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Construction and Characterization of a Recombinant Herpes Simplex Virus Type 1 Which Overexpresses the Transrepressor Protein ICP0R

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ICP0R is a truncated form of the herpes simplex virus type 1 (HSV-1) transactivator protein ICP0 that is synthesized at low levels during infection through an alternative splicing mechanism. In transient expression assays, ICP0R has been shown to inhibit the transactivation function of several HSV-1 regulatory proteins, suggesting that an antiviral strategy which alters normal ICP0 mRNA splicing and thereby stimulates the synthesis of ICP0R protein may have potential in suppressing HSV-1 infections. To explore the feasibility of this approach, a recombinant virus was constructed which expressed high levels of ICP0R instead of ICP0. Surprisingly, overexpression of the ICP0R protein in this virus, HSV-KST, had no detectable effect on virus replication, since the growth properties of HSV-KST were indistinguishable from those of the ICP0/ICP0R null mutant dl1403, and HSV-KST was no more efficient than dl1403 at inhibiting the replication of an ICP0-expressing wild-type virus. The absence of a demonstrable phenotype in HSV-KST was not due to the acquisition of an inactivating mutation in the gene encoding ICP0R, since copies of the gene rescued from this virus retained full transrepression capability in transient expression assays. These results indicate that the ability of ICP0R to act as a transrepressor is significantly reduced if not completely eliminated in the context of a productive HSV-1 infection and suggest that this protein may not represent an exploitable target for the development of novel antiviral therapies.

ICP0 (infected cell polypeptide 0) is an immediate early polypeptide encoded by herpes simplex virus type 1 (HSV-1) that represents a key regulatory protein in the life cycle of this pathogen. ICP0 is not absolutely essential for replication in cell culture; however, mutant viruses containing deletions of the gene encoding ICP0 do exhibit an impaired ability to replicate in low multiplicity infections, as exemplified by reduced virus yields and inefficient expression of all classes of viral genes (Sacks and Schaffer, 1987; Stow and Stow, 1986). These defects are particularly pronounced in experimental situations involving *de novo* virus production from viral DNA in the absence of the virion transactivator protein VP16, including transfection of naked viral DNA into cells (Cai and Schaffer, 1989), reactivation from latent infections in trigeminal ganglia explant cocultivation experiments (Cai *et al.,* 1993; Clements and Stow, 1989; Leib *et al.,* 1989), and reactivation from in vitro latency models (Harris *et al.,* 1989; Zhu *et al.,* 1990). Consistent with its proposed role in upregulating viral genes during productive infection and reactivation from latency, ICP0 has been shown to behave as a potent transactivator in transfection assays, where it activates a wide variety of promoters in a manner that is independent of specific promoter elements (Everett, 1984; Gelman and Silverstein, 1985; Mosca *et al.,* 1987; O'Hare and Hayward, 1985; Ostrove *et al.,* 1987; Quinlan and Knipe, 1985; Sekulovich *et al.,* 1988; Shapira *et al.,* 1987). The mechanism by which ICP0 mediates transactivation has not yet been determined, but is likely to involve interactions with one or more host cell proteins, including a 135-kDa cellular protein of unknown function (Meredith *et al.,* 1995, 1994) and a complex nuclear structure called the ND10 domain (Everett and Maul, 1994; Maul and Everett, 1994; Maul *et al.,* 1993).

The mRNA encoding ICP0 is spliced, such that its protein-coding sequences are contained within three exons (Fig. 1) (Perry *et al.,* 1986). Splicing of the primary mRNA occurs with high efficiency during productive infection, and translation of this fully processed transcript yields a 775-amino-acid polypeptide with an apparent molecular weight of 110 kDa (Fig. 1). However, alternative splicing of the primary transcript can occur at low frequencies to yield a processed mRNA in which the second intron has been retained (Everett *et al.,* 1993). This results in the synthesis of a novel truncated 41-kDa polypeptide which contains all 241 amino acids encoded by exons 1 and 2 of the ICP0 gene plus an additional 21 amino acids resulting from readthrough into the second intron (Fig. 1). This protein was first characterized in transient expression assays, where it was found to behave as a potent dominant negative repressor of ICP0 transactivation and

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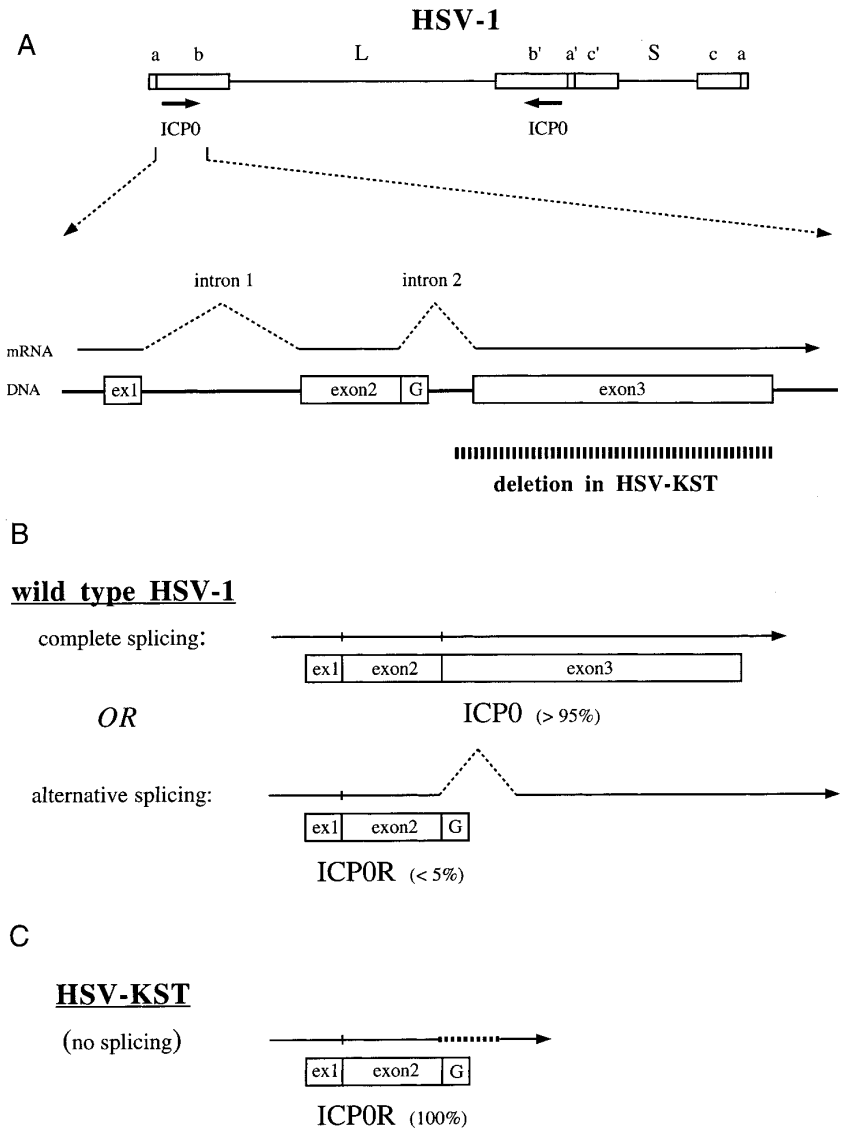

