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Adenovirus Vector Expressing Functional Herpes Simplex Virus ICP0

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ICP0, one of the five immediate-early (IE) gene products of herpes simplex virus (HSV), is a potent activator of transcription. To assess the biological activities of ICP0 and to explore its mechanisms of action, two helper-independent recombinant adenoviruses were constructed. In each recombinant, the E1 region was substituted with the ICP0-encoding genomic segment under the control of either the adenovirus major late promoter (MLP-0) or the HSV IE-0 promoter (0_{PRO}-0). Infection of HeLa cells or 293 cells (a human embryo kidney cell line expressing adenovirus 5 E1a and -b functions) with the MLP-0 recombinant results in the synthesis of more IE-0 mRNA and ICP0 protein than did infection with the 0_{PRO}-0 recombinant. Although 293 cells infected with MLP-0 accumulate 5- to 10-fold more IE-0 mRNA late in the infection than cells infected with HSV, the level of the protein product, ICP0, increased only slightly. In 293 cells, both recombinants could replicate, albeit at a slower rate and with lower final yields than wild-type adenovirus. Neither virus replicates its DNA in HeLa cells, and thus ICP0 cannot substitute for adenovirus E1a; however, the level of ICP0 that accumulates in MLP-0-infected HeLa cells was comparable to that of HSV-infected HeLa cells. In a functional test, we demonstrated that the adeno-ICP0 recombinant viruses can transactivate a transfected TK-CAT cassette, indicating that the ICP0 is biologically active.

During the course of productive infection, herpes simplex virus (HSV) genes are coordinately regulated and expressed in a cascade fashion (24). This temporal program is composed of at least three groups of viral proteins: immediate-early (IE; α), early (β), and late (γ) (25). Genetic and biochemical experiments have shown that at least two of the five IE gene products are required to complete the replicative cycle. Studies with temperature-sensitive mutants have shown that ICP4 is required in an active form throughout the course of infection for the maintenance of both β and γ gene expression (9–11, 41, 57). Similar studies with temperature-sensitive mutants of ICP27 have shown that this protein is involved in the coordination of late protein synthesis (45). Transient expression analyses have shown that ICP27 is involved in the regulation of expression of the gD promoter and in autoregulation (14, 19).

ICP0, one of the five HSV α gene products, is a 110-kilodalton (kDa) nuclear phosphoprotein (39, 59). Genetic studies with mutant viruses from which portions of the IE-0 gene have been deleted indicate that they can be propagated without helper virus (46, 48). However, several parameters of infection by these viruses are abnormal, e.g., low burst size and slow growth rate (46, 48). Therefore, the role of this α gene product is not clearly established in productive infection. Using a transient transfection assay, we and others have shown that ICP0 is a potent transactivator (13, 17, 37, 44). Moreover, when present together with ICP4, ICP0 exhibits a synergistic effect on activating β genes, suggesting a functional interaction between these two products (13, 17, 37, 44).

Purification of ICP0 from HSV-infected cells has several disadvantages, namely, low levels of ICP0 synthesis and high background levels of other HSV gene products. To avoid these problems, we explored the use of an adenovirus-based vector for the expression of ICP0. The adenovirus genome is easily manipulated. Large quantities of soluble proteins are produced during the infectious cycle, and high levels of infected cells can be achieved. These and other features have made it an attractive vector for expressing eucaryotic gene products, e.g., the successful overproduction of papovavirus tumor antigens (4, 8, 47, 53, 54). Moreover, the expression of ICP0 using an adenovirus vector would allow us to determine whether a transcriptional activator from HSV can substitute for the function of E1a in the infectious cycle of adenovirus.

We report here the construction of two adeno-ICP0 recombinants. In each construct, the E1a and most of the E1b regions were substituted with the ICP0-encoding genomic segment under the control of the adenovirus major late promoter (MLP-0) or the HSV IE-0 promoter (0_{PRO}-0), respectively. In both 293 and HeLa cells, MLP-0 directed the synthesis of more ICP0 than did 0_{PRO}-0. In 293 cells, there was a disparity between the level of IE-0 mRNA synthesis after MLP-0 infection and the synthesis of the protein product. In MLP-0-infected HeLa cells, ICP0 accumulated to levels comparable to those seen in HSV-infected cells. ICP0 produced in the recombinant-infected cells was functional as judged by its ability to transactivate a TK-CAT target in a short-term transient expression assay. No DNA or viral replication could be detected with the recombinants in HeLa cells. Therefore, ICP0 does not appear to be able to substitute for E1a in the viral growth cycle.

MATERIALS AND METHODS

Cells and viruses. HeLa cells were grown in Dulbecco modified Eagle medium supplemented with 8% calf serum and 2% fetal calf serum, and 293 cells were grown in the same medium supplemented with 5% fetal calf serum. All of the adenoviruses and adeno-ICP0 recombinant viruses were propagated in 293 cells, a human embryo kidney cell line expressing adenovirus type 5 (Ad5) E1a and E1b functions (21). Viruses were released from infected cells by three cycles of freezing and thawing and titrated by fluorescent focus assay (12) on 293 cells. H5*dl*327 is an E1 wild-type

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derivative of *dl*324 (52). H5*in*340 is a derivative of *dl*309 which carries its packaging signal at the right end of the genome (22). H5*dl*312, a strain from which the E1a promoter and most of the coding region was deleted, has been described previously (27).

