Nucleic Acids Research, Vol. 20, No. 9 2233–2239

Constitutive and enhanced expression from the CMV major IE promoter in a defective adenovirus vector

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Received February 26, 1992; Revised and Accepted March 25, 1992

ABSTRACT

A defective adenovirus (Ad) type 5 E1⁻ vector has been combined with the powerful constitutive cytomegalovirus (CMV) major immediate early (IE) promoter to produce a novel eukaryotic expression system. The Ad vector can replicate to high titres in 293 cells and then be used to infect a wide variety of non-permissive cell types. The *Escherichia coli lacZ* and CMV IE1 genes have been cloned to generate the Ad recombinants RAd35 and RAd3I respectively. In human fibroblasts infected with RAd35 β-galactosidase (β-gal) expression could be detected in virtually 100% of target cells, there was no detectable transcription from the Ad genome and extremely high levels of expression could be achieved with β-gal representing the predominant cytoplasmic cellular protein. Additionally, a number of agents, including the CMV IE1 gene product (in RAd31) and forskolin, significantly enhanced expression from RAd35-infected human fibroblasts. Lower levels of constitutive β-gal expression were obtained in RAd35-infected HeLa cells but again expression could be enhanced (up to 60 fold) by chemical inducing agents. Expression from the IE promoter in the Ad vector could be repressed by co-infection with CMV.

INTRODUCTION

High level expression of recombinant proteins can readily be achieved using Adenovirus (Ad) vectors when expression is being driven by the Ad major late promoter (reviewed in 4). Ad vectors have been produced based on E1 deletion mutants. Since the E1 gene trans-activates early phase gene expression, Ad E1⁻ mutants can replicate in helper cell lines carrying integrated E1 helper function but in other cell types early phase gene expression is not activated. This paper describes the novel combination of the powerful constitutive CMV major immediate early (IE) promoter with a defective Ad vector. The Ad vector acts as an extremely efficient gene delivery system to non-permissive target cell populations where high level expression of genes cloned under the control of the CMV IE promoter can be achieved.

The strength of the CMV major IE promoter lies primarily in its enhancer element which contains a remarkable array of 16, 18, 19 and 21 bp imperfect direct repeats (1,5). The 18 and 19 bp repeats bind transcription factors with the properties of NF-κB and the cAMP responsive binding protein (CREB) respectively (10,27,30). Induction of NF-κB-binding activity in Jurkat cells by phorbol ester treatment stimulates the IE promoter (27), while agents which increase CREB activity have been shown to enhance expression via the 19 bp repeat (6,15,22,30). Additionally the IE promoter can be stimulated in trans by the products of the CMV major IE gene itself (8) and the Ad E1a gene (9,11,22). We have exploited the inducible properties of the CMV promoter in the Ad vector to significantly enhance yields of an expressed protein.

MATERIALS AND METHODS

Cells

Primary human lung fibroblasts (MRC5 cells) and 293 cells (13) were grown in Glasgow's modified minimal essential media (Imperial Laboratories, Andover, UK) containing 8% foetal calf serum. All Ad stocks were titrated in 293 cells. MRC5 cells were stimulated by incubating in the presence of 50 ng/ml phorbol-12-myristate-13-acetate (PMA) and 4 μg/ml phytohemagglutinin (PHA), 10 μM forskolin, 1mM dibutyryl cAMP or 2mM sodium butyrate.

Construction of RAd35 and RAd31

Ad recombinants were produced according to the methodology described by McGrory and co-workers (19). The *E. coli lacZ* gene was inserted into a transient expression vector under the control of the CMV major IE promoter (−299 to +69) and upstream of a polyadenylation signal (+2757 to +3025). Nucleotide sequence numbering of the CMV strain Ad169 major IE gene is as previously described (1). The CMV IE promoter/*lacZ* expression cassette was excised from the transient expression vector on a *Hin*dIII fragment and inserted into the Ad transfer vector pMV60 to generate the plasmid pMV35 (Fig 1a). pMV60 is identical to pXCX2 (29) except that a linker (containing the *Hin*dIII cloning site) has been inserted at its unique *Xba*I cleavage site.

pMV35 and pJM17 were co-transfected into 293 cells and after 7 days Ad plaques were detected. Plasmid pJM17 contains the entire Ad5dl309 genome with the prokaryotic vector pBRX inserted into the E1a gene. The prokaryotic vector insertion makes pJM17 too large to package into Ad nucleocapsids (19).