FIG. 1. Expression of the ICP0 and ICP0R proteins in HSV-1. (A) Organization of the ICP0 gene of HSV-1. A map of the HSV-1 genome is shown at the top which identifies the long (L) and short (S) components; the inverted repeat sequences *a*, *b*, and *c*; and the location of the two copies of the ICP0 gene in the *b* sequences. The primary transcript and the intron–exon structure of the ICP0 gene are shown below. The sequences deleted from the HSV-KST genome, which included the splice acceptor site of intron 2 and the entire coding sequences of exon3, are indicated at the bottom. Note that since the latency-associated transcript overlaps these deleted sequences, HSV-KST is technically an ICP0/LAT double mutant. (B) Generation of the ICP0R protein through alternative splicing of the primary mRNA transcript of the ICP0 gene. The two potential splicing products of the primary mRNA transcript of the ICP0 gene, their intron–exon structures, and their relative frequencies in HSV-1 infected cells [based on (Everett *et al.,* 1993)] are shown. Complete splicing of the mRNA yields the full-length protein ICP0, which is encoded by all three exons of the gene. A failure to splice out intron 2 yields the truncated protein ICP0R, which is encoded by the first two exons of the gene plus a short stretch of unspliced intron 2 sequence that is rich in glycine codons ("G"). (C) Forced expression of the ICP0R protein in HSV-KST. The predicted mRNA transcript and intron–exon structure of the mutated ICP0 gene of HSV-KST are shown. Note that only expression of ICP0R and not ICP0 is possible in this virus, since splicing of the intron 2-encoded carboxy-terminal sequences of ICP0R are directly prevented by the removal of the splice acceptor site of intron 2, and the exon 3 coding sequences of ICP0 have been completely deleted from the viral genome.

was therefore designated ICP0R (Weber *et al.,* 1992). However, like other dominant negative mutants of ICP0 to which it is structurally very similar (Weber and Wigdahl, 1992), ICP0R is able to promiscuously inhibit transactivation by not only ICP0 but also other HSV-1 regulatory proteins such as ICP4 and VP16. It was subsequently detected in HSV-1-infected cells at levels that were 5% or less than those of the full-length ICP0 protein (Everett *et al.,* 1993). The mechanism by which ICP0R mediates transrepression in transient expression assays is unknown; however, the results of a recent mutational analysis indicate that sequences encompassing a RING finger metal binding motif (Freemont *et al.,* 1991; Lovering *et al.,* 1993) encode a domain critical for this function (Spatz *et al.,* 1996). It is likely that this domain interacts with a host cell factor(s) which is critical for transactivation by

HSV-1 regulatory proteins, so that transrepression results from the titration or sequestration of this factor by ICP0R.

The role that ICP0R plays in the HSV-1 life cycle is unclear, since a mutant virus which expressed ICP0 but not ICP0R was found to exhibit normal replication in cell culture and normal infections in mice (Everett, 1991; Natarajan *et al.,* 1991). Although this mutant was demonstrated to reactivate normally from latency in mouse trigeminal ganglia explant cocultivation experiments (Natarajan *et al.,* 1991), it has not yet been examined in more relevant animal models which assess the capability for reactivation *in vivo.* Moreover, the inefficiency of the alternative splicing reaction that is required for ICP0R expression makes it impossible to assess what effect higher concentrations of this protein would have on HSV-1 replication. This question is of particular relevance from a therapeutic standpoint, since novel antiviral strategies have been proposed which involve upregulation of the expression of this transrepressor protein (Weber *et al.,* 1992). To address these issues, a recombinant virus was constructed which contained a deletion that prevented splicing of the intron 2 sequences from the primary transcript of ICP0. This manipulation was designed to force HSV-1 to synthesize high levels of ICP0R instead of ICP0 (Fig. 1), so that the influence of this protein on virus replication could be readily addressed.

The source of this deletion was the plasmid pKST, which had been used in the original characterization of the ICP0R protein in transfection assay experiments (Weber *et al.,* 1992). This construct contained a mutant ICP0 gene in which all sequences downstream of the *Sau*3AI site of intron 2, including the intron 2 splice acceptor site, exon 3 coding sequences, and polyadenylation signals of ICP0, were replaced by the polyadenylation signals of the SV40 early transcription unit. Since this mutation eliminated splicing of intron 2, the ICP0 gene in pKST expressed ICP0R instead of ICP0 (Weber *et al.,* 1992). To construct a recombinant HSV-1 which similarly expressed ICP0R instead of ICP0, the mutation in pKST was incorporated into the HSV-1 genome through the use of the marker transfer vehicle pKST-BX/SP. pKST-BX/SP was constructed in two steps: first, the 1.0-kb *Bam*HI/*Xba*I fragment of pKST (Weber *et al.,* 1992) was inserted into the *Bam*HI and *Xba*I sites of pUC19 to yield plasmid pKST-BX. The 2.2-kb *Sal*I/*Pst*I fragment of pIGA15 (Gelman and Silverstein, 1985) was then inserted into the *Sal*I and *Pst*I sites of pKST-BX to yield plasmid pKST-BX/SP. This final construct therefore contained all of the ICP0 sequences from the *Bam*HI site in intron 1 to the *Pst*I site downstream of the gene, with an internal deletion in which the sequences from the *Sau*3AI site of intron 2 to the *Sal*I site mapping immediately upstream of the ICP0 polyadenylation signals were replaced by a short stretch of polylinker from pKST containing *Sst*I and *Xba*I sites. The ICP0 gene in pKST-BX/SP therefore contained a mutation that is essentially identical to the one in pKST, except that it now utilizes the native polyadenylation signals of the ICP0 gene rather than those of the SV40 early transcription unit (Fig. 1). Moreover, the sequences upstream of the *Bam*HI site in intron 1 of the ICP0 gene had been intentionally deleted in pKST-BX/SP to prevent this plasmid from expressing ICP0R during marker transfer transfection, which had been previously shown to severely inhibit progeny virus production (Weber *et al.,* 1992). However, successful incorporation of pKST-BX/SP into the HSV-1 genome should restore these upstream sequences and thereby confer the ability to overexpress ICP0R protein.

