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The α protein ICP0 does not appear to play a major role in the regulation of herpes simplex virus gene expression during infection in tissue culture

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ABSTRACT

The herpes simplex virus type 1 (HSV-1) α protein ICP0 trans-activates HSV-1 early genes in transient expression assays. To investigate the function of ICP0 during HSV-1 infection, we have lowered the level of ICP0 by use of a recombinant plasmid that has been engineered to express the antisense message. Cell lines were constructed which stably carry the antisense plasmid. Total protein profiles from infected antisense cell lines showed that the level of ICP0 was reduced to less than 10% of the wild type level in two of the cell lines. However, reducing the level of ICP0 did not have a significant effect on the expression of HSV-1 early or late genes. The polypeptide patterns for the remaining infected cell polypeptides were similar in that no bands were absent although there were some quantitative differences. The level of two early proteins, glycoprotein B and glycoprotein D was reduced in one of the cell lines, however, levels were nearly equivalent to the control infection for two other cell lines tested. Virus yields were the same for the antisense cell lines and for parent cells. Decreased ICP0 levels did not lead to more restrictive phenotypes for an α 4 or α 27 mutant as protein patterns were similar for these mutants in antisense and parent cells. Therefore, while ICP0 has been demonstrated to be a strong inducer of gene expression in transient expression assays, it does not appear to have a major role as an activator during the productive infection of tissue culture cells.

INTRODUCTION

The genome of herpes simplex virus type 1 (HSV-1) encodes at least three sets of genes termed α, β and γ whose expression is coordinately regulated and sequentially ordered in a cascade fashion (1-5). The α or immediate early genes are expressed first after viral infection (1,3). The α genes include α 4 (IE 3), α 0 (IE 1), α 27 (IE 2), α 22 (IE 4) and α 47 (IE 5). The α proteins appear to be involved primarily in the regulation of the later classes of herpes genes, namely the β or early genes and the γ or late genes. The α 4 gene (IE 3) which encodes ICP4 (Vmw 175) has been shown through analysis of temperature sensitive and deletion mutants to be essential for early and late gene expression in HSV-1 infected cells (6-11). The α 27 and α 22 genes appear to be involved in late gene expression as γ gene products are severely diminished in infections with mutants defective in ICP27 or ICP22 (12,13), although a host cell factor can apparently substitute for the function of ICP22 in some cell lines (13). The functions of ICP47 and ICP0 during

infection have not been defined, although a mutant in which the α 47 gene was deleted was shown to grow normally in several cell lines tested (14). It has been demonstrated that ICP4 and ICP0 are strong trans-activators of early gene promoters in transient expression assays (15-18). Under these conditions, ICP4 and ICP0 can act synergistically thus increasing the expression of the indicator gene to greater levels than can be observed with either product alone (15-19). It is not known whether ICP0 functions in this role during infection, however. To determine the function of ICP0 during infection, we chose to use antisense mRNA to reduce the levels of ICP0. This approach, which was originally described by Izant and Weintraub (20,21), has been used successfully to inhibit gene expression in Dictyostelium (22), Xenopus oocytes (23,24), Drosophila (25,26), mammalian cells (20,21,27) and most recently in plants (28). In addition, antisense RNA regulation of gene expression has been reported to occur naturally in bacteria (29-35). The antisense approach was chosen to avoid difficulties involved in isolating mutants in the ICP0 gene. These difficulties are due to the fact that the ICP0 gene is present in two copies in the viral genome, whereas only one copy is necessary for viral replication (36). It would therefore be necessary to mutate both copies of the gene to see an effect.

In this paper we show that a recombinant plasmid which expresses an mRNA antisense to ICP0 RNA reduces the ICP0 trans-activation of two early indicator genes. We further show that in cell lines in which this plasmid is stably integrated, the levels of ICP0 are severely reduced upon infection. Despite the reduction in ICP0 levels however, there are no significant reductions in early or late gene expression or in viral production. From this we conclude that ICP0 does not play an important role in inducing the expression of early and late genes during productive HSV-1 infection in tissue culture.