Following recombination the IEP/*lacZ* expression cassette replaced both the prokaryotic vector and the Ad E1a gene region thus generating a smaller DNA molecule which could replicate and be packaged in the helper cell line (Fig 1a). A preliminary analysis of the Ad recombinant RAd35 has shown it to express β-gal in both MRC5 and 293 cells (33).

A second Ad recombinant containing the CMV major immediate early gene (IE1) under the control of its own promoter was constructed using the same methodology. A cDNA copy of the IE1 gene (1) was used and the IEP/IE1 expression cassette was inserted into pMV60 to produce pMV31. IEP/*lacZ* and IEP/IE1 were cloned into the Ad transfer vector in opposite orientations. Consequently, in RAd31, transcription of the IE1 gene is being driven in a right to left direction with respect to the conventional orientation of the Ad genome (Fig 1b).

β-Galactosidase assay

The expression of β-galactosidase (β-gal) was detected either by direct histological staining of cells with the chromogenic substrate X-gal (5-bromo-4-chloro-3-indolyly-β-D-galactoside) (35) or by performing a quantitative enzyme assay on cell extracts using ONPG (*o*-nitrophenyl- β-D-galactopyranoside) according to a standard protocol (26) except the reaction volume was scaled down to 100μl. The assay was performed in a microtitre plate and absorbence measured using a Multiskan MCC plate reader at 414 nm.

Immunofluorescence

MRC5 cells grown on glass coverslips were infected with RAd31 (10 PFU/cell). Acetone-fixed cells were incubated with the anti-IE1 monoclonal antibody L14.94 (kindly provided by Dr Jay Nelson) for 1h at 37°C, washed in PBS then incubated with FITC-conjugated goat anti-mouse IgG (Sigma) diluted 1:50 in PBS for 30 min at 37°C. Coverslips were washed in PBS before mounting.

Northern transfer

Total cytoplasmic RNA was purified and Northern transfer experiments were performed as described previously (34).

RESULTS

Construction of Ad recombinants and constitutive expression

In the construction of the recombinant viruses RAd35 and RAd31 the Ad E1 gene region was replaced by the expression cassettes IEP/*lacZ* and IEP/IE1 as detailed in Fig 1. Consequently, both Ad recombinants are E1 deletion mutants which require the provision of a helper function for replication. The recombinant viruses were plaque purified and high titre virus stocks prepared in 293 cells. Restriction endonuclease cleavage of DNA purified from both recombinant viruses confirmed the insertion of appropriately-sized fragments (results not shown).

The host range of CMV is extremely limited with an efficient productive infection in vitro being observed only in primary human fibroblasts. In contrast, the CMV IE promoter itself can function in a wide variety of cell types (17,21,28). Regulation of expression from the IE promoter in the Ad recombinants was investigated both in cell lines permissive (MRC5 cells) and non-permissive (HeLa cells) for CMV. HeLa cells are unusual in that following CMV infection there is no detectable IE gene expression. Nevertheless, the CMV IE promoter will function in HeLa cells when introduced by DNA transfection or in a