The ICP0 null mutant dl1403 (Stow and Stow, 1986) (generously provided by R. Everett, MRC Virology Unit, Glasgow, UK) was chosen for use as the parental virus in marker transfer experiments instead of wild-type HSV-1, since it was reasoned that any reduction in replicative ability conferred by the overexpression of ICP0R protein might be minimized if the parental virus was already unable to make a functional ICP0 protein. The dl1403 genome contained a deletion of ICP0 sequences between the *Xho*I site of exon 2 and the *Sal*I site of exon3, while the pKST-BX/SP plasmid contained a deletion between the *Sau*3AI site of intron 2 and the *Sal*I site of exon 3, so that successful marker transfer was predicted to result in the net acquisition of 0.5 kb of ICP0 sequences between the *Xho*I and *Sau*3AI sites (Fig. 2A). To construct the desired recombinant virus, a mixture of 5 μg of cesium chloride gradient-purified pKST-BX/SP plasmid DNA that had been linearized with *Pst*I and 10 μg of cesium chloride gradient-purified dl1403 genomic DNA was cotransfected into Vero cells using calcium phosphate precipitation and a 15% glycerol shock as described previously (Weber *et al.,* 1988). DNA from individual plaques of the resulting progeny virus were dot blotted onto nylon membranes and probed with the radiolabeled 0.5-kb *Xho*I–*Xba*I fragment of pKST-BX/SP to screen for recombinant viruses that had successfully acquired these plasmid sequences. Virus stocks from hybridization-positive plaques were then amplified and subjected to further rounds of plaque purification and dot blot analysis. Three independently constructed versions of the desired recombinant virus were eventually isolated and characterized. Since all three possessed properties that were essentially identical (unpublished observations), the characterization of only one of these viral recombinants, HSV-KST, will be presented in this study.

The incorporation of the deletion in the pKST-BX/SP plasmid into the genome of HSV-KST was confirmed by extensive restriction site mapping and Southern blot analysis of viral DNA (unpublished observations); two examples of this characterization are illustrated in Fig. 2. The *Bam*HI–*Pst*I fragment of the ICP0 gene of wild-type HSV-1 (strain 17) is 4.6 kb in size (Fig. 2A). The corresponding fragment in dl1403 is predicted to be only

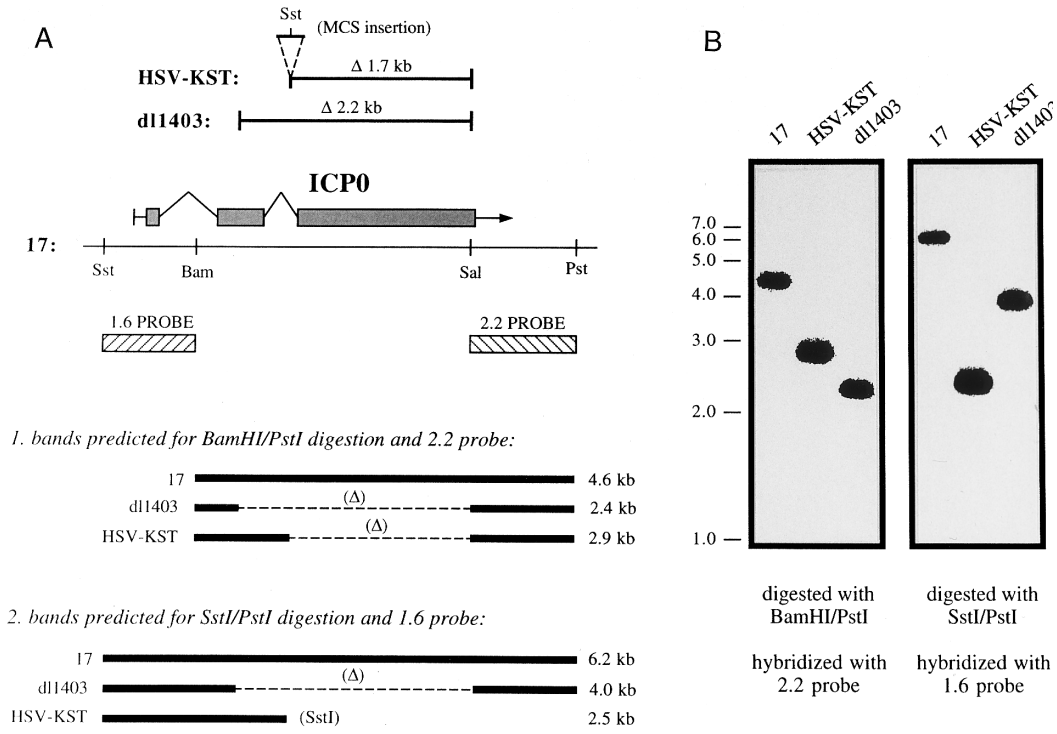

FIG. 2. Southern blot analysis of the genome structure of HSV-KST. (A) Restriction map of the ICP0 gene. The restriction map of the ICP0 gene of wild-type HSV-1 (strain 17) is shown at the top and includes sites for *Bam*HI, *Pst*I, *Sal*I, and *Sst*I. The locations and sizes of the deletions introduced into the HSV-KST and dl1403 genomes are indicated above the restriction map; note that the sequences deleted in HSV-KST are replaced by a novel *Sst*I site derived from the multicloning site (MCS) of a plasmid vector. The two restriction fragments that were radiolabeled and used as probes in Southern blot hybridizations are identified below the restriction map; these include the 1.6-kb *Sst*I/*Bam*HI fragment ("1.6 probe") and the 2.2-kb *Sal*I/*Pst*I fragment ("2.2 probe"). The locations and sizes of *Bam*HI/*Pst*I and *Sst*I/*Pst*I restriction fragments from each virus that are predicted to hybridize with the 2.2 and 1.6 probes, respectively, are shown at the bottom. (B) Southern blot analysis of the HSV-1 ICP0 gene. Genomic DNA of wild-type HSV-1 (strain 17), HSV-KST, and dl1403 was digested with the indicated restriction enzymes, blotted, and hybridized with the indicated probes. Autoradiography was performed using a Betagen phosphoimager, and the final image was adjusted to allow for subtraction of background signal. A molecular weight scale (in kb) is included at the left.

