INTRODUCTION

Although Orthopoxviruses can infect a wide variety of mammalian and avian cells, there are host-range mutants that fail to replicate in specific lines derived from pig kidney (Fenner and Sambrook, 1966; Lake and Cooper, 1980; Moyer et al., 1980; Moyer and Graves, 1982), Chinese hamster ovary (CHO) (Drillien et al., 1978; Njayou et al., 1982), or human sources (Drillien et al., 1981; Meyer et al., 1991; Perkus et al., 1990). These mutants provide a unique opportunity to investigate specific virus/host interactions. The CHO host-range defect of laboratory strains of vaccinia virus (VV) can be complemented by the cowpox CHO hr gene, also called CP77 (Spehner et al., 1988). VV has a CP77 homolog but the open reading frame is interrupted in strain WR (Kotwal and Moss, 1988) and deleted in strain Copenhagen (Goebel et al., 1990). A spontaneous human host-range mutant VV, isolated by Drillien et al. (1981), contains a large deletion near the left end of the genome. Marker transfer experiments indicated that the human host-range defect could be overcome by insertion of the K1L gene, one of several open reading frames contained within the deleted DNA segment (Gillard et al., 1985, 1986). Subsequently, Perkus et al. (1990) discovered a functionally equivalent human host-range gene, C7L, within the same segment of deleted DNA but further showed that K1L was specifically required for replication in rabbit kidney (RK13) cells. Deletions of other Orthopoxvirus genes have produced host-specific effects that will not be considered here (Ali et al., 1994; Brooks et al., 1995; Takahashi-Nishimaki et al., 1991).

Initial host-range studies with the Copenhagen strain of VV in CHO cells demonstrated a block at an early stage of the replication cycle accomplished by rapid inhibition of host and viral protein synthesis (Drillien et al., 1978; Njayou et al., 1982). More recent investigations with the WR strain of VV indicated that synthesis of vaccinia viral early mRNA and proteins, DNA, and intermediate stage mRNAs occurred but neither intermediate viral proteins nor late mRNAs or proteins were made in CHO cells (Ramsey-Ewing and Moss, 1995). Ink et al. (1995) reported that apoptosis occurred in CHO cells infected with VV or VV recombinants expressing the cowpox CP77 gene but was delayed under the latter conditions. Relationships between apoptosis, inhibition of viral intermediate protein synthesis and host restriction remain to be determined, since only a minority of cells appear to undergo this type of cell death (Ramsey-Ewing, unpublished).

Drillien et al. (1981) reported that the VV mutant with the large deletion encompassing both K1L and C7L was blocked at an early stage of replication in human cells: cytoplasmic RNA synthesis and viral protein synthesis occurred transiently and cytoplasmic DNA synthesis was
greatly reduced. Both ectromelia virus and vaccinia virus with K1L deletions exhibited transient synthesis of viral proteins in RK13 cells (Chen et al., 1993; Sutter et al., 1994). Using the modified vaccinia virus Ankara (MVA) strain which has a truncated K1L gene (Meyer et al., 1991), Sutter et al. (1994) noted that viral DNA replication was blocked and that intermediate mRNAs were not made in RK13 cells, suggesting a difference from the host restriction phenotype obtained with the WR strain of VV in CHO cells. Although the MVA phenotype in RK13 cells was reversed by expression of the K1L gene, MVA has multiple deletions and remains host restricted for other mammalian cell lines even after restoration of K1L, invalidating direct comparisons with more standard VV strains such as WR or Copenhagen.

Perhaps the most remarkable feature of the host-range genes CP77, K1L, and C7L is their apparent functional equivalence despite the absence of any evident sequence similarities. Perkus et al. (1990) discovered that: (i) C7L is functionally equivalent to K1L for replication in human cells; (ii) C7L can be distinguished from K1L by its inability to replace K1L for replication in RK13 cells; (iii) CP77 can substitute for K1L in RK13 cells; and (iv) K1L, C7L, or CP77 allows replication of VV in pig kidney cells. The efficiency of complementation, however, cannot be deduced from this initial report, since only the plaque titers in various cell lines were reported with no information regarding plaque sizes or virus yields. Virus yield experiments of Oguiura et al. (1993) confirmed that K1L and C7L were functionally equivalent in human cells, although in some lines the K1L–C7L gave higher yields than the K1L–C7L+ . They also confirmed that C7L could not replace K1L in RK13 cells and discovered that both K1L and C7L were required in rat NRK cells and that C7L was specifically required in hamster Dede cells. The functional relationship of CP77 with the other host-range gene, that is, K1L, was not evaluated in the latter study.

