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RNA Synthesis in Cells Infected with Herpes Simplex Virus

X. Properties of Viral Symmetric Transcripts and of Double-Stranded RNA Prepared from Them

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HEp-2 cells infected with herpes simplex 1 virus contain RNA transcripts capable of forming double-stranded (DS) RNA on annealing. The properties of purified DS RNA were as follows: (i) DS RNA is resistant to depolymerization by RNase A or T_1 in 2× 0.15 M NaCl, plus 0.015 M sodium citrate (SSC) but not 0.1× SSC or following thermal denaturation. (ii) The T_m of the viral DS RNA was 100 C in 0.1× SSC. (iii) Undenatured DS RNA does not hybridize with viral DNA; upon denaturation, excess unlabeled RNA drove 50 to 55% of labeled DNA into DNA-RNA hybrid. The kinetics of hybridization indicate that the DS RNA consists of at least two populations of transcripts arising from 29 and 26% of viral DNA and differing 40-fold in molar concentration.

This paper details a series of experiments designed to detect and analyze viral RNA sequences capable of forming double-stranded structures by base pairing in HEp-2 cells infected with human herpesvirus 1 (herpes simplex 1, HSV-1). The circumstances which led to this study are as follows.

(i) In principle, analyses of the processes which regulate transcription require some information concerning the process of transcription itself. We could envision several possibilities, i.e., asymmetric transcription of one or both strands of DNA, partially symmetric transcription resulting from overlapping transcription of both strands, and totally symmetric transcription. The first and last imply entirely different regulatory mechanisms for making transcripts available for function as mRNA.

(ii) Transcripts arising from symmetric transcription and therefore capable of base pairing have been detected in cells infected with a number of DNA viruses but especially with poxviruses, adenoviruses, and papovaviruses (1, 2, 4, 12). Suggestive evidence that complementary, self-annealing viral RNA sequences exist in HSV-1-infected cells emerged from the observations that nuclear RNA from 8-h infected cells preannealed under conditions, which promoted self-annealing, drove only 35% of viral DNA into DNA-RNA hybrid, i.e., 15% less than the nonpreannealed RNA. However, the capacity to drive 50% of viral DNA into hybrid was restored by heat denaturing the preannealed RNA prior to hybridization. Moreover, the self-annealing of RNA was dependent on RNA concentration and was not affected by mild alkaline hydrolysis which decreases the size of the RNA. These data suggested the presence of abundant symmetric transcripts in the nuclei of infected cells (11) but did not reveal the extent to which viral DNA was symmetrically transcribed or the characteristics of the double-stranded (DS) RNA formed by self-annealing the RNA.

The present studies were designed to analyze the properties of the DS RNA prepared by self-annealing RNA from infected cells and to estimate the extent of the DNA giving rise to symmetric transcripts.

MATERIALS AND METHODS

Solutions and chemicals. Standard saline citrate consisted of 0.15 M sodium chloride plus 0.015 M sodium citrate (1× SSC); reticulocyte standard buffer consisted of 0.01 M sodium chloride-0.0025 M magnesium chloride-0.01 M Tris-hydrochloride (pH 7.5). *Escherichia coli* DNA was the kind gift of A. Markovitz of the University of Chicago. Sodium dodecyl sulfate and formamide were obtained from Matheson, Coleman and Bell; sarkosyl (NL97) from Geigy Chemical Company, sodium deoxycholate from Schwarz Mann BioResearch, Orangeburg, N.Y.; crystallized DNase free of RNase, RNase T_1, and RNase A, from Worthington Biochemicals, Freehold, N.J.; and [5-^{3}H]uridine (specific activity 28 Ci/mmol) from New England Nuclear, Boston, Mass.

Cells and virus. The procedures for propagation and maintenance of human epidermoid carcinoma no. 2 (HEp-2) cells and production, assay, and pertinent

properties of the F strain of herpes simplex 1 virus [HSV-1(F)] were described elsewhere (2, 8, 11, 15, 16).

