Expression of Immediate-Early Genes in HerpesSimplex Virus
Type 1-infected XC Cells: Lack of ICP22 (68K) Polypeptide

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SUMMARY

The expression of immediate-early (IE) genes of herpes simplex virus type 1 (HSV-1, MP strain) in non-permissive rat XC cells was analysed and compared with the expression of IE genes in permissive HEp-2 cells by the following three methods: analysis of virus polypeptide synthesis in infected cells, Northern blot hybridization between poly(A) nuclear or cytoplasmic RNA and in vitro labelled virus DNA or plasmid-cloned fragments corresponding to IE genes, and ability of poly(A) cytoplasmic RNAs to direct synthesis of virus polypeptides in vitro. ICP4 (175K), ICP0 (110K) and ICP27 (62K) were synthesized in XC cells although in smaller amounts than in HEp-2 cells; ICP4 is functional since early and late polypeptides could be observed. Their corresponding mRNAs were present at low levels in nuclei and in cytoplasm and are functional since the polypeptides were synthesized in a rabbit reticulocyte system. ICP22 (68K) was not detectable in infected XC cells; its mRNA was present in nuclei and in cytoplasm, but it is not functional since the corresponding polypeptide was not synthesized in a rabbit reticulocyte system. This suggests some structural differences in the ICP22 mRNA molecules in infected XC and HEp-2 cells and implicates cellular determinants in the control of the expression of HSV-1 IE genes.

INTRODUCTION

Herpes simplex virus type 1 (HSV-1) replicates efficiently in many cell cultures but grows poorly in some mouse or rat cell cultures and is unable to multiply in XC cells (Docherty et al., 1973), a rat cell line transformed by Rous sarcoma virus (Svoboda, 1960). Using this cell line, we have previously shown that variants of HSV-1 can be divided into two classes according to their penetration capacity (Epstein et al., 1984). When the virus penetrates, virus polypeptides are seen and are regulated in a cascade fashion as in permissive cells. However, the virus cycle is somehow delayed and most polypeptides dependent on virus DNA synthesis cannot be observed (Epstein & Jacquemont, 1983). To investigate whether the absence or diminution of some immediate-early gene products might be responsible for this block, we have compared immediate-early gene expression in XC and HEp-2 cells.

HSV expression is divided into three successive steps corresponding respectively to expression of immediate-early (IE), early (E) and late (L) genes. IE genes give rise to five polypeptides designated ICP4 (175K), ICP0 (110K), ICP22 (68K), ICP27 (62K) and ICP47 (12K) (Honess & Roizman, 1973; Watson et al., 1979; Preston, 1979). These genes have been mapped inside or close to inverted repeat sequences, except ICP27 which maps in the unique sequences of the ‘L’ component of the genome (Jones et al., 1977; Morse et al., 1978; Preston et al., 1978; Watson et al., 1979; Watson & Clements, 1980) and IE mRNA are transcribed from both strands of the virus DNA (Clements et al., 1979). Each IE gene is transcribed independently and is regulated by its own promoter (Mackem & Roizman, 1980). ICP4 appears to have a central role: it is implicated in the switch from IE to E genes and its function is required continuously throughout the virus growth cycle (Watson & Clements, 1980). Synthesis of other IE gene products is blocked very early in infection. ICP22 and ICP47 mRNAs have in
common a 5' end and a small splice located in IRs and TRs respectively (Watson et al., 1981). The size of the intron varies according to the strain (Rixon & Clements, 1982). Finally, the ICP22 gene has been shown to be non-essential for virus replication, since a mutant lacking a complete copy of this polypeptide could grow in Vero cell cultures (Post & Roizman, 1981).

In this report, IE expression was analysed in cells infected with HSV-1 strain MP in the presence of cycloheximide, a protein synthesis inhibitor. Our results show differences in the relative amounts of nuclear and cytoplasmic IE RNAs between HEp-2 and XC cells as well as functional differences. Functional ICP4, ICP0 and ICP27 mRNAs were detected both by translation in vivo and in vitro. ICP22 mRNA was synthesized but it is not functional since it could not be detected either in vivo or in vitro.