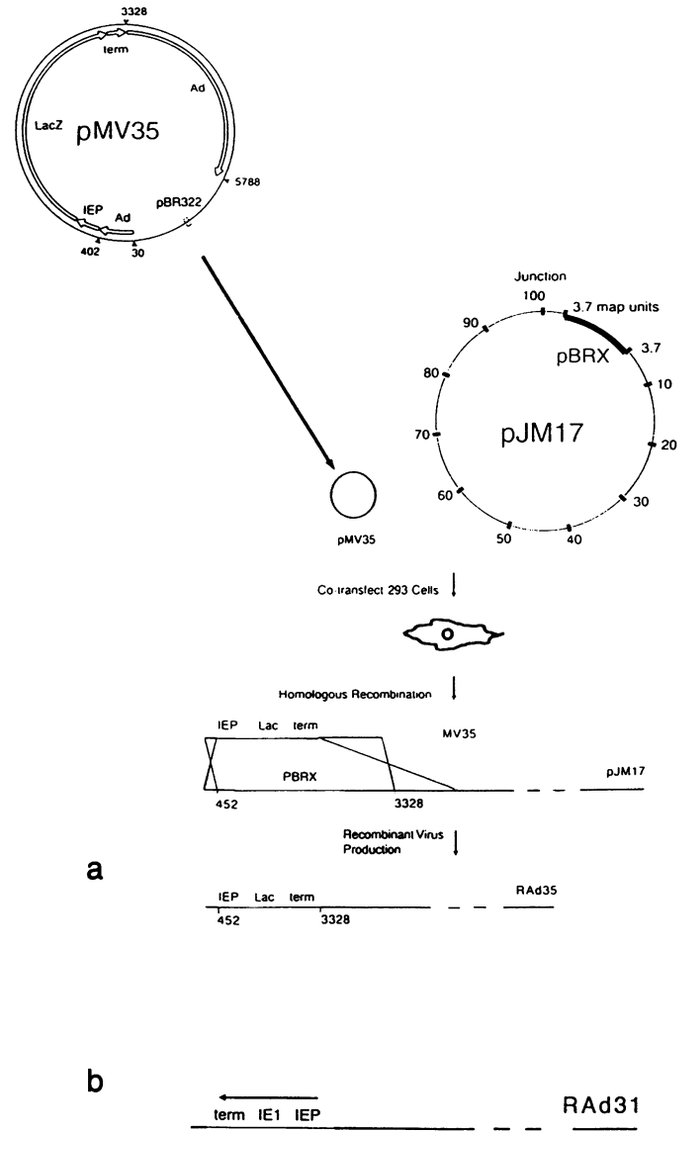


Figure 1. Generation of recombinant viruses RAd35 and RAd31. (a) The CMV IE promoter/*lacZ* expression cassette was inserted into an Ad transfer vector to generate the plasmid pMV35. The transfer vector provides the expression cassette with flanking homology from either side of the Ad E1a gene region. Numbers indicated on pMV35 refer to the Ad5 genomic sequence. pJM17 contains the entire Ad5dl309 genome. Plasmids pJM17 and pMV35 were co-transfected into 293 cells. Homologous recombination between the plasmids resulted in the IEP/*lacZ* cassette replacing both the E1a gene region (bases 402–3328) and the intervening prokaryotic vector. The resulting E1a$^-$ recombinant was designated RAd35. (b) The same protocol was used to generate RAd31. The CMV/IE1 cassette was inserted into the Ad transfer vector producing the plasmid pMV31 which was co-transfected into 293 cells with pJM17. The expression cassettes were inserted into RAd35 and RAd31 in opposite orientations.

replicating Ad vector (14). MRC5 and HeLa cells were infected with RAd35 and expression of the *lacZ* reporter gene assayed by directly staining cells with the chromogenic substrate X-gal. When MRC5 cells were infected at high multiplicity of infection (MOI) histological staining demonstrated β-gal expression in virtually 100% of the target cell population (Fig 2a&b). The Ad vector clearly functions as a highly efficient gene delivery system in primary human fibroblasts. However, expression of β-gal in

