- and 60-min samples were confirmed to be ribonuclease resistant), these results suggested that probably no more than 15% of the adsorbed virus was transcribed in this experiment.

Consequently, it appeared that there was a considerable portion of untranscribed VSV ge-

nomes associated with the BHK cells although whether they just adsorbed to the cell surface, or had an intracellular location was not determined.

Time-course for complete transcription of the VSV genome. From the foregoing experiment it was concluded that the criterion of ribonuclease resistance of the input template RNA was a reasonable indication of the number of genomes which had been transcribed. Although this was probably not true for RNA extracted at very early time points, it has been noted that most of the increase in ^3H -resistance occurred, for samples incubated above 36.5 C, between 0 and 20 min of cell incubation. Consequently, the rate of intracellular VSV transcription could be calculated. For example, with the 38 or 39.5 C samples described in Fig. 1, approximately one-fifth of the adsorbed viral RNA was ribonuclease resistant at 20 min, whereas after annealing 100% was ribonuclease resistant. If one-fifth of the virus was able to synthesize enough complementary RNA to anneal to all the viral RNA present in the extracted nucleic acids, then the average length of time to synthesize one copy was at most 4 min. In this particular case the time might have been even shorter since no determination was made of the total number of complementary genome equivalents present in these infected cell RNA samples.

Effect of drugs upon the primary parental transcription of VSV. The effect of three drugs, cycloheximide, puromycin, and actinomycin D, upon the transcription of the input genome of VSV was examined. Monolayers of BHK-21 cells were incubated with 5 μg of actinomycin-D, 50 μg of cycloheximide, or 100 μg of puromycin per ml of Eagle medium for 1 h prior to infection with ^3H VSV. The three drugs were present in similar proportions during infection and subsequent incubations at 38 C. Infected cell nucleic acids were extracted at 0, 10, 20, and 60 min postinitiation of incubation and the ribonuclease-resistant ^3H -RNA was determined before or after annealing (Fig. 3). The results indicate that there was no reduction of the primary transcription process in the presence of any of these three drugs by comparison to an untreated control.

Effect of virus grown in different cell lines upon adsorption and transcription in another cell line. An experiment was performed to determine if there was a detectable effect of virus grown in BHK cells upon adsorption and transcription in CEF cells, and vice versa. Virus preparations, from VSV normally passaged in BHK cells, were obtained by growth in CEF cells (VSV_{CEF}) or BHK cells (VSV_{BHK}). Identifi-

