Expression of the Genome of Defective Interfering Pseudorabies Virions in the Presence or Absence of Helper Functions Provided by Standard Virus

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SUMMARY

The synthesis of virus RNA and proteins in cells infected with two populations of defective virions [Pr(I)53 and Pr(2)53] which vary in the overall composition of their DNA, but which share some structural and biological characteristics, have been examined. The experiments were done under two sets of conditions: (1) at high multiplicity of infection. In this case, practically all the cells in the cultures were co-infected with defective and infectious virions; (2) at low multiplicity of infection. In this case, 75% of the cells in the cultures were infected with defective virions only and 25% were co-infected with defective and infectious virions. The relative abundance of RNA classes complementary to different regions of the virus genomes that were synthesized under various conditions of infection were determined by the Southern (1975) technique; the synthesis of virus proteins was determined by polyacrylamide gel electrophoresis (PAGE). In cells co-infected with standard and defective virions, RNA complementary to the regions that are reiterated in the defective genomes is present in larger amounts than in cells infected with standard virions alone, indicating that the genomes of the defective virions are transcribed. Furthermore, the transcriptional controls that operate normally in the infected cells also operate in cells co-infected with standard and defective virions. The over-abundant accumulation of transcripts of some regions of the virus genome in cells co-infected with defective virions is not necessarily accompanied by an overproduction of some virus proteins. No difference in the PAGE profiles of the proteins synthesized was detected in cells co-infected with Pr(I)53 and standard virions. However, cells co-infected with standard and Pr(2)53 overproduced three polypeptides. Transcription of the virus genome is detectable in cells infected with Pr(2)53 alone but not in cells infected with Pr(I)53 alone. Virus protein synthesis is also detectable under these conditions in Pr(2)-, but not in Pr(I)-infected cells. Thus, despite the similarities in the biological characteristics of the two populations of defective virions described previously, similarities with respect to the expression of their genomes were not found.

INTRODUCTION

In the course of our studies on defective pseudorabies (Pr) virions that accumulate upon serial passage at high multiplicity, we have derived two populations of particles that have become enriched for different parts of the Pr virus genome (Rixon & Ben-Porat, 1979).

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Although the overall composition of the DNA in the two populations of defective virions is different (Ben-Porat & Kaplan, 1976), the genomes of these particles share some common structural characteristics (Rixon & Ben-Porat, 1979). Furthermore, the two populations of defective virions interfere with the growth of standard viruses in a similar fashion. Neither population interferes significantly with the adsorption of standard virus nor with the replication of standard virus DNA. A significant increase in the degree of breakage and reunion of standard parental DNA strands is also not observed in cells co-infected with either population of defective virions. However, in cells co-infected with standard virus and either population of defective virions, there is a delay in the maturation of concatemeric nascent forms of virus DNA to unit size molecules, as well as a reduction in the number of virus particles produced compared to cells infected with standard virus alone (Ben-Porat & Kaplan, 1975, 1976; Kaplan et al. 1976).

One of the aims of the studies described in this paper was to elucidate the mechanism by which defective Pr virions interfere with the growth of standard virus. Interference may occur by two mechanisms: (1) interference could be due to a property of defective DNA. Thus, it has been postulated that defective DNA molecules may have a selective advantage over standard DNA molecules because they are enriched for origins of DNA replication. However, the two populations of defective virions studied here do not interfere with standard virus DNA replication (Kaplan et al. 1976) and the mechanism of their interference is therefore probably not related to any selective advantage for DNA replication. Alternatively, defective DNA may lack specific sites necessary for the processing of concatemeric DNA to unit size genomes. We have suggested previously that the behaviour of the defective virions of Pr virus is consistent with this possibility (Kaplan et al. 1976). A refined analysis of the termini of the virus genome would be required to ascertain whether this is indeed the case. (2) Interference with the growth of standard virus would be required to ascertain whether this is indeed the case.

The experiments described in this paper deal with the expression of the functions encoded in the DNA of the defective particles under the following two sets of conditions: (a) in cells co-infected with standard virus; (b) in cells infected with defective virions only. These studies were initiated with the expectation that they would shed some light on the mechanisms controlling the expression of the virus genome during the normal course of infection, as well as elucidate the basis for the interference of defective virions with the growth of standard virus.

**METHODS**

**Virus and cell culture.** The properties of standard pseudorabies [Pr(s)] virus and cultivation of rabbit kidney (RK) cells have been described previously (Kaplan, 1957). Defective Pr virus strains were derived by repeated passage of undiluted standard virus in RK cells. The methods of propagation and the nomenclature applied to the two populations of defective virions have been described previously (Ben-Porat & Kaplan, 1976). Characterizations of the defective strains have been reported (Ben-Porat et al. 1974; Rubenstein & Kaplan, 1975; Ben-Porat & Kaplan, 1976; Rixon & Ben-Porat, 1979).