Preparation and labeling of viral DNA. The procedure for preparation and purification of viral DNA was the same as previously described (8) except that the final product was digested with RNase and extracted with phenol and chloroform. All batches of DNA used in this study were repurified by isopycnic banding in CsCl solution. HEp-2 DNA was extracted by the method of Marmur (13).

The procedure for in vitro labeling of HSV-1(F) DNA by repair synthesis using *E. coli* polymerase I was described elsewhere (7). The *E. coli* polymerase I was the kind gift of A. Kornberg, Stanford University.

RNA purification. Total RNA from infected and uninfected cells was extracted as follows: cells were washed in phosphate buffered saline, suspended in reticulocyte standard buffer, lysed by the addition of sodium deoxycholate (0.5% final concentration), and digested briefly with RNase-free DNase (50 μg/ml, room temperature) to diminish viscosity. The material was then extracted with sodium dodecyl sulfate, phenol and chloroform, precipitated with ethanol, dissolved in 0.01 M Tris (pH 7.5) plus 0.025 M $MgCl_2$, dialyzed against the same buffer, and digested again with DNase. The RNA was then again extracted with sodium dodecyl sulfate and phenol. This cycle was repeated once again. In the last step of the extraction procedure, the RNA was dialyzed against the hybridization buffer for 3 days.

Purification of DS RNA. The RNA was extracted as described above, then allowed to reanneal at 50 C for 24 h in 0.01 M Tris (pH 7.5)-0.75 M sodium chloride-1 mM EDTA-50% (vol/vol) formamide. After reannealing the RNA was diluted and precipitated with 2 volumes of ethanol. The RNA pellet was solubilized in 0.01 M Tris (pH 7.5) plus 2.5 mM magnesium chloride, dialyzed against this buffer, and digested with DNase I (50 μg/ml) for 1 h at 37 C. Then 0.1 volumes of 10 mM EDTA in 0.01 M Tris (pH 7.5) was added, the salt concentration was adjusted to 0.35 M NaCl, and the RNA was digested for 1 h at 37 C with 50 μg of RNase A and 10 U of RNase T_1 per ml. To remove degradation products, RNA was passed through Sephadex G50 (1 by 60 cm) columns, and the excluded material was precipitated with ethanol.

Hybridization of labeled RNA to DNA on filters. In several experiments, the RNA was denatured in 0.1× SSC at 115 C for 5 min and hybridized to *E. coli*, HEp-2, and HSV-1 DNAs fixed to nitrocellulose filters. The DNAs were fixed to 25-mm Schleicher and Schuell B6 filters as previously described (9). Small disks (6 mm in diameter) were then punched out. A set of disks containing *E. coli*, HEp-2, and HSV-1 DNAs, respectively, plus a blank were placed in a Beem capsule containing the denatured RNA in 200 μl of hybridization buffer (0.75 M NaCl-5 nM EDTA-0.25% sodium dodecyl sulfate-0.01 M Tris, pH 7.5). After incubation for 20 h at 66 C, the disks were removed, washed two times for 30 min each in 2× SSC, digested with RNase A (50 μg/ml) for 1 h at room temperature, washed as before, and dried.

Hybridization of unlabeled RNA to labeled DNA in solution. Excess unlabeled RNA and sheared in vitro labeled DNA were denatured by heating for 7 min at 115 C, then hybridized in 0.3 M Na^+-0.04 M phosphate buffer at 75 C. The amount of DNA driven into DNA-RNA hybrid was monitored by digestion with a *Neurospora crassa* nuclease (14) which depolymerizes single-stranded DNA but does not attack DNA-RNA hybrid. The procedures for preparation of the enzyme and digestion of the hybridization mixture were as previously described (6, 11). In these experiments, the amount of denatured DNA resistant to enzyme digestion did not exceed 5% of input DNA counts.