2.4 kb, due to the presence of a 2.2-kb deletion in the ICP0 gene in this virus. As discussed earlier, the transfer of the sequences in pKST-BX/SP into the dl1403 genome should reduce the size of this deletion by 0.5 kb; thus, the *Bam*HI–*Pst*I fragment of HSV-KST is predicted to be 2.9 kb in size (Fig. 2A). Southern blot analysis of *Bam*HI–*Pst*I-digested DNA from HSV-1 (strain 17)-, dl1403-, and HSV-KST-infected cells demonstrated the existence of the predicted 4.6-, 2.4-, and 2.9-kb fragments, respectively (Fig. 2B).

Additional corroboration of the genomic structure of HSV-KST was provided by analysis of *Sst*I–*Pst*I digests of viral DNA. This pair of restriction enzymes is predicted to generate a 6.2-kb ICP0 gene-containing fragment in wild-type HSV-1 (strain 17), but only a 4.0-kb fragment in dl1403, due to the presence of its 2.2-kb deletion (Fig. 2A). The corresponding fragment in HSV-KST would again be expected to be 0.5 kb larger than that of dl1403, or 4.5 kb, were it not for the acquisition of a novel *Sst*I site in the second intron of the ICP0 gene from pKST-BX/SP. This new site splits the 4.5-kb *Sst*I–*Pst*I fragment into a 2.5-kb *Sst*I fragment and a 2.0-kb *Sst*I–*Pst*I fragment (Fig. 2A). Hybridization of *Sst*I–*Pst*I-digested DNA from HSV-1 (strain 17)-, dl1403-, and HSV-KST-infected cells with a radiolabeled fragment corresponding to the 5′ end of the ICP0 gene confirmed the presence of the predicted 6.2-kb *Sst*I–*Pst*I, 4.0-kb *Sst*I–*Pst*I, and 2.5-kb *Sst*I fragments in these viruses, respectively (Fig. 2B).

Final verification of the structure of the HSV-KST genome was provided by the direct cloning of the ICP0R gene from viral DNA as a 2.4-kb *Not*I–*Xba*I fragment into the 3.0-kb vector-containing fragment of pHXT (Weber and Wigdahl, 1992). The resulting construct was identical in structure to pKST, so that the properties of this rescued gene could be directly compared to those of the original ICP0R gene in transfection assays. Two independent isolates of this rescued gene, pKST-resc-1 and pKST-resc-2, were characterized extensively by restriction site mapping and found to be indistinguishable from pKST (unpublished observations). This not only provided the definitive verification of the genomic structure of HSV-KST, but also demonstrated that a recombinant HSV-1 could be engineered to express the transrepressor protein ICP0R instead of the transactivator protein ICP0 and still be propagated in cell culture. However, it was still formally possible that the ICP0R gene in HSV-KST had acquired an

TABLE 1

Transrepression Capabilities of ICP0R Genes Rescued from HSV-KST

Plasmids cotransfected with pTAAT-CAT[a]	CAT activity (units $\times 10^{-3}$)[b]	Transactivation (fold increase)[c]	Transactivation (% of pHXT control)[d]
None	0.77 (0.05)	1.0 (0.1)	0 (1)
p111 + pHXT	6.37 (1.04)	8.3 (1.4)	100 (19)
p111 + pKST	1.05 (0.25)	1.4 (0.3)	5 (4)
p111 + pKST-resc-1	1.09 (0.09)	1.4 (0.1)	6 (2)
p111 + pKST-resc-2	1.15 (0.19)	1.5 (0.3)	7 (3)

[a] Vero cells were transfected in triplicate with equimolar amounts of the indicated plasmid constructs using Lipofectin reagent (GIBCO-BRL Life Technologies, Gaithersburg, MD) as described previously (Spatz *et al.,* 1996).

[b] Cells were lysed 48 hr posttransfection and extracts prepared using a detergent extraction procedure (Weber and Wigdahl, 1992). The extracts were then analyzed in CAT assays where the *n*-butyrylated chloramphenicol products were extracted with mixed xylenes and quantified by liquid scintillation (Weber and Wigdahl, 1992).

[c] Transactivation of pTAAT-CAT by the ICPO gene in p111 as measured by the fold increase in CAT activity over that of pTAAT-CAT transfected alone.

[d] Transactivation of pTAAT-CAT by the ICPO gene in p111 interpolated from a scale where the control transfections of pTAAT-CAT + p111 + pHXT and pTAAT-CAT alone represent 100 and 0% transactivation, respectively.

inactivating point mutation during earlier passages of the virus; this would yield a rescued gene that was indistinguishable from pKST by simple restriction site mapping. Moreover, the previous finding that ICP0R can act as a potent transrepressor of HSV-1 gene expression (Weber *et al.,* 1992) suggested that overexpression of a functional ICP0R protein was likely to have a negative effect on virus replication, so that faster growing variants of HSV-KST expressing inactive forms of this polypeptide may very well have arisen during plaque purification of virus stocks.

Since it was of interest to ascertain whether the rescued ICP0R genes encoded proteins that were indeed nonfunctional, these plasmids were examined for their ability to repress ICP0-mediated transactivation in transfection assays. A recently developed transient transrepression assay (Spatz *et al.,* 1996) was employed which utilized cotransfection of the reporter plasmid pTAAT-CAT, which contains the promoter of the HSV-1 immediate early gene ICP4 fused to the chloramphenicol acetyltransferase (CAT) gene (Weber and Wigdahl, 1992); the ICP0R-expressing plasmid pKST (Weber *et al.,* 1992); and the ICP0-expressing plasmid p111 (Everett, 1987). Control transfections in which the ICP0R null mutant-encoding plasmid pHXT (Weber and Wigdahl, 1992) was substituted for pKST determined the level of transactivation by ICP0 that would be expected to occur in the presence of a defective ICP0R protein; an eightfold induction of the ICP4 promoter in pTAAT-CAT was observed in these control experiments (Table 1). However, this induction was reduced by 95% in transfections in which p111 was cotransfected with pKST; more importantly, a comparable inhibition of transactivation was observed when p111 was cotransfected with either of the two ICP0R genes that had been rescued from the HSV-KST genome, pKST-resc-1 and pKST-resc-2 (Table 1). Thus, the transrepression-competent phenotype of these plasmids confirmed that the ICP0R gene which had been incorporated into the HSV-KST genome had not been inactivated by a point mutation during the construction of this virus and that HSV-KST should express a fully functional ICP0R protein.

