Inhibition of transient gene expression with plasmids encoding herpes simplex virus type 1 UL55 and alpha genes

Timothy Block, 1* Robert Jordan, 1 Daniel H. Farkas 2,3 and Robert G. Hughes, Jr 2

1 Department of Microbiology, Jefferson Medical College, 1020 Locust Street, Philadelphia, Pennsylvania 19107, 2 Department of Molecular and Cellular Biology, Roswell Park Cancer Institute, Carlton at Elm Street, Buffalo, New York 14263 and 3 Department of Pathology, St Barnabas Medical Center, Livingston, New Jersey 07039, U.S.A.

Herpes simplex virus type 1 (HSV-1) subgenomic sequences from 0-743 to 0-782 map units have been molecularly cloned as plasmid AT1 and shown to inhibit stable DNA-mediated gene transformation of Ltk- cells with the HSV-1 thymidine kinase (tk) gene. Here it is shown that AT1 also inhibits transient gene expression. Expression from the chloramphenicol acetyltransferase (CAT) gene under the control of either the HSV-1 tk gene or the Rous sarcoma virus (RSV) promoter was inhibited when cotransfected into Ltk- and CV-1 cells with equimolar amounts of AT1. AT1 was subcloned as three overlapping plasmids called ATla, ATlb and ATlc. The ATlb plasmid encodes the HSV-1 immediate early gene, z27; ATla possesses sequences that specify an open reading frame in HSV-1 strain KOS used in these studies, although the significance of this open reading frame is unknown; ATlb possesses the sequences for UL55 and UL56, also genes for which no function has been reported. No single subclone or pair of subclones demonstrated significant inhibition of transient gene expression. Cotransfection of all three subclones did result in inhibition of RSV-CAT gene expression, suggesting that information from each subclone is necessary. One of the three subclones, ATla, contains the HSV-1 immediate early gene, z27, so the possibility that other immediate early genes could substitute for z27 was tested. Inhibition of RSV-CAT gene expression was also achieved by cotransfection of ATla and ATlb with either an 0- or z4-containing plasmid, suggesting that the role of the z27-containing plasmid can be replaced by other alpha genes with trans-regulating capability. Finally, ATla and ATlb linker insertion mutants have been constructed and used to study the role these plasmids play in mediating inhibition. These results suggest that AT1 contains HSV-1 functions in addition to that of z27 that interfere with gene expression.

Introduction

Herpes simplex virus types 1 and 2 (HSV-1 and -2) are important human pathogens that cause a variety of diseases ranging from benign superficial cutaneous lesions to life-threatening encephalitis (Whitley, 1985). The normal course of infection is thought to be characterized by the ability of the virus to establish latent infections, whereby it persists in the peripheral nervous system for the life of the affected individual, often in the absence of any clinical manifestation (Whitley, 1985).

In tissue culture, however, infection of permissive cells provides a model for both positive and negative regulation of gene expression. Shortly after infection in vitro, host macromolecular synthesis is inhibited and replaced by the synthesis of virus-specified gene products (Honess & Roizman, 1974). Transcription of the large double-stranded 1 x 108 Molecule genome has been generally divided into three temporal classes called immediate (or 0), early ( 3 ) and late (or 3 ) (Fenwick, 1984; Roizman & Batterson, 1985). Alpha polypeptides appear first and are believed to be necessary for expression of the next group, the 3 genes. The 3 polypeptides are the final group of viral polypeptides to be made in the infected cell.

Positive regulation of viral gene expression has been extensively studied. For example, genes carrying 0 and 3 class promoter regulatory regions have been shown to be sensitive to trans-activation by particular viral gene products. This has been demonstrated using transfection of DNA to achieve transient gene expression. Specifically, the bacterial gene for chloramphenicol acetyltransferase (CAT) was linked to the promoter regulatory domain of a 3 gene and shown to be trans-activated by cotransfection with plasmids expressing the 0 genes ICP-4 and ICP-0 (Everett, 1986; O'Hare & Hayward; 1985a, b) in a transient expression system. Similarly, CAT genes linked to an 0 gene promoter regulatory region...
have been shown to be trans-activated by a virion structural protein, Vmw63 (also called VP16) (Campbell et al., 1984; Pellett et al., 1985). There is, therefore, considerable information about functions that trans-activate viral genes.

Less is known about the way that negative regulation of viral and host functions is achieved. Since HSV infection of permissive cells results in rapid host 'shut down' and cell death, this is an important area to study. We have been trying to identify regions of the HSV-1 genome that encode functions that inhibit gene expression. In our first series of experiments, we tried to determine whether isolated subfragments of the viral genome could inhibit formation of stably transformed cells in a DNA-mediated cotransformation assay (Farkas et al., 1987). That is, Ltk- cells were cotransfected with either the HSV-1 thymidine kinase (tk) gene or a simian virus 40 (SV40) early promoter-regulated mycophenolic acid resistance gene, in the presence of subgenomic fragments of the HSV-1 genome. Most fragments had no (or little) effect on the transformation frequency. However, two regions were extremely effective inhibitors, reducing the number of transformed colonies observed by greater than 90%, when as little as 30 fmol/ml of fragment were used. We now call these 'antitransformation' (AT) sequences because their presence in the calcium phosphate precipitate inhibits DNA-mediated transformation of L cells. The two AT sequences are contained in the EcoR1 fragments of the viral genome called JK and EK (Goldin et al., 1981); EK was studied in most detail. Antitransformation is therefore defined as the inhibition of the formation of stable biochemical transformants in a DNA-mediated gene transfer assay.