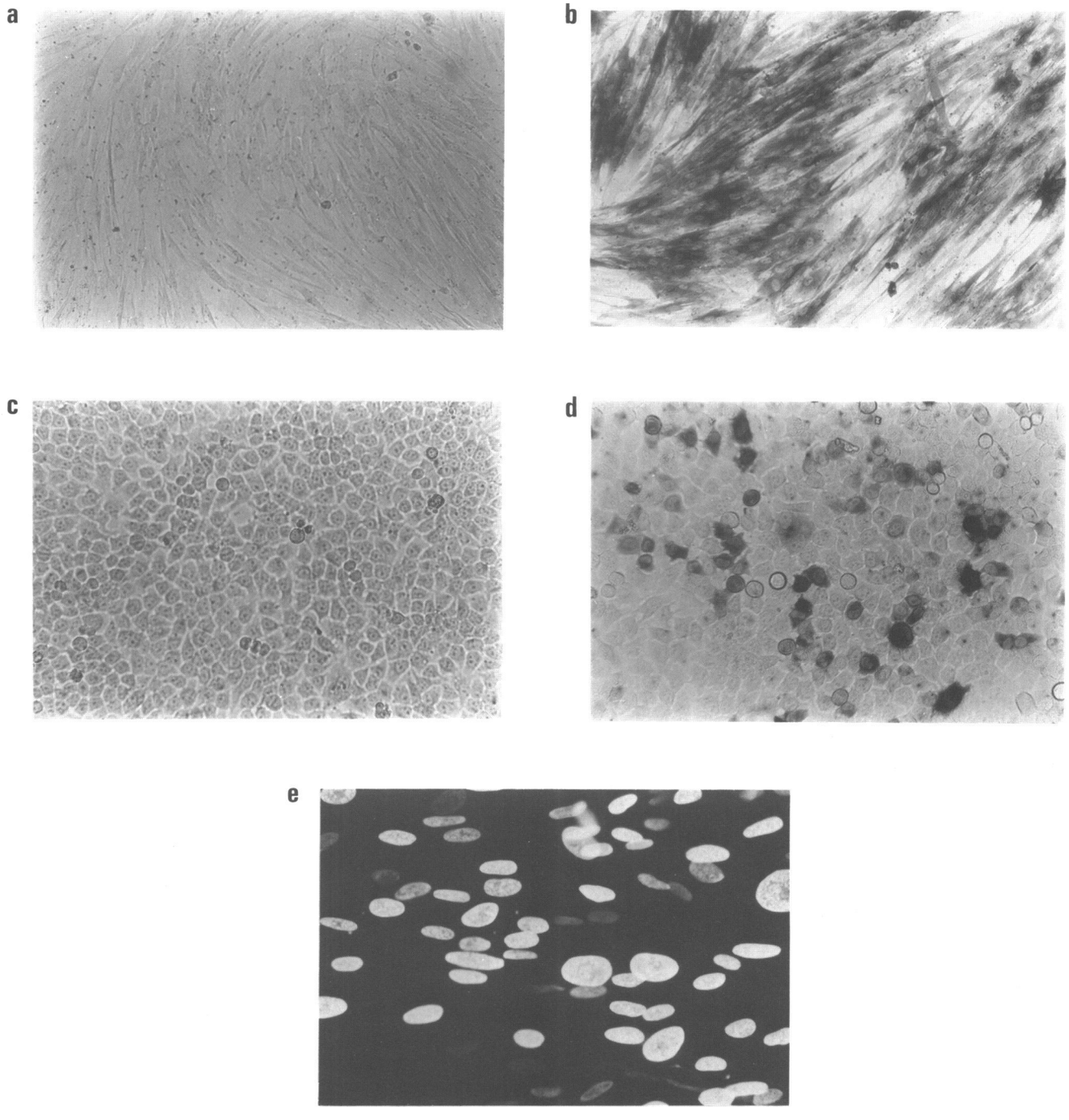


Figure 2. Expression from RAd35 and RAd31 in non-permissive cells. (a) Uninfected MRC5, (b) RAd35-infected (30 PFU/cell) MRC5 cells, (c) uninfected HeLa cells and (d) RAd35-infected (30 PFU/cell) HeLa cells were stained 48h p.i. for β-gal activity using the chromogenic substrate X-gal. The RAd35-infected cells stained a dark blue colour. (e) Nuclear expression of the CMV IE1 gene product in RAd31-infected MRC5 cells (10 PFU/cell) detected 16h p.i. by indirect immunofluorescence.

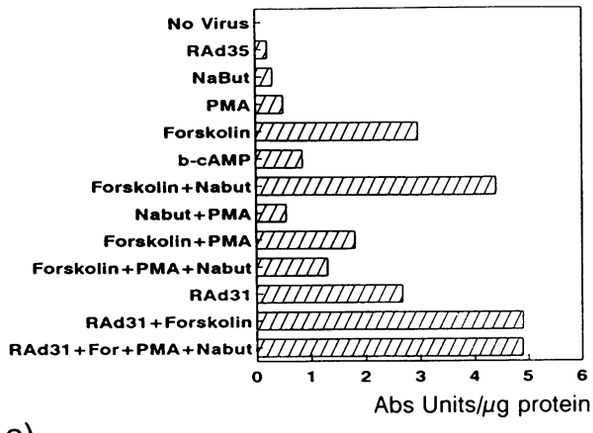
RAd35 infected HeLa cells was relatively low. Direct staining of monolayers with X-gal revealed that even at high MOI only approximately 10% of cells expressed β-gal at a detectable level (Fig 2 c&d).

Although prepared by the same methodology used to generate RAd35, the IEP/IE1 expression cassette was inserted into RAd31 in the opposite orientation (Fig 1b). To test for constitutive expression, RAd31-infected MRC5 cells were stained with an anti-IE1-specific monoclonal antibody. The characteristic nuclear immunofluorescence pattern typical of the CMV major IE protein was observed in almost all cells (Fig 2e). In a parallel control experiment performed with uninfected and RAd35-infected MRC5 cells, no significant immunofluorescence could be detected (results not shown). Infection of MRC5 cells with RAd31 was observed to induce a cytopathic effect (CPE) even at low MOI. The CPE was distinct from that induced by a wild-type Ad

infection and the results of RNA hybridisation experiments (described below) indicated the CMV IE1 gene product was unable to complement the Ad E1 deletion. This result is in agreement with published data from DNA transfection experiments (32).