**Media and solutions.** ES: Earle's saline. EDS: Eagle's synthetic medium (Eagle, 1959), plus 3% dialysed bovine serum. EDS ∩ PO₄: the same as EDS but without PO₄. EDS ∩ AA: EDS without amino acids and containing 3% dialysed bovine serum, 2 mM-glutamine and 0.6 mM-arginine. Denhardt's solution: 0.02% Ficoll, 0.02% polyvinyl pyrrolidone, 0.02% bovine serum albumin (Denhardt, 1966) in 2× SSC. RSB: 2% SDS, 0.01 M-KCl, 0.0015 M-MgCl₂, 0.1 M-tris, pH 7.4 (Warner et al. 1963), plus 2%, sodium dodecyl sulphate. SSC: 0.15 M-NaCl, 0.015 M-sodium citrate, pH 7.4. NET buffer: 150 mM-NaCl, 5 mM-EDTA, 0.02% sodium azide, 50 mM-tris, pH 7.4.

**Enzymes and chemicals.** Restriction endonucleases Kpn-I and BamH-I were obtained from
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New England Biolabs, Inc., DNase I and RNase A from Worthington Biochemical Corporation, and inorganic $^{32}$P (carrier-free) from ICN. $^3$H-leucine (62 Ci/mmol) was purchased from Schwartz/Mann.

Southern blot hybridization. Filter strips to which restriction fragments of Pr virus DNA were fixed were prepared by the method of Southern (1975). The hybridization conditions were the same as described previously (Rixon & Ben-Porat, 1979). Labelling, purification and hybridization of RNA to the filter strips have been described elsewhere (Feldman et al. 1979). The amount of $^{32}$P-labelled RNA hybridized to the different bands was measured by scanning the autoradiograms (after different exposure times to ensure linearity of exposure of the film with a Joyce-Loebl microdensitometer). The areas under the peaks were estimated with a planimeter.

Labelling of polypeptides. Cultures of primary RK cells were infected 7 days after seeding at either high or low multiplicity for 1 h (see Table 1). The virus inoculum was removed and the cultures further incubated in EDS. At various times, EDS AA containing $^{3}$H-leucine (30 $\mu$Ci/ml) was added. Following the labelling period, the medium was removed, the cells were collected by scraping them into a solution of 0.05-tris (pH 6.8), 4 M-urea and 2 % SDS. The samples were quick frozen and stored at $-20^\circ$C until required.

To label immediate-early proteins (IEP), infection was carried out in medium containing cycloheximide (50 $\mu$g/ml). After 1 h, the virus inoculum was replaced with 5 ml of EDS plus cycloheximide (50 $\mu$g/ml) and the cultures were further incubated for 4 h. The cells were then washed six times with ES to remove the cycloheximide and incubated for 30 min in EDS $\delta$ AA followed by incubation in the same medium containing $^3$H-leucine (50 $\mu$Ci/ml) for a 2 h period. At the end of the labelling period, the cells were scraped into 0.2 ml phosphate-buffered saline (pH 7.4). NaCl was added (final concentration, 1 M) and the samples were quick frozen and stored at $-20^\circ$C until required.

Immune precipitation of immediate-early proteins. Two-tenths ml of sample, prepared as described above, was mixed with 0.1 ml of either pre-immune rabbit or anti-IEP serum (Ben-Porat et al. 1975); 45 $\mu$l of 2-mercaptoethanol (5 mM) and EDTA (10 mM) were also added. The samples were incubated at 37 $^\circ$C for 1 h. Two-tenths ml of formalin-treated Staphylococcus aureus (Kessler, 1975), which had been washed the same day with NET buffer containing 0.5 %, Triton X-100, was then added and the samples further incubated for 2 h at 37 $^\circ$C and overnight at 4 $^\circ$C. The samples were then centrifuged at 2000 g for 10 min and the pellets washed four times in NET buffer containing 0.05 % Triton X-100. After the final wash, 0.12 ml of 6 M-urea plus 2 % SDS was added to the pellets, which were then boiled for 2 min. After a further centrifugation at 2000 g for 10 min at 27 $^\circ$C, the supernatant fluid was removed, a sample was counted directly in a scintillation spectrometer, and the remainder quick-frozen and stored at 20 $^\circ$C until required.

Polyacrylamide gel electrophoresis (PAGE) of proteins. Urea and SDS (final concentrations 4 M and 2 %, respectively) were added to all samples. The samples were sonicated (to reduce viscosity) and boiled for 2 min. Dithioerythritol (final concentration, 0.025 M) was added and the samples were incubated at 37 $^\circ$C for 15 min. The samples were then electrophoresed on a slab of acrylamide gel with a resolving gel consisting of 10 % polyacrylamide (ratio acrylamide/bisacrylamide, 36.5:1); 0.375 M-tris buffer (pH 8.8), 0.1 % SDS, and a stacking gel consisting of 5 % polyacrylamide in 0.125 M-tris buffer (pH 6.8) and 0.1 % SDS. Electrophoresis was at 30 mA for 5 h using a running buffer consisting of 0.05 M-tris-HCl, 0.38 M-glycine and 0.1 % SDS.

