High mol. wt. RNA (HMW-RNA) extracted from nuclei of HEp-2 cells 5 h post infection with herpes simplex 1 virus has been shown to have the following characteristics: (i) the amount of HMW-RNA hybridizing to viral DNA fixed on filters increased with input multiplicity as well as following heat denaturation prior to hybridization; (ii) purified self-annealed HMW-RNA was enriched for viral RNA sequences which hybridized to viral DNA following denaturation; (iii) hybridization of excess unlabelled HMW-RNA which labelled viral DNA fragments followed by isopycnic centrifuging in C5SO4 led to the partitioning of a fraction of DNA with HMW-RNA. This DNA self annealed at a faster rate than the parental DNA population from which it was derived indicating that the HMW-RNA contained transcripts derived from symmetric transcription of a fraction of viral DNA; (iv) excess unlabelled, heat-denatured, HMW viral RNA drove 50% of viral DNA into DNA-RNA hybrid in hybridization tests with trace amounts of labelled viral DNA. Analysis of the kinetics of hybridization indicated that HMW-RNA consisted of 2 classes arising from 24 to 26% of viral DNA and differing 5000-fold in molar concentration. Since HMW-RNA contains symmetric viral transcripts which self anneal during the hybridization this is probably a minimal estimate of the amount of viral DNA represented in HMW-RNA.

INTRODUCTION

RNA molecules considerably longer than those found in polyribosomes have been known to exist and to undergo rapid turnover in eukaryotic cells (Soeiro, Birnboim & Darnell, 1966). Viral RNA molecules larger than those found in polyribosomes have been found in cells infected with human herpesvirus 1 (herpes simplex virus 1: HSV-1; Wagner & Roizman, 1969; Roisman et al. 1970) and subsequently in cells infected with other DNA viruses (Martin & Byrne, 1970; Parsons, Gardiner & Green, 1971; Wall, Philipson & Darnell, 1972). Since the RNA sequences contained in the high mol. wt. RNA included those found in polyribosomes, it was concluded that the high mol. wt. RNA serves as a precursor to the RNA directing the synthesis of viral proteins (Wagner & Roizman, 1969; Roizman et al. 1970).

The purpose of the studies described in this paper was to elucidate the characteristics of the high mol. wt. RNA labelled within a short interval and accumulating in the infected cell.

* Paper No. 11 in the series.
The study was designed to permit accurate assessments of the homogeneity in the concentration of the RNA and the size of the DNA template from which the RNA was derived and take cognizance of the observation that nuclei of infected cells contain transcripts arising by symmetric transcription (Jacquemont & Roizman, 1975).

**METHODS**

_Solutions and chemicals._ Standard saline citrate (1 × SSC) consisted of 0.15 M-sodium chloride and 0.015 M-sodium citrate; reticulocyte standard buffer (RSB) consisted of 0.01 M-sodium chloride, 0.0025 M-magnesium chloride and 0.01 M-tris hydrochloride, pH 7.5; isotonic high pH (IH) buffer consisted of 0.14 M-NaCl, 0.01 M-tris, pH 8.4, 1.5 mM-MgCl₂. Dimethyl sulphoxide, formamide and sodium dodecyl sulphate (SDS) were obtained from Matheson, Coleman and Bell; sarkosyl (NL 97) from Geigy Chemical Company; sodium deoxycholate from Schwarz Mann BioResearch, Orangeburg, N.Y.; Nonidet P₄₀ (NP₄₀) from Shell Oil Company, New York; crystallized DNase free of RNase, RNase T₁ and RNase A, from Worthington Biochemicals, Freehold, N.J.; [5-³H]-uridine (sp. act. 28 Ci/mmol) from New England Nuclear, Boston, Mass. *Escherichia coli* DNA was the kind gift of Professor A. Markovitz of the University of Chicago.