RESULTS

Preparation of DS RNA from productively infected cells. In this series of experiments HEp-2 cells were infected with a multiplicity of 100 PFU/cell, labeled from 4.8 to 5 h postinfection with [^{3}H]uridine (50 μCi/ml), then harvested. The extraction of RNA and purification of DS RNA were as described above. DS RNA purification was monitored by digesting in 2× SSC samples taken throughout the purification procedure with RNase A or with both RNase A and RNase T_1. The salt concentration was chosen on the basis of the report (3) that DS RNA is depolymerized with RNase in the presence of $\leq$0.02 M Na^{2+} but not at concentrations of $Na^+ \geq 0.2$ M. The results of a typical experiment are as shown in Table 1. In this experiment, the fraction of labeled RNA resistant in 2× SSC to nucleases rose from 2% before self-annealing to 5 to 10% after self-annealing and 60 to 70% after nuclease treatment. The RNA included in the G50 Sephadex was 28% resistant to RNase A. The excluded fraction was 90 to 95% resistant in the same salt concentration to RNase A, 93% resistant to digestion with RNase A + T_1, and 87% resistant to digestion with DNase followed by RNase A and T_1. The same RNA was only 1.7% resistant to RNase A in 0.1× SSC and only 1.8% resistant to the same enzyme in 2× SSC after denaturation in 0.1× SSC.

Demonstration of virus-specific DS RNA. DS RNA prepared as described above was tested for the presence of virus-specific RNA sequences. In the experiment summarized in Table 2, the DS RNA was denatured by heating in 0.1× SSC at 115 C for 5 min, then hybridized to HSV-1(F), *E. coli*, and HEp-2 DNA fixed to nitrocellulose filters as described in the footnote to Table 2. The data show that 32% of the labeled RNA hybridized to HSV-1(F) DNA bound to filters. This is probably a minimal estimate since a test of the fluid phase after hybridization showed that 16% of the labeled RNA in solution was resistant to digestion in 2× SSC with 50 μg of RNase A per ml for 30 min at 37 C. The DS RNA formed during the incuba-

TABLE 1. *Purification of DS RNA from HSV-1-infected cells*

Preparation tested	Nuclease	Salt	^{3}H-labeled RNA resistant to digestion with nucleases (%)	
			Native	Denatured[a]
Initial RNA	RNase A[b]	2× SSC	2	
After self-annealing (1st digestion)	RNase A[b]	2× SSC	5–10	
After RNase A + T_1 + DNase (2nd digestion)	RNase A[b]	2× SSC	60–70	
After G50 Sephadex chromatography				
Included material	RNase A[b]	2× SSC	27.5	3.7
Excluded material	RNase A[b]	2× SSC	90–94	1.8
	RNase A[b]	0.1× SSC	1.7	
	RNase A + T_1[c]	2× SSC	93	
	DNase[d]	RSB[e]		
	Followed by RNases A + T_1[c]	2× SSC	87	3

[a] Denatured by heating at 115 C for 5 min, then cooled quickly in dry ice.
[b] 50 μg of RNase A per ml, 30 min at 37 C.
[c] 50 μg of RNase A and 10 U of RNase T_1 per ml, 30 min at 37 C.
[d] 50 μg of ribonuclease-free DNase per ml, 1 h at 37 C.
[e] RSB, Reticulocyte standard buffer.

TABLE 2. *Hybridization on filters of viral DNA and HEp-2 DNA with denatured labeled DS RNA from infected cells*[a]

DNA on filter	DNA (μg)	Input (counts/min)	Counts/min bound to filter	Input bound (%)
None	0	5,436	4	0.072
E. coli	20	5,436	2	0.037
HEp-2	20	5,436	778	14.3
F	5	5,436	1,740	32

[a] Labeled DS RNA (G50 Sephadex-excluded fraction) prepared from infected cells as described in Materials and Methods was denatured by heating at 115 C in 0.1× SSC for 5 min and chilled in dry ice. HSV-1(F), HEp-2, and *E. coli* DNAs bound to filters were prepared as described and incubated together for 20 h at 66 C in 200 μl of hybridization buffer containing, in addition to the denatured RNA, 0.75 M NaCl, 5 mM EDTA, 0.2% sodium dodecyl sulfate and 0.01 M Tris (pH 7.5). The filters were washed, digested with RNase A (50 μg/ml, 2 h at room temperature in 2× SSC), washed again, and dried. Radioactivity was measured in a Packard scintillation spectrometer.

tion was therefore unavailable for hybridization to either host or viral DNA on filters.

