*RNA SYNTHESIS IN CELLS INFECTED WITH HERPES SIMPLEX VIRUS, II. EVIDENCE THAT A CLASS OF VIRAL mRNA IS DERIVED FROM A HIGH MOLECULAR WEIGHT PRECURSOR SYNTHESIZED IN THE NUCLEUS**

By Edward K. Wagner† and Bernard Roizman

DEPARTMENT OF MICROBIOLOGY, UNIVERSITY OF CHICAGO, ILLINOIS

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Abstract.—Viral RNA extracted from the cytoplasmic polyribosomes of human epidermoid carcinoma no. 2 cells infected with herpes simplex virus (mRNA) had a sedimentation coefficient between 10*S* and 20*S* while that from nuclei of infected cells varied in size from 10*S* to >80*S*. Estimates of the maximum molecular weight of viral RNA from its sedimentation coefficients suggest that at least 10 per cent of the viral genome is transcribed as a single molecule. The ratio of RNA of different sizes found in the nuclei of cells pulse labeled for 12 minutes was approximately the same as those found in cells labeled for longer intervals implying that either some classes of viral mRNA were made as small molecules or that the large viral RNA molecules were cleaved soon after synthesis. Cytoplasmic mRNA competed to a level of at least 80 per cent in viral DNA-RNA hybridization tests with >50*S* RNA extracted from nuclei of infected cells. This is consistent with the hypothesis that viral mRNA is produced by cleavage of a large precursor RNA molecule.

Introduction.—In the first paper of this series[1] we reported that the size distribution of herpes simplex virus RNA in the nucleus of infected human epidermoid carcinoma no. 2 cells differed from that found in the cytoplasm. Viral RNA associated with polyribosomes had a sedimentation coefficient in the range between 10*S* and 40*S* as determined by sucrose density gradient centrifugation. However, the major portion of cytoplasmic RNA had a sedimentation coefficient of 14*S* to 20*S* and less than 5 per cent sedimented faster than 28*S*. Nuclear viral RNA on the other hand sedimented in a very broad range, from <10*S* to >80*S;* however, a large portion was greater than 28S.

In the work described in this communication, viral DNA-RNA hybridization competition experiments showed that at least part of the cytoplasmic viral mRNA is indeed derived from a high molecular weight nuclear precursor RNA. We have not been able to determine from the kinetics of labeling whether the large nuclear RNA molecules are rapidly cleaved or whether at least some of the products of transcription of viral DNA are relatively small. However, calculations based on the size range of the large RNA molecules indicate that a single RNA molecule is the transcription product of at least 10 per cent of the herpes simplex virus genome.

Materials and Methods.—Cells and virus: The details of the growth and infection of human epidermoid carcinoma no. 2 cells with herpes simplex virus has been described previously.[1-3] The F (Type 1) strain of this virus[2] was used in all experiments. The patterns of inhibition of host cell RNA synthesis following infection with the F strain are similar to that described previously.[1]

Radioactive labeling and cell fractionation: At 4 hr after infection with 20 to 40 plaque forming units (P.F.U.)/cell the cells were labeled with ^{3}H-uridine (spec. act. 20 c/mM, Schwarz BioResearch Inc.) and fractionated into nuclei and cytoplasm by low-speed centrifugation following incubation in RSB[4] containing 0.5% Nonidet P-40[5] (Shell Chemical Company). Cytoplasmic polyribosomes were obtained by a modification of the method of Soeiro and Amos.[6] The cytoplasmic extract containing the Nonidet P-40 was layered on a 2 ml pad of 50% (w/w) sucrose in RSB and centrifuged at 60,000 rpm for 2.5 hr in the Spinco 65 rotor. Preliminary experiments have shown that the polyribosomes contained in the pellet had the same sedimentation coefficients as those contained in the cytoplasmic extract but that the pellet contained only $^1/_4$ of the free ribosomes.