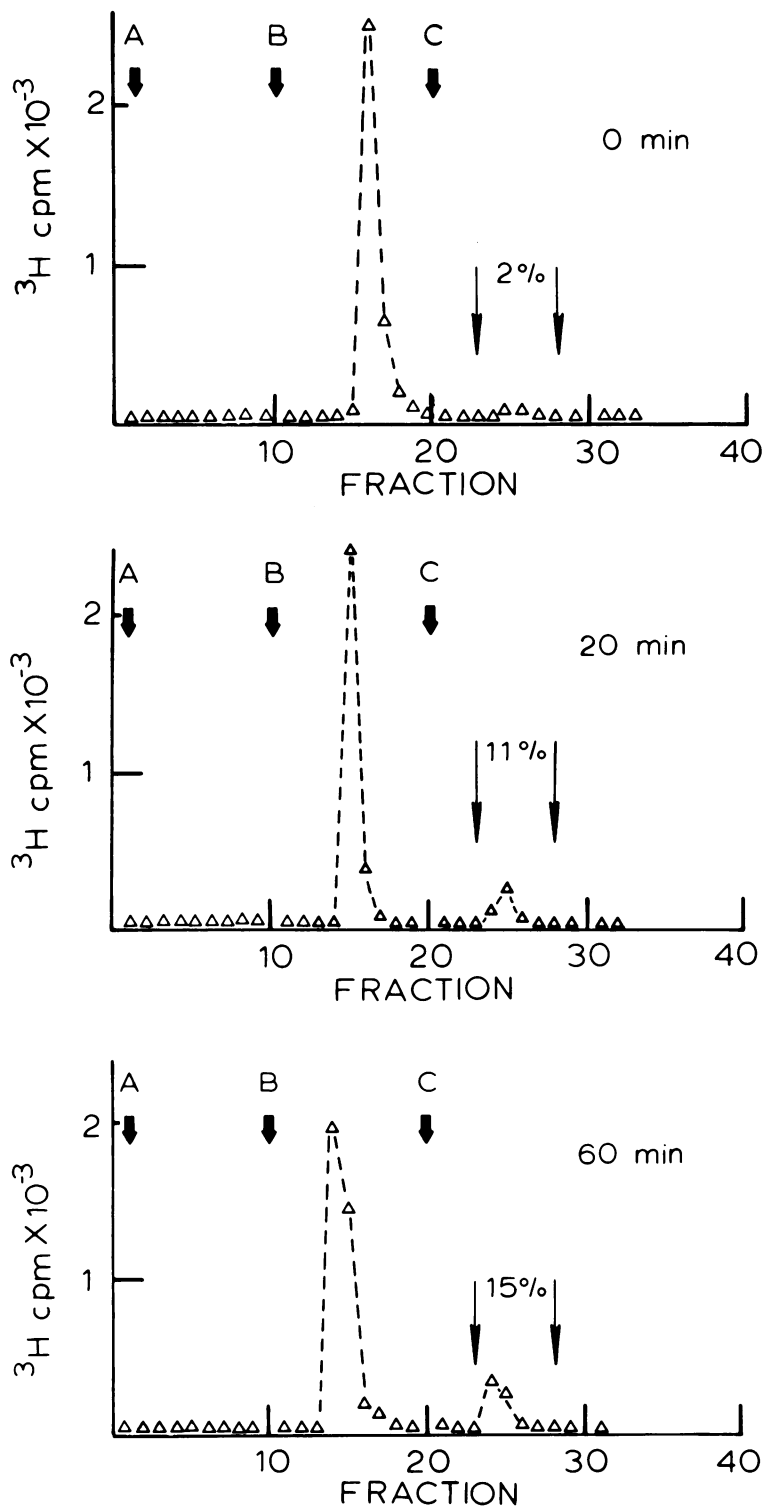


FIG. 2. Cellulose chromatography of nucleic acids recovered from ^3H -VSV infected BHK cells. Monolayers of BHK cells were infected at 4 C with ^3H -VSV (3.2×10^6 counts per min per monolayer) and then incubated in media at 38 C for 0, 20, or 60 min. The infected cell nucleic acids were extracted (1.9×10^4 counts/min for the 0 time sample, 1.0×10^4 for the 20-min sample and 1.7×10^4 counts/min for the 60-min sample) and the ^3H ribonuclease resistance was determined (4, 14, and 14%, respectively). Samples of each nucleic acid were chromatographed on cellulose (11) and eluted by (A) 65% TSES: 35% ethanol, then (B) 85% TSES: 15% ethanol and (C) 100% TSES. The distribution of acid insoluble label was determined. The initial virus preparation was found to possess a particle to PFU ratio of 700:1.

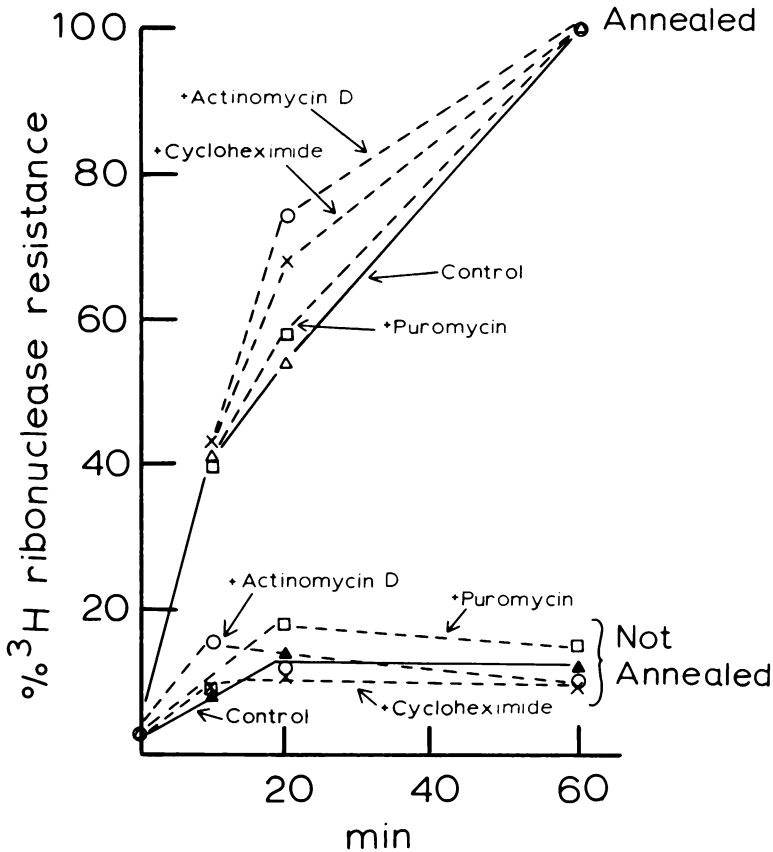


FIG. 3. Effect of drugs upon the *in vivo* transcription of VSV. Monolayers of BHK cells were pretreated with $5 \mu\text{g}$ of actinomycin D per ml of Eagle medium or $50 \mu\text{g}$ of cycloheximide, or $100 \mu\text{g}$ of puromycin for 1 h at 37°C prior to infection with 3.5×10^6 counts/min of ^3H -labeled VSV. The virus possessed a particle to PFU ratio of 700:1 and virus adsorption was performed in the presence of the same amounts of each drug. Infected monolayers were incubated in Eagle medium at 38°C for 0, 10, 20, or 60 min again in the presence of drugs, and the infected cell nucleic acids extracted. The ^3H percentage of ribonuclease resistance was determined before or after annealing. The mean ^3H (\pm standard deviation) recovered from the various monolayers was 1.5×10^4 ($\pm 0.5 \times 10^4$) counts/min. In control experiments it was found that the incorporation of ^{14}C -labeled amino acids into uninfected cell acid-insoluble product was inhibited 98% by either the puromycin or cycloheximide treatment, whereas the incorporation of ^3H -uridine was inhibited 89% by the actinomycin D treatment.

