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Hybridization and Sedimentation Studies on "Early" and "Late" Vaccinia Messenger RNA

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The vaccinia virus multiplication cycle can be divided into two portions: the early portion, which precedes viral DNA replication and lasts for about one-and-a-half hours; and the late portion which follows until maturation of progeny is complete. Vaccinia messenger RNA transcribed during these two periods, that is, early and late messenger RNA, has been studied by means of hybridization and density gradient sedimentation. The following results were obtained:

(1) Early vaccinia messenger RNA is distinctly smaller than late messenger RNA (10 to 12 s against 16 to 20 s). Late messenger RNA contains nucleotide sequences not present in early messenger RNA; however, all the sequences transcribed early are also transcribed late. If late messenger RNA is transcribed from the entire viral genome, one-half to two-thirds is transcribed early.

(2) Messenger RNA transcribed in the presence of cytosine arabinoside, which completely inhibits viral DNA replication, appears to be identical to early messenger RNA. The cytosine arabinoside RNA transcribed in HeLa and L cells contains the same base sequences.

(3) The pattern of transcription of early and late vaccinia messenger RNA in HeLa and L cells is quite different. In HeLa cells the total amount of late messenger RNA synthesized greatly exceeds the amount of early; in L cells the position is reversed.

(4) The effect of varying the multiplicity of infection on the pattern of early and late messenger RNA transcription has been studied. The rate of early mRNA synthesis is proportional to the multiplicity over a certain limited range. Once the maximum rate of mRNA transcription has been reached, which occurs the earlier the higher the multiplicity, the rate of transcription of messenger RNA falls to a steady low level equal to no more than 5% of the maximum value; this rate of transcription then persists until at least 19 hours after infection.

(5) The large messenger RNA molecules transcribed late contain nucleotide sequences also present in small early messenger RNA molecules. Some small messenger RNA molecules are also transcribed late: these contain at least some sequences characteristic of late messenger RNA.

(6) At five hours after infection the messenger RNA molecules present in polyribosomes contain all the sequences characteristic of early messenger RNA molecules. By eight hours after infection messenger RNA in polyribosomes is very significantly depleted with respect to sequences characteristic of early messenger RNA.

(7) The stability of early and late vaccinia messenger RNA in the presence of actinomycin D has been studied in HeLa and L cells. Early vaccinia messenger RNA is very stable in HeLa cells. Those late messenger RNA molecules which contain sequences characteristic of late messenger RNA are mostly unstable

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(half-life less than one hour); but those containing sequences characteristic of early messenger RNA are as stable as early messenger RNA itself. In L cells early and late vaccinia messenger RNA are equally stable (half-life two to three hours). mRNA is considerably more stable in extracts of HeLa cells than of L cells; and early mRNA is somewhat more stable in extracts of HeLa cells than is late mRNA.

These results are discussed in relation to the pattern of macromolecular biosynthesis during the vaccinia virus multiplication cycle.

1. Introduction

The genome of vaccinia virus is a DNA molecule with a molecular weight of about 160,000,000. This amount of DNA is capable of coding for about 500 proteins. Very few of these are known; however it seems quite clear that they are not all synthesized at the same time during the infection cycle. Thus virus-induced enzymes are formed between one and four hours after infection (McAuslan & Joklik, 1962; McAuslan, 1963*a*,*b*; Jungwirth & Joklik, 1965); structural viral proteins which coat progeny genomes are synthesized sequentially from about three hours on (Joklik & Becker, 1964; Salzman, Shatkin & Sebring, 1963); and various proteins capable of reacting with antiserum to vaccinia virus are synthesized at different times during the infection cycle (Appleyard, Hume & Westwood, 1965). Thus there probably exists a mechanism for sequential gene expression. Such a mechanism could operate at the level of transcription or of translation of genetic information, or possibly by a combination of these. Evidence that there are controls at both levels in the vaccinia virus-infected cell has recently been reviewed (Joklik, 1966).

Control of the expression of genetic information has been extensively studied in bacteriophage-infected bacteria. In essence, interest has centered around the question whether mRNA coding for late functions is transcribed during the early part of the infection cycle or not. Hall, Nygaard & Green (1964) showed that about 27% of T2 mRNA molecules transcribed during the late period are copied from DNA sequences not expressed during the early period. Dove (1966) showed that late lambda gene action is dependent both upon chromosomal replication and a general inducer of late functions, and presented models for the interaction of replication and transcriptability from genes for late functions. Protass & Korn (1966*a*) showed that the formation of mRNA coding for T4 lysozyme (a late enzyme) does not significantly precede the time at which the enzyme first becomes detectable, which is also the time when first progeny DNA molecules arise, and concluded that the mechanism regulating early and late protein synthesis operated at the level of transcription. Further, in lambda-infected *Escherichia coli* exonuclease synthesis begins early and is independent of the formation of progeny DNA, while endolysin formation coincides with and is dependent on the appearance of progeny DNA (Protass & Korn, 1966*b*). Parallel observations on the control of the synthesis of the early enzyme dCMP deaminase and the late enzyme lysozyme in *Bacillus subtilis* infected with phage 2C were made by Pène & Marmur (1967). Finally, the work of Ebisuzaki (1965,1966) on the ultraviolet light sensitivity of early and late phage functions is in accord with this concept that certain regions only of parental genomes are transcribed and that additional sequences are available for transcription from progeny genomes. Evidence that some mRNA coding for late functions is transcribed from parental genomes has been advanced by Edlin (1965) studying phenotypic reversion of T4 amber mutants by 5-fluorouracil and by Bautz, Kasai, Reilly & Bautz (1966) who

found that gene *e* of T4, which codes for lysozyme, is transcribed to a limited extent immediately after infection, not transcribed for the remainder of the early period, and then extensively transcribed once progeny genomes are synthesized.

We have now carried out similar studies on the mRNA transcribed in mammalian cells infected with vaccinia virus. This system possesses a considerable advantage over other systems, namely that early and late mRNA differ in size and can therefore be identified by density-gradient centrifugation. We describe in this paper a study of early and late vaccinia mRNA both by hybridization and sedimentation analysis. Both HeLa and L cells were investigated since it had become obvious from other work that the pattern of transcription differs considerably in these two cell lines. Among the questions investigated were the kinetics of transcription of early and late mRNA at various multiplicities of infection, the identity of early mRNA transcribed in HeLa and L cells, the nucleotide sequence content of the large late mRNA molecules as compared with the small early ones, the characterization of the mRNA molecules associated with polyribosomes at various times after infection and the intracellular stability of early and late vaccinia mRNA.

2. Materials and Methods

(a) *Cells, medium and virus*

HeLa S3 cells were grown in suspension culture in Eagle's medium (Eagle, 1959) supplemented with 5% calf serum. L cells were grown similarly in medium supplemented with 10% fetal calf serum. The WR strain of vaccinia virus was used. Highly purified preparations (Joklik, 1962*a*) containing 2 to 3×10^{11} elementary bodies and 5 to 10×10^{9} plaque forming units/ml. on chick embryo fibroblasts were used.

(b) *Infection of cells*

Cells were infected at a concentration of 10^7 cells/ml. in adsorption medium (Becker & Joklik, 1964). Adsorption was allowed to proceed for 15 min at 37°C with stirring; this resulted in 50% adsorption. The cells were then diluted to 8×10^5 cells/ml. with growth medium. The instant of dilution was designated as time zero.

(c) *Pulse-labeling*

[^{14}C]UR† (30 mc/m-mole, New England Nuclear Corporation) was used at the rate of 2 μc/10^7 cells. [^{3}H]UR (21 to 25 c/m-mole, New England Nuclear Corporation) was used at the rate of 5 to 10 μc/10^7 cells. For the labeling of nascent protein chains on polyribosomes a reconstituted mixture of uniformly ^{14}C-labeled amino acids (New England Nuclear Corporation, 1·5 mc/mg) was used.

(d) *Breaking open of cells*

Cells were collected by centrifugation, washed once with Earle's saline, and allowed to swell for 10 min (for HeLa cells) or 5 min (for L cells) at 0°C in hypotonic medium RSB (10^{-2} M-NaCl–10^{-2} M-Tris (pH 7·4)–1·5 mM-Mg^{2+}; Warner, Knopf & Rich, 1963). The cells were broken in a Dounce homogenizer calibrated for the number of strokes necessary to break over 98% of HeLa cells (in lots of 4×10^7 cells in 2ml.). The efficiency of breaking L cells was somewhat lower, but exceeded 85%. The homogenate was then centrifuged at 800 *g* for 2 to 3 min, and the supernatant solution used as the cytoplasmic fraction.

(e) *Sucrose density-gradient centrifugation and counting of radioactivity*

The techniques have been fully described (Becker & Joklik, 1964; Joklik & Becker, 1964).

† Abbreviations used: UR, uridine; dT, thymidine; CAR, cytosine arabinoside; dCMP, deoxycytidylate; dFU, 5′-fluorodeoxyuridine; SDS, sodium dodecyl sulfate; HeLa CAR.RNA and L cell CAR.RNA, vaccinia messenger RNA transcribed in the presence of cytosine arabinoside in HeLa and L cells, respectively.

(f) *Preparation of RNA for hybridization*

The following procedure was found to be superior to extraction with phenol. Cytoplasmic extracts were rendered 1% with respect to SDS and 0·2 M with respect to NaCl. After standing for 15 min at room temperature 2 vol. of ethanol were added and the mixture kept overnight at — 18°C. The precipitates were then centrifuged down at 1000 *g* for 10 min, and suspended in 0·05 M-sodium acetate–0·01 M-EDTA pH 5·1, at the rate of 1 ml./10^7 cells from which the cytoplasmic extract had been prepared. SDS was added to a final concentration of 1%; the precipitates dissolved on thorough mixing. Sodium perchlorate was then added to a final concentration of 0·5 M. The mixtures were shaken for 1 to 2 min with an equal volume of chloroform–isoamyl alcohol (24 : 1), chilled for 15 min and centrifuged at 1000 *g* for 10 min. The organic phase was removed and the procedure repeated. After the second centrifugation the aqueous phase was made 0·2 M with respect to NaCl and 2 vol. of ethanol were added. The mixtures were again chilled at — 18°C overnight and then centrifuged. The precipitates then dissolved instantaneously in 0·1 × SSC (SSC is 0·15 M-NaCl–0·015 M-sodium citrate pH 7). This method removed over 98% of the protein and gave recoveries of RNA of over 95%. No degradation of RNA was observed; RNA samples could be kept indefinitely without degradation at — 18°C in 67% ethanol.

