Development and use of a 293 cell line expressing lac repressor for the rescue of recombinant adenoviruses expressing high levels of rabies virus glycoprotein

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An expression cassette designed for high-level production of rabies virus glycoprotein (RG) could not be rescued into a replication-defective, adenovirus-based vector using standard procedures. To overcome this difficulty, a 293-based cell line, designated 293LAP13, was constructed that contained and expressed a derivative of the lac repressor protein. The lac operator sequence, to which the repressor binds, was incorporated into an expression cassette, containing a promoter and intron, designed for high-level production of RG. Insertion of a single operator sequence immediately downstream of the transcription start site and the use of the 293LAP13 cell line allowed recombinant viruses that could not be isolated with 293 cells to be rescued efficiently. The operator-containing virus reached higher titres in 293LAP13 than in parental 293 cells and also produced plaques more efficiently in 293LAP13 cells. Moreover, in non-complementing human and canine cell lines, adenovirus vectors with a promoter–intron expression cassette expressed RG at much higher levels than vectors lacking the intron. These observations, together with the demonstration that expression of RG by operator-containing vectors was repressed markedly in 293LAP13 cells and that this inhibition was relieved at least partly by IPTG, suggest that the 293LAP13 cell line may be useful for the rescue and propagation of many vectors in which high expression of the desired protein prevents vector rescue in 293 cells.

Introduction

Human adenoviruses (Ad) have become one of the vectors of choice for the delivery and expression of foreign proteins for gene therapy, immunotherapy and vaccination (for a recent review see Hitt et al., 1997). In particular, Ad vectors have been shown to be effective vaccines for the delivery of rabies virus glycoprotein (RG) antigens to non-human hosts. Protective immune responses have been induced in animals immunized with infectious (Prevec et al., 1990; Charlton et al., 1992; Yarosh et al., 1996) and defective (Xiang et al., 1996) Ad vectors delivered by parenteral and oral routes. Using vectors containing the RG gene in E3, we showed that the level of protective immunity induced in treated animals correlated with the amount of antigen produced in cell culture (Yarosh et al., 1996). Because high-level antigen expression by recombinant virus vaccines allows for the use of lower doses and gives greater efficacy, and because one of our principal objectives is to use Ad-based vectors expressing RG as oral vaccines for wildlife, it is clearly important to obtain the highest possible level of production in infected cells.

In general, replication-defective Ad vectors have the early transcription regions E1 and E3 deleted and cassettes inserted containing an appropriate exogenous promoter and the gene(s) to be expressed. Such viruses, often called ‘first-generation vectors’, are produced and replicated in E1-complementing cell lines such as the human 293 cell line (Graham et al., 1977). We have described procedures employing bacterial plasmids and in vivo recombination to generate E1−, E3− vectors with inserts in E1 or E3 (Graham & Prevec, 1995). Basically, the foreign gene (or promoter–gene cassette) is cloned into one of a series of shuttle plasmids and co-transfected into 293 cells with a complementing plasmid encoding the majority of the Ad genome, in order to rescue recombinant Ad vectors containing the desired transgene. This procedure has been used successfully to produce many recombinant viruses expressing proteins from a wide variety of sources.

While the ease with which particular vectors are generated...
varies, only rarely can the desired expression cassette not be rescued into the virus vector. One such situation presented itself when we tried to generate a recombinant vector expressing RG under the control of a murine cytomegalovirus (MCMV) promoter with an intron sequence upstream of the RG open reading frame. Our results suggested that high-level expression of RG was responsible and to solve this problem, we developed a 293-derived cell line (293LAP13) expressing lac repressor and expression cassettes containing the lac operator to down-regulate transgene expression. This approach allowed us to isolate vectors that express very high levels of RG and may have general application for the rescue and replication of other Ad vectors that are difficult to produce by the procedures that are currently employed.