Gels were prepared for fluorography by the method of Bonner & Laskey (1975). The gels were washed twice for 30 min with dimethyl sulphoxide (DMSO), placed in DMSO containing 25 % (w/v) 2.5-diphenyloxazole for 4 h and then washed for 24 h in several changes of water. The gels were dried and exposed for autoradiography.
RESULTS

During the normal course of infection, RNA transcribed from the genome of Pr virus can be divided into at least three main classes (Feldman et al. 1979) as summarized in Fig. 1. The first of these classes, immediate-early (IE) RNA, has been well characterized (Feldman et al. 1979) and is transcribed exclusively from a limited region of the genome located at the end of the inverted repeats. The second class, early RNA, is transcribed from regions throughout most of the virus genome but the sequences at or near the junction between the short unique and repeat regions are most abundantly represented. A region of the genome which is not transcribed early but which is transcribed at later times after infection (6 to 9 h) is the middle
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Fig. 2. Summary of the patterns obtained by hybridizing 32P-labelled Pr(1) and Pr(2) DNA to filters containing electrophoretically separated fragments of Kpn-I or BamH-I digested Pr(s) DNA. DNA was isolated from purified virions obtained from cells infected with defective virus and labelled with 32P. The 32P-labelled DNA was fragmented and hybridized to filters to which standard DNA digested by Kpn-I or BamH-I had been fixed. The relative amount of 32P-labelled DNA bound to each fragment was determined with the help of a planimeter and the percentage of the total DNA on the filter present in each fragment was determined. The values obtained with the various defective DNA preparations were divided by those obtained from standard DNA. Under or over-representation of sequences from different regions of the genome in the DNA of Pr(1) and Pr(2) are therefore shown by comparing the relative hybridization to fragments from that region to the value of t which represents the standard DNA control (horizontal line at 1).

of the inverted repeat. At late times after infection, most of the genome seems to be transcribed with a relatively uniform abundance distribution (as determined by the degree of hybridization of RNA to about 30 different restriction fragments of virus DNA).

The two populations of defective virions under study are enriched for different parts of the virus genome. One population of defective particles, Pr(1), is enriched for sequences originating from the middle and the end of the long unique region of the standard virus genome. The other population, Pr(2), is enriched mainly for sequences originating from the inverted repeats and the short unique sequence (Rixon & Ben-Porat, 1979). Fig. 2 illustrates the degree of enrichment of the two populations of defective virions for various parts of the standard virus genome, as determined by the pattern of hybridization of their DNA to restriction fragments of standard DNA according to the technique of Southern (1975).

Because the sequences of DNA for which the defective DNA molecules are enriched originate from regions of the genome that vary in the degree of their transcription at different stages of the infective process, these populations of defective virions offered an opportunity to determine whether the controls of transcription which operate in cells infected with standard virus alone also function in cells infected with defective virions. In the experiments to be described this was tested under the following two sets of conditions: (1) in cells co-infected with standard (helper) virus (high multiplicity infection), and (2) in cells infected with defective particles only (low multiplicity infection).
Fig. 3. Pattern of hybridization of 'early' RNA synthesized by cells infected with standard and defective virions to Kpn-I-digested Pr DNA fixed to filters. Phosphate-starved primary RK cells were infected with either Pr(s), Pr(1) or Pr(2) at a multiplicity of 5 p.f.u./cell (see Table 1 for details) and labelled with $^{32}P$ (200 µCi/ml) between 0 and 2 h p.i. as described in Methods. The RNA was extracted and hybridized to filters containing separated fragments of Kpn-I-digested Pr DNA. The filters were autoradiographed and the autoradiograms were scanned with a Joyce-Loebl microdensitometer.

**Table 1. Ratio of physical to infectious particles in standard and defective stocks and definition of conditions of infection**

<table>
<thead>
<tr>
<th>Virus</th>
<th>PP/ml*</th>
<th>p.f.u./ml†</th>
<th>PP/p.f.u. at high m.o.i. (5 p.f.u./cell)‡</th>
<th>Particles/cell at low m.o.i. (0.25 p.f.u./cell)§</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pr(s)</td>
<td>$2.5 \times 10^9$</td>
<td>$1.3 \times 10^8$</td>
<td>19</td>
<td>95</td>
</tr>
<tr>
<td>Pr(1)53</td>
<td>$1.7 \times 10^9$</td>
<td>$5.0 \times 10^8$</td>
<td>3400</td>
<td>940</td>
</tr>
<tr>
<td>Pr(2)53</td>
<td>$2.6 \times 10^9$</td>
<td>$3.0 \times 10^8$</td>
<td>867</td>
<td>1376</td>
</tr>
</tbody>
</table>