**METHODS**

**Cells and virus.** Serially propagated human epidermoid carcinoma no. 2 (HEp-2) cells were grown in Eagle's MEM supplemented with 10% inactivated newborn calf serum. XC cells were grown in MEM supplemented with 10% tryptose phosphate buffer and 8% inactivated foetal calf serum. Both cell lines were obtained from the American Type Culture Collection.

HSV-1 MP strain (Hoggan & Roizman, 1959) was obtained from B. Roizman (University of Chicago, U.S.A.). Stocks were produced by growth in HEp-2 cells at very low multiplicity of infection.

**Purification of virus DNA and labelling in vitro.** Virus DNA was purified from the cytoplasm of infected HEp-2 cells by banding in NaI equilibrium gradients according to Walboomers & Ter Scheggett (1976).

Intact HSV-1 DNA or virus DNA fragments were labelled by nick translation to $5 \times 10^7$ c.p.m./µg by using *Escherichia coli* DNA polymerase I and [$\alpha$-$^32$P]dCTP (300 Ci/mmol; Amersham) (Rigby et al., 1977).

**Cloning procedure.** BamHI B, Y and N DNA fragments from HSV-1 MP DNA were purified by electrophoresis in a low melting agarose gel (Sigma) and eluted by solubilization at 65 °C in 0-5 M-NaCl, 10 mM-Tris–HCl pH 7.5, 5 mM-EDTA. The vector pBR322 was cleaved with BamHI and diphosphorylated with calf intestinal alkaline phosphatase (Boehringer Mannheim) according to Maniatis et al. (1982). Two-hundred or 300 ng of fragment and 100 ng of diphosphorylated pBR322 were ligated by T4 DNA ligase (Maniatis et al., 1982) and the recombinant DNA used to transform recipient strain *E. coli* C600 by the calcium chloride procedure (Mandel & Higa, 1970). Ampicillin-resistant bacteria were selected and screened for sensitivity to tetracycline. The colonies were transferred from agar plates onto Schleicher & Schüll BA85 nitrocellulose paper, denatured with 0-5 M-NaOH, neutralized to pH 7-4, rinsed in 2 × SSC (1 × SSC = 0-15 M-sodium chloride, 0-015 M-sodium citrate), dried and baked at 80 °C for 2 h. After incubation at 66 °C for 4 h in modified Denhardt's solution (0-02% bovine serum albumin, 0-02%, Ficoll, 0-02%, polyvinylpyrrolidone, 6 × SSC), filters were hybridized at 66 °C for 22 h with HSV-1 MP DNA labelled in vitro in 30% formamide, 0-5% SDS, 50 µg/ml calf thymus DNA in modified Denhardt's solution. Plasmid recombinant DNAs were checked by digestion with restriction endonucleases. A schematic representation of IE genes, showing the location of cloned fragments, is shown in Fig. 1. Biohazards associated with these experiments have been examined previously by the French National Control Committee and the experiments were carried out according to the rules established by this committee.

**Cell fractionation and isolation of RNA.** Nuclear and cytoplasmic RNAs were prepared by lysis of infected cells as previously described (Jacquemont et al., 1980).

**Selection of poly(A) RNA.** Nuclear and cytoplasmic RNAs were recovered by centrifugation and dissolved in a small volume of water, denatured for 5 min at 65 °C and diluted after cooling in loading buffer (0-5 M-sodium chloride, 1 mm-EDTA, 10 mm-Tris–HCl pH 7-6, 0-1% SDS). The RNA was applied to a column of oligo(dT)- cellulose (Collaborative Research, Waltham, Mass., U.S.A.), washed with 5 column vol. of loading buffer, then 5 vol. of loading buffer with 0-15 M-sodium chloride. Poly(A) RNA was eluted with 10 mm-Tris–HCl pH 7-6, 1 mm-EDTA, 0-05% SDS and precipitated with 2 vols. ethanol in 0-3 M-sodium chloride.

