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Restriction of Vaccinia Virus Replication in CHO Cells Occurs at the Stage of Viral Intermediate Protein Synthesis

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Vaccinia virus (VV) and cowpox virus (CPV) differ in their abilities to replicate in Chinese hamster ovary (CHO) cells because VV has a disrupted host range (*hr*) gene. To facilitate an examination of the molecular events associated with abortive infection of CHO cells with VV, we constructed two sets of recombinant viruses that contain a viral early promoter regulating the *cat* gene encoding chloramphenicol acetyltransferase and viral intermediate or late promoters regulating the *lacZ* gene encoding β-galactosidase. The first set has the disrupted *hr* gene and the second set has the intact CPV homolog, allowing replication in CHO cells. Reporter chloramphenicol acetyltransferase and β-galactosidase assays demonstrated that early gene expression was unperturbed, whereas intermediate and late gene expression were severely inhibited under abortive conditions. Metabolic labeling studies confirmed the absence of viral late protein synthesis. The accumulation of viral DNA under abortive conditions was consistent with the synthesis of viral early proteins and established that inhibition of late protein synthesis was not primarily due to a replicative block. Analysis of steady state levels of viral mRNAs revealed substantial quantities of early and intermediate species but only very small amounts of late mRNAs under nonpermissive conditions. Despite the presence of viral intermediate mRNAs, the corresponding intermediate proteins, which function as late transcription factors, were not detected by immunoprecipitation of lysates from metabolically labeled infected CHO cells. Furthermore, when expression of *lacZ* was regulated by an intermediate promoter, no β-galactosidase was detected even though *lacZ* transcripts were present. Thus, the abortive phenotype in CHO cells can be explained by a block to translation of intermediate mRNAs which prevents the synthesis of late transcription factors.

INTRODUCTION

Vaccinia virus (VV) can replicate in a broad range of mammalian and avian cells. Chinese hamster ovary (CHO) cells are not, however, permissive for VV replication (Drillien *et al.*, 1978; Hruby *et al.*, 1980). The discovery of this phenomenon was followed by other examples of host range restriction. To date, at least three genes have been identified that play roles in VV host range. When recombined into the VV genome at the thymidine kinase (*tk*) locus, a cowpox virus (CPV) gene (CHO *hr*) encoding a 77-kDa protein (CP77) allows replication of VV in CHO cells (Spehner *et al.*, 1988). This CHO host range (*hr*) gene is disrupted in (Kotwal and Moss, 1988) and entirely absent from (Goebel *et al.*, 1990) VV strains WR and Copenhagen, respectively. Similarly the CHO *hr* open reading frame (ORF) is disrupted in ectromelia virus (Chen *et al.*, 1992), another member of the orthopoxvirus genus. The K1L ORF of VV is required for productive infection of rabbit and pig kidney cells and also human cells under certain conditions (Gillard *et al.*, 1985, 1989; Perkus *et al.*, 1989, 1990). The C7L ORF of VV can substitute for the K1L ORF for productive infection of some human cells and pig kidney cells, but not rabbit kidney cells (Perkus *et al.*, 1990). Despite the functional relationship of the three host range genes, they have no evident sequence similarity.

Although some viral genes involved in host range restriction have been identified, the molecular events that occur during abortive infections have not been precisely characterized. The original description of the failure of VV to replicate in CHO cells included an examination of its relationship to the shutoff of host protein synthesis (Drillien *et al.*, 1978). The authors described an abrupt cessation of both viral and host protein synthesis shortly after infection and suggested that this was the result of unregulated action of a viral early gene product. A subsequent analysis revealed changes in the gross uptake of [^{35}S]methionine and [^{14}C]uridine into acid-precipitable material, suggesting that viral and cellular proteins as well as cytoplasmic RNAs are synthesized to a lesser degree during abortive infection (Hruby *et al.*, 1980). More detailed investigation of the molecular basis of this host range restriction was not possible with the information and techniques available at the time.

Recent progress in delineation of the regulatory cascade that governs VV replication (reviewed in Moss, 1990) prompted a reexamination of the VV host range negative (HR−) phenotype in CHO cells. To determine the precise stage at which the host range defect is manifested, we examined individual steps in the regulation of VV replication during abortive and productive infections. Recombinant viruses that contain reporter genes driven

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by viral early, intermediate, or late promoters were used to quantitatively monitor protein synthesis during the course of virus infection. Control viruses containing the CPV CHO *hr* gene, as well as the reporter genes, were also constructed. We found that transcription of early genes, synthesis of early proteins, viral DNA replication, and transcription of intermediate genes all occurred under nonpermissive conditions. Translation of viral intermediate mRNAs and all subsequent events, however, were blocked.

MATERIALS AND METHODS

Cells and viruses

BS-C-1 cells and CV-1 cells were grown in minimal essential medium (MEM) supplemented with 2.5% fetal calf serum. CHO cells were grown in Ham's F-12 medium supplemented with 5% fetal calf serum. HuTK- 143 cells were grown in MEM supplemented with 10% fetal calf serum and infected with recombinant viruses in the presence of 25 μg of 5-bromodeoxyuridine per milliliter. VV strain WR and recombinants thereof were propagated as described (Earl *et al.*, 1991).