Thermal denaturation profiles of DS RNA. Thermal denaturation profiles of DS RNA purified from infected and uninfected cells were based on susceptibility to RNase A in 2× SSC. Specifically, the G50-excluded fraction of DS RNA prepared from infected cells as described above and in the footnotes to Table 1, as well as from uninfected cells pulse labeled for the same length of time, were heated at various temperatures for 5-min intervals. Samples taken after heating at various temperatures were quickly chilled in dry ice, made 2× SSC by the addition of concentrated solution, and digested with RNase A (50 μg/ml, 30 min at 37 C). Corresponding DS RNA heated, but untreated with RNase, served as a control. Figure 1 shows the fraction of total labeled DS RNAs depolymerized by RNase A as a function of the temperature of denaturation. The thermal denaturation of virus-specific sequence in the infected cell DNA were analyzed in a similar fashion, but in this instance the denaturation was monitored by measuring the ability of the heated RNA to hybridize to viral DNA fixed to filters. Specifically, samples heated to various temperatures as above were quickly chilled in dry ice and hybridized to HSV-1(F) and *E. coli* DNAs bound to filters. The hybridization conditions were as those described in the footnote to Table 2. Figure 1 shows the fraction of the virus-specific sequences made available for hybridization to viral DNA as a function of temperature of denaturation. The data were corrected for nonspecific binding to filters by subtracting the counts bound to *E. coli* DNA and normalized with respect to the maximum amount of RNA hybridizing to DNA on filters observed after

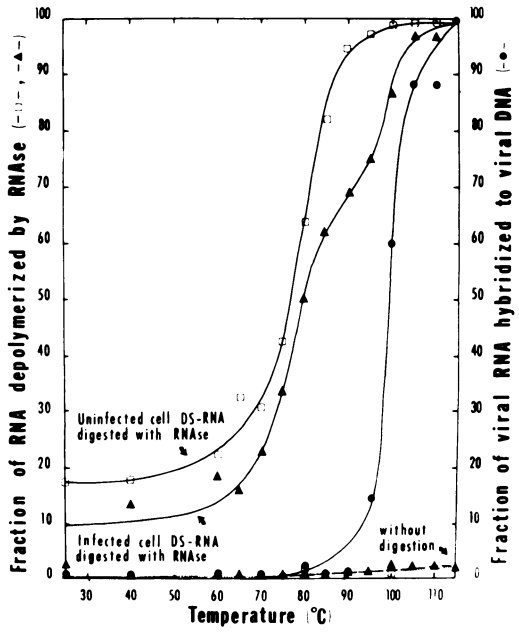

FIG. 1. *Thermal transition of self-annealed ^{3}H-labeled RNA from HSV-1-infected cells in 0.1× SSC. Thermal transition was measured by sensitivity to RNase A (▲ and □) in 2× SSC and by hybridization to viral DNA fixed to filters (●). Controls included RNA heated to indicated temperatures but not digested by nucleases.*

denaturation of the DS RNA at 115 C for 5 min. The DS RNA from infected cells shows three thermal transitions, i.e., one between 20 and 40 C, probably resulting from poorly matched base pairing, one at approximately 78 C, corresponding to that of uninfected cells, and one at approximately 100 C. The virus-specific sequences show one thermal transition with a T_m of approximately 100 C (Fig. 1).

Estimation of the fraction of viral DNA giving rise to DS RNA. In the preceding sections, we demonstrated that infected cells contain virus-specific DS RNA. The purpose of these experiments was to determine the fraction of the DNA from which the DS RNA arose. Unlike the preceding section, in which we used labeled DS RNA and hybridized it to DNA on filters, in the experiments described below we hybridized in solution excess unlabeled RNA to trace amounts of labeled DNA. This technique, described in detail by Frenkel and Roizman (6), allows direct estimate of the amount of DNA which served as a template to the RNA with which it hybridizes.