_Cells and virus._ The procedures for propagation and maintenance of human epidermoid carcinoma No. 2 (HEp-2) cells, production, assay and pertinent properties of the F strain of HSV-1 [HSV-1 (F)] were described elsewhere (Roizman & Spear, 1968; Wagner & Roizman, 1969; Roizman et al. 1970; Kieff, Bachenheimer & Roizman, 1971; Kieff et al. 1972; Silverstein et al. 1973).

_Prep and labelling of viral DNA._ The procedure for preparation and purification of viral DNA was the same as previously described (Kieff et al. 1971) 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 (1963).

The procedure for *in vitro* labelling of HSV-1 (F) DNA by repair synthesis using *Escherichia coli* polymerase I was described elsewhere (Frenkel et al. 1972). The *E. coli* polymerase I was the kind gift of Professor A. Kornberg, Stanford University.

_RNA purification._ Total RNA from infected and uninfected cells was extracted as described by Silverstein et al. (1973). Nuclear RNA was made as follows: cells were washed in phosphate buffered saline, suspended in IH buffer, made 0.5% with respect to NP₄₀ and incubated at 4 °C for 15 min. Nuclei were removed by low speed sedimentation and washed with 0.5% NP₄₀ in IH buffer. The nuclei were then resuspended in RSB, 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 SDS phenol and chloroform, precipitated with ethanol, dissolved in 0.01 M-tris, pH 7.5, 0.02 M-MgCl₂, dialysed against the same buffer, and digested again with DNase. The RNA was then again extracted with SDS and phenol. This cycle was repeated once again. In the last step of the extraction procedure the RNA was dialysed against the hybridization buffer for 3 days.

_Prep of high mol. wt. RNA (HMW-RNA)._ Nuclei were lysed as described above and the deoxycholate nuclear extract was then digested with DNase treated with 0.1 M-sodium acetate, 0.01 M-sodium iodoacetate, pH 5.3, at 55 °C for 40 min to inactivate residual traces of RNase (Zimmerman & Sandeen, 1966). The RNA was then extracted with a mixture of SDS, phenol-chloroform (4:1, v/v) as described elsewhere (Penman, 1966).
RNA in herpes simplex virus infected cells

The pellet was resuspended in 0.05 M-tris, pH 7.5, 0.1 M-NaCl, 5 mM-EDTA and layered on to pre-formed 20 to 45 % (w/w) sucrose density gradients prepared in the same buffer containing in addition 0.5 % SDS and 0.5 % sarkosyl and centrifuged for 15 h at 20000 rev/min in a SW27 rotor at 25 °C. The fractions, containing 1 ml each, were measured for extinction at 254 nm. Nuclear RNA > 45S was pooled and precipitated with ethanol. For hybridization in liquid the RNAs were digested with DNase followed by SDS phenol-chloroform treatment as described above.

Purification of double-stranded 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 and 50 % (v/v) formamide. After annealing the RNA was diluted and precipitated with 2 vol. of ethanol. The RNA pellet was solubilized in 0.01 M-tris, pH 7.5, 2.5 mM-magnesium chloride, dialysed against this buffer and digested with DNase I (50 µg/ml) for 1 h at 37 °C. Then 0.1 vol. of 10 mM-EDTA, 0.01 M-tris, pH 7.5, was added, salt concentration adjusted to 0.35 M-NaCl and the RNA digested for 1 h at 37 °C with 50 µg/ml of RNase A and 10 U/ml of RNase T1. In order to remove degradation products, RNA was passed through Sephadex G50 (1 x 60 cm) columns and the excluded material was precipitated with ethanol.

Hybridization of labelled RNA to DNA on filters. In several experiments, the RNA was denatured in 0.1 x SSC at 115 °C for 5 min and hybridized to Escherichia coli, HEp-2, and HSV-1 DNAs fixed to nitrocellulose filters. The DNAs were fixed to 25 mm Schleicher and Schuell B6 filters as described by Kieff et al. (1972). Small discs (6 mm in diam.) were then punched out. A set of discs containing E. coli, HEp-2, HSV-1 DNA plus a blank were incubated together at 66 °C for 20 h in 200 µl of hybridization buffer containing the denatured RNA, 0.75 M-NaCl, 5 mM-EDTA, 0.25 % SDS, 0.01 M-tris, pH 7.5. The discs were then removed, washed twice for 30 min each in 2 x SSC, digested with RNase A (50 µg/ml) for 1 h at room temperature, washed as before and dried. The purpose of the filter hybridization was to demonstrate the presence and relative amounts of labelled viral RNA sequences in the cell fractions. All measurements cited in the text were at below saturation levels.