cal amounts of virus were applied to confluent monolayers of primary CEF cells or BHK-21 cells and nucleic acids extracted at 0, 20, or 60 min postinitiation of incubation at 38°C . The results are presented in Fig. 4. VSV_{CEF} was adsorbed and transcribed better in BHK cells than in CEF cells as determined by the amount of label recovered from the cell monolayers and the percentage of ribonuclease resistance postannealing (Fig. 4 legend). VSV_{BHK} also was adsorbed and transcribed better in BHK cells than in CEF cells, based on the same two criteria. However, it appeared that VSV_{CEF} was adsorbed and transcribed better in chick cells than VSV_{BHK} virus in the same cells. Similar results were obtained when secondary CEF

monolayer cultures were used instead of primary cultures. Moreover, virus normally passaged in CEF cells were also better adsorbed and transcribed in BHK than CEF monolayers.

Primary parental transcription of temperature-sensitive mutants of VSV as a function of the incubation temperature. Selected mutants from five complementation groups of VSV were grown in BHK cells at 31°C in the presence of ^3H -nucleosides and the virus preparations were purified as usual. Monolayers of BHK cells were infected with samples of each preparation and incubated at 31°C (for ts IV, 100) or 34°C (for the other mutants) as well as at 39.5°C for all mutants. The infected cell nucleic acids were in BHK cells—the amount of adsorbed virus and

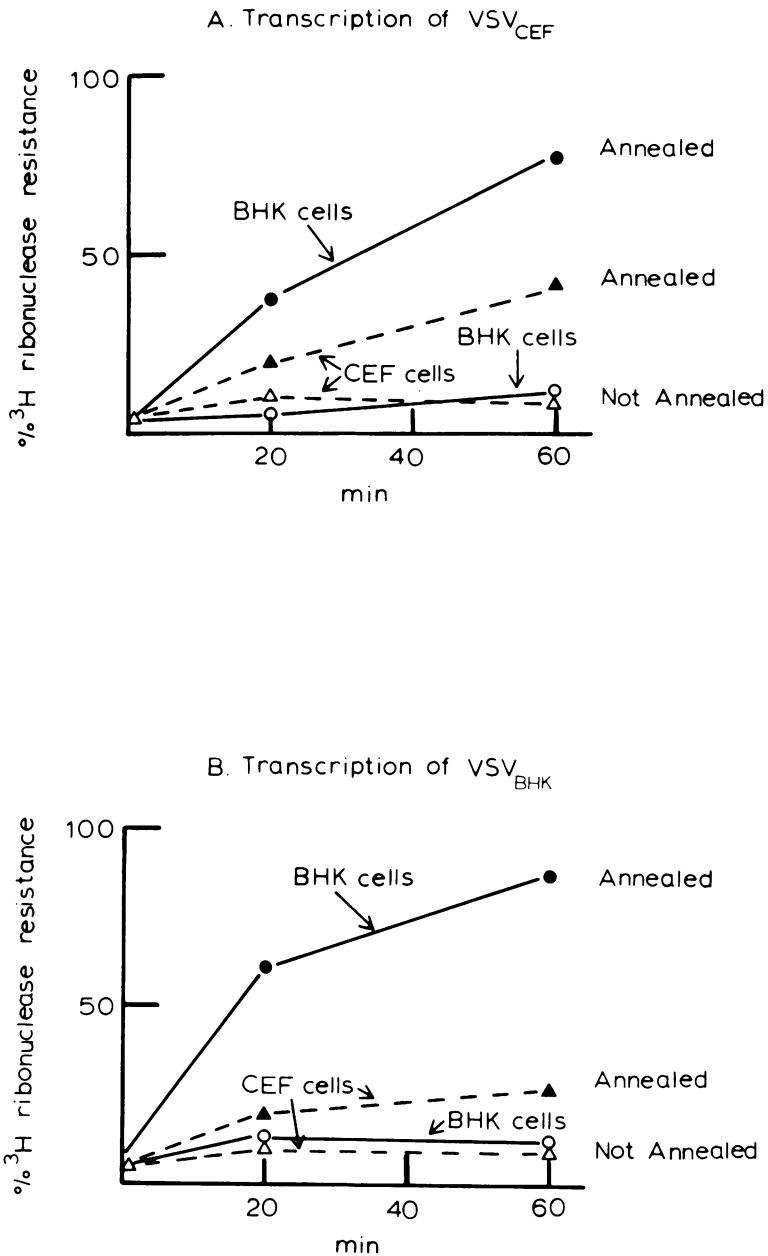


FIG. 4. Effect of virus grown in one cell type upon transcription in another. In (A) VSV was grown in primary chicken embryo fibroblasts (VSV_{CEF}) and used to infect BHK or CEF cells by the standard procedure. Infected cell monolayers were extracted for nucleic acids and the 3H percentage of ribonuclease resistance was determined before or after annealing. The total 3H recovered from the CEF cells was 10^4 counts/min (0 min) and 4×10^3 counts/min (20 and 60 min). From the BHK cells the total 3H recovered was 1.3×10^4 counts/min (0 min) and 1.2×10^4 counts/min (20 and 60 min). In (B) VSV was grown in BHK cells (VSV_{BHK}) and used to infect BHK or CEF monolayers. The total label recovered from the CEF cells was 1.6×10^4 counts/min (0 min), 7.4×10^3 counts/min (20 min), 6.5×10^3 counts/min (60 min), whereas the label recovered from the BHK cells was 4.5×10^4 counts/min (0 min), 3.9×10^4 counts/min (20 min), 4.1×10^4 counts/min (60 min).

extracted at various times after the initiation of incubation and the 3H -ribonuclease resistance determined before or after annealing. The re-

sults are presented in Fig. 5. For each mutant at either temperature-representing permissive or not permissive conditions for virus production

percentage of ^3H -ribonuclease resistance before or after annealing was not notably different (see Discussion).