Recombinant virus construction

Protocols for insertion of foreign genes into the VV genome by homologous recombination at the *tk* gene locus have been published (Earl and Moss, 1991a). For construction of the vREβCAT and vRECP77 recombinant viruses, we first engineered the pREβCAT dual reporter donor plasmid. Recombinant polymerase chain reaction (PCR) was performed with pSV$_2$CAT (Gorman *et al.*, 1982) as a template and oligo ALR-1, which added the vaccinia virus growth factor (VGF) gene promoter to the chloramphenicol acetyltransferase (CAT) coding region. Oligonucleotide ALR-1 contained 5′ *Bam*HI–*Sal*I sites followed by VGF promoter sequences (Yuen and Moss, 1986) and then sequences corresponding to the 5′ coding sequences of the CAT open reading frame (ORF). The other primer, oligo ALR-2, provided the *Bam*HI site just 3′ to the end of the CAT ORF. The fragment generated by recombinant PCR, 5′*Bam*HI–*Sal*I–VGF promoter–CAT–*Bam*HI 3′, was subcloned into the *Bam*HI site of pMJ455 (Davison and Moss, 1989) adjacent to and in the opposite orientation of the resident F17R promoter-regulated *lacZ* ORF, resulting in a dual reporter cassette flanked by viral *tk* sequences. The pREβCAT plasmid was then coprecipitated with VV WR viral DNA and transfected into VV WR-infected CV-1 cells as described (Earl and Moss, 1991a). Recombinant virus vREβCAT was selected in HuTK- 143 cells in the presence of 5-bromodeoxyuridine and the plaques were stained with X-gal. After three rounds of plaque purification, virus stocks were prepared (Table 1). Recombinant virus vP30lacZ containing the *lacZ* gene regulated by the G8R intermediate promoter (Table 1) was previously described (Baldick *et al.*, 1992).

To construct the CHO HR+ vRECP77 and vP30CP77 recombinant viruses containing intact copies of the CP77 ORF instead of the disrupted VV copy, we first subcloned the 2.3-kb *Eco*RI/*Pst*I fragment derived from pEA36 (Spehner *et al.*, 1988), which contains the entire ORF encoding CPV CP77, into the *Eco*RI/*Pst*I sites of pUC19. The resultant plasmid, pRECP77, was used as a donor in homologous recombination with vP30lacZ (Baldick *et al.*, 1992) or vREβCAT to create vP30CP77 and vRECP77, respectively (Table 1). Recombinant HR+ viruses were selected by five passages in CHO cells then plaque purified in BS-C-1 cells.

One-step growth of vREβCAT and vRECP77

The vREβCAT and vRECP77 recombinant viruses were used to infect BS-C-1 or CHO cells at a multiplicity of 1 plaque forming unit (PFU) per cell. Progeny viruses were harvested at various times after infection and their titers determined by plaquing on BS-C-1 cells as described (Earl *et al.*, 1991).

Viral DNA replication

CHO and BS-C-1 cells were infected with recombinant viruses vREβCAT and vRECP77 at a multiplicity of 10 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 m*M* Tris–HCl (pH 8.0), 10 m*M* EDTA, 0.75% sodium dodecylsulfate (SDS), 570 μg/ml proteinase K] and viral DNA was isolated by phenol/chloroform extraction and ethanol precipitation as described (Earl and Moss, 1991b). Viral DNA samples were applied to a nylon filter using a slot blot apparatus as described by the manufacturer (Hoefer), and hybridized to vaccinia viral K1L ORF DNA sequences that had been labeled by random oligonucleotide priming using the labeling and Quikhyb kits as specified by the manufacturer (Promega).

Analysis of reporter gene expression

Cells were infected with recombinant viruses at a multiplicity of 30 PFU/cell. At various times after infection, the cells were washed twice with PBS, harvested in TEN [40 m*M* Tris–HCl (pH 7.5), 1 m*M* EDTA, 150 m*M* NaCl], centrifuged, and then resuspended in 0.25 *M* Tris–HCl (pH 8.0), followed by three rounds of freeze-thawing. The lysate was centrifuged and the supernatant retained for further analysis. Protein content of each lysate was determined colorimetrically using the Pierce Coomassie blue reagent. Either equal volumes of lysates or equal amounts of protein were used in CAT and beta-galactosidase (βGAL) assays as described by the manufacturer (Promega). Standard curves of both protein content and enzyme activity were prepared for quantitative analysis of assay results.

TABLE 1

Structural and Functional Properties of Recombinant Viruses

Virus	Promoter[a]/ reporter(s)	CHO hr gene	HR[b] phenotype
WR	NONE	–	HR–
vREβCAT	E/CAT;L/βGAL	–	HR–
vRECP77	E/CAT;L/βGAL	+	HR+
vP30lacZ	I/βGAL	–	HR–
vP30CP77	I/βGAL	+	HR+

[a] E, early; I, intermediate; L, late.
[b] HR, host range.

Analysis of viral protein synthesis

To examine viral protein synthesis, we seeded 5 × 10^5 permissive or nonpermissive cells in normal growth medium. After 16 to 24 hr, the cells were infected with recombinant viruses at a multiplicity of 30 PFU/cell. From 30 to 60 min before each labeling period, cells were washed twice and incubated in medium without methionine. The cells were then incubated in the presence of 75 μCi of [^{35}S]methionine in 250 μl 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 m*M* Tris–HCl (pH 8.0), 10 m*M* NaCl, 0.5% Nonidet P-40]. The lysate was then collected and centrifuged for 2 min at 12,000 *g* to pellet nuclei. The supernatants containing ^{35}S-labeled polypeptides were stored at −20°C. A portion of each sample was mixed with one-fifth volume of SDS/βME sample buffer (5′ to 3′, Inc.) and boiled for 5 min. The samples were resolved by electrophoresis in 10% SDS–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 PBS then lysed and the RNA extracted by the guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987) using RNAzol B (Tel-Test). The yield of total infected-cell RNA was typically 100 to 150 μg per 10^7 cells.