Hybridization of unlabelled RNA to labelled DNA in solution. Excess unlabelled RNA and in vitro labelled DNA were denatured by heating, then hybridized in liquid as previously described (Frenkel & Roizman, 1972). The amount of DNA driven into DNA-RNA hybrid was monitored by digestion with Neurospora crassa nuclease (Rabin, Preiss & Fraser, 1971) which depolymerizes only single stranded DNA. The procedures for preparation of the enzyme and digestion of the hybridization mixture were as previously described (Frenkel & Roizman, 1972; Silverstein et al. 1973). In these experiments, the amount of denatured DNA resistant to enzyme digestion did not exceed 5 % of input DNA counts. The procedure for analysis of hybridization kinetics by non-linear least squares regression was published elsewhere (Frenkel & Roizman, 1972).

RESULTS

Size and composition of heterogeneous RNA from nuclei of infected cells

In this series of experiments HEp-2 cells were infected at 2 multiplicities, i.e. 10 and 100 p.f.u./cell, then pulse labelled for 20 min with 50 µCi of uridine per ml at 5 h post infection. The results of analyses of the extracted RNA may be summarized as follows.

The sedimentation profiles of the RNA in sucrose density gradients are shown in Fig. 1(a). The RNA extracted from cells infected with a multiplicity of 10 p.f.u./cell shows two major peaks sedimenting at 32 and 45S respectively. The RNA extracted from cells...
infected with a multiplicity of 100 p.f.u./cell shows only one major peak sedimenting at 45S. In both gradients 22% of total counts have a sedimentation coefficient greater than 58S.

Secondly RNA from cells infected with 100 p.f.u./cell and sedimenting in the region of 58S but without the pellet was pooled, heated for 10 min at 60 °C in 0.1 M-phosphate buffer, pH 7.5, containing 1.1 M-formaldehyde, and recentrifuged at 30000 rev/min for 15 h in 15 to 30% (w/w) sucrose density gradient containing 1.1 M-formaldehyde to minimize the aggregation of RNA during centrifuging. In these centrifugations (Fig. 1b) the 18S and 28S ribosomal RNAs were used as sedimentation markers. Based on the relationship between the logarithm of the mol. wt. and distance of migration (Boedtker, 1968) we estimate that a substantive fraction of the RNA has a mol. wt. greater than 10^7, which corresponds to 20% of the intact single strand of viral DNA.

A sample of the same RNA was denatured in 99% dimethyl sulphoxide, prepared in
RNA in herpes simplex virus infected cells

Table 1. Hybridization of labelled infected cell nuclear RNA fractionated by centrifuging in sucrose density gradients to viral DNA fixed to filters*

<table>
<thead>
<tr>
<th>Multiplicity of infection (p.f.u./cell)</th>
<th>ct/min bound to filters</th>
<th>% of input ct/min bound specifically to HSV-1(F) DNA</th>
<th>Ratio of % ct/min bound: HSV-1(F) DNA hybridized to HSV-1 DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli DNA</td>
<td>HSV-1(F) DNA</td>
<td>&gt;58S/(38-58S)</td>
<td>&gt;58S/(38-58S)</td>
</tr>
<tr>
<td>38-58S</td>
<td>38-58S</td>
<td>&gt;58S</td>
<td>&gt;58S/(38-58S)</td>
</tr>
<tr>
<td>10</td>
<td>32769</td>
<td>12</td>
<td>0.064</td>
</tr>
<tr>
<td></td>
<td>39164</td>
<td>11</td>
<td>0.078</td>
</tr>
<tr>
<td>100</td>
<td>139576</td>
<td>11\3</td>
<td>0.64</td>
</tr>
<tr>
<td></td>
<td>127507</td>
<td>29</td>
<td>0.59</td>
</tr>
</tbody>
</table>

* The RNA comprising the sedimentation ranges 38 to 58S and > 58S were collected from regions of the sucrose density gradients outlined by horizontal bars in Fig. 1 (a).