We considered that further experimental work was needed to more precisely determine the stage at which a specific K1L–VV mutant is blocked in RK13 cells and the ability of the CP77 gene to relieve this defect. In the present investigations, we compared viral replication and gene expression in RK13 cells infected with recombinant VV (strain WR) that contained reporter genes and were K1L–CP77− (wild type), K1L–CP77−, or K1L–CP77+. A preliminary report of this work was presented at the 14th Annual Meeting of the American Society for Virology, 1995.

**MATERIALS AND METHODS**

Cells and viruses

BS-C-1 cells (kidney, African green monkey) and CV-1 (kidney, African green monkey) cells were grown in minimum essential medium (MEM) supplemented with 2.5% fetal calf serum (FCS). For guanine phosphoribosyltransferase (gpt) selection, viruses were isolated by plaqueing on BS-C-1 monolayer cultures in the presence of 25 μg/ml mycophenolic acid (MPA), 250 μg/ml xanthine, and 15 μg/ml hypoxanthine as described (Falkner and Moss, 1988). RK13 cells were grown in MEM supplemented with 10% FCS. CHO cells were grown in Ham's F-12 medium supplemented with 5% FCS. HuTK− 143 (osteosarcoma, human) cells were grown in MEM supplemented with 10% FCS and recombinant viruses selected in the presence of 25 μg of 5-bromodeoxyuridine per milliliter. VV strain WR and recombinants thereof were propagated and plaque assayed as described (Earl and Moss, 1991).

**Recombinant viruses**

Protocols for construction of recombinant VV by homologous recombination at the thymidine kinase (tk) locus have been published (Earl and Moss, 1991). For recombinant viruses in this study, we used a dual reporter plasmid (pREβCAT) with TK flanking sequences in which the cat gene, encoding chloramphenicol acetyltransferase (CAT), is regulated by a viral early promoter (virus growth factor, VGF), and the lacZ gene, encoding β-galactosidase (βGAL), is regulated by a viral late promoter (11K structural protein) (Ramsey-Ewing and Moss, 1995). The pREβCAT plasmid was then coprecipitated with VV WR viral DNA and transfected into VV WR-infected CV-1 cells producing the recombinant virus vK1L−CP77− in which the dual reporter cassette is recombined into the tk locus (Fig. 1). To prepare the K1L− knockout virus, we first reassembled the K1L gene of VV strain WR from two plasmids obtained from M. Merchlinsky, pHindIIIck and pHindIIIM, that contain the HindIII K and M fragments of VV strain WR, respectively. Plasmid pHindIIIM was digested with HindIII and EcoRV and the resultant 847-bp fragment containing the 5′ end of the K1L gene was isolated. Plasmid pHindIIIM was digested with HindIII and SphiI and the resultant 1506-bp fragment containing the coding region and 3′ end of the K1L gene was isolated. The K and M fragments were both subcloned into pUC19 that had been linearized with Smal and SphiI. The resultant plasmid, pREK1L, was digested with BgIII and recircularized to produce plasmid pREK1L−, which contains a K1L gene from which the major portion of the coding region (amino acids 1 thru 253) is deleted. A BgIII–gpt–BgIII cassette, in which the Escherichia coli gpt gene is regulated by the VV early promoter (7.5K) and followed by a VV early termination signal, was prepared in a recombinant PCR reaction using plasmid pGEMgpt (Blasco et al., 1991) as template. The isolated fragment was digested with BgIII and ligated to pREK1L− that had been linearized with BgIII to produce plasmid pK1Lgpt. To construct recombinant virus vK1L−CP77−, plasmid pK1Lgpt was used as a donor in homologous recombination with vK1L−CP77+. The resultant recombi-
niant virus, vK1L−CP77+, was isolated as a TK− βGAL+ MPA-resistant plaque. To construct the vK1L+CP77+ and vK1L−CP77+ recombinant viruses, we first subcloned the 2.3-kb EcoRI/PstI fragment derived from pEA36 (Sperhac et al., 1988), which contains the entire CHO hr gene encoding the CP77 protein, into the EcoRI/PstI sites of pUC19. The resultant plasmid, pRECP77, was used as a donor in homologous recombination with either vK1L+CP77+ or vK1L−CP77+ to create vK1L+CP77+ (Ramsey-Ewing and Moss, 1995) and vK1L−CP77+, respectively (Fig. 1). In addition to the dual reporter cassette at the tk locus, recombinant viruses vK1L+CP77+ and vK1L−CP77+ have an intact copy of the CHO hr gene instead of the disrupted WR copy (Kotwal and Moss, 1988) at the corresponding region of the HindIII C fragment.