To quantitate the steady-state levels of viral mRNAs, a nuclease protection assay was performed using total infected-cell RNA and ^{32}P-labeled RNA probes. The DNA templates, *in vitro* synthesized RNAs and protected products were previously described (Keck *et al.*, 1990; Baldick *et al.*, 1992). Linearized plasmid DNA was transcribed by bacteriophage T7 RNA polymerase to generate ^{32}P-labeled RNA probes as described by the distributor of the enzyme (Promega). Total infected-cell RNA (10 μg) was hybridized to gene-specific RNA probes in 30 μl of hybridization buffer [40 m*M* PIPES (pH 6.4), 1 m*M* EDTA, 400 m*M* NaCl, 80% formamide] for 12 to 16 hr at either 37 or 45°C. Unhybridized RNA was digested at 37°C for 1 hr in 0.3 ml of digestion buffer [10 m*M* Tris–HCl (pH 7.5), 5 m*M* EDTA, 200 m*M* Na acetate] containing RNase ONE (Promega) at 17 to 25 units/ml for probes to VGF and G8R ORF transcripts or with RNase A and RNase T1 at 4 μg/ml and 10 units/ml, respectively, for probes to F17R, A1L, and *lacZ* ORF transcripts. RNA was precipitated directly from RNase ONE digestion mixes by the addition of 0.7 ml of cold absolute ethanol, while RNase A + T1 digestions were terminated by the addition of 5 μl of 10 mg/ml proteinase K and 20 μl of 10% SDS. After incubation at 37°C for 15 min, the undigested RNAs were phenol/chloroform extracted. Samples were electrophoretically resolved on 8% polyacrylamide gels containing 8 *M* urea.

Immunoprecipitation

Cells were infected with recombinant viruses at a multiplicity of 30 PFU/cell, labeled with [^{35}S]methionine as described above, and harvested at various times. Lysates were prepared in isotonic lysis buffer [50 m*M* Tris–HCl (pH 8.0), 150 m*M* NaCl, 0.02% sodium azide, 0.1% SDS, 100 μg/ml phenylmethylsulfonyl fluoride, 1% Nonidet P-40], centrifuged to remove nuclei, and then incubated with the indicated antiserum at a 1:10 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. Polyclonal rabbit antisera to the *Escherichia coli*-expressed products of the following viral ORFs were used: G8R, A2L, and A1L (Keck *et al.*, 1990). Immune complexes were washed once in Triton buffer [300 m*M* NaCl, 50 m*M* Tris–HCl (pH 7.4), 0.1% Triton X-100]. Proteins from immunoprecipitation reactions were recovered in Laemmli buffer then resolved by electrophoresis in 15% SDS–polyacrylamide gels.

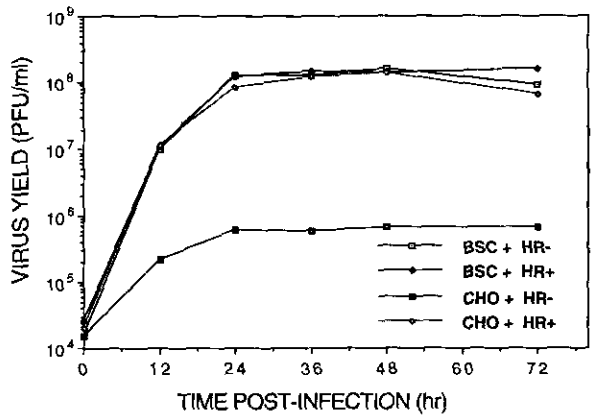

FIG. 1. Replication of recombinant viruses vREβCAT and vRECP77 in permissive and nonpermissive cells. BS-C-1 or CHO cells were infected with recombinant viruses vREβCAT or vRECP77 at a multiplicity of 1 PFU/cell. At the indicated times after infection, the cells were harvested and infectious titers determined by plaquing on BS-C-1 cells. HR–, vREβCAT; HR+, vRECP77.

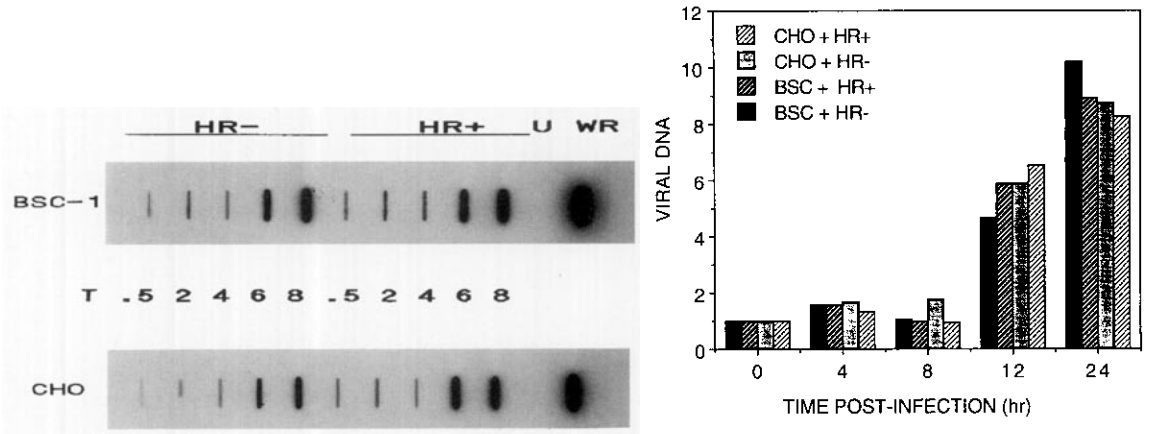

FIG. 2. Viral DNA replication under permissive and nonpermissive conditions. Permissive (CHO) and nonpermissive (BS-C-1) cells were infected with vREβCAT (HR−) or vRECP77 (HR+) at a multiplicity of 10 PFU/cell. Viral DNA was isolated from cells harvested at the indicated hours after infection, immobilized on a nylon membrane, and hybridized to a ^{32}P-labeled vaccinia virus K1L ORF DNA fragment. Control DNAs from uninfected cells (U) and wild type VV-infected cells (WR) were analyzed simultaneously. (Left) An autoradiogram is shown. (Right) Densitometric analysis of the autoradiogram expressed as fold increase over 0-time values.