Enhanced expression

Expression from the CMV IE promoter can be stimulated by a number of agents; most notably the IE1 gene product and by inducers of the transcription factors NF-κB (e.g. PMA/PHA) and CREB (e.g. forskolin and dibutyryl cAMP) which are believed to bind to the enhancer. The effect of such inducing agents on the CMV IE promoter in the Ad vector was investigated. Substantial enhancement of expression was achieved in chemically stimulated RAd35-infected MRC5 and HeLa cells (Fig 3). The most pronounced effect by a single agent was produced by forskolin in MRC5 cells where a 14-fold stimulation in β-gal expression levels was observed. Dibutyryl-cAMP routinely stimulated RAd35 expression less efficiently than forskolin. In MRC5 cells PMA/PHA treatment produced only a slight stimulation in expression and, indeed, had a negative effect when used in combination with forskolin. The pattern of stimulation by chemical agents in HeLa cells was quite different. None of the agents alone increased expression dramatically, PMA/PHA treatment producing the most significant increase (4-fold). There was, however, a synergistic effect when any two agents were used in combination. When sodium butyrate, forskolin and PMA/PHA were added together expression was stimulated 60-fold (Fig 3b). Sodium butyrate is an inhibitor of DNA replication which is known to modify chromatin structure. It has been shown to both stimulate expression from the SV40 and RSV promoters in DNA transfection experiments (12) and to induce CMV infection of epithelial cells (24,31). In both MRC5 and HeLa cells sodium butyrate had a positive effect on expression levels, especially when used in combination with other inducing agents.



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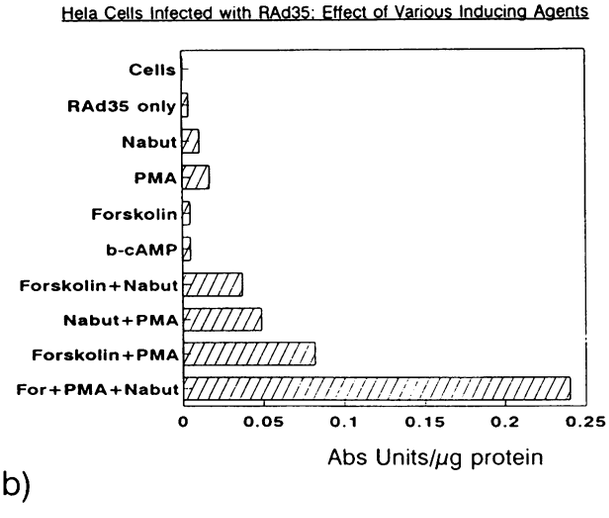
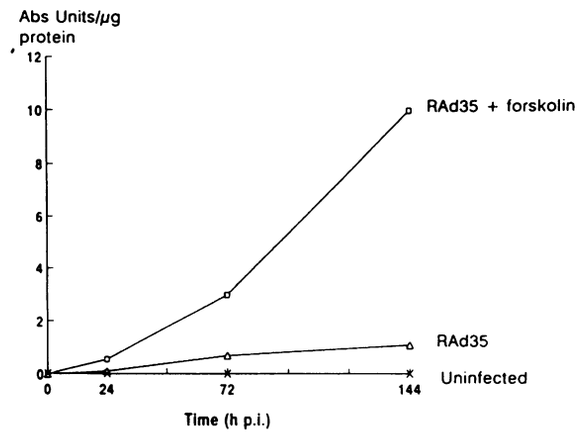


b)

Figure 3. Enhanced expression in RAd35-infected cells. a) MRC5 cells and b) HeLa cells infected with RAd35 (30 PFU/cell) and treated with inducing agents: 2 mM sodium butyrate (NaBut), 50ng/ml PMA was used in combination with 4 μg/ml PHA (PMA), 10 μM forskolin and 1 mM dibutyryl c-AMP (b-cAMP). Inducing agents were added immediately after Ad infection. In co-infection experiments RAd31 was also used at 30 PFU/cell. ONPG assays were performed on cellular extracts taken 48h p.i.



