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Ribonucleic Acid Synthesis in Cells Infected with Herpes Simplex Virus: Controls of Transcription and of RNA Abundance*

(hybridization/nucleases/mammalian cells)

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ABSTRACT **Analysis of the kinetics of hybridization in liquid of labeled herpes simplex virus-1 DNA and excess viral RNA revealed the following: (*i*) Cells infected by herpes simplex virus-1 for 2 hr (before DNA synthesis) contain two classes of RNA molecules differing 140-fold in molar concentrations. The abundant and scarce RNAs are transcribed from 14 and 30% of the DNA, respectively. RNA extracted at 8 hr after infection (late RNA) also contains abundant and scarce classes differing 40-fold in molar concentrations; these are transcribed from 19 and 28% of viral DNA, respectively. Abundance competition hybridization tests indicate that the abundant RNA at 2 hr is a subset of the 8-hr abundant RNA. (*ii*) The abundant RNAs probably specify structural proteins, as indicated by estimates of DNA template required for structural proteins and by experiments showing that 19 of 24 proteins (corresponding to 68% of genetic information for structural proteins) are already made between 0.5 and 2 hr after infection. We conclude that there are two types of transcriptional controls, i.e., on-off and abundance controls, and that the synthesis of most structural components is an early viral function.**

In this paper, we are reporting a procedure for measuring the amounts of viral DNA transcribed, the number of viral RNA classes differing in abundance, and the molar ratios of the RNA differing in abundance. We are also reporting a new procedure for RNA abundance competition to indentify whether RNA species of specific abundance present in cells at different times after infection are transcribed from the same DNA template. The techniques are based on kinetics of conversion of single-stranded DNA fragments to DNA–RNA hybrids under carefully controlled conditions. We have applied these techniques to the study of the transcriptional program of herpes simplex virus subtype 1 (HSV-1) in human epidermoid carcinoma (HEp-2) cells.

Pertinent to the understanding of the experimental designs used are the following data: (*i*) HSV-1 DNA are linear double-stranded molecules of molecular weight $(99 \pm 5) \times 10^6$ (1). Reassociation kinetics indicated the absence of appreciable repetitive sequences and yielded an estimate of $(95 \pm 1) \times 10^6$ daltons for the sum of unique sequences (2). (*ii*) Viral DNA synthesis begins about 3 hr after infection (3); viral progeny can be seen at 4 hr and are present in appreciable amounts at 6 hr after infection (3). RNA extracted at 2 hr after infection (i.e., after exposure to virus) should contain predominantly RNA made before the onset of DNA synthesis, whereas viral RNA extracted at 8 hr after infection should contain all the species synthesized after the onset of DNA synthesis and required for virus maturation.

Abbreviations: HSV-1, herpes simplex virus subtype 1; HEp-2 cells, human epidermoid carcinoma cells.

* Paper no. V in the series. No. IV is ref. 22.

MATERIALS AND METHODS

Cell, Virus, and Infection. The procedure for propagation and maintenance of HEp-2 cells and the pertinent properties of HSV-1 have been reported elsewhere (1, 3–5). In the experiments described here, HEp-2 cells were infected with a multiplicity of 8 plaque forming units per cell.

Analysis of Virion Proteins. The procedure for (*i*) purification of the herpes virion, (*ii*) the solubilization and high resolution polyacrylamide gel electrophoresis of viral proteins, and (*iii*) fixation, staining, and autoradiography of the polyacrylamide gels were described elsewhere (4).

DNA Preparation. The procedure for preparation of intact viral DNA from cytoplasmic virions was published (1). For these experiments, DNA was treated with 0.3 N NaOH for 6 hr to hydrolyze residual RNA, sheared by sonication, and dialyzed against 0.05 M Tris·HCl (pH 8.05)–0.07 N NaCl.