RESULTS

Construction of recombinant vaccinia viruses carrying reporter and host range genes

To assess the level of expression of VV early, intermediate, and late genes, we constructed recombinant viruses in which the *cat* and *lacZ* genes were regulated by well characterized stage-specific viral promoters. In vREβCAT (Table 1), the early promoter regulating *cat* was derived from the VGF gene which is maximally expressed during the prereplicative phase of the VV life cycle (Yuen and Moss, 1986; Baldick *et al.*, 1993) and the late promoter regulating *lacZ* was derived from F18R ORF which encodes the 11-kDa structural protein (Bertholet *et al.*, 1985). In vP30lacZ (Baldick *et al.*, 1992; Table 1) the intermediate promoter regulating *lacZ* was derived from the G8R gene which encodes a late transcription factor (Keck *et al.*, 1990).

Neither vREβCAT nor vP30lacZ can replicate in CHO cells because the genomic region of VV strain WR corresponding to the CPV CHO *hr* gene contains several deletions and in frame stop codons (Kotwal and Moss, 1988). The disrupted CHO *hr* genes of vREβCAT and vP30lacZ were repaired by homologous recombination with the cloned CPV CHO *hr* gene, which contains approximately 100 bp of sequences 5′ of the initiation codon and 200 bp of 3′ untranslated sequences (Spehner *et al.*, 1988). The selection method employed was growth in nonpermissive CHO cells. Hybridization of a CHO *hr* gene probe to DNA from cells infected with plaque-purified recombinant VV revealed fragments of predicted length (data not shown). Complete sequence analysis of the CHO *hr* region in another recombinant VV isolated in a similar manner indicated that recombination had occurred 66 nucleotides 5′ of the initiation codon and at nucleotide 1329 within the conserved 3′ region at residue 444. The presence of an intact CHO *hr* gene in vRECP77 and vP30CP77 (Table 1) was consistent with the ability of these viruses to grow in CHO cells. Therefore, we refer to these recombinants as host range positive (HR+). Northern blot analysis revealed that transcription of the CHO *hr* gene in cells infected with vRECP77 was maximal at early times after infection (data not shown).

The CHO *hr* gene from CPV overcomes growth restriction of VV in CHO cells

Growth of vREβCAT (HR−) and vRECP77 (HR+) in BS-C-1 cells and CHO cells was compared. The virus yields at various times were determined by plaque assay. The HR+ recombinant VV replicated similarly in BS-C-1 and CHO cells (Fig. 1). In contrast, the HR− recombinant VV replicated well in BS-C-1 cells, but the virus yield was reduced by more than 99% in CHO cells. Although some replication of HR− virus was detected in CHO cells, an attempt to select spontaneous mutants capable of more efficient replication by continued passage was unsuccessful (data not shown) as previously reported (Drillien *et al.*, 1981). This strongly suggests that the CHO *hr* gene was directly responsible for conversion of the VV HR− phenotype to HR+ in CHO cells.

Defective growth of vaccinia virus in CHO cells is not due to a block in DNA replication

To determine whether viral DNA replication occurs during abortive infection of CHO cells by VV, we infected either BS-C-1 or CHO cells with recombinant viruses vREβCAT (HR−) or vRECP77 (HR+). Hybridization with a radioactive probe specific for VV sequences (K1L ORF) indicated that viral DNA replicated to similar extents under permissive or nonpermissive conditions (Fig. 2).

Viral late protein synthesis is blocked during abortive infection of CHO cells by vaccinia virus

Viral and cellular protein synthesis was monitored by *in vivo* metabolic labeling with [^{35}S]methionine and SDS−

