Gene Therapy (1997) 4, 258–263

Isolation and characterization of packaging cell lines that coexpress the adenovirus E1, DNA polymerase, and preterminal proteins: implications for gene therapy

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Current generation adenovirus (Ad) vectors are deleted for the E1 region of genes and require propagation in E1 expressing 293 cells. Expression of genes delivered by Ad vectors into immunocompetent hosts is generally transient since the current vectors are not completely replication defective. Viral proteins expressed by Ad vectors, in part, induce a rapid, T cell-mediated loss of the transduced cells. Introduction of temperature-sensitive point mutations into new Ad vectors may be of limited usefulness in prolonging transduced gene expression in vivo. Isolation of new Ad vectors deleted for genes required for normal Ad growth may further prevent Ad protein expression. These new vectors will need to be grown in 293 cells capable of coexpressing other Ad genes. Unfortunately, many of the Ad genes are toxic when coexpressed in 293 cells. We describe the isolation of E1 expressing 293 cells which also express both the Ad polymerase and preterminal proteins, both of which are essential to normal Ad growth. The isolation of new Ad vectors deleted for the E1, polymerase and preterminal proteins are predicted to have many advantageous properties, including the prolongation of transduced foreign gene expression in vivo.

Keywords: *adenovirus; gene therapy; 293 cells; polymerase; preterminal protein; packaging*

Introduction

Adenovirus (Ad) vectors have been extensively utilized as vectors for the delivery of foreign genes to mammalian cells *in vitro* and *in vivo*. There are many advantages when utilizing Ad vectors, however, shortfalls have become apparent with their use *in vivo*. This is due in part because current Ad vectors are deleted for only the early region 1 (E1) genes. These vectors are crippled in their ability to replicate normally without the transcomplementation of E1 functions provided by human 293 cells.[1] Unfortunately, with the use of high titers of E1 deleted vectors, and the fact that there are E1-like factors present in many cell types, E1 deleted vectors can overcome the block to replication and express other viral gene products.[2–4] The expression of viral proteins in the infected target cells elicits a swift host immune response, that is largely T cell mediated.[5,6] The transduced cells are subsequently eliminated, along with the transferred foreign gene. In immuno-incompetent animals, Ad delivered genes can be expressed for periods of up to 1 year.[6–8] Another shortcoming is that a single recombination event between the genome of an Ad vector and the integrated E1 sequences present in 293 cells can generate replication competent Ad (RCA), which can readily contaminate viral stocks. In order to further cripple viral protein expression, and also to decrease the frequency of generating RCA, we have begun to determine which genes of the virus can be constitutively expressed in 293 cells along with the E1 gene products. With every gene that can be constitutively expressed in 293 cells comes the opportunity to generate new versions of Ad vectors deleted for the respective genes. This would have immediate benefits; increased carrying capacity and a decreased incidence of RCA generation, since two or more independent recombination events would be required to generate RCA. In addition, when genes critical to the viral life cycle are deleted, a further crippling of Ad to replicate and express other viral gene proteins would occur. This should decrease immune recognition of virally infected cells, and allow for extended durations of foreign gene expression.

We have addressed the limitations of current generation Ad vectors by isolating new 293 cell lines coexpressing critical viral gene functions. As a first step, we previously described the isolation and characterization of 293 cell lines capable of constitutively expressing the Ad polymerase protein.[9] We now report the isolation of 293 cells which not only express the E1 and polymerase proteins, but also the Ad preterminal protein. The isolation of this cell line demonstrates that three genes critical to the life cycle of Ad can be constitutively coexpressed. The potential of these cell lines to allow the isolation of new Ad vectors deleted for all three gene functions is discussed.