Viral DNA prepared by this method is free of host DNA as determined by isopycnic banding in the model E centrifuge and by hybridization with excess host DNA (6). It has a kinetic complexity of $(95 \pm 1) \times 10^6$ daltons (2).

RNA Purification. RNA was extracted from whole infected cells at 2 and 8 hr after infection by the method of Lee *et al.* (7) to ensure recovery of HSV–RNA molecules covalently bound to poly(A)sequences (8). RNA obtained by this method was digested with DNase, extracted with phenol and chloroform–isoamyl alcohol, and precipitated with ethanol. This process was repeated once more. After the second ethanol precipitation the RNA pellet was solubilized in, and dialyzed against, 0.07 N NaCl–0.05 M Tris·HCl (pH 8.05).

Hybridization. For hybridization, 143 ng of sonicated labeled viral DNA were mixed with various amounts of RNA, and brought to 0.1 ml volume containing a final concentration of 0.05 M Tris·HCl (pH 8.05) (room temperature)–0.07 N NaCl. The mixture was sealed in 100-μl micropipettes, immersed for 7 min in an ethylene glycol bath heated to 115°, then incubated at 66° (25° below the T_m). The time of incubation varied but did not exceed 26 hr.

The buffer system was selected to permit digestion of the DNA–RNA mixture after hybridization by nucleases specific for single strands without prior dialysis. At the temperature used for hybridization, the pH of the solution was 7.4.

Enzymatic Digestion of Hybridization Mixture. The hybridization mixture was digested with shark-liver endonuclease (9)

and *Neurospora crassa* nuclease (10) to differentiate between the residual single-strand DNA and the DNA in hybrid form. The specificity of these enzymes for single-stranded DNA as opposed to DNA–DNA and DNA–RNA hybrids has been reported (9–11). The enzymatic digestion consisted of two steps. In the first step, 100 μl of hybridization mixture was diluted in appropriate solution so as to yield 300 μl containing 0.02 M Tris·HCl (pH 8.05)–0.12 M NaCl–1.5 mM $MgCl_2$. Two aliquots of 40 μl each were removed for precipitation with trichloroacetic acid to determine the amount of total labeled DNA. A third aliquot containing 200 μl was mixed with 1 unit of shark-liver endonuclease and incubated first for 1 hr at 37° to allow digestion and then for 25 min at 60° to inactivate the enzyme. In the second step, the reaction mixture was diluted with 200 μl of appropriate solution to yield 400 μl containing 0.1 M Tris·HCl (pH 8.05)–10 mM $MgCl_2$, 60 mM NaCl and 1 unit of *N. crassa* nuclease. The mixture was incubated at 37° for 2 hr, then precipitated with trichloroacetic acid. The shark-liver enzyme was inactivated because, at the lower NaCl concentrations suitable for *N. crassa* nuclease, it is less specific and attacks both single-stranded and double-stranded DNA (9). The specificity of the nucleases for single-stranded DNA under our test conditions is shown in Fig. 1. Briefly, denatured and native labeled DNAs were mixed in various proportions, then digested with the enzymes in the presence of 400 μg of yeast RNA as described above. The data show that the enzymatic digestion is specific, complete, and unaffected by yeast RNA at the concentrations tested.

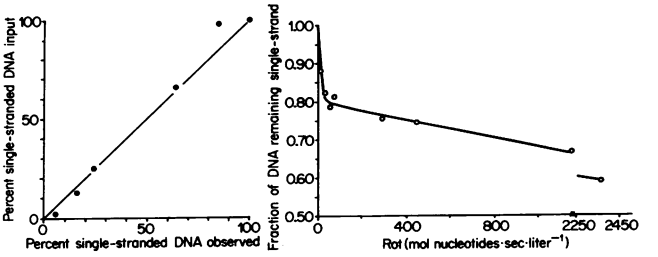

FIG. 1. (*left*) Specificity of the nucleases used to differentiate between single-stranded DNA and DNA in duplexes.