MATERIALS AND METHODS

1. Bacteria, Cells and Viruses

Escherichia coli K-12 strain 1100 derivative DH-1 (recA1 hsdM$^+$ nalA96^r thi-1 endA1 supE44, 37) was used as the host for the propagation of all chimeric plasmids. Vero cells and rabbit skin fibroblasts (ATCC) were grown in Eagle minimal essential medium supplemented with nonessential amino acids, 100 μg of streptomycin per ml, 100 U of penicillin per ml and 10% fetal calf serum (GIBCO). Selection for G418 resistant colonies and growth of G418 resistant cell lines was done in the presence of 750 μg of G418 per ml of the above medium. Viruses used were HSV-1 (KOS), ts606 (38) and tsLG4 (39). Viruses were grown and assayed as described previously (40).

2. Plasmids Used and Construction of Recombinant Plasmids

The plasmid pTK-CAT consists of the promoter-regulatory sequences of the HSV-1 thymidine kinase gene fused to the gene encoding chloramphenicol acetyl transferase. It was constructed by inserting a Kpn I to Bgl II fragment of the tk gene from approxi-

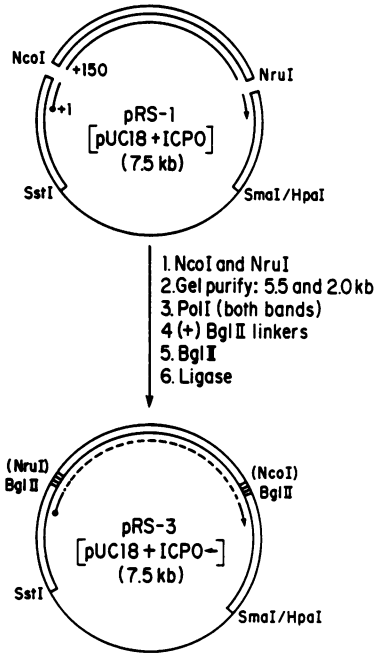

Figure 1. Construction of the antisense plasmid. The plasmid pRS-1 contains a 4.8 Kb Sst I to Hpa I fragment which encodes the ICP0 gene (coordinates 0.78 to 0.80) inserted into the Sst I and Sma I sites of pUC18. This plasmid was cut with Nco I and Nru I. The Nco I site is approximately 150 bp downstream from the transcriptional start site (45,46) and the Nru I site is in the middle of the ICP0 gene. Both the 2.0 Kb fragment and the vector were modified with Bgl II linkers and the fragment was reinserted in the opposite orientation. The resulting plasmid was designated pRS-3. Plasmids are not drawn to scale.

mately -650 to +50 (41) into the Hind III site of the promoterless pSV0-CAT which was obtained from Bruce Howard (42). The plasmid pgD-CAT contains the promoter-regulatory region of the HSV-1 glycoprotein D gene from an Sst I site at -700 to a Hind III site at +8 (43,44) in the Hind III site of pSV0-CAT. The plasmid pRS-1 consists of the 4.8 Kb Sst I to Hpa I fragment containing the ICP0 gene cloned into the vector pUC18 (Figure 1). To construct the antisense plasmid, pRS-1 was cut with Nco I and Nru I and the 2 Kb fragment and the remaining part of the plasmid were each gel purified. The Nco I site is approximately 150 bp downstream from the start site of transcription at the start of translation (45,46) and the Nru I site is in the middle of the ICP0 gene. Both fragments were treated with the Klenow fragment of Pol I and ligated to Bgl II linkers. After digestion with Bgl II, the 2 Kb fragment was ligated into the remaining plasmid and clones were checked for orientation of the fragment. The construct containing the