Methods

**Cells and viruses.** The 293 cells (Graham et al., 1977) and 293-cell derivatives were maintained as previously described (Hitt et al., 1995). Isolated colonies of cells transformed to G418 resistance (described below) were maintained in α-MEM supplemented with foetal bovine serum (10% v/v; Gibco BRL), penicillin (100 U/ml), streptomycin (100 µg/ml) and G418 (200 µg/ml, Gibco BRL). Ad dl70-3, used as a control, is derived from Ad5 dl309 (Jones & Shenk, 1979) but has a deletion of E1 and a deletion/substitution in E3 (Bett et al., 1994, 1995). The rescue, isolation, propagation, purification and titration of recombinant viruses were performed as described previously (Hitt et al., 1998). Names applied to particular virus stocks were simplified from the corresponding laboratory designation for purposes of consistency and simplicity in this paper. The laboratory designations of AdE3Fx, Ad(l)E3Fx and Ad(l)E3Fx(o) are Ad5BHGE3MH5Fx, AdMH5(l)E3Fx and AdMH5(l)E3Fx(o), respectively. The number of virus particles in gradient-purified stocks was determined from the absorbance at 260 nm using the relationship 1 absorbance unit = 1·1 × 10¹² particles/ml (Mittereder et al., 1996).

**Plasmid construction.** Molecular cloning techniques were as described by Sambrook et al. (1989). All DNA fragments were separated by agarose gel electrophoresis and eluted from the agarose with GeneClean (Bio 101). The RG gene from a red fox isolate (Nadin-Davis et al., 1990) was obtained from S. Nadin-Davis (Canadian Food Inspection Agency, Nepean, Ontario) and modified by removing an ATG sequence downstream of the transcription start site. Unlabelled portions of the sequence with or without a copy of the lac operator sequence (o) just downstream of the transcription start site. Unlabelled portions of the circles represent plasmid or Ad5 sequences. The sequences of linkers AB9483 and AB9484, which comprise the operator sequence, are given in Methods.

![Diagram](image)

Fig. 1. Construction of plasmids containing the RG gene (Fx, indicated on maps as fx-g) under the control of the MCMV promoter, an intron (I) sequence with or without a copy of the lac operator sequence (o) just downstream of the transcription start site. Unlabelled portions of the circles represent plasmid or Ad5 sequences. The sequences of linkers AB9483 and AB9484, which comprise the operator sequence, are given in Methods.

moter/enhancer, all flanked by a BamHI site and a BglII site (Fig. 2a; D.A. Matthews & F.L. Graham, unpublished data). Plasmid pHCMVLPAP348 (Labow et al., 1990; kindly provided by M. Rudnicki, McMaster University) contains a modified lac repressor gene containing a nuclear localization signal from the SV40 large T antigen and the transcription activation domain from herpes simplex virus type 1 (HSV-1) protein VP16 (LAP348). This plasmid was cut with HindIII and the 2·5 kbp fragment containing the HCMV promoter and the LAP348 gene was ligated in a series of steps with other plasmids (not shown). The final product, pMPG3, contains the HCMV promoter, the gene for protein LAP348 and the SV40-derived polyadenylation sequence and a head-to-tail copy of the Ad inverted terminal repeats (ITRs) derived from plasmid pFG140 (Graham, 1984), all flanked by BglII and BglII sites, as depicted in Fig. 2(a).
GenBank entry DQ278490.3 from G418-resistant colonies from two independent experiments were isolated.

**Southern blotting.** DNA from transformed cell lines was digested with appropriate restriction enzymes, fractionated by electrophoresis in 1% (w/v) agarose gels and transferred to positively charged nylon membranes (Boehringer) by capillary transfer (Stratagene), and then UV cross-linked with a BioRad GS GeneLinker. DNA fragments for hybridization were labelled with digoxigenin (DIG) using the DIG HighPrime system (Boehringer) and detection of DNA was performed with High SDS buffer and the DIG detection kit (Boehringer) according to the manufacturer’s protocols.