* The number of physical particles (PP) was determined by counting the number of particles relative to a known number of latex particles using an electron microscope.  
† The number of plaque-forming units (p.f.u.) was estimated by plaque assay (Kaplan, 1957).  
‡ RK cultures ($4 \times 10^6$ cells/culture) were infected with 2 ml of virus. Standard virus was diluted to give a multiplicity of 5 p.f.u./cell ($2 \times 10^6$ p.f.u./culture). To concentrated defective stocks, sufficient standard virus was added to give a multiplicity of 5 p.f.u./cell.  
§ Standard and Pr(2)53 stocks were diluted to give a multiplicity of 0.25 p.f.u./cell ($10^6$ p.f.u./culture); Pr(1)53 was used undiluted.
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Fig. 4. Pattern of hybridization of late RNA synthesized by cells infected with standard and defective virions to Kpn-I-digested fragments of Pr DNA fixed to the filters. The experiment was performed as described in the legend to Fig. 3 except that the cells were labelled between 6 and 9 h p.i.

The ratio of infectious to non-infectious particles in populations of Pr standard, Pr(1)53 and Pr(2)53 virions is summarized in Table 1. The number of particles infecting the cells under the two sets of conditions is also indicated. Non-infectious and infectious Pr virions adsorb similarly to cells (Ben-Porat et al. 1976).

Transcription of the virus genome in cells co-infected with standard and defective virions – high multiplicity infection

In these experiments the cells were infected with 5 p.f.u./cell as well as with a varying number of particles unable to give rise to plaques (see Table 1). The RNA synthesized by the infected cultures at early (0 to 2 h) and at late (6 to 9 h) times after infection was labelled with 32P, extracted and hybridized, using the Southern procedure (Southern, 1975), to filter strips to which virus DNA digested by various restriction enzymes had been fixed. Fig. 3 and 4 show scans of the autoradiograms obtained with RNA preparations hybridized to filter strips containing Kpn-I-digested Pr DNA. While the overall amount of virus RNA synthesized was approximately the same (data not given), the abundance of RNA complementary to the different DNA fragments which was synthesized by cells infected with the
three different types of virion preparations varied. Results which are consistent with those illustrated in Fig. 3 and 4 were also obtained when the RNA was hybridized to filter strips to which BamH-I-digested Pr(s) virus DNA was fixed (data not shown). From the cumulative data obtained with the Kpn-I- and BamH-I-digested DNA, the accumulation of transcripts from various regions of the standard virus genome in cells infected with standard and either one of the two types of defective particles was deduced. These data are summarized in Fig. 5 and 6.

The RNA synthesized between 0 and 2 h p.i. in cells co-infected with standard and either Pr(1) or Pr(2) virions exhibited an overall pattern of hybridization which is similar to that of cells infected with standard virus alone. The most salient difference is that in Pr(2) infected cells the RNA hybridizing to the regions adjacent to the inverted repeats, which are the sequences normally transcribed abundantly between 0 and 2 h p.i. in cells infected with standard virus alone, were even more abundantly represented. These are the sequences which are reiterated in this defective DNA. This is not due to the greater number of particles with which the cells were infected, since the pattern of transcription in cells infected with Pr standard virus did not change even when these cells were infected at a tenfold higher multiplicity (results not shown).

It is interesting to note that neither the regions of the DNA that specify for IE RNA
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### Late RNA (6-9 h)

<table>
<thead>
<tr>
<th>Map units</th>
<th>0.1</th>
<th>0.2</th>
<th>0.3</th>
<th>0.4</th>
<th>0.5</th>
<th>0.6</th>
<th>0.7</th>
<th>0.8</th>
<th>0.9</th>
<th>1.0</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pr (s)</strong></td>
<td></td>
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<tr>
<td><strong>Pr (1) 53</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Pr (2) 53</strong></td>
<td></td>
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<td></td>
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</tbody>
</table>

Fig. 6. Summary of the patterns obtained by hybridizing late virus RNA to filters containing electrophoretically separated fragments of Kpn-I- and BamH-I-digested Pr standard DNA. The results are expressed as described in the legend to Fig. 1.