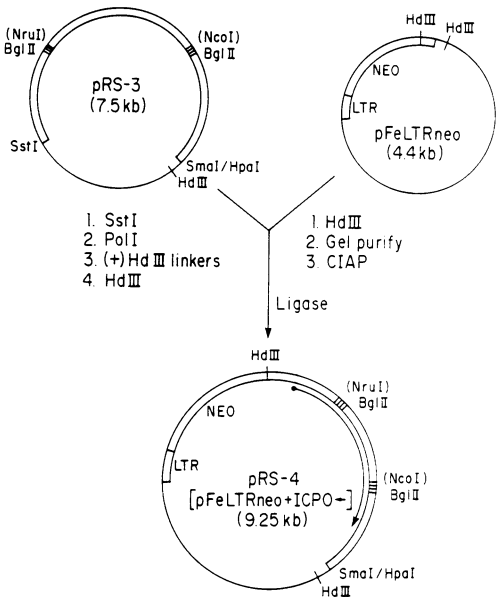

Figure 2. Construction of the antisense-neo plasmid. The plasmid pRS-3, which has a 2.0 kB fragment of the ICP0 gene from near the 5' end of the message to the middle of the gene in the reverse or antisense orientation, was cut with Sst I. This site was modified to a Hind III site and the plasmid was then digested with Hind III which also cut at a Hind III site in the polylinker of pUC18 downstream from the Hpa I/Sma I junction. The 4.8 Kb antisense fragment was inserted into the Hind III site of pFeLTR neo (47) which is 3' to the neo gene. The resulting plasmid was designated pRS-4. Plasmids are not drawn to scale.

fragment in the antisense or opposite orientation from pRS-1 was designated pRS-3 (Figure 1).

The plasmid pRS-4 containing the antisense ICP0 gene and the neo marker (Figure 2) was constructed by cutting pRS-3 with Sst I, treating with Klenow then ligating with Hind III linkers. The plasmid was then cut with Hind III which cut the Hind III linkers and also cut at a Hind III site in the polylinker of pUC18 downstream from the Sma I site. The ICP0 antisense fragment was then ligated into the vector pFeLTR neo which contains the neo gene under the control of the LTR from feline leukemia virus and was kindly provided by Nevis Fregien (47).

3. Transfection

For transient expression assays, subconfluent monolayers of rabbit skin fibroblasts (RSF) in 60 mm dishes were transfected with 5 μg of indicator plasmid and various amounts of pRS-1 or pRS-3 as described in the Figure legends. The total amount of DNA

in each transfection was adjusted to 25 μg by adding pUC18 DNA where necessary. Transfections were performed as described (48) and cells were shocked with 15% glycerol 4 hours after the addition of DNA. In most cases, cells were harvested 48 hours after transfection and CAT activity was assayed as described by Gorman et al. (42). When cells were infected with HSV-1 KOS, infections were performed at an moi of 1 at 24 hours after transfection and cells were harvested 16 hours after infection.

For the isolation of cell lines containing the antisense construct, both Vero cells and rabbit skin fibroblasts in 25 cm^2 flasks were transfected with 20 μg of pRS-4 DNA. After 24 hours, selection was initiated using medium containing 750 μg of G418. Colonies which were clearly visible by 3 weeks were picked and expanded. G418 resistant cells were screened for the presence of antisense sequences by Southern blot hybridization of cellular DNA as previously described (38,49).

4. Extraction of Proteins, Immunoprecipitation and Polyacrylamide Gel Electrophoresis

Vero cells, rabbit skin fibroblasts or antisense cell lines were infected with KOS, ts606 or tsLG4 at an moi of 1. Where KOS alone was used infections were performed at 37°C. In experiments with the ts mutants, infections were performed at 39.5°C. Four hours after infection, 20 μCi/ml of ^{35}S-methionine was added to infected cells which were harvested 12 hours later. Cells were scraped into PBS, centrifuged and resuspended in electrophoresis sample solution (50). Aliquots were electrophoresed on 10% polyacrylamide gels as described previously (50). When samples were to be immunoprecipitated, cells were resuspended in extraction buffer containing 1% nonidet 40 (50) and immunoprecipitations were performed as described by Holland et al. (50). Monoclonal antibodies to glycoprotein B and glycoprotein D were generously provided by J.C. Glorioso (University of Michigan, Ann Arbor).

RESULTS

Trans-activation of HSV-1 Early Genes by ICP0 Can Be Inhibited by Cotranfection with an ICP0-Antisense Plasmid.