Construction of plasmids and recombinant viruses. The plasmid mpcv2 (35) was obtained through the courtesy of John Logan and Thomas Shenk (Princeton University). It contains the left-end 356 base pairs of the Ad5 genome, a module composed of the Ad2 major late promoter, the cDNA copy of the complete first two and two-thirds of the third tripartite leader, and Ad5 sequences (from 7.8 to 16.1 map units) with two unique sites (*Xho*I and *Bgl*II) that are convenient for the insertion of desired foreign DNA (Fig. 1A). An HSV-1 genomic segment encoding ICP0 from the plasmid p0XB, starting from the first AUG codon and containing the IE-0 polyadenylation signals, was cloned into mpcv2 as illustrated in Fig. 1A. First, the *Nco*I site containing the 5′ translation initiation codon was changed to an *Hin*dIII site after digestion with *Nco*I, end-filling with the Klenow fragment of *Escherichia coli* DNA polymerase I, and addition of *Hin*dIII linkers (12-mer). The 3′ *Hpa*I site, which is beyond the polyadenylation signal of IE-0, was converted to a *Bgl*II site by insertion of a *Bgl*II linker (10-mer) after cleavage with *Hpa*I. Similarly, the *Xho*I site in mpcv2 was changed to a *Hin*dIII site after cleavage with *Xho*I, digestion with mung bean nuclease, and insertion of a *Hin*dIII linker. This altered mpcv2 derivative was digested with *Hin*dIII and *Bgl*II, and a *Hin*dIII-*Bgl*II fragment containing the genomic segment encoding the ICP0 from HSV KOS was inserted between these sites. The resulting plasmid pDS-2 contains the hybrid transcription unit, which consists of the major late promoter of Ad2, the cDNA copy of the first two and two-thirds of the third tripartite leader, and the complete genomic segment encoding ICP0.

The plasmid GEM Ad60.1 (5) contains the left end of adenovirus up to an *Xho*I site at nucleotide (nt) 5778. However, the region between nt 199 and 3322, which contains the E1a gene and most of the E1b gene, is deleted (5). A *Bam*HI-*Bgl*II fragment containing the entire IE-0 transcription unit was excised from pDS-7 and inserted at the *Bgl*II site in GEM Ad60.1 to generate pDS-8 (Fig. 1B). The *Stu*I site about 650 base pairs upstream of the transcription initiation site (32) of IE-0 in pDS-7 was converted to a *Hin*dIII site, and the *Bam*HI site within IVS I was destroyed by digestion with *Bam*HI, end-filling with the Klenow fragment, and ligation. A *Bgl*II linker was inserted at the *Hpa*I site that is just downstream of the polyadenylation site for IE-0.

To construct the virus MLP-0, pDS-2 was linearized by cleavage with *Cla*I and cotransfected with *Cla*I-cut *dl*327 DNA into 293 cells. MLP-0 was generated by in vivo homologous recombination (56) between the overlapping sequence on the linearized plasmid and *dl*327. 0_{PRO}-0 was isolated after cotransfection of *Eco*RI-digested pDS-8 with *Cla*I-digested *in*340. Isolated plaques were picked and used to infect 293 cells. Infection was performed in a 24-well plate seeded with 10^5 cells per well. Lysates from the infected cells were screened by dot blot hybridization with ^{32}P-labeled IE-0-specific DNA. Positive plaques were then further analyzed and confirmed by restriction mapping and Southern blot hybridization. The adeno-ICP0 recombinant viruses were plaque purified twice on 293 cells, and virus stocks were prepared and titrated. By a similar approach, 0_{PRO}-0 was constructed by cotransfecting the linearized pDS-8 and adenoviral DNA from H5*in*340 cut with *Xba*I and *Cla*I into 293 cells.

RNA isolation and analysis. Total cytoplasmic RNA was isolated at early (6 h) or late (20 or 24 h) times postinfection from 293 cells and HeLa cells infected with HSV, *dl*327, MLP-0, or 0_{PRO}-0. RNAs were then analyzed by electrophoresis through 0.8% (wt/vol) agarose gels prepared in 2.2 M formaldehyde as described previously (38). The RNA was transferred to nitrocellulose paper and probed with a ^{32}P-labeled DNA fragment of IE-0.

Polypeptide analysis. For analysis of polypeptides synthesized in cells infected with wild-type or recombinant viruses, 60-mm-diameter dishes of HeLa or 293 cells (4×10^6) were infected at a multiplicity of infection of 10 PFU per cell. At selected times postinfection, cells were harvested and nuclear extracts were prepared as described earlier (59). Polypeptides from the nuclear extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (30) and then electrophoretically transferred to nitrocellulose paper. The nitrocellulose paper was blocked by incubation overnight with gentle agitation at 4°C in phosphate-buffered saline containing 0.05% Tween 20 and 10% nonfat dry milk. The filter was subsequently incubated with a mouse monoclonal antibody against ICP0 (generously provided by Lenore Pereira) at a 1:1,000 dilution in buffer A (0.05% Tween 20 and 2% nonfat dry milk in phosphate-buffered saline) at room temperature for 2 h. This was followed by three 20-min washes with buffer B (0.05% Tween 20 and 0.1% Nonidet P-40 in phosphate-buffered saline). The filter was then reacted with the second antibody (goat anti-mouse immunoglobulin G conjugated to alkaline phosphatase at a 1:2,000 dilution with buffer A) at room temperature for 2 h. The filter was again washed three times with buffer B and two more times with buffer C (100 mM Tris, pH 9.5; 100 mM NaCl; 5 mM $MgCl_2$). Finally, the filter was developed in 0.01% (wt/vol) nitroblue tetrazolium and indoxyl phosphate dissolved in buffer C.

Immunofluorescence. HeLa cells were infected with different viruses at a multiplicity of infection of 1. At 24 to 48 h postinfection, cells were fixed and analyzed for the presence of ICP0 by immunofluorescence as previously described (18).

Transfection and CAT assays. HeLa cells plated at 5×10^6 per 10-cm-diameter dish were transfected (17, 58) with 10 μg of a TK-CAT chimera and infected with virus 24 h later. Chloramphenicol acetyltransferase (CAT) activity was determined as previously described (18).

DNA analysis and virus growth. HeLa and 293 cells were infected with *dl*327, *dl*312, MLP-0, and 0_{PRO}-0. At different times postinfection, cells were harvested and lysed by three cycles of freezing and thawing. The supernatant was used either to assay for DNA replication by slot blot hybridization or to assay for viral replication by fluorescent focus assay on 293 cells. For slot blot analysis, lysates were adjusted to 100 mM Tris (pH 7.5)–12.5 mM EDTA–150 mM NaCl–0.1% SDS–10 μg of proteinase K per 125 μl and incubated at 65°C for 30 min. The viscous lysate was then adjusted to 25 mM NaOH at 65°C for 10 min and then loaded into the wells of a slot blot apparatus. The lysates were allowed to stand for 30 min and a very gentle vacuum was applied. The membrane was then rinsed six times with 0.4 M Tris (pH 7.5) and air dried prior to hybridization. The DNA probe used to detect DNA replication was a cloned E2B fragment (nt 8523 to 10579 obtained from Paul Freimuth) from Ad5 which had been ^{32}P labeled by random priming.