Analysis of viral genome replication

RK13 cells were infected with recombinant viruses at a multiplicity of 10 plaque-forming units (PFU)/cell. At various times, cells were washed twice and scraped in phosphate-buffered saline (PBS). The cell pellet was resuspended in lysis buffer [20 mM Tris–HCl (pH 8.0), 10 mM EDTA, 0.75% sodium dodecyl sulfate (SDS), 570 mg/ml proteinase K] and viral DNA was isolated by phenol/chloroform extraction and ethanol precipitation as described (Earl and Moss, 1991). Viral DNA samples were applied to a nylon filter using a slot blot apparatus as described by the manufacturer (Promega). Either equal volumes of lysates or equal amounts of protein were used in βGAL assays as described by the manufacturer (Promega). Standard protein and enzyme activity curves were prepared for quantitative analysis of assay results.

For in situ analysis of βGAL expression, RK13 cells were infected with serial dilutions of recombinant virus stocks. At 24 hpi, infected cell monolayers were washed twice with PBS and then fixed in 1% gluteraldehyde in 0.1 M sodium phosphate (pH 7.0), 1 mM magnesium chloride for 15 min at room temperature. Assay buffer (0.2% 5-bromo-4-chloro-3-indolyl-β-d-galactosidase (X-Gal) in 10 mM sodium phosphate (pH 7.0), 1 mM magnesium chloride, 150 mM sodium chloride, 3.3 mM potassium ferricyanide, 3.3 mM potassium ferricyanide trihydrate) was added and the plates were incubated an additional 12 to 16 hr at 37°C. Plates were washed several times and individual representative plaques photographed under PBS.

Analysis of viral protein synthesis

RK13 cells (5 × 10^5) were seeded into 24-well plates; at 16 to 24 hr, the cells were infected with recombinant viruses at a multiplicity of 30 PFU/cell. From 10 to 20 min before each labeling period, cells were washed twice and incubated in prewarmed medium without methionine. The cells were then incubated in the presence of 75 µCi of [35S]methionine in 0.25 ml of methionine-free medium for 30 min. The labeling medium was removed and the cells were washed twice with ice-cold PBS and then incubated at 37°C for 3 to 5 min with hypotonic lysis buffer [20 mM Tris–HCl (pH 8.0), 10 mM NaCl, 0.5% Nonidet P-40 (NP-40)]. The lysate was collected and centrifuged for 2 min at 12,000 g to pellet nuclei. The supernatants containing 35S-labeled polypeptides were stored at −20°C. A portion of each sample was mixed with an appropriate volume of SDS/2-mercaptoethanol sample buffer and boiled for 5 min. The samples were resolved by electrophoresis in SDS 10% polyacrylamide gels.

Western blot analysis

Pellets from 10^5 infected cells were incubated with 100 ml lysis buffer [20 mM Tris–HCl (pH 7.0), 0.5% Triton X-100 in PBS] for 5 min at 37°C. Lysates were centrifuged at 14,000 g for 5 min and the supernatants stored at −20°C. A portion was mixed with 5× SDS/2-mercaptoethanol sample buffer (5’ to 3’, Inc.), boiled for 5 min, and proteins were resolved in SDS 10% polyacrylamide gels. Proteins were electrotransferred to nitrocellulose membranes and incubated with polyclonal antiserum to vaccinia virus at 1:500 overnight at 4°C and then 125I-protein A overnight at 4°C.