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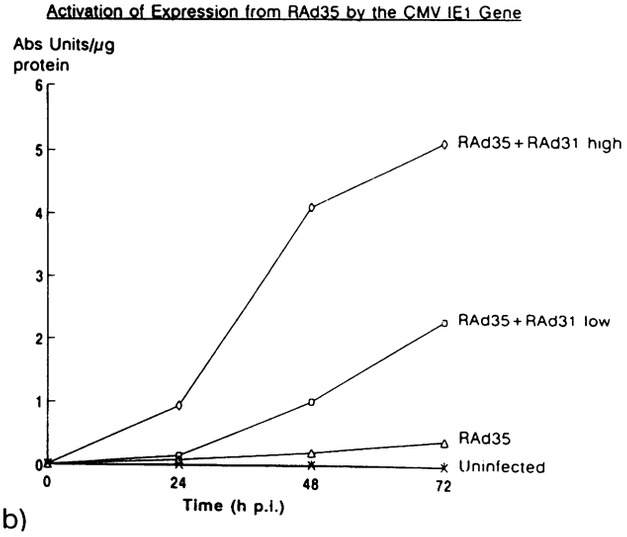

b)

Figure 4. Temporal control of expression from RAd35. RAd35 infected (30 PFU/cell) MRC5 cells were treated with (a) 10 μM forskolin or (b) co-infected with RAd31 at low (3 PFU/cell) and high (30 PFU/cell) MOI and samples taken at the times indicated. Samples were also taken for mock-infected and RAd35-infected cells in both experiments. β-Gal enzyme activity in cell extracts was measured using an ONPG assay.

In MRC5 cells, RAd31 co-infection stimulated expression from RAd35 12-fold indicating that the expressed IE1 gene product was biologically active in the context of a virus infection. The IE1 gene product (expressed by RAd31) can stimulate the IE promoter in human fibroblasts, possibly via the 18bp repeat (8), while forskolin presumably stimulates the interaction of CREB with the 19 bp repeat (6,15,30). In MRC5 cells, the highest level of β-gal expression from RAd35 was observed when RAd31 and forskolin were used in combination (23-fold stimulation).

Temporal regulation of expression

During a productive CMV infection the IE promoter is active only for the first 6 h after which transcription is repressed (20), possibly by the action of the CMV IE2 gene product. A series of time course experiments were performed to investigate temporal regulation of expression from the IE promoter in the Ad vector. The level of β-gal expressed by RAd35 in MRC5 cells continues to increase until at least 144h p.i. (Fig 4&5). In transient DNA/calcium phosphate transfection experiments maximum expression levels are reached at approximately 48 h post transfection. The Ad vector, however, appears to hold the IEP/*lacZ* expression cassette in a transcriptionally active state much longer. Furthermore forskolin-enhanced expression continued throughout the duration of the time course experiment (Fig 4a). Co-infection of RAd31 with RAd35 also enhanced expression of β-gal from the IE promoter up to 72h p.i., with the stimulatory effect of the IE1 gene product being dosage dependent (Fig 4b). After the 72h time point RAd31 co-infection began to induce cell death.

CMV encodes factors which can both stimulate and repress expression from the IE promoter. CMV co-infection effectively repressed β-gal expression from RAd35 (Fig 5). Since CMV infection can complement an Ad E1a defect (32), cytosine β-D-arabinofuranoside (ara-C) was used in this experiment to inhibit Ad replication. The repressive effect of CMV co-infection on RAd35 expression was even more pronounced when Ad replication was being inhibited. By contrast, treatment with ara-C by itself stimulated expression from RAd35-infected cells 2-fold in the absence of CMV co-infection (result not shown). The IE promoter used in the Ad vector extends from −299 to +69 and thus contains the cis-acting element identified as the site of CMV IE2-induced repression (7,18,23). The IE promoter in the RAd35 genome was being negatively regulated during the early and late phases of infection co-ordinately with the IE promoter in the CMV genome.

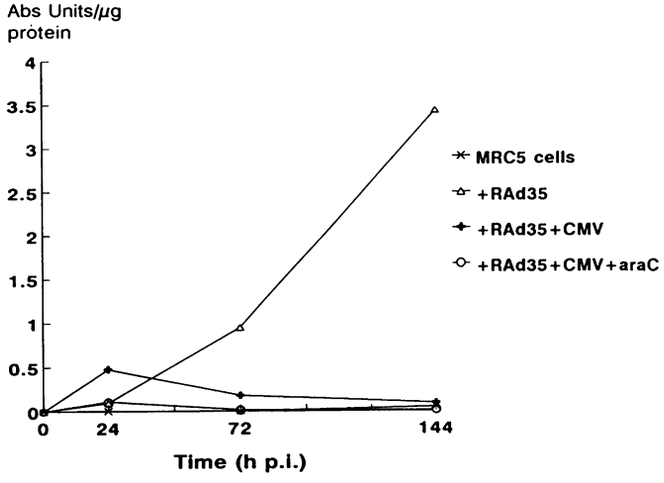

Figure 5. Effect of CMV co-infection on expression from RAd35. In a time course experiment the effect of CMV infection (5 PFU/cell), with and without Ara-C (20 μg/ml), on β-gal expression in RAd35-infected (30 PFU/cell) MRC5 cells was investigated. β-Gal enzyme activity in cell extracts were measured using an ONPG assay.