**Size fractionation and transfer of RNA.** Poly(A) RNA was electrophoresed on agarose slab gels after glyoxal denaturation according to Thomas (1980). Briefly, the RNA pellet as selected above was incubated in 5 µl of the mixture containing 135 µl deionized glyoxal, 400 µl DMSO, 20 µl 0-4 M-sodium phosphate buffer pH 7, 245 µl water at 50 °C for 1 h. The reaction mixture was cooled and mixed with 5 µl of loading buffer. The samples were electrophoresed on horizontal 1-2% agarose gels in 10 mm-sodium phosphate buffer pH 7 at 110 V for 6 h with constant circulation of buffer. Ribosomal RNA from HEp-2 cells and from *E. coli*, used as controls, was stained with ethidium bromide and poly(A) RNA was directly transferred from agarose to nitrocellulose (Schleicher & Schüll BA85) by using 20 × SSC overnight at 4 °C. Blots were dried and baked under vacuum for 2 h at 80 °C.

**Hybridization to blots.** Nitrocellulose filters were incubated at 52 °C for 24 h in prehybridization buffer containing 50% (v/v) deionized formamide (Bethesda Research Laboratories), 0-5 M-sodium chloride, 5 mm-EDTA, 0-1% SDS, 100 mm-Tris–HCl pH 7-5, 5 × Denhardt's solution, 100 µg/ml yeast RNA, 100 µg/ml calf thymus DNA.
HSV-1 IE expression in XC cells

Fig. 1. Schematic representation of the short region of herpes simplex virus type 1 genome and map location of immediate-early mRNAs. The first line shows a diagrammatic representation of the unique sequence Us limited by the two reiterated sequences IRs and TRs and the right-hand part of the unique sequence Ul limited on the right by the reiterated sequence IRl. The second line shows the size and the position of HSV-1 DNA (F strain) fragments generated by BamHI restriction enzyme (Post et al., 1980). HSV-1 DNA fragments contained in plasmids are denoted by a thicker line. The third line indicates the probes used, designated by the thicker line, on the restriction endonuclease map of HSV-1 DNA (b, BamHI; s, SacI). One SacI site in BamHI N was observed by Watson et al. (1983) in the Patton strain. This enzyme cleaves the 5000 bp BamHI N fragment from MP strain into 1600 bp and 3400 bp fragments but does not cleave pBR322. The 3400 bp subfragment, which complements the ICP22 mRNA (mRNA-4) located by Rixon & Clements (1982) on a 500 bp fragment, was used as probe. IE mRNA corresponds to immediate-early transcripts as located on HSV-1 DNA by Clements et al. (1979). Interrupted arrows indicate the splice junctions as described by Rixon & Clements (1982) and the designated size as obtained in hybridization experiments. IE polypeptides show the size of immediate-early polypeptides obtained in HEp-2 cells infected by HSV-1 strain MP (Hones & Roizman, 1975).

RESULTS

Synthesis of IE virus polypeptides in XC cells

XC and HEp-2 cells were infected at 33 °C with 50 p.f.u./cell of HSV-1 (MP) in the presence of 50 μg/ml cycloheximide (added 1 h before infection) and labelled with [35S]methionine between 5 and 6 h post-infection, after removal of cycloheximide. Virus polypeptides were analysed on 9% SDS–polyacrylamide slab gels. Fig. 2 shows that in infected HEp-2 cells (lane 4) there was an accumulation of ICP4, 6, 0, 22 and 27. In infected XC cells (lane 2) ICP4, 6 and 0...
were also detected, though in smaller amounts than in HEp-2 cells. The situation was less clear regarding ICP22 and 27 because of the presence of host proteins migrating in the same region as the virus polypeptides. Nevertheless, ICP27 seemed to be present: it migrated slightly slower than the host polypeptide and, furthermore, its synthesis was strongly stimulated by infection. In contrast, ICP22 did not seem to be present. A polypeptide slightly larger than ICP22 was observed in both uninfected and infected XC cells; this polypeptide, which seemed to be only slightly stimulated by infection, probably corresponds to the 70K heat-shock protein, as its synthesis is increased at 39 °C (Epstein & Jacquemont, 1983). The absence of ICP22 was also previously noted in XC cells infected with strain 13VB4tsC75 at non-permissive temperature...
HSV-1 IE expression in XC cells

(Epstein & Jacquemont, 1983; their Fig. 3, lane 8). ICP47 was not observed at any time in infected HEp-2 cells; consequently, we did not analyse it in the present paper.