**Radioimmunoprecipitation assays (RIPA).** Virus-infected cells were labelled at various times post-infection for 2 h with [35S]methionine (50 µCi per 60 mm dish) and then harvested, and radiolabelled RG was immunoprecipitated as previously described (Graham & Prevec, 1995). When required, 10 mM IPTG was added to the cells 24 h before infection. Mouse anti-RG monoclonal antibody was provided by A. Wandel (Canadian Food Inspection Agency, Nepean, Ontario). The immunoprecipitated proteins were separated by SDS–PAGE and visualized by exposure to Kodak XAR5 X-ray film.

**Results**

The presence of an intron within transcripts has been shown to increase the expression of some genes significantly (Choi et al., 1991; Petitclerc et al., 1995; Yew et al., 1997) and we also have obtained evidence that expression of a number of proteins in Ad vectors can be greatly enhanced by placing an intron upstream of the protein coding sequences (L. Prevec & F. L. Graham, unpublished observations). Consequently, we attempted to produce replication-defective Ad vectors in which the expression of RG was driven by the strong MCMV promoter (Dorsch-Hasler et al., 1985; Addison et al., 1997) in combination with an intron sequence previously used in Ad vectors (Berkner & Sharp, 1985). The construction of plasmid pMH5(Fx) is described in Methods and shown in Fig. 1. Numerous attempts to rescue this cassette into a virus were unsuccessful, despite the use of co-transfection protocols that routinely allowed rescue of transgenes, including RG, in other expression cassettes. Over a dozen independent experiments, including some with slightly modified constructs and representing over 300 dishes (60 mm diameter) of transfected 293 cells, failed to generate a single recombinant with the correct structure. The only successful rescue resulted from a plasmid that had a fortuitous deletion of 59 nucleotides from the start of the RG open reading frame (not shown), which prevented expression, but was otherwise intact. This result strongly suggested that the failure to rescue functional vectors was due to toxicity of the over-expressed RG. To overcome this problem, we constructed a 293-based cell line which expressed a repressor protein and expression cassettes containing repressor-binding sequences in order to reduce RG expression, and attendant toxicity, in transfected cells.
Isolation and screening of cells

The first step was to produce a 293-based cell line expressing a lac repressor-derived protein (LAP348) to allow rescue and propagation of vectors containing RG expression cassettes down-regulated by the lac operator. Low-passage 293 cells were transfected with a ligation mixture of BglII–BamHI fragments from pMPG2 and pMPG3 (Fig. 2) and resistant clones were selected in the presence of G418 as described in Methods. Twenty-eight resistant colonies were isolated, propagated and assayed by Western blotting for expression of the LAP348 protein. Two lines, 293LAP10 and 293LAP13, that expressed high levels of repressor were selected for further study. Transient transfections of these cell lines with plasmids pMH5(I)Fx(o) and pMH5(I)Fx, which contain or lack the lac operator sequence, respectively, indicted that both lines expressed functional lac repressor protein, as RG production from the plasmid containing the operator was significantly reduced in 293LAP10 and 293LAP13 cells but not in 293 cells (data not shown).

Characterization of LAP cell lines by Southern blotting

Because the 293LAP lines had been transfected with a ligation mixture containing BglII–BamHI fragments from pMPG2 and pMPG3, it was of some interest to determine the structure of the integrated sequences in these lines. DNA was extracted from 293 cells and 293LAP10 and 293LAP13 and analysed by Southern blot hybridization, using as probes the BamHI–BglII fragment of pMPG2 (probe A) and the Ndel–HindIII fragment of pMPG3 (probe B) (Fig. 2a). The pattern of fragments detected in both 293LAP10 and 293LAP13 cells corresponded to that predicted for integration of a DNA segment with the structure indicated in Fig. 2(a). Both probes hybridized to Xbal fragments of about 8.6 kbp in 293LAP10 cells and about 7.4 kbp in 293LAP13 cells, suggesting a single insertion at distinct sites within the two cell lines, since Xbal does not cut within either the pMPG2- or pMPG3-derived fragments. As shown in Fig. 2(b), probe A primarily detected different fragments in the HindIII digestion products of 293LAP13 and 293LAP10 cells. These are presumably the fragments resulting from HindIII cleavage at the site depicted at nt 1592 in Fig. 2(a) and a HindIII site, unique to each cell line, in DNA sequences leftward of the insertion site. The fragments of about 2.3 kbp in lanes 1 and 2 of Fig. 2(b) presumably represent the 2333 bp HindIII fragment (from nt 1592 to 3925 in Fig. 2a) that is detected by virtue of the HCMV sequences present in probe A. These HindIII fragments are more strongly detected by probe B (Fig. 2c), as expected. As predicted by the structure indicated in Fig. 2(d), probes A and B hybridized to a PstI fragment of approximately 2.7 kbp generated by cleavage at nt 634 and nt 3401 in Fig. 2(d), as well as PstI fragments unique to each cell line due to cleavage at nt 634 and at sites in the cellular DNA leftward of the insertion site. Probe B consistently detected DNA fragments of 10 kbp or larger but these fragments were also detected in 293 DNA.