To confirm that the mutation that was introduced into the HSV-KST genome did enable this virus to produce high levels of ICP0R instead of ICP0, extracts from Vero cells that had been infected for 18 hr with either HSV-KST or its dl1403 parent or wild-type HSV-1 (strain 17) were examined for the levels of ICP0R protein detectable by Western blot analysis (Fig. 3A). The blotted lysates were probed with the ICP0 exon 2-specific monoclonal antibody 11060 (a generous gift from R. Everett, MRC Virology Unit, Glasgow, UK), which had been previously shown to recognize the ICP0R protein in HSV-1-infected cells (Everett *et al.,* 1993). Analysis of extracts from cells that had been infected with HSV-KST revealed specific recognition of a prominent 41-kDa protein by the 11060 antibody (Fig. 3A, lane 3). The molecular weight of this species was comparable to that reported previously for ICP0R in wild-type HSV-1-infected cells (Everett *et al.,* 1993), and its identity as ICP0R was confirmed by the absence of this protein in lysates from cells infected with dl1403 (Fig. 3A, lane 2).

In contrast, a 110-kDa protein was readily apparent in lysates from wild-type HSV-1 (strain 17)-infected cells (Fig. 3A, lane 1) that was not detected in cells infected with either HSV-KST or dl1403; the identity of this protein as ICP0 was consistent with the absence of the ICP0 exon 3 coding sequences in the latter two mutants. Interestingly, the dl1403 virus synthesized low levels of a 32-kDa protein which reacted with the 11060 monoclonal antibody (Fig. 3A, lane 2). Although the existence of such a polypeptide had been predicted in the original characterization of this virus, it had never been detected (Stow

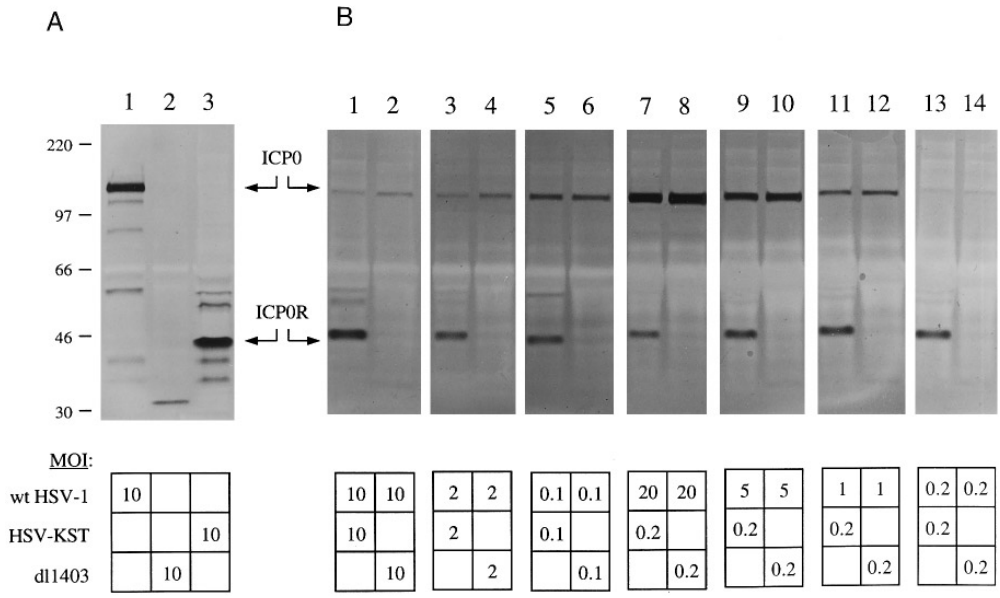

FIG. 3. Western blot analysis of ICP0 and ICP0R protein expression in HSV-1-infected cells. Extracts from Vero cells that were infected individually with wild-type HSV-1 (strain 17), HSV-KST, or dl1403 (A) or with the indicated mixtures of these viruses (B) were prepared, blotted, and probed with the ICP0 exon 2-specific monoclonal antibody 11060 (Everett *et al.,* 1993) as described previously (Spatz *et al.,* 1996). The multiplicity of infection employed for each virus is indicated below each lane. A molecular weight scale is included at left. The molecular weights of ICP0 and ICP0R are 110 and 41 kDa, respectively; both are indicated by the arrows between the panels. The group of negatively staining bands of 50–70 kDa molecular weight corresponds to residual fetal bovine serum proteins (unpublished observations).

and Stow, 1986). This 32-kDa protein is likely to represent the product of the mutated ICP0 gene in dl1403, whose deletion fuses the first 105 codons of ICP0 to 56 codons derived from noncoding sequences downstream of the gene. Consistent with its lack of function in dl1403 infections, this 32-kDa protein failed to mediate any detectable effects on reporter gene expression in transient expression assays (unpublished observations).

Although wild-type HSV-1 (strain 17) has been previously demonstrated to synthesize low levels of ICP0R through alternative splicing of the ICP0 mRNA (Everett *et al.,* 1993), no ICP0R protein was detected for this virus in these experiments (Fig. 3A, lane 1). This was because only trace amounts of ICP0R are synthesized when Vero cells are employed as the host cells for infection (Everett *et al.,* 1993; unpublished observations), and even these low levels were visible only if the infection was allowed to proceed past 24 hr (unpublished observations). Thus, the HSV-KST mutant was capable of synthesizing dramatically higher quantities of the ICP0R protein than wild-type HSV-1 (strain 17). In fact, the level of ICP0R that was generated during HSV-KST infections was comparable to that of ICP0 in wild-type HSV-1 (strain 17) infections (Fig. 3A); moreover, the kinetics of expression of these two proteins by their respective viruses were found to be essentially identical (unpublished observations). These observations demonstrated that the deletion introduced into HSV-KST had indeed been successful in forcing this virus to synthesize ICP0R instead of ICP0.