The concentration of RNA was measured spectrophotometrically, using Adams' modification of the method of Warburg & Christian (1942) (distributed by the California Corporation for Biochemical Research). One optical density unit at 260 mμ corresponds to 45 μg RNA and 41·5 μg RNA when the absorbancy ratio at 280 mμ/260 mμ is 0·5 and 0·6, respectively; this was the range for the RNA preparations used here.

For the preparation of RNA from whole cells, phenol extraction according to Scherrer & Darnell (1962) was used.

(g) *Preparation of vaccinia virus DNA*

Vaccinia virus DNA was isolated essentially according to the method of Joklik (1962*b*) with slight modifications. The defatted virus (60 mg) was suspended in 3 ml. of SSC and 0·1 ml. of 2-mercaptoethanol (14 M) was added. After 1 hr at room temperature 1 ml. of pronase (2 mg/ml.) pre-incubated at 37°C for 2 hr was added and the mixture incubated at 37°C overnight. SDS was then added to a final concentration of 0·5% and incubation at 37°C continued for 1 hr. DNA was then twice extracted with an equal volume of phenol saturated with 0·15 M-NaCl–0·1 M-EDTA, pH 8, in an Erlenmayer flask, with very gentle stirring. After centrifugation, the supernatant solution was transferred carefully into dialysis tubing and dialyzed against 3 changes of 1 liter of SSC for a total of 24 hr at 4°C. The $S_{20,w}$ of the DNA thus prepared ranged from 12 to 16 corresponding to a mol. wt of 2 to 3·5 × 10^6. The yield of DNA was about 70%.

(h) *Hydridization*

In essence, the method of Gillespie & Spiegelman (1965) was used. The following points concerning hybridization parameters are of relevance.

(i) *Immobilization of DNA*

DNA was denatured at 0°C for 10 min at pH 12·5 in 0·1 × SSC at a concentration of 20 μg/ml. The solution was then neutralized by the addition of HCl and diluted 40-fold in 6 × SSC. Filtration of DNA through pre-soaked Millipore membrane filters (15/16ths inches diameter) was carried out very slowly. Filters were then washed with 10 ml. 6 × SSC. Usually filtration and washing took over 30 min. Approximately 90% of DNA was retained on the filters.

(ii) *Duration of hybridization*

Membrane filters containing 1 μg of immobilized DNA were incubated with portions of RNA at 66°C in 6 × SSC (final vol. 5 ml.). Figure 1 shows the relation between the duration of hybridization and the amount of RNA hybridized. A plateau is reached at about 30 hr; after more than 40 hr the amount of RNA hybridized decreased slightly. Usually hybridization was carried out for 24 hr, when approximately 90% of the plateau level was reached. In every hybridization experiment non-specific binding was determined by incubating an identical portion of the RNA being tested with a membrane filter

containing the same amount of immobilized *E. coli* K12 DNA as vaccinia DNA. This background was subtracted from the value obtained for hybridization with vaccinia DNA. After incubation membrane filters were rinsed with 6 × SSC, incubated at room temperature for 2 hr with 5 ml. of pancreatic RNase (20 μg/ml., heated at 85°C at pH 5 for 10 min in 2 × SSC), and then washed with 50 ml. of 6 × SSC by means of filtration, with the DNA-containing surface uppermost.

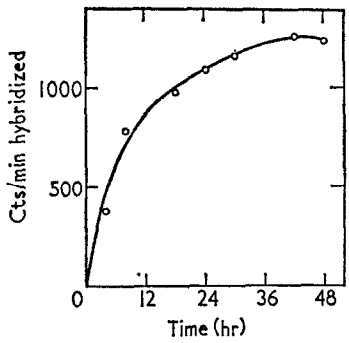

FIG. 1. Time-course of saturation of vaccinia DNA with mRNA.

HeLa cells (2×10^8) were infected with vaccinia virus WR at a multiplicity of 200. The cells were labeled with 200 μc of [^{3}H]UR for 30 min at 5 hr after infection. RNA was prepared from the cytoplasmic fraction as described in Materials and Methods. The specific activity of the RNA was 407 cts/min/μg. Membrane filters containing 1 μg vaccinia DNA were incubated with 57 μg of RNA, and the amount of RNA hybridized was assayed at the times indicated.

(iii) *Hybridization to DNA pre-saturated with cold RNA*

Membrane filters containing immobilized DNA were pre-incubated with saturating amounts of cold RNA in 6 × SSC for 20 hr at 66°C, and the labeled RNA solution (in 0·3 ml. or less) was then added. The filters were incubated for another 24 hr so as to allow unsaturated regions of DNA molecules to hybridize with labeled RNA. Saturating amounts of RNA were determined by incubating 1 μg of immobilized DNA with varying amounts of identical labeled RNA. Pre-incubation of immobilized DNA in 6 × SSC for 20 hr at 66°C did not affect its ability to hybridize with RNA.

3. Definitions and Background Information

The following definitions and background information will be found useful for following the experiments and arguments presented below:

(1) mRNA transcribed before or in the absence of viral DNA replication is "early mRNA". mRNA transcribed after the initiation of viral DNA replication is "late mRNA". The nucleotide sequences in early mRNA molecules are those "characteristic of early mRNA"; and those sequences present in late mRNA which are not present in early mRNA are those "characteristic of late mRNA". Late mRNA contains nucleotide sequences characteristic of early mRNA as well as those characteristic of late mRNA.

(2) The RNA preparations used for hybridization contain other RNA species besides vaccinia mRNA. There are present in addition 28 s and 16 s ribosomal RNA, tRNA, and very small amounts of host-cell mRNA. Pulse lengths throughout this work have been so arranged that the two ribosomal RNA species were *never* labeled (except for the experiments determining the stability of vaccinia mRNA (see below), where some labeled ribosomal RNA entered the cytoplasm during the chase period). tRNA was labeled progressively less as infection proceeded; its hybridization to vaccinia DNA was not specifically studied, but is insignificant compared with that of vaccinia mRNA. Over 90% of the total amount of RNA in the preparations used here was thus unlabeled ribosomal RNA, and vaccinia mRNA most probably never exceeded 1%. Throughout the period of the infection cycle examined the rate of incorporation of label into mRNA correlated well with the total

amount of mRNA present (as determined by saturation–competition experiments), indicating that there occurred no marked changes in precursor pool levels. However, the amount of mRNA per cell did vary somewhat from experiment to experiment, and from preparation to preparation. However, this variability, which is not surprising, did not exceed ± 33%.

(3) Multiplicity is always expressed in terms of the number of virus particles adsorbed per cell.

(4) HeLa and L cells are compared in many of the experiments described below. L cells are distinctly smaller; the mass of an L cell is probably only about one-half that of a HeLa cell. The virus yields in L cells (up to 10,000 virions/cell) were generally about 50% higher than in HeLa cells.

4. Results

(a) *Operational identification of early and late vaccinia mRNA*

The starting point of this investigation is the demonstration that early vaccinia mRNA is unable to block sequences of the vaccinia genome capable of hybridizing with late vaccinia mRNA. However, late vaccinia mRNA is as effective as early vaccinia mRNA in preventing hybridization of early vaccinia mRNA.

The saturation characteristics of early and late vaccinia mRNA labeled with [^{3}H]UR are illustrated in Fig. 2. The steeper curve for late mRNA reflects the larger number of mRNA molecules per μg RNA; evidence that the rate of viral mRNA synthesis is greater at five hours than at one hour after infection is presented below (see Fig. 11).

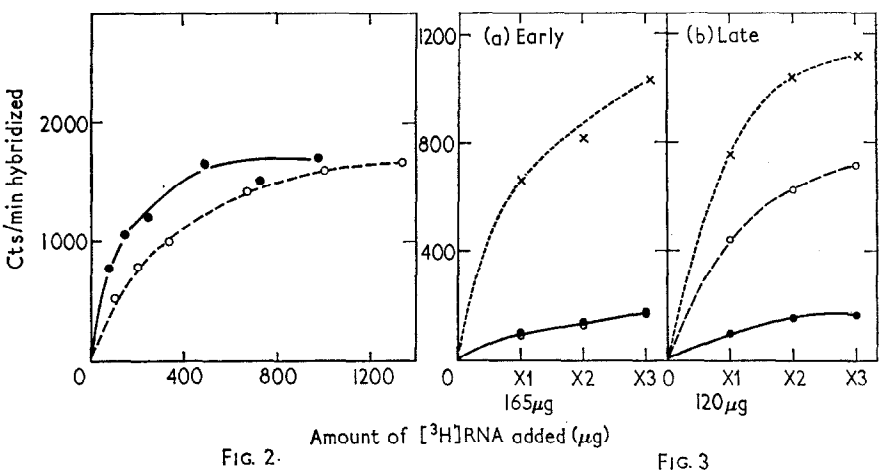

FIG. 2. FIG. 3

FIG. 2. Saturation of vaccinia DNA with early and late vaccinia mRNA synthesized in HeLa cells.

HeLa cells (7×10^6) were infected with vaccinia virus at a multiplicity of 300. Samples of 4×10^6 and 3×10^6 cells were labeled for 30 min with 300 μc and 200 μc of [^{3}H]UR at 1 and 4 5/6 hr after infection, respectively. RNA was extracted from the cytoplasm as described in Materials and Methods. The specific activity of early and late RNA was 137 cts/min/μg and 129 cts/min/μg, respectively. Membrane filters containing 1 μg of vaccinia DNA were used. —○——○—, Early mRNA; —●—●—, late mRNA.

FIG. 3. Hybridization analysis of early and late vaccinia mRNA.

Membrane filters containing 1 μg of immobilized vaccinia DNA were pre-incubated with 1060 μg of cold early mRNA —○—○— or with 790 μg of cold late mRNA —●—●—, and increasing amounts of ^{3}H-labeled early or late mRNA were added. The specific activity of early and late [^{3}H]mRNA was the same as specified in Fig. 2. The curve with (×) describes the course of hybridization to filters not pre-incubated with cold mRNA.

The hybridization-blocking experiment is shown in Fig. 3. Labeled early and late viral mRNA was hybridized to filters containing 1 μg immobilized vaccinia DNA alone, as well as to such filters which had been pre-exposed to saturating amounts of unlabeled early and late viral mRNA. Pre-saturation with unlabeled early or late mRNA prevented hybridization of labeled early vaccinia mRNA by at least 85%. Similarly, hybridization of late vaccinia mRNA was almost completely inhibited by pre-saturation with cold late mRNA, but pre-saturation with early mRNA inhibited only by about 45%. This figure was somewhat variable; values between 45 and 65% were encountered. This experiment shows that *all* nucleotide sequences transcribed early are represented in mRNA present late; but that there are also transcribed sequences at late stages which are not represented in early mRNA. The simplest model, and the one most heuristically useful, is that all sequences, or at least the major portion, of the viral genome are transcribed at late periods of the infection cycle, but that only about one-half of them are transcribed early.