Extraction and fractionation of RNA: Cytoplasmic and nuclear RNA was extracted by the phenol-SDS method.[1, 7] Unlabeled RNA for hydridization competition experiments was digested with 50 μg/ml of electrophoretically purified DNase (Schwarz BioResearch) and excluded from a 1.5 $\times$ 40 cm Sephadex G-100 column in 0.15 *M* sodium chloride and 0.015 *M* sodium citrate (1 $\times$ SSC), precipitated with ethanol, and dissolved at a concentration of 2 to 4 mg/ml in 4 $\times$ SSC prior to use. Phenol extracted nuclear RNA from ^{3}H-uridine labeled cells was fractionated by centrifugation on 16 ml 0.5% SDS, 15–30% sucrose (w/w) gradients[8] at 25° in the Spinco SW 25.3 rotor. Gradients were collected into 1 ml fractions and 50 μl aliquots precipitated with TCA and assayed for radioactivity as described previously.[1]

DNA-RNA hybridization: Herpes simplex virus (F strain) DNA was prepared by two cycles of isopycnic centrifugation in CsCl solution. Hybridization tests were done as previously described.[1, 9] Hybridization competition was carried out by preincubating the DNA with the unlabeled competing RNA for 12 hr at 68° prior to the addition of ^{3}H-uridine labeled RNA. The RNase digestion and washing of the discs were carried out as described and background radioactivity bound to blank discs was less than 0.01% of input radioactivity.

Results.—The size range of the heavy viral RNA in the nucleus of the infected host: Radioactive nuclear RNA from herpes simplex virus infected cells was prepared as follows: Approximately 5 $\times$ 10^7 infected cells were suspended in 10 ml of labeling medium containing 100 μc of ^{3}H-uridine and incubated at 37° for one hour. The nuclear RNA was then extracted and centrifuged in 0.5 per cent SDS, 15–30 per cent sucrose gradients. The profile of the labeled nuclear RNA (Fig. 1) shows considerable amounts of radioactivity sedimenting more rapidly than 60*S*.

The following experiment was done in order to establish more precisely the sedimentation coefficients of the large species of nuclear RNA which pellet in the

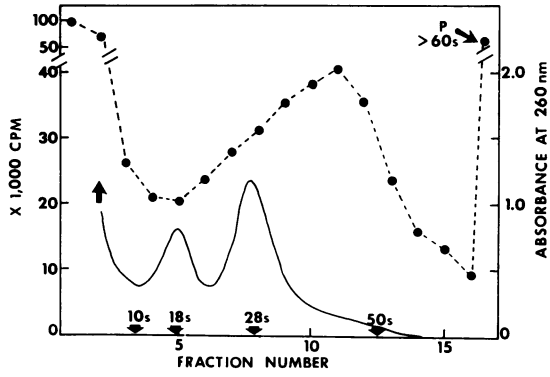

FIG. 1.—Sucrose density gradient fractionation of the nuclear RNA from human epidermoid carcinoma no. 2 cells infected with the F strain of herpes simplex virus. Approximately 5 $\times$ 10^7 of 4-hr infected cells were labeled for an hour at 37° with ^{3}H-uridine. Nuclear RNA was centrifuged for 14 hr at 20,500 rpm and collected as described.

15–30 per cent sucrose gradients used in these studies. RNA extracted from 5×10^7 cells infected and labeled as described in the legend to Figure 1 was centrifuged on a 0.5 per cent SDS, 15–50 per cent sucrose density gradient. The radioactivity profile indicated a broad band of radioactivity within the gradient with sedimentation coefficients centering around 60*S*. It can be calculated that this corresponds to an approximate molecular weight of 8×10^6 assuming that the relationship between the sedimentation coefficient and molecular weight determined by Studier[10] applies to this type of RNA and that 45*S* RNA has a molecular weight of 4.1×10^6.[11] This experiment also showed that the proportion of radioactivity found in the pellet at the bottom of the gradient is similar to that found in the pellet obtained after centrifugation in a 0.5 per cent SDS, 15–30 per cent sucrose density gradient (Fig. 1). Based on the assumption that the conformation of the RNA in the pellet is the same as that sedimenting more slowly in the gradients, it can be calculated that this material must have a sedimentation coefficient of at least 80*S* corresponding to a minimum molecular weight of 12×10^6.

Further studies are being carried out to establish the maximum size of the viral RNA in the nucleus. Preliminary centrifugation in high salt buffer sucrose density gradients of Penman *et al.*[12] revealed that classes of viral RNA sedimenting as high as 200*S* are present in the nucleus of infected cells.