...
Fig. 7. Autoradiograms of Pr virus proteins synthesized by cultures infected with Pr(s), Pr(1) or Pr(2) at high m.o.i. Cells were infected at high multiplicity (5 p.f.u./cell), as indicated in Table 1, labelled between 6 and 9 h p.i. with ³H-leucine and electrophoresed as described in Methods. The three polypeptides which are present in Pr(2)-infected cells in relatively higher amounts than Pr(s)- or Pr(1)-infected cells are marked with an open circle. Other virus-induced polypeptides are indicated by squares. MI = mock infected.

in Pr(2)-infected cells either at early or at late times after infection, despite the over-representation of these regions in the DNA. Thus, for at least two classes of RNA, IE RNA (end of repeat) and some late RNA (middle of the repeat), the temporal restrictions on transcription found with Pr(s) virus DNA are maintained in cells co-infected with standard virus and Pr(2).
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Synthesis of virus proteins in cells co-infected with standard and defective virions - high multiplicity infection

The results described above show that in cells co-infected with Pr(s) and defective particles, the genomes of the latter are transcribed as indicated by an overabundant synthesis of RNA complementary to some of the regions of the virus genome for which the populations of defective virions are enriched. To determine whether this is reflected in an altered pattern of synthesis of virus-induced polypeptides, the proteins synthesized by these cells were analysed by PAGE (Fig. 7).

Cells infected with Pr(s) synthesize a large number of virus-induced proteins, not all of which are detectable in these autoradiograms because of the background of cellular protein synthesis which is not completely arrested under the conditions of infection used (5 p.f.u./cell). Comparison between the PAGE profiles of cells infected with Pr(s) alone and those of cells co-infected with Pr(1) revealed no detectable differences. This was true for cells labelled at early times p.i. (2 to 5 h, data not shown), as well as for cells labelled at late times p.i. (6 to 9 h). In cells co-infected with Pr(2) virions, however, three polypeptides (67K, 56K and 27K) were synthesized in larger amounts than in cells infected with standard virus alone.

Thus in Pr(2) virus-infected cells, the overabundant transcription of some RNA sequences is reflected by an overabundant synthesis of some polypeptides. This does not appear to be true of Pr(1) virus-infected cells.

Transcription of the DNA of defective virions in the absence of helper functions provided by Pr(s) - low multiplicity infection

To determine whether or not information contained in the defective genome is expressed in the absence of helper functions provided by co-infecting Pr(s), cultures were infected with either Pr(s), Pr(1), or Pr(2) at 0.25 p.f.u./cell. Under these conditions, each cell in the cultures infected with standard virus was infected with approx. 5 physical particles, but each cell in the cultures infected with defective virions received either 850 [Pr(1)] or 217 [Pr(2)] physical particles (see Table 1). The RNA synthesized by the cultures at early and late times after infection was labelled, extracted and hybridized to Kpn-I- and BamH-I-digested Pr virus DNA fixed to filters. The results are illustrated in Fig. 8. Under the experimental conditions used, the synthesis of virus RNA was not detectable in cultures infected with standard virus, even though the cells in the cultures were infected with 0.25 p.f.u. and an average of 5 physical particles/cell. It is probable therefore that no virus RNA is synthesized by cells infected with non-infectious standard virus. Indeed, immediate-early antigen synthesis (the earliest antigen synthesized in the infected cells) cannot be detected in cells infected with non-infectious standard virus although the virions adsorb to the cells and their DNA reaches the cell nucleus (DeMarchi et al. 1979).

In Pr(1)-infected cultures, virus RNA synthesis was also not detectable at early times but was detectable at late times after infection when RNA complementary to Kpn-I DNA fragment B, as well as a small amount of RNA complementary to fragment D, appeared. These DNA fragments contain sequences which are reiterated in the DNA of Pr(1) defective virions and, as described above, these sequences are overabundantly transcribed in cells co-infected with Pr(1) and standard virus (See Fig. 2). It is possible therefore that the synthesis of this RNA is detectable in cultures infected with Pr(1) at low multiplicity because of its overproduction in those cells in the culture which have been co-infected with Pr(1) and standard virus rather than in cells infected with Pr(1) alone.

There was considerable hybridization of RNA synthesized by the Pr(2)-infected cultures to BamH-I DNA fragments 5, 10, and 12 and to Kpn-I fragments I and J [all these fragments
Fig. 8. Autoradiograms of filters containing Kpn-I- or BamH-I-digested Pr virus DNA to which RNA synthesized by cells infected at low m.o.i. had been hybridized. Phosphate-starved cultures were infected with either Pr(s), Pr(1) or Pr(2) at a multiplicity of 0.25 p.f.u./cell (see Table 1 for details) and labelled between 0 and 2 h or 6 and 9 h p.i. with $^{32}$P (200 µCi/ml). The RNA was extracted and
hybridized to the filters which were then exposed for autoradiography. (a) RNA hybridized to \textit{Kpn-I} filters; (b) RNA hybridized to \textit{BamH-I} filters. The tracks at the right (a) or left (b) hand of the figure are autoradiograms of the filter strips that have been hybridized to $^{32}$P-labelled standard Pr DNA.
Fig. 9. PAGE proteins synthesized by cultures infected with Pr(s), Pr(1) and Pr(2) at low m.o.i. RK cells were infected at 0.25 p.f.u./cell (see Table 1 for details) and labelled between 6 and 9 h with \(^{3}H\)-leucine. The proteins were electrophoresed as described in Methods. As a control, proteins synthesized by cells infected with Pr(s) at high multiplicity [Pr(s) high] and proteins present in a purified preparation of mature virions (v.p.) were also electrophoresed. The three polypeptides which were present in relatively higher amounts in Pr(2) than in Pr(s)-infected cells are marked with open circles.