DS RNA was prepared from RNA extracted from 8-h infected cells as described above, but since the RNA was not labeled the purification was done as follows. The RNA was divided into two unequal portions. To the small portion were added small volumes of labeled viral DNA and RNA from 8-h infected cells. The two portions were then processed as described above except that RNA was diluted for RNase digestion and chromatographed on G50 Sephadex. The small portion served as a check for the completeness of digestion and purity of the DS RNA in the larger, unlabeled RNA portion. The hybridization tests were then done with the unlabeled DS RNA excluded from the G50 Sephadex column as described above. The results, summarized in Fig. 2A, show the following.

(i) Undenatured DS RNA hybridizes with at most 5% viral DNA. This could represent contaminating single-stranded viral RNA, or DS RNA with poorly matched base pairs, such that could arise from intrastrand base pairing of short segments.

(ii) Denatured DS RNA drove 55% of viral DNA into DNA-RNA hybrid. The labeled DNA hybridized in the presence of the same concentration of uninfected cell RNA reassociated to only 1% during the 8 h of hybridization required for the maximum R_0t values reached in this test.

(iii) To test the possibility that the DNA was driven into DNA-DNA hybrid by trace amounts of viral DNA contaminating the preparation, DS RNA at a concentration of 500 μg/ml was denatured at 115 C for 5 min, chilled, mixed with DNase-free RNase (50 μg/ml), and incubated for 2 h at 37 C. After digestion labeled viral DNA was added, the concentration of salt was increased to 0.3 M final concentration, and the mixture was allowed to hybridize to the R_0t value shown in Fig. 2. The amount of DNA in hybrid was found to be approximately 1%, i.e., the same as that reassociating in the absence of viral RNA.

(iv) The hybridization kinetics of denatured DS RNA with labeled viral DNA were analyzed by two different methods to determine whether all viral sequences in DS RNA were present at the same molar concentration. In the first, we applied the analytical treatment of Frenkel and Roizman (6). Specifically, if $\alpha_1 \ldots \alpha_n$ is the fraction of DNA serving as template for n classes of RNA, i.e., $R_1 \ldots R_n$ differing in abundance expressed in moles of RNA per liter, K is the hybridization rate constant, and D_v/D_0 is the fraction of DNA remaining single stranded, then the hybridization of excess nuclear RNA with trace amounts of labeled DNA, under conditions in which the reassociation of DNA is insignificant, is described by the following equation (6).