The region of EK that was responsible for the observed antitransformation was subcloned as plasmid AT1 (formerly called pEK-P3P4). AT1 contains a 6 kb PstI fragment, mapping from 0-743 to 0-782 map units on the HSV-1 strain KOS genome; this region includes the immediate early gene a27. We have divided AT1 into three overlapping subclones called AT1a, a27 and AT1b (called pEK-P3S1, pKHX-BH and pEK-S1P4, respectively, in Farkas et al., 1987). No single subclone or pair of subclones could inhibit transformation; however, the three subclones mixed together were as effective as the intact parental AT1 in antitransformation activity.

Since AT1 and its derivatives could inhibit stable DNA-mediated transformation, we asked whether transient gene expression would also be affected. Transient gene expression systems have been developed in which transfected target cells are harvested within days after transfection and analysed for the uptake and expression of the donated genetic material. Transient expression systems have several advantages over the stable transfor-

mation system. First, the results of transfection can be obtained within days, rather than weeks. In addition, transfectants in which the antitransformation events are occurring are the cells being studied. In the transformation inhibition assay, on the other hand, the only surviving colonies are those that presumably did not take up or express the AT sequences. Finally, transient expression systems are flexible; we can easily transfect a variety of different cell lines without consideration of selection for dominant markers.

The CAT gene has been successfully used as a measure of transient gene expression. In this report we show that expression of CAT genes linked to the promoter regulatory domains of either the HSV-1 tk gene (β-CAT) or the RNA promoter from the Rous sarcoma virus (RSV) long terminal repeat (LTR) (RSV-CAT) is inhibited when cotransfected into either Ltk- or CV-1 cells with AT1.

Methods

Cells, medium and virus. African green monkey kidney CV-1 and mouse Ltk- cells were cultured in a modified Eagle's medium containing 5% newborn calf serum (Block et al., 1985). All mammalian cultures were grown in 5% CO₂ incubators at 37 °C. HSV-1 strain KOS was used to infect CV-1 cells in RNA isolation experiments. Virus preparation was performed as described previously (Block et al., 1985).

Plasmids. pHFBA-3'-UT contains DNA corresponding to the 3' untranslated region of the human β-actin gene and was provided by Dr L. Kedes. CAT-containing plasmids were obtained from the following sources. RSV-CAT (Alwine, 1985), containing the CAT gene linked to a promoter from the RSV LTR regions molecularly cloned into a pBR322 plasmid derivative, was provided by Dr J. Alwine. β-CAT, called TK CAT elsewhere (DeLuca & Schaffer, 1985), containing the promoter regulatory domain from the HSV-1 tk gene linked to the CAT gene (DeLuca & Schaffer, 1985), was provided by Dr P. Schaffer. pKHX-BH, called a27 here, has been described previously (Bond & Person, 1984) and was provided by Dr S. Person. pIGA-15, containing the a0 gene, and pRHP6, containing the a4 gene (Gelman & Silverstein, 1987), were from Dr S. Silverstein. JE-CAT, containing the HSV-1 (ICP4, a4) promoter regulatory region linked to the CAT gene, has been described previously (DeLuca & Schaffer, 1985). AT1 and its subclones AT1a and AT1b (previously called P3P4, P3S1 and S1P4 respectively) have been described (Farkas et al., 1987). Plasmids were maintained and prepared by standard methods (Maniatis et al., 1982) using lysA by lysozyme.

Mutant plasmids (ATM), shown in Fig. 1, were constructed as follows. The HSV-1 sequences from AT1a and AT1b were cloned into pUC-13*, a pUC13 derivative which lacks BamHI and SacI sites. AT1a/pUC-13* was digested with BamHI and the sticky ends were filled in and ligated by standard methods (Maniatis et al., 1982). The resultant plasmid, called AT1aM, contains a ClaI site in place of the BamHI site found in AT1a/pUC-13*. AT1b/pUC-13* was digested with SacI and ligated to a 10 bp oligonucleotide, synthesized by the RPMI biopolymer facility to introduce a KpnI site; the resultant plasmid was called AT1bM. Enzymes were used according to manufacturer’s instructions.
DNA-mediated gene transfer. Calcium phosphate precipitates containing 10 μg/ml calf thymus DNA carrier were prepared as described previously (Wigler et al., 1978). Generally, 200 fmol CAT per ml plasmid (approximately 0.56 μg/ml) was coprecipitated with the indicated ‘challenger’ plasmid in equimolar amounts unless otherwise stated. All transfections were normalized such that the concentration of total DNA (plasmid and carrier) was always 10 μg/ml (unless indicated otherwise). After formation of calcium precipitates, a 10-fold excess of medium with calf serum was added and, usually, 5 ml of diluted precipitate was added to 4 x 10⁶ cells. After 4 h incubation at 37 °C, cells were treated for 3 min with DMSO diluted in growth medium; Ltk⁻ and CV-1 cells received 4% and 20% DMSO, respectively. DMSO was removed, monolayers were washed twice with growth medium and cells were cultured as usual until collection for CAT assay. RSV-CAT was routinely transfected into CV-1 cells because activity there was much greater than in transfected Ltk⁻ cells (data not shown). Stable DNA-mediated gene transfer, in which transformants were selected in HAT medium, was performed exactly as described previously (Farkas et al., 1987).

