RNA Synthesis in Cells Infected with Herpes Simplex Virus

VII. Control of Transcription and of Transcript Abundancies of Unique and Common Sequences of Herpes Simplex Virus 1 and 2

NIZA FRENKEL, SAUL SILVERSTEIN, ENZO CASSAI, AND BERNARD ROIZMAN

Department of Microbiology, The University of Chicago, Chicago, Illinois 60637

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Analysis of the kinetics of hybridization in liquid of labeled herpes simplex virus (HSV) 1 and 2 DNAs with excess unlabeled RNA extracted at 2 (early) and 8 (late) h postinfection revealed the following. (i) The RNA transcripts present in the HSV-1-infected cells at 2 and 8 h postinfection are complementary to 44 and 48% of HSV-1 DNA. The RNA transcripts present in the HSV-2-infected cells at 2 and 8 h postinfection are complementary to 21 and 50% of HSV-2 DNA. (ii) The transcripts present in 2-h HSV-1- or HSV-2-infected cells treated with cycloheximide are complementary to 44 and 45% of the respective DNAs. (iii) The RNA transcripts present in the HSV-1-infected cells at 2 h postinfection and in HSV-2-infected cells at 8 h postinfection form 2 classes, abundant and scarce, differing in molar concentrations. The RNA transcripts present in the HSV-2-infected cells at 2 h postinfection form only one abundance class. (iv) The transcripts present in the HSV-1-infected cells at 8 h postinfection are complementary to 24% of HSV-2 DNA and therefore 50% of the transcribed HSV-1 sequences are shared by the two viruses. Of the RNA sequences complementary to HSV-2 DNA, 13% arise from HSV-1 templates specifying abundant RNA and 11% arise from HSV-1 templates specifying scarce RNA. Thus, the DNA sequences shared in common by HSV-1 and HSV-2 DNAs constitute 71% of the HSV-1 templates specifying abundant RNA and 39% of sequences specifying scarce RNA.

Herpes simplex viruses (HSV) 1 and 2 are closely related genetically (9, 11). In a preceding paper of this series (5) we described a novel technique for analysis of transcriptional programs and we applied it to analysis of the transcription of HSV-1. The techniques involve hybridization of labeled DNA with excess RNA in a liquid phase. The parameters amenable to analysis by this technique are (i) the total amount of DNA sequences transcribed during the course of infection, (ii) the number of RNA classes differing in molar concentrations and their molar ratios, and (iii) the amount of DNA serving as templates for each class of RNA. The techniques were applied to analyses of HSV-1 infection of human epidermoid carcinoma (HEp-2) cells. In this paper we extended the analysis to the transcriptional program of HSV-2 DNA and of sequences shared by HSV-1 and HSV-2 DNAs.

MATERIALS AND METHODS

Cells and virus. All experiments were done in HEp-2 cells. The general procedures for maintenance and infection of these cells have been described elsewhere (15). An isolate from a facial infection (3) passaged at low (>0.01 PFU/cell) multiplicity four times and designated as the F strain served as a prototype of HSV-1. An isolate from a genital infection (3) passaged in a similar fashion and designated as the G strain served as a prototype of HSV-2. In addition, we also tested strain 174 of HSV-2 isolated by Maurizio Terni at the University of Ferrara, Ferrara, Italy. The G and 174 strains cannot be differentiated with respect to any of the properties relevant to the results presented here. The general properties of HSV-1 and HSV-2 prototypes have been reported in several publications from this laboratory (6, 8; B. Roizman, P. G. Spear, and E. D. Kieff, *Persp. Virol.*, in press).

Preparation of labeled HSV-1 and HSV-2 DNA. The procedure for the preparation of ^3H -thymidine-labeled HSV-2 DNA was the same as that described

previously. The DNA produced in this fashion has a mol wt of 100 million (8) and is free of host DNA sequences (9). The final product was dialyzed extensively against 0.05 M Tris (pH 8.05) and 0.07 M NaCl, and was sheared in a French press at 18,000 lb/in². The specific activities of the DNAs used in these studies ranged from 70,000 to 100,000 counts per min per μ g.