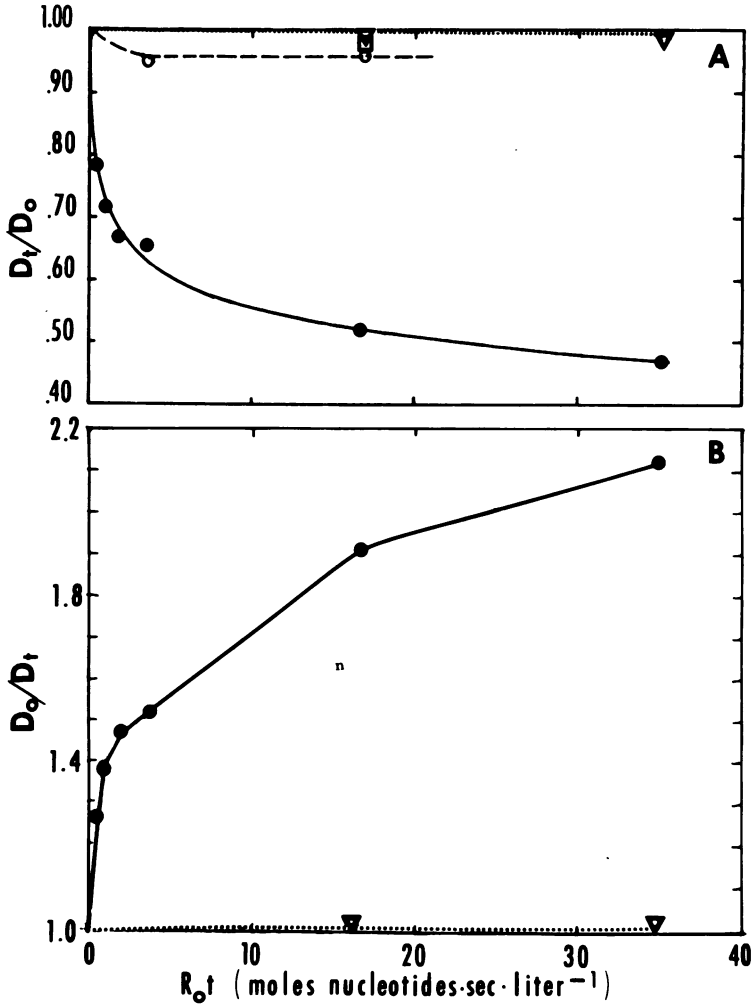

FIG. 2. *Hybridization of excess DS RNA to in vitro labeled, sheared, and denatured viral DNA. Symbols:* O, *Reassociation of viral DNA in the presence of undenatured DS RNA;* ●, *denatured DS RNA;* ▽, *reassociation of viral DNA in the presence of uninfected cell RNA during the same time interval as the experimental points;* □, *reassociation of viral DNA in the presence of denatured DS RNA depolymerized by RNase.* D_t/D_0, *Fraction of DNA remaining single stranded. The basis for the plots in A and B is stated in the text.*

$$\frac{D_t}{D_0} = \alpha_1 e^{-KR_1t} + \ldots \alpha_n e^{-KRnt} + 1 - (\alpha_1 \ldots \alpha_n)$$

The values $\alpha \ldots \alpha_n$ and the ratios of R_1 to $R_2 \ldots R_n$ were estimated by determining the best fit of a nonlinear regression of D_t/D_0 against t for $n = 1, 2, 3$, etc. Analysis of the data by this method shown in Fig. 2A indicates that viral DS RNA consisted of at least two components. The abundant component arose from 28.7% of viral DNA and was 40 times more abundant than the scarce component complementary to 26.0% of viral DNA. One critique of the application of this anslysis is that the equation is based on the assumption that the concentration of single-stranded RNA remains unchanged during hybridization, i.e., that the concentration of RNA in hybrid is small compared to that of the single-stranded RNA left. Since the RNA sequences are derived from denatured DS RNA, which probably reassociates during the hybridization, the validity of this assumption is in doubt.

In the second analytical procedure, we assumed that the hybridization of labeled viral DNA serves as an indicator and reflects the reassociation of unlabeled symmetric viral RNA sequences. The relation which best describes this situation, again under conditions in which the reassociation of DNA is insignificant, is a

modification of Britten's (4) second-order equation for the reassociation of DNA.

$$\frac{D_t}{D_0} = \frac{1}{1 + KR_0t} \text{ or } \frac{D_0}{D_t} = 1 + KR_0t$$

The equation predicts that if all viral RNA sequences present in DS RNA were equimolar in concentration, a plot of D_0/D_t against R_0t would yield a straight line with an intercept of 1. This is obviously not the case. The plot of the data shown in Fig. 2B indicates that the DS RNA contains at least two components, of which the most abundant hybridizes with approximately 32% of DNA. Application of mathematical analyses to be dealt with elsewhere (N. Frenkel, B. Cox and B. Roizman, manuscript in preparation) indicate that this component is 70-fold more abundant than the scarce species.

DISCUSSION

The salient features and significance of the results described in this paper may be summarized as follows.

(i) Infected cells accumulate RNA capable of self-annealing. Analyses of several properties of the product of self-annealing, notably its resistance to depolymerization by nucleases in high salt and sensitivity to these nucleases in low salt and upon heat denaturation, indicate that it conforms with properties of DS RNA (3).

(ii) Excess unlabeled, denatured DS RNA drove slightly more than 50% of labeled viral DNA into DNA-RNA hybrid under conditions in which the amounts of DNA-DNA hybrid formed were insignificant. Moreover, analyses of the hybridization kinetics indicated that the viral RNA sequences were nonhomogeneous and consisted of at least two components differing in molar concentration.