Both LAP cell lines therefore appeared to contain a single copy of the LAP348 expression cassette (from pMPG3) linked to a single copy of the neo expression cassette (from pMPG2), as indicated in Fig. 2(d). This combined cassette was integrated into distinct sites within the 293LAP10 and the 293LAP13 cell lines. For most of the subsequent studies, we arbitrarily chose the 293LAP13 cell line.

LAP348 protein levels increase after infection of 293LAP cells with Ad

Cells of line 293LAP13 were infected with Ad dl70-3 and samples of infected cells were harvested at various times post-infection. Levels of LAP348 protein were assayed by Western blotting and compared with protein levels present in uninfected cells. At 24 h post-infection there was significantly more LAP348 protein in infected samples than in the uninfected samples (Fig. 3) and levels of LAP348 remained high for 3 days post-infection. In contrast, levels of an unknown cellular protein, of about 45 kDa, that cross-reacted with the antiserum had dramatically declined. This is as expected for a typical
Table 1. Relative efficiency of rescue of Ad recombinants expressing RG on 293LAP13 cells with plasmids with or without the operator sequence

<table>
<thead>
<tr>
<th>Plasmid combination</th>
<th>No. of plaques observed (no. of dishes)</th>
<th>Average no. of plaques per dish</th>
<th>No. of plaques analysed (no. of plaques with the correct structure)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 μg pMH5(I)Fx + 5 μg pBHGE3</td>
<td>4 (5)</td>
<td>0.8</td>
<td>2 (2)</td>
</tr>
<tr>
<td>5 μg pMH5(I)Fx(o) + 5 μg pBHGE3</td>
<td>68 (8)</td>
<td>8.5</td>
<td>4 (4)</td>
</tr>
<tr>
<td>10 μg pMH5(I)Fx(o) + 10 μg pBHGE3</td>
<td>45 (6)</td>
<td>7.5</td>
<td>ND</td>
</tr>
<tr>
<td>1 μg pFG140</td>
<td>129 (4)</td>
<td>32.3</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, Not determined.

RG expression in 293 and 293LAP13 cell lines

To determine the kinetics of synthesis of RG, 293LAP13 and 293 cells were infected with 2 p.f.u. per cell (titres determined in 293LAP13 cells) of Ad(I)E3Fx or Ad(I)E3Fx(o) or of the control ‘intron-less’ virus AdE3Fx, and then labelled with [35S]methionine at 8–10 or 17–19 h post-infection. The infected cultures were harvested and radiolabelled RG was immunoprecipitated and analysed by SDS–PAGE (Fig. 4a). Ad(I)E3Fx and Ad(I)E3Fx(o) expressed comparable amounts of RG in 293 cells, whereas in 293LAP13 cells, the expression of RG by Ad(I)E3Fx(o) was markedly reduced compared with Ad(I)E3Fx, particularly at 8–10 h (compare lanes 6 and 5). Moreover, pre-treatment of the 293LAP13 cells with IPTG relieved this blockage of RG expression by Ad(I)E3Fx(o) (Fig. 4b). It is also clear from the results shown in Fig. 4(a) that the intron sequence in Ad(I)E3Fx and Ad(I)E3Fx(o) greatly increased RG expression over that produced by the comparable intron-less vector AdE3Fx.