**Nuclear and cytoplasmic RNA synthesis in XC cells**

Since virus polypeptides were detected in small amounts or not at all in XC cells, we analysed IE transcripts by Northern blot hybridization. XC and HEp-2 cells were infected with MP strain in the presence of cycloheximide up to 5 h after infection. Cells were fractionated into nuclei and cytoplasm, and RNA was extracted from each fraction. Poly(A) RNA was purified on oligo(dT)-cellulose. Equal amounts of total RNA from XC and from HEp-2 cells were electrophoresed in parallel tracks on agarose gels after glyoxal denaturation. RNAs were blotted onto nitrocellulose paper and hybridized with nick translation-labelled MP DNA.

Results in Fig. 3 show that in the nuclei of HEp-2 cells (Fig. 3a, lane 1) IE mRNA was divided into three populations of 4-8 kb, 3 kb and 1-9 kb, consistent with cytoplasmic RNA previously described by Watson *et al.* (1979) and Anderson *et al.* (1980). These three populations correspond respectively to mRNA for ICP4, for ICPO and for ICP22, 27 and 47 (Watson *et al.*, 1979) (Fig. 1). Fig. 3 also shows that, in the nuclei of XC cells, these three populations of RNA were also observed, the quantity of RNA being smaller than in HEp-2 cells but the relative proportions of the bands were roughly the same. Hybridization experiments with poly(A)−RNA did not allow the detection of any virus RNA population; this suggests that the low level of poly(A) RNA reflects a low level of transcription.

In the cytoplasm of HEp-2 cells (Fig. 3b, lane 1) the molecular weight of viral RNA was the same as in nuclei but the relative proportion of the bands was significantly different. The 3 kb and 1-9 kb populations were more abundant than the 4-8 kb population. In the cytoplasm of XC cells, the 4-8 kb mRNA was difficult to observe and the ratio of 3 kb to 1-9 kb mRNA was higher than in HEp-2 cells (Fig. 3b, lane 2).

Since the 4-8 kb mRNA was difficult to observe in cytoplasmic RNA and the 1-9 kb population is a mixture of different mRNAs, we used fragments, labelled in vitro, purified from plasmids as probes for the following hybridizations. (i) When the RNA extracted from the cytoplasm of XC cells was hybridized with *BamHI* Y fragment as a probe for 4-8 kb RNA (Fig. 1) a weak band was observed (Fig. 3b, lane 4). This band seemed to migrate faster than the 4-8 kb band. However, when smaller concentrations of poly(A) RNA were used, this band migrated at the same level in XC as in HEp-2 cells because the ratio of cellular and viral poly(A) to total RNA is higher in XC than in HEp-2 cells (data not shown). (ii) When nuclear and cytoplasmic RNAs were hybridized with *BamHI* B fragment as a probe for both the 3 kb mRNA (corresponding to ICPO), and the 1-9 kb RNA (corresponding to ICP27) (Fig. 1) these RNAs were observed (Fig. 3). However, whereas in the nuclei of XC cells the amounts of ICP27 and ICPO mRNA seemed similar, in the cytoplasm of XC cells (but not in HEp-2 cells), the amount of ICPO mRNA was higher than that of ICP27 consistent with the observation made with MP DNA probe. (iii) When nuclear and cytoplasmic RNAs were hybridized with *SacI–BamHI* subfragment, isolated from a plasmid containing *BamHI N* (Fig. 1), as a probe for mRNA corresponding to ICP22, this mRNA was present in both cases.