represent DNA sequences which are reiterated in Pr(2) DNA]. These transcripts were detectable both at early and at late times after infection. Because the over-representation of RNA complementary to these DNA sequences is small at early times after infection in cells co-infected with Pr(2) and standard virus (See Fig. 3 and 5) and because transcripts complementary to \(Kpn-I\) fragment K [which are transcribed overabundantly at late times after
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Fig. 10. Autoradiograms of filters containing Kpn-I-digested DNA which had been hybridized to IE RNA made by cells infected at high and low m.o.i. The cultures were infected in the presence of cycloheximide and the RNA labelled and extracted as described in Methods. The RNA was hybridized to filters containing Kpn-I-digested Pr DNA. High, indicates infection with 5 p.f.u./ml; low, 0.25 p.f.u./ml (see Table 1).

infection in cells co-infected with Pr(s) and Pr(2)] were barely detectable, it is likely that this RNA was synthesized in cells infected with Pr(2) alone.

Synthesis of virus proteins in cells infected with defective virions in the absence of helper functions provided by Pr(s) – low multiplicity infection

Analysis of the PAGE profiles of the polypeptides made in cells infected at low multiplicity infection with defective or standard Pr virions yielded results which are consistent with those obtained from the studies of RNA transcription. In cultures infected with Pr(s) and Pr(1) at 0.25 p.f.u./cell, very low levels of virus polypeptides were detectable (Fig. 9). In Pr(2)-infected cells, however, a polypeptide with a mol. wt. of 27K was greatly overproduced and synthesis of several other polypeptides including those which are overproduced in cells co-infected with Pr(s) (67K and 56K) was also detectable. These results indicate that the DNA of defective Pr(2) virus is expressed in the absence of co-infecting standard virus and induces the synthesis of some virus proteins. Similar conclusions have
Table 2. Synthesis of immediate-early proteins in cells infected with standard and defective virions at low multiplicity of infection*

<table>
<thead>
<tr>
<th>Proteins reactive with:</th>
<th>Pre-immune serum†</th>
<th>Anti-IEP serum (ct/min × 10⁻⁶)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells infected with:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uninfected</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pr(1)</td>
<td>0·25</td>
<td>2·3</td>
</tr>
<tr>
<td>Pr(2)</td>
<td>0·25</td>
<td>2·3</td>
</tr>
<tr>
<td>Pr(s) (low)</td>
<td>0·25</td>
<td>2·3</td>
</tr>
<tr>
<td>Pr(s) (high)</td>
<td>5·0</td>
<td>2·7</td>
</tr>
</tbody>
</table>

* RK cells were infected in EDS containing cycloheximide (50 µg/ml) as described in Methods and incubated up to 5 h in cycloheximide medium. The culture was then washed to remove the cycloheximide and the proteins synthesized by the cultures were labelled with ³H-leucine between 5·5 and 7·4 h and immune precipitated as described in Methods.

† Normal rabbit serum (NRS).
‡ Anti-IEP (immediate-early proteins) serum adsorbed unspecifically a greater amount of proteins than did NRS. The reason for this is unclear.

also been drawn from the results of experiments in which the synthesis of virus proteins and DNA was examined in individual cells infected with Pr(2) virions using a technique which combines autoradiography and immunofluorescence (DeMarchi et al. 1979).

Synthesis of IE RNA and proteins in cells infected with defective virions in the absence of co-infecting 'helper' infectious virus

Although it is possible to detect Pr IE RNA at early stages of infection during the normal course of infection, this RNA is usually made in small amounts (Feldman et al. 1979). However, IE RNA will accumulate when cells are infected in the presence of an inhibitor of protein synthesis, such as cycloheximide (Rakusanova et al. 1971; Feldman et al. 1979). To detect the synthesis of IE RNA, both in cells co-infected with standard and defective virions (high multiplicity infection) and in cells infected with defective virions alone (low multiplicity infection), we analysed the RNA synthesized in infected cultures treated with cycloheximide. The results are illustrated in Fig. 10. Cells infected in the presence of cycloheximide at high m.o.i. (5 p.f.u./cell) with standard virions as well as with Pr(1) or with Pr(2) synthesized RNA that hybridized to Kpn-I fragments E and H only (these fragments represent the extreme ends of the inverted repeats). The relative abundance of this RNA in Pr(2)-infected cells was greater than that in either Pr(1)-or Pr(s)-infected cells and probably reflects the over-representation of IE sequences in the DNA of Pr(2) (see Fig. 2).