Analysis of CAT enzyme. Monolayers of transfected cells were washed twice with phosphate-buffered saline (PBS) and scraped into PBS. Cell pellets were resuspended in 10 volumes of 0.25 M Tris–HCl pH 7.4. Extracts were made by five cycles of freezing and thawing, followed by 15 min centrifugation at 10000 g at 4 °C; supernatants from this sedimentation were the source of enzyme. With the exception of the experiment in Table 3, CAT was assayed as described by Alwine (1985) using 0.1 μCi [¹⁴C]chloramphenicol (NEN) (54.2 mCi/mmol) per reaction vessel and acetyl-CoA (Boehringer-Mannheim). Acetylated chloramphenicol was recovered by ethyl acetate extraction and resolved by silica gel thin-layer chromatography (Baker). After visualization of [¹⁴C]-labelled product by autoradiography of the TLC plates, tape was placed over both forms of acetylated product and cut, dissolved in counting fluor and the radioactivity was then determined (Sedmak & Grosberg, 1977); all values presented are the average from two different precipitates. All experiments were done at least three different times and most experiments were done more than three times.

In Table 3, CAT activity was determined as in Neuman et al. (1987). Briefly, the reaction mixture was 150 mM-Tris–HCl pH 7.8, 1 mM-chloramphenicol, 60 μM-acetyl-CoA, 0.1 μCi [³H]acyethyl-CoA (16 Ci/mmol); ICN). After 1 h incubation, the 250 μl reaction was overlayed with 5 ml Beta-fluor scintillation fluid (NEN) and diffused product was counted immediately in an LKB liquid scintillator.

Isolation of RNA from transfected cells. After transfection, cells were harvested into PBS and centrifuged. Cell pellets (approximately 10⁷ cells) were resuspended as described (Ausubel et al., 1987) in 10 mM-Tris–HCl pH 7.4, 10 mM-NaCl, 3 mM-MgCl₂, 0.5% NP40. This lysate was centrifuged at 4 °C at 500 r.p.m. for 5 min. The supernatant was made 5 mM in vanadylribonucleoside (BRL) and was added to an equal volume of 0.2 M-Tris–HCl pH 7.5, 0.44 mM-NaCl, 2% SDS, 25 mM-EDTA and incubated with 200 μg/ml proteinase K (BRL). RNA was recovered from this material by phenol extraction and ethanol precipitation and nucleic acid was then digested with RNase-free DNase I (Promega), re-extracted with phenol and ethanol-precipitated; these methods are described in Ausubel et al. (1987). Cytoplasmic RNA was diluted in sodium citrate-formaldehyde and applied to nitrocellulose paper using a Schleicher & Schuell dot-blot apparatus, as in White & Bancroft (1982). Probes were labelled by nick translation according to Rigby et al. (1977).

Results

AT1 Inhibits β-CAT

AT1 is a plasmid that contains a 6 kb PstI fragment spanning map units 0.743 to 0.782 of the HSV-1 genome (Fig. 1). We have previously shown that cotransfer of AT1 with dominant selectable markers to Ltk⁻ cells results in an inhibition of transformation (Farkas et al., 1987). That is, coprecipitation of fm amounts of AT1 with pHHSV106, the plasmid containing the HSV-1 tk gene, caused a greater than 90% reduction in the number of stably transformed colonies appearing in selective HAT medium. We therefore called these regions AT sequences.

Since AT1 is an effective inhibitor of stable gene transfer to mouse Ltk⁻ cells, we wanted to determine whether it would also affect transient gene expression. Transient gene expression can be studied using the bacterial CAT gene ligated to appropriate mammalian RNA polymerase binding sites (Gorman et al., 1982). Cells which had been transfected with calcium phosphate precipitates of CAT and test plasmids were harvested after 48 h and assayed for expression of CAT.

β-CAT contains the CAT gene under the control of the HSV-1 tk promoter (DeLuca & Schaffer, 1985). Fig. 2 shows that transfection of β-CAT into Ltk⁻ cells resulted in production of low but detectable levels of CAT enzyme (row 1). When β-CAT was cotransfected with the z0 gene-containing plasmid (pIGA-15), a nearly five-fold increase in activity was observed (row 3). The enhancing effect of z genes on β gene-directed expression has been demonstrated by others (Block & Jordan, 1988; Everett, 1986; Gelman & Silverstein, 1987; O’Hare & Hayward, 1985b) and reflects the fact that certain β genes, such as the tk gene, are positively regulated by z0 or z4 gene expression.

Fig. 2 also shows that CAT levels are 85% lower in cells cotransfected with equimolar amounts of AT1 and β-CAT and z0 compared to those receiving just β-CAT and z0 plasmids (compare row 4 with row 3). Cotransfection of equimolar amounts of either pBR325 or control plasmid pSG-5 with β-CAT and z0 did not significantly influence CAT levels. pSG-5 contains HSV-1 EcoRI fragment O molecularly cloned into pBR325 (Goldin et al., 1981). Therefore, inhibition is not due to the presence of additional plasmid or HSV sequences in general. It is also interesting to note that AT1 inhibition of β-CAT occurs in the absence of z0.