It has been demonstrated that ICP0 can trans-activate early gene promoters in transient expression assays (15-19). We first determined whether RNA antisense to ICP0 could inhibit this function. As a source of antisense RNA, we constructed a plasmid in which a portion of the ICP0 gene from approximately 150 bp downstream from the start site of transcription at the start of translation (45,46) to a site near the middle of the gene was excised and reinserted in the opposite orientation (Figure 1). This results in a construct in which the mRNA is under the control of the ICP0 promoter-regulatory sequences and which would be polyadenylated correctly, however, a portion of the transcript from near the 5' end to near the middle of the coding sequences would be in the

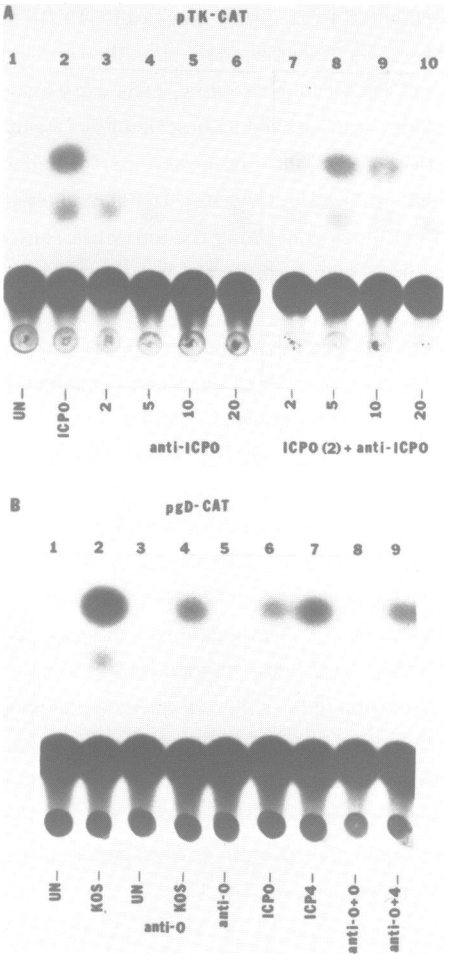

Figure 3. Activation of an early promoter by HSV-1 in the presence or absence of the ICP0 antisense plasmid. A) Rabbit skin fibroblasts (RSF) were transfected with pTK-CAT (5 μg/ml) either alone (lane 1) or with pRS-1 (2 μg/ml) which contains the ICP0 gene (lanes 2 and 7-10). The antisense ICP0 plasmid pRS-3 was added to the transfection mixes at 2 (lanes 3 and 7), 5 (lanes 4 and 8), 10 (lanes 5 and 9) or 20 μg/ml (lanes 6 and 10). The total concentration of DNA in each transfection was adjusted to 25 μg/ml by adding pUC18 DNA where necessary. Transfections were incubated for 48 hours at which time CAT activity was measured (42). B) RSF cells were transfected with pgD-CAT at 5 μg/ml. pRS-3 DNA at 20 μg/ml was added to transfections shown in lanes 3, 4, 5, 8 and 9. pRS-1 was added at 2 μg/ml to transfections shown in lanes 6 and 8. A plasmid containing the ICP4 gene (pSG28 K/B) was added at 2 μg/ml to transfections shown in lanes 7 and 9. Transfections in lanes 5-9 were incubated for 48 hours at which time CAT activity was assayed. In lanes 1-4, transfections were incubated for 24 hours, at which time cells were either mock infected or infected with HSV-1 KOS at an moi of 1. Cells were harvested 16 hours later and assayed for CAT activity.

opposite or antisense orientation to the authentic ICP0 mRNA. This plasmid was called pRS-3. In the experiment shown in Figure 3A, rabbit skin cells were transfected with an indicator plasmid containing the promoter region from the HSV-1 β gene encoding thymidine kinase linked to the chloramphenicol acetyltransferase gene (pTK-CAT). When pRS-1, a plasmid containing the ICP0 gene was cotranfected with pTK-CAT (lane 2), the level of CAT expression was induced relative to the constitutive level seen with pTK-CAT alone (lane 1). In this experiment pTK-CAT was added at 5 μg per plate and pRS-1 was added at 2 μg. When pRS-3 was cotranfected with pTK-CAT at concentrations of 2, 5, 10, or 20 μg per plate (lanes 3-7), no differences were seen in the low constitutive level of expression of pTK-CAT indicating that pRS-3 alone had no effect on pTK-CAT expression. However, when pRS-3, at a concentration of 20 μg, was added to transfections with pTK-CAT in the presence of pRS-1 (2 μg, lane 10), the level of pTK-CAT expression was equivalent to the uninduced level seen in the absence of ICP0 (lanes 1 and 3-6). Therefore, the presence of the antisense plasmid at a ratio of 10:1 to the sense plasmid significantly reduced the induction of pTK-CAT seen with ICP0 alone (lane 2). The combination of antisense to sense at ratios of 1:1, 2.5:1 or 5:1 still resulted in some induction of pTK-CAT expression by ICP0 (lanes 7-9). These results are consistent with other studies using antisense RNA which showed that the antisense to sense ratio must be high to inhibit function (27,28).