Once it had been demonstrated that the HSV-KST recombinant overexpressed a functional ICP0R protein, it was then of interest to determine what effect this had on virus replication. Since ICP0R can act as a powerful repressor of several HSV-1 transactivator proteins in transient expression assays (Weber *et al.,* 1992; Weber and Wigdahl, 1992), it was anticipated that the expression of high levels of this protein during infection would have a dramatic effect on virus replication. The growth properties of HSV-KST in both high and low multiplicity infections were therefore examined and compared with those of its dl1403 parent and wild-type HSV-1 (strain 17). Vero cells were infected with each virus at a multiplicity of infection of either 10 or 0.01; after 24 and 48 hr, respectively, the cells were frozen and thawed, and the titers of virus in the resulting supernatants were determined (Table 2).

The dl1403 virus produced yields that were just 10-fold lower than those of wild-type HSV-1 (strain 17) in high multiplicity infections, but 5500-fold lower in low multiplicity infections (Table 2). These results were representative of the multiplicity-dependent defect that would be expected for a virus which is incapable of expressing ICP0 and were comparable to those that have been reported previously for dl1403 and other ICP0 null mutants (Sacks and Schaffer, 1987; Stow and Stow, 1986). Since it not only fails to synthesize ICP0 but also overexpresses the transrepressor protein ICP0R, it was expected that the replicative abilities of HSV-KST would be even more impaired than those of dl1403. However, the yields of these two viruses were virtually indistinguishable (Table

TABLE 2

Replicative Abilities of HSV-KST

Virus	Yield (PFU/ml)[a] 24-hr infection (m.o.i. = 10)	48-hr infection (m.o.i. = 0.01)
HSV-1 (strain 17)	2.0×10^7 ($\pm 2.0 \times 10^6$)	2.7×10^7 ($\pm 1.1 \times 10^6$)
dl1403	1.6×10^6 ($\pm 3.0 \times 10^5$)	5.0×10^3 ($\pm 2.4 \times 10^3$)
HSV-KST	2.5×10^6 ($\pm 2.1 \times 10^5$)	4.6×10^3 ($\pm 3.9 \times 10^2$)

[a] Yield represents the average number of plaque-forming units/ml generated in triplicate infections of 1×10^6 Vero cells in 6-well dishes using the indicated multiplicities and lengths of infection.

2). In agreement with these results, there were no apparent differences in the efficiency of gene expression between the two viruses, based on Western blot quantification of representative immediate early, early, and late viral proteins synthesized during infection (unpublished observations).

The overexpression of ICP0R protein during HSV-KST infection therefore appeared to have no detectable effect on the replication of this virus. This result was particularly surprising for the yield experiments that had been carried out at a high multiplicity of infection, since significantly elevated levels of ICP0R accumulation had been detected under these conditions (Fig. 3A). However, defects involving the ICP0 protein have been shown to be dramatically more pronounced in *de novo* virus infections, that is, infections in which progeny virus is produced from naked DNA in the absence of the virion transactivator protein VP16 (Cai and Schaffer, 1989). It was therefore possible that the presence of VP16 in the virus yield determination experiments may have masked an inhibitory effect on replication mediated by the ICP0R protein. The growth properties of HSV-KST were therefore examined in *de novo* virus infections employing transfection of infectious cesium chloride gradient-purified viral DNA. Varying amounts of HSV-KST, dl1403, and wild-type HSV-1 (strain 17) genomic DNA were transfected into Vero cells and overlaid with media containing methylcellulose to allow plaque formation. After 3 days, the total numbers of infectious centers arising from each concentration of DNA transfected were determined.

The results obtained were plotted as the number of plaques per transfection versus concentration of DNA transfected and subjected to linear regression analysis. The linear range for each of the three viruses was determined to fall between 0.5 and 6 μg of DNA transfected; within these limits, the data generated lines with very good fit ($r > 0.93-0.99$, $P < 0.007-0.0003$) (Fig. 4). The slopes of these lines were then used to calculate the number of plaques generated per microgram of transfected DNA. Just as in infections employing intact virus particles, the ICP0 null mutant dl1403 exhibited a significant *de novo* replication defect compared to wild-type HSV-1 (strain 17), as evident by the 63% reduction in the number of infectious centers that it generates per microgram of DNA (Fig. 4). However, nearly identical slopes were obtained for the plots of the HSV-KST and dl1403 data (59 versus 79 plaques/μg DNA, respectively; Fig. 4). Moreover, no significant differences in plaque formation were observed between the two viruses at each individual concentration of DNA tested; in contrast, statistically significant differences were apparent between wild-type HSV-1 (strain 17) and either of the two mutants for each DNA concentration ($P < 0.05-0.02$). Thus, *de novo* virus production in HSV-KST and its dl1403 parent were virtually identical, indicating that overexpression of ICP0R during infection had no detectable effect on virus replication in the absence of VP16 transactivation.

The analyses presented above clearly demonstrate that overexpression of ICP0R does not appear to inhibit the replication of HSV-KST relative to its dl1403 parent. However, it is important to note that neither of these viruses express the ICP0 protein, whereas high levels of this transactivator are typically produced in a wild-type HSV-1 infection. The fact that normal HSV-KST replication could be observed in a high multiplicity infection (Table 2), which represents conditions where the absence of ICP0 has been shown to be of little if any consequence (Sacks and Schaffer, 1987; Stow and Stow, 1986), would seem to argue that this distinction may not be relevant. Nevertheless, it is formally possible that ICP0R may be able to exert a deleterious effect on virus replication only if the infection is actively utilizing the ICP0 transactivator protein. To test this hypothesis, mixed infections of wild-type HSV-1 (strain 17) and either HSV-KST or dl1403 were performed to determine whether elevated levels of the ICP0R protein selectively inhibited virus infections in which ICP0 was present.