**Translation in vitro of poly(A) IE RNA**

Since all IE mRNAs seem to be present in the cytoplasm of infected XC cells, we examined the ability of poly(A) RNA to be translated in a translation system in vitro. XC and HEp-2 cells were infected with HSV-1 (MP) in the presence of cycloheximide as previously described. RNA was extracted from the cytoplasm of infected cells and poly(A) RNA purified on oligo(dT)–cellulose. Equal amounts of RNA were introduced into a reticulocyte system in vitro with [35S]methionine and the polypeptides synthesized were analysed by electrophoresis on 9% SDS–polyacrylamide gel. Different times of infection were tested and the best result, corresponding to the least cellular polypeptide synthesis and the greatest virus polypeptide synthesis, was obtained at 5 h post-infection.
Fig. 3. Hybridization analysis of IE mRNA isolated from infected HEp-2 and XC cells. HEp-2 and XC cells preincubated with 50 μg/ml cycloheximide for 1 h were infected with 20 p.f.u. HSV-1 (MP) per cell and incubated in the presence of the drug. At 5 h post-infection, nuclear and cytoplasmic RNAs were extracted and poly(A) RNAs, purified on oligo(dT)-cellulose columns, were fractionated on 1.2% agarose gels after glyoxal denaturation. They were then transferred to nitrocellulose paper and hybridized with nick-translation-labelled virus DNA or pBR322 cloned fragments specific for each major IE region. *BamHI* Y for ICP4 mRNA, *BamHI* B for ICP0 and ICP27 mRNAs and *SacI*-BamHI (3400 bp) from the *BamHI* N fragment for ICP22 mRNA (as indicated in Fig. 1). Hybridization with poly(A) nuclear (a) and cytoplasmic (b) RNAs is shown. HEp-2 rRNA (5.2 kb and 3 kb) and *E. coli* rRNA (3 kb and 1.6 kb) served as molecular size standards (ethidium bromide staining shown in lane 7). The approximate sizes in kb of mRNA species, estimated from the plot of log size versus migration with stained bands of rRNA, are shown on the left.
Fig. 4. Autoradiographic images of electrophoretically separated polypeptides translated \textit{in vitro} from IE poly(A) RNA extracted from cytoplasm of infected XC and HEp-2 cells. XC and HEp-2 cells were preincubated with 50 \( \mu \text{g/ml} \) cycloheximide for 1 h, then infected with 20 p.f.u. HSV-1 (MP) per cell and incubated in the presence of the drug. At 5 h post-infection, cytoplasmic RNAs were extracted and poly(A) RNA fractionated on oligo(dT)-cellulose columns. Two \( \mu \text{g} \) of these RNAs was used to programme a reticulocyte translation system supplemented with \([35\text{S}]\)methionine for 1 h at 37 \( ^\circ\text{C} \). The products of translation were separated on a 9\% SDS–polyacrylamide gel together with the products of translation of poly(A) cytoplasmic RNA extracted from uninfected XC cells treated with cycloheximide (lane 1). HEp-2 cells infected in the same conditions in the presence of cycloheximide and labelled with \([35\text{S}]\)methionine between 5 and 6 h post-infection, following the removal of cycloheximide (lane 4) or infected without cycloheximide and labelled between 5 and 6 h post-infection (lane 5) were used as controls (translation \textit{in vivo}). The arrow indicates the expected position of ICP22 in XC cells.

As shown in Fig. 4, with infected HEp-2 cells (lane 3), ICP4, 6, 0, 22 and 27 were observed as \textit{in vivo} following reversal of a cycloheximide block (lane 4). In XC cells, under the same conditions (lane 2), ICP4, 0 and 27 were detected, though in smaller amounts than in HEp-2 cells. Again, as for translation experiments \textit{in vivo}, a polypeptide of cellular origin migrated very close and slightly faster than ICP27. However, ICP22 was undetectable. Identical results were
obtained in several labelling experiments in vitro. ICP6 could not be observed in these experiments; however, in contrast with ICP22, ICP6 could be readily observed in infected XC cells (this Fig. 2; Epstein & Jacquemont, 1983).