That the antisense plasmid specifically interfered with induction by ICP0 was demonstrated by the experiment shown in Figure 3B. When pRS-3 (20 μg) was added to transfections with the indicator plasmid pgD-CAT, containing the early gD promoter fused to CAT, and pRS-1 (2 μg), induction of gD-CAT expression was not observed (lane 8), although, ICP0 alone induced gD-CAT activity (lane 6). However, the presence of pRS-3 had no effect on induction of gD-CAT expression by ICP4 (lane 9) which stimulated CAT expression whether or not pRS-3 was present (lanes 7 and 9). Figure 3B also shows that pRS-3 significantly reduced trans-activation of gD-CAT by superinfection with HSV-1 KOS (compare lanes 2 and 4). This result implies that ICP0 specified by the virus is involved in the trans-activation of indicator genes seen upon HSV-1 infection as pRS-3 does not interfere with induction by ICP4.

The Level of ICP0 is Reduced in Cell Lines Which Stably Carry the Antisense Sequences.

The effect of the antisense RNA was presumably due to a decrease in the level of ICP0 protein translated from the sense message. To confirm that this was the case, we first isolated cell lines containing the antisense plasmid so that the entire cell population would express antisense RNA. As shown above, antisense levels must be high relative to sense levels to observe an effect. We considered it likely that this would be the case upon superinfection of antisense lines with HSV-1, because the ICP0 promoter was used

in the construction of pRS-3. HSV-1 is known to contain a trans-acting virion stimulatory protein which induces α gene expression (51-57). Trans-activation by superinfection of α chimeric genes stably carried in cell lines has been demonstrated to result in high levels of expression of the resident α genes (51,58-61). Therefore, cells were transfected with pRS-4 which contains the antisense ICP0 gene and the neo gene (Figure 2), and

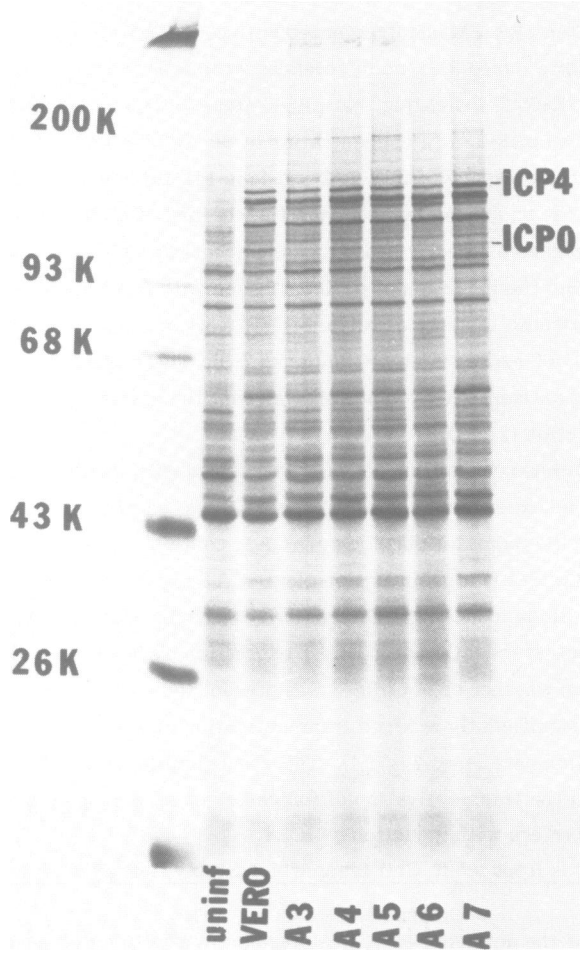

Figure 4. Analysis of infected cell polypeptide patterns in cell lines which stably carry the antisense plasmid. Vero cells and the antisense derivatives A3, A4, A5, A6 and A7 were infected with KOS at an moi of 1 or left uninfected (lane 1). Four hours later, 20 μCi of ^{35}S-methionine was added. Cells were harvested 12 hours later. Aliquots in electrophoresis sample solution (50) were run on a 10% SDS-polyacrylamide gel as described (50).