In these experiments, Vero cells were coinfected with the wild-type and mutant viruses at varying multiplicities for 24 hr, at which time the culture supernatants were collected and lysates prepared from the remaining cells. The supernatants were used to determine the titers of wild-type progeny virus by selective enumeration of large plaques (Table 3), whereas the lysates were used in Western blot analyses to examine the relative concentrations of ICP0 and ICP0R proteins in these infections (Fig. 3B). Two sets of virus mixture strategies were employed in these experiments. In the first, Vero cells were coinfected with wild-type HSV-1 (strain 17) and either mutant at equal multiplicities of infection (10, 2, or 0.1). However, Western blot analyses of lysates of these cells revealed that the concentration of ICP0 protein in these infections was significantly reduced in the presence of a coinfecting virus, particularly at the higher multiplicities (Fig. 3B, lanes 1 to 6). This was likely due to the elevated particle to PFU ratio that has been described for ICP0-deficient

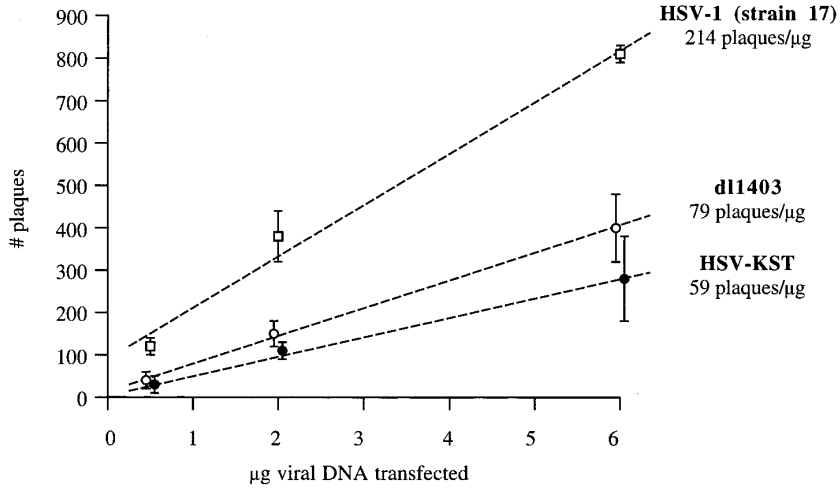

FIG. 4. *De novo* virus production assay. The number of infectious centers that resulted from transfection of the indicated amounts of wild-type HSV-1 (strain 17), HSV-KST, or dl1403 genomic DNA into Vero cells was plotted. Linear regression analysis was used to calculate the best fit lines for each set of data, and the slopes of these lines were used to calculate the numbers of plaques generated per microgram of transfected DNA that are listed for each virus.

mutants such as HSV-KST and dl1403 (Sacks and Schaffer, 1987; Stow and Stow, 1986): coinfection with equal PFUs of wild-type HSV-1 and an ICP0 null mutant actually results in a much higher particle number (and therefore mutant ICP0 gene dosage) for the latter virus, leading to a reduction in the number of genomes capable of ICP0 expression in the infected cell. Thus, a second mixing strategy was employed in which the mutant virus infection was held to a low multiplicity (0.2, which is still a high particle multiplicity for an ICP0 mutant virus), while the wild-type HSV-1 infection was carried out at increasing multiplicities (0.2, 1, 5, and 20). Western blot analyses of lysates of these cells indicated that this approach achieved a much greater variation in the relative levels of the ICP0 and ICP0R proteins (Fig. 3B, lanes 7 to 14) and even resulted in infections where the ICP0 protein was the predominant species (Fig. 3B, lane 7) or where the concentrations of the two proteins were equal (Fig. 3B, lane 9).

Previous studies have shown that such coinfections with wild-type HSV-1 and an ICP0 null mutant generally result in a modest decrease in titer for the former virus (Sacks and Schaffer, 1987). This is presumably due to a reduction in the available ICP0 transactivator protein caused by the difference in particle to PFU ratio between the two viruses, as discussed earlier, and is typically less than a single order of magnitude in size. In agreement with these earlier studies, coinfection with HSV-1 (strain 17) and dl1403 also resulted in a reduction in wild-type virus yield: in six of the seven virus mixtures that were tested, the titer of wild-type HSV-1 (strain 17) was reduced from 57 to 94% in the presence of coinfecting dl1403 when compared to an infection in the absence of the mutant (Table 3). However, this reduction in yield was not increased when HSV-KST was used as the coinfecting virus; in fact, no statistically significant differences in wild-type HSV-1 titers were detected for each of the seven mixtures of wild-type HSV-1/HSV-KST and wild-type HSV-1/dl1403 tested (Table 3). Consistent with these results, coinfection with HSV-KST did not appear to have any detectable effect on the efficiency of wild-type HSV-1 gene expression, based on Western blot quantification of representative immediate early, early, and late viral proteins synthesized during infection (unpublished observations). The only protein whose expression was affected by the presence of HSV-KST was ICP0 (Fig. 3B); however, comparable reductions in ICP0 expression were observed in coinfections with dl1403 for each virus mixture. As discussed earlier, this is likely the result of the high particle to PFU ratios in these viruses when compared to wild-type HSV-1. Thus, there was no apparent difference between the effect of HSV-KST or dl1403 on the replication of wild type HSV-1 (strain 17). From these results, it can be concluded that the overexpression of ICP0R protein not only failed to mediate a detectable inhibition of HSV-1 infection in the absence of ICP0, it had no obvious effect in the presence of this protein as well. These experiments formally eliminated the hypothesis that ICP0R might inhibit HSV-1 gene expression through direct interaction with the ICP0 protein; however, this possibility was already inconsistent with the previous demonstration that dominant negative mutants like ICP0R could mediate transrepression in the absence of ICP0 (Weber and Wigdahl, 1992) and with the failure to detect ICP0–ICP0R interactions through a variety of *in vitro* binding assays (unpublished observations).