DISCUSSION

The initial phase of intracellular transcription of the genome of VSV has been followed by using highly labeled preparations of virus. It has been found that the primary transcription process is optimal between 36 and 39.5 C in BHK-21 cells as well as repetitive and complete both in the presence or absence of drugs known to inhibit protein or DNA-directed RNA synthesis.

Temperature-sensitive mutants of all five complementation groups of VSV were found capable of primary genome transcription at temperatures which were permissive or nonpermissive for virus production.

Virus adsorption. In most of the experiments described here, about 5% of the virus inoculum was adsorbed to BHK-21 cell monolayers during 30 min of incubation at 4 C. A two- to fivefold larger percentage has been obtained by the use of DEAE-dextran (unpublished observations). Although incubation at room temperature was also effective in increasing the percentage of adsorbed virus (unpublished observations), to obtain transcription synchrony, the adopted temperature for virus adsorption was 4 C.

Based on the unannealed ^3H ribonuclease-resistance data, of the virus particles which remained adsorbed during incubation at elevated temperatures, between one-tenth and one-third were transcribed intracellularly. Whether the rest of the virus was adsorbed to the other surface of the cells or represented virus trapped between cells in the monolayer has not been determined. Nor is it known how many virions penetrated but were untranscribed within the cells.

It appears that virus adsorption to chicken embryo fibroblasts was generally less efficient than for BHK-21 cell monolayers.

Virus desorption. Virus desorption can be defined as the loss of virus upon incubation of washed, infected cell monolayers. In all the experiments described here using wild-type VSV, the mean amount of desorbed virus from BHK cells (\pm standard deviation), was 20% (\pm 10%) of the initial adsorption. With VSV mutants of groups III and V, the percentages of desorbed virus from BHK cells was higher—about 45% (\pm 35%) (see Fig. 5 legend)—, whereas for mutants of groups I, II, and IV, the amounts of desorbed virus were about 20% (\pm 10%).

Virus desorption from CEF monolayers infected with wild-type VSV was often greater than with BHK cells (see Fig. 4 legend), al-

though essentially similar results were obtained whether the virus inoculum was prepared from BHK or CEF cells. The mean virus desorption (\pm standard deviation) from CEF monolayers was 60% (\pm 10%) of the initial adsorbed virus.

In experiments in which infected and washed CEF monolayers were incubated at 38 C and the supernatant fluids precipitated by trichloroacetic acid, it was found that the label desorbed from CEF monolayers could be recovered in an acid-insoluble form (unpublished data), suggesting that the loss of label from these monolayers represented desorbed virus and not intracellular degradation of the virion genomes.

Particle to plaque-forming unit ratios of virus preparations. It has been calculated that in the virus preparations used, 1 count/min of ^3H label was equivalent to approximately 2×10^5 virion genomes. When virus preparations were assayed for both plaque-forming capacity and particle content, most of the viral preparations possessed particle to PFU ratios of around 500 to 1 (see Fig. 2, 3, and text). The PFU determinations were usually performed by using CEF monolayers (12), whilst the particle counts were based on the total label present in a virus inoculum. We have shown that somewhat less than 5% of the label in a virus inoculum was normally adsorbed by CEF monolayers (see Fig. 4 legend), but of that label, half was subsequently desorbed. Approximately 10% of the remaining adsorbed virus appeared to be transcribed in the chick cells (see Fig. 4). Consequently, the percentage of virion inoculum which was observed to be transcribed intracellularly corresponded reasonably well with the number of PFU originally assayed in the virus preparations.

In the case of BHK cells, the percentage of virus inoculum usually adsorbed by a monolayer was greater than for CEF monolayers, whereas the amount of desorption was less and the number of genomes transcribed intracellularly also greater than for chick cells. In parallel with these observations we have always observed that the efficiency of plating a given VSV inoculum on BHK monolayers was two to fivefold higher than on CEF monolayers (unpublished observations). We do not know if the 70 to 90% untranscribed virion genomes were impotent for virus production or represented virus particles which were latent in development or cell penetration.