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Received 1 July 1996; accepted 13 November 1996

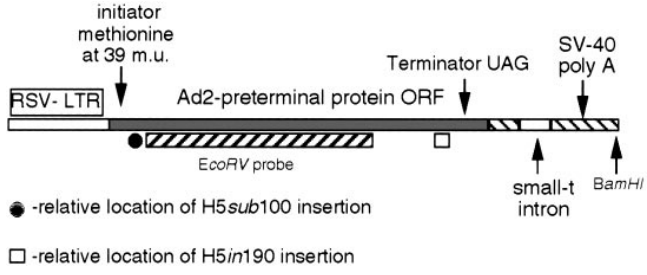

Figure 1 *The expression plasmid pRSV-pTP. Note the location of the EcoRV subfragment utilized as a probe in the genomic DNA and cellular RNA evaluations, as well as the initiator methionine codon present from map unit 39 in the Ad5 genome.[10] The locations of the H5in190 and H5sub100 insertions are shown relative to the preterminal protein open-reading frame.*

Results

Isolation of Ad polymerase and preterminal protein expressing 293 cells

As we have previously described, LP-293 cells were cotransfected with pRSV-pol and a hygromycin resistance plasmid to isolate 20 Ad polymerase expressing cell lines.[9] In addition, some of the cell lines were also initially cotransfected with the preterminal protein expression plasmid, pRSV-pTP (Figure 1).[10] All cell lines were then grown in the presence of hygromycin, and individual clonal isolates were expanded. Seven cell lines originally transfected with pRSV-pol and pRSV-pTP were further analyzed.

Analysis of cell line DNA and RNA

We screened those cell lines which had received the preterminal protein expression plasmid for the presence of pRSV-pTP sequences. One of the transfected cell lines, C-7, was found to contain the preterminal protein coding sequences within its genomic DNA. Genomic DNA from C-7, B-6 (transfected only with pRSV-pol), and the parental LP-293 cell lines were analyzed by Southern blot analysis for the presence of both E1 and preterminal protein sequences. As shown in Figure 2a, only the C-7 cell line genomic DNA had preterminal coding sequences, unlike the parental LP-293 cells, or the Ad polymerase expressing B-6 cells. In addition, all cell lines have E1-specific sequences present at nearly equivalent amounts, demonstrating that the selection design has not caused the loss of the E1 sequences originally present in the LP-293 cells. We have previously demonstrated that both the B-6 and C-7 cell lines contain polymerase-specific sequences within their genomes, unlike the parental LP-293 cells.[9] To confirm that transcription of preterminal protein was occurring, total RNA was isolated from each of the cell lines, transferred to Nylon membranes, and probed to detect preterminal protein-specific mRNA transcripts. As shown in Figure 2b, a single mRNA of the expected size (approximately 3000 nucleotides in length) is only detected in the C-7 cell line-derived RNA. We have previously demonstrated that the C-7 cell line also expresses high levels of the Ad polymerase mRNA,[9] therefore demonstrating that the constitutive expression of both of these mRNAs along with E1 transcripts is without significant toxicity.

Plaquing efficiency of pTP mutants on pTP-expressing cell lines

We next screened the C-7 cell line for the ability to transcomplement the growth of previously described pre-