The finding that overexpression of ICP0R did not appear to mediate any deleterious effect on the replicative abilities of HSV-KST was clearly unexpected, given the

TABLE 3

Effect of Coinfecting HSV-KST or dl1403 on Wild-Type HSV-1 (Strain 17) Replication

Virus mixture	Wild-type HSV-1 progeny titer (PFU/ml)[a]	Percentage of wild-type HSV-1 alone[b]
(A) $MOI_{wtHSV-1} = 10$, $MOI_{mutant} = 10$		
Wild-type HSV-1 alone	$3.9 (\pm 0.4) \times 10^5$	100
Wild-type HSV-1 + dl1403	$8.7 (\pm 1.7) \times 10^4$	22 (±4)
Wild-type HSV-1 + HSV-KST	$5.2 (\pm 0.6) \times 10^4$	13 (±1)
(B) $MOI_{wtHSV-1} = 2$, $MOI_{mutant} = 2$		
Wild-type HSV-1 alone	$1.4 (\pm 0.2) \times 10^5$	100
Wild-type HSV-1 + dl1403	$1.1 (\pm 0.1) \times 10^4$	8 (±1)
Wild-type HSV-1 + HSV-KST	$8.9 (\pm 3.7) \times 10^3$	6 (±3)
(C) $MOI_{wtHSV-1} = 0.1$, $MOI_{mutant} = 0.1$		
Wild-type HSV-1 alone	$1.1 (\pm 0.3) \times 10^4$	100
Wild-type HSV-1 + dl1403	$2.6 (\pm 0.8) \times 10^3$	23 (±7)
Wild-type HSV-1 + HSV-KST	$2.1 (\pm 0.1) \times 10^3$	18 (±1)
(D) $MOI_{wtHSV-1} = 20$, $MOI_{mutant} = 0.2$		
Wild-type HSV-1 alone	$1.0 (\pm 0.1) \times 10^6$	100
Wild-type HSV-1 + dl1403	$8.6 (\pm 2.2) \times 10^5$	83 (±22)
Wild-type HSV-1 + HSV-KST	$1.0 (\pm 0.1) \times 10^6$	98 (±6)
(E) $MOI_{wtHSV-1} = 5$, $MOI_{mutant} = 0.2$		
Wild-type HSV-1 alone	$3.6 (\pm 0.3) \times 10^5$	100
Wild-type HSV-1 + dl1403	$1.9 (\pm 0.3) \times 10^5$	51 (±8)
Wild-type HSV-1 + HSV-KST	$1.5 (\pm 0.1) \times 10^5$	43 (±1)
(F) $MOI_{wtHSV-1} = 1$, $MOI_{mutant} = 0.2$		
Wild-type HSV-1 alone	$1.7 (\pm 0.1) \times 10^5$	100
Wild-type HSV-1 + dl1403	$2.7 (\pm 0.1) \times 10^4$	16 (±1)
Wild-type HSV-1 + HSV-KST	$2.4 (\pm 0.5) \times 10^4$	14 (±3)
(G) $MOI_{wtHSV-1} = 0.2$, $MOI_{mutant} = 0.2$		
Wild-type HSV-1 alone	$2.9 (\pm 0.5) \times 10^4$	100
Wild-type HSV-1 + dl1403	$7.0 (\pm 2.8) \times 10^3$	24 (±10)
Wild-type HSV-1 + HSV-KST	$4.1 (\pm 0.5) \times 10^3$	14 (±2)

[a] Titer of wild-type HSV-1 progeny resulting from 24-hr infections with the indicated mixture of viruses as determined by selective enumeration of large plaques.

[b] Titer of wild-type HSV-1 present in coinfection progeny as a percentage of the titer of wild-type HSV-1 in the absence of coinfecting virus.

demonstrated ability of this protein to function as a powerful suppressor of viral gene expression in transfection assays (Weber *et al.,* 1992). The simplest interpretation of these results is that a fundamental difference exists between the ICP0R protein generated during HSV-KST infection and that from a transfected plasmid. The nature of this distinction is far from apparent, as immunofluorescence analysis indicated that ICP0R synthesized by either route has an identical localization within the cell, and additional experiments did not reveal any obvious differences in the stability or processing of this protein (unpublished observations). Although it is possible that infection by HSV-1 could alter the cellular environment so as to prevent transrepression by ICP0R, the finding that transient expression assay results are generally not influenced significantly by the presence of superinfecting virus (unpublished observations) would argue against this hypothesis.

One potential explanation for the puzzling behavior of viral expressed ICP0R involves the actual level of protein expressed during infection. Previous studies have indicated that transrepression can be achieved in the presence of very little ICP0R protein, based on the finding that functional wild-type ICP0R accumulates to levels that are significantly lower than those of its transrepression-defective mutants (Spatz *et al.,* 1996). However, the levels of ICP0R protein synthesized during infection by HSV-KST appear to be even lower still, since quantitative immunofluorescence and Western blot analyses have indicated that the amount of ICP0R protein present in HSV-KST-infected cells could be as much as 40-fold lower than that of pKST-transfected cells (unpublished observations). This finding suggests the intriguing possibility that transrepression requires a threshold level of ICP0R protein and that the ICP0R generated during HSV-KST infection fails to influence viral gene expression simply because it accumulates at concentrations that are well below this level. This is in fact consistent with the previous observation that a 20-fold dilution of a plasmid containing an ICP0 dominant negative mutant similar to ICP0R was found to eliminate transrepression completely (Weber *et al.,* 1992). Since the deletion engineered into the HSV-KST genome would be expected to generate the same level of ICP0R protein that would result if splicing of intron 2 from the normal ICP0 transcript were to be completely inhibited during infection, then even under condi-

tions of maximal alternative splicing, wild-type HSV-1 would never have the capacity to synthesize the amounts of ICP0R required for downregulation of gene expression. Thus, antiviral strategies which are designed to inhibit HSV-1 replication by disrupting intron 2 splicing would be effective only from the standpoint that they serve to reduce the available concentration of ICP0 transactivator, not because they act to create ICP0R transrepressor.

Characterization of the HSV-KST virus has so far failed to identify a specific function of the ICP0R protein during HSV-1 infection, and any evidence for its suspected role in the downregulation of viral genes was not generated by the results of this study. Nevertheless, the dramatic ability of ICP0R to mediate transrepression when overexpressed in transfection assays is a phenomenon whose underlying mechanism is likely to be of some relevance in understanding the molecular biology of HSV-1 gene expression. It is still plausable that this protein titrates a cellular factor required by HSV-1 regulatory proteins for transactivation and that continued studies of ICP0R may eventually reveal the identity of a host cell function critical for expression of HSV-1 genes. The phenomenon of transrepression itself is equally puzzling. Since the original description of dominant negative mutants of ICP0 (Weber *et al.,* 1992; Weber and Wigdahl, 1992), transdominant mutants of other RING finger-containing proteins have been described (Hay *et al.,* 1995; Le *et al.,* 1996; Moriuchi *et al.,* 1994). Further studies on how the overexpression of RING finger metal binding domains from a number of diverse sources mediates an inhibitory effect on cells will almost certainly provide valuable information on how this protein motif functions in such a wide variety of different contexts.

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