A Genetic Test for Expression of a Functional Herpes Simplex Virus DNA-Binding Protein from a Transfected Plasmid

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The major DNA-binding protein, ICP8, encoded by herpes simplex virus is localized to the infected cell nucleus where it plays a role in viral DNA replication and control of viral gene expression. To identify the parts of the ICP8 protein that are important for its localization and functions, we have developed a system to test the ability of recombinant plasmids to express functional ICP8. A recombinant plasmid containing the wild-type ICP8 gene was transfected into cells. The cells were later infected with a temperature-sensitive ICP8 mutant virus at the nonpermissive temperature. Sufficient wild-type ICP8 was expressed from the transfected plasmid to complement the replication of the mutant virus. This provides a genetic system to test the properties of ICP8 expressed from mutagenized plasmids without the establishment of a stable cell line or the reintroduction of the ICP8 gene into the herpes simplex virus genome.

The herpes simplex virus (HSV) DNA-binding protein, ICP8, is essential for viral DNA replication. In vitro, the protein binds preferentially to single-stranded DNA (1, 8, 15, 17) and lowers the melting temperature of polydeoxyadenylic acid-polythymidylic acid helices (14). In infected cells before viral DNA replication, ICP8 is localized to specific sites in the nucleus designated as prereplicative sites. When viral DNA replication occurs, ICP8 is localized to large globular replication compartments in the nucleus (18) where it is bound to progeny or replicating DNA (12, 13). ICP8 also plays a role in regulating the level of viral mRNA in infected cells (3; P. Godowski and D. Knipe, submitted for publica-

expression of its mRNA requires HSV immediate early gene products during lytic infection (2, 6, 9, 16, 19, 23). The pSG18 plasmid (4; Fig. 1) contains the ICP8 gene (5, 19) and can express ICP8 upon introduction into cells but at low levels in the absence of viral immediate early gene products (18, 18a, 20). In this study, ICP8 expression was monitored by immunoprecipitation of labeled proteins from cells transfected with the recombinant plasmid pSG18. A low level of ICP8 protein was expressed in cells transfected with this DNA (Fig. 2, lane 2). ICP8 expression from the plasmid was increased by infection of the transfected cells. Cells were transfected with pSG18, and after 24 h they were infected

FIG. 1. Origin of the viral DNA inserts in recombinant plasmids. The pSG18 plasmid (4) contains the HSV type 1 KOS1.1 *EcoRI* F DNA fragment (0.314 to 0.420 map units). Shown are the positions of the *EcoRI* (E) and *SalI* (S) sites and the approximate position and direction of transcription of the ICP8 mRNA. Plasmid pSG18-SalIA (12) contains the *SalI* fragment indicated.

tion). To determine which parts of the ICP8 protein are important for these functions, we developed a system to test the ability of recombinant plasmids to express functional ICP8.

ICP8 is a beta (early) viral gene product; therefore,

with KOS1.1 *ts*⁺ or *ts*18 (5) virus. The mutant *ts*18 encodes a thermolabile ICP8 molecule (12) that, when synthesized at 39.5°C, is poorly immunoprecipitated by the 39S monoclonal antibody (21), as compared with the wild-type protein (Fig. 2, lanes 5 and 6). This mutant expresses viral immediate early and early gene products (P. Godowski and D. Knipe, unpublished data) but is defective for viral DNA replication (5). A large increase in wild-type ICP8 expression was observed when cells transfected with pSG18 were infected with *ts*18 at 39.5°C (Fig. 2, lane 4). Evidence presented below indicated that there was little recombination between

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FIG. 2. Immunoprecipitation of ICP8 from extracts of transfected and infected cells. The transfection mix was prepared as described previously (10). The total amount of DNA in each mix (0.5 ml) was adjusted to 10 μ g by the addition of salmon sperm DNA. Vero cells were trypsinized and concentrated in Dulbecco modified Eagle medium with 10% heat-inactivated VSP neonate calf serum (Biocell Labs), and samples of 2×10^6 cells were collected by centrifugation at $1,200 \times g$ for 10 min at 22°C. The cells were resuspended in 0.1 to 0.2 ml of medium to which 0.5 ml of the transfection mix was added. This mixture was incubated for 20 to 40 min at 22°C with occasional agitation. Dulbecco modified Eagle medium–2% heat-inactivated VSP serum was then added. The cells were plated in 25-cm² flasks and incubated for 3 to 5 h at 37°C. This medium was replaced with Dulbecco modified Eagle medium–6% heat-inactivated VSP, and the cultures were incubated at 37°C. Some cultures (see below) were infected after 24 h. All cultures were maintained at 39.5°C and labeled from 1 to 4 h after infection with 25 μ Ci of [³⁵S]methionine per ml of minimal essential medium containing 5% of the normal concentration of methionine and 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.4). Immunoprecipitations were performed as previously described (18), with the addition of 10 μ M *N*- α -*p*-tosyl-L-lysine chloromethyl ketone to all the buffers. Shown is a fluorogram of a sodium dodecyl sulfate-polyacrylamide gel demonstrating the polypeptides immunoprecipitated by 39S antibody from cells transfected and superinfected as indicated. Lanes: 1, transfection with 0.5 μ g of HSV type 1 mP *ts*⁺ virus, no super-infection; 2, transfection with 5 μ g of pSG18, no infection; 3, transfection with 5 μ g of pSG18, infection with KOS1.1 *ts*⁺ virus; 4, transfection with 5 μ g of pSG18, infection with KOS1.1 *ts*18; 5, no plasmid transfected, infection with KOS1.1 *ts*⁺ virus; 6, no plasmid transfected, infection with KOS1.1 *ts*18 virus.