DISCUSSION

The expression of IE genes of HSV-1 was analysed in non-permissive XC cells, and compared with the expression of IE genes in permissive HEp-2 cells. The differences observed in the two systems were both quantitative and qualitative. In nuclear RNA, all IE mRNAs seemed to be synthesized in relative proportions similar to those found in the permissive system, but in much smaller amounts. This low level of transcription might result either from lower levels of penetration into XC cells, or from the absence of efficient activation of IE genes, or by both. In a previous work (Epstein et al., 1980) we analysed the fate of the HSV-1 genome by infecting XC and HEp-2 cells with labelled virus. Although the amount of labelled virus DNA that enters the cells, and even the nuclei, is not significantly different in the two systems, the method of entry in XC cells, as revealed by electron microscopy, is mainly by pinocytosis and not by fusion as in HEp-2 cells. Thus, it is possible that this type of penetration does not allow IE gene expression and that only the fraction of virus that enters the cells by fusion with cell membranes gives rise to the synthesis of virus RNA. This hypothesis is supported by the observation that promoting virus penetration by fusion with XC cell membranes, employing polyethylene glycol as a fusogen, greatly enhances the expression of HSV-1 in these cells (Epstein et al., 1984). On the other hand, different observations indicate that expression of IE genes is activated by a factor that is a virion component (Post et al., 1981; Hertz & Roizman, 1983). This inducer, which seems to bind to regulatory sequences of the ICP4 gene (Cordingley et al., 1983), is located outside the capsid (Batterson & Roizman, 1983). If the fusion step is required for an efficient activation of the IE functions, the lack of this event could help to explain why transcription is very low even if a significant fraction of the genome actually reaches the nuclei.

In cytoplasmic RNA, the ratio between the three populations of mRNA molecules was different in XC compared to HEp-2 cells. Since these differences were not detected at the level of nuclear RNA, these results suggest some variation in the processing or in the stability of RNA molecules between the two cell types. It is possible, for example, that cellular gene products can act as regulators for the accumulation of virus mRNA.

For polypeptide synthesis, the low level of synthesis can be explained by the low level of transcripts. However, some differences between the two systems were also qualitative. Whereas ICP4, ICP0 and ICP27 were detected both in vivo and in vitro, ICP22, in contrast, could not be detected by any system although its corresponding mRNA was present in the cytoplasm. This suggests a difference in the ICP22 mRNA structure itself. It is tempting to relate the failure to direct the synthesis of ICP22 with splicing events and interesting to compare this situation with host range cases of other viruses, especially papova- and adenoviruses. Segal et al. (1979) described unspliced AgT mRNA in undifferentiated murine teratocarcinoma cells infected with SV40, and Klessig & Chow (1980) observed the same type of block on fibre mRNA in adenovirus-infected monkey cells. These two observations show that control of gene expression by RNA splicing may be a common mechanism in determining the host range of DNA viruses. Our hybridization experiments could not allow us to distinguish an unspliced from a spliced RNA in XC cells. Stable unspliced RNA might exist since Rixon & Clements (1982) have observed the presence of both these structures in the cytoplasm of permissive cells. Whatever the mechanism, our results show the involvement of cellular determinants in the control of the expression of HSV-1 IE genes at the post-transcriptional level.

IE expression of HSV-1 in XC cells is an abnormal situation. In this cell line ICP22 is lacking and ICP4, described to be the only essential IE polypeptide, is synthesized in significant amounts and is functional since E and L polypeptides can be synthesized (Epstein & Jacquemont, 1983). Thus, E and L polypeptides observed in XC cells are probably not dependent on the synthesis of ICP22. Conversely, the absence of some E and L polypeptides in XC cells might be related to the absence of ICP22 in this cell line. These results are consistent with, although do not prove, the existence in parallel of different cascade regulations, as proposed by Pereira et al. (1977).
Although it is tempting to relate the abortive infection in XC cells with the lack of ICP22, this still remains an open question. In some permissive systems, like BHK cells, essentially no ICP22 can be detected in vivo (I. W. Halliburton, personal communication); however, this system appears to differ from XC cells in that mRNA taken from infected BHK cells is able to direct the synthesis of ICP22 in vitro (Watson et al., 1979). On the other hand, Post & Roizman (1981) have suggested that ICP22 is not essential for normal replication since a deletion mutant lacking this polypeptide could grow in Vero cells. However, in this case a polypeptide fragment of about 20K was synthesized which might be responsible for the essential functions of ICP22 and, interestingly, I. W. Halliburton & B. Roizman (personal communication) have recently observed that in BHK cells infected with the deletion mutant, the 20K polypeptide fragment cannot be observed and that this correlates with a highly decreased replication of the virus, even if most virus functions in these cells seemed normal.

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