Kinetics of synthesis of herpes simplex virus nuclear and cytoplasmic RNA: Approximately 1.5×10^8 human epidermoid carcinoma no. 2 cells were infected with 40 PFU/cell and incubated for four hours. The cells were then scraped, washed, and resuspended at 37° in 30 ml of labeling medium containing 360 μc of ^{3}H-uridine. At 12, 25, and 45 minutes after addition of the label 10 ml ali-

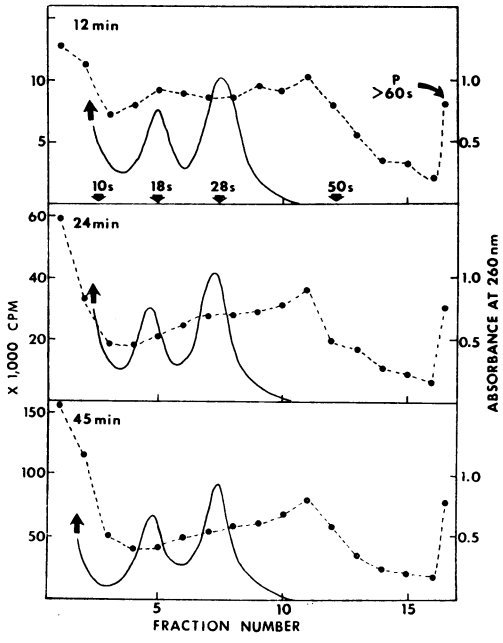

FIG. 2.—The kinetics of labeling of different classes of nuclear RNA in herpes-virus infected human epidermoid carcinoma no. 2 cells. 1.5×10^8 cells were incubated at 4 hr after infection in 30 ml of medium containing 360 μc of ^{3}H-uridine for the time intervals shown. The nuclear RNA was centrifuged for 14 hr at 20,500 rpm.

quots were removed and poured over crushed frozen phosphate buffered saline. The nuclear and cytoplasmic RNA were then extracted, and the nuclear RNA from these samples was centrifuged on SDS-sucrose gradients. The profiles of radioactivity in the gradients are shown in Figure 2; the data show that there is no preferential incorporation of ^{3}H-uridine into any particular size range of the nuclear RNA during these labeling intervals.

The relative proportions of virus specific RNA of different size ranges were determined by pooling fractions of Figure 2 to yield RNA of size ranges of 10*S* to 28*S;* 28*S* to 50*S;* and >50*S* (including the pelleted material). These fractions were precipitated, digested with purified DNase, and resuspended in 4xSSC. RNA from each class was hybridized with 2 μg of viral DNA as described. The amount of radioactivity incubated with the viral DNA was approximately the same for each time point within each size range and was well below the amount necessary to saturate viral DNA as shown by the fact that the amount of each class of RNA bound to the discs was proportional to input for at least 1.5 times as much as used in these experiments. On the basis of these findings and assuming that the specific radioactivity of the newly synthesized viral RNA does not change appreciably during the labeling intervals in this experiment, the percentage of input counts bound to the viral DNA is an approximate measure of the relative proportion of viral RNA in the size range being tested. The results of this experiment are shown in Figure 3; it is apparent that at the time intervals chosen there is little difference in the rate of appearance of radioactivity into nuclear viral RNA of any size range. The data show, however, that there is a definite lag in the appearance of viral RNA into the cytoplasmic fraction which implies that the transport of RNA from the nucleus to the cytoplasm is slow compared to the synthesis and processing of viral RNA.

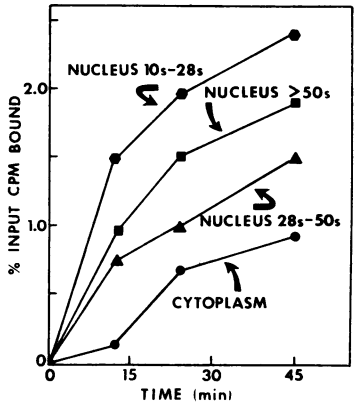

FIG. 3.—Kinetics of appearance of viral RNA in the nucleus and cytoplasm of herpes virus infected human epidermoid carcinoma no. 2 cells. The percentage of the input radioactive RNA of the size ranges shown annealed to 2 μg of viral DNA is plotted versus the time of incubation of the cells with ^{3}H-uridine. The amounts of RNA of the various size ranges incubated with viral DNA were: nuclear RNA 10*S*–28*S*; 12 min–35,000 cpm, 24 min–46,000 cpm, 45 min–52,000 cpm. Nuclear RNA 28*S*–50*S*; 12 min–54,000 cpm, 24 min–56,000 cpm, 45 min–65,000 cpm. Nuclear RNA 50*S*–>60*S*; 12 min–25,000 cpm, 25 min–32,000 cpm, 45 min–36,000 cpm. Cytoplasmic RNA; 12 min–75,000 cpm, 24 min–95,000 cpm, 45 min–83,000 cpm.