(b) *Identity of early viral mRNA and mRNA transcribed in the presence of cytosine arabinoside*

It is clear that measuring the ability of mRNA to hybridize with immobilized DNA without and with pre-saturation with cold early mRNA provides an estimate of the relative amounts of early and late sequences: hybridization to normal vaccinia DNA gives an estimate of the total amount of vaccinia mRNA, hybridization to DNA pre-saturated with cold early mRNA gives an estimate of the amount of mRNA only transcribed late, and the difference gives the amount of early viral mRNA. This is true provided one uses amounts of mRNA well below saturating levels; in order to be certain that sufficiently low amounts of mRNA were in fact used, mRNA samples were usually tested at three concentration levels. This ability to quantitate the sequences characteristic of early and late mRNA permitted work on the relative kinetics of transcription of early and late mRNA; on the characterization of large and small mRNA molecules; on the characterization of the type of mRNA associated with polyribosomes at various stages of the infection cycle; on the half-life of early and late mRNA; and on the patterns of transcription in two different cell lines, L cells and HeLa cells. However it was difficult in practice to prepare from normally infected HeLa cells the large amounts of unlabeled early vaccinia mRNA needed for all these projects. Only relatively small amounts of vaccinia mRNA are transcribed in HeLa cells at early stages of infection; further, since early vaccinia mRNA must be harvested before *any* progeny DNA has been synthesized, one tends to collect mRNA earlier than might actually be necessary. Early vaccinia mRNA was therefore prepared from cells infected in the presence of CAR, that is, in cells in which DNA replication was inhibited. It was anticipated that under these conditions early vaccinia mRNA, which, as will be shown below, is very stable, would accumulate in the cell.

At a concentration of 10 μg/ml., CAR inhibits DNA synthesis in uninfected HeLa or L cells, as well as in HeLa or L cells infected with vaccinia virus, by at least 99% (Fig. 4). Transcription of early mRNA commences normally in the presence of this concentration of CAR and then continues for a time which is remarkably different for HeLa and L cells. Figure 5 shows the density gradient profiles of vaccinia mRNA transcribed as infection of HeLa and L cells progresses in the presence and absence

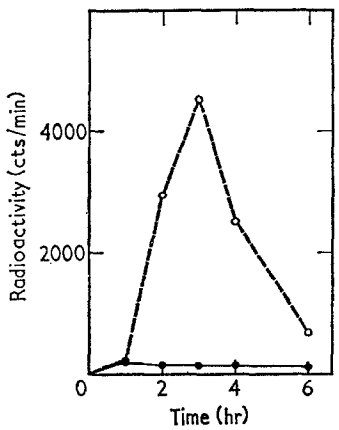

FIG. 4. Inhibition of viral DNA synthesis by CAR.

HeLa cells (6×10^7) were infected with vaccinia virus at a multiplicity of 200 in the presence and absence of 10 μg/ml. of CAR ($3{\cdot}5 \times 10^{-5}$ M). Samples of 10^7 cells were pulse-labeled with 4 μc of [^{3}H]dT (6·5 c/m-mole) for 10 min at the times indicated. Cells were disrupted in 1 ml. of RSB, and 0·5 ml. of the cytoplasmic fractions was counted. —○—○—, no CAR; —●—●—, CAR present.

of CAR. In normally infected HeLa cells the early mRNA is small, with a median sedimentation coefficient of about 10 to 12 S. Its rate of synthesis is exceeded two- to threefold by that of late mRNA, the median S of which is 16 to 20. In the presence of CAR early mRNA is transcribed at the normal rate for about 100 minutes, but by 2·5 hours its rate of synthesis is already barely detectable.

The pattern is quite different in L cells. Here early 10 to 12 S mRNA is transcribed very rapidly. Synthesis of late mRNA is all but undetectable at the multiplicity of 500 which was used in this experiment. Sedimentation profiles obtained from cells infected in the absence and presence of CAR are very similar: one may conclude that in L cells infected at a multiplicity of 500 most of the vaccinia mRNA is transcribed from *parental* genomes. Portions of the infection cycle later than the 228 minutes shown in Fig. 5 were then examined. The following is a typical finding: the relative rates of mRNA transcription in L cells at 1, 4, 14 and 19 hours after infection at a multiplicity of 500 were 100, 25, 1, and 1. The median sedimentation coefficient of the mRNA at 1 and 4 hours was 10 to 12 S, at 14 and 19 hours 16 to 18 S. When *lower* multiplicities are used (50 to 100), then the rate of early 10 to 12 S mRNA transcription is lower, and transcription of late 16 S mRNA becomes more clearly apparent (this has also been discussed briefly by Joklik & Merigan, 1966). However the rate of late mRNA transcription is always only a small fraction of that in HeLa cells.

Thus, only 10 to 12 S type mRNA is synthesized in either HeLa or L cells infected in the presence of CAR, and the amount of label in such mRNA is much larger in L cells than in HeLa cells. This could be due either to a larger number of mRNA molecules transcribed or to a higher specific activity (due to a lower precursor pool level) of the molecules transcribed. Evidence will be presented below which indicates that the first alternative is the predominant one.

With this background established, large amounts of cold CAR.RNA (early vaccinia mRNA harvested from cells infected in the presence of CAR) were prepared, as well

as smaller amounts of labeled CAR.RNA. Figure 6 shows that HeLa CAR.RNA inhibits the hybridization of early and late vaccinia mRNA in the same manner as early mRNA: hybridization of early mRNA is inhibited by over 90%, hybridization of late mRNA by only about 60%. CAR.RNA thus contains the same species of RNA as early vaccinia mRNA transcribed in cells infected in the absence of CAR and since much larger amounts of CAR.RNA could conveniently be collected, CAR.RNA was used in all subsequent experiments in place of unlabeled early vaccinia mRNA.

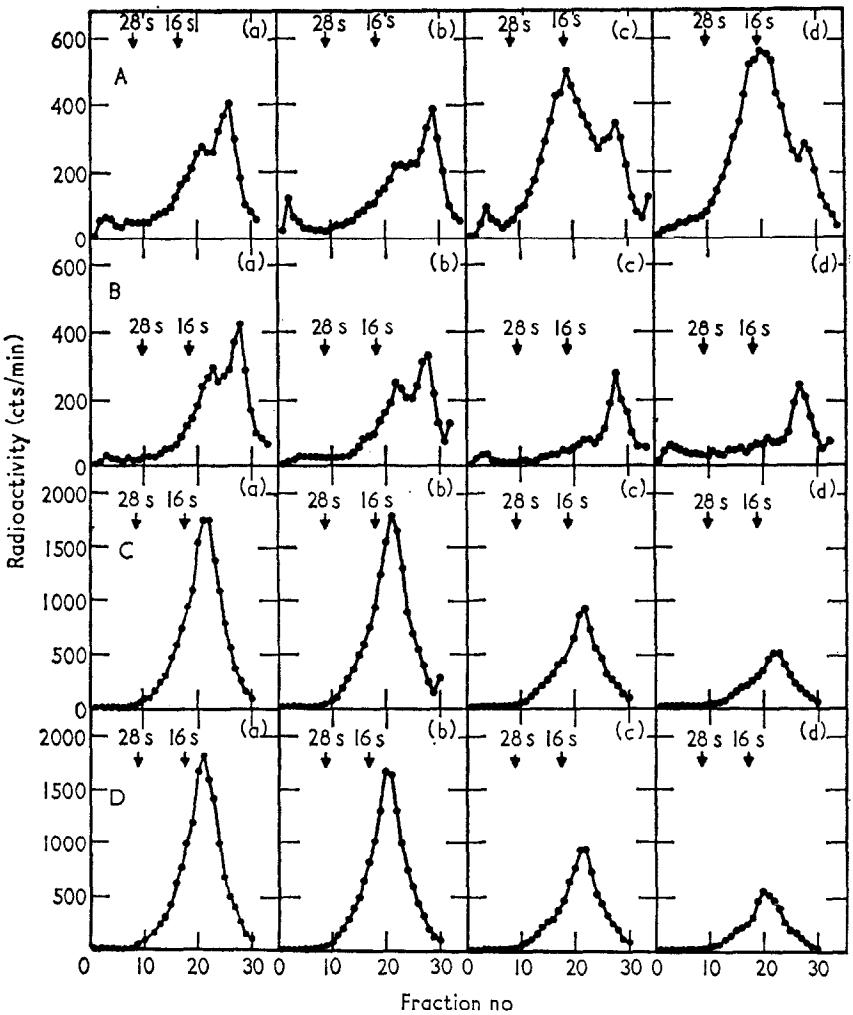

FIG. 5. Size distribution of pulse-labeled vaccinia mRNA prepared from cells infected in the presence and absence of CAR. HeLa and L cells (each 8×10^7) were infected with vaccinia virus at multiplicities of 300 and 500, respectively, in the presence and absence of 10 μg/ml. of CAR. Samples of 2×10^7 cells were pulse-labeled with 5 μc of [^{14}C]UR for 12 min at the times indicated. Cells were disrupted in 1·2 ml. RSB, and the cytoplasmic fractions rendered 1% with respect to SDS and analyzed by SDS sucrose density gradient centrifugation. (A) HeLa cells, (B) HeLa cells plus CAR, (C) L cells, (D) L cells plus CAR. (a), (b), (c), (d) indicate addition of label at 25, 78, 138 and 228 min after infection, respectively.

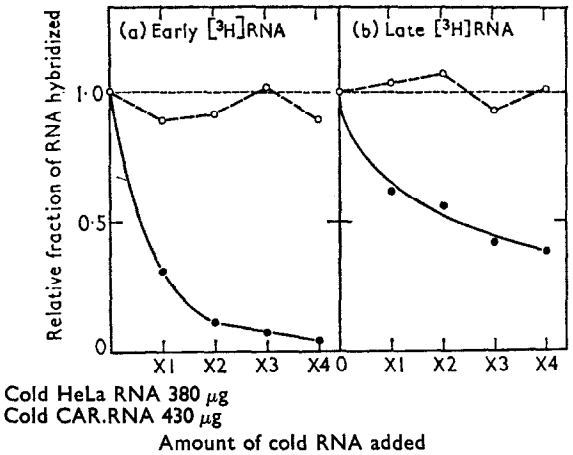

FIG. 6. Hybridization-competition between CAR.RNA and early and late vaccinia mRNA.