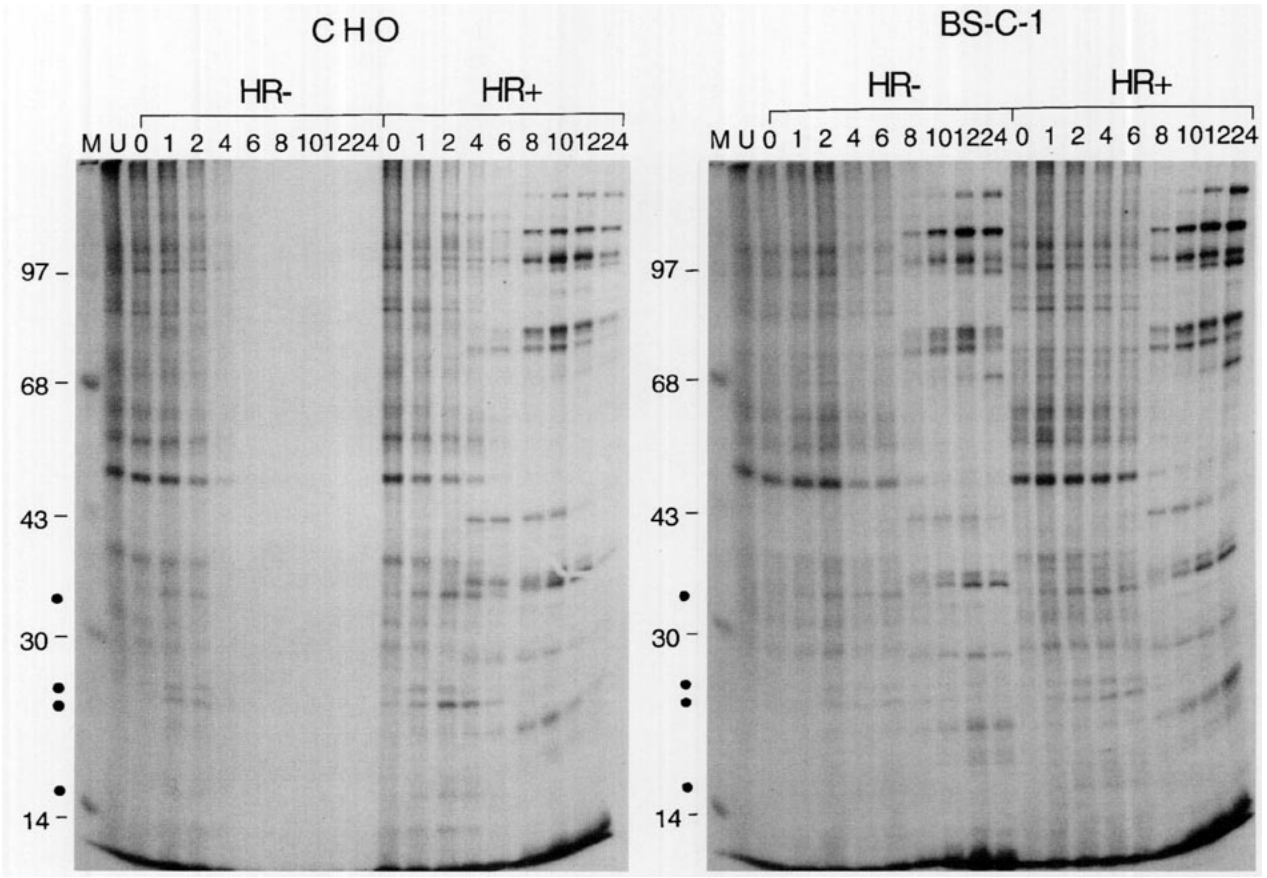

FIG. 3. Analysis of viral protein synthesis during productive or abortive infection. BS-C-1 or CHO cells were infected at a multiplicity of 30 PFU/cell with recombinant viruses vREβCAT (HR−) or vRECP77 (HR+). Infected cells were pulse-labeled with [^{35}S]methionine for 30 min starting at the indicated times after infection. The cells were lysed and labeled proteins analyzed by polyacrylamide gel electrophoresis. Polypeptides synthesized under both permissive and nonpermissive conditions are indicated by filled circles. The positions of protein standards of indicated molecular mass (kDa) are indicated on the left of the autoradiogram. The dots are adjacent to putative viral early proteins synthesized during abortive infection of CHO cells.

polyacrylamide gel electrophoresis (PAGE). When BS-C-1 cells were infected with either vREβCAT or vRECP77 recombinant viruses, we observed the anticipated temporal shift from cellular to viral protein synthesis (Fig. 3, right). Faint new bands representing early viral proteins were detected during the first 2 hr after infection. By 8 hr after infection, the predominant bands were viral late proteins, while synthesis of cellular proteins had essentially ceased. A similar labeling pattern was obtained when CHO cells were infected with the HR+ virus (Fig. 3, left). Viral early protein synthesis and shutoff of host protein synthesis occurred when CHO cells were infected with the HR− virus. However, no late proteins were labeled.

Cessation of host and viral protein synthesis during abortive infection does not require DNA replication

Metabolic labeling experiments suggested that CHO cellular protein synthesis was inhibited similarly by HR− and HR+ viruses even though the major viral late proteins were not detected in the latter case. This result, however, did not preclude a requirement for synthesis of minor undetected viral intermediate or late proteins for the cessation of CHO protein synthesis. Because intermediate and late genes are transcribed from replicated templates, infection of cells in the presence of the DNA synthesis inhibitor cytosine arabinoside (AraC) prevents the formation of intermediate and late viral proteins. We infected BS-C-1 or CHO cells with vREβCAT in the presence or absence of 40 μg/ml AraC and monitored protein synthesis by [^{35}S]methionine incorporation and SDS−PAGE (Fig. 4). The results indicated that the shutdown of cellular and viral protein synthesis occurred in the absence of DNA replication and thus does not require viral intermediate or late gene products.

Expression of reporter genes regulated by viral promoters during productive or abortive infection

To further examine and quantitate the molecular defect(s) associated with host range restriction of VV in CHO cells, we monitored expression of reporter genes at various times after infection with recombinant viruses. In recombinant viruses vREβCAT and vRECP77 the *cat* gene is regulated by an early promoter and the *lacZ* gene

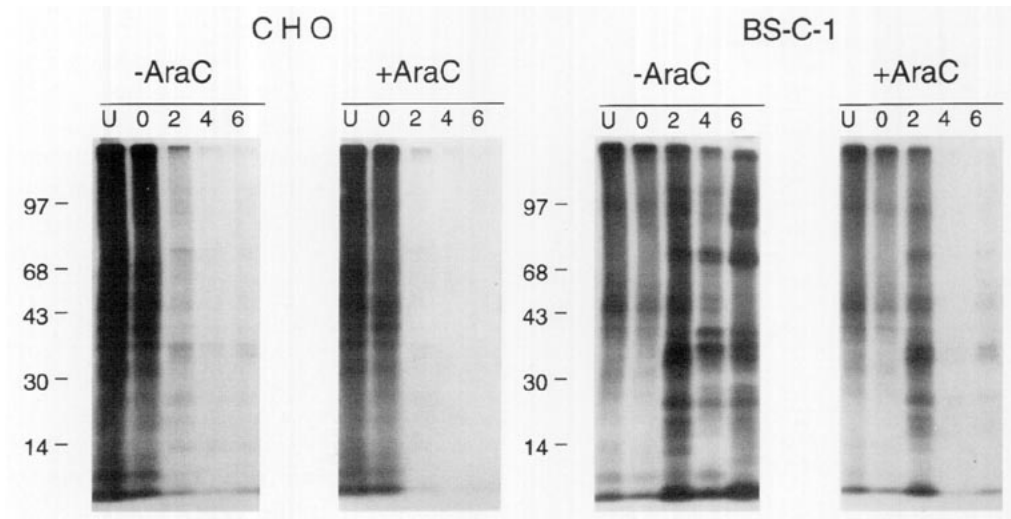

FIG. 4. Analysis of viral protein synthesis in the presence or absence of AraC. CHO and BS-C-1 cells were infected at a multiplicity of 30 PFU/cell with recombinant virus vREβCAT in the presence or absence of 40 μg/ml AraC. Infected cells were pulse-labeled with [^{35}S]methionine for 30 min starting at the indicated times after infection. The cells were lysed and labeled proteins analyzed by SDS–PAGE. The positions of protein standards of indicated molecular mass (kDa) are shown on the left of the autoradiogram.