FIG. 2. (*right*) Hybridization of HSV-1 DNA with excess RNA extracted from cells 2 hr after infection. *Open circles*, experimentally determined points. The *line* is a computer plot calculated by Eq. V and fitted by nonlinear least squares method. The computer analysis involved the determination of parameters α_n and R_n in Eq. 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 $n = 3$, α_1 was less than 0.001, whereas α_2 and α_3 retained the same values as α_1 and α_2 for $n = 2$. Since various amounts of RNA were hybridized for different lengths of time, the data are presented as a plot of the fraction of single-stranded DNA as a function of the input concentration of RNA (Ro) in moles of nucleotides per liter × the time of hybridization (t).

Experimental design and analytical treatment of the data

We studied kinetics of hybridization of labeled viral DNA with a high excess of unlabeled whole-cell RNA.

The results obtained by this technique are readily amenable to analytical treatment. Specifically, let $D + R \rightarrow D\text{–}R$ be the reaction of single-stranded DNA fragments with homologous RNA, and further, let the concentration of DNA be such that the reassociation of DNA is negligible. It follows:

$$-\frac{dD}{dt} = k \cdot R \cdot D \qquad \text{(I)}$$

where t is the length of hybridization, D is the molar concentration of single-stranded DNA at time t, R is the molar concentration of single-stranded RNA, and k is the rate constant. In high RNA excess, where the concentration of RNA in hybrid is small compared to that of the single-stranded RNA left, R can be assumed constant and equals R_o—the input RNA concentration. Equation I upon integration yields:

$$\frac{D_t}{D_o} = e^{-k \cdot R_o \cdot t} \qquad \text{(II)}$$

where D_t is the concentration of single-stranded DNA at time t, and D_o is the initial concentration of single-stranded DNA. Similar first-order kinetics and the term *Rot* were applied by Mayfield and Bonner (12).

Eq. II assumes that all the DNA is transcribed to yield RNA with a single abundance R_o. The equation can be applied to the more general case with n classes of RNA, each appearing in molar concentration R_n. Such reaction can be actually visualized as the sum total of the independent reactions of each RNA class with the DNA homologous to it. For each such class the fraction of the DNA remaining single-stranded is:

$$\frac{D_{tn}}{D_{on}} = e^{-k_n \cdot R_n \cdot t} \qquad \text{(III)}$$

However, D_{on} equals $D_o \cdot \alpha_n$ where α_n is the fraction of the total viral DNA serving as a template for this RNA class, and D_o is the total DNA input. It follows therefore that:

$$\frac{D_{tn}}{D_o \cdot \alpha_n} = e^{-k_n \cdot R_n \cdot t} \qquad \text{(IV)}$$

The observed fraction of single-stranded DNA would be:

$$\frac{D_t}{D_o} = \frac{D_{t1}}{D_o} + \ldots \frac{D_{tn}}{D_o} + [1 - (\alpha_1 + \ldots \alpha_n)]$$

where $1 - (\alpha_1 + \ldots \alpha_n)$ is the fraction of the DNA that is not transcribed and will therefore remain single-stranded throughout the hybridization.

$$\text{Hence: } \frac{D_t}{D_o} = \alpha_1 \cdot e^{-k_1 \cdot R_1 \cdot t} + \ldots \alpha_n \cdot e^{-k_n \cdot R_n \cdot t} + 1 - (\alpha_1 + \ldots \alpha_n) \qquad \text{(V)}$$

The calculation of the parameters α and R requires the numerical value of the hybridization rate constants in addition to the technique for quantitative separation of DNA duplex from single-stranded DNA described earlier in the text. When $R_1 \ldots R_n$, the concentration of RNA in Eq. V, is expressed in mol RNA per liter, the numerical values of the rate constants are independent of the complexity (sum of unique sequences) of the RNA species represented in the different terms of the equation. Assuming the base composition of the various RNA species do not vary appreciably, the