Competition between nuclear RNA and polyribosomal mRNA extracted from infected cells in hybridization tests with viral DNA: Two series of experiments were done. In the first series viral DNA was incubated first with unlabeled RNA extracted from viral polyribosomes, infected nuclei or from uninfected cells, and later with labeled large nuclear RNA (>50*S*). In the second

series hybridization competition experiments were done between unlabeled RNA extracted from viral polyribosomes and labeled nuclear RNA of different sizes. The radioactive nuclear RNA used in these experiments was prepared from 1.5 $\times$ 10^8 cells infected with a multiplicity of 20 PFU/cell, incubated for 4 hours, scraped, and suspended for one hour in 30 ml of labeling medium containing 300 μc of ^{3}H-uridine. The nuclear RNA was centrifuged in three 0.5 per cent SDS, 15–30 per cent sucrose gradients as described in Figure 1. The fractions containing RNA with sedimentation coefficients of 10*S*–28*S*, 28*S*–50*S*, 50*S*–60*S*, and the pellets (>60*S*) from the three gradients were pooled. The RNA in the four pools was each precipitated with 2 mg of purified yeast RNA, digested for 1 hour at 37° in RSB containing 50 μg of purified DNase/ml, extracted twice with chloroform-isoamyl alcohol to remove the DNase, precipitated again, and finally dissolved in 4xSSC. Unlabeled RNA for the competition experiments was extracted from three sources, i.e., uninfected cells, cytoplasmic polyribosomes of infected cells, and infected cell nuclei.

In the first series of experiments varying amounts of unlabeled RNA were pre-incubated in vials with two filter discs each bearing 0.5 μg of herpes simplex virus DNA and one blank filter disc. The final concentration of RNA in each vial was brought to 2.5 mg with purified yeast RNA. After pre-incubation 68,000 ^{3}H CPM of high molecular weight nuclear RNA, obtained by pooling the RNA >50*S* including the pelleted material, was added to each vial. The hybridization tests were then performed as described in *Materials and Methods*. Figure 4 shows the fraction of RNase-resistant radioactivity bound to 1 μg of herpes simplex virus DNA in each vial as a function of the total unlabeled infected nuclear, infected polyribosomal, or uninfected cell RNA with which the DNA was pre-incubated. The data obtained from this experiment show that the highest concentrations of unlabeled viral polyribosomal and nuclear RNA saturate 80 and 92 per cent, respectively, of the viral DNA complementary to large nuclear RNA. Since complete saturation of viral DNA with the unlabeled RNA has not been attained it is probable that the extent of homology between large nuclear RNA and the cytoplasmic RNA is higher than the estimates obtained in this experiment.

In the second series of experiments, discs containing viral DNA were pre-incubated either with 2 mg of yeast RNA or 1.6 mg of unlabeled polyribosomal

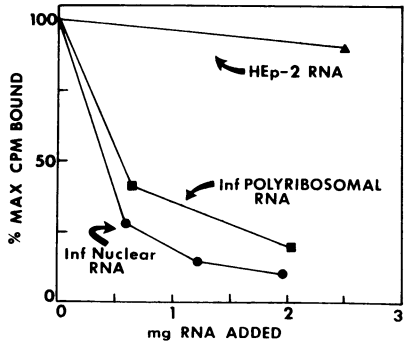

FIG. 4.—The competition of unlabeled viral nuclear and polyribosomal RNA with radioactive heavy viral nuclear RNA in hybridization to viral DNA. The amounts of RNA of the different species shown were pre-incubated for 12 hr with 2 discs bearing 0.5 μg each of viral DNA and then radioactive nuclear RNA >50*S* from infected cells was added to each vial and the hybridization continued for 20 hr. The 100% hybridization point (500 cpm/μg of viral DNA) was determined for a sample preincubated with 1.25 mg of uninfected human epidermoid carcinoma no. 2 cell RNA.

RNA and then incubated with labeled nuclear RNA fractionated according to size. The results of this competition experiment are shown in Table 1. The data show that viral RNA associated with polyribosomes competes with equal efficiency against all size ranges of nuclear RNA.