Cells transfected with β-CAT and AT1 contain substantially less CAT than those transfected with β-CAT alone (compare row 2 with row 1). Similar results have also been observed using CV-1 cells as transfection
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(a)

HSV

(b)

AT1

AT1a

AT1aM

x27

AT1b

AT1bM

Fig. 1. The HSV-1 sequences in AT1 and its subclones. The HSV-1 genome and sequences cloned into various plasmids that are used in these studies is presented. (a) The HSV-1 genome is labelled and bold boxes show the location of the repeat sequences. (b) The HSV region cloned as AT1 is presented as an expansion map. The letters to the left of the bar drawings are the plasmid names. The numbers under the AT1 map are the map units along the HSV-1 strain KOS genome. Restriction endonuclease cleavage sites are indicated: P, PstI; B, BamHI; S, SacI; H, HpaI. The location and direction (arrow) of the ct27 transcript, based on Sacks et al. (1985), is shown above AT1. The open reading frame (ORF) is based upon Debroy et al. (1985). Other transcripts are also shown, based on McGeoch et al. (1988). (c) AT1 subclones.

Table 1. Inhibition of RSV-CAT-induced enzyme activity by plasmid AT1

<table>
<thead>
<tr>
<th>Plasmid*</th>
<th>CAT activity†</th>
<th>Inhibition‡ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RSV-CAT alone</td>
<td>66.6 ± 2.9</td>
<td>0</td>
</tr>
<tr>
<td>RSV-CAT + pBR325</td>
<td>49.5 ± 5.0</td>
<td>25.7</td>
</tr>
<tr>
<td>RSV-CAT + AT1</td>
<td>10.6 ± 1.5</td>
<td>84.1</td>
</tr>
</tbody>
</table>

* 200 fmol/ml each plasmid was transfected into CV-1 cells in the usual manner. See Methods.
† CAT levels, presented as pmol acetylated chloramphenicol per 50 μg protein per 30 min of assay, were determined from cell extracts 2 days after transfection, as described in Methods. Values are the average of assays done on two duplicate samples ± s.d.
‡ Percentage inhibition is the fraction of CAT activity relative to that observed in cells transfected with only CAT plasmids.

Targets. These data suggest that HSV sequences present in AT1 encode an activity that suppresses β-CAT gene expression independently of α0 function.

AT1 inhibits RSV-CAT

Inhibition of β-CAT could have been due to a negative regulatory effect of AT1 on the HSV β promoter governing CAT expression. In addition, because α27, present in AT1, may have a negative regulatory effect on α0 (Block & Jordan, 1988; Sekulovich et al., 1988), a target other than β-CAT was sought. We therefore wished to determine whether CAT expression directed by non-HSV promoters would be affected. RSV-CAT contains the CAT gene under the control of an RSV promoter (Gorman et al., 1982) and can efficiently direct the synthesis of CAT in appropriately transfected target cells. Table 1 shows that transfection of CV-1 cells with 200 fmol/ml RSV-CAT resulted in easily detectable levels of CAT. CV-1 cells were used as target cells for transfection because greater amounts of CAT were produced following RSV-CAT transfection into CV-1 than Ltk- cells (data not shown). Table 1 also shows that an equimolar amount of AT1 in the calcium phosphate precipitate with RSV-CAT resulted in a greater than 80% reduction in observed CAT levels.

The ability of AT1 to inhibit RSV-CAT was dose dependent. Fig. 3 shows that CAT inhibition occurred as a function of increased AT1 concentration and that effective inhibition is observed at concentrations as low as 50 fmol/ml.

Since AT1 contains an HSV-1 DNA fragment in pBR325, the effect of pBR325 on RSV-CAT expression is also presented. At concentrations at which AT1 was an effective inhibitor, pBR325 had a negligible effect; similarly, cotransfer of pSG-5 with RSV-CAT had no
significant effect. Since pSG-5 had not been associated with AT activity in our previous study (Farkas et al., 1987), this result is not surprising and suggests that HSV sequences specific to AT1 mediate anti-CAT activity.

CAT RNA levels in transfectants

The AT1-mediated inhibition of CAT activity could be due to either an effect on the level of CAT mRNA or a depression in protein synthesis. To distinguish between these possibilities, cytoplasmic cellular RNA was isolated from cells transfected with RSV-CAT in the absence and presence of an equimolar amount of AT1. RNA preparations were digested with DNase I, serially diluted and applied to nitrocellulose paper using a microfilter apparatus (see Methods). RNA dot-blots were analysed with radioactive probes recognizing either RSV-CAT or β-actin. Panel (a) was probed with radioactive RSV-CAT and shows that cells receiving RSV-CAT and AT1 (column 3) contained less than 20% of the amount of RSV-CAT RNA compared to those receiving RSV-CAT alone (column 2), as measured by liquid scintillation analyses of the radioactivity. Cells that were mock-transfected (column 1) or untransfected (column 4) are presented to demonstrate background levels of hybridization to the probe. Other samples were probed for actin as loading controls. Similar amounts of actin were present in all samples, demonstrating equal loading of RNA onto nitrocellulose paper (Fig. 4b). Therefore, cotransfection of AT1 with RSV-CAT does result in an 80% depression in RSV-CAT-specific transcript production. Since these experiments measure steady-state levels of RNA, they do not distinguish between an effect on the rate of CAT RNA transcription and an effect on the stability of CAT RNA.