Comparative plaque-forming efficiency on 293 cells and 293LAP13 cells

We have suggested above that high-level expression of RG resulted in inhibition of virus replication and hence prevented efficient rescue of vectors. To see if this affected plaque formation, we measured the relative plaque-forming efficiency
Fig. 4. (a) Expression of RG by different vector viruses in 293 and 293LAP13 cells. Monolayer cultures of 293 or 293LAP13 cells were infected at 2 p.f.u. per cell with one of the following viruses: AdE3Fx, which lacks intron or operator sequences but is otherwise identical to the other vectors (lanes 1, 4, 7, 10); Ad(I)E3Fx, which has the intron but no operator sequences (lanes 2, 5, 8, 11); and Ad(I)E3Fx(o), which has the intron and one operator sequence (lanes 3, 6, 9, 12). The infected monolayers were labelled with [35S]methionine at 8–10 h or 17–19 h post-infection and then the cells were harvested and extracts prepared, immunoprecipitated with anti-RG antibody and analysed by SDS–PAGE, all as described in the text. Lane 13 contains extracts from uninfected 293LAP13 cells. Two different exposures, 18 h (top) and 2 h (bottom), are presented for comparison. (b) Operator-dependent inhibition of RG expression in 293LAP13 cells and its reversal by IPTG. Monolayer cultures of 293LAP13 cells, with (lanes 4, 7) or without (lanes 3, 6) pre-treatment with IPTG, were infected with Ad(I)E3Fx, the vector lacking an operator sequence (lanes 3–4), or with Ad(I)E3Fx(o), the vector having one operator sequence (lanes 6–7). As a control, 293 cells were also infected with Ad(I)E3Fx (lane 2) or Ad(I)E3Fx(o) (lane 5). All infections were at 2 p.f.u. per cell and all infected cultures were labelled with [35S]methionine at 8–10 h post-infection and analysed as described in Methods.

Fig. 5. Expression of RG by different vectors in non-complementing human (MRC-5) or canine (MDCK) cell lines. Monolayer cultures of MDCK cells (lanes 1–5) or MRC-5 cells (lanes 6–10) were infected with one of the following: 10 p.f.u. (lanes 1, 6) or 50 p.f.u. (lanes 2, 7) per cell AdE3Fx; 10 p.f.u. per cell Ad(I)E3Fx (lanes 3, 8); or 10 p.f.u. per cell Ad(I)E3Fx(o) (lanes 4, 9); or were not infected (lanes 5, 10). The infected cultures were labelled at 8–10 h post-infection and were harvested, immunoprecipitated and analysed by SDS–PAGE, as described in Methods. The long exposure was 20 h (top) and the short exposure was 4 h (bottom).

on 293 cells or 293LAP13 cells of CsCl gradient-purified viruses Ad(I)E3Fx, Ad(I)E3Fx(o) and the control vector lacking the intron sequence, AdE3Fx (Table 2). As predicted, both of the high-level RG producers, Ad(I)E3Fx and Ad(I)E3Fx(o), were an order of magnitude less efficient than control virus in forming plaques on 293 cells, as reflected in the particle to p.f.u. ratio. More importantly, the Ad(I)E3Fx(o) vector, which contains the operator sequence, was as efficient at forming plaques on 293LAP13 cells as was the control virus. Thus, decreasing the level of RG expression by this vector, through binding of the lac repressor, resulted in a plaque-forming efficiency equal to that of control virus. This is consistent with the suggestion that high-level RG expression has an inhibitory effect on virus replication. The vector lacking the operator sequence also had an increased plaque-forming efficiency on 293LAP13 cells (approximately 3-fold compared to 293 cells). This suggests that the small reduction in RG synthesis in 293LAP13 cells compared with 293 cells (Fig. 4b, compare lane 2 and lanes 3–4) may be sufficient to allow for replication of the vector not containing the operator.