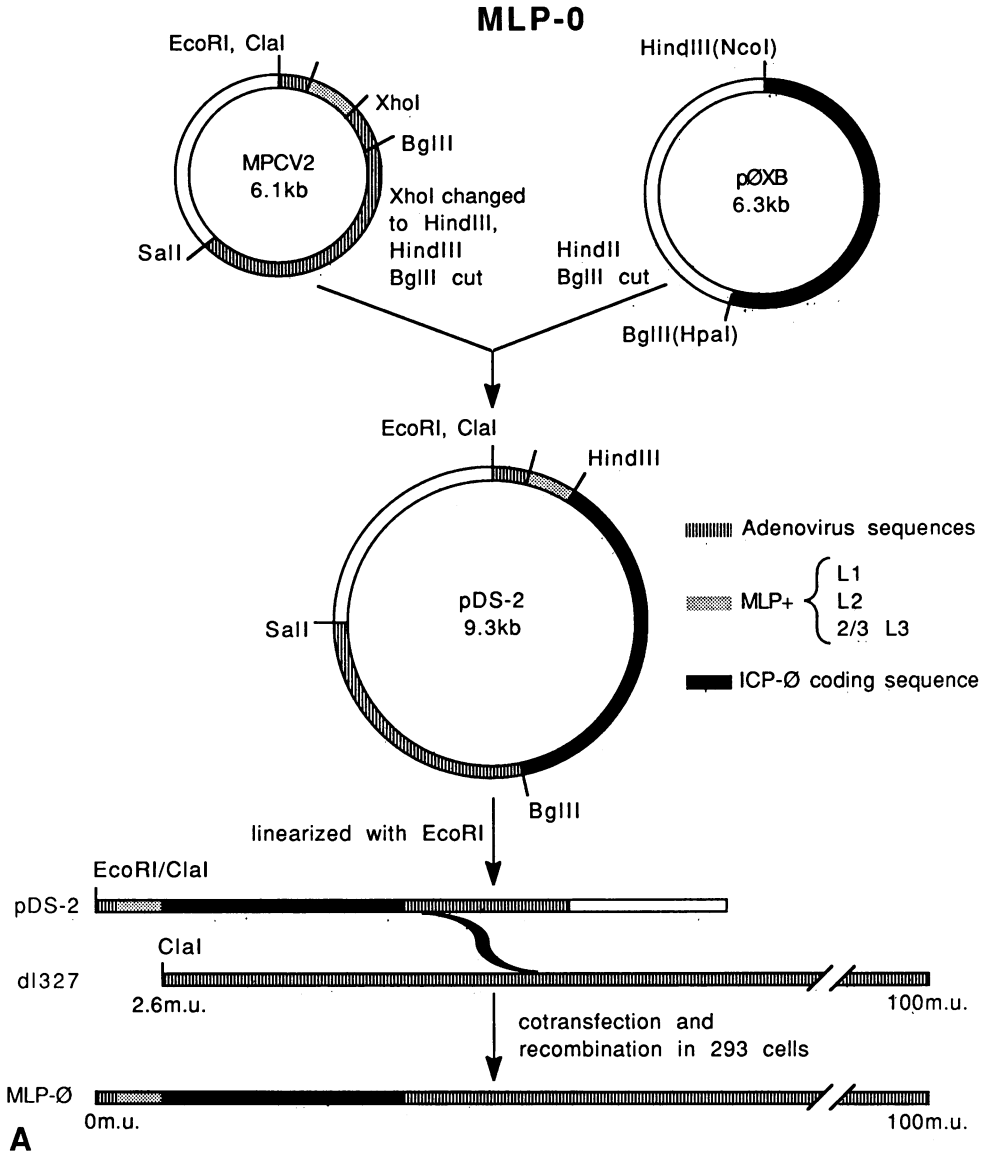
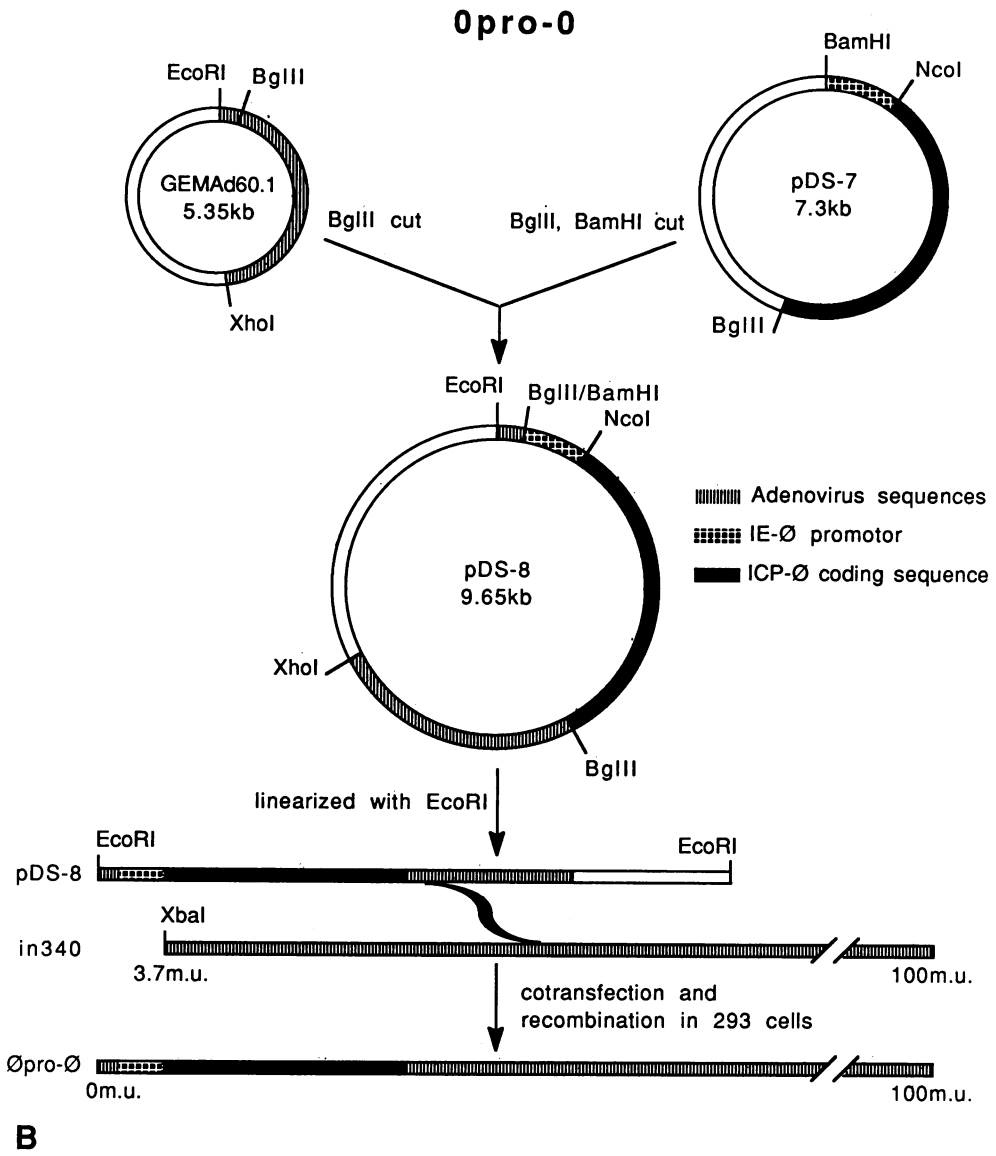