is regulated by a late promoter. In this set of experiments CAT and βGAL activities were monitored in lysates of BS-C-1 and CHO cells infected with vREβCAT and vRECP77. The results indicated that the presence of the CHO *hr* gene made no significant difference in the level or kinetics of accumulation of CAT during abortive or productive infection (Fig. 5, top). By contrast, βGAL activity, which reflects late gene expression, was reduced to background levels under abortive conditions (Fig. 5, bottom). These results were consistent with [^{35}S]-methionine incorporation into viral proteins.

Viral intermediate proteins were not detected under nonpermissive conditions

In order to examine the status of viral intermediate gene expression under nonpermissive conditions, we infected CHO cells with vREβCAT or vRECP77. The synthesis of three intermediate proteins, 17, 26, and 30 kDa, encoded by ORFs A1L, A2L, and G8R, respectively, was monitored using polyclonal antisera for immunoprecipitation of lysates from metabolically labeled cells (Fig. 6). Under permissive conditions, the three proteins were synthesized at the highest rates between 4 and 12 hr after infection. Synthesis of the intermediate proteins continued at a diminished rate up to 24 hr after infection under these conditions. On the other hand, under nonpermissive conditions synthesis of the three intermediate proteins was not detected at any time up to 24 hr after infection.

Selective inhibition of late transcription under nonpermissive conditions

Because of the deficiency in viral intermediate and late protein synthesis under nonpermissive conditions, it was important to determine whether the corresponding mRNAs were present. Nuclease protection analysis was used to measure the levels of viral mRNAs representative of the three temporal gene classes, early, intermediate, and late. (Fig. 7). To assess early gene transcription, we employed a probe derived from the VGF gene. Transcripts of the VGF gene were present in similar amounts in CHO or BS-C-1 cells infected with HR+ (vRECP77) or HR– (vREβCAT) viruses. Two probes derived from the intermediate viral genes G8R and A1L were used to measure the level of viral intermediate mRNAs in CHO or BS-C-1 cells infected with HR+ (vRECP77) or HR– (vREβ-CAT). Similar levels of the intermediate mRNAs were detected under permissive or nonpermissive conditions. Therefore our inability to detect synthesis of intermediate proteins under nonpermissive conditions could not be attributed to the absence of intermediate mRNAs. The level of late gene mRNA was examined using a probe derived from the F18R late structural protein gene. In contrast to early and intermediate mRNAs, the level of late mRNA was specifically reduced when CHO cells were infected with HR– virus.

Expression of a reporter gene regulated by an intermediate promoter

To confirm the defect in translation of viral intermediate mRNAs, we infected BS-C-1 and CHO cells with vP30*lacZ* (HR–) or vP30CP77 (HR+), in which the *lacZ* gene is regulated by the G8R intermediate promoter. β-galactosidase assays indicated that intermediate reporter gene expression in CHO cells was dependent on the CHO *hr* gene (Fig. 8, top). However, similar levels of *lacZ* mRNA were present in CHO cells infected with HR+ or HR– virus (Fig. 8, bottom). Thus, expression of the intermediate promoter-regulated reporter gene mimicked that of viral intermediate genes.

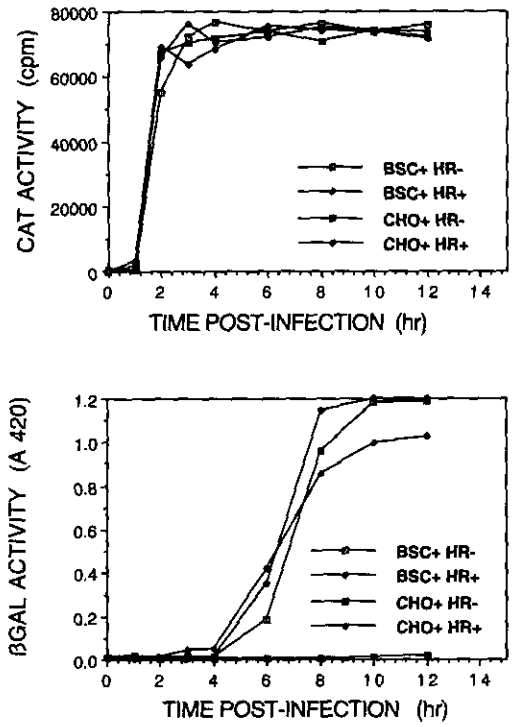

FIG. 5. Reporter gene expression under permissive and nonpermissive conditions. (Top) Viral early gene expression. CAT assays were performed on extracts prepared from BS-C-1 and CHO cells infected with vREβCAT or vRECP77. Cells were harvested at the indicated times after infection and the protein contents of extracts determined. CAT activity was quantitated by liquid scintillation counting. (Bottom) Viral late gene expression. Spectrophotometric βGAL assays were performed on extracts prepared from infected cells. BSC, BS-C-1 cells; CHO, CHO cells; HR−, vREβCAT; HR+, vRECP77; A 420, absorbance at 420 nm.