In cultures infected at low m.o.i. (0·25 p.f.u./cell), IE RNA synthesis was not detectable in cells infected with Pr(s) (Fig. 10), probably because this RNA was synthesized only in those cells which were infected with infectious virus and the level of synthesis of the RNA in the cultures was therefore too low to be detected by the methods used. This was also the case for cultures infected with Pr(1), despite the much greater number of defective particles with which the cells in these cultures were infected. In cultures infected with Pr(2) at low multiplicity IE RNA was, however, readily detectable.

The accumulation of IE RNA in cells infected with Pr(2) defective particles was confirmed by the results of an experiment in which the level of synthesis of IE proteins in those cells was determined. If IE RNA is allowed to accumulate by incubating the cells with cycloheximide and the drug is then removed, IE proteins are synthesized in much greater quantities in cultures infected with Pr(2) than either in cells infected with Pr(1) or Pr(s) at the same m.o.i. Immune precipitation with anti-IEP serum of the proteins synthesized by the cells (Table 2)
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![Table and Image]

MI Anti-IEP NRS Anti-IEP NRS Anti-IEP NRS Anti-IEP NRS
Pr(s) low Pr(1)53 Pr(2)53 Pr(s) high

Fig. 11. Autoradiogram of the immune-precipitated IE proteins synthesized by cells infected with standard or defective virus at low m.o.i. The samples were prepared as described in the legend to Table 2 and electrophoresed as described in Methods. MI = mock infected; NRS = normal rabbit serum.

Infected with 0.25 p.f.u./cell showed that only Pr(2)-infected cultures produced significant levels of IE proteins. Indeed, the amount of the IE proteins synthesized by cultures infected at a low multiplicity (0.25 p.f.u./cell) of Pr(2) and a high multiplicity (5 p.f.u./cell) of Pr(s) was approximately the same. Analysis of the precipitated proteins by PAGE (Fig. 11) confirmed that the proteins were Pr virus IE proteins (Rakusanova et al. 1971; Ben-Porat et al. 1974).

Thus, the IE sequences which are reiterated in Pr(2) virions and which, under normal conditions of infection are not overabundantly transcribed (see Fig. 5 and 6), are abundantly transcribed when virus protein synthesis is inhibited, i.e. in cycloheximide-treated, infected cells. The data in Fig. 10 and 11 in themselves do not allow one to conclude that the IE RNA is transcribed in cells infected with Pr(2) alone rather than in cells co-infected with Pr(s) and Pr(2). However, the data in Fig. 8 indicate that the genome of Pr(2) is transcribed in the absence of helper functions provided by Pr(s). Furthermore, experiments in which the
The synthesis of immediate-early proteins was followed in single cells showed that this was the case (DeMarchi et al. 1979).

**DISCUSSION**

The experiments described in this paper deal with the type of virus transcripts that accumulate and the type of virus proteins that are synthesized in cells infected with two populations of defective virions under the following two sets of conditions: (1) Cells co-infected with standard and defective virions. These experiments were performed to determine the expression of the defective genomes in the cells, in an attempt to determine whether there is a correlation between gene expression and the ability to interfere with the growth of standard virus. (2) Cells infected with defective virions alone. These experiments were performed to determine the degree to which the expression of the genome of the defective virions is dependent on helper functions provided by standard virions.

Overproduction of transcripts from some of the regions for which the populations of defective virions are enriched was observed in cells co-infected with Pr(s) and either Pr(1) or Pr(2). Thus, in cells co-infected with Pr(s) at late times after infection, transcripts of regions of the genome, for which the defective DNA is enriched, are abundantly synthesized. The pattern of hybridization of the RNA reflects in general the composition of the defective DNA, suggesting that the DNA is transcribed. The RNA transcribed from the defective DNA may, however, not be functional. This conclusion is derived from the observation that the PAGE profiles of the proteins synthesized by cells infected with Pr(s) alone and by cells co-infected with Pr(1) or Pr(s) were the same, indicating an apparent lack of translation of the transcripts that were overproduced as a result of co-infection with Pr(1). Thus, an overproduction (or the lack of synthesis) of some proteins as a result of co-infection with Pr(1), which could account for the interfering ability of these defective virions, was not detected.

In cells co-infected with Pr(s) and Pr(2), an overproduction of some proteins as compared to cells infected with Pr(s) alone was observed, however. Three protein bands with mol. wt. of 67K, 56K and 27K accumulated in greater abundance in cells co-infected with Pr(s) and Pr(2) than in cells infected with Pr(s) alone. Polypeptides with the same PAGE migration characteristics were also found as components of purified virions (Fig. 9). Thus, co-infection with Pr(2) results in the overproduction of some proteins which appear to be structural virus proteins. An overproduction of a non-structural virus protein (175K or ICP4) in cells co-infected with standard and defective virions of HSV has been previously reported (Frenkel et al. 1975; Murray et al. 1975). The overproduction of some virus proteins in cells co-infected with standard and defective virions is, however, not a prerequisite for interference since such an overproduction was not detectable in cells co-infected with Pr(s) and Pr(1). Thus, despite the fact that Pr(1) and Pr(2) interfere similarly with the production of infectious virus, we have been unable to detect similarities in the expression of their genomes that might account for their ability to interfere.