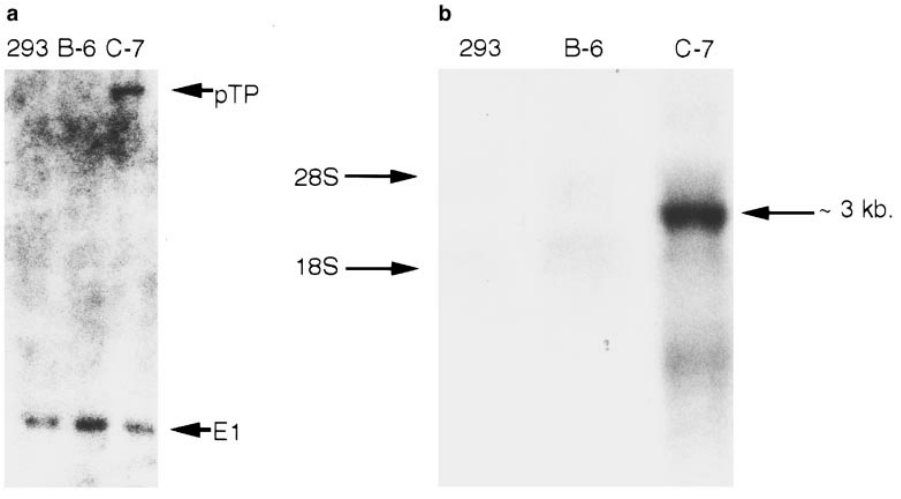

Figure 2 *(a) DNA analysis. Two micrograms of DNA from each of the indicated cell lines was prepared and digested with XbaI and BamHI, electrophoresed, transferred to a nylon membrane, and probed with E1 and preterminal protein coding sequence-specific probes simultaneously. The preterminal specific sequences migrated as an approximately 11.0 kb DNA fragment while the E1 containing band migrated as a 2.3 kb DNA fragment. Note the lack of pTP hybridization to DNA isolated from either the LP-293 or B-6 cell lines. (b) RNA analysis. Fifteen micrograms of total RNA from each of the indicated cell lines was prepared, electrophoresed, transferred to a nylon membrane, and probed with the EcoRV subfragment of Ad5 DNA, which is complementary to the preterminal protein coding region. The preterminal protein mRNA migrated as an approximately 3000 nt message. Note the lack of hybridization in the LP-293 or B-6 cell line-derived RNAs.*

terminal mutant viruses. The virus H5*in*190 (contains a 12 base pair insertion located within the carboxy-terminus of the preterminal protein,) has been shown to have a severe growth and replication defect, producing less than 10 plaque-forming units (p.f.u.) per cell.[11] When equivalent dilutions of H5*in*190 were utilized, the plaquing efficiency of the C-7 cell line was at least 100-fold greater than that of the B-6 or LP-293 cells (Table 1). This result demonstrates that the C-7 cell line produces a functional preterminal protein, capable of transcomplementing the defect of the H5*in*190-derived preterminal protein. We next screened the cell lines with the temperature-sensitive virus, H5*sub*100, at nonpermissive temperatures. H5*sub*100 has a codon insertion mutation within the amino-terminus of the preterminal protein, as well as an E1 deletion. The mutation is responsible both for a temperature-sensitive growth defect, as well as a replication defect.[11,12] The plaquing efficiency of the cell line C-7 was found to be at least 1000-fold greater than that of the LP-293 cells (at nonpermissive temperatures), see Table 1. Interestingly, the cell line B-6 was *also* capable of producing large numbers of H5*sub*100-derived plaques at 38.5°C, even though it does not express any preterminal protein. This result suggested that the high level expression of the polymerase protein was allowing plaque formation of H5*sub*100 in the B-6 cell line. We therefore further examined the nature of H5*sub*100 growth in the various cell lines.