Experiments have been performed to determine if the initial unadsorbed virus was still infectious. Monolayers of BHK cells were infected with an inoculum of ^3H -uridine labeled VSV in Eagle medium containing ^{32}P -sodium phosphate. After adsorption at 4 C for 30 min,

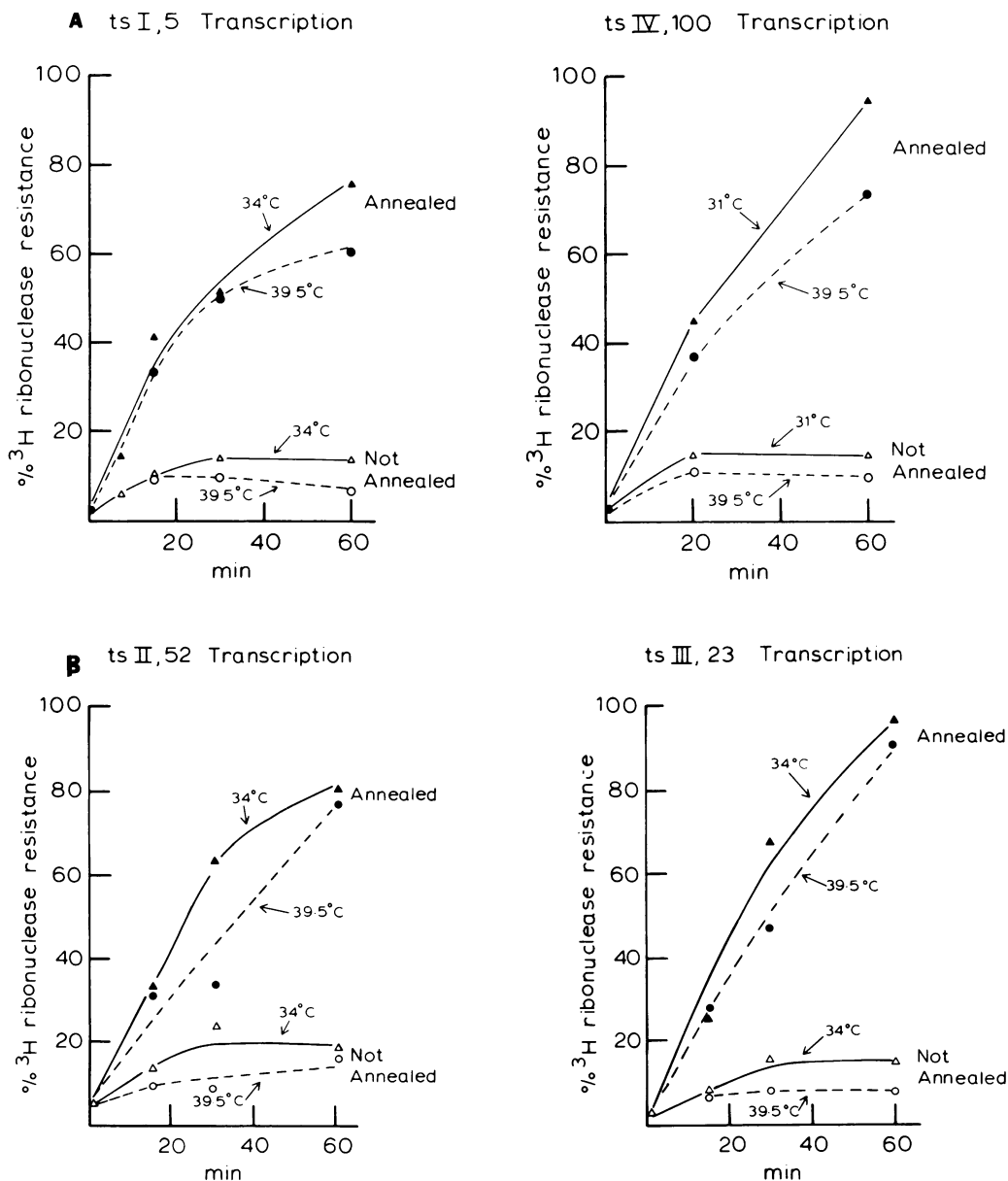


FIG. 5. Primary transcription of selected temperature-sensitive mutants from five complementation groups of VSV. Preparations of temperature-sensitive mutants of VSV labeled by ^3H nucleosides were purified and used to infect confluent monolayers of BHK cells. Infected monolayers were incubated at 39.5 and 31 C (or 34 C) and nucleic acids were extracted after the prescribed times. The ^3H -ribonuclease resistance before or after annealing was determined. For each of the viruses examined, the amounts of virus adsorbed were similar to those given in Fig. 1 legend. However, for group III and V mutants the amounts of desorbed virus were greater than for group I, II, and IV or wild-type viruses. For example, with *ts III, 23*, a sample of 3.2×10^6 counts/min of labeled virus was used to infect each monolayer and the recovered infected cell nucleic acids contained 1.3×10^4 counts/min for the 0 time sample, 6.5×10^3 counts/min, 3.3×10^3 counts/min, and 1.5×10^4 counts/min for the 15-, 30-, and 60-min samples of the 34 C incubations and 4.4×10^3 , 4.0×10^3 , and 6.4×10^3 counts/min for the 15-, 30-, and 60-min samples of the 39.5 C incubations. The annealing time at 60 C was 60 h.

the inoculum was removed and samples of the supernatant fluids were assayed for PFU, as well as content of ^3H and ^{32}P label, and then

were used to infect fresh monolayers of BHK cells. The process was repeated for 12 successive passages. The ratios of $^3\text{H}/^{32}\text{P}$ and PFU/ ^{32}P