Finally RNA sedimenting in the region of 38-58S and greater than 58S (but without the pellet) were pooled and hybridized to viral DNA fixed to filters. The results shown in Table 1 indicate that the RNA > 58S contained a larger fraction of virus specific sequences than 38 to 58S RNA and moreover, the amount of virus specific sequences appeared to be higher in cells infected at the high input multiplicity.

Secondary structure and symmetry of viral transcripts in high mol. wt. RNA from infected cells

Three types of experiments were done to determine the nature of the viral transcripts in HMW-RNA.

In the first, pooled RNA from two regions of the sucrose density gradients were denatured by heating at 115 °C for 5 min in 0.1 x SSC, then rapidly chilled in dry ice. Comparison of the hybridization of native and denatured RNAs (Experiment 1, Table 2) showed that denatured RNA hybridized 3 times more efficiently than native RNA.

We reported elsewhere (Jacquemont & Roizman, 1975) that a substantive fraction of viral transcripts in infected cells anneal to form double stranded (DS) RNA. In the second experiment we therefore prepared DS RNA from RNA > 45S pooled from sucrose density gradients as described in Methods. This RNA was at least 80 % resistant to RNase A (50 µg/ml, 30 min at 37 °C in 2 x SSC). Undenatured DS RNA from infected cells prepared in this fashion does not hybridize to viral DNA (Jacquemont & Roizman, 1975). This RNA was then denatured by heating at 115 °C for 5 min in 0.1 x SSC and hybridized to viral DNA fixed to filters. As shown in (Experiment 2, Table 2) approx. 7.1 % of denatured DS RNA hybridized to viral DNA fixed to filters.

The third experiment in this series was done as follows. Unlabelled RNA > 50S was collected from sucrose gradients, pooled, concentrated and dialysed against a solution containing 1 M-NaCl, 50 % (v/v) formamide, and 0.05 M-tris, pH 8.05. The RNA was then hybridized in this solution with denatured, in vitro labelled HSV-1 (F) DNA for 70 h at 50 °C. A portion of the hybridization mixture was dialysed to remove the formamide, then digested with Neurospora crassa nuclease; this test indicated that approx. 40 % of viral DNA was driven into hybrid. The remainder of the hybridization mixture was added in a solution
Table 2. Hybridization of labelled high mol. wt. infected cell nuclear RNA to viral DNA fixed to filters

<table>
<thead>
<tr>
<th>DNA fixed to filters</th>
<th>Infected cell RNA class</th>
<th>Nature of RNA</th>
<th>ct/min input</th>
<th>ct/min bound</th>
<th>% input bound</th>
<th>% virus specific</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli</strong></td>
<td>10 µg</td>
<td>38–58S</td>
<td>Undenatured</td>
<td>32769</td>
<td>12</td>
<td>0.037</td>
</tr>
<tr>
<td></td>
<td>10 µg</td>
<td>38–58S</td>
<td>Denatured</td>
<td>27416</td>
<td>11.6</td>
<td>0.042</td>
</tr>
<tr>
<td></td>
<td>10 µg</td>
<td>&gt;58S</td>
<td>Undenatured</td>
<td>39164</td>
<td>11.6</td>
<td>0.030</td>
</tr>
<tr>
<td></td>
<td>10 µg</td>
<td>&gt;58S</td>
<td>Denatured</td>
<td>51160</td>
<td>10.9</td>
<td>0.021</td>
</tr>
<tr>
<td><strong>HSV-1(F)</strong></td>
<td>1 µg</td>
<td>38–58S</td>
<td>Undenatured</td>
<td>32769</td>
<td>33</td>
<td>0.101</td>
</tr>
<tr>
<td></td>
<td>1 µg</td>
<td>38–58S</td>
<td>Denatured</td>
<td>27416</td>
<td>58</td>
<td>0.211</td>
</tr>
<tr>
<td></td>
<td>1 µg</td>
<td>&gt;58S</td>
<td>Undenatured</td>
<td>39164</td>
<td>42</td>
<td>0.107</td>
</tr>
<tr>
<td></td>
<td>1 µg</td>
<td>&gt;58S</td>
<td>Denatured</td>
<td>51160</td>
<td>120</td>
<td>0.235</td>
</tr>
</tbody>
</table>