G418 resistant cell lines were isolated. Five G418 resistant cell lines which were derived from transfections with Vero cells, and two cell lines derived from transfections with rabbit skin fibroblasts (RSF) were found to contain between one and five copies of the plasmid pRS-4 integrated into the cellular genome by Southern blot hybridization analysis (data not shown).

To examine the level of ICP0 in these cells, Vero cells and the antisense cell lines A3, A4, A5, A6 and A7 were infected with KOS. ICP0 was identified on polyacrylamide gels on the basis of its apparent molecular weight (120 Kd). Figure 4 shows that ICP0 levels were reduced to different extents in each of the antisense cell lines compared to the control Vero cells. The greatest reductions were seen in cell lines A3 and A6 where ICP0 was reduced to 10% or less of the wild type level. This estimate was based on densitometer scans of appropriate exposures of the autoradiograph.

Since ICP0 has been been shown to trans-activate viral genes in transient expression assays, it seemed likely that it would have a role in the regulation of early or late gene expression during infection. To determine if this was the case, we also examined the expression of other viral polypeptides during infection of the antisense cell lines. The polypeptide patterns of the infected antisense cell lines appeared to be qualitatively

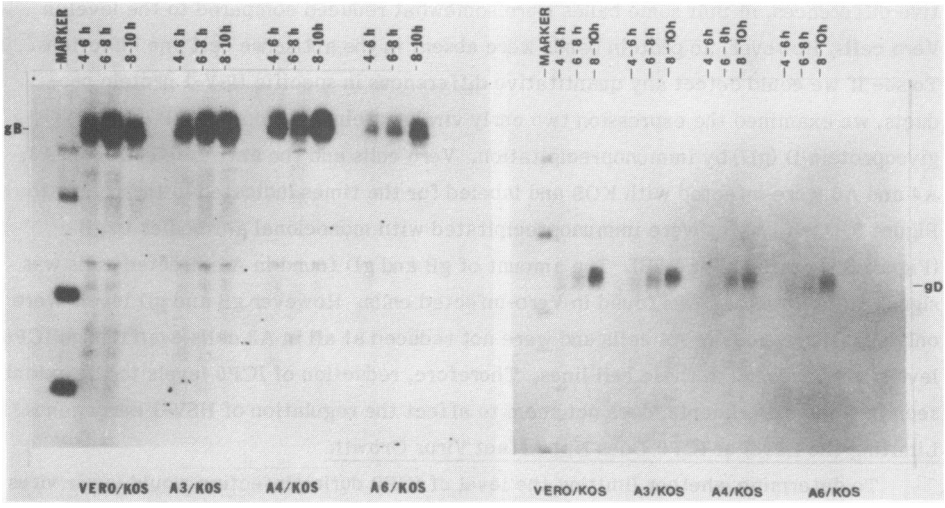

Figure 5. Immunoprecipitation of two HSV-1 early proteins from antisense infected cell lysates. Vero cells and antisense cell lines A3, A4 and A6 were infected with KOS at an moi of 1. ^{35}S-methionine (20 µCi/ml) was added either at 4, 6 or 8 hours post infection. Cells were harvested 2 hours after addition of label. Aliquots of infected cell lysates were immunoprecipitated with monoclonal antibody to A) glycoprotein B or B) glycoprotein D. Samples were electrophoresed in a 10% SDS-polyacrylamide gel.

Table 1
Measurement of Infectivity of HSV-1 KOS[1] on
Antisense Cells and Control Cells

	12 hours	24 hours
Vero	2.2×10^6	2.5×10^7
A3[2]	1.5×10^6	5.7×10^6
A4	1.1×10^6	9.1×10^6
A5	1.8×10^6	1.1×10^7
A6	1.0×10^6	6.9×10^6
A7	1.1×10^6	9.8×10^6
RSF	6.2×10^5	5.5×10^6
A1[3]	7.1×10^5	5.8×10^6
A2	7.6×10^5	6.7×10^6

[1] The multiplicity of infection was 1.