FIG. 1. Construction of adenovirus-ICP0 recombinants. (A) MLP-0. (B) 0_{PRO}-0. The plasmids pDS-2 and pDS-8 were constructed as described in Materials and Methods and inserted into either *dl*327 or *in*340 by overlap recombination in 293 cells. Plaques were isolated and scored for the presence and structure of the IE-0 sequences by first slot blot and then Southern blot hybridization.

RESULTS

Construction of adeno-ICP0 recombinant viruses. Two helper-independent adenovirus ICP0 recombinant viruses were constructed using the same strategy. In pDS-2, the MLP-0 construct, the genomic segment encoding ICP0 was placed behind the adenovirus major late promoter and a cDNA copy of the complete first two and two-thirds of the third tripartite leader (Fig. 1). The junction was located at an artificial *Hin*dIII site that juxtaposed an *Xho*I site (within the third leader) and an *Nco*I site containing the first potential translational initiation codon for ICP0. The remaining ICP0 sequences extended to a *Bgl*II site that was inserted at an *Hpa*I site beyond the IE-0 polyadenylation signal. The linearized plasmid pDS-2 was cotransfected with *dl*327 DNA cut with *Cla*I into 293 cells. Since adenovirus can package approximately 105% (20) of its genome size and the inserted ICP0 encoding sequence is about 2.7 kilobases long, we chose to use *dl*327, an E3 deletion mutant (35, 52), to ensure that the recombinant could be packaged. Recombinants arising by in vivo homologous recombination between the overlapping sequences on the plasmid and adenovirus genomes resulted in the generation of MLP-0, a recombinant virus capable of helper-independent growth on 293 cells. Similarly, to construct 0_{PRO}-0, the entire IE-0 transcription unit (which included the IE-0 promoter, the 5′-untranslated region, and the ICP0 coding sequence) was recombined into the left end of the adenoviral genome of *in*340. Because insertion of IE-0 into the left end of the viral genome at nt 199 would disrupt the packaging signal, we used *in*340 as the parental virus since the packaging signal was transposed to its right end (22).

Viable viruses were first screened by DNA dot blot hybridization for the presence of IE-0 sequences (49). This approach facilitated rapid screening of large numbers of



plaques. In the MLP-0 × *dl*327 cross 3 of 25 plaques were positive, and in the 0_{PRO}-0 × *in*340 cross 12 of 90 plaques were positive. Of the positive plaques, two MLP-0 and five 0_{PRO}-0 isolates were further analyzed by restriction endonuclease mapping and Southern blot hybridization (data not shown) to demonstrate the integrity and structure of the insert. All of the positive plaques analyzed had the expected structure.

IE-0 mRNAs transcribed from the recombinants. RNA analysis by Northern (RNA) blot hybridization was done to determine the kinetics of IE-0 mRNA accumulation, the relative abundance, and the size of the IE-0 transcript. Cells were infected with HSV, *dl*327, MLP-0, and 0_{PRO}-0, and total cytoplasmic RNAs were isolated at different times postinfection and analyzed for the presence of IE-0 RNA by Northern blot hybridization with an IE-0-specific DNA probe (Fig. 2). A higher level of IE-0 RNA accumulated in both 293 and HeLa cells infected with MLP-0 than in cells infected with 0_{PRO}-0. Moreover, in 293 cells at late times postinfection, MLP-0 directed the synthesis of large amounts of IE-0 mRNA, which was estimated to be 5 to 10 times higher than that in HSV-infected cells based on the intensity of the hybridization signal. In HeLa cells infected with MLP-0, IE-0 mRNA only accumulated to a level comparable to that found in HSV-infected cells. If the adenovirus major late promoter and IE-0 polyadenylation signal were correctly recognized, the processed hybrid transcription unit in MLP-0 should result in the synthesis of an RNA molecule of about 2.7 kilobases containing 168 nt of adenovirus late leader, the junction sequence, and the remainder (which is the ICP-0 coding sequence). Within the limits of resolution of Northern analysis, MLP-0 directed the synthesis of a single RNA species that migrated with the same R_f as the IE-0 mRNA that accumulated in cells infected with either HSV or 0_{PRO}-0.