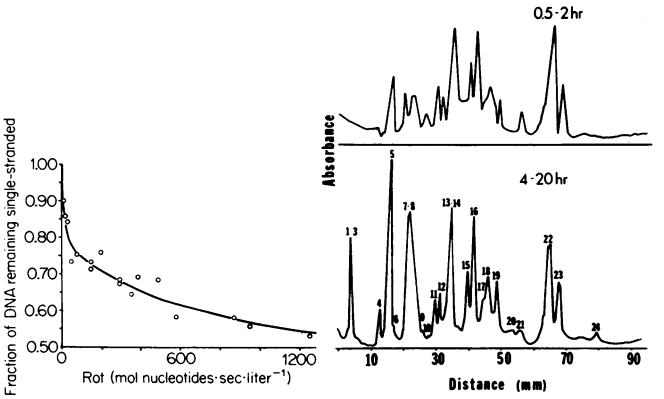

FIG. 3. (*left*) Hybridization of viral DNA with excess RNA extracted from cells 8 hr after infection. Computation procedure and presentation of data are the same as described in Fig. 2. Although visual inspection suggests more than two classes of RNA differing in abundance, attempts to fit three or more classes of RNA yielded one set of two αs with dimensions corresponding to α_1 and α_2 for $n = 2$ and an additional set of αs where aggregate dimensions were smaller than 0.001. $n = 2$ yielded the best fit.

FIG. 4. (*right*) Absorbance tracing of autoradiograms of structural proteins in purified HSV-1 virions subjected to electrophoresis in polyacrylamide gels. *Top:* proteins labeled 0.5–2 hr after infection. *Bottom:* 4–20 hr after infection.

numerical values of rate constant $k_1 \ldots k_n$ appearing in the different terms of the equation should be about the same. The rate constant, k, was calculated from the reassociation kinetics of the same batch of DNA as the one used in the DNA–RNA hybridizations on the assumption that the hybridization rate constant for RNA–DNA is the same as that of DNA–DNA reassociation.† The reassociation was monitored by the nuclease digestion described earlier in the text. The experimentally determined value of k for DNA–DNA hybridization in 0.07 M NaCl–0.05 M Tris·HCl and for sonicated DNA fragments with an average sedimentation constant of 5 S was 1.12×10^5 liter · mol^{-1} · sec^{-1}.

† The rate constant for DNA–DNA hybridization is defined by

$$\frac{dD}{dt} = -kD^2$$

where D is the concentration of DNA and is expressed as mol DNA per liter, i.e., $\frac{\text{g/liter}}{100 \cdot 10^6 \text{ g/mol}}$

Intuitively it could be predicted that the rate constant for DNA–DNA reassociation might be different from that for DNA–RNA hybridization. Melli *et al.* (13), on the basis of hybridization of excess *Escherichia coli* DNA to complementary RNA prepared *in vitro*, calculated that the observed rate constants differ by a factor of 0.43. The applicability of this finding to our analyses is not entirely clear. Gelderman *et al.* (23) also assumed that the DNA–DNA and DNA–RNA hybridization constants are the same. It should be noted, however, that the absolute numerical value of the hybridization rate constant is required for calculations of the *absolute* values of RNA concentrations in the cell. It is not required for calculation of relative concentrations of RNA species or for determination of the size of template from which they are transcribed.

RESULTS

Abundance classes of RNA after infection

Unlabeled RNA extracted from whole cells at 2 hr and at 8 hr after infection was hybridized to labeled sheared viral DNA. Fig. 2 and 3 show the residual single-stranded DNA as a function of *Rot*, i.e., the product of RNA concentration in the reaction mixture and the time of hybridization for RNA extracted from cells at 2 and 8 hr after infection.