TABLE 1. Complementation of an ICP8 mutant virus by a transfected plasmid^a

Plasmid DNA used for transfection	Virus used for infection	Titer ^b of virus yield (10 ³ PFU) at:	
		33°C	39.5°C
None	KOS1.1 <i>ts</i> 18	1.7, 1.5	0.45, 0.23
pSG18	KOS1.1 <i>ts</i> 18	49, 54	0.26, 0.28
pSG18-SalA	KOS1.1 <i>ts</i> 18	1.5, 1.5	0.17, 0.32

^a Cells (2×10^6) were transfected as described in the legend to Fig. 2 with salmon sperm DNA and the plasmid DNAs as indicated. The transfected cells were plated in 25-cm² flasks. They were infected 24 h after transfection with KOS1.1 *ts*18 at a multiplicity of infection of 5 PFU per cell. The virus was allowed to adsorb to the cells at 0 to 4°C for 60 min. The monolayers were washed four times with phosphate-buffered saline at 0 to 4°C. The cells were then overlaid with medium 199 containing 1% heat-inactivated calf serum. The infected cultures were incubated in 39.5°C water baths. The cells were harvested at 18 h postinfection, and the progeny virus was titrated at 33 and 39.5°C.

^b The two values represent titers from replicate cultures in the same experiment.

the plasmid and the viral genome in this system. Therefore, expression of the ICP8 gene on the pSG18 plasmid appeared to be increased by viral gene products acting in *trans*. Further studies on the activation of ICP8 expression in transfected cells by viral gene products will be presented elsewhere (18a).

To examine the properties of the ICP8 molecules expressed in transfected cells, we performed two types of experiments. First, we examined the intranuclear distribution of the protein by indirect immunofluorescence. As described previously (18), ICP8 expressed from pSG18 transfected alone shows a punctate intranuclear distribution similar to the prereplicative sites in lytically infected cells (Fig. 3A). With this technique, no ICP8 was detected in cells mock transfected with salmon sperm DNA and infected with *ts*18 at 39.5°C (Fig. 3B). When cells were transfected with pSG18 and infected with *ts*18 at 39.5°C, many exhibited nuclear ICP8. In some of these nuclei, ICP8 was visible in large globular structures resembling the replication compartments observed in infected cells (Fig. 3C). This suggested that in the last situation the ICP8 expressed from the plasmid was participating in viral DNA replication.

To examine the possibility that the ICP8 expressed from the transfected plasmid was promoting viral DNA replication, we tested the ability of the pSG18 plasmid to complement the growth of *ts*18 at 39.5°C. At 24 h after transfection of pSG18, cultures were infected with *ts*18 at 39.5°C. The progeny virus was harvested at 18 h after infection and titrated at 33 and 39.5°C. The yield of *ts*18 was increased 25- to 35-fold by the prior transfection of the pSG18 plasmid (Table 1). No increase in the number of progeny capable of forming plaques at 39.5°C was observed. The constant level of plaques observed at 39.5°C was probably due to revertant viruses in the *ts*18 stock. This mutant virus shows a very tight temperature block, but a reversion rate of approximately 10^{-4} to 10^{-5} (P. Godowski, E. Richards, and D. Knipe, unpublished data). Because the presence of pSG18 did not increase the 39.5°C titer of the progeny virus, we concluded that recombination or an increase in the reversion rate did not contribute to the increased yield of *ts*18 virus. In other situations, the frequency of recombination between a transfected plasmid and the HSV genome is very high (11, 22). The lack of recombination in this system may be due to the assembly of the transfected plasmid into nucleosomal particles (7) before infection with the mutant virus. This

FIG. 3. Intranuclear distribution of ICP8 in transfected and infected cells. Transfections and infections were performed as described in the legend to Fig. 2. Cells (2×10^5) were plated on 18-mm circular no. 1 cover slips. At 5 h postinfection the cells were fixed and processed for immunofluorescence with 39S as described previously (18). (A) Transfection with 5 μ g of pSG18, mock infection; (B) no plasmid transfected, infected with KOS1.1 *ts18* at 39.5°C; (C) transfection with 5 μ g of pSG18, infection with KOS1.1 *ts18* at 39.5°C. Bar, 5 μ m.

might decrease its ability to participate in recombination with the infecting viral genome. Therefore, the increase in the yield of *ts18* seems to be due to complementation of the growth of *ts18* by ICP8 expressed from the plasmid. Similar complementation has been observed with subclones of pSG18 containing a complete ICP8 gene (data not shown). By this assay, the ICP8 expressed from these plasmids appears to be fully functional.

To further characterize this system, we tested complementation of *ts18* by a plasmid containing a truncated ICP8 gene. The pSG18-SalA plasmid contains a deletion of 750 base pairs or less from the 3' end of the ICP8 transcriptional unit (5). Nevertheless, this plasmid has been used for marker rescue of nearly all of the ICP8 *ts* mutants, including *ts18* (5,

12, 24). When cells were transfected with pSG18-SalA and infected with *ts18*, a low level of a truncated form of ICP8 with a molecular weight of approximately 117,000 was immunoprecipitated from the extracts (data not shown). The pSG18-SalA plasmid did not complement the growth of *ts18* at 39.5°C (Table 1). This could be due to the low level of accumulation, to improper localization; or to impaired function of the truncated polypeptide. The observations that this plasmid can rescue *ts18* (5, 12) but not complement it in this assay (Table 1) argue against a role for recombination in the growth of *ts18* at 39.5°C in this system.

Sandri-Goldin et al. (20) previously demonstrated complementation of *ts18* by cell lines containing stably incorporated sequences from pSG18. The system presented here provides a new approach to the study of this and other cloned viral genes. In this system the functional properties of the gene product expressed from mutated plasmids can be tested without establishing stable cell lines or reintroducing the gene into the viral genome.

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