ICP0 synthesis in cells infected with the recombinants. The protein products synthesized in cells infected with the recombinants, wild-type HSV, and adenoviruses were examined by Western blot (immunoblot) analysis with a monoclonal antibody specific for ICP0 (Fig. 3 and 4). ICP0 produced

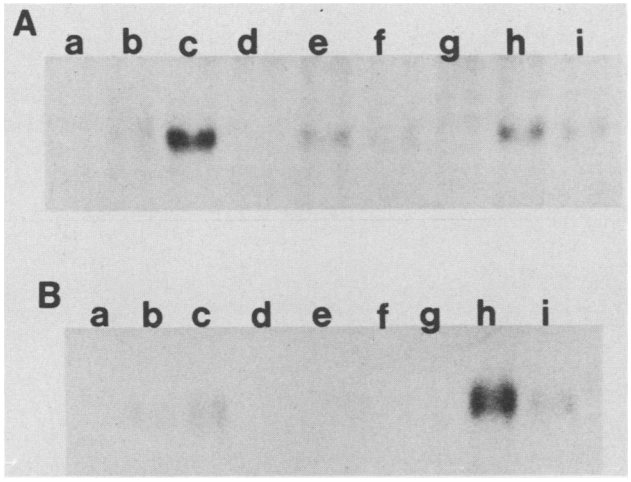


FIG. 2. Analysis of RNA from cells infected with MLP-0 or 0_{PRO}-0. (A) HeLa cells. (B) 293 cells. Total cytoplasmic RNA (20 μg) extracted from HeLa or 293 cells infected with wild-type and recombinant viruses was fractionated on a 0.8% agarose gel prepared in 2.2 M formaldehyde. The RNA was then transferred to nitrocellulose and hybridized with IE-0-specific DNA. Lanes: a, mock infection; b, HSV-1 (3 h); c, HSV-1 (6 h); d, *dl*327 (6 h); e, MLP-0 (6 h); f, 0_{PRO}-0 (6 h); g, *dl*327 (24 h); h, MLP-0 (24 h); i, 0_{PRO}-0 (24 h).

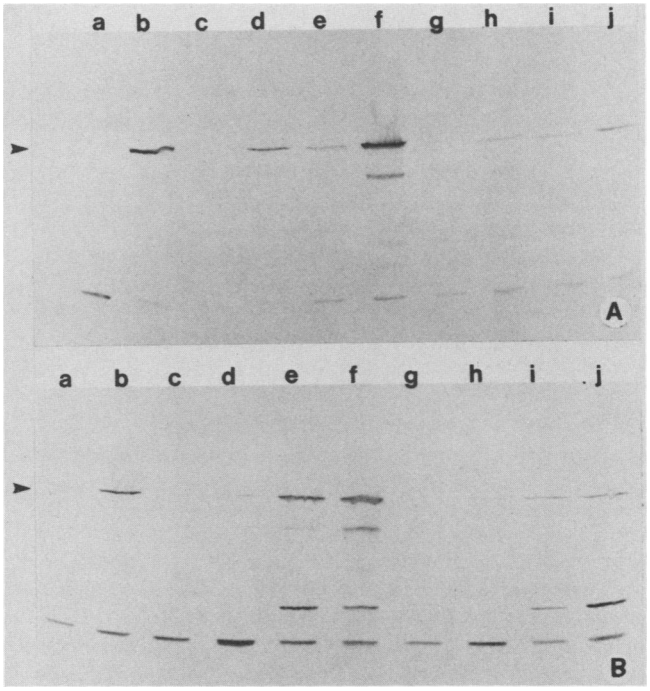


FIG. 4. Kinetics of ICP0 accumulation. HeLa (A) or 293 (B) cells were infected with the recombinants or HSV-1, and at intervals nuclei were isolated and analyzed for the presence of ICP0 by Western blot analysis. (A) Lanes: a, mock infected; b, HSV-1 (6 h); c to f, MLP-0 (6, 20, 24, and 48 h, respectively); g to j, 0_{PRO}-0 (6, 20, 24, and 48 h, respectively). (B) Lanes: a and b, HSV-1 (3 and 6 h, respectively); c to f, MLP-0 (6, 20, 24, and 28 h, respectively); g to j, 0_{PRO}-0 (6, 20, 24, and 28 h, respectively). The arrows denote the migration position of ICP0 from cells infected with HSV-1.

in HSV-1-infected cells has an apparent molecular size of 110 kDa, as determined from its electrophoretic mobility in SDS-PAGE, and is located in the nucleus (1, 39). A single species of ICP0 with an electrophoretic mobility indistinguishable from authentic ICP0 was detected in isolated nuclei from both HeLa and 293 cells infected with either MLP-0 or 0_{PRO}-0 (Fig. 3). Previous work from this laboratory showed that ICP0 is distributed as punctate granules in the nucleus of cells transfected with plasmids encoding ICP0 (18). The distribution pattern of ICP0 in cells infected with the recombinants was analyzed by immunofluorescence by using a monoclonal antibody specific for ICP0 (1). At 36 h postinfection, ICP0 was in the nucleus and distributed as punctate granules in HeLa cells infected with MLP-0 or 0_{PRO}-0 (data not shown).

Cells infected with MLP-0 contained a higher level of ICP0 than did cells infected with 0_{PRO}-0 (Fig. 3, lanes d and e, respectively). This is true for both cell types (Fig. 3A and B).