DISCUSSION

Replication of vaccinia virus requires successful progression through a series of temporally regulated steps. Viral early gene expression begins immediately after infection, since the enzymes and templates are contained within the virion. The products of viral early genes include *trans*-acting factors required for DNA replication and intermediate gene transcription. Concomitant with the onset of DNA replication is the expression of intermediate genes, which encode late gene transcription factors. Late gene products include structural proteins and early gene transcription factors, which are packaged in progeny virions. To complete the cycle, the early gene transcription factors are activated following infection of new cells.

Our studies point to the stage of intermediate viral protein synthesis as the first detectable molecular defect during host range restriction of VV in CHO cells. We found that: (1) viral early proteins and mRNAs were synthesized, (2) viral DNA replication occurred, (3) viral intermediate mRNAs were produced, (4) viral intermediate proteins were not detected, (5) viral late mRNAs were present in low amounts and late proteins were not detected.

In a previous study, a decreased rate of total DNA synthesis in infected cells was determined by thymidine incorporation into radiolabeled acid-precipitable material (Hruby *et al.*, 1980). When accumulation of viral DNA was directly measured by slot blot analysis, we found substantial quantities under nonpermissive conditions, excluding a replicative block as the major defect. Our observation that DNA replication occurred during abortive infection is consistent with (1) the synthesis of viral early proteins which include requisite factors for DNA replication, and (2) the synthesis of intermediate mRNAs, which is dependent upon DNA replication.

Examination of overall protein synthesis patterns in infected cells revealed that both cellular and viral protein synthesis ceased within 6 hr after abortive infection. We detected the synthesis of several viral early proteins, but no late proteins, in agreement with the results of others (Hruby *et al.*, 1980; Drillien *et al.*, 1978). While the CHO *hr* gene product was required to sustain viral protein synthesis in CHO cells, we observed no difference in the timing or extent of the shutoff of cellular protein synthesis in CHO cells infected with HR+ or HR− viruses. The shutoff of host protein synthesis was not due to leaky intermediate or late viral protein synthesis, since it was unaffected by an inhibitor of DNA replication. This result indicates that viral early gene expression was sufficient for the cessation of host protein synthesis under permissive and nonpermissive conditions in CHO cells.

Only four specific intermediate gene products have

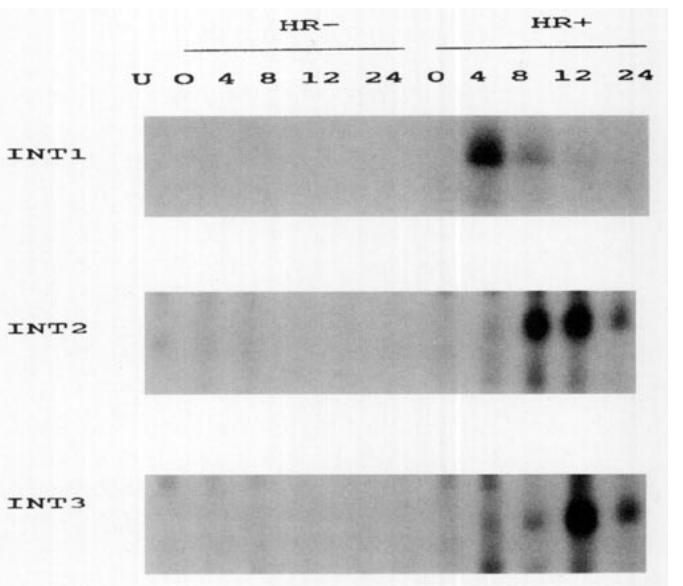

FIG. 6. Immunoprecipitation of viral intermediate proteins. CHO cells were infected with recombinant viruses vREβCAT (HR−) or vRECP77 (HR+) at a multiplicity of 30 PFU/cell. Infected cells were pulse-labeled with [^{35}S]methionine for 30 min starting at the indicated hr after infection. The cells were lysed and radiolabeled proteins incubated with individual polyclonal rabbit antisera specific for each of the three intermediate viral proteins. Immune complexes were analyzed by SDS–PAGE. INT1, A1L ORF-encoded 17-kDa protein; INT2, G8R ORF-encoded 30-kDa protein; INT3, A2L ORF-encoded 26-kDa protein.

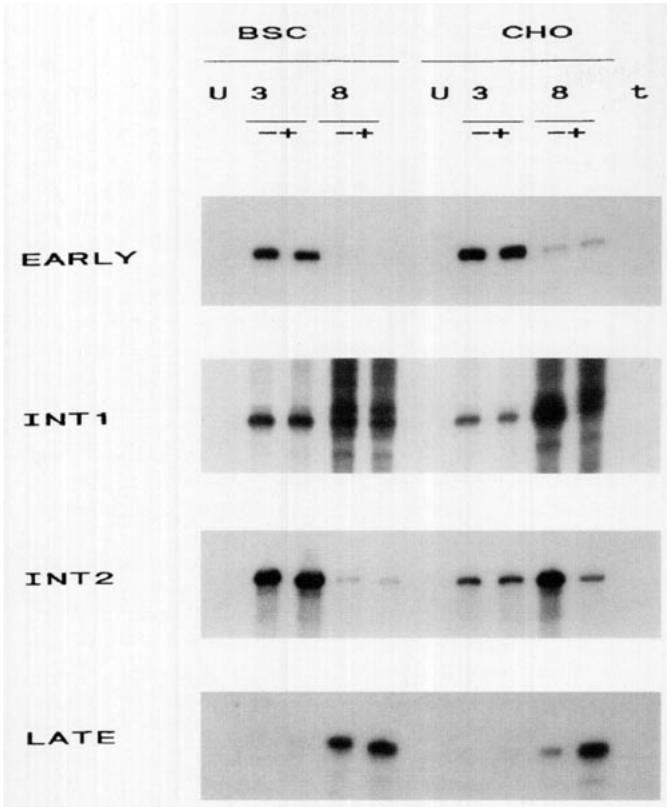

FIG. 7. *Analysis of steady state levels of viral mRNAs during abortive* or productive infection. BS-C-1 cells and CHO cells were infected with recombinant viruses vREβCAT (−) or vRECP77 (+) at a multiplicity of 30 PFU/cell. At 3 and 8 hr after infection, total infected cell RNAs were isolated. Nuclease protection analysis was performed on 10 μg of RNA with radiolabeled antisense RNA probes complementary to the following viral mRNAs: VGF (early), INT1 and INT2 (intermediate A1L and *G8R*, respectively), and *F17R (late)*. *Nuclease resistant products* were resolved on 8% acrylamide/8 *M* urea gels. RNAs prepared from uninfected cells (U) and tRNA (t) were used as negative controls.