Extraction of RNA from infected cells. RNA was extracted from infected cells by the method of Lee et al. (10) to permit maximal recovery of HSV-RNA covalently linked to poly A sequences (2), then digested with DNase (RNAse free, Worthington Biochemical Corp., Freehold, N.J.), and was phenol extracted repeatedly. The final product was dialyzed extensively against 0.05 M Tris (pH 8.05) and 0.07 M NaCl. The RNA concentrations used in the hybridization tests varied from 0.2 to 14 mg/ml.

Hybridization tests. The hybridization tests were done in 0.05 M Tris (pH 8.05) and 0.07 M NaCl in 100- μ liter micropipettes sealed at both ends and immersed in ethylene glycol baths maintained at temperatures 25 C below the melting temperature of the DNAs, as previously described (5). After hybridization, the hybridization mixture was treated with single-stranded specific nucleases. Included in each experiment were mixtures containing known amounts of denatured and native DNA mixed with RNA extracted from uninfected cells; these served as controls for the nuclease digestion. In the initial experiments we used two nucleases, i.e., shark liver endonuclease (1) followed by *Neurospora crassa* nuclease (12). In latter experiments *Neurospora crassa* nuclease was used alone with identical results, i.e., under the conditions it was used the enzyme did not attack native DNA and digested more than 99% of the denatured, single-strand DNA. The appropriate control experiments were published elsewhere (5).

Experimental design. The RNA was extracted from infected cells at 2 and at 8 h postinfection. The time intervals were selected on the basis of observations that viral DNA synthesis begins roughly at 3 h postinfection and that infectious progeny appears at 6 h postinfection and is present in appreciable levels at 8 h (14). The 2-h RNA should therefore contain the bulk of the RNA made before viral DNA synthesis, whereas the 8-h RNA should contain all of the species made late in infection and required for the synthesis of infectious progeny.

As described elsewhere in detail (5), the kinetics of hybridization of labeled DNA with excess unlabeled RNA under conditions which preclude the formation of appreciable amounts of DNA-DNA hybrids are predicted by the following equation:

$$Dt/Do = \alpha_1 \cdot e^{-kR_1t} + \dots \alpha_n \cdot e^{-kR_nt} + 1 - (\alpha_1 + \dots \alpha_n)$$

where Dt/Do is the fraction of DNA remaining single stranded; subscripts 1 $\dots$ n refer to classes of RNA differing in molar concentrations; $R_1 \dots R_n$ represents RNA concentrations in moles per liter; and $\alpha_1 \dots \alpha_n$ are corresponding fractions of the total DNA serving as templates for the classes of RNA differing in abundance. The parameters n, $\alpha_1 \dots \alpha_n$, and $R_1 \dots R_n$ were determined from nonlinear least

squares regression of Dt/Do against t, calculated by a computer instructed to test the fitness of n = 1, 2, 3, etc.

RESULTS

Transcriptional program of HSV-1 and HSV-2 in productively infected cells. The results of the hybridization tests are shown in Fig. 1. The computed parameters n, $\alpha_1 \dots \alpha_n$, and $R_1 \dots R_n$ are summarized in Table 1. The salient features of the data may be summarized as follows.

FIG. 1. Hybridization of labeled HSV-1 DNA with excess RNA extracted from HEP-2 cells at 2 and 8 h postinfection. Since variable amounts of RNA were hybridized for different time intervals as described in the text, the data are presented as a plot of the fraction of single-stranded DNA as a function of the input concentration of RNA (R_0) in moles of nucleotides per liter times the time of hybridization (t). Experimentally determined points are shown as circles. The line is a computer plot calculated by the equation given in Materials and Methods and fitted by nonlinear least squares method. The computer analysis involved the determination of parameters α_n and R_n in equation V in which the number of classes of RNA (n) was assumed to be 1, 2, 3, 4, etc. The fit for n = 2 was better than that for n = 1. When n was greater than 2, the results were meaningless. Thus, for the hybridization of RNA extracted at 2 h postinfection, for n = 3 α_1 was less than 0.001, whereas α_2 and α_3 retained the same values as α_1 and α_2 in the case of n = 2. Similar results were obtained with RNA extracted from 8-h infected cells.