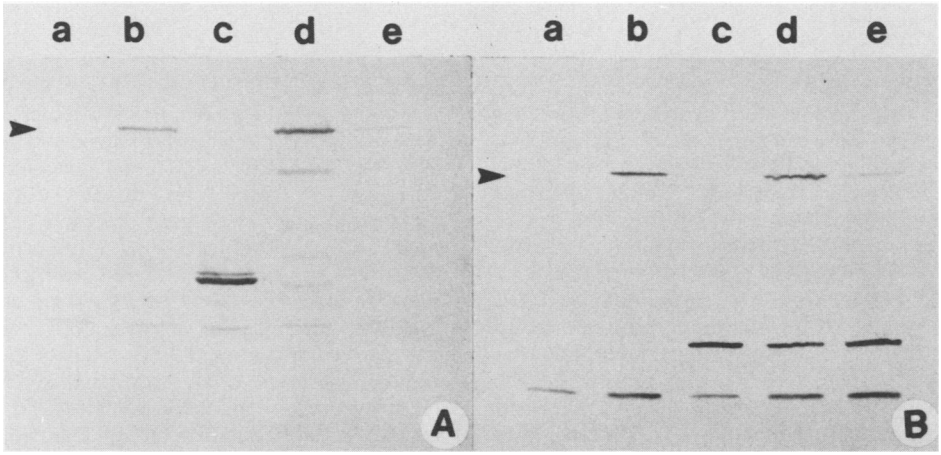


FIG. 3. ICP0 synthesis in cells infected with MLP-0 or 0_{PRO}-0. HeLa (A) and 293 (B) cells were infected with parental and recombinant adenoviruses or HSV-1, and nuclear extracts were prepared from equal numbers of cells at the indicated time postinfection and subjected to SDS-PAGE. The separated proteins were electrophoretically transferred from the gel to nitrocellulose paper and analyzed for the presence and size of ICP0 by Western blot analysis with a monoclonal antiserum to ICP0 as described in Materials and Methods. Lanes: a, mock infection; b, HSV-1 (6 h); c, *dl*327 (24 h); d, MLP-0 (24 h); e, 0_{PRO}-0 (24 h). The arrows denote the migration position of ICP0 from cells infected with HSV-1.

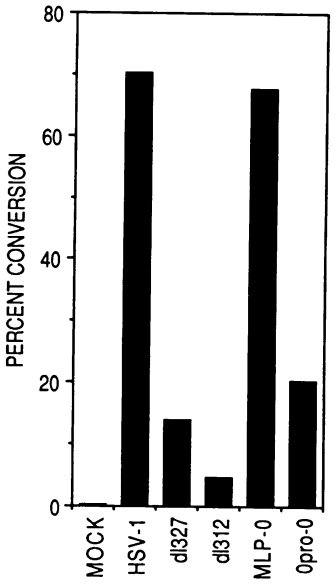


FIG. 5. Biologic activity of recombinant ICP0. HeLa cells were transfected with a TK-CAT cassette; 24 h later they were infected and incubated for 12 h if infected with HSV or for 24 h if infected with adenovirus or one of the recombinants. Cell extracts were prepared and analyzed for CAT activity as described previously (18).

However, the data on the kinetics of protein accumulation (Fig. 4) reveal two unexpected observations. First, despite the presence of IE-0 mRNA early in the infectious cycle, we have not detected protein until late times postinfection in both cell types with both constructs (Fig. 4). Second, at late times postinfection, when 293 cells were infected with MLP-0 the accumulation of ICP0 was increased only slightly when compared with that seen in HSV-1-infected 293 cells (Fig. 4B), despite the marked increase in IE-0-specific RNA (Fig. 2B, lanes c and h). At late times postinfection (20 to 48 h) in HeLa cells, MLP-0 accumulated ICP0 at a level comparable to that of HSV-1-infected HeLa cells.

The presence of more rapidly migrating species detected in the Western blots (Fig. 3 and 4) may be attributed to the proteolytic cleavage products of ICP0. Similar bands are observed when the protein is expressed in *E. coli* and when vaccinia virus recombinants are used to infect HeLa cells (data not shown).

Functional analysis of ICP0 synthesized by the recombinants. ICP0 is a potent transcriptional activator of HSV promoters when examined in short-term transient expression assays. Therefore, the ability of ICP0 synthesized by the recombinant viruses to transactivate a virus promoter linked to a reporter gene (the TK promoter linked to the CAT gene) was examined. Briefly, a plasmid containing this TK-CAT cassette was first transfected into HeLa cells, and 24 h later the cells were infected with the different viruses. At various times postinfection (HSV, 12 h; adeno and adeno-ICP0 recombinants, 24 h), cells were harvested and cellular extracts were prepared and assayed for CAT activity. If ICP0, produced during the course of infection, retained its transactivating ability it would activate TK-CAT and result in higher levels of CAT activity. Extracts from cells infected with both recombinants exhibited higher CAT activity than did extracts from cells infected with *dl*327 (Fig. 5). Moreover, the level of CAT activity in MLP-0-infected cells was higher than that in 0_{PRO}-0-infected cells. This was consistent with the level of ICP0 produced by these two recombinants.

Replication of recombinant viruses. The IE gene products of pseudorabies and cytomegaloviruses can substitute for E1a in the activation of adenoviral early gene expression (15, 26, 50, 51). By using a cell line that constitutively expresses ICP4, it has been shown that ICP4 can activate the expression of the adenovirus 72-kDa early gene product (55). However, it is unknown whether ICP0, when expressed in the context of the adenoviral genome, can substitute for the function of E1a. In particular, can ICP0 complement the defect of an E1a deletion mutant? To address these questions, we used the adeno-ICP0 recombinants because they differed from the wild-type adenovirus only in that the E1a region and part of the E1b region were replaced with the ICP0-encoding segment.

First, we examined DNA replication of each recombinant in both HeLa and 293 cells. Figure 6 shows that neither recombinant replicates its DNA in HeLa cells, whereas both do so in 293 cells. Control viruses replicate as expected. *dl*327, the replication-competent parent for MLP-0, replicates efficiently in both HeLa and 293 cells, whereas *dl*312, which has a deletion in E1a (27), replicates its DNA only in 293 cells (Fig. 6). Therefore, ICP0 cannot substitute for the deficiency in E1a and E1b.

In HeLa cells, *dl*312 did not replicate until 48 h postinfection (Fig. 7). For 0_{PRO}-0, very low levels of viral growth were detected at 48 and 72 h postinfection, as assayed by a fluorescent focus assay. To determine whether the limited amount of replication resulted from recombination with the indigenous adenoviral sequences in 293 cells, we analyzed the supernatants from 0_{PRO}-0-infected HeLa cells (48 and 72 h) by titration on HeLa cells. Five plaques were picked, and a modified Hirt supernatant (23, 56) was prepared from cells infected with these viruses. The Hirt DNA was analyzed by restriction mapping and found to contain E1a sequences (data not shown). Therefore, the low level of viral growth that was detected in 0_{PRO}-0-infected HeLa cells was, at least in part, a result of the generation of wild-type revertants during their passage in 293 cells. Although both MLP-0 and 0_{PRO}-0 grow efficiently in 293 cells, their rate of viral replication was delayed and their final yields were lower than that of *dl*327 (Fig. 7B). The replicative defects of 0_{PRO}-0 were more dramatic than those of MLP-0.