The results of these studies summarized in Table 1 indicate the following: (*i*) about 48% of viral DNA serves as a template for the RNA present in the cell at 8 hr after infection. This amounts to 96% of the asymmetric informational content of the viral genome. (*ii*) the amount of viral DNA transcribed by 2 hr is 44%, only slightly less than that transcribed at 8 hr after infection. (*iii*) The cells infected for 2 and 8 hr contain two classes of RNA differing in abundance. The data thus clearly indicate control of the transcriptional process with respect to abundance of the transcripts. (*iv*) The most abundant class at 2 hr after infection is transcribed from 14% of the viral DNA and that of 8 hr after infection from 19% of the DNA. A further determination of the homogeneity of these most abundant classes was not attempted. Thus, we cannot state at present whether the most abundant species at 8 hr after infection is actually a homogeneous class or whether it consists of two or more classes of RNA having slightly different abundancies. (*v*) About 30% of the viral DNA serves as a template for the lower abundance groups. Assuming the total RNA content in the cell is about constant throughout infection, this group of RNA is more abundant at 8 hr than at 2 hr by a factor of 3.2.

Abundance competition test for most abundant RNA 2 hr after infection

The presence of two distinct abundance classes in RNA 2 hr after infection raised the question whether the nucleotide sequences in the most abundant class at 2 hr are identical to those in the most abundant class present at 8 hr after infection. To answer this question, we performed a series of abundance

TABLE 1. *Viral RNA transcripts and their abundance 2 and 8 hr after infection*

	Time of RNA extraction (hr after infection)	
	2 hr	8 hr
Fraction of DNA transcribed		
α_1	0.14	0.19
α_2	0.30	0.28
Total	0.44	0.48
RNA abundance		
R_1 (nmol/liter)*	7.9	7.1
R_1 (μmol/liter)†	355	457
R_2 (nmol/liter)*	0.058	0.183
R_2 (μmol/liter)†	5.88	16.91
R_1/R_2		
Molar RNA ratio	136.2	40.3
Molar nucleotide ratio	60.0	27.0

* mol of viral RNA of $0.99 \times 10^8 \cdot \alpha_n$ daltons (complexity).
† mol of ribonucleotides in viral RNA.

competition tests. This type of analysis is designed to answer the general question whether any class of RNA with a given abundance contains the same nucleotide sequences as any other class of RNA with the same or different abundance. As shown in Table 2, two sets of hybridization tests were done. In the control set (Table 2), the RNA from 2- and 8-hr infected cells were each hybridized to single-stranded DNA at a concentration (*Ro*) and time (*t*) such that all of the single-stranded DNA fragments complementary to the most abundant RNA were driven into DNA–RNA hybrids. In the abundance competition set (Table 2), the RNA from 8- and 2-hr infected cells were mixed. However, the concentration of each RNA was adjusted so that for a common time of incubation, t_c, the product $Ro \cdot t_c$ was exactly the same as the product $Ro \cdot t$ for each of the RNAs in the control set.

Table 2 shows the amount of single-stranded DNA expected to be driven into DNA–RNA hybrid if the most abundant sequences at 2 and 8 hr were identical and if they were different. The data clearly demonstrate that the sequences in the most abundant RNA at 2 hr are also present in the most abundant RNA at 8 hr after infection, i.e., the most abundant RNA at 2 hr is a subset of the abundant class of late RNA.‡

Nature of proteins specified by viral RNA present in high abundance

One hypothesis concerning the function of viral RNA present in high abundance is that it specifies structural proteins. This hypothesis is based not only on the expectation that the most abundant RNA should specify the most abundant viral proteins, but also on the estimated size of the DNA template required to specify the aminoacid sequence of viral structural proteins. Briefly (4, 14), the herpes virion consists of 24 structural proteins. Based on the aggregate molecular weights of the proteins, it was previously estimated that 23.5% of viral DNA was required to specify their aminoacid sequences. This figure is an overestimate since half the proteins are glycosylated (4, 14) and the polysaccharide moieties, while contributing to the molecular weight, should be excluded from the calculation. Nevertheless, even this amount of DNA template (23.5%) is in good agreement with the DNA template for the abundant RNA (19%). A direct test for the information content of the abundant viral RNA would require *in vitro* synthesis of proteins with that RNA as messenger. What proportion of the 24 viral structural proteins is made early after infection, particularly since a subset of high abundance RNA corresponding to 14% of the DNA is present then.