AT1 can be divided into three functional domains

Several restriction endonuclease cleavage sites located within AT1 are shown in Fig. 1. Gene z27 (ICP-27) is located entirely within AT1. Fig. 1 also shows that AT1 has been divided into three overlapping subclones called AT1a, AT1b and pKHX-BH. Plasmid pKHX-BH contains the intact z27 gene (Bond & Person, 1984), whereas AT1a and AT1b contain the left and right ends of AT1 (called pEK-P3S1 and pEK-S1P4 by Farkas et al., 1987). Thus, AT1a and AT1b bisect the z27 gene and do not contain any previously reported functions.

We have recently shown that inhibition of stable DNA-mediated gene transfer of the HSV tk gene to Ltk- cells could not be achieved with any single one or paired combination of the AT1 subclones (Farkas et al., 1987). However, when all three subclones (AT1a + AT1b + z27) were cotransfected with the HSV tk gene into Ltk- cells, inhibition was achieved,
modest inhibition of CAT, it was not comparable to that transfected (rows 3, 4 and 5 respectively). Although these subclones would affect transient gene expression. RSV-CAT alone is an effective inhibitor of RSV-CAT-induced activity with AT1 subclones. The results of these experiments are shown in various combinations of equimolar amounts of AT1 and its subclones, AT1a, AT1b, and z27 (pKHX-BH), are shown in Fig. 1. CAT activity is presented as pmol acetylated chloramphenicol per 50 μg protein assayed per 30 min (see Methods). Narrow lines indicate S.D.

It was therefore of interest to determine how the AT1 subclones would affect transient gene expression. RSV-CAT was transfected into CV-1 cells alone or with various combinations of equimolar amounts of AT1 subclones. The results of these experiments are shown in Fig. 5. Clearly, AT1 is an effective inhibitor of RSV-CAT, causing an 85% inhibition of CAT activity (row 2). On the other hand, neither pBR325, AT1a or AT1b had any significant effect on CAT levels when separately transfected (rows 3, 4 and 5 respectively). Although these concentrations of z27-containing plasmid did result in a modest inhibition of CAT, it was not comparable to that seen with AT1 (compare rows 2 and 6). Similarly, pairs of the subclones were only moderately active (rows 7, 8 and 9). However, when all three subclones were added together, substantial inhibition of RSV-CAT was observed (row 12). These results suggest that AT1 does contain three separate functions that act together to maximally inhibit transient gene expression and that information from each subclone is needed to effect inhibition.

Other z genes can substitute for z27 in the inhibition assay

Since z27 appeared to be at least one of the functions active in the inhibition of transient gene expression, it was of interest to determine whether other z genes could substitute for z27. In some in vitro systems, z genes can provide similar functions. For example, stimulation of β-CAT can be achieved by cotransfection of either z4 or z0 genes (O'Hare & Hayward, 1985a, b). To test the possibility that either z4 or z0 functions could replace the role of z27 in the inhibition of transient gene expression, RSV-CAT was cotransfected into CV-1 cells in the presence and absence of the AT1 subclones, AT1a and AT1b. Plasmids containing either z0 or z4 were substituted for z27 where indicated. Fig. 6 shows the results of CAT assays performed on these transfectants. When the AT1 subclones (AT1a + AT1b + z27) were added together with RSV-CAT, a substantial reduction in CAT activity occurred. As shown previously in Fig. 5, AT1a and AT1b together were insufficient to prevent CAT expression. Inhibition occurred only when all three AT1 subclones, AT1a, AT1b, and z27, were included. Fig. 6 also shows that although z0 and z4 plasmids themselves did not inhibit CAT activity (rows 7 and 8), they can effectively substitute for z27 if AT1a and AT1b are also present (rows 3 and 4). Transfection of z4 with either AT1a or AT1b separately (data not shown). These CAT (rows 5 and 6); both AT1a and AT1b had to be present with an z gene. Similarly, no significant RSV-CAT inhibition was observed after transfecting z0 with either AT1a or AT1b, separately (data not shown). These results confirm that AT1a, AT1b and z27 functions are needed to produce CAT inhibition. Furthermore, the contribution from the z27-containing plasmid could be replaced by either z0 or z4 gene-containing plasmids.
Fine structure mapping of AT1 sequences essential to achieve antitransformation and CAT inhibition

The sequences critical to achieve AT have been characterized by restriction enzyme analyses. AT1 subclones have been digested with restriction enzymes prior to use in the AT assay (Farkas et al., 1987). These experiments revealed that digestion of AT1a with BamHI and digestion of AT1b with SacI eliminated their ability to participate in AT (Farkas et al., 1987). This suggested that the BamHI site in AT1a and the SacI site in AT1b are located within genetic information essential to the AT process and that intact AT1a and AT1b are needed to achieve AT. Fig. 1 shows where these sites are located within the AT1 subclones AT1a and AT1b. Note that neither BamHI or SacI cleaves within the a27 structural gene.