**Virus replication in 293LAP13 cells and 293 cells**

In view of the effects seen on transfection and on plaque formation, we were interested to determine whether the replication of recombinant vectors containing the promoter–intron cassette with or without the operator was substantially different in 293 or 293LAP13 cells. Fig. 6 shows one-step growth curves for Ad(I)E3Fx and Ad(I)E3Fx(o) over 48 h, at which time CPE was complete. As expected, Ad(I)E3Fx and Ad(I)E3Fx(o), which produced high levels of RG, also produced the lowest yields from infected 293 cells. The Ad(I)E3Fx(o) vector reached a final titre some 20-fold higher in 293LAP13 cells than in 293 cells. Interestingly, but not entirely unexpectedly, based on the results of the plaque-forming efficiency data shown in Table 2, Ad(I)E3Fx, lacking the operator sequence, also replicated better in 293LAP13 than 293 cells. A possible explanation for this effect is discussed below.
**Discussion**

The lac repressor system has been extensively characterized and used in prokaryotic and eukaryotic systems for both inducible expression and conditional repression of proteins (reviewed by Matthews & Nichols, 1998). In this study, we used this system to rescue and propagate E1-deleted recombinant Ads containing an expression cassette that could not be rescued previously due to the apparent toxicity of the over-expressed transgene product.

Both the cell lines that were isolated and characterized by Southern blot hybridization appeared to contain a single insertion of the neo cassette linked to the cassette containing the LAP348 gene. Ad infection of permissive cells results in down-regulation of transport of host mRNA to the cytoplasm and shut-off of the majority of host cell protein synthesis (Beltz & Flint, 1979). In view of this, it was somewhat surprising that infection of 293LAP13 cells enhanced the expression of LAP348 protein. We assume that the increase in LAP348 protein is due to up-regulation of transcription from the HCMV promoter. Such an up-regulation would only have to be temporary, since Yang et al. (1996) have shown that heat-shock genes induced artificially shortly after Ad infection escape the general shut-off of host cell mRNA transport. In any event, the increase in LAP348 protein levels in response to Ad infection is a desirable property, since it undoubtedly leads to more effective down-regulation of transgene expression.

We constructed several plasmids containing different RG gene expression cassettes. Previous reports have shown that the presence of an intron in a transcript increases the levels of protein expression, presumably by favouring processing and transport to the cytoplasm of the transcript (Choi et al., 1991; Petitclerc et al., 1995; Yew et al., 1997). Since the presence of the intron in our construct precluded efficient rescue into recombinant Ad vectors in 293 cells, we reasoned that increased levels of RG expression might be inhibitory to Ad replication processes. When the intron-containing cassette was modified by the insertion of the lac operator sequence, we were able to rescue viruses with high efficiency by using 293LAP13 cells. In addition, virus containing the operator sequence replicated more rapidly and to higher titres in 293LAP13 cells than in 293 cells. These properties undoubtedly result from the ability of the 293LAP13 cell line to repress expression of RG as a result of repressor-binding at the operator site, a conclusion that was confirmed through the use of IPTG. Our results clearly showed that RG synthesis from vectors containing the operator was greatly repressed at 8 h post-infection in 293LAP13 cells and was slightly reduced even late in infection, when abundant viral DNA replication would be expected to titrate out the lac repressor. It is possible that RG synthesis or processing competes for some function or pathway essential for Ad replication and that efficient down-regulation of RG expression is most important early in infection. Difficulty in rescuing vectors expressing high levels of glycoprotein is not unique to RG. Yoshida et al. (1997) reported a similar difficulty in rescuing the glycoprotein gene of vesicular stomatitis virus (VSV) and we have found that vectors expressing HSV-1

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**Table 2. Relative plaque-forming titres and particle to p.f.u. ratios of recombinant Ad expressing RG on 293 or 293LAP13 cells**