The experiments in this paper clarify some aspects of the controls that operate at the level of transcription of the virus genome. Most informative in this respect is the pattern of transcription of the virus genomes in cells infected with Pr(2) alone or in cells co-infected with Pr(2) and Pr(s).

The DNA of Pr(2) contains a reiteration in which one of the inverted repeat regions is conserved in intact form (Rixon & Ben-Porat, 1979). Our results indicate that the reiterated DNA in Pr(2) is subject to the same transcriptional controls as is the DNA of standard virus. The DNA in the inverted repeat is part of three distinct transcriptional classes (see Fig. 1). The extreme ends of the inverted repeats are transcribed exclusively as IE RNA; the middle is transcribed exclusively as late RNA; the junctions of the inverted repeat and the short unique sequence are transcribed both at early and late times after infection.

At early times after infection in cells co-infected with Pr(s) and Pr(2), RNA complementary
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to the regions bordering the junction between the short unique and the repeat regions forms an even larger percentage of the total RNA transcribed than in cells infected with Pr(s) alone. This is due, presumably, to the presence of these sequences in reiterated form in this defective DNA. However, other DNA sequences, which are also reiterated in this defective DNA, such as the region that specifies IE RNA (end of repeats) or the region transcribed exclusively at late times (Kpn-I fragment K, middle of repeat) are not transcribed in great abundance at early times after infection. Under other conditions of infection, these regions of the defective genome are, however, transcribed abundantly. For example, in the presence of cycloheximide, IE RNA accumulates in greater abundance in cells co-infected with Pr(s) and Pr(2) than in cells infected with Pr(s) alone (Fig. 19). Also at late times after infection, transcripts from the middle of the repeat (Kpn-I fragment K) are transcribed more abundantly in cells co-infected with Pr(s) and Pr(2) than in cells co-infected with Pr(s) alone (Fig. 4 and 6). These results imply that the genome of Pr(2) is transcribed in the cells co-infected with Pr(2) and Pr(s) and that the same transcriptional controls operate in cells co-infected with defective virions as in cells infected with standard virus alone.

These conclusions are reinforced by the data obtained from experiments in which most of the cells in the cultures (75%) were infected with defective virions only. Under these conditions, Pr(1) virions, as well as the non-infectious virions present in populations of standard virus, seem to be transcriptionally inert. Pr(2)-infected cells, on the other hand, made appreciable amounts of virus RNA and the pattern of hybridization of the RNA synthesized at early and at late times after infection was approximately the same. This RNA hybridized mainly to regions of the genome from which early RNA is normally transcribed. The fact that at late times after infection, RNA hybridizing to the middle of the repeat region [which at late times after infection is a region of extensive transcription in cells co-infected with Pr(2) and Pr(s)] was synthesized in barely detectable amounts indicates that a function(s) provided by the standard genome is required to allow this region of the genome to be transcribed. It is also interesting that IE RNA is not overproduced in Pr(2)-infected cells (unless an inhibitor of protein synthesis is present), even though the sequences that specify for this RNA are reiterated in Pr(2) DNA (see Fig. 2 and Rixon & Ben-Porat, 1979). These results show that the controls of transcription over these segments of DNA have been retained.

Our data are consistent with the following interpretation: the presence of an intact IE region allows the earliest event in infection to take place, as it does in Pr(s)-infected cells. The products of the IE genes then direct the transcription of RNA from the early genes and the synthesis of some virus proteins. This sequence of events is as far as the infective process advances in cells infected with Pr(2) only. In cells co-infected with Pr(s) and Pr(2), the late genes which are reiterated in Pr(2) are also switched on, probably as a result of a helper function(s) provided by Pr(s), with the consequent appearance of large amounts of RNA which hybridize to the middle of the repeat region. The need for helper functions to trigger these events suggests that these functions are not directed by any of the genes which are reiterated in Pr(2) DNA but by that part of the genome which is absent from, or not expressed in, this defective DNA. Interestingly, in cells co-infected with Pr(2) and standard virus, we could not detect an overproduction of some polypeptides which could be correlated with the overproduction of this late RNA class; the same proteins are overproduced both at early and late times after infection. This is similar to the situation that prevails in cells co-infected with Pr(s) and Pr(1) in which overproduction of proteins resulting from an overabundant accumulation of some RNA species could not be detected.

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