Experiment 1*

| **E. coli**          | 20 µg                   | >45S          | Denatured DS RNA | 25252       | 14            | 0.055          |               |
| **HSV-1(F)**         | 5 µg                    | >45S          | Denatured DS RNA | 25252       | 1826          | 7.2            | 7.15          |

* The cells were infected with a multiplicity of 10 p.f.u./cell. RNA was fractionated as described in legends to Fig. 1(a).

† The cells were infected with a multiplicity of 100 p.f.u./cell. RNA was extracted from 5 h infected cells as described in Methods.

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Fig. 2. Distribution of labelled viral DNA on isopycnic banding in Cs2SO4 solution following hybridization with high mol. wt. RNA (●●●●), infected cell nuclear RNA (○○○○) and uninfected HEp-2 cell RNA (■■■■).
of Cs$_2$SO$_4$ to yield a density of 1.526 g/ml then centrifuged to equilibrium at 37000 rev/min for 60 h in an SW39 rotor. To minimize losses of single stranded DNA, the polyallomer tubes were pre-soaked in 0.1% SDS, 0.05 M-tris, pH 7.5 and then in a solution 0.05 M-tris, pH 7.5, containing 25 µg of HEp-2 RNA per ml. The distribution of the labelled DNA sequences in the gradient are shown in Fig. 2. The position of double stranded, single stranded and HMW-RNA were determined from several independent sedimentations of these materials in similar gradients; the buoyant densities were 1.457 g/ml for double stranded DNA, 1.498 g/ml for single stranded DNA, and 1.679 g/ml for HMW-RNA. The data indicate that 12 and 6% of labelled DNA hybridized to HMW-RNA and to the total nuclear 8 h infected cell RNA, respectively, and banded at a density of RNA and that this fraction was absent from hybridization mixtures of labelled DNA and uninfected HEp-2 cell RNA. It should be pointed out that the DNA banding in this region probably represents DNA sequences hybridized to long stretches of viral RNA. This conclusion is suggested by the general shape of the broad DNA band in the buoyant density range of 1.45 to 1.60 g/ml. Thus the labelled DNA hybridized to uninfected HEp-2 bands in the region of single stranded DNA. The DNA hybridized to nuclear RNA, which is highly heterogeneous in size, bands in part in the position of single stranded DNA and at higher densities. The DNA hybridized to HMW-RNA partitioned largely in the positions occupied by single stranded DNA and by HMW-RNA.

In the next step, the fractions in the buoyant density range of 1.650 and 1.750 g/ml from tubes containing the hybridization mixtures of HMW-infected cell RNA and viral DNA were pooled, and the RNA depolymerized by treatment with 0.3 M-KOH overnight at 37 °C. The mixture was then neutralized, concentrated and dialysed against 0.7 M-NaCl, 0.05 M-tris, pH 8.05. This DNA (HMW-RNA-DNA) which is capable of reannealing with viral DNA was then compared with the DNA from which it was derived (parental DNA) with respect to ability to renature and to hybridize with unlabelled RNA extracted from nuclei of 8 h infected cells. The results summarized in Table 3 show the following: (i) the HMW-RNA-DNA is able to self anneal. In fact, 43.4% of the DNA self annealed at a $C_0$ of 0.024. This indicates that at least 43% of the DNA was hybridized to RNA capable of self annealing; by extension, this RNA was transcribed from complementary DNA sequences. (ii) The rate of reassociation of HMW-RNA-DNA is faster than that of parental DNA from which it is derived. This finding indicates that HMW-DNA is not representative of the parental DNA but rather is enriched for a selected set of sequences. Implicit in this conclusion is that sequences in HMW-RNA arose from a limited portion of the genome or that
HMW-RNA consists of 2 or more classes of transcripts differing greatly in abundance.