Immunoprecipitation

Cells were infected with recombinant viruses at a multiplicity of 30 PFU/cell, labeled with [35S]methionine as described above, and harvested at various times. Lysates were prepared in isotonic lysis buffer [50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, 100 mg/ml phenylmethylsulfonyl fluoride, 1% NP-40], centrifuged to remove nuclei, and then incubated with polyclonal antiserum to vaccinia virus at 1:500 dilution at 4°C overnight. An equal volume of 20% Protein A–Sepharose beads in PBS was added and incubation continued at room temperature for an additional 2 to 3 hr or at 4°C overnight. Immune complexes were washed twice in Triton buffer [300 mM NaCl, 50 mM Tris–HCl (pH 7.4), 0.1% Triton X-100]. Proteins from immunoprecipitation reactions were recovered by boiling in sample buffer and then..
resolved by electrophoresis in SDS 10% polyacrylamide gels.

Preparation and analysis of viral RNA

Viral RNA was isolated from cells that had been infected with recombinant viruses at a multiplicity of 30 PFU/cell. At various times after infection, the cells were washed twice in ice-cold PBS and then pelleted. Approximately 10^6 infected cells were lysed in 100 μl of Direct Protect lysis buffer and aliquots of the lysates added to 50-μl hybridization reactions and processed as specified by the manufacturer (Ambion). Single-stranded 32P-labeled antisense RNA probes (Riboprobes) were prepared by in vitro transcription of linearized plasmid or PCR-generated DNA templates as described by the manufacturer (Promega). Probes used in these studies were transcribed from the following templates: CAT and βGAL, plasmids pTRI-cat and pTRI-βGAL (Ambion); and A1L, plasmid pGEM17K (Baldick et al., 1992).

RESULTS

Construction of recombinant vaccinia viruses

To facilitate comparisons, the recombinant viruses used in these studies (Fig. 1) had a common WR genome and contained two reporter genes in the tk locus, cat and lacZ under early (VGF) and late (11K) promoters, respectively. In two viruses, an intact CP77 gene replaced the original interrupted homolog. These CP77 recombinant viruses were isolated by passage in CHO cells ensuring their functionality; in addition, PCR copies of the gene were sequenced. In two viruses, the K1L gene was almost entirely deleted by insertion of a gpt selectable marker. Similar titr stocks of the four recombinant viruses K1L-CP77-, K1L-CP77+, K1L-CP770, and K1L-CP77+ were prepared in BS-C-1 cells which are permissive for all of them.

Cell-dependent replication of recombinant vaccinia viruses

To examine the functional relationship between the K1L and CP77 genes, we infected BS-C-1 or RK13 cells with individual recombinant viruses and stained the plaques by an in situ βGAL assay 24 hr later. The staining assay depends on late expression of the lacZ gene and can measure virus spread even in the absence of strong cytopathic effects. The recombinant viruses generated plaques of similar size and staining intensity on BS-C-1 cells indicating that neither K1L nor CP77 was required (Fig. 2). On RK13 cells, however, K1L-CP77- and K1L-CP770 gave similar-size stained plaques, K1L-CP77 gave no plaques, and K1L-CP77+ gave small stained plaques (Fig. 2). In Fig. 2, the photographs of the RK13 cells were enlarged fourfold relative to the BS-C-1 cells in order to better show the small K1L-CP77+ plaques. These results indicated that CP77 compensated for the absence of K1L but that virus spread, as revealed by plaque formation, was inefficient under these conditions.

To quantitate virus replication, RK13 cells were infected with recombinant viruses and the yields were determined at 12, 24, 48, and 72 hr (Fig. 3). The growth curves of K1L-CP77+ and K1L-CP770 were virtually superimposable, indicating that the CP77 gene does not provide an enhancing effect when the K1L gene is functional. By contrast, the presence of the CP77 gene enhanced the replication of K1L- virus by 1 to 2 logs (Fig. 3). Replication of K1L-CP77+, however, was delayed and never reached the level of K1L- viruses. By contrast, the 24-hr yields of K1L-CP77-, K1L-CP770, and K1L-CP77+ were identical in BS-C-1 cells, indicating the cell-specific nature of the mutations (Table 1). The functionality of the CP77 gene in K1L-CP77 was demonstrated by a 2- to 3-log increase in virus titer, compared to the corresponding CP77- virus in CHO cells (Table 1). We concluded that the CP77 gene enhanced replication of a K1L-VV in RK13 cells but to a lesser extent than in CHO cells.