Cold CAR.RNA was prepared from the cytoplasm of HeLa cells infected with vaccinia virus at a multiplicity of 200 for 2 hr in the presence of 10 μg/ml. of CAR. Early and late vaccinia mRNA labeled with [^{3}H]UR were prepared from the cytoplasm of $2{\cdot}5\times10^8$ and $1{\cdot}8\times10^8$ infected HeLa cells (multiplicity 200) labeled for 30 min with 200 μc and 150 μc of [^{3}H]UR at 1 and 4 5/6 hr after infection, respectively. Membrane filters containing 1 μg of immobilized vaccinia DNA were incubated with (a) 240 μg of ^{3}H-labeled early mRNA (52 cts/min/μg) or (b) 150 μg of ^{3}H-labeled late mRNA (65 cts/min/μg) in the presence of increasing amounts of cold HeLa cell cytoplasmic RNA (i.e. from uninfected cells) (—○—○—), or cold HeLa CAR. RNA (—●—●—) at 66°C for 24 hr. ——— indicates the level of hybridization without any cold RNA. This corresponds to 450 cts/min for (a) and 309 cts/min for (b).

An important control is included in this experiment, namely the demonstration that cytoplasmic RNA from uninfected HeLa cells has no effect on the ability of either early or late vaccinia mRNA to hybridize. This control is important because, as pointed out above, all mRNA preparations contain large amounts (relative to the amount of vaccinia mRNA) of ribosomal RNA as well as tRNA and variable amounts (depending on the time after infection) of host cell mRNA. However none of these RNA species interferes in any way with the ability of vaccinia mRNA to hybridize.

(c) *Comparison of the base sequence content of HeLa CAR.RNA and L cell CAR.RNA*

It was of considerable interest to determine whether the same base sequences were transcribed from parental genomes in HeLa and L cells. First, saturation plateaux were determined for labeled preparations of HeLa CAR.RNA and L cell CAR.RNA (Fig. 7). The plateau is reached far more quickly with L cell CAR.RNA than with HeLa CAR.RNA, indicating many more molecules of mRNA per μg RNA in the L cell preparation than in the HeLa cell preparation; the increased amount of label in the gradients in Fig. 5 thus reflects a larger number of mRNA molecules transcribed in the presence of CAR in L cells than in HeLa cells, rather than merely a difference in some precursor pool size.

In Figs 8 and 9 we compare HeLa CAR.RNA and L cell CAR.RNA for identity of base sequence content. In Fig. 8 a direct competition experiment is described; in Fig. 9 an experiment employing the pre-saturation technique. The results of the two experiments were identical. Cold L cell CAR.RNA competes equally efficiently

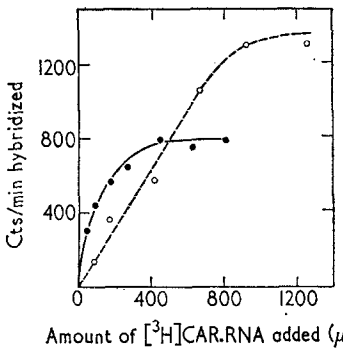

FIG. 7. Saturation of vaccinia DNA with HeLa and L cell CAR.RNA.

HeLa and L cells were infected with vaccinia virus at a multiplicity of 400 in the presence of 10 μg/ml. of CAR. Cells were labeled with [^{3}H]UR for 30 min at 1 hr after infection. RNA was extracted from the cytoplasm as described in Materials and Methods. The specific activities of ^{3}H-labeled HeLa and L cell CAR.RNA were 428 cts/min/μg and 228 cts/min/μg, respectively. Membrane filters containing 1 μg of immobilized vaccinia DNA were incubated with ^{3}H-labeled HeLa CAR.RNA (—○—○—) or ^{3}H-labeled L cell CAR.RNA (—●—●—).

with CAR.RNA transcribed in either HeLa or L cells; for HeLa CAR.RNA the curves are also similar, although competition is less efficient. The similarity of the two sets of curves in each Figure indicates that HeLa and L cell CAR.RNA contain the same base sequences; quantitative differences are explicable by the established fact that there are more mRNA molecules synthesized in L cells in the presence of CAR than in HeLa cells.

The possibility that the high efficiency with which L cell CAR.RNA (relative to HeLa CAR.RNA) inhibited hybridization of both homologous and heterologous CAR.RNA was due to some non-specific hybridization inhibitor was ruled out by the experiment shown in Fig. 10. L cell CAR.RNA inhibits hybridization of late vaccinia mRNA transcribed in HeLa cells to the same extent (about 50%) as does early vaccinia mRNA transcribed in HeLa cells.

(d) *Kinetics of transcription of early and late vaccinia mRNA in HeLa and L cells*

The results obtained so far enable one to measure the rate of formation of early and late vaccinia mRNA under a variety of conditions.

(1) In Fig. 11 we show the rate of formation in HeLa cells of total vaccinia mRNA (curve B) and of mRNA hybridizing to membranes containing DNA pre-saturated with cold HeLa CAR.RNA (curve A), that is, of mRNA containing sequences not present in early mRNA. Also plotted is the ratio A/B. This ratio is 0·4 or more for late mRNA and 0·1 or less for early mRNA (see Fig. 3). Late mRNA sequences begin to be transcribed slightly before three hours after infection, which coincides with the time when viral DNA replication is proceeding at its most rapid rate (Joklik & Becker, 1964). Figure 12 shows the corresponding curves for L cells. In L cells only very little late mRNA is detectable, in agreement with the density gradient analysis presented in Fig. 5.

Table 1 shows the effect of multiplicity on the amount of early and late mRNA transcribed at one, four and six hours after infection in HeLa and L cells. The pattern of transcription in the two cell lines is drastically different. In HeLa cells the amount

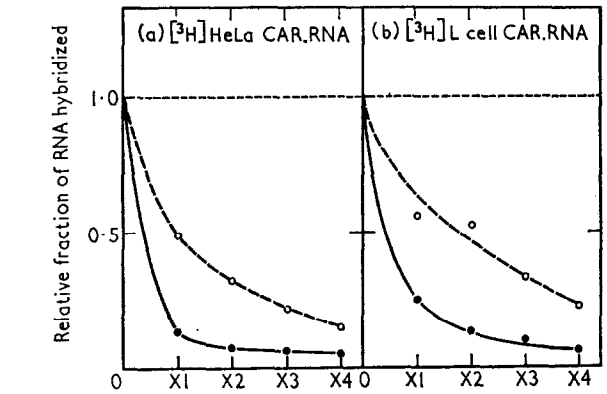

FIG. 8

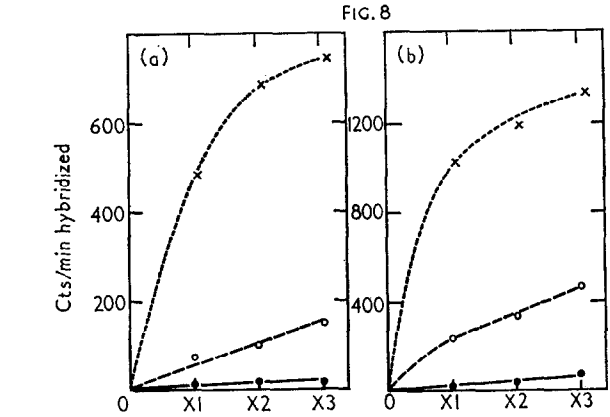

FIG. 9

FIG. 8. Comparison of the base sequence content of CAR.RNA transcribed in HeLa and L cells.

[^{3}H]CAR.RNA was prepared from the cytoplasm of $5{\cdot}2 \times 10^8$ HeLa cells and $6{\cdot}5 \times 10^8$ L cells, both infected at a multiplicity of 250 and labeled for 30 min with 200 μc of [^{3}H]UR at 1 hr after infection in the presence of 10 μg/ml. of CAR. Cold CAR.RNA was prepared from cells similarly infected for 2 hr. Filters containing 1 μg of immobilized vaccinia DNA were hybridized with (a) 468 μg of ^{3}H-labeled HeLa CAR.RNA (46 cts/min/μg) or (b) 207 μg of ^{3}H-labeled L cell CAR. RNA (209 cts/min/μg) in the presence of increasing amounts of cold HeLa CAR.RNA (—○—○—), or cold L cell CAR.RNA (—●—●—). ———— indicates the level of hybridization without any cold RNA. This corresponds to 370 cts/min for (a) and 697 cts/min for (b).

FIG. 9. Comparison of the base sequence content of CAR.RNA transcribed in HeLa and L cells.

Increasing amounts of (a) ^{3}H-labeled HeLa CAR.RNA (85 cts/min/μg) or (b) ^{3}H-labeled L cell CAR.RNA (213 cts/min/μg) were hybridized to filters containing 1 μg of immobilized vaccinia DNA pre-incubated with 1·5 mg of cold HeLa CAR.RNA (—○—○—) or cold L cell CAR.RNA (—●—●—) or without RNA (—×—×—).

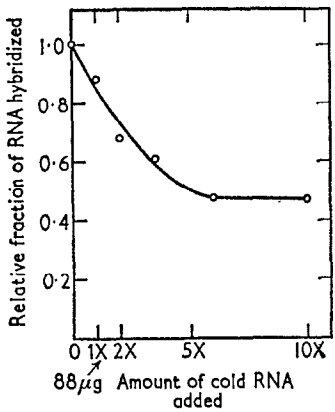

FIG. 10. Effect of cold L cell CAR.RNA on the hybridization of late vaccinia mRNA prepared from HeLa cells. Late [^{3}H]mRNA was prepared from the cytoplasm of HeLa cells by labeling with [^{3}H]UR for 30 min at 5 hr after infection. The specific activity of this RNA was 407 cts/min/μg. Cold L cell CAR.RNA was prepared as described in Fig. 6. Late [^{3}H]mRNA (115 μg) was incubated with membrane filters containing 1 μg of immobilized vaccinia DNA for 24 hr at 66°C in the presence of increasing amounts of cold L cell CAR.RNA. 1276 cts/min hybridized to a filter in the absence of cold L cell CAR.RNA.