been identified to date and they are difficult to resolve by direct analysis of labeled proteins by gel electrophoresis. Antisera to three intermediate proteins were used to isolate them from metabolically labeled lysates of infected cells. This analysis showed that synthesis of viral intermediate proteins in CHO cells is dependent on the CHO *hr* gene. Analysis of viral RNA, however, revealed that both early and intermediate mRNAs were present at the same levels during permissive and abortive infection. The discrepancy between intermediate mRNAs and proteins suggested a translational defect. By contrast, the absence of viral late protein synthesis could be explained by reduced levels of late mRNAs. In addition to the RNA *polymerase, at least three virus-encoded trans-acting* factors are needed for the synthesis of late mRNAs. These late transcription factors are products of the intermediate genes A1L, A2L, and *G8R*. Thus, the failure to translate these mRNAs accounts for the block in late gene expression.

Analysis of reporter gene expression was used to confirm that the host range defect is promoter-specific and does not depend on structural features within the viral ORFs. Reporter gene assays were useful for several reasons: (1) they allowed quantitative analysis of expression *from well-characterized early, intermediate, and late promoters*, (2) they were not dependent on uptake or pool sizes of labeled amino acids, and (3) they largely eliminated the possibility that specific viral mRNA or protein sequences are targeted.

In RK13 cells infected with K1L- recombinant VV, strains MVA (Sutter *et al.*, 1994) and WR (Ramsey-Ewing, unpublished), only viral early mRNAs are present. This result suggests that while host range restriction of VV in CHO cells and K1L- VV in RK13 cells share a general shutoff of cellular and viral protein synthesis, the two situations differ in important aspects. The K1L defect was accompanied by a block in both DNA replication and intermediate gene transcription, while nonpermissive infection of CHO cells by VV resulted in a later block, after DNA replication and intermediate gene transcription have occurred.

Having identified the level at which host range restric-

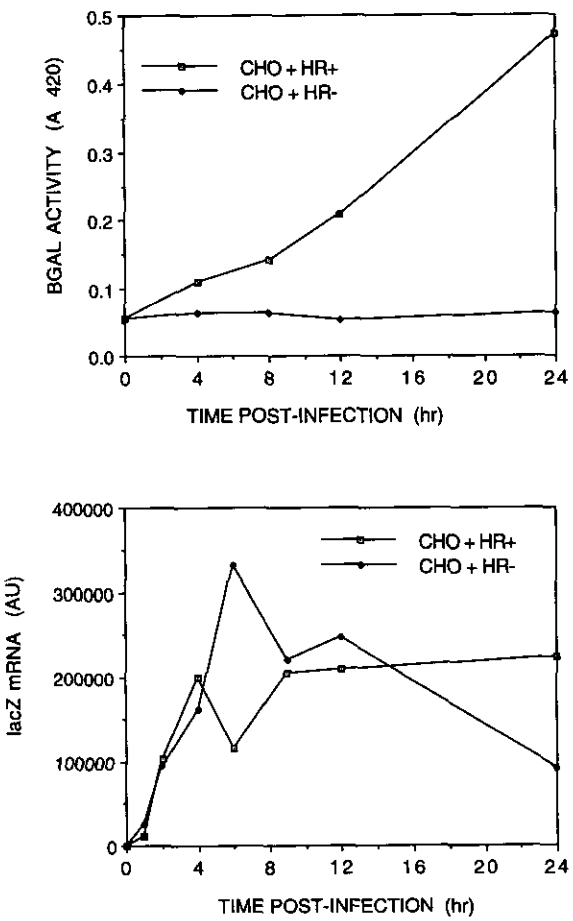

FIG. 8. Viral intermediate-*lacZ* reporter gene expression. CHO cells were infected with vP30lacZ or vP30CP77 and βGAL enzymatic activity (top) or *lacZ* mRNA levels (bottom) measured as described in the legends to Figs. 5 and 7, respectively. Electrophoreticaly resolved nuclease-resistant products were quantitated on a Phosphoimager and intensity of the *lacZ*-specific product plotted. HR−, vP30lacZ; HR+, vP30CP77; A 420, absorbance at 420 nm.

tion of VV in CHO cells occurs, the examination of the mechanism(s) that mediates these events can begin. The absence of viral intermediate proteins in the presence of intermediate transcripts suggests that the defect occurs by translational control at the stage of intermediate gene expression. Previous studies of poxvirus host range restriction have suggested a translational block at the stage of early gene expression during abortive infection with rabbitpox virus (Brown and Moyer, 1993) and a general inhibition of translation during vaccinia virus host range restriction (Njayou *et al.*, 1982). *In vivo* and *in vitro* experimentation are needed to investigate such a mechanism. There are several ways by which an apparent translational defect may be induced. First, the viral mRNAs may be modified so that they are nontranslatable. Alternatively, the mRNAs may be functional but the translational machinery may be altered. Third, the translational machinery may not have access to appropriate mRNA sequences, for example due to sequestration of mRNA. We are currently undertaking experiments to determine which, if any, of these mechanisms are operative during host range restriction of VV on CHO cells.

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