Complementation of the replication and growth defects of H5sub100

The cell lines B-6 and C-7 have been previously demonstrated to overcome the replication defect of H5ts36.[9] Since the preterminal and polymerase proteins are known to physically interact with each other,[10] we investigated whether the expresssion of the Ad polymerase could overcome the replication defect of H5*sub*100 at nonpermissive temperatures. The results depicted in Figure 3 demonstrate that *both* cell lines B-6 and C-7 could transcomplement the replication defect of H5*sub*100. This result demonstrated that the expression of the Ad polymerase in B-6 cells was able to overcome the preterminal protein mediated replication defect of H5*sub*100, possibly because of a direct physical interaction of the polymerase with the amino-terminus of the H5*sub*100-derived preterminal protein. In support of this hypothesis, we have also noted that the H5*sub*100 replication defect could be overcome when LP-293 cells were infected with a 100-fold greater amount of H5*sub*100 (data not shown). However, complementation of the H5*sub*100 replication defect is *not* sufficient to overcome the growth defect of H5*sub*100, since temperature shift-up experiments have demonstrated that the H5*sub*100 growth defect is not dependent upon viral replication.[12] Therefore, the overexpression of the Ad polymerase must have allowed a very low level but detectable production of infectious H5*sub*100 particles in the B-6 cell line. The reduced growth of H5*sub*100 is therefore not due to a replication defect, but rather some other critical activity that the preterminal protein has a role in, such as augmentation of viral transcription by association with the nuclear matrix.[13,14] This was confirmed by assessing the ability of the C-7 cell line to overcome the growth defect of H5*sub*100 utilizing one-step growth assays (Figure 4). The results demonstrated that even though the B-6 cell line allowed normal replication and plaque formation of H5*sub*100 at 38.5°C (B-6 cells were utilized to determine the plaque titers depicted in Figure 4) they could not allow high-level growth of H5*sub*100 and only produced titers of H5*sub*100 equivalent to that produced by the LP-293 cells. The C-7 cell line produced 100-fold more virus than the LP-293 or B-6 cells (Figure 4). Encouragingly, the titer of H5*sub*100 produced by the C-7 cells approached titers produced by LP-293 cells infected with the wild-type virus, Ad5.

When the H5*sub*100 virions produced from infection of

Table 1 Plaquing efficiency of preterminal protein-mutant viruses

Virus	*Mutation location*	*Plaque titers (p.f.u./ml)*		
		LP-293	*B-6*	*C-7*
H5*in*190	C-terminus	$<1\times10^{2}$	$<1\times10^{2}$	1.4×10^{5}
H5*sub*100	N-terminus	$<1\times10^{4}$	9.0×10^{8}	4.5×10^{8}

LP-293, B-6 or C-7 cells were seeded at a density of $2.0–2.5\times10^{6}$ cells per plate. The cells were infected with limiting dilutions of lysates derived from the preterminal protein-mutant viruses H5*in*190 or H5*sub*100, incubated at 38.5°C, and plaques counted after 6 days. Only the C-7 cell line allowed efficient plaque formation of H5*in*190 at 38.5°C (the H5*in*190 lysate used to infect the cells was of a low titer, relative to the high titer H5*sub*100 stock) while both the B-6 and C-7 cell lines had nearly equivalent plaquing efficiencies when H5*sub*100 was utilized as the infecting virus.

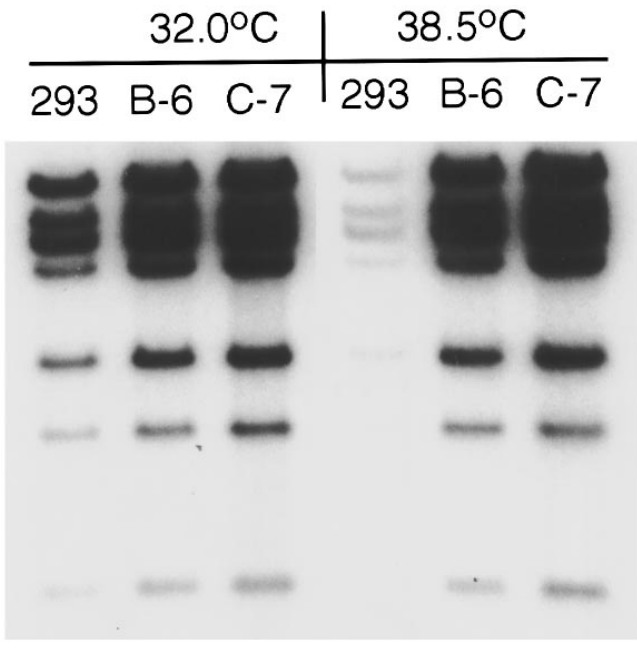

Figure 3 *Replication-complementation assay. Cells (2.0×10^{6}) of each of the cell lines were infected at an MOI of 0.25 with H5sub100. Four micrograms of total DNA was harvested from the cells after 16 h of incubation at 38.5°C (or after 40 h at 32.0°C), digested with HindIII, and transferred to a nylon membrane. The membrane was probed with ^{32}P-labeled Ad5 virion DNA. Note the H5sub100 replication defect when grown in LP-293 cells at 38.5°C, which is not present when the virus is grown at the same temperature in either B-6 or C-7 cells.*