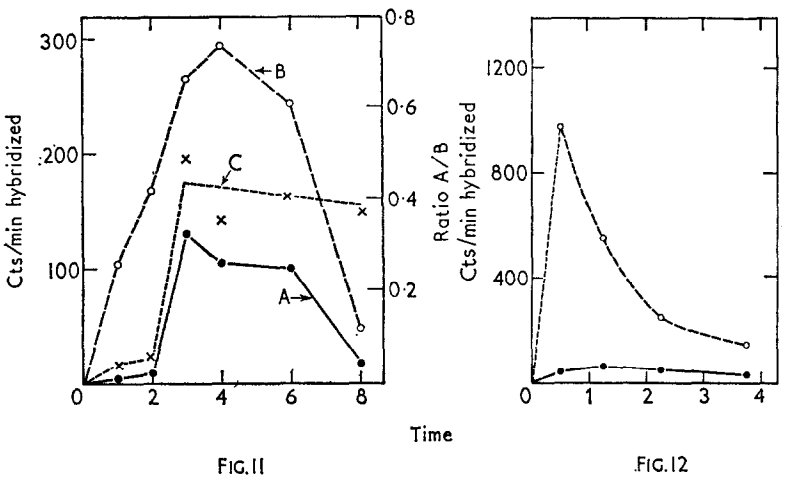

FIG. 11. Kinetics of transcription of early and late vaccinia mRNA in HeLa cells. HeLa cells ($3{\cdot}2 \times 10^8$) were infected with vaccinia virus at a multiplicity of 200. Samples of 5×10^7 cells were labeled with 30 μc of [^{3}H]UR for 20 min at the times indicated. RNA was extracted from the cytoplasm of infected cells as described in Materials and Methods. The RNA was dissolved in 1·1 ml. of 0·1 × SSC and portions of 0·3 ml. (approx. 180 μg of RNA) were added to 1 μg of immobilized vaccinia DNA pre-incubated with (curve A) or without (curve B) 1·3 mg of cold HeLa CAR.RNA. The broken curve (C) plots the ratio curves A/B.

FIG. 12. Kinetics of transcription of early and late vaccinia mRNA in L cells. L cells ($1{\cdot}6 \times 10^8$) were infected with vaccinia virus at a multiplicity of 500. Samples of $3{\cdot}8 \times 10^7$ cells were pulse-labeled with 35 μc of [^{3}H]UR for 15 min at the times indicated. RNA extracted from the cytoplasmic fractions was dissolved in 1·1 ml. of 0·1 × SSC, and portions of 0·3 ml. (approx. 150 μg of RNA) were added to filters with 1 μg of immobilized vaccinia DNA pre-incubated in the presence (—●—●—) or absence —○—○— of 1·1 mg L cell CAR.RNA.

TABLE 1

Effect of multiplicity on the rate of transcription of early and late vaccinia mRNA

Infected cells	Multiplicity	Time (hr after infection)	Amount of RNA added (μg)	Amount of RNA hybridized to DNA (cts/min)		A/B
				Not pre-incubated B	Pre-incubated with L cell CAR.RNA A	
HeLa cells	75	1	114	48	<10	<0·1
		4	114	226	102	0·45
		6	110	181	69	0·38
	200	1	100	126	13	0·10
		4	120	281	136	0·48
		6	112	232	101	0·44
	600	1	102	197	38	0·19
		4	114	372	200	0·54
		6	110	213	118	0·55
L cells	75	1	96	45	<10	<0·1
		4	53	43	<10	<0·1
	200	1	94	213	14	<0·1
		4	53	41	<10	—
	600	1	86	326	26	0·08
		4	25	61	<10	—

Samples of HeLa cells ($1{\cdot}2 \times 10^8$) and L cells (8×10^7) were infected with vaccinia virus at multiplicities of 75, 200 and 600. Samples of 4×10^7 cells were pulse-labeled with 30 μc of [^{3}H]UR for 20 min at 1 and 4 hr and for HeLa cells also at 6 hr after infection. RNA was extracted from the cytoplasmic fractions as described in Materials and Methods and dissolved in 1·2 ml. of 0·1 × SSC. Membrane filters containing 1 μg of immobilized vaccinia DNA were incubated with portions of 0·2 ml. of the RNA solution (approx. 100 μg of RNA) with (A) and without (B) pre-incubation with 1·2 mg of cold L cell CAR.RNA. The column listing the amount of RNA added is included to illustrate the loss of cytoplasmic material from L cells; this loss was particularly marked in this experiment. Where no value for the ratio A/B is given, the number of counts in column A was so low as to preclude computation of an accurate value. However it was quite clear that in no case did the value approach 0·4.

of early mRNA transcribed is a fraction of that transcribed late. The higher the multiplicity of infection the faster do events in the infection cycle proceed: the earlier is early mRNA transcribed, the earlier is late mRNA transcribed, the earlier does the decline in the rate of late mRNA transcription set in. In L cells this multiplicity effect is also observed, superimposed on the characteristic L cell mRNA transcription pattern, with its very high rate of early mRNA transcription and its extremely low rate of late mRNA transcription. In HeLa cells the rate of transcription of early, but not of late, mRNA is proportional to the multiplicity of infection over a range from at least 75 to 200; in L cells this relationship is much more complex.

It should be pointed out that L cells infected at high multiplicities tend to lose cytoplasm after about four hours after infection; this phenomenon can be quantitated by measuring the decrease in the amount of cell-associated ribsomal RNA. Microscopic observation shows that the edges of a certain proportion of cells become ragged. This proportion may amount to as much as 30% of a population of L cells infected for six hours at a multiplicity of 500. This effect has been described previously (Joklik & Merigan, 1966). It is conceivable that this disruptive effect of a large number of virus particles is responsible for the low capacity of L cells to synthesize late mRNA. It that were so, one would also expect a depression in the virus yield. The virus yields in cells infected with a range of multiplicities was therefore determined. The yields in terms of PFU/cell at 24 hours were: n†=50, 150; n=125, 200; n=250, 250; n=500, 200; n=1000, 50. Infection with very high multiplicities therefore does indeed result in a reduction of the yield: but this effect is only just starting to operate at the highest multiplicity used in this experiment.

(2) It is of some interest that the pattern of transcription of viral mRNA in HeLa cells infected in the presence of dFU differed from that exhibited by cells infected in the presence of CAR. The rate of transcription of total mRNA in the presence of CAR decreased more rapidly than in the presence of dFU and the amount of mRNA not blocked by CAR. RNA was greater in the presence of dFU. These results suggest that a very small amount of replication is permitted in HeLa cells in the presence of dFU. The preferred inhibitor of DNA replication is CAR.

(3) Hybridization provides a means for examining the effect of infection with vaccinia virus on the rate of transcription of host cell mRNA. The results (Fig. 13) show that the rate of transcription of RNA capable of hybridizing with host cell DNA, which represents principally though not entirely messenger RNA, is halved by two hours after infection and inhibited by 80% by four hours. The fact that infection markedly inhibits the transport of host cell mRNA to the cytoplasm has been reported previously (Becker & Joklik, 1964; Salzman, Shatkin & Sebring, 1964).

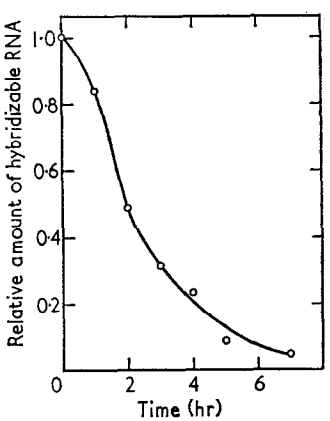

FIG. 13. Synthesis of host RNA in HeLa cells infected with vaccinia virus.

HeLa cells (3×10^6) were infected with vaccinia virus at a multiplicity of 200. At the times indicated samples of 5×10^7 cells were pulse-labeled for 20 min with 30 μc of [^{3}H]UR. RNA was extracted from whole cells, dissolved in 1·1 ml. of 0·1 × SSC and portions of 0·3 ml. (approx. 200 μg of RNA) were hybridized with 20 μg of immobilized HeLa cell DNA. At time 0 (cells immediately before infection) 240 cts/min hybridized.

† n is the multiplicity of infection.

(e) *Characterization of large and small viral mRNA*

The series of profiles depicted in Fig. 5A illustrates the remarkable change in size of vaccinia mRNA as infection progresses. At early times only 10 to 12 s mRNA molecules are transcribed, at late times both these and additional larger molecules, up to 28 s, are transcribed. Hybridization data indicate that at early times early mRNA sequences are transcribed, and that at late times both these and additional sequences are transcribed. It was therefore of interest to determine whether the sequences characteristic of early mRNA are always in small 10 to 12 s molecules, and whether the large molecules transcribed late during the infection cycle contain predominantly nucleotide sequences not present in early mRNA.

Figure 14 shows the size distribution of vaccinia mRNA at one, five and eight hours after infection of HeLa cells. The fractions indicated were pooled, the RNA isolated from them and its hybridization characteristics to normal DNA and to DNA pre-saturated with homologous CAR.RNA determined (Fig. 15). 1S.RNA is typical early mRNA; it does not hybridize with DNA pre-saturated with CAR.RNA. At five hours the larger fraction (about 17 to 26 s) is typically late mRNA; while the smaller fraction (about 7 to 15 s) behaves in a manner explicable most easily as follows: there are present molecules not present in CAR.RNA, but they are transcribed from relatively few cistrons since they saturate the DNA at a very low concentration. Similar behavior is shown by the viral mRNA transcribed at eight hours infection. There the largest molecules behave like 5L.RNA molecules, the smallest like 5S.RNA molecules, and those of intermediate size show an intermediate behavior pattern.

One may conclude that the largest mRNA molecules transcribed at late stages of the infection cycle are transcribed both from sequences *not* transcribed at early

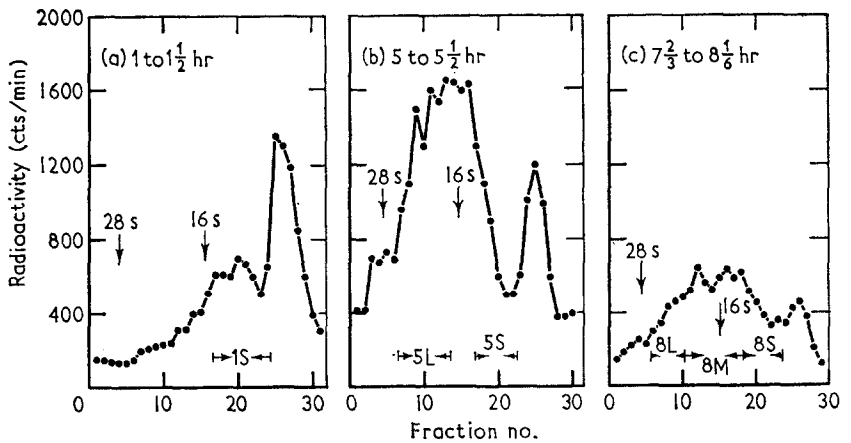

FIG. 14. Change in size distribution of vaccinia virus mRNA with time after infection.