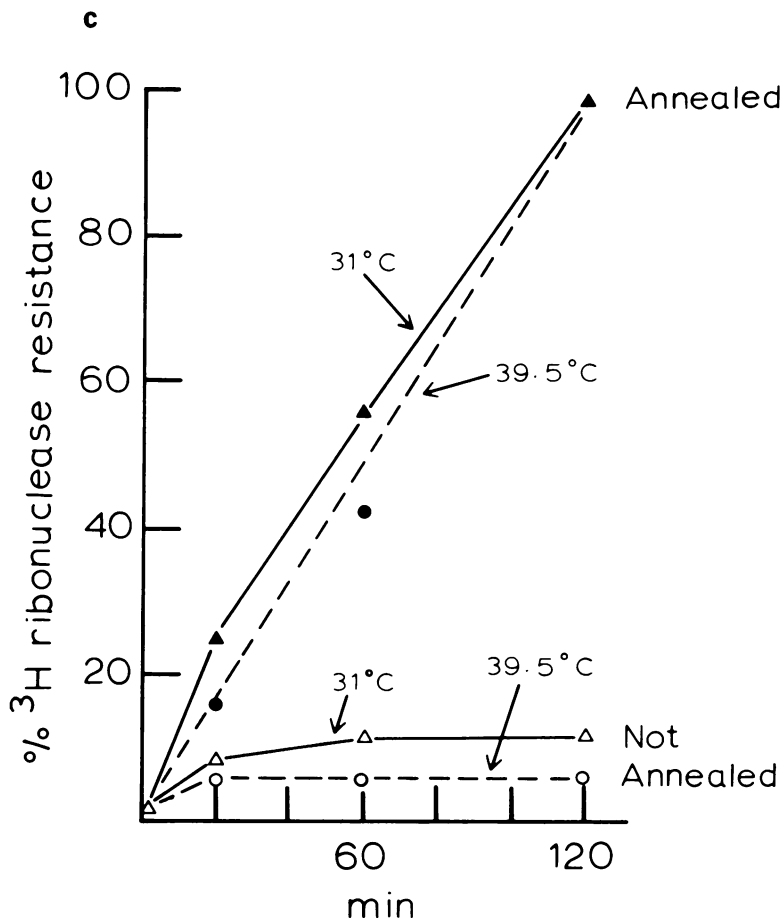


FIG. 5—continued.

were then compared for each passage and to the initial inoculum and found to be indistinguishable. It should be mentioned, however, that the ^{32}P content per milliliter decreased through dilution during the successive passages over the monolayers, to about 10% of its initial value. Hence, comparison of the ratios of labels and PFU/ ^{32}P for the initial and subsequent inocula were made and not the gross values.

It was concluded from these results that virus which remained unadsorbed to BHK monolayers during incubation at 4 C was probably as infectious as the initial virus preparation. No selection through adsorption of a minority of infectious versus a majority of noninfectious particles was observed by this procedure.

Intracellular transcription of the VSV genome. In agreement with results reported by Huang and Manders (18), we find that the transcription process of VSV is dependent upon the infected cell incubation temperature and is not inhibited by the presence of actinomycin D, puromycin, or cycloheximide. We have been

able to show that the average time taken to synthesize complete transcripts of the VSV genome is a matter of a few minutes. Also, transcription is essentially similar for large variations in multiplicities of adsorption or in contact inhibited cells.

The process which has been studied represents the primary transcription of the infecting virion genome. Kinetic experiments involving hybridization of nucleic acids extracted from cells infected in the presence or absence of cycloheximide, to different amounts of labeled viral RNA, are in progress to delineate primary from other forms of VSV RNA transcription.

Phases of RNA synthesis in infected cells. The present studies have been directed at the initial synthesis of RNA (presumably mRNA) by VSV in infected cells.

For the purposes of definition, we would suggest the following phases of RNA synthesis in infected cells.

(i) Primary parental transcription: transcription of the parental incoming viral genome

by the virion polymerase molecules and synthesis of messenger RNA. (ii) Secondary parental transcription: transcription of the parental viral genome by newly-synthesized polymerase molecules—synthesized under the direction of messenger RNA made by the primary parental transcription. (iii) Parental replication: the synthesis of a continuous, complete complementary copy of the virion genome (either directly, or by the action of a ligase enzyme on aligned messenger RNA molecules), followed by the subsequent synthesis of RNA identical to the input genome. (iv) Tertiary transcription: transcription of the newly synthesized viral-type RNA into more messenger RNA species. (v) Amplified replication and transcription: several rounds of replication and transcription on various templates.

We do not as yet know if all of these particular stages of RNA synthesis in fact occur in infected cells (for instance, it is conceivable that individual messenger RNA species could be replicated). However, these stages should be operationally distinguishable and thereby delineated through the use of drugs, e.g., those known to inhibit protein synthesis, and possibly by the use of temperature-sensitive mutants of the RNA-negative types.

Temperature-sensitive mutants of VSV. Of the five known complementation groups of VSV, mutants in three produce less RNA at nonpermissive temperatures than the wild-type virus (12-14, 16, 20, 23, 26-28, 32).