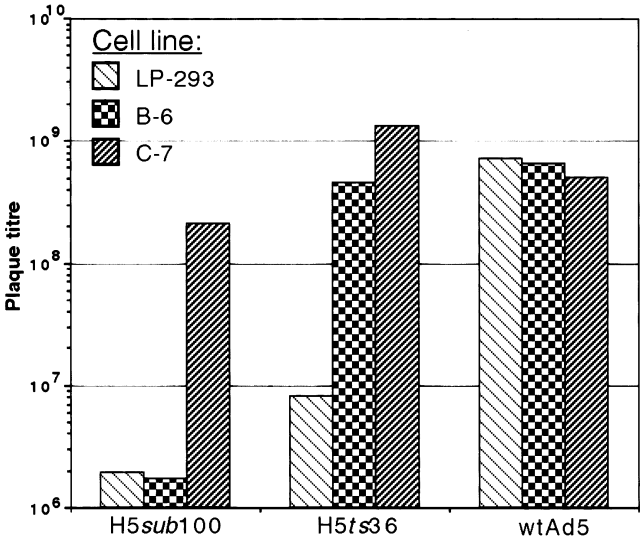

Figure 4 *One step growth curves of wtAd5, H5ts36 and H5sub100. Cells (2.0×10^6) of each of the cell lines were infected at an MOI of 4 with either wt Ad5, H5ts36 or H5sub100, and incubated at 38.5°C for 40 h. The total number of virion particles produced from each infection was determined by limiting dilutions on the B-6 cell line. Note the high-level production of H5sub100 only in the C-7 cell line at 38.5°C.*

the C-7 cells were used to infect LP-293 cells at 38.5°C, all virus produced retained the *ts* mutation, *ie* at least a 1000-fold drop in pfu was detected when LP-293 cells were infected at 38.5°C *versus* 32.0°C. This finding effectively rules out the theoretical possibility that the H5*sub*100 input virus genomes recombined with the preterminal protein sequences present in the C-7 cells. In addition, the C-7 cell line allowed the high-level growth of H5*ts*36, demonstrating that adequate amounts of the Ad polymerase protein were also being expressed. The C-7 cell line was capable of transcomplementing the growth of both H5*ts*36 and H5*sub*100 after 4 months of serial passaging, demonstrating that the coexpression of the E1, preterminal, and polymerase proteins was not toxic. Finally, it is clear that the constitutive expression of both the polymerase and preterminal proteins is not detrimental to normal virus production, which might have occurred if one or both of the proteins had to be expressed only during a narrow time period during the Ad life cycle. In summary, these results demonstrated that the C-7 cell line may be able to allow the high-level growth of E1, preterminal, and polymerase-deleted Ad vectors.

Discussion

The utilization of Ad vectors for gene therapy has great potential due to the normal biology of Ad. Ad-mediated gene transfer into mammalian cells is extremely efficient, relative to even the most efficient polycation or liposome-mediated delivery methods. Delivery and subsequent foreign gene expression by Ad vectors does not require cell division (unlike retrovirus-mediated gene transfer) which is required when attempting gene transfer into quiescent, terminally differentiated cells, such as skeletal muscle. Despite these attributes, there are several drawbacks that must be addressed when contemplating the use of presently available Ad vector systems *in vivo*, which have also raised questions concerning clinical gene therapy trials in general. These concerns include: (1) the generation of RCA; (2) suboptimal carrying capacity, especially when considering gene transfer of larger cDNAs (such as dystrophin[15]); and (3) the transient expression of transduced genes, partly due to Ad protein expression inducing an immune-system-mediated elimination of virally infected cells.[6,16]