<table>
<thead>
<tr>
<th>Virus</th>
<th>293 cells</th>
<th>293LAP13 cells</th>
<th>Titre (p.f.u./ml)</th>
<th>Particle/p.f.u. ratio</th>
<th>Titre (p.f.u./ml)</th>
<th>Particle/p.f.u. ratio</th>
<th>Titre ratio*</th>
</tr>
</thead>
<tbody>
<tr>
<td>AdE3Fx</td>
<td>5.0 × 10&lt;sup&gt;10&lt;/sup&gt;</td>
<td>120</td>
<td>5.0 × 10&lt;sup&gt;10&lt;/sup&gt;</td>
<td>120</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ad(I)E3Fx</td>
<td>1.3 × 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>2300</td>
<td>3.5 × 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>860</td>
<td>2.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ad(I)E3Fx(o)</td>
<td>3.5 × 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>1000</td>
<td>2.4 × 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>150</td>
<td>6.9</td>
<td></td>
<td></td>
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</table>

*Ratio of titre on 293LAP13 cells to titre on 293 cells.

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**Fig. 6.** Growth curves of recombinant Ads expressing RG in either 293 or 293LAP13 cell lines. Replicate monolayer cultures each containing approximately 1 × 10<sup>6</sup> cells (either 293 or 293LAP13) were infected with the recombinant viruses at an m.o.i. of 1 p.f.u. per cell. At the indicated times post-infection, duplicate cells and supernatants were harvested and assayed for p.f.u. on 293LAP13 cells. Titres are plotted on an exponential scale. Open symbols are yields from 293 cells for Ad(I)E3Fx (○) and Ad(I)E3Fx(o) (□). Closed symbols are yields from 293LAP13 cells for Ad(I)E3Fx (●) and Ad(I)E3Fx(o) (■).
glycoproteins often replicate poorly (unpublished observations).

The observations that expression cassettes containing the intron but without the operator could also be rescued into virus by using 293LAP13 cells, albeit at very low efficiency, and that such vectors also replicated better in 293LAP13 than in 293 cells, need some explanation. We suggest that this is due to a reduction in RG expression as a consequence of transcriptional suppression or ‘squelching’ by the HSV-1 VP16 transactivation domain on the LAP348 protein. Natesan et al. (1997) have shown that transcription of episomal DNA is preferentially inhibited by ‘squelching’. Thus, inhibition might be most pronounced during transfection with plasmids, permitting the generation of virus in co-transfected 293LAP13 cells, and might be sufficiently strong early in virus replication to account for the enhanced replication evident from the results shown in Fig. 6. As a consequence, the 293LAP13 cell line may be preferred over 293 cells for the rescue of transgenes even without incorporation of the operator, though the presence of the latter in the expression cassette clearly resulted in the highest efficiency of virus rescue and replication.

A number of other solutions to the problem of overexpression of toxic genes by virus vectors have been explored. Yoshida & Hamada (1997) placed the VSV glycoprotein gene under the control of a minimal HCMV promoter fused to several tet operator sequences. The operator sequences serve as potential binding sites for a chimeric transactivator protein consisting of the C-terminal domain of the HSV-1 VP16 protein fused to the tet repressor protein. In the presence of tetracycline, the transactivator cannot bind to the promoter region, while in its absence, expression of transcripts from this promoter is a function of the amount of transactivator present. In order to provide adequate levels of transactivator, Yoshida & Hamada (1997) used a second recombinant Ad vector. Massie et al. (1998) also exploited the tetracycline-controllable expression system, developing 293 cells that expressed the tet-regulated transactivator to obtain high-level expression of a toxic protein. In yet another approach, Tomanin et al. (1997) developed Ad vectors containing cassettes regulated by a T7 promoter in combination with vectors expressing the T7 RNA polymerase to express toxic proteins.

All these methods, while potentially useful for the production of proteins in cell culture, do not lend themselves to an in vivo expression system. In contrast, our approach, which relies on repression of transgene expression during vector isolation and replication, was clearly very successful in producing recombinant Ad capable of expressing RG at high levels in other host cells and in animals. Preliminary studies in mice indicate that a very high level of rabies virus-neutralizing antibody can be induced by the intron-containing vectors following a single intraperitoneal injection (data not shown).

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