To characterize further the sequences in AT1a and AT1b needed to inhibit CAT activity in transient gene expression assays, plasmids in which the HSV sequences from AT1a and AT1b had been disrupted were constructed. AT1a was disrupted at the BamHI site and AT1b was disrupted at the SacI site (see Methods for details). Both these changes result in the disruption of translation in all reading frames. The AT1a and AT1b linker insertion mutants are called AT1aM and AT1bM respectively and are shown in Fig. 1. Since AT1a and AT1b are needed to achieve AT, Table 2 shows that DNA-mediated gene transfer of the HSV tk gene to Ltk- cells resulted in a significant reduction of tk+ colonies when compared with the control tk frequency.

However, the number of tk+ colonies was not significantly affected by cotransfer of AT1aM, AT1b and a27 (pKHX-BH), or AT1a, AT1bM and a27, with the HSV tk gene. That is, AT1aM cannot substitute for AT1a and AT1bM cannot substitute for AT1b in the production of tk transformation inhibition. Although the DNA sequences in AT1aM and AT1bM have not yet been determined by sequence analysis, it seems likely that their inability to complement in the transfection inhibition assay is due to disruptions at the BamHI and SacI sites, respectively.

The AT1aM and AT1bM plasmids were also tested for their ability to participate in the transient gene inhibi-

Table 2. AT1aM and AT1bM plasmids do not inhibit stable gene transfer

<table>
<thead>
<tr>
<th>HSV DNA*</th>
<th>Average no. colonies (± error)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>109 (±3)</td>
<td>0</td>
</tr>
<tr>
<td>AT1</td>
<td>151 (±2)</td>
<td>86</td>
</tr>
<tr>
<td>AT1a</td>
<td>112 (±8)</td>
<td>-3</td>
</tr>
<tr>
<td>AT1b</td>
<td>99 (±6)</td>
<td>9</td>
</tr>
<tr>
<td>pKHX-BH (a27)</td>
<td>120 (±5)</td>
<td>5</td>
</tr>
<tr>
<td>AT1a + AT1b</td>
<td>111 (±4)</td>
<td>-2</td>
</tr>
<tr>
<td>AT1a + pKHX-BH</td>
<td>106 (±6)</td>
<td>3</td>
</tr>
<tr>
<td>AT1b + pKHX-BH</td>
<td>109 (±8)</td>
<td>0</td>
</tr>
<tr>
<td>AT1a + AT1b + pKHX-BH</td>
<td>110 (±7)</td>
<td>-1</td>
</tr>
<tr>
<td>AT1aM + AT1b + pKHX-BH</td>
<td>101 (±7)</td>
<td>7</td>
</tr>
<tr>
<td>AT1a + AT1bM + pKHX-BH</td>
<td>110 (±7)</td>
<td>0</td>
</tr>
</tbody>
</table>

* Precipitates containing 200 ng/ml HSV106 and 30 fmol/ml of each HSV DNA-containing plasmid were transfected into Ltk- cells as described. pbR325 was added as necessary such that each precipitate contained bacterial plasmid sequences at a concentration of 90 fmol/ml.

† Average number of tk+ colonies based on one precipitate per sample and two 25 cm2 flasks per precipitate.

Table 3. AT1aM and AT2aM plasmids do not inhibit transient gene expression

<table>
<thead>
<tr>
<th>Plasmids*</th>
<th>CAT activity (± S.D.)*†</th>
</tr>
</thead>
<tbody>
<tr>
<td>RSV-CAT alone</td>
<td>61.0 ± (± 8.5)</td>
</tr>
<tr>
<td>+ pUC18</td>
<td>52.5 ± (± 4.1)</td>
</tr>
<tr>
<td>+ AT1</td>
<td>13.8 ± (± 3.6)</td>
</tr>
<tr>
<td>+ AT1a + AT1b + a27</td>
<td>19.2 ± (± 6.4)</td>
</tr>
<tr>
<td>+ AT1aM + AT1bM + a27</td>
<td>46.8 ± (± 2.0)</td>
</tr>
<tr>
<td>+ AT1aM + AT1b + a27</td>
<td>46.4 ± (± 3.2)</td>
</tr>
<tr>
<td>+ AT1bM + AT1a + a27</td>
<td>55.0 ± (± 4.3)</td>
</tr>
<tr>
<td>No plasmid transfected</td>
<td>6.8 ± (± 0.6)</td>
</tr>
</tbody>
</table>

* 200 fmol/ml of the indicated plasmids were transfected into CV-1 cells as described in Methods. Plasmid identities are described in the test.

† CAT activity is presented as pmol [3H]chloramphenicol and was determined as in Methods.

‡ No plasmid transfected refers to 'H-labelled product recovered from cell preparations that did not receive any plasmids and represents a background that has been subtracted from the CAT activity values above.
assay. These results support the theory that gene products encoded by AT1a and AT1b are involved in mediating CAT inhibition and that DNA sequences spanning the BamHI site in AT1a and the SacI site in AT1b are essential to the integrity of these coding sequences.