We have attempted to address each of these concerns by capitalizing upon normal Ad biology. The Ad life cycle is a complex and coordinated interaction of at least 70 virally encoded proteins interacting with a myriad of host cellular factors. An initial hypothesis of ours was that the deletion of critical viral genes (in an E1-deleted Ad vector) involved in genome replication should have the potential to decrease overall viral protein expression (and diminish the host immune response) *in vivo*.[9] Deletion of viral coding regions would also increase carrying capacity and decrease the frequency of generating RCA. To delete critical genes from future Ad vectors, the proteins encoded by the targeted genes have first to be coexpressed in cells together with the E1 proteins. Therefore, only those proteins that are nontoxic when coexpressed constitutively (or toxic proteins inducibly expressed) can be utilized. Coexpression in 293 cells of the E1 and E4 genes has been demonstrated (utilizing inducible promoters),[17,18] as well as 293 cells coexpressing E1 and protein IX regions (a virion structural protein),[19] and 293 cells expressing E1, E4 and protein IX genes.[20]

We have investigated a different region of the viral genome, E2-b, which encodes the viral replication proteins which are absolutely required for Ad genome replication.[21,22] We have previously described the isolation of 293 cells constitutively expressing the 140 kDa Ad polymerase.[9] Other researchers have reported the isolation of 293 cells which express the Ad preterminal protein utilizing an inducible promoter.[12] We now report the high-level, constitutive coexpression of the E1, polymerase, and preterminal proteins in 293 cells, without toxicity. We have demonstrated the ability of these cells to allow the normal growth of both the polymerase mutant virus, H5ts36, and the preterminal protein mutant viruses, H5*in*190 and H5*sub*100. The results suggest that the isolation of new Ad vectors deleted for the E1, polymerase, and preterminal proteins is now possible. Several benefits will be achievable with these new vectors, including a decreased ability to generate RCA. Carrying capacity will also be significantly increased, but the deletion of the polymerase and preterminal protein genes will not be straightforward, since other viral regulatory elements are also present in this area, including the second and third tripartite leader sequences, the i-leader, portions of the major–late promoter intronic sequences (required for high-level transcription from the major–late promoter (MLP)), and the IVa2 gene. Despite this complexity, we anticipate that deletions of subportions of the polymerase and preterminal protein genes can theoretically approach at least 4.6 kb. Therefore, the C-7 cell line has the potential to allow the propagation of new Ad vectors with a carrying capacity approaching 13 kb without the need for a contaminating helper virus.[23,24] The most important attribute of E1, polymerase and preterminal protein deleted vectors, however, will be their inability to express

the respective proteins, as well as a predicted lack of expression of most of the viral structural proteins. For example, the MLP of Ad is responsible for transcription of the late structural proteins L1 to L5.[21] Though the MLP is minimally active before Ad genome replication,[21,25,26] the rest of the late genes get transcribed and translated from the MLP only after viral genome replication has occurred.[25] This *cis*-dependent activation of late gene transcription is a feature of DNA viruses in general, such as in the growth of polyoma and SV-40. The polymerase and preterminal proteins are absolutely required for Ad replication (unlike the E4 or protein IX proteins) and we hypothesize that their deletion will be extremely detrimental to Ad vector late gene expression. Experiments to prove these theories are currently underway.