The effect of host-range genes on cell viability was determined using a trypan blue exclusion assay. The data indicated a correlation between loss of viability and virus replication (Table 1). Most significantly, viability of RK13 cells was less after infection with either K1L-CP77- or K1L-CP77+ compared to K1L-CP77-. The same correlation held true in CHO cells: viability was less after infection with K1L-CP77+ compared to either K1L-CP77- or K1L-CP77+.

Viral DNA replication in RK13 cells

VV DNA replication was measured by applying lysates of infected RK13 cells to a membrane which was then
hybridized with a labeled DNA probe. In cells infected with K1L+/CP77− or K1L+/CP77+ VV, the amount of viral DNA increased within 4 hr and thereafter (Fig. 4). In the case of K1L−CP77+, viral DNA was not above the 0 time level except perhaps for a small increase at 48 hr. In cells infected with K1L−CP77+ VV, viral DNA steadily increased after a lag of 8 to 16 hr. Both the lag period and the recovery of less viral DNA in the case of K1L−CP77+ VV than K1L+/CP77− or K1L+/CP77+ VV were consistent with the virus yield experiment. Control experiments demonstrated that the K1L gene was not required for DNA replication in CHO cells (data not shown).

A lag in DNA replication could arise from a delay in uncoating of the viral genome to provide the template or in formation of the replication apparatus. To distinguish between these alternatives, we took advantage of previous investigations that had demonstrated non-sequence-specific replication of transfected plasmid DNA by viral enzymes in VV-infected cells (DeLange and McFadden, 1986; Merchlinsky and Moss, 1988). The assay depends on the stringent preference of the DpnI restriction endonuclease for the methylated input plasmid DNA; replication is revealed by the accumulation of high-molecular-weight DpnI-resistant DNA. High-molecular-weight DpnI-resistant DNA accumulated between 4 and 8 hr after infection of RK13 cells with wild-type K1L−CP77+ VV (Fig. 5). The absence of such DNA after infection with K1L−CP77+ VV indicated a global replicative block. Significantly, DpnI-resistant DNA was synthesized between 12 and 24 hr after infection with K1L−CP77+ VV. The similar time of onset of replication of the viral genome and transfected plasmid suggested a delay in production of the replication apparatus rather than (or in addition to) uncoating of the viral genome.

Viral RNA synthesis in RK13 cells

VV gene expression can be divided into early, late, and intermediate phases regulated by stage-specific promoters and transcription factors (Moss et al., 1991). The viral DNA brought into the cell to initiate infection serves as the template for early transcription
prior to uncoating, whereas replicated viral DNA is the template for intermediate and late transcription. RK13 cells were infected with recombinant VV and steady-state RNA levels were determined by nuclease protection assays. The cat gene, regulated by the early VGF promoter, was chosen to measure early mRNA. Accordingly, a $^{32}$P-labeled antisense RNA probe was prepared. The nuclease protection data indicated that CAT mRNA peaked at 2 hr after infection then dramatically declined in wild-type (K1L$^{+}$CP77$^{-}$) VV-infected cells (Fig. 6). The reappearance of CAT RNA at later times is probably due to read-through late transcription, rather than virus spread to uninfected cells, since the virus multiplicity was 30. In cells infected with K1L$^{+}$CP77$^{-}$ VV, CAT RNA levels were also detected at 2 hr but persisted until 6 hr before declining, suggesting delayed shutoff of early transcription. The failure of CAT transcripts to reappear at late times can be attributed to a block in late transcription. A pattern similar to that of K1L$^{-}$CP77$^{-}$ and K1L$^{+}$CP77$^{-}$ was seen in cells infected with K1L$^{-}$CP77$^{-}$; CAT RNA persisted for 3 hr, declined, and then reappeared at later times.