(iii) At a $R_{at}$ of 916, more of the HMW-RNA-DNA is driven into DNA-RNA hybrid than parental DNA. The data indicate that HMW-RNA sequences which hybridized to the DNA are present in abundant amounts in nuclei of 8 h infected cells.

**Extent of representation of HSV-1 (F) DNA in HMW-RNA**

The objective of these experiments was to determine what fraction of HSV-1 DNA was represented among viral RNA sequences contained in HMW-RNA accumulating in 5 h infected cells. In these experiments $> 45S$ was collected from sucrose density gradients, pooled, concentrated and dialysed against 0.07 M-NaCl, 0.05 M-tris, pH 8.05, and hybridized with *in vitro* labelled viral DNA in liquid as described in Methods. The results, shown in Fig. 3, indicate that the HMW-RNA drove approx. 50% of the DNA into DNA-RNA hybrid. The analysis of the hybridization kinetics by non-linear least squares regression indicated that the HMW-RNA contained at least 2 sets of sequences. One set, derived from 24% of viral DNA was 5000-fold more abundant than the second, scarce set, derived from 26% of viral DNA.

**DISCUSSION**

In this study we analysed two populations of HMW-RNA prepared by sucrose density gradient fractionation of RNA extracted from nuclei of HEp-2 cells infected with HSV-1. The first population comprised molecules labelled with $[^3H]$-uridine during a 20 min interval and therefore we are concerned with RNA molecules made and accumulating within a relatively short interval. The second population was not labelled; we were concerned therefore with RNA molecules which accumulated between the intervals of 0 and 5 h post infection when the cells were harvested for extraction.

The salient characteristics of the labelled HMW-viral RNA are as follows: (i) the increase in the amount of HMW-RNA hybridizing to viral DNA on filters upon heat denaturation suggests that a large fraction of HMW-RNA is base-paired and heating either denatures double stranded RNA formed during extraction or more likely, denatures intrastrand base-pairing which is characteristic of many informational RNA molecules (Strauss & Sinsheimer, 1963; Lodish, 1971; Gralla & Delisi, 1974; Jelinek et al. 1974). (ii) Of particular interest are the observations that viral RNA sequences were capable of self annealing and that
RNA in herpes simplex virus infected cells

DS-RNA defined by its resistance to RNase in $2 \times$ SSC and several other properties described elsewhere (Jacquemont & Roizman, 1975) was considerably enriched for viral RNA sequences. The enrichment could be due to accumulation of symmetric transcripts of viral genome in preference to those of the host DNA. (iii) Labelled RNA populations are not suitable for accurate measurements of the amount of DNA template from which they are derived and none was attempted. It should be noted however that hybridization competition tests with HSV-DNA fixed on filters showed that at least 80% of the viral RNA sequences present in polyribosomes of infected cells were represented in labelled HMW-nuclear RNA (Wagner & Roizman, 1969).

The salient features of the studies on the unlabelled HMW-RNA extracted from 5 h infected cells are as follows: firstly, this RNA population contained transcripts arising from symmetric transcription of a fraction of the genome and, moreover, it is likely that these transcripts were present in abundant quantities. This conclusion is based on the results of experiments involving hybridization at the HMW-RNA to labelled viral DNA, harvesting of the hybrid banding at a density of the RNA, and characterization of the DNA which hybridized to the HMW-RNA and banded with it. Since the initial hybridization of labelled viral DNA to unlabelled HMW-RNA did not reach completion, it is likely that only DNA fragments homologous to abundant RNA species were driven into DNA-RNA hybrid. This hypothesis is supported by the observation that the HMW-RNA-DNA consists of a selected population comprising approx. 30% of DNA – in good agreement with the amount of DNA template (29%) giving rise to abundant symmetric transcripts (Jacquemont & Roizman, 1975).