TABLE 1. *Analysis of the transcripts of HSV-1 and HSV-2 DNA in productively infected HEp-2 cells at 2 and 8 h postinfection*

Virus	Time of extraction (h post infection)	Fraction of viral DNA transcribed			RNA abundance		
		a_1	a_2	Total	R_1^a nm/liter	R_2^a nm/liter	R_1/R_2
HSV-1	2	0.14	0.30	0.44	7.9	0.058	136.2
	8	0.19	0.28	0.48	7.1	0.183	40.3
HSV-2	2	0.21	0.0	0.21	0.51	0.0	
	8	0.31	0.19	0.50	2.23	0.28	7.96

^a Moles of viral RNA fragments of $0.99 \times 10^6 \times a_n$ mol wt.

(i) As we previously reported, the virus-specific transcripts present in RNA extracted from HSV-1-infected cells at 2 and 8 h postinfection were complementary to 44 and 48% of total HSV-1 DNA, respectively (Fig. 1). The virus-specific transcripts present in RNA extracted from HSV-2-infected cells at the same time intervals postinfection were complementary to 21 and 50% of the total HSV-2 DNA, respectively. The same results were consistently obtained with several preparations of HSV-1 RNA and with RNA extracted from cells infected with two different prototypes of HSV-2. The transcriptional programs of HSV-1 and HSV-2 prototypes tested differ in the extent of transcription of their DNAs at 2 h postinfection.

(ii) Analysis of the kinetics of hybridization (Table 1) indicated that RNA transcripts in RNA extracted from HSV-1-infected cells at 2 and 8 h postinfection and RNA extracted from HSV-2-infected cells at 8 h postinfection each comprised at least two classes differing in molar concentration. HSV-1 RNA extracted at 2 h postinfection comprised two classes of RNA complementary to 14 and 30% of viral DNA, respectively. The most abundant class complementary to 14% of the DNA was present in a 136-fold molar excess over the scarce RNA complementary to 30% of the DNA. At 8 h postinfection, HSV-1 RNA transcripts formed two classes of RNA, abundant and scarce, complementary to 19 and 28% of viral DNA, respectively, and differing 40-fold in molar ratio. HSV-2 RNA extracted from 8-h infected cells also formed two classes of RNA, abundant and scarce, complementary to 31 and 19% of viral DNA, respectively, and differing only eightfold in molar ratio. By contrast, the virus-specific transcripts contained in RNA extracted from 2-h infected cells were complementary to 21% of viral DNA and formed only one abundance class. Pertinent here is the observation that analyses of several preparations of RNA

extracted from both HSV-1- and HSV-2-infected cells indicate that the number of classes (n) and the size of DNA template complementary to each class resolved with this technique are highly reproducible. On the other hand, the absolute molar amounts of viral RNA in each class and, hence, the molar ratios of RNA differed somewhat from preparation to preparation. We do not know at present whether the amount of RNA transcripts in each abundance class reflects differences in the recovery of RNA in the course of the extraction procedure or the physiological condition of the cell.

Transcription of HSV-1 and HSV-2 DNA in the presence of inhibitors of protein synthesis. The difference between the transcriptional programs of HSV-1 and HSV-2 are so striking that they raise questions as to whether the RNA extracted from 2-h infected cells is indeed early RNA, i.e., RNA made before the onset of viral DNA synthesis. The problem arises from the fact that determinations of the onset of DNA synthesis are imprecise; the experimental determination hinges on the ability to detect trace amounts of viral DNA. The alternatives open to us were to test the transcripts made in the presence of inhibitors of either DNA or protein synthesis. The latter alternative is based on observations that viral DNA synthesis requires prior protein synthesis (14) and is not initiated in cells treated with inhibitors of protein synthesis from the time of exposure of cells to virus. Of the two alternatives, we choose the inhibition of protein synthesis largely because in our hands most inhibitors of DNA synthesis reduce but do not completely inhibit DNA synthesis, whereas the inhibition of protein synthesis appears to be almost complete.