We used an antisense RNA probe to the intermediate A1L gene (Keck et al., 1990) to measure intermediate transcripts. A1L RNA was detected at 4 hr after infection during wild-type (K1L$^{+}$CP77$^{-}$) VV infection of RK13 cells and remained at high levels (Fig. 6), in contrast to the more rapid decline previously seen in synchronously infected HeLa suspension cells (Baldick and Moss, 1993). During infection with K1L$^{-}$CP77$^{-}$ VV, only a minute amount of A1L mRNA was detected at 24 hr. By contrast, in RK13 cells infected with K1L$^{+}$CP77$^{-}$, A1L RNA was readily detected at 12 hr and increased in amount at later times (Fig. 6). The delay in intermediate mRNA, compared to K1L$^{+}$CP77$^{-}$, follows the difference in kinetics of DNA replication.

The lacZ reporter gene, regulated by the VV 11K promoter was used to measure late transcription. Late $\beta$GAL transcripts were present between 6 and 24 hr after wild-type (K1L$^{+}$CP77$^{-}$) infection of RK13 cells, but were not detected during infection with K1L$^{+}$CP77$^{-}$ (Fig. 6). In RK13 cells infected with K1L$^{+}$CP77$^{+}$, $\beta$GAL RNA accumulated between 12 and 24 hpi.

### TABLE 1

<table>
<thead>
<tr>
<th>Virus</th>
<th>RK13</th>
<th>CHO</th>
<th>BS-C-1</th>
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<tr>
<td></td>
<td>Yield$^a$</td>
<td>Viability$^b$</td>
<td>Yield</td>
</tr>
<tr>
<td>K1L + CP77$^{-}$</td>
<td>$7 \times 10^8$</td>
<td>22</td>
<td>$5 \times 10^5$</td>
</tr>
<tr>
<td>K1L + CP77$^{+}$</td>
<td>—</td>
<td>25</td>
<td>—</td>
</tr>
<tr>
<td>K1L - CP77$^{-}$</td>
<td>$9 \times 10^3$</td>
<td>68</td>
<td>$3 \times 10^5$</td>
</tr>
<tr>
<td>K1L - CP77$^{+}$</td>
<td>$2 \times 10^5$</td>
<td>24</td>
<td>$1 \times 10^6$</td>
</tr>
</tbody>
</table>

*a Virus yield after 24 hr (PFU/ml).

*b Cell viability after 24 hr (percentage).
Viral protein synthesis in RK13 cells

For initial experiments, we took advantage of the late promoter-regulated lacZ gene to compare βGAL synthesis in RK13 cells infected with the recombinant viruses. In cells infected with K1LCP77 VV, βGAL activity was detected by 8 hr and progressively increased over a 24-hr period (Fig. 7). By contrast, βGAL activity remained at nearly background levels up to 24 hr after infection with K1LCP77 VV. When RK13 cells were infected with K1LCP77 VV, βGAL synthesis was considerably delayed and the activity at 24 hr was substantially less than that in cells infected with vK1LCP77 in accordance with the measurements of βGAL RNA in the previous section.

Metabolic pulse-labeling followed by SDS–PAGE was carried out to obtain a global view of protein synthesis in VV-infected cells. When RK13 cells were infected with the wild-type virus (K1LCP77), the expected pattern of viral polypeptide synthesis was observed by autoradiography (Fig. 8A). During the first few hours of infection, several bands increased in intensity, suggesting that they are viral early proteins. Viral late proteins were dominant from 3.5 hr on. In contrast, when RK13 cells were infected
FIG. 8. Analysis of metabolically labeled viral proteins. RK13 cells were incubated with 30 PFU/cell of K1L CP77− (A), K1L CP77− (B), or K1L CP77+ (C, D) for 30 min. At the end of this incubation period, the cells were pulse-labeled with [35S]methionine for 30 min (0 time) and at the subsequent indicated hours after infection. The cells were lysed and radiolabeled proteins analyzed by SDS–PAGE. The positions of protein standards of indicated molecular mass (kDa) are indicated on the left of the autoradiogram. Putative early proteins are indicated by dots.

with either K1L CP77− VV (Fig. 8B) or K1L CP77+ VV (Fig. 8C), a short period of early protein synthesis was followed by a dramatic reduction of viral and host protein synthesis, so that by 2 hr little metabolic labeling was evident. In RK13 cells infected with K1L CP77− VV, protein synthesis never recovered, whereas a remarkable recovery occurred by 24 hr in cells infected with K1L CP77+. A closer examination of the recovery period revealed that protein synthesis resumed at about 9 hpi, at which time the pattern of bands was similar to that just prior to shutoff and appeared to include some early proteins (Fig. 8D). By 12 hr, the dominant late pattern of proteins was present.