Secondly, analyses of the kinetics of hybridization (Frenkel & Roizman, 1972) of unlabelled HMW-RNA to labelled viral DNA in liquid indicate that the RNA was homologous to approx. 50% of viral DNA and that it consisted of at least 2 classes arising from 24 and 26% of viral DNA and differing 5000-fold in molecular concentration. These results should be taken with some caution. Since HMW-RNA contained symmetric transcripts, it is likely that both RNA-RNA and RNA-DNA hybrids formed during the hybridization and therefore the amount of RNA available to drive DNA into DNA-DNA hybrid changed drastically during the hybridization. We may conclude therefore that the HMW-RNA was derived from at least 50% of viral DNA and probably more. Furthermore, it seems likely that although the conclusions concerning the inhomogeneity of the RNA are probably correct, the numerical estimates may not be.

Finally we should like to draw attention to several observations for which we have as yet no definitive explanation. First, the experiment with HMW-RNA-DNA indicates that the abundant RNA species in HMW-RNA is also the abundant species in total 8 h nuclear RNA. We are puzzled therefore by the observation that the ratio of abundant to scarce in HMW-RNA is 5000:1 whereas in 8 h nuclear RNA it is usually about two orders of magnitude lower. Eventually, studies on the mechanisms regulating RNA abundance might discriminate between the various hypotheses which could explain the data; we could envisage, for instance, that scarce RNA species are transcribed less often or that they are processed into mRNA, utilized and degraded more rapidly than abundant species. The second observation concerns the amount of DNA which arises by symmetric transcription and the arrangement of the sequences in the DNA. The preceding paper in this series (Jacquemont & Roizman, 1975) clearly established that at least 30% of the transcribed DNA is capable of self annealing. It is likely that this figure is an underestimate and that the actual amount of DNA transcribed symmetrically is higher. For this reason we doubt that the self annealing transcripts arise from the internal inverted repetitions of the terminal sequences reported.
by Sheldrick & Berthelot (1974) and extended by our laboratory (Roizman et al. 1974; Wadsworth, Jacob & Roizman, 1975) since terminal sequences and their inversions do not exceed 21% of the DNA. However, at the moment we cannot exclude the possibility that the symmetric transcripts arise as a consequence of more extensive inversions in the DNA. Specifically current data (Roizman et al. 1974, Hayward et al. 1975) indicate that HSV-DNA are represented by 4 molecules differing solely in the relative orientation of two regions, L and S, comprising 82 and 18% of the DNA respectively. Because of the inversions, readthrough from the promotor regions would almost necessarily ensure symmetric transcription of nearly the entire HSV-DNA.

The fundamental assumption underlying many of the studies on HMW-RNA, both eukaryotic cell and viral, is that it serves as a precursor for RNA functioning as messenger (Darnell, Jelinek & Molloy, 1973). The model does not necessarily predict that all HMW-RNA is processed into mRNA and, in fact, in eukaryotic cells a substantial amount of the heterogeneous nuclear RNA appears to turn over rapidly (Sceiro et al. 1966). The fate of viral HMW-RNA is not clear. Because of the limitations in the available techniques for analyses of unlabelled accumulated viral RNA and of pulse labelled RNA we cannot tell whether the viral RNA molecules labelled within a short time have the same composition as those accumulating in the infected cell over several hours. The only valid conclusion that can be made at this time is that pulse labelled RNA must consist, at least in part, of symmetric transcripts since these accumulate in abundant amounts. Based on the observations that viral RNA sequences not translated on polyribosomes are selectively retained in the nucleus and the apparent absence of abundant quantities of symmetric viral transcripts in the cytoplasm (Kozak & Roizman, 1974), it seems likely that the viral RNA sequences complementary to viral messenger RNA are probably restricted to the nucleus.

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