Discussion

Our previous studies have revealed that a 6 kb region of the HSV-1 strain KOS genome can reduce the efficiency of stable DNA-mediated gene transfer of the HSV-1 tk gene to mouse Ltk− cells (Farkas et al., 1987). This subgenomic region was molecularly cloned into pBR325 and is called AT1. AT1 contains DNA from 0·743 to 0·782 HSV-1 map units and is called the AT sequence. The AT region was further subcloned as three overlapping pieces called AT1a, AT27 and AT1b [called pEK-P3S1, pKHX-BH and pEK-S1P4, respectively, in Farkas et al., 1987]. Although no single subclone or pair of AT1 subclones could inhibit transformation, all three added simultaneously to the calcium phosphate precipitate were as effective as AT1.

This report extends those findings in several important regards. First we show that transient gene expression is inhibited by AT1. That is, cells transfected with CAT genes contained greatly reduced levels of CAT enzyme when AT1 was included in the calcium phosphate precipitate. Therefore the AT sequence effect is not limited to the formation of stable transformed colonies. It is valuable to know that both stable DNA-mediated gene transfer and transient gene expression are inhibited by the AT sequences because stable transformation of target cells occurs in only a fraction of the transfectants (Block et al., 1985; Wigler et al., 1978); usually, about 1 in 1000 cells will stably acquire a transfection phenotype. In addition, in the stable transformation systems studied here and in Farkas et al. (1987), integration of the selectable marker into the host chromosome is presumably required for transformation because the marker genes do not possess mammalian origins of replication. Transient gene expression reflects activity in a large number of the transfectants. As many as 5 to 10% of the transfectants express the donated phenotype (Gelman & Silverstein, 1987). These two systems of transfection may therefore reflect different cellular processes. Since the HSV-1 AT sequences in AT1 inhibit both processes, a common component of the systems may be affected. This is not a trivial distinction since the AT activity in JK (Farkas et al., 1987) inhibits stable but not transient gene expression (unpublished results).

Furthermore, two different promoter systems in two different cellular environments were inhibited. CAT genes linked to either the HSV-1 tk or the RSV LTR promoter were sensitive to AT1-mediated inhibition. The inhibition of β-CAT was studied in mouse Ltk− cells, whereas RSV-CAT was studied in monkey CV-1 cells. Significantly, when the AT1 plasmid was subdivided into subclones with overlapping HSV-1 DNA sequences, one subclone containing the intact HSV-1 immediate early gene for AT27 and the other two subclones not having any assigned function, no single or pair of clones inhibited CAT activity. Therefore AT27 function by itself is not inhibitory. Only when all three subclones were transfected together was efficient inhibition observed. This suggests that genetic information encoded separately by each subclone is necessary for activity.

It has recently been shown that AT27 can negatively regulate HSV α gene expression and, therefore, does itself have a negative regulatory potential (Block & Jordan, 1988; Rice et al., 1989; Sekulovich et al., 1988; Su & Knipe, 1989). This negative regulatory effect is specific for certain HSV functions (e.g. the AT0 function). However, it is shown here that AT27 gene expression itself does not significantly inhibit CAT gene expression under the control of HSV β- or RSV LTR-derived promoters at the plasmid concentrations used here. It is noted, however, that AT27-containing plasmids do inhibit these CAT constructs at higher plasmid concentrations. The role of AT27 in helping AT1a and AT1b to inhibit HSV β-CAT and RSV-CAT gene expression is discussed below.

The AT27-containing plasmid, pKHX-BH, encodes a functional AT27 polypeptide (Sacks et al., 1985). AT27 is one of the five HSV-1 α genes which are the first to be transcribed in productively infected cells (Roizman & Batterson, 1985). The likelihood that an AT27 function is necessary but not sufficient to mediate inhibition of transient gene expression is supported by the following observations. Cotransfer of the MC1 plasmid with AT1 results in more efficient inhibition of transient gene expression than that seen with AT1 in the absence of MC1 (unpublished observation). MC1 encodes the HSV-1 protein Vmw65 (Campbell et al., 1984) (also called VP16 and α TIF), which trans-activates HSV α gene expression. If AT27 is involved in the inhibition, cotransfer of MC1 with AT1 or its subclones would result in stimulation of expression of the AT27 gene in AT1 and, thus, more efficient inhibition. The enhancement of AT1’s inhibitory effect by MC1 also suggests that AT1 is not outcompeting the CAT plasmids for essential transcription factors; 35 fmol/ml AT1 was as effective as 200 fmol/ml when MC1 was present.

Moreover, plasmids containing AT0 or AT4 genes could substitute for AT27 in the AT1 subclone-mediated CAT gene expression inhibition assay. That is, the combination of AT0 or AT4 plasmids with AT1a and AT1b was as effective in inhibiting CAT gene expression as AT27 + AT1a + AT1b. This suggests that AT0, AT4 and AT27 gene products provide functionally similar contributions to
the inhibition assay. Since each of these \( \alpha \) functions has been shown, by themselves or in combination, to trans-stimulate the expression of other HSV genes (Block & Jordan, 1988; Everett, 1986; Gelman & Silverstein, 1987; O'Hare & Hayward, 1985; Su & Knipe, 1989), it seems likely that their role in the inhibition assay, described here, is to trans-activate HSV information in either or both of the other herpesvirus plasmids, AT\( \lambda a \) and AT\( \lambda b \). It is important to emphasize that, by themselves, the \( \alpha \) genes do not inhibit transient gene expression of \( \beta \)-CAT or RSV-CAT. Inhibition occurs only if the \( \alpha \) genes are cotransferred with AT\( \lambda a \) and AT\( \lambda b \).