In these experiments, HEp-2 cells were infected in the presence of 100 μ g of cycloheximide per ml of medium and maintained in the presence of the drug for the entire duration of the experiment. This concentration of cyclohex-

imide reduced amino acid incorporation by 99% during 2, 5, and 6 h of labeling relative to untreated controls. The RNA was extracted at 2 h postinfection and hybridized with labeled viral DNA. The results of the hybridization tests shown in Table 2 indicated that the transcription of HSV-1 at 2 h postinfection was not dependent on protein synthesis and, hence, the RNA extracted from 2-h infected cells contained only transcripts made before the onset of DNA synthesis. However, the most striking observation was that the RNA extracted from cycloheximide-treated cells at 2 h postinfection with HSV-2 cycloheximide was complementary to 45% of HSV-2 DNA, i.e., considerably more than the RNA extracted from the untreated, 2-h infected cells.

Transcription of unique and common sequences of HSV-1 and HSV-2 DNA. In these experiments RNA extracted from 8-h HSV-1-infected cells was hybridized with labeled HSV-2 DNA. The results (Fig. 2; Table 3) may be summarized as follows.

(i) Comparisons of the hybridization of RNA extracted at 8 h from HSV-1-infected cells with homologous and heterologous DNA indicate that HSV-1 RNA is complementary to 24% of the HSV-2 DNA. Since the same RNA hybridized with 48% of HSV-1 DNA, we conclude that 50% of the transcribed HSV-1 sequences are shared by HSV-2. Assuming that transcription is asymmetrical (i.e., either only one strand or alternate regions of both strands are transcribed), almost all (96%) of HSV-1 genetic information is transcribed. It follows that the extent of homology between HSV-1 and HSV-2 DNA is 48 to 50%, i.e., the same value as that obtained from DNA-DNA hybridization (9).

(ii) In the preceding sections we have shown that the transcribed HSV-1 DNA contained two sets of templates complementary to abundant and scarce species of virus-specific RNA, respectively. It was of interest, therefore, to determine the distribution of DNA sequences shared in common among these two sets of templates. These calculations are based on two considerations. First, since the same RNA was hybridized to the homologous and heterologous DNAs, we calculated first the molar concentrations (R_1 and R_2) of the abundant and scarce HSV-1 RNA and used these to obtain the fractions (α_1 and α_2) of HSV-2. Second, the thermal elution profiles of sheared HSV-1 DNA—HSV-2 DNA heterohybrids (9) showed a uniform matching of base pairs even though it was somewhat lower than those of homohybrids. We conclude from this finding that the matching of base pairs of HSV-1 RNA—HSV-2 DNA hybrids should also

TABLE 2. *Effect of cycloheximide on the extent of transcription of HSV-DNA early in infection^a*

Virus	Treatment	Percent of total DNA transcribed	R_{st}^b
HSV-1	None	44	2,350
	Cycloheximide	44	2,500 ^c
HSV-2	None	21	800–2,000 ^c
	Cycloheximide	45	3,020

^a The cycloheximide in concentrations of 100 μ g of medium per ml was present during and after exposure of cells to virus. The hybridization procedures are described in Materials and Methods.

^b The product of RNA concentration (R_0) in moles of nucleotide per second and time of hybridization to which the hybridizations were carried out.

^c No further hybridization occurred in this range.

Fig. 2. Hybridization of HSV-2 DNA with excess RNA extracted from cells 8 h postinfection with HSV-1. The procedure for the hybridization, etc., were the same as described in the legend to Fig. 1 except that the hybridization was carried out at a temperature of 33 C below the melting temperature of the HSV-2 DNA. The solid line is the computer plot for homologous DNA-RNA hybridization shown in upper panel of Fig. 1. The dashed line is the computer plot for the heterologous hybridization calculated according to the equation given in Materials and Methods except that the values of R_1 and R_2 were taken to be the same as those for 8-h RNA reactive in the homologous DNA-RNA hybridization (Fig. 1).

be uniform. On the basis of these considerations we calculated (Table 3) that 13% of HSV-2 DNA is complementary to HSV-1 RNA of high abundance. Correspondingly, 11% of HSV-2 DNA is complementary to HSV-1 RNA of low abundance. However, the HSV-1 DNA sequences shared in common by HSV-1 and HSV-2 DNA represent 71% of the HSV-1 templates specifying abundant RNA and only 39% of sequences specifying scarce RNA.