DISCUSSION

We report here the construction and characterization of two adenovirus recombinants, MLP-0 and 0_{PRO}-0, that express ICP0, an HSV α gene product. In both recombinants, the E1 region was substituted with the ICP0-encoding genomic segment under the control of the adenovirus major late promoter or the HSV IE-0 promoter, respectively.

MLP-0 was constructed so that the ICP0 coding sequence was under the control of a promoter that is expressed at high levels late in infection (for a review, see reference 3). Although at late times of MLP-0 infection in 293 cells, IE-0 mRNA accumulates to a level that is 5 to 10 times higher than that found in HSV-infected 293 cells, the level of the protein product (ICP0) is not correspondingly elevated. These results differ from those of Davidson and Hassell (8), who, using the same strategy and plasmid construct (mpcv2), showed that the expression level of polyoma middle T antigen from their recombinants was 10 times higher than that found in cells infected with polyomavirus. It is

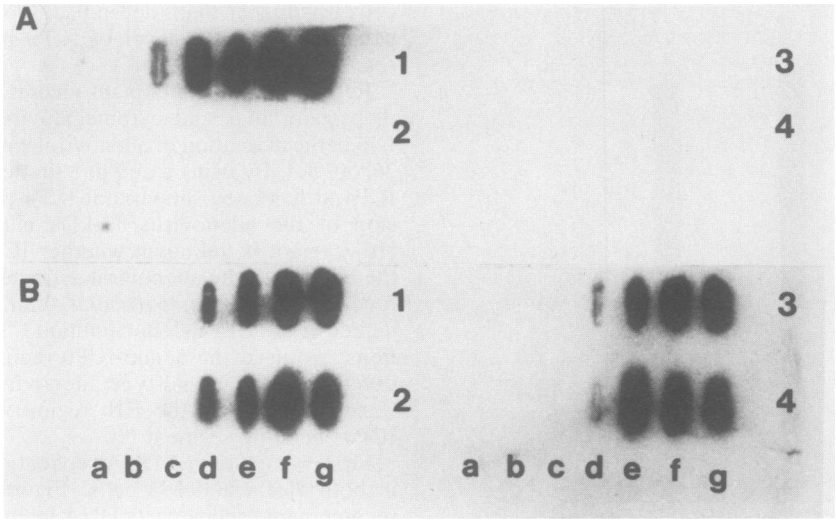


FIG. 6. Time course of DNA replication. Cells were infected with 10 PFU of each recombinant virus and at various times postinfection were harvested and analyzed for replication of virus DNA by slot blot hybridization with a cloned E2B fragment from adenovirus as the probe. (A) HeLa cells. (B) 293 cells. The cells were infected with *dl*327 (row 1), *dl*312 (row 2), MLP-0 (row 3), and 0_{PRO}-0 (row 4). Samples were removed for analysis at 0, 2, 14, 18, 22, 26, and 48 h postinfection (lanes a to g, respectively).

conceivable that this discrepancy results from differences between the natural expression level of these proteins (middle T antigen from polyomavirus-infected cells versus ICP0 from HSV-infected cells).

Adenovirus mRNAs are selectively translated during the late times of infection. The 5′ noncoding sequences present in front of most late mRNAs (tripartite leaders) are important for the efficient translation late after infection (31). Moreover, the translation efficiency is dependent upon the proximity of the initiation codon to the tripartite leaders (4). However, it remains unclear at which step during translation the tripartite leader sequences act to facilitate this process.