HeLa cells ($8{\cdot}2\times10^8$) were infected with vaccinia virus at a multiplicity of 300. Samples of $1{\cdot}5\times10^8$, $2{\cdot}7\times10^8$ and 4×10^8 cells were labeled with 100 μc, 200 μc and 200 μc of [^{3}H]UR for 30 min at (a) 1 hr, (b) 5 hr and (c) 7 2/3 hr after infection, respectively. RNA was extracted from the cytoplasm, dissolved in 2,4 and 6 ml. of $0{\cdot}1\times$SSC, respectively, and 1/10 vol. of 10% SDS was added. Samples of 2 ml. were centrifuged in 15 to 30% (w/w) sucrose–SDS density gradients for 16 hr at 25,000 rev./min. Samples of 0·05 ml. from each fraction were counted after trichloroacetic acid precipitation. Fractions indicated by the arrows were used in the following experiments.

stages and from sequences which *are* transcribed early. Further, in the population of the smallest molecules which are transcribed late there are present molecules not represented among the early mRNA population, but the hybridization behavior of these molecules suggests that they are transcribed from relatively few cistrons only.

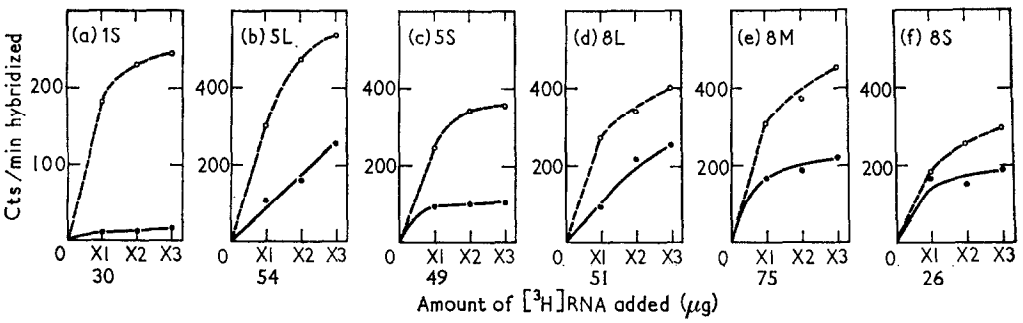

FIG. 15. Hybridization characteristics of large and small vaccinia mRNA.

The fractions indicated by arrows in Fig. 14 were pooled. RNA was precipitated by the addition of 1/10 vol. of 2 M-NaCl and 2 vol. of ethanol. After centrifugation at 10,000 *g* for 30 min, the pellets were dissolved in 2 ml. of 0·1 × SSC. Specific activities (cts/min/μg RNA) were as follows: 1S, 98; 5L, 196; 5S, 172; 8L, 62; 8M, 75; 8S, 108. RNA was incubated with 1 μg of immobilized vaccinia DNA with (—●—●—) and without (—○—○—) pre-incubation with 1·3 mg of cold HeLa CAR.RNA. (a) 1S. RNA, (b) 5L. RNA, (c) 5S. RNA, (d) 8L. RNA, (e) 8M. RNA, (f) 8S. RNA.

(f) *Characterization of mRNA associated with polyribosomes at early and late stages of the infection cycle*

Up to now we have been concerned with the viral mRNA which is being transcribed; we now consider the nature of the mRNA present in polyribosomes as infection progresses. Figures 16 and 17 describe the optical density profiles of polyribosomes of HeLa cells at two, five and eight hours after infection, as well as the distribution of newly-transcribed mRNA molecules (molecules transcribed in the preceding 30-minute period) and of nascent polypeptide chains. The number of polyribosomes decreases as infection progresses, as noted previously (Becker & Joklik, 1964; Joklik & Merigan, 1966). The distribution of newly-formed mRNA in polyribosomes is what would be expected from the O.D. profiles except for some accumulation of mRNA in the small polyribosome region at five hours; this was observed repeatedly, but the reason for it is not clear. The distribution of label in nascent polypeptide chains is quite characteristic at the three times examined. The major factors influencing the position of this label in the gradient are (a) the number of ribosomes in the particular polyribosome being examined and (b) the size of the polypeptide chain being synthesized. The difference in the amino acid label distribution pattern in the two-hour and five-hour gradients indicates either that the functioning polyribosomes at five hours are considerably larger, or that larger polypeptides are being synthesized at five hours on a fraction of a polyribosome population the size distribution of which is similar to that at two hours, or a combination of these. The accumulation of mRNA in the small polyribosome region in which relatively little protein is being synthesized is thus particularly striking. The distribution profiles at eight hours after infection were qualitatively similar to those at five hours.

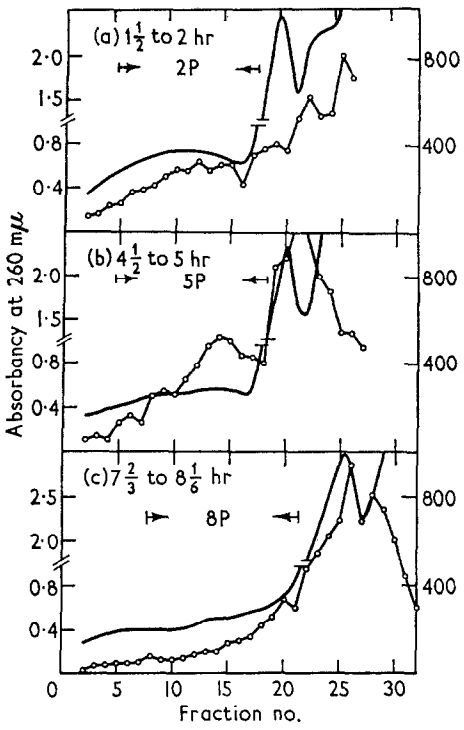

FIG. 16

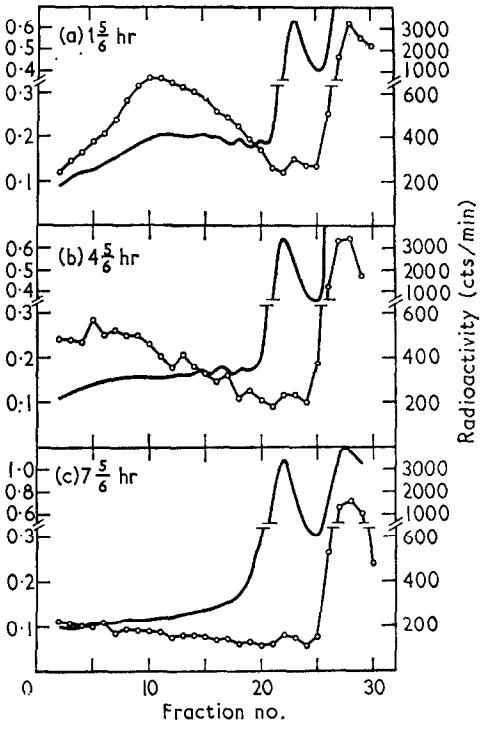

FIG. 17

FIG. 16. Distribution of pulse-labeled vaccinia mRNA in polyribosomes.

HeLa cells ($7{\cdot}7\times10^8$) were infected with vaccinia virus at a multiplicity of 300. Samples of $3{\cdot}5\times10^8$, 3×10^8 and $2{\cdot}2\times10^8$ cells were labeled with 200 μc of [^{3}H]UR for 30 min at (a) 1 1/2 hr; (b) 4 1/2 hr; and (c) 7 2/3 hr after infection respectively. Cells were disrupted in 10, 10 and 6·5 ml. of RSB, respectively, and the cytoplasmic fractions centrifuged in lots of 3 ml. in 15 to 30% (w/w) sucrose density gradients in RSB for 2 hr at 25,000 rev./min. Samples of 0·05 ml. from each fraction were counted after trichloroacetic acid precipitation. Fractions indicated by arrows were pooled and used in the following experiments. —○—○—, Radioactivity; solid lines, absorbancy.

FIG. 17. Distribution of nascent vaccinia proteins in polyribosomes.

HeLa cells (10^8) were infected with vaccinia virus at a multiplicity of 200. At (a) 1 5/6 hr, (b) 4 5/6 hr and (c) 7 5/6 hr after infection samples of 3×10^7 cells were centrifuged and resuspended in 30 ml. of warmed medium lacking amino acids but containing 5% calf serum. The cells were then pulse-labeled for 80 sec with 30 μc of [^{14}C]amino acid mixture (1·5 mc/mg) and chilled rapidly. After disruption of the cells in 1·5 ml. RSB, the cytoplasmic fractions were centrifuged in sucrose density gradients as described in Fig. 16. —○—○—, Radioactivity; solid lines, absorbancy.

Messenger RNA was prepared from the pooled fractions indicated in Fig. 16 and its hybridization properties determined. Hybridization to normal vaccinia DNA, as well as to DNA pre-saturated with CAR.RNA, 5P.RNA and 8P.RNA (RNA isolated from the same polyribosome gradient fractions as indicated in Fig. 16) was carried out. The amounts of the latter two necessary to saturate the amount of DNA used (1 μg or 0·5 μg as indicated) were determined in separate experiments.

Figure 18(a) shows that all the sequences present in HeLa CAR.RNA are present in mRNA in polyribosomes at five hours after infection; it prevents hybridization by CAR.RNA as efficiently as homologous RNA. In agreement with this finding,

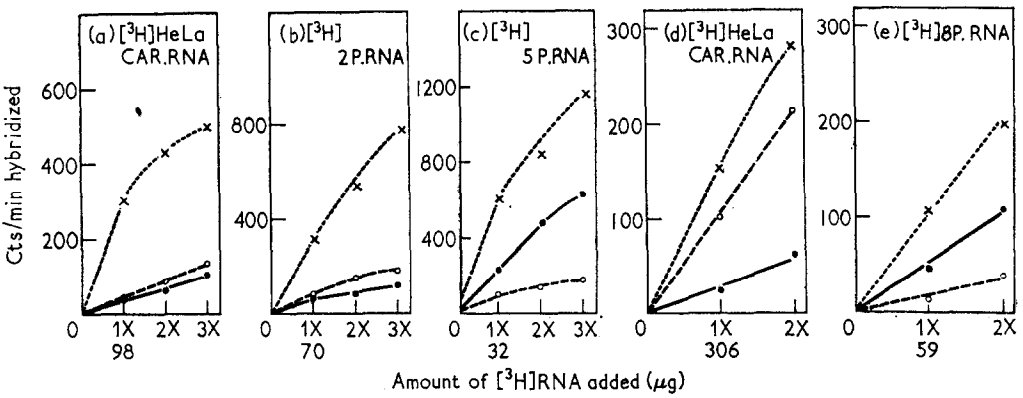

FIG. 18. Hybridization of vaccinia mRNA associated with early and late polyribosomes.