Western blot analysis with rabbit antiserum against vaccinia virus was carried out to compare the accumulation of viral proteins synthesized in RK13 cells infected with the different recombinant VV and to rule out any labeling artifacts due to changes in amino acid transport or pool sizes. In addition, it is difficult to distinguish between cellular proteins and early viral proteins by metabolic labeling. With RK13 cells infected with wild-type (K1L CP77−) VV, only early viral proteins were detected at 1 to 2 hr and late proteins accumulated between 4 and 24 hr (Fig. 9). In cells infected with K1L CP77+ VV, the late protein pattern was established between 12 and 24 hr, whereas that pattern did not occur in cells infected with K1L CP77− VV (Fig. 9).

Recovery of viral early gene expression mediated by the CP77 gene product

The data thus far suggested that the absence of the K1L gene product imposed a premature arrest of transla-
FIG. 9. Western blot analysis. RK13 cells were infected with K1L-CP77-, K1L-CP77+, or K1L-CP77+ VV at a multiplicity of 30 PFU/cell and harvested at the indicated hours after infection. Detergent lysates were electrophoretically resolved by SDS-PAGE, transferred to nitrocellulose membrane, and then incubated with anti-VV polyclonal antiserum at a 1:500 dilution. Antibody binding was visualized by autoradiography of the membranes after exposure to 125I-protein A. Early proteins are indicated by dots.

Discussion

Over the years, several investigators noted defects in protein synthesis during abortive replication of Orthopoxvirus host-range mutants in a variety of cell lines (Brown and Moyer, 1983; Chen et al., 1992; Drillien et al., 1978; Moyer et al., 1980; Njayou et al., 1982; Sutter et al., 1994). Evaluation of these results is difficult because of varied genetic backgrounds of the Orthopoxviruses, some having multiple deletions, and because many of the studies preceded present realization of the complexity of Orthopoxvirus gene regulation. In the most detailed analysis to date, we reported that in CHO cells infected with VV (strain WR) viral early mRNAs, early proteins, DNA, and intermediate mRNAs were made, but that synthesis of intermediate proteins, late mRNAs, and late proteins did not occur (Ramsey-Ewing and Moss, 1995). The presence of intermediate mRNAs but not intermediate proteins led us to consider a translational block. We have now carried out a similar analysis of the host-range defect in RK13 cells infected with a K1L deletion mutant of VV (strain WR). While metabolic labeling studies indicated a block in intermediate and late protein synthesis, as occurs in CHO cells, further experiments revealed an important difference. Host-range restriction in RK13 cells prevented viral DNA replication and synthesis of intermediate mRNAs. However, early mRNAs persisted despite the cessation of early protein synthesis, again suggesting a block in translation.

The different stages of host restriction in RK13 and CHO cells might be explained if early and intermediate mRNAs contained unique structural elements that serve as targets for translational inhibition. No early- or intermediate-specific sequences have been noted in either the coding or 3'-terminal sequences of VV mRNAs; moreover, reporter genes regulated by early or late promoters exhibit the same host-range expression defects as authentic VV mRNAs. There may, however, be a difference in the 5' ends of most early and intermediate mRNAs. The three intermediate mRNAs examined by Baldick and Moss (Baldick and Moss, 1993) had a 5' poly(A) leader, presumably formed by VV RNA polymerase slippage on
FIG. 10. Immunoprecipitation of metabolically labeled early viral proteins. RK13 cells were infected with K1L’CP77-, K1L’CP77-, or K1L’CP77+ VV at a multiplicity of 30 PFU/cell in the presence of AraC. At the indicated hour after infection, the cells were labeled with [35S]methionine for 30 min and then lysed. Radiolabeled proteins were incubated with anti-VV polyclonal antiserum. Immune complexes were captured with Protein A-Sepharose beads. The bound proteins were washed, eluted by boiling in SDS sample buffer, and analyzed by SDS-PAGE. An autoradiogram is shown; early proteins are indicated by dots.