We conclude from these experiments that at least some of the viral mRNA in polyribosomes is synthesized in the nucleus of the host cell as a class of high molecular weight RNA which must then be cleaved into the molecules of the size of cytoplasmic viral mRNA. It is also apparent that there are smaller species of viral RNA in the nucleus which have a nucleotide sequence identical to that of viral cytoplasmic mRNA.

Discussion.—Site of synthesis of viral RNA: Viral RNA appears first in the nucleus and only later in the cytoplasm. Hybridization competition experiments indicate that the cytoplasmic and nuclear RNA specified by the virus have the same nucleotide sequence. We conclude, therefore, that viral mRNA is made in the nucleus and that it is transported into the cytoplasm.

TABLE 1. *Hybridization competition between ^{3}H-labeled nuclear RNA and unlabeled polysomal RNA from herpes simplex virus infected human epidermoid carcinoma cells.*

Nuclear ^{3}H-RNA	CPM Incubated with 1 μg DNA	Net RNase Resistant CPM Bound to 1 μg Viral DNA: Pre-incubated with 2 mg yeast RNA	Pre-incubated with 1.6 mg polysomal RNA	Competition (%)
10*S*–28*S*	90,000	750	150	80
28*S*–50*S*	180,000	800	200	75
50*S*–60*S*	72,000	500	100	80
>60*S* (pelleted RNA)	42,000	670	140	78

The size of viral RNA accumulating in the nucleus: The size of viral RNA in the nucleus varies from 10*S* to greater than 80*S*. The experiments described in this paper do not give the exact size of the largest species of nuclear RNA hybridizable with viral DNA. However, the most conservative estimate for the molecular weight of the large RNA species is at least 8×10^6 daltons calculated for 60*S* RNA. We estimate, therefore, that the class of large nuclear RNA represents the transcription product of at least 10 per cent of the viral genome and perhaps a considerably larger percentage of the genome.

The relationship between the large and small classes of viral RNA accumulating in the nucleus: If the large RNA class were processed at the same rate as ribosomal RNA, it would be expected that only the large species would have been labeled with ^{3}H-uridine during the 12-minute pulse.[13] It follows that either the high molecular weight RNA is cleaved faster than ribosomal RNA or that the transcription products of viral RNA vary considerably in size. It is of interest to recall at this point that in uninfected eukaryotic cells the class of large RNA molecules with a nucleotide composition similar to that of cellular DNA decay at a rate considerably faster than the rate of processing of ribosomal RNA.[12, 14–18]

Relationship between the large class of nuclear viral RNA and viral mRNA extracted from polyribosomes: The main point of the paper is that viral RNA

extracted from cytoplasmic polyribosomes competes with much larger classes of nuclear viral RNA for homologous sites on the viral DNA. The implication of the data are as follows: First, it necessarily follows that at least a portion of the viral mRNA is derived from large nuclear precursor RNA by cleavage. Calculations based on the probable molecular weight of >60*S* precursor and of mRNA of 10*S* to 20*S* suggest that the precursor may be cleaved into 10 or more mRNA molecules. The fact that viral mRNA competes for at least 80 per cent of the DNA sites homologous with large nuclear RNA suggests that little, if any, of the RNA cleaved in the processing of the precursor RNA does not function as mRNA. Second, it has always been assumed that there are two kinds of punctuation. The first resides in the DNA and specifies initiation and termination of transcription of DNA. The second resides in mRNA and specifies initiation and termination of protein synthesis during translation. Since the cleavage of precursor RNA molecules cannot be random and must necessarily be specified, there must also be a third type of punctuation which also resides in the mRNA and specifies the site of cleavage. Since to date each type of punctuation was found to have a regulatory function, it can be expected that a regulatory mechanism is also embodied in the processing of mRNA precursor molecules. Lastly, it is of interest to point out that the mechanism of formation of herpes simplex virus mRNA may be a general feature of all eukaryotic cells. Large rapidly turning-over RNA species with a base composition similar to cellular DNA have been known for many years.[14-18] Recently, Stevenin *et al.*[19] showed that in rat brain cells, 65 per cent of this nuclear RNA can be related to microsomal mRNA and is synthesized as a high molecular weight species. Recent experiments (Wagner and Roizman, in preparation) have shown that heating the nuclear RNA from infected cells to 65° in the presence of 0.5 per cent formaldehyde or treatment with dimethyl sulfoxide does not appreciably alter the size distribution of this RNA. These results indicate that the large sedimentation values obtained for nuclear viral RNA are due to the size and not to the confirmation of the RNA.

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