[^{3}H]RNA was extracted from the polyribosome fractions indicated by arrows in Fig. 16. Fractions were pooled and 1/10 vol. of 2 M-NaCl and 2 vol. of ethanol were added. The suspensions were kept at −20°C for at least 2 hr and then centrifuged at 10,000 *g* for 30 min. The pellets were suspended in 0·05 M-sodium acetate buffer (pH 5·1) containing 0·01 M-EDTA and SDS was added to a final concentration of 1%. RNA was then deproteinized as described in Materials and Methods. The specific activities of the RNA prepared from 2P, 5P and 8P were 85, 212 and 115 cts/min/μg, respectively. Cold HeLa 5P.RNA and 8P.RNA were prepared in the same way. ^{3}H-labeled HeLa CAR.RNA and cold HeLa CAR.RNA were prepared as described in Fig. 7.

Hybridization:

(a) ^{3}H-labeled HeLa CAR.RNA (92 cts/min/μg), 1 μg of vaccinia DNA;
(b) [^{3}H]2P.RNA, 1 μg of vaccinia DNA;
(c) [^{3}H]5P.RNA, 1 μg of vaccinia DNA;
(d) ^{3}H-labeled HeLa CAR.RNA (98 cts/min/μg), 0·5 μg of vaccinia DNA;
(e) [^{3}H]8P.RNA, 0·5 μg of vaccinia DNA.

—×—×—, Normal hybridization; —●—●—, hybridization after pre-incubation of immobilized DNA with 1·2 mg of cold HeLa CAR.RNA; —○—○—, hybridization after pre-incubation of immobilized DNA with 285 μg of cold 5P.RNA (for (a), (b) and (c)), or with 275 μg of cold 8P.RNA (for (d) and (e)).

Fig. 18(b) shows that hybridization by 2P.RNA is also blocked by pre-saturation of DNA with 5P.RNA; mRNA sequences present in polyribosomes at two hours are therefore present at five hours also. From Fig. 18(c) it is clear on the other hand that mRNA transcribed at five hours and forming polyribosomes at that time contains sequences not present in HeLa CAR.RNA, that is, it contains typical late mRNA. Figure 18(d) and (e) show the corresponding results for 8P.RNA. This RNA is inefficient in blocking hybridization by CAR.RNA when compared with 5P.RNA: there are therefore present in polyribosomes at eight hours fewer sequences identical with early mRNA than at five hours. However, the mRNA transcribed at eight hours and entering polyribosomes contains as high a content of the sequences characteristic of early mRNA as the mRNA transcribed and forming polyribosomes at five hours (Fig. 18(e)).

In summary, mRNA transcribed and forming polyribosomes at five and eight hours after infection is typical late mRNA and contains sequences characteristic of early as well as of late mRNA. Further, there are present in polyribosomes at five hours mRNA sequences capable of (almost) completely inhibiting hybridization of early (CAR) mRNA; but at eight hours after infection there is a very significant reduction in the amount of these sequences. Similar studies could not be carried out with L cells owing to the unfavorable pattern of mRNA transcription.

(g) *Stability of early and late vaccinia mRNA in HeLa and L cells*

The hybridization technique was used to determine the intracellular stability of early and late vaccinia mRNA (transcribed between 70 and 90 minutes and 300 and 320 minutes after infection, respectively). Transcription was arrested with actinomycin D and the relative amounts of RNA capable of hybridizing with viral DNA were determined over the next 5·5 hours. The results are shown in Fig. 19(a). Early HeLa cell mRNA is very stable: less than 20% is degraded over a 5·5 hour period. Late vaccinia mRNA is much more labile. Measuring the capacity to hybridize to DNA presaturated with HeLa CAR.RNA revealed that the sequences of mRNA characteristic of late mRNA are very labile, with a half-life of less than one hour; but that the sequences in late mRNA characteristic of early mRNA are much more stable, as stable as early mRNA itself. (It seems that *some* of the mRNA molecules containing sequences characteristic of late mRNA are relatively stable; but these cannot amount to more than about 25%.)

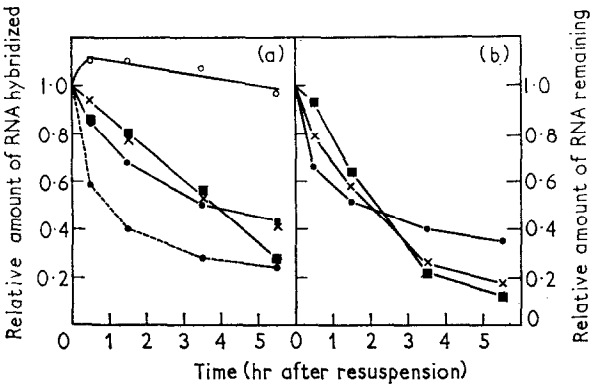

FIG. 19. Stability of early and late vaccinia mRNA.

HeLa and L cells (each 10^9) were infected with vaccinia virus at multiplicities of 200 and 400, respectively. Samples of 5×10^8 cells were labeled with 300 μc of [^{3}H]UR for 20 min at 1 1/6 and 5 hr after infection, respectively, and poured onto crushed frozen saline (0·8%) containing 5 μg/ml. of actinomycin D. After centrifugation, the cells were resuspended in fresh medium containing 5 μg/ml. of actinomycin D. At 5, 30, 90, 210 and 330 min after resuspension, samples of $9{\cdot}5 \times 10^7$ cells were harvested and disrupted in 4 ml. RSB. After centrifugation at 800 *g* for 3 min, 0·4 ml. of 10% SDS was added to the cytoplasmic fractions (final volume about 4·2 ml.) and portions of 3 and 1 ml. were used for the preparation of RNA for hybridization (a) and for sucrose density gradient centrifugation (b), respectively.

(a) RNA was prepared as described in Materials and Methods. After ethanol precipitation the RNA was dissolved in 1·2 ml. of 0·1 × SSC, and portions of 0·2 ml. (approx. 170 μg of RNA) were incubated with 1 μg of immobilized vaccinia DNA. The specific activities (in cts/min/μg) of the RNA at 5 min after resuspension were (i) early mRNA transcribed in HeLa cells, 98; (ii) late mRNA transcribed in HeLa cells, 158; (iii) early mRNA transcribed in L cells, 147; (iv) late mRNA transcribed in L cells, 16. The number of cts/min hybridized at 5 min after resuspension were (i) 468; (ii) 542; (iii) 692; (iv) 109. 255 cts/min of late mRNA transcribed in HeLa cells hybridized to DNA pre-saturated with cold HeLa CAR.RNA. These values were taken as 1·0 in Fig. 19(a).

(b) Each point was derived from summation of the appropriate area of sucrose–SDS density gradient profiles. The total cts/min in mRNA at 5 min after resuspension were (ii) 35850; (iii) 47797; (iv) 4085, where (ii), (iii), etc., have the same meaning as in (a). These values are again taken as 1·0.

—○—○—, (i); —●—●—, (ii); —×—×—, (iii); —■—■—, (iv). The broken curve represents hybridization of late vaccinia mRNA to immobilized DNA pre-saturated with 1·5 mg of cold HeLa CAR.RNA.

In L cells the situation is different. The stabilities of early and late mRNA are very similar, the half-life being two to three hours.

These results were compared with those obtained by density gradient centrifugation analysis (Fig. 19(b)). This technique can be used whenever the contribution to the amount of label in the mRNA region by labeled ribosomal RNA emerging from the nucleus after addition of actinomycin D is negligible. This is the case for HeLa cells at five hours after infection, but not for HeLa cells during the early period of the infection cycle. It is also the case at all stages of the infection cycle in L cells at the multiplicity used here (400); probably it is not so if the multiplicity is very much lower. Thus in Fig. 19(b) there are plotted the relative rates of disappearance after addition of actinomycin D of label in early vaccinia mRNA in L cells and in late vaccinia mRNA in both L and HeLa cells. The results agreed well with those obtained with the hybridization technique.

The difference in the intracellular stability of early vaccinia mRNA in HeLa and L cells is striking, as is the difference between that of early and late vaccinia mRNA in HeLa cells. Experiments with cell extracts were therefore carried out in order to assess the relative importance for vaccinia mRNA degradation of the enzymic complement of the cell sap and the intrinsic sensitivity to breakdown of the mRNA molecules themselves. Extracts were prepared from HeLa and L cells infected for 3·5 hours and these were incubated at 37°C for one and three hours with early and late vaccinia mRNA transcribed in HeLa cells. Controls were samples which were not incubated but to which SDS was added immediately. The results of the sucrose density gradient analysis are shown in Table 2. Both early and late vaccinia mRNA are more stable in extracts of HeLa cells than of L cells. The cytoplasm of L cells

TABLE 2

The stability of early and late vaccinia mRNA in extracts of infected HeLa and L cells

Vaccinia mRNA	% remaining after incubation with HeLa cell extract		L cell extract	
	1 hr	3 hr	1 hr	3 hr
Early	60	40	25	<10
Late	60	25	25	<5

HeLa cells and L cells (10^8 cells of each) were infected with vaccinia virus at a multiplicity of 150 and 300, respectively. After 3½ hr the cells were homogenized in 4 ml. each and the supernatants after centrifugation for 3 min at 800 *g* were used as the cell extracts. For the preparation of early and late vaccinia mRNA HeLa cells (6×10^7) were infected at a multiplicity of 200. At 65 and 240 min, one-half of the culture was pulse-labeled with 10 μc [^{14}C]UR for 20 min. At each time the cells were homogenized in 0·9 ml. and the supernatant after centrifugation as above was used as the mRNA preparation. The incubation mixtures contained 0·2 ml. of the mRNA preparation plus 0·6 ml. of the cell extract. After incubation at 37°C for 0, 1 and 3 hr 0·1 ml. of SDS (10%) was added and the mixtures centrifuged on sucrose (15 to 30% w/w)–SDS density gradients at 25,000 rev./min for 18 hr at 27°C. Appropriate areas on the density gradient profiles were then summed for the computation of the amount of label in mRNA. All figures have been rounded to the nearest 5%.

thus certainly contains a more active mechanism for degrading mRNA than that of HeLa cells. Further, early vaccinia mRNA was only slightly more stable in a cell-free extract of HeLa cells than late mRNA, in contrast to the situation *in vivo*. Two observations are pertinent: (1) the cell extract was derived from cells infected for 3·5 hours, that is, from cells in which a late nuclease could already have been present. Such an enzyme could not be formed under the conditions of the *in vivo* experiment. (2) The late mRNA remaining after incubation for one and three hours with cell extracts had a median sedimentation coefficient of 12 S, in contrast to 16 S when not incubated. Either partial breakdown had occured, or the molecules degraded preferentially were the larger ones characteristic of late mRNA. The latter alternative would be in line with the finding (see above) that sequences characteristic of late mRNA are less stable *in vivo* than those characteristic of early mRNA.