The mechanism of the AT\( 1 \)-mediated inhibition of transient gene expression is not yet known. AT\( 1 \) is composed of an \( \alpha \) gene as well as at least two other domains which are contained in AT\( \lambda a \) and AT\( \lambda b \). We have identified RNA transcripts homologous to AT\( \lambda a \) and AT\( \lambda b \) which are produced in transfected cells (unpublished observations). In infected cells, a 1.5 kb transcript homologous to AT\( \lambda a \) and 0.9 and 1.1 kb transcripts homologous to AT\( \lambda b \), have been observed (unpublished results). Similar transcripts have been observed or predicted by others (MacLean & Brown, 1987; Spivack & Fraser, 1988). The 0.9 and 1.1 kb transcripts may encode the polypeptide products predicted by the open reading frames present in the sequences in AT\( \lambda b \) called UL55 and UL56 (McGeoch et al., 1988). Debroy et al. (1985) have described a right-to-left open reading frame spanning the BamHI site of AT\( \lambda a \) and report that the HSV-1 strain KOS sequence to the right of this site is homologous, without additions or deletions, to that of HSV-1 strain F determined by Mackem & Roisman (1982). Sequences to the left of the BamHI site of HSV-1 strain KOS (Debroy et al., 1985) and HSV-1 strains MP and mP (Pogue-Geile & Spear, 1987), coupled with that to the right of the site, permits the description of a right-to-left open reading frame of 201 amino acids. Interestingly, this open reading frame is also present in HSV-1 strain 17 (McGeoch et al., 1988). Although Debroy et al. (1985) have detected RNA transcribed in the right-to-left direction, no evidence is available to indicate whether the transcript is translated. However, it should be noted that McGeoch et al. (1988) did not assign a gene number to this open reading frame because its sequences did not meet their standards for consideration as a translated gene. Therefore, all of the AT\( 1 \) subclones used in these experiments have RNA- and polypeptide-encoding potential, although it is not known whether gene products are produced from all of the indicated open reading frames. It remains to be seen whether these transcripts are mediators of the gene transfection inhibition reported here and whether any are actually positively regulated by \( \alpha 27 \), as our model predicts.

The linker insertion experiments make several important points. Insertion of only 10 bp in the UL55 coding region of AT\( \lambda b \) and 4 bp in the BamHI site of AT\( \lambda a \) prevents these plasmids from mediating inhibition. Therefore, it is possible that polypeptide gene products are involved, although biological activity due to the transcript itself or a cis-acting effect of the DNA cannot be excluded. Furthermore, the insertion mutation in AT\( \lambda b \) confirms that the UL55 coding region is important for inhibition; we do not know whether the UL56 region is also necessary.

There are several ways that inhibition could be achieved. AT\( 1 \) may encode functions that prevent stable integration of transfected markers within host chromatin. The marker plasmids would then exist transiently as episomes. This would result in an inhibition of the formation of transformed colonies following stable DNA-mediated gene transfer because the marker plasmids do not possess a mammalian origin of replication and do not, therefore, replicate as extrachromosomal elements. Since AT\( 1 \) inhibits transient gene expression as well as stable DNA-mediated gene transfer, other possible mechanisms seem more likely. For example, AT\( \lambda a \) and/or AT\( \lambda b \) may encode gene products which are cytotoxic. Alternatively, AT\( 1 \) might cause degradation of transfected DNA. Our work in progress, however, has shown that transfected DNA is no less abundant in AT\( 1 \)-treated than in untreated cells. Therefore, AT\( 1 \)-encoded degradation of transfected DNA does not occur at detectable levels.

Another possible mechanism of inhibition would be that AT\( 1 \) interferes with the transcription or translation of the CAT gene. Since the level of CAT transcript is much lower in cells transfected with AT\( 1 \) (Fig. 5), CAT gene transcription may be inhibited or CAT mRNA may be rapidly degraded. In this regard, HSV infection is known to cause degradation of host mRNA and this is thought to be due to a \( \beta \) or \( \gamma \) function (Read & Frenkel, 1983). However, this function has been mapped to 0-605 map units, corresponding to UL41 (Kwong et al., 1988; McGeoch et al., 1988, Fenwick & Everett, 1990), distant from either AT\( \lambda a \) or AT\( \lambda b \) HSV-1 sequences. Of course, it is still formally possible that AT\( \lambda a \) or AT\( \lambda b \) may encode a different product which degrades mRNA and which would therefore decrease transient gene expression as well as inhibit formation of stable transfectants.

Although cytoplasmic CAT RNA levels are reduced by 85\% in cells receiving AT\( 1 \), this does not distinguish between an AT\( 1 \)-mediated gene repression of CAT gene transcription and the production of a lethal product. Additional experiments are needed to distinguish between these mechanisms of action.

Other possibilities exist as well and remain to be tested. The experiments described here firmly identify certain HSV-1 sequences as mediating negative regula-
tory events. Moreover, prior to this report, no specific functions had been associated with HSV gene UL55. Additional experiments, which address the mechanisms of this negative regulation more directly, are needed and are currently under way.

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References


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