TABLE 2. *Abundance competition between most abundant RNA 2 hr after infection and RNA 8 hr after infection*

Competition experiment	RNA source	*Rot**	Observed % DNA in hybrid	Predicted % DNA in hybrid (*a*)	Predicted % DNA in hybrid (*b*)
1	2-hr	71.6	18.8		
	8-hr	72.5	24.8		
	8-hr	144.9	28.8		
	8 hr	72.5			
	+		22.6	24.8–28.8	43.6
	2-hr	71.6			
2	2-hr	71.6	18.8		
	8-hr	289.9	32.6		
	8-hr	362.4	35.6		
	8-hr	289.9			
	+		35.6	32.6–35.6	51.4
	2-hr	71.6			

(*a*) All abundant sequences in 2-hr RNA are present in competing 8-hr RNA. (*b*) Most abundant species in 2-hr RNA different from those in competing 8-hr RNA. * Expressed as mol nucleotides·sec per liter. 2-hr RNA, 8-hr RNA, or both were incubated with labeled DNA to *Rot* specified.

In these experiments, 4×10^8 cells were labeled between 0.5 and 2 hr after exposure to virus with 10 μCi of [^{14}C]amino-acid mixture (reconstituted protein hydrolysate, New England Nuclear Copr., Boston, Mass.) per ml of Eagle's minimal essential medium lacking amino acids. At the end of the pulse, the cells were washed and replenished with mixture 199 supplemented with 1% calf serum. Replenishment with medium containing unlabeled amino acids precludes further incorporation of labeled amino acids into proteins (15, 16). An infected cell culture labeled continuously beginning 4 hr after infection served as control. Both the pulsed and the control cultures were harvested 20 hr after infection. The virus was purified, solubilized, and subjected to electrophoresis on polyacrylamide gels. The absorbance profiles of the autoradiogram are shown in Fig. 4. The data show the following: (*i*) structural proteins are already made between 0.5 and 2 hr after infection; however, only proteins 5 through 24 become labeled at that time. (*ii*) All viral proteins are labeled, and therefore synthesized, after 4 hr after infection. (*iii*) Based on the molecular weights of the proteins, we calculated that as much as 68% of the estimated DNA template for structural proteins is expressed between 0.5 and 2 hr after infection. This is in good agreement with the proportion of the DNA template for abundant RNA transcribed (74%).

DISCUSSION

Analytical procedures for estimation of RNA abundance

The procedures used in this paper involved analysis of kinetics of hybridization of labeled viral DNA with excess of unlabeled RNA. Analyses of kinetics of DNA–RNA hybridization have been covered in numerous publications. In principle, most analyses reported to date were of hybridizations

‡ The results obtained in this experiment cannot be explained by the hypothesis that the templates for abundant RNA 2 and 8 hr after infection are on opposite strands and that late in infection the transcription of abundant 8-hr RNA extends into the region of the DNA opposite the template for abundant 2-hr RNA. The hypothesis predicts that the DNA sequences, which are transcribed symmetrically, would follow in hybridization the kinetics of RNA–RNA hybrid formation with a correction for the rate constants. However, abundant RNA comprises about 3% of total RNA (calculated on the basis of the assumption that the rate constant for DNA–DNA reassociation measured as indicated in the text is similar to that of DNA–RNA hybridization. This value is even higher if the rate constant for RNA–DNA hybridization is lower than that for DNA–DNA reassociation), i.e., the *Rot* value for abundant RNA in exp. 1, Table 2, is about 2.2 mol nucleotides·sec per liter. This value is sufficiently high for complete hybridization of RNA–RNA complementary regions (with maximal complexity of 20×10^4 daltons), even if the rate constant for RNA–RNA hybridization is as much as 5-fold lower than that for DNA–DNA hybridization. Therefore, the DNA sequences, which are transcribed symmetrically, are expected to be fully hybridized and the predicted percent of DNA in hybrid would be expected to be higher than that found.