[2] A3-A7 cell lines were derived from Vero cells.

[3] A1 and A2 cell lines were derived from RSF cells.

similar to those for infected Vero cells (Figure 4). There did appear to be some quantitative differences, in that some bands were somewhat reduced compared to the level in Vero cells. However, no protein bands were absent in the antisense cell line infections. To see if we could detect any quantitative differences in specific HSV-1 protein products, we examined the expression two early viral proteins, glycoprotein B (gB) and glycoprotein D (gD) by immunoprecipitation. Vero cells and the antisense cell lines A3, A4 and A6 were infected with KOS and labeled for the times indicated in the legend to Figure 5. Cell lysates were immunoprecipitated with monoclonal antibodies to gB (Figure 5A) or gD (Figure 5B). The amount of gB and gD found in A6 infected cells was significantly less than was found in Vero-infected cells. However gB and gD levels were only slightly reduced in A4 cells and were not reduced at all in A3 cells even though ICP0 levels were reduced in these cell lines. Therefore, reduction of ICP0 levels to the extent seen in these experiments, does not seem to affect the regulation of HSV-1 early genes.

Limiting the Level of ICP0 Does Not Affect Virus Growth.

To determine whether limiting the level of ICP0 during infection would lower virus yields, the antisense cell lines A3, A4, A5 and A6, as well as the parental Vero cells, and cell lines A1 and A2 and parent RSF cells were infected with HSV-1 KOS at an moi of 1. Progeny virus were assayed at 12 and 24 hours after infection. Table 1 shows that there

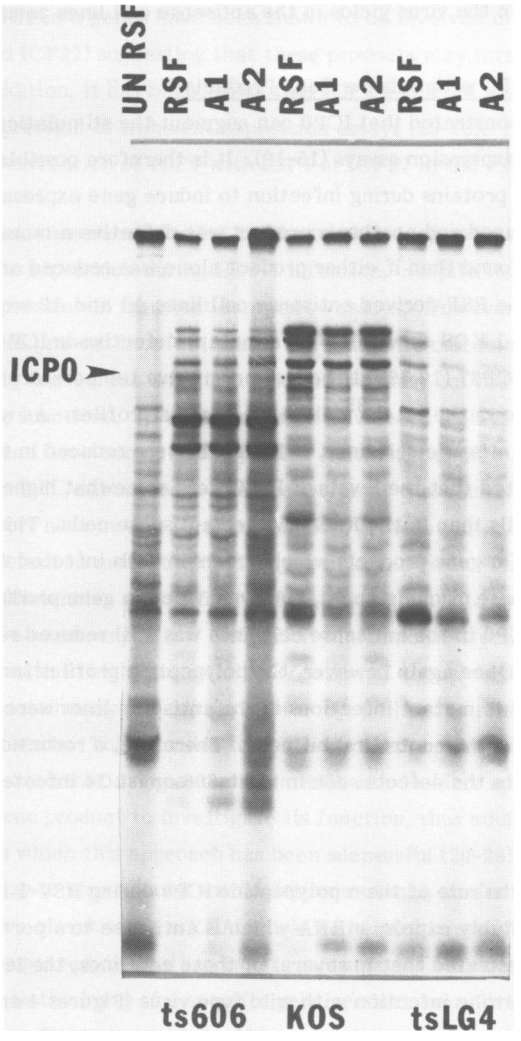

Figure 6. Polypeptide patterns from antisense cell lines infected with an ICP4 or ICP27 ts mutant. Rabbit skin fibroblasts and two derivative antisense cell lines A1 and A2 were infected with the ICP4 mutant ts606 (lanes 2-4), wild type KOS (lanes 5-7) or the ICP27 mutant tsLG4 (lanes 8-10) at an moi of 1. All infections were performed at 39.5°C, the non-permissive temperature for the ts mutants. Four hours after infection, ^{35}S-methionine (20 μCi/ml) was added and cells were harvested 12 hours later. Samples were electrophoresed in a 10% polyacrylamide gel.

were no differences in the virus yields in the antisense cell lines compared to the control cells.

The Interaction of ICP0 with Other α Gene Products.