It has been suggested from studies involving the *in vitro* analysis of VSV transcription using virions of temperature-sensitive mutants of group I and IV, that the virion polymerase is also temperature sensitive since, for some mutants, the enzyme functions poorly at high temperatures—even more poorly than the wild-type enzyme (7, 28, 32). We have confirmed the results of Printz-Ané, Combard, and Martinet (28) that the transcriptases of mutants I-5 and IV-100 used in these studies function less well at 38 C than at 31 C. However, because the wild-type enzyme does not function well at 37 C (3-5) and the polymerases of these mutants also transcribe poorly by comparison to the wild-type enzymes at low temperatures, these observations could have other interpretations, and need an alternate means of verification.

In contrast to *in vitro* experiments on the virion associated polymerase of temperature-sensitive mutants of groups I and IV of VSV (4, 28, 32), the kinetics of *in vivo* transcription of selected mutants of groups I and IV were not too different from those of other *ts* mutants at 31, 34, or 39.5 C. From the results presented, it is

apparent that for all mutants, the transcription process was repetitive at either permissive or nonpermissive temperatures, but that the number of adsorbed virions which were transcribed intracellularly was generally less at the higher temperature—which was not true for the wild-type virus (Fig. 1). Since the number of temperature-insensitive revertants present in the virion preparations were (except for mutant II-52) less than 0.01% of the temperature-sensitive plaque-forming population (12-15), it is unlikely that the observed transcription at high temperatures was due to such revertants. In *in vivo* transcription experiments employing two other group I and one other group IV mutants, we have obtained similar results to those reported in Fig. 5.

We conclude from these results that the virion polymerases of these *ts* mutants when grown at permissive temperatures are not temperature sensitive at nonpermissive temperatures *in vivo*. These results suggest the possibility that if one or more of these mutants represent altered transcriptase gene(s), then the temperature-sensitive defect(s) could be the result of proteins which are nonfunctional when they are synthesized at nonpermissive temperatures, perhaps due to some conformational problem.

It would appear from the data presented in Fig. 5 that parental transcription of mutants from group III and IV was probably complete at both 34 and 39.5 C, since at either temperature the ³H-ribonuclease resistance postannealing was between 90 and 100%. During more extensive incubations with the group I and IV mutants we have been able to obtain between 80 and 95% ³H-ribonuclease resistance postannealing for infected cells growing for 2 h at 39.5 C (unpublished observations) which indicates that the transcription process of these mutants was not only repetitive but also probably complete.

Initial phases of virus development in infected cells. By tracing the fate of the infecting genome of a single-stranded RNA virus it should be possible to realize the answers to several questions which are at present only poorly understood. For instance, we would like to determine the intracellular location of the virion genome and its associations with cellular structures. We would also be interested to know if the infecting virus genome is released from other virion components, particularly the nucleocapsid and membrane proteins—release from the former being extremely difficult to achieve *in vitro* (6, 10). We would also like to know if the products of primary transcription are the same

as those which have been identified at later periods of infection (17-19, 24, 31). Experiments are in progress on these aspects of the intracellular development of VSV and also to determine if the primary parental transcription of the viral genome is inhibited by poly I: poly C, or interferon pretreatment of cells (22).

Since there are many factors which affect virus infection of a particular cell (see for example 33), analysis of cell infection by tracing the fate of the infecting virion genome in permissive or nonpermissive cells gives us a valuable tool for delineating some of the processes involved, particularly for oncornaviruses and those virus groups which possess or are thought to possess virion nucleic acid polymerases.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grant AI-10692 from the National Institute of Allergy and Infectious Diseases.

We thank Roger Van Deroef for excellent technical assistance, and Polly Roy for the preparation of the VSV transcription product RNA.