5. Discussion

This study of vaccinia mRNA transcription and functioning in two established cell lines by hybridization and sedimentation analysis has yielded findings which may be fitted into the general framework of the viral multiplication cycle in the following manner:

(1) The mRNA transcribed from parental genomes during the early phase of the multiplication cycle differs from that transcribed when progeny genomes are also present both in the extent of DNA sequences copied and in size. As far as one can tell all the nucleotide sequences transcribed early are also transcribed late; in addition there are transcribed at late stages further sequences which are not transcribed early. Assuming that all the viral genome is transcribed late, between one-half and two-thirds is transcribed early. The situation is thus very similar to that described by Hall *et. al.* (1964) for T2.

It should be pointed out here that not all adsorbed vaccinia virus particles are uncoated (Joklik, 1964). Recent experiments (McAuslan, personal communication) have indicated that mRNA may be transcribed from parental DNA which is not uncoated as judged by the criterion of susceptibility to deoxyribonuclease; and we have ourselves observed that there exists no correlation between the rate of early mRNA transcription and the number of completely uncoated parental genomes. It has therefore yet to be determined whether any of the sequences characteristic of early mRNA which are synthesized at late times of the infection cycle are transcribed from residual incompletely uncoated virus particles.

(2) Early mRNA accumulates in cells infected in the presence of CAR. As far as one can tell the same sequences of the viral genome are transcribed in HeLa cells as in L cells. However the rate of transcription of this mRNA in L cells greatly exceeds that in HeLa cells. The reason for this difference is not clear and is being investigated by us now.

(3) Whereas the extent of the rate of transcription of early mRNA in L cells greatly exceeds that in HeLa cells, the reverse is true for late mRNA. Far more late mRNA is transcribed in HeLa cells than early mRNA, but in L cells the synthesis of late mRNA is barely detectable. These patterns of the rates of early and late mRNA transcription in HeLa and L cells are themselves profoundly influenced by the multiplicity of infection. The general conclusion one can draw is that the higher the multiplicity the more telescoped become the successive stages of the infection cycle: uncoating proceeds more rapidly (Joklik, 1964), DNA replication commences

earlier and the early period is therefore shorter, and the rate of transcription of late mRNA decreases earlier to the low level which then persists until maturation of viral progeny is complete.

We thus have the situation that in L cells most of the viral mRNA synthesis taken over the whole infection cycle is of the early type which does *not* contain all of the sequences which can be transcribed; whereas in HeLa cells most of the mRNA synthesized is of the late type, which contains, as we have seen, sequences in addition to those characteristic of early mRNA. Yet in both cell types high yields of virus are formed; in fact, the yields in the L cells used here are higher than those in HeLa cells and often exceed 10,000 virus particles per cell. The question thus arises as to the nature of the information encoded in early and late mRNA, respectively. The following facts are at hand. (1) Early enzymes are encoded in early mRNA; (2) at least some structural viral proteins are also encoded in early mRNA (this is shown by the fact in the presence of isatin-β-thiosemicarbazone, which allows expression of early viral mRNA but prevents that of late mRNA (Woodson & Joklik, 1965; Joklik, Jungwirth, Oda & Woodson, 1967), some structural viral proteins are formed (Appleyard *et al.*, 1965); (3) certain structural viral proteins are only encoded in late mRNA; and (4) there are about 15 to 20 structural viral proteins, but the genome of vaccinia virus is large enough to code for about 500 average size proteins (Joklik, 1966). These facts have to be handled so as to account for a pattern of mRNA synthesis such that synthesis of a very large number of early mRNA molecules plus a small number of late mRNA molecules yields the same end result as synthesis of a small number of early mRNA molecules plus a very large number of late ones. It is unprofitable to speculate on this topic at this time: the situation will be clarified greatly by our current studies relating the total amount of virus-coded proteins synthesized throughout the infection cycle to the amount of structural viral protein synthesized in both HeLa and L cells, infected in the absence and presence of CAR (Holowczak & Joklik, results to be published).

(4) All the sequences characteristic of early mRNA are present in late mRNA. However the median sedimentation coefficients of early and late mRNA are different: that of early molecules is 10 to 12 S, that of late molecules is 16 to 20 S. Since there are some 10 to 12 S molecules among those transcribed late, it is tempting to postulate that among a population of late mRNA molecules the largest ones will be the ones not present in a population of early ones and the small ones would be those also found in an early population. This however is not the case: the largest molecules still contain sequences characteristic of early mRNA, and the small ones also contain some sequences characteristic of late mRNA in addition to those characteristic of early mRNA. This raises the possibility that sequences of the viral genome which are transcribed monocistronically during the early phase of the infection cycle are transcribed polycistronically later on. The molecular basis for such an effect is unknown.

(5) The stability of early mRNA in HeLa cells is very high; in L cells it is less, the half-life being two to three hours. Late mRNA is rather unstable in HeLa cells (half-life less than 60 minutes for most of the molecules, although there may be some—not more than 25%—which are much more stable); late mRNA seems to be as stable as early mRNA in L cells. These estimates come from both hybridization and sedimentation studies which agree well. A very important factor determining the stability of mRNA is undoubtedly the enzymic complement of the cell cytoplasm, since vaccinia mRNA transcribed in HeLa cells is considerably more stable if mixed *in vitro* with

HeLa cell cytoplasm than with L cell cytoplasm. However, it seems that early mRNA molecules themselves are more stable towards the degradative mechanism than late ones; this is shown especially by the figures relating to the three-hour incubation period in Table 2. A difference between early and late mRNA molecules is evident also in the switch-off phenomenon (McAuslan, 1963*a*): early mRNA molecules coding for early enzymes are prevented from functioning from the time when late mRNA molecules are formed, which are themselves able to function perfectly well (Joklik *et al.*, 1966).

These results raise important questions regarding the nature of the mRNA in polyribosomes as the infection cycle progresses. In particular, the question of the fate of early mRNA is an intriguing one, since it is intrinsically both chemically and functionally stable (Results, section (g), this paper, and Jungwirth & Joklik, 1965), yet its ability to function is switched off after three to four hours of the normal infection cycle (McAuslan, 1963*b*; Jungwirth & Joklik, 1965). Three possible explanations are: (a) early mRNA is inactivated in some way, possibly degraded, by enzymes (the switch-off protein?) coded by late mRNA which would not be formed in the presence of actinomycin D, so that the stability of early mRNA under normal conditions would be much less than that suggested by the experiments employing actinomycin D; (b) early mRNA is prevented from combining with ribosomes at the end of the early period of the infection cycle and continues to persist in the free state; and (c) the polyribosomes formed by early mRNA cease synthesizing protein, but remain physically intact. In order to investigate these alternatives, we investigated the type of mRNA in polyribosomes at two, five and eight hours after infection. The size distribution of polyribosomes changes as infection progresses: this is revealed not only by the optical density profiles, but also by the amino acid incorporation profiles. The most likely interpretation of these profiles is that the polyribosomes are larger at five hours (and at eight hours) than at two hours and that larger polypeptides are being synthesized. This would be in line with the demonstrated larger size of late as compared with early mRNA. Hybridization studies revealed that at five hours all the sequences present in early mRNA were present in polyribosomes; at eight hours however this was not the case since 8P.RNA competed only inefficiently with CAR.RNA. Thus by eight hours most of the mRNA in polyribosomes only contains those nucleotide sequences *not* found in early mRNA. This is a remarkable state of affairs since new mRNA forming polyribosomes at five and eight hours is very similar with respect to content of sequences characteristic of early mRNA. This situation suggests that the *functional* life of mRNA molecules containing mostly the characteristic late sequences is greater than that of those containing mostly early ones. The situation is clearly complex; again analysis of the spectrum of proteins synthesized at various times should be of value.

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REFERENCES

Appleyard, G., Hume, V. B. M. & Westwood, J. C. N. (1965). *Ann. N.Y. Acad. Sci.* **130**, 92.

Bautz, E. K. F., Kasai, P., Reilly, E. & Bautz, F. A. (1966). *Proc. Nat. Acad. Sci., Wash.* **55**, 1081.

Becker, Y. & Joklik, W. K. (1964). *Proc. Nat. Acad. Sci., Wash.* **51**, 577.

Dove, W. F. (1966). *J. Mol. Biol.* **19**, 187.
Eagle, H. (1959). *Science*, **130**, 432.
Ebisuzaki, K. (1965). *Virology*, **26**, 390.
Ebisuzaki, K. (1966). *J. Mol. Biol.* **20**, 545.
Edlin, G. (1965). *J. Mol. Biol.* **12**, 363.
Gillespie, D. & Spiegelman, S. (1965). *J. Mol. Biol.* **12**, 829.
Hall, B. D., Nygaard, A. P. & Green, M. H. (1964). *J. Mol. Biol.* **9**, 143.
Joklik, W. K. (1962*a*). *Virology*, **18**, 9.
Joklik, W. K. (1962*b*). *J. Mol. Biol.* **5**, 265.
Joklik, W. K. (1964). *J. Mol. Biol.* **8**, 263.
Joklik, W. K. (1966). *Bact. Rev.* **30**, 33.
Joklik, W. K. & Becker, Y. (1964). *J. Mol. Biol.* **10**, 452.
Joklik, W. K., Jungwirth, C., Oda, K. & Woodson, B. (1966). In *The Molecular Biology of Viruses*, ed. by J. S. Cotter & W. Paranchych. New York: Academic Press, in the press.
Joklik, W. K. & Merigan, T. C. (1966). *Proc. Nat. Acad. Sci., Wash.* **56**, 558.
Jungwirth, C. & Joklik, W. K. (1965). *Virology*, **27**, 80.
McAuslan, B. R. (1963*a*). *Virology*, **20**, 162.
McAuslan, B. R. (1963*b*). *Virology*, **21**, 383.
McAuslan, B. R. & Joklik, W. K. (1962). *Biochem. Biophys. Res. Comm.* **8**, 486.
Pène, J. & Marmur, J. (1967). *J. Virol.* **1**, 86.
Protass, J. J. & Korn, D. (1966*a*). *Proc. Nat. Acad. Sci., Wash.* **55**, 832.
Protass, J. J. & Korn, D. (1966*b*). *J. Biol. Chem.* **241**, 4175.
Salzman, N. P., Shatkin, A. J. & Sebring, E. D. (1963). *Virology*, **19**, 542.
Salzman, N. P., Shatkin, A. J. & Sebring, E. D. (1964). *J. Mol. Biol.* **8**, 405.
Scherrer, K. & Darnell, J. E. (1962). *Biochem. Biophys. Res. Comm.* **7**, 486.
Warburg, O. & Christian, W. (1942). *Biochem. Z.* **310**, 385.
Warner, J. R., Knopf, P. & Rich, A. (1963). *Proc. Nat. Acad Sci., Wash.* **49**, 122.
Woodson, B. & Joklik, W. K. (1965). *Proc. Nat. Acad. Sci., Wash.* **54**, 946.