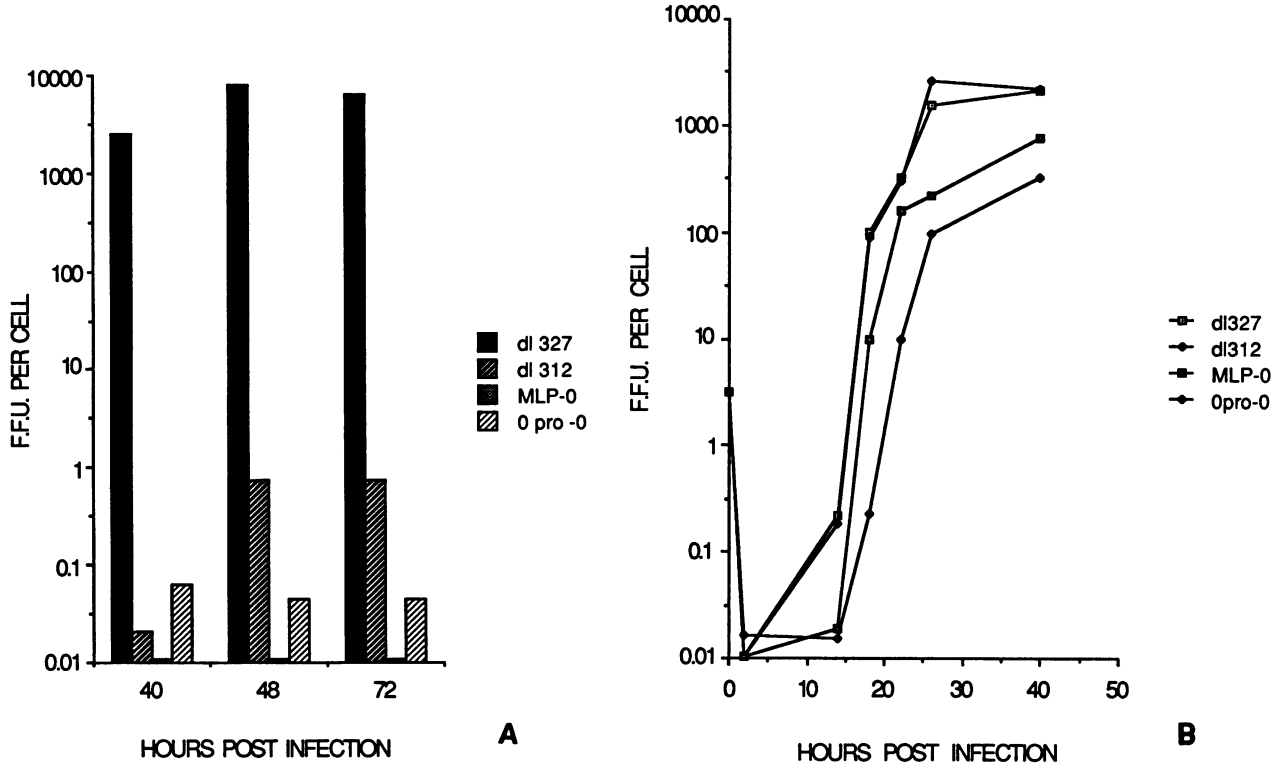


FIG. 7. Replication of recombinant viruses. An aliquot of the freeze-thawed lysate from each time point in the experiment described in the legend to Fig. 6 was assayed by fluorescent focus assay on 293 cells to determine the level of virus replication. An additional time point reading was taken at 72 h in infected HeLa cells. The yields from HeLa cells infected with the indicated viruses (A) and from 293 cells (B) are shown. F.F.U., fluorescent focus unit.

pmcv2 contains the first two leaders in their entirety and two-thirds of the last leader sequence. Whether this is sufficient for efficient translation of the chimeric ICP0 mRNA expressed by the MLP-0 recombinant remains to be determined.

The 0_{PRO}-0 recombinant was originally constructed for two reasons. First, the coding sequences were left under the control of their own promoter, which is normally expressed immediately after infection, in the hope that ICP0 might be expressed earlier in the infectious cycle and therefore accumulate to greater levels. The second reason was to examine the role of the HSV-1 leader sequence in translation of this polypeptide. This recombinant expresses IE-0 mRNA as early as 6 h postinfection but did not accumulate high levels of mRNA at late times. This result was unexpected because the IE-0 promoter has a high constitutive level of expression when assayed by transient expression (19). However, high-level expression from the IE-0 promoter in HSV-infected cells results from the interaction between a virion-associated transactivating factor α-TIF (7, 28, 29, 40), a host factor NFIII (28, 29, 34, 36, 42, 43), and a specific nucleotide sequence TAATGARATTC that is located in the upstream promoter regulatory regions of all HSV α genes (6, 16, 32, 33). Under the conditions of infection with the recombinant adenovirus, α-TIF is absent and the gene is no longer in its normal environment. This may explain in part the failure to detect high levels of IE-0 mRNA. The level of ICP0 is correspondingly low. This suggests that the HSV-encoded leader is no more efficient in translation than the tripartite leader under these experimental conditions.

The ICP0 produced by each recombinant virus is biologically active. This conclusion is based on the following experimental data. First, using the HeLa cell transient transfection assay, we have shown that a transfected TK-CAT target is activated by infection with the recombinant viruses, but not with either *dl*312 or *dl*327. The relatively higher CAT activity following *dl*327 infection compared with *dl*312 is probably a result of adenovirus early gene products expressed by the replication-competent *dl*327. Second, the level of CAT activity that accumulates in MLP-0-infected cells is higher than that in 0_{PRO}-0-infected cells. This is consistent with the relative level of ICP0 accumulation in these two recombinants. Third, using the adeno-ICP0 recombinants described here, both we and J. Russell and C. M. Preston (unpublished studies) have reactivated a latent HSV genome in a model in vitro latency system.

Using viruses as model systems to study gene regulation has greatly furthered our understanding of eucaryotic gene expression. Adenovirus and HSV are DNA viruses that are coordinately regulated in a temporal fashion. The IE gene products in both viruses serve as important regulators of expression of gene families that are expressed late in the infectious cycle (3, 13, 17, 37, 44). What mechanism is involved in this process? Will the IE gene products from different viruses regulate gene expression in a similar way? In an attempt to address these questions, Feldman and co-workers (15, 26) have shown that the IE gene product of pseudorabies virus can complement the defects of *dl*312, an E1a deletion mutant of adenovirus. In this study we tested the ability of ICP0 to substitute for adenovirus E1a and E1b gene products. The results were negative: no virus DNA replication occurred in HeLa cells infected with either recombinant. We do not believe that this was a consequence of a temporal delay in synthesis because the same results were obtained when infection was extended to 72 h. This complex regulatory pathway is not reconstituted by ICP0, a transcriptional activator from HSV which has no sequence homology with E1a. Similar results were obtained when KB18, a cell line which constitutively expresses E1b (2), was used (data not shown). Therefore, the failure of ICP0 to substitute for E1a is not a result of the absence of E1b in the recombinants. Moreover, in HeLa cells infected with the recombinants, expression of the adenovirus 72-kDa product, a protein involved in adenoviral DNA replication, could not be detected (data not shown). Therefore, the failure to activate the expression of 72-kDa product is at least one of the many possible reasons why ICP0 cannot substitute for E1a in viral replication. We have also shown that the recombinants are somewhat impaired in their replicative abilities in 293 cells (e.g., longer eclipse, delayed replication, and lower final yields). It is unlikely that this impairment results from defects at the packaging level because the genome size of the recombinants is within the packaging limit (20). Moreover, the defects were also detectable at the level of DNA replication. It is conceivable that the expressed ICP0 titrated out some specific transcription factors or acted directly on adenovirus early promoters to inhibit their expression. Complex interactions between HSV-1 ICP4 and adenovirus early gene expression have been demonstrated by Tremblay et al. (55). The more marked replication defects of 0_{PRO}-0 might have resulted at least in part because of the translocation of the packaging signal to the right end of the viral genome (22).

Although MLP-0 did not replicate in HeLa cells, it did accumulate ICP0 at late times postinfection to a level comparable to that detected in cells infected with HSV. This provides us with the opportunity to detect the transcriptional activating property of ICP0 and to assay the interactions with specific regulatory sequences by using crude nuclear extracts from MLP-0-infected HeLa cells.

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