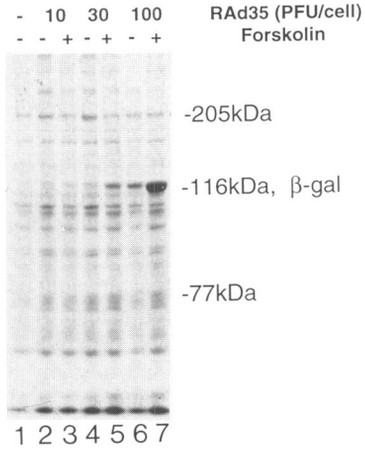

Figure 6. Visualisation of expressed protein on a polyacrylamide gel. MRC5 cells in 6 cm diameter dishes were infected with RAd35 at 10, 30 and 100 PFU/cell with and without forskolin (10μM) induction and incubated 144h. Total cytoplasmic cell extracts were prepared and samples subjected to SDS-PAGE (7.5% gel). Protein gels were stained using Coomassie blue.

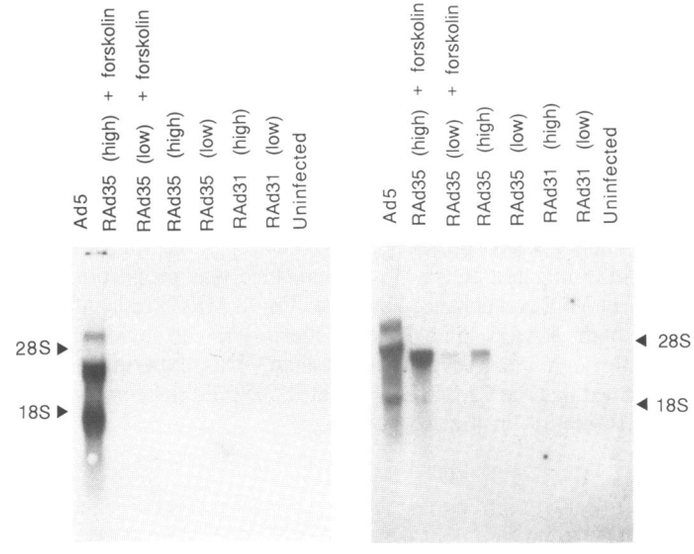

Figure 7. RNA hybridisation experiment to test for Ad transcription in nonpermissive human fibroblasts. Total cytoplasmic RNA was extracted from uninfected cells and MRC5 cells infected with: non-defective Ad5dl309 (1 PFU/cell), RAd35 at low (10 PFU/cell) and high (100 PFU/cell) MOIs, with and without forskolin induction, and RAd31 at low (3 PFU/cell) and high (30 PFU/cell) MOIs. RNA was prepared 72h p.i. The RNA was then subjected to electrophoresis in 1% formaldehyde agarose gels, blotted on to nitrocellulose membranes and hybridised with radiolabelled DNA probes. The resulting autoradiographs are shown: a) hybridised with a pJM17 probe and b) hybridised with a pJM17 probe then rehybridised with a probe containing the *lacZ* gene (pON3).

Expression Levels

Expression from the CMV IE promoter in the Ad vector has been shown to increase appreciably with time and to be strongly stimulated by certain inducing agents. We were interested in determining what levels of expression could be achieved with this vector system. MRC5 cells were therefore infected at 10, 30 and 100 PFU/cell with RAd35 then incubated for 144h both with and without forskolin induction. Cytoplasmic protein extracts were prepared and analysed by SDS-PAGE. Expression from RAd35 was proportional to the size of the input virus inoculum with or without forskolin induction (Fig 6). The level of expression detected in non-permissive cells from the constitutive CMV IE promoter was extremely high for a non-replicative system. An induced protein corresponding to β-gal can be clearly identified in Fig 6 lanes 5&6 and in lane 7 it is the predominant protein species. A densitometric analysis of lane 7 indicated that β-gal represented 17.5% of total cytoplasmic cellular protein giving an estimated yield of approximately 27μg β-gal for a 6 cm diameter tissue culture dish in this sample. In similar experiments β-gal expression levels of greater than 35% total cytoplasmic cell protein have been achieved (not shown).