the TAAA sequence at the RNA start site, similar to that suggested for the poly(A) leader on late mRNAs (Davison and Moss, 1989b; Stunnenberg et al., 1989). Although more intermediate mRNAs need to be analyzed, the feature will likely be a common one since the TAAA is an important promoter motif (Baldick and Moss, 1993). By contrast, only a few early mRNAs have a 5′ poly(A) leader (Ahn et al., 1990; Ink and Pickup, 1990) and the TAAA is not an early promoter element (Davison and Moss, 1989a). The function of the the 5′ poly(A) leader has not been determined although a role in translational initiation has been suggested (Schnierle and Moss, 1992). An alternative possibility to explain the host-range differences is that early or intermediate mRNAs per se are not targeted but that the effects are manifested at early or intermediate times after infection. Thus, a translational defect at early times will effect early mRNA and a translational effect at intermediate times will effect intermediate mRNAs just because they are present.

The host-range defect in CHO cells can be overcome by inserting the cowpox virus CP77 gene into the tk locus of VV (strain Copenhagen) which entirely lacks the homologous gene (Spehner et al., 1988) or by repairing the homologous gene of VV (strain WR) which has multiple frame-shifts (Ramsey-Ewing and Moss, 1995). Perkus et al. (1990) reported that the CP77 and K1L genes are functionally equivalent with regard to replication of the Copenhagen strain of VV in RK13 cells. We were especially curious about this result since the genes have no evident sequence similarity and our data indicated that the steps at which host restriction occurs in CHO cells and RK13 cells are different. If CP77 specifically enhances the translatability of intermediate stage mRNAs, then it would be difficult to understand how the block to translation of early mRNAs in RK13 cells is overcome.

Using recombinant VV derived from the WR strain, we confirmed the ability of the CP77 gene under its native promoter to permit replication of a K1L deletion mutant in RK13 cells. However, plaques formed in RK13 cells within 24 hr by K1L’CP77+ VV were much smaller than those formed by K1L’CP77− and the virus yield also was considerably less. Despite this difference, K1L’CP77+ VV grew as well in BS-C-1 cells as wild-type virus and the yield of K1L’CP77+ VV was nearly 3 logs higher than that of wild-type virus in CHO cells. In addition, the sequence of a PCR copy of the CP77 gene, from K1L’CP77+ VV, was verified. Next, we compared the synthesis of viral DNA, RNA, and proteins in RK13 cells infected with wild-type (K1L’CP77−), K1L’CP77−, and K1L’CP77+ VV. For the first 6 to 12 hr after infection, the presence of the CP77 gene in the K1L deletion mutant had no detectable effect; cessation of viral and cellular protein synthesis occurred at the same time and to the same extent. Unfortunately, we have not yet obtained suitable antiserum to measure the synthesis of the CP77 gene product during this period. The first detectable effect of the CP77 gene was the resumption of early protein synthesis at 8 to 10 hr after infection. DNA replication occurred later, suggesting either that the genomic template had to be uncoated or that the replication machinery had not yet been made or assembled. The latter interpretation was supported by finding a similar delay in the replication of naked plasmid DNA transfected into RK13 cells infected with K1L’CP77+ VV. Following DNA replication, intermediate and late mRNAs and proteins were made.

Both pulse-labeling and Western blotting suggested that the remarkable resumption of protein synthesis in RK13 cells infected with K1L’CP77+ VV started with early species. This phenomenon was verified by carrying out the infection in the presence of an inhibitor of DNA replication. Under these conditions, synthesis of viral early proteins appeared to stop only to start again several hours later, consistent with a translational block that is eventually overcome even without DNA replication. The explanation as to why K1L permits
a smooth transition through the early, intermediate, and late stages of VV gene expression whereas protein synthesis is temporarily suspended when K1L is replaced by CP77 is most intriguing. The CP77 product may simply accumulate less rapidly or be less potent than the K1L gene product. These possibilities might be tested by increasing the level of CP77 expression. Alternatively, since K1L is neither needed for VV replication in CHO cells nor can compensate for CP77 in that system, the targets or actions of the two proteins may differ. In addition, both K1L and CP77 may have multiple roles as the former has been implicated in activating viral transcription (Rosales et al., 1994) and the latter in preventing apoptosis (Ink et al., 1995).

It seems likely that these host-range proteins are interacting with cellular regulators of complex metabolic pathways and that further studies will be illuminating.

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