It has been demonstrated that ICP0 can augment the stimulation of gene expression by ICP4 in transient expression assays (15-19). It is therefore possible that ICP0 may interact with other α proteins during infection to induce gene expression. In this case, if ICP0 levels were reduced and another α product was defective a more limited pattern of expression might be found than if either product alone was reduced or altered. To examine this possibility, the RSF derived antisense cell lines A1 and A2 were infected with either wild-type HSV-1 KOS or with ts606, a mutant defective in ICP4 (38), or tsLG4, a mutant defective in ICP27 (12,39) at the non-permissive temperature for the ts mutants. Figure 6 shows the total ^{35}S-labeled protein profiles. As with the infections of the Vero-derived antisense cell lines, ICP0 levels were reduced in the antisense cell lines. It should be noted that the levels of ICP0 were somewhat higher in the ts606 infected antisense cells than in the KOS infected antisense cells. This is probably due to the overproduction of α gene products which occurs in cells infected with an ICP4 mutant (9,10). Even under these circumstances, however, where α gene products were overproduced, the level of ICP0 in the antisense cell lines was still reduced relative to the control rabbit cells. Once again however, the polypeptide profiles for either the wild type infections or the ts mutant infections in the antisense lines were not significantly different from those in the control rabbit cells. Therefore, a reduction in ICP0 levels did not alter or exacerbate the defects seen in the ts606 or tsLG4 infected cells.

DISCUSSION

To investigate the role of the α polypeptide ICP0 during HSV-1 infection, cell lines were isolated which stably express mRNA which is antisense to a portion of the ICP0 mRNA. It was demonstrated that in several of these cell lines, the level of ICP0 was severely reduced following infection with wild type virus (Figures 4 and 6). This reduction in ICP0 levels did not have a consistent effect on the expression of HSV-1 early genes, however, as there was only a small reduction in the levels of some early gene products and this did not occur in all the antisense cell lines. This result was surprising because ICP0 trans-activation of early genes in transfection experiments was inhibited by the antisense plasmid whether the source of ICP0 was from a plasmid or from the virus (Figure 3). Virus yields on the antisense cell lines were equivalent to yields on parental cell lines indicating that this degree of ICP0 reduction was not detrimental to virus growth. Furthermore, no differences were seen in infection of antisense cell lines with mutants defective in ICP4 or ICP27 so that decreased ICP0 levels did not result in even more restrictive phenotypes. We investigated the possiblity of interactions among

the α genes because three α genes have been shown to be involved in late gene expression (ICP4, ICP27 and ICP22) suggesting that these products may interact during infection (12,13,62). In addition, it has been shown that ICP0 and ICP4 act synergistically to induce early gene expression in transient expression assays (15-19). However, we did not see any evidence of interaction of ICP0 with ICP4 or ICP27 in the experiment using the ts mutants.

From these results we conclude that ICP0 probably does not play a major role in the regulation of gene expression during HSV-1 infection, despite its activity as a transactivator of gene expression in transient expression assays. However, because a low level of ICP0 was still present in infections of the antisense cell lines, we cannot exclude the possibility from these data that this residual level of ICP0 was sufficient to perform any inducing functions. Recently mutants have been isolated in which both copies of the ICP0 gene have been deleted (Sacks, W.R. and Schaffer, P.A., submitted; Stow, N.D. and Stow, E.C., submitted). The phenotype of these mutants is very similar to that which we report here in that viral replication, protein synthesis and packaging are not significantly different in mutant and wild type infected cells. These mutants do produce plaques at efficiencies around 50-100 fold lower than wild type virus suggesting that ICP0 may only be necessary in low multiplicity infections. Because ICP0 is not essential for virus growth in high multiplicity infection of tissue culture cells, it has been suggested that this protein may play a role in latency, perhaps in reactivation of the virus from the latent state. This remains to be determined, however.

We have shown here that using antisense mRNA is a feasible approach to reducing the level of a viral gene product to investigate its function, thus adding to a growing number of systems in which this approach has been successful (20-28). The results which we obtained are similar to those found with deletion mutants, confirming the validity of this approach. The use of antisense mRNA should be helpful in studying the functions of genes for which it has proved difficult to isolate mutants. In addition, antisense mRNA could be used to reduce the level of a specific gene product even further in cases where leaky ts mutants are available but which are too permissive to fully evaluate the phenotype.

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