LITERATURE CITED

- Aaslestad, H. G., H. F. Clark, D. H. L. Bishop, and H. Koprowski. 1971. Comparison of the ribonucleic acid polymerases of two rhabdoviruses, Kern Canyon virus and vesicular stomatitis virus. *J. Virol.* **7**:726-735.
- Baltimore, D., A. S. Huang, and M. Stampfer. 1970. Ribonucleic acid synthesis of vesicular stomatitis virus. II. An RNA polymerase in the virion. *Proc. Nat. Acad. Sci. U.S.A.* **66**:572-576.
- Bishop, D. H. L. 1971. Complete transcription by the transcriptase of vesicular stomatitis virus. *J. Virol.* **7**:486-490.
- Bishop, D. H. L., and P. Roy. 1971. Kinetics of RNA synthesis by vesicular stomatitis virus particles. *J. Mol. Biol.* **57**:513-527.
- Bishop, D. H. L., and P. Roy. 1971. Properties of the product synthesized by vesicular stomatitis virus particles. *J. Mol. Biol.* **58**:799-814.
- Bishop, D. H. L., and P. Roy. 1972. Dissociation of vesicular stomatitis virus and relation of the virion proteins to the viral transcriptase. *J. Virol.* **10**:234-243.
- Cairns, J. E., A. F. Holloway, and D. V. Cormack. 1972. Temperature sensitive mutants of vesicular stomatitis virus: in vitro studies of virion associated polymerase. *J. Virol.* **10**:1130-1135.
- Deutsch, V., and A. Berkaloff. 1971. Analyse d'un mutant thermolabile du virus de la stomatite vésiculaire (VSV). *Ann. Inst. Pasteur* **121**:101-106.
- Eagle, H. 1959. Amino acid metabolism in mammalian cell culture. *Science* **130**:432-437.
- Emerson, S. U., and R. W. Wagner. 1972. Dissociation and reconstitution of the transcriptase and template activities of vesicular stomatitis B and T virions. *J. Virol.* **10**:297-309.
- Erickson, R. S. 1969. Procedures for the purification of intermediate forms of viral RNA from RNA virus-infected cells, p. 451-459. *Fundamental techniques in virology*. Academic Press, Inc. New York.
- Flamand, A. 1969. Etude des mutants thermosensibles du virus de la stomatite vésiculaire. Mise au point d'un test de complémentation. *C. R. Acad. Sci. Paris* **268D**:2305-2308.
- Flamand, A. 1970. Etude génétique du virus de la stomatite vésiculaire: classement de mutants thermosensibles spontanés en groupes de complémentation. *J. Gen. Virol.* **8**:187-195.
- Flamand, A., and F. Lafay. 1973. Etude des mutants thermosensibles du virus de la stomatite vésiculaire appartenant au groupe de complémentation II. *Ann. Inst. Pasteur* **124**:261-269.
- Flamand, A., and C. R. Pringle. 1971. The homologies of spontaneous and induced temperature-sensitive mutants of vesicular stomatitis virus isolated in chick embryo and BHK-21 cells. *J. Gen. Virol.* **11**:81-85.
- Holloway, A. F., P. K. Y. Wong, and D. V. Cormack. 1970. Isolation and characterization of temperature-sensitive mutants of vesicular stomatitis virus. *Virology* **42**:917-926.
- Huang, A. S., D. Baltimore, and M. Stampfer. 1970. Ribonucleic acid synthesis of vesicular stomatitis virus. III. Multiple complementary messenger RNA molecules. *Virology* **42**:946-957.
- Huang, A. S., and E. K. Manders. 1972. Ribonucleic acid synthesis of vesicular stomatitis virus IV. Transcription by standard virus in the presence of defective interfering particles. *J. Virol.* **9**:909-916.
- Kiley, M. P., and R. R. Wagner. 1972. Ribonucleic acid species of intracellular nucleocapsids and released virions of vesicular stomatitis virus. *J. Virol.* **10**:244-255.
- Lafay, F. 1969. Etude des mutants thermosensibles du virus de la stomatite vésiculaire. Classification de quelques mutants d'après des critères de fonctionnement. *C. R. Acad. Sci. Paris* **268D**:2385-2388.
- Lafay, F. 1971. Etude des fonctions du virus de la stomatite vésiculaire altérées par une mutation thermosensible: Mise en évidence de la protéine structurale affectée par la mutation *ts* 23. *J. Gen. Virol.* **13**:449-453.
- Marcus, P. I., D. L. Engelhardt, J. M. Hunt, and M. J. Sekellick. 1971. Interferon action: inhibition of vesicular stomatitis virus RNA synthesis induced by virion-bound polymerase. *Science* **174**:593-598.
- Martinet, C., and C. Printz-Ané. 1970. Analyse de l'ARN viral du virus de la stomatite vésiculaire (VSV). Utilisation de mutants thermosensible. *Ann. Inst. Pasteur* **119**:411-419.
- Mudd, J. A., and D. F. Summers. 1970. Polysomal ribonucleic acid of vesicular stomatitis virus infected HeLa cells. *Virology* **42**:958-968.
- Pringle, C. R. 1970. Genetic characteristics of conditional lethal mutants of vesicular stomatitis virus induced by 5-fluorouracil, 5-azacytidine and ethyl methane sulphinate. *J. Virol.* **5**:559-567.
- Pringle, C. R., and I. B. Duncan. 1971. Preliminary physiological characterization of temperature-sensitive mutants of vesicular stomatitis virus. *J. Virol.* **8**:56-61.
- Printz, P., and R. R. Wagner. 1971. Temperature-sensitive mutants of vesicular stomatitis virus: synthesis of virus specific proteins. *J. Virol.* **7**:651-662.
- Printz-Ané, C., A. Combard, and C. Martinet. 1972. Study of the transcription and the replication of vesicular stomatitis virus by using temperature sensitive mutants. *J. Virol.* **10**:889-895.
- Roy, P., and D. H. L. Bishop. 1972. The genome homology of vesicular stomatitis virus and defective T particles and evidence for the sequential transcription of the virion ribonucleic acid. *J. Virol.* **9**:946-955.
- Roy, P., and D. H. L. Bishop. 1973. Initiation and direction of RNA transcription by vesicular stomatitis virus virion transcriptase. *J. Virol.* **11**:487-501.
- Stampfer, M., D. Baltimore, and A. S. Huang. 1969.

- Ribonucleic acid synthesis of vesicular stomatitis virus.
1. Species of ribonucleic acid found in Chinese hamster ovary cells infected with plaque-forming and defective particles. *J. Virol.* **4**:154-161.
 32. Szilagyi, J. F., and C. R. Pringle. 1972. Effect of temperature-sensitive mutations on the virion-associated RNA transcriptase of vesicular stomatitis virus. *J. Mol. Biol.* **72**:281-292.
 33. Valle, M. 1971. Factors affecting plaque assay of animal viruses, with special reference to vesicular stomatitis and vaccine virus. *Acta Pathol. Microbiol. Scand.* **219**:7-69.