in which DNA was in excess (see for example refs. 13, 17, 18). The objectives of those studies were to determine whether RNA was transcribed off unique or repetitive DNA sequences or both. Hybridization of DNA with excess RNA (saturation studies) are common. Analysis of the saturation kinetics of unlabeled DNA on filters with excess labeled RNA based on assumptions that all RNA sequences are present in about the same concentration have been reported (12). Analytical studies of kinetics of saturation, particularly with the objectives of determining the number of classes of RNA differing in abundance and the size of the DNA template from which the RNA classes were transcribed have not been reported. In this paper, we are reporting such a procedure and its application to analyses of transcription of viral RNA.

Transcriptional controls in HSV-infected cells

There are two kinds of transcriptional controls of viral DNA. One control regulates off-on transcription. Evidence for this control is based not on the overall DNA transcribed before 2 and 8 hr after infection (44 and 48%, respectively), but rather on the basis of comparisons of amounts of DNA template transcribed at high abundance at these intervals (14 and 19%, respectively). While we cannot exclude the possibility that a scarce RNA species becomes abundant, the mechanism for such a control is no less complicated than that required for off-on control. Additional weight in support of our contention that the abundant RNA absent in 2-hr infected cells is transcribed late in infection, and comes from considerations of the function of this RNA discussed below.

The second control regulates abundance of viral transcripts. Thus, the RNA extracted at 2 hr after infection contains two classes of RNA differing in molar concentrations. We have no information on how these controls operate.

Nature of the products specified by abundant and scarce HSV-RNA

We suspect that abundant RNA specifies structural proteins. The chain of reasoning that led us to this conclusion is stated earlier in the text. In general, if abundant RNA specifies structural proteins, it could be expected that the fraction of structural proteins made early in infection would correspond to the fraction of the template for abundant RNA transcribed at that time. This appears, in fact, to be the case in that both the total DNA transcribed and the DNA template for abundant RNA increase by 4 to 5 $\times$ 10^6 daltons (4–5% of total DNA), and this increase corresponds to the amount of information for structural proteins expressed at 8 hr, but not at 2 hr, after infection. It should be pointed out that there is independent evidence that synthesis of most structural proteins is an early function. Thus, in addition to the evidence presented in this paper that most viral structural proteins are made between 0.5 and 2 hr after infection, it has also been shown by us and by others that inhibitors of DNA synthesis do not block synthesis of viral proteins, assembly of proteins into capsids, or the appearance of structural components of the virus on the surface of the cells (19–21). In this respect, there is a substantial difference between the transcriptional program of herpesvirus and that of other DNA viruses infecting animal cells.

If our suspicions concerning the function of abundant RNA are correct, it follows that the scarce RNA specifies nonstructural proteins, i.e., enzymes involved in the synthesis and processing of viral structural components (DNA, proteins, glycoproteins, lipids, and polyamines) and in the modification of the host, which involves inhibition of host DNA and protein synthesis, alteration of the synthesis, processing and transport of host RNA, and alteration of the structure and function of cellular membranes. Most of the viral functions listed here are early functions, expressed independently of viral DNA synthesis (3, 20, 22).

Nature of late viral functions

On the basis of comparisons between total RNA at 2 and 8 hr, it would seem that the difference between early and late functions consists of transcription of a small percentage of DNA template at high abundance. The function of this RNA could be to specify maturation proteins, etc. We cannot exclude the possibility that the temporal programming of viral functions is also determined at the level of processing and transport of the RNA from nucleus to cytoplasm.

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