Materials and methods

Tissue culture and virus propagation

The use of LP-293 cells (Microbix Biosystems, Toronto, Canada), Ad polymerase expressing cell lines, and plaquing efficiency assays of Ad viruses has been described previously.[9] All cells were maintained in 10% fetal bovine serum supplemented DMEM media (GIBCO, Gaithersburg, MD, USA) in the presence of antibiotics. The virus H5*sub*100 (provided by Dr H Ginsberg[11]) has a temperature sensitive (ts) mutation caused by a three base pair insertion within the amino-terminus of the preterminal protein, in addition to a deletion of the E1 sequences (see Figure 1). H5*sub*100 was propagated and titrated at 32.0°C in LP-293 cells; the leakiness of this stock was less than 1 per 1000 p.f.u. at the nonpermissive temperature of 38.5°C. A lower titer cell lysate containing the virus H5*in*190 (which contains a 12 base pair insertion within the carboxy-terminus of the preterminal protein as well as a deletion of the E1 region, see Figure 1) was provided by Dr P Freimuth.[11] The polymerase and preterminal protein expressing cell lines were always maintained in media supplemented with hygromycin (Sigma, St Louis, MO, USA) at 100 μg/ml.

Isolation of Ad polymerase and preterminal protein expressing 293 cells

We utilized the preterminal protein expression plasmid pRSV-pTP, provided by Dr K Padmanabhan.[10] Briefly, the Ad2 preterminal protein mRNA (including the amino terminal peptides encoded by the exon at map unit 39 of the Ad genome) was placed under the transcriptional control of the Rous sarcoma virus LTR/promoter element, and flanked on its 3′ end by the SV-40 small-t intron and SV-40 polyadenylation signals (see Figure 1 and Ref. 10). The cell lines C-1, C-4, C-7, C-13 and C-14 (previously found to express the Ad polymerase protein) were also transfected with the *Bam*HI linearized plasmid pRSV-pTP. Each of the cell lines were selected in hygromycin containing media and screened for the ability to support the growth of H5ts36 (ts for the Ad polymerase), H5*in*190 and H5*sub*100.

Analysis of genomic DNA and cellular RNA

Total DNA was isolated from the LP-293, B-6 or C-7 cell lines, 2 μg of each DNA was codigested with the restriction enzymes *Xba*I and *Bam*HI, electrophoretically separated in a 0.6% agarose gel, and transferred on to a nylon membrane. The membrane was UV crosslinked, probed with both the 1.8 kb *Bln*I–*Xba*I fragment (spans the E1 coding region) isolated from the plasmid pFG140,[27] and the 1.8 kb *Eco*RV subfragment of Ad serotype 5 (spans the preterminal protein coding sequences), both of which were random-primer radiolabeled with ^{32}P to a specific activity greater than 3.0×10^8 c.p.m./μg. The membrane was subsequently exposed to radiographic film with enhancement by a fluorescent screen. Cellular RNA was isolated from the respective cell lines, transferred to nylon membranes, and probed with the 1.8 kb *Eco*RV radiolabeled subfragment of Ad5 (see Figure 1) as described previously.[9,28] A total of 15 μg of genomic RNA was evaluated per cell line.

Replication-complementation assay of H5sub100

LP-293, B-6, or C-7 cells were seeded on to 60 mm dishes at a density of 2×10^6 cells per dish and infected the next day with H5*sub*100 at a multiplicity of infection (MOI) of 0.25, and incubated at 38.5°C for 16 h or 32.0°C for 40 h. The cells from each infected plate were then harvested and total DNA extracted as described previously.[9] Four micrograms of each DNA sample was restriction enzyme digested with *Hin*dIII, electrophoresed through a 0.7% agarose gel, transferred to a nylon membrane, and probed with ^{32}P-labeled H5ts36 virion DNA.

One-step growth curve assays

Each of the cell lines were seeded on to 60 mm dishes at 2.0×10^6 cells per dish. The cell lines were infected at an MOI of 4 with each of the appropriate viruses (wtAd5, H5ts36 or H5*sub*100), and incubated at 38.5°C for 40 h. The total virus produced in each 60 mm dish was released from the cell lysates by three cycles of freeze–thawing, and the titer determined by limiting dilution and plaque assay on B-6 cells at 38.5°C.

Acknowledgements

We thank CA Begy and A Saulino for excellent technical support and Drs MA Hauser and R Kumar-Singh for helpful discussions. This work was supported by a grant from the Muscular Dystrophy Association (USA) to JSC.

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