DISCUSSION

Comparison of the transcriptional programs of HSV-1 and HSV-2. As summarized in Table 4, the transcriptional programs of HSV-1 and HSV-2 share two features in common. Both exhibit "off-on" controls of transcription and controls of RNA abundance described previously (5). They differ, however, in detail and, more specifically, in four major respects as follows: (i) the extent of DNA transcribed at 2 h postexposure of cells to virus, i.e., prior to the onset of DNA synthesis; (ii) the composition of RNA transcripts and, more specifically, the number of classes of RNA templates differing in molar ratios; (iii) the size of the templates specifying abundant RNA; and (iv) the extent of DNA transcribed in 2-h infected cells treated with cycloheximide.

Three points should be made in connection

TABLE 3. *Complementary of HSV-1—HSV-2 DNAs serving as templates for abundant and scarce RNA species*

Fraction of total DNA hybridizing with 8-h HSV-1 RNA	HSV-1 DNA	HSV-2 DNA	HSV-2/HSV-1
<i>a</i> ^a	19	13	0.71
<i>a</i> ^b	28	11	0.39

^a Fraction of DNA complementary to RNA of high abundance (see footnotes, Fig. 1).

^b Fraction of DNA complementary to RNA of low abundance.

with these data. The first point arises from the observation that early in infection, i.e., before the onset of DNA synthesis, HSV-1 and HSV-2 DNAs are transcribed to 44 and 21%, respectively. However, in the presence of cycloheximide, which inhibits protein synthesis, this difference disappears and both DNAs are transcribed to the extent of 44 to 45%. These observations indicate the following. (i) 44 to 45% DNA or, assuming the transcription is asymmetrical, 88 to 90% of the informational content of the DNA is transcribed in the absence of protein synthesis. The results indicate that the bulk of the DNA is transcribed either by a preexisting host enzyme or by an enzyme introduced into the cell by the virus. The recent finding that naked DNA is infectious (P. Sheldrick, personal communication) would favor the hypothesis that the virus is transcribed by a host enzyme. (ii) The mechanism by which treatment with cycloheximide results in an apparent increase in the amount of transcribed HSV-2 DNA is not clear. The possibilities are as follows. First, the transcription of the 24% of DNA, i.e., the difference between 45 and 21% transcribed in treated and untreated infected cells, respectively, could be regulated by a negative control which involves proteins made after infection. This regulatory function could either be absent in HSV-1-infected cells or it could occur very early, i.e., before 2 h postinfection. The second possibility is that 24% of HSV-2 DNA is transcribed and the transcripts are very rapidly degraded. Parenthetically, the

TABLE 4. *Summary of the transcriptional programs of HSV-1 and HSV-2 in productively infected HEP-2 cells*

Features of the program	HSV-1	HSV-2
Early (2 h postinfection)		
Percent of DNA transcribed (total)	44	21
Number of RNA classes differing in abundance	2	1
Percent of DNA specifying abundant class	14	21
Percent of DNA specifying scarce class	30	
Percent of DNA transcribed in the presence of cycloheximide	44	45
Late (8 h postinfection)		
Percent of DNA transcribed (total)	48	50
Number of RNA classes differing in abundance	2	2
Percent of DNA specifying abundant class	19	31
Percent of DNA specifying scarce class	28	19
Percent of DNA hybridizing with RNA from 8-h infected cell	48	24
Percent of DNA hybridizing with abundant RNA	19	13
Percent of DNA hybridizing with scarce RNA	28	11
Percent of HSV-1 DNA template specifying abundant HSV-1 RNA shared in common by HSV-1 and HSV-2 DNAs	100	71
Percent of HSV-1 DNA template specifying scarce RNA shared in common by HSV-1 and HSV-2 DNAs	100	39