(iii) The abundant class of symmetric transcripts is of particular interest from two points of view. First, the measurements of the T_m of the viral DS RNA were done by heating labeled DS RNA and hybridizing it to DNA on filters. In principle, hybridization of labeled RNA in solution to DNA fixed on filters measures primarily abundant RNA species and it is likely, therefore, that the T_m measurements apply to the abundant symmetric transcripts which arise from at least 28 to 32% of viral DNA. Application of the equation of Billeter et al. (3), within the range of values to which the equation is applicable and assuming that the linear relationship between base composition at melting point observed for other DS RNAs can be extrapolated to HSV-1 RNA as well, predicts that the guanine plus cysteine content of HSV-1 DS RNA is only slightly higher than the average guanine plus cysteine content of HSV-1 DNA (Fig. 3). Second, the estimate that the abundant DS RNA arises from at least 29% of viral DNA is significant in reference to the observation (11) that preannealed nuclear RNA lost the capacity to drive 15% of DNA into DNA-RNA hybrid but that denaturation of the preannealed RNA fairly restored the ability of the RNA to drive the DNA into hybrid. There were two possible explanations for this observation (11). One postulated that complementary transcripts arose from 15% of the DNA; i.e., sequences derived from 7.5% of each DNA strand accumulate at nearly equimolar concentrations in the infected cell nuclei. The alternative was that complementary RNA sequences were derived from 15% of each DNA strand, but the transcripts arising from one strand were present at a lower concentration than the corresponding RNA sequences derived from the opposite strand. Preannealing would sequester the less abundant RNA in an RNA-RNA hybrid, but the complementary sequences would remain available for hybridization with DNA, although their concentration would have been reduced. The data presented in this paper appear to discriminate between the two hypotheses and support the second. They would indicate that the transcripts annealing to make the DS RNA do not accumulate in equimolar concentrations in the infected cells.

(iv) We have little information on the nature

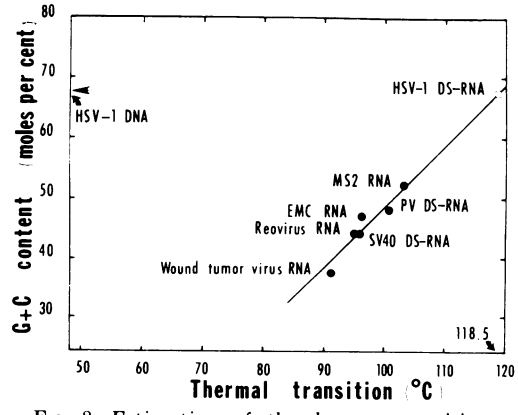

FIG. 3. *Estimation of the base composition of HSV-1 DS RNA from its thermal transition. The thermal transition of HSV-1 DNA in 1× SSC was calculated from the equation of Billeter et al. (3) and plotted on the line derived from the linear regression of the relationship between base composition and thermal transition in 1× SSC for DS RNA of other viruses (3). PV, Polyomavirus; EMC, encephalomyocarditis virus.*

of the scarce DS RNA species. We cannot exclude the possibility that this RNA arises by intrastrand base pairing even though the RNA is excluded from G50 Sephadex columns, is stable to prolonged incubation at 20 C below the T_m of DS RNA, and shares with the abundant species the ability to resist digestion by RNase in 2× SSC.

(v) The accumulation of symmetric transcripts arising from at least 29% and possibly from as much as 55% of the DNA suggests that transcription of HSV-1 DNA is largely symmetrical and implies the existence of a post-transcriptional mechanism operating in the nucleus for discrimination of transcripts giving rise to mRNA from those derived from the opposite strand of DNA. This conclusion is supported by the observation that only trace amounts of viral RNA sequences accumulating in the cytoplasm become unavailable to hybridization with viral DNA upon self-annealing (11) and that viral RNA sequences which are not translated in polyribosomes of infected cells are selectively retained in the nucleus (10).

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