Restriction of Ad Vector Expression

The primary purpose in developing the vector system was to use Ad as an efficient gene delivery system to produce expression only of the gene cloned under the control of the CMV IE promoter. It was important, therefore, to investigate whether there was significant breakthrough to Ad early and late phase gene expression in the non-permissive cell line. Total cytoplasmic RNA was prepared from MRC5 cells infected with RAd35, RAd31 and a non-defective Ad5. In a hybridisation experiment, a radiolabelled pJM17 probe (containing the complete Ad genome) was able to detect Ad-encoded RNAs in MRC5 cells infected with the non-defective Ad but not in cells infected with RAd35 (with or without forskolin stimulation) or RAd31 (Fig 7a). Ethidium bromide staining demonstrated similar amounts of RNA were present in each track (not shown). To further test that mRNAs purified from RAd35-infected cells were intact a hybridisation probe containing the *lacZ* gene was also used (Fig 7b). A major RNA species of approximately 3.3kb was detected in RAd35-infected cells whose abundance was proportional to the input MOI and enhanced by forskolin. In MRC5 cells infected with both RAd35 and RAd31 there was no evidence of breakthrough into Ad gene expression. This experiment also confirmed that the CMV IE1 gene in RAd31 did not complement the E1 deletion in the Ad vector.

DISCUSSION

The CMV IE promoter was combined with a defective Ad vector to express recombinant gene products in the target cell population without: (i) having to accommodate the artificial conditions and inefficiency associated with DNA transfection, (ii) the need to clonally select continuous cell lines or (iii) interference from vector gene functions. Ad type 5 is a particularly appropriate vector to use as a gene delivery system. High titre Ad5 stocks ($>10^9$ PFU/ml) can readily be produced, an efficient technology has been developed to generate recombinants (19) and the virus can infect an exceptionally wide range of cell types. RAd35 has been shown to infect and induce β-gal expression in human lymphocytic lines (JM, C8166 and U937 cells), primary mouse macrophages, primary chick embryo fibroblasts, 293, Vero, porcine kidney and Chinese hamster ovary cells (unpublished results).

Expression from RAd35, detectable in virtually 100% of infected MRC5 cells, was proportional to the size of the virus inoculum and continued to increase with time up until at least 144h p.i. The efficiency of expression was much less efficient in HeLa cells. Since they are highly susceptible to Ad infection, HeLa cells presumably either lack factors necessary for activating or contain factors capable of repressing the IE promoter. Levels of expression attainable in different cell types will be dependent on the susceptibility of cells to Ad infection combined with either the constitutive or the inducible activity of the CMV IE promoter.

We were interested in determining whether the expression system could be used to study the control of gene expression. The ability of (i) the CMV IE1 gene product encoded by RAd31 to trans-activate expression from RAd35 and (ii) CMV co-infection to repress expression was clearly demonstrated. The cytotoxicity associated with RAd31 infection suggests the IE1 gene product may have a role in generating the CPE associated with the early phase of CMV infection (2). In addition to being a transcriptional trans-activator, the CMV IE1 gene also encodes a primary target for cytotoxic T lymphocytes (3). The recombinant RAd31 will facilitate further studies into the biological properties of the CMV major IE protein.

The CMV IE promoter in the context of the Ad vector was susceptible to stimulation by a variety of chemical agents. Interestingly, the effect of such inducing agents on the promoter in MRC5 and HeLa cells was quite different. Forskolin clearly significantly enhanced expression from the Ad-based expression system in MRC5 cells (although not in HeLa cells) and potentially could be used to increase the yield of any recombinant protein. Results obtained with RAd35 are not in complete agreement with data from DNA transfection experiments. Most notably, Stamminger and co-workers (30) observed that cAMP stimulation increased expression 5.2-fold in HeLa cells but no enhancement was detected in human fibroblasts. If such differences are due to the method of gene delivery, then the results obtained with RAd35 are important to studies of CMV pathogenesis. However, the Ad vector contains a relatively weak enhancer element, normally associated with the Ad E1 gene, located upstream of the CMV enhancer. This additional enhancer element may influence expression from the CMV promoter.

The combination of the CMV IE promoter with a defective Ad vector has definite advantages over comparable expression systems and can clearly be applied to studies of gene regulation, gene function and antigen presentation. A similar Ad recombinant has recently been used to generate an antibody response in vaccinated mice and provide protection against a viral challenge (16). This novel, efficient expression system has great potential for use in vaccine development and possibly also for use in somatic gene therapy (25). The defective Ad vector inflicts minimal damage on the target cell population, compared with lytic vaccinia systems, and provides a degree of biological containment. The exceptionally high levels of β-gal detected in human fibroblasts infected with RAd35 exceeded expectation and makes the system attractive simply to achieve high level production of recombinant proteins.

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