effects of cycloheximide noted in our studies differ materially from those deduced by Rakusanova et al. (13), who concluded that the RNA made in the presence of cycloheximide was complementary to a smaller template than that made "early." We cannot account for the differences in our respective conclusions except to point out that their results could also be interpreted as indicating that transcripts accumulate in larger amounts in the presence of the drug and that techniques involving hybridization in liquid of excess unlabeled RNA with labeled DNA is free of errors due to ribonucleotide pool size and is more amenable to analytical treatment than hybridization tests involving labeled RNA in liquid to DNA bound to filters.

The second point concerns the number of "on" controls in the transcriptional program of HSV-1 and HSV-2 DNAs. Evidence for one "on" control arises from observations that early in infection the extent of transcription of HSV-1 DNA is 44% and that HSV-2 DNA is transcribed to the same extent in the presence of cycloheximide. Yet, late in infection both HSV-1 and HSV-2 DNAs are transcribed to 48 to 50%. The difference between 44% of HSV-1 DNA transcribed early in infection and the 48 to 50% transcribed late is significant particularly since it is reproducible, and, as reported previously (5), it corresponds roughly to the amount of template required for the synthesis of several structural proteins made late in infection. As pointed out earlier, a second "on" control may be operative at 2 h postinfection in HSV-2-infected cells and either much earlier or not at all in HSV-1-infected cells. The existence of a second "on" control requires further verification. Parenthetically, a second "on" control limiting transcription to a relatively small region of HSV-2 DNA would help explain the observed transcription of 5% of HSV-2 DNA in the cervical carcinoma tissue, even though 39% of the genome was present (7) and could explain the survival of the cell in nonproductive infections in which few viral functions may be expressed.

Lastly, the abundant HSV-1 RNA extracted late in infection is transcribed off 19% of HSV-1 DNA. Several lines of evidence cited elsewhere in detail (5) led us to suggest that, in HSV-1-infected cells, the abundant RNA specifies structural proteins. The conclusions were based in part on the size of the DNA template required for structural proteins, their abundance relative to nonstructural proteins, and analyses of the structural proteins made at different times in the reproductive cycle. By contrast, the late abundant HSV-2 RNA is

complementary to 31% of HSV-2 DNA. Since HSV-1 and HSV-2 DNA are closely related, it is not likely that HSV-2 structural proteins require 50% more DNA template (31% of total) than the HSV-1 structural proteins (19% of total). One possible explanation is that some nonstructural proteins as well as structural proteins may be specified by abundant RNA species. Another possible explanation of this discrepancy arises from the observation that HSV-1 DNA consists largely (at least 95%) of unique DNA sequences (4). By contrast, both the G and 174 prototypes of HSV-2 isolated and propagated a continent apart contained repetitive sequences (7). The nature of the functions specified by the repetitive sequences are now known; they could be a subset of the sequences specifying the abundant RNA late in infection.

Transcription of DNA sequences common to HSV-1 and HSV-2. The transcription of the DNA sequences shared in common by HSV-1 and HSV-2 are of particular interest from the following point of view. In a preceding paper of this series, we listed several lines of evidence which suggested that the HSV-1 template for abundant RNA specifies the structural proteins of the virus. The evidence was based on the amounts of DNA required for the structural proteins based on their cumulative molecular weight, on pulse-chase experiments which indicated that 20 out of 24 structural proteins of the virus were made and correspond in cumulative molecular weight to the amount of DNA which served as template for abundant RNA early in infection, and on the observation that the cumulative molecular weight of the structural proteins made in infection corresponded to the amount of DNA which served as template for abundant RNA and was transcribed late but not early. In the present study we have shown that, although the shared sequences are divided among the templates for abundant and scarce RNA, those contained in the template for abundant RNA constitute 71% of that set. If our interpretation of the function of the abundant RNA species is correct, it would follow that, in the course of evolution of HSV-1 and HSV-2 from a common ancestor, the sequences coding structural proteins diverged less, on the average, than those specifying nonstructural proteins.

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