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Improved adenovirus packaging cell lines to support the growth of replication-defective gene-delivery vectors

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ABSTRACT **Adenovirus (Ad) vectors have been extensively used to deliver recombinant genes to a great variety of cell types *in vitro* and *in vivo*. Ad-based vectors are available that replace the Ad early region 1 (E1) with recombinant foreign genes. The resultant E1-deleted vectors can then be propagated on 293 cells, a human embryonal kidney cell line that constitutively expresses the *E1* genes. Unfortunately, infection of cells and tissues *in vivo* results in low-level expression of Ad early and late proteins (despite the absence of E1 activity) resulting in immune recognition of virally infected cells. The infected cells are subsequently eliminated, resulting in only a transient expression of foreign genes *in vivo*. We hypothesize that a second-generation Ad vector with a deletion of viral genes necessary for Ad genome replication should block viral DNA replication and decrease viral protein production, resulting in a diminished immune response and extended duration of foreign gene expression *in vivo*. As a first step toward the generation of such a modified vector, we report the construction of cell lines that not only express the *E1* genes but also constitutively express the Ad serotype 2 140-kDa DNA polymerase protein, one of three virally encoded proteins essential for Ad genome replication. The Ad polymerase-expressing cell lines support the replication and growth of H5ts36, an Ad with a temperature-sensitive mutation of the Ad polymerase protein. These packaging cell lines can be used to prepare Ad vectors deleted for the E1 and polymerase functions, which should facilitate development of viral vectors for gene therapy of human diseases.**

The role of viral replication has come under increased scrutiny with the use of adenovirus (Ad)-based vectors for *in vivo* gene transfer. Ad vectors can deliver recombinant genes to a great number of diverse cell types, including mitotically quiescent cells, with high efficiency. Ad-based vectors have a large carrying capacity, allowing for the transfer of genes up to 8.3 kb in size (1). One major drawback, however, has been the limited duration of foreign gene expression *in vivo*. This lack of persistence appears to be primarily immune-mediated because newborn immunotolerant mice, as well as immune-deficient animals, allow long-term expression of Ad-delivered genes (2, 3). Immunocompetent animals only allow transient expression of transferred genes, usually for 2–3 weeks (3, 4). The immune-mediated loss of foreign gene expression may in part be due to the limited replication and subsequent expression of viral epitopes by current-generation Ad vectors in target cells (5).

Ad replication has been extensively analyzed, using both viral mutants of replication and *in vitro* replication assays (6–8). In the presence of the viral inverted terminal repeats at the ends of the linear viral genome, three viral-encoded proteins, the 72-kDa single-stranded DNA-binding protein (ssDBP), the 80-kDa precursor terminal protein (pTP), and the 140-kDa DNA polymerase (pol) protein, interact with at least three host nuclear factors to allow priming and elongation of the viral DNA *in vitro* (9). The E2 precursor RNA is differentially spliced to yield the mRNAs encoding the three viral replication proteins (10).

In Ad-infected cells the Ad polymerase mRNA is expressed at very low levels (11), suggesting that viral packaging cell lines constitutively expressing very low levels of the 140-kDa polymerase protein might be capable of supporting the replication and growth of Ad pol mutants. One well-characterized pol mutant is H5ts36, an Ad5-based virus with a C $\rightarrow$ T transition at position 7623 of the Ad serotype 5 (Ad5) genome (12). Under nonpermissive conditions, the temperature sensitive (ts) form of the pol cannot initiate DNA replication at the viral origin (7).

An approach to block the replication of Ad vectors *in vivo* would be to construct an Ad vector deleted not only for the *E1* genes, but also for the Ad pol gene. Such a vector would require the presence of adequate amounts of the Ad pol protein supplied *in trans* in 293 cells. As a first step toward this goal, we describe the isolation and characterization of multiple 293-derived cell lines that constitutively express a functional Ad pol protein. These cell lines support the replication and growth of H5ts36 (at the nonpermissive temperature) as well as E1-defective viruses. Their use in the future isolation of replication-defective Ad vectors for gene therapy of human disorders is discussed.

MATERIALS AND METHODS

Tissue Culture and Virus Growth. LP-293 cells (Microbix Biosystems, Toronto) were grown and serially passaged as suggested by the supplier. Plaque assays were done in 60-mm dishes containing cell monolayers at ≈90% confluency. The appropriate virus dilution in a 2% Dulbecco's modified Eagle's medium (DMEM) solution was dripped onto the cells, and the plates were incubated at the appropriate temperature for 1 hr. The virus-containing medium was aspirated; the monolayer was overlaid with 10 ml of a prewarmed Eagle's minimal essential medium agar overlay solution (0.8% Noble agar/4% fetal calf serum/antibiotics) and allowed to solidify. After the appropriate incubation time (usually 7 days for incubations at 38.5°C and 10–12 days for incubations at 32°C) 5 ml of the agar-containing solution containing 1.3% neutral red was overlaid onto the infected dishes, and plaques were counted the next day. An aliquot of the virus H5ts36 (provided by H. Ginsberg, Columbia University) was used to produce high-titer stocks after infection of 293 cells at 32°C. The infected cells were harvested after the onset of extensive cytopathic effect,

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Abbreviations: Ad, adenovirus; Ad2 or Ad5, adenovirus serotypes 2 or 5; pol, DNA polymerase; pTP, precursor terminal protein; ssDBP, single-stranded DNA-binding protein; ts, temperature sensitive; MOI, multiplicity of infection; pfu, plaque-forming units; RSV-LTR, Rous sarcoma virus-long terminal repeat.

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pelleted by centrifugation, and resuspended in 10 mM Tris·Cl, pH 8.0. The lysate was freeze-thawed three times and centrifuged to remove the cell debris. The cleared lysate was applied to $CsCl_2$ step gradients (heavy CsCl at a density of 1.45 g/ml, the light CsCl at density of 1.20 g/ml), ultracentrifuged, and purified as described (13). The concentration of plaque-forming units (pfu) of this stock was determined at 32°C as described above. Virion DNA was extracted from the high-titer stock by Pronase digestion, phenol-chloroform extraction, and ethanol precipitation. The leakiness of this stock was found to be <1 in 2000 pfu at the nonpermissive temperature, consistent with previous reports (12).

Isolation of Ad Pol-Expressing 293 Cell Lines. The expression plasmid pRSV-pol (provided by R. Padmanabhan, University of Kansas) has been described (8). Briefly, the Ad2 pol mRNA (including the start codon from the exon at map unit 39) was placed under the transcriptional control of the Rous sarcoma virus-long terminal repeat (RSV-LTR) promoter element, and flanked on its 3′ end by the simian virus 40 small tumor antigen intron and simian virus 40 polyadenylylation addition site (see Fig. 1). LP-293 cells were cotransfected with *Bam*HI-linearized pRSV-pol and pCEP4 (a plasmid containing a hygromycin expression cassette, Invitrogen), at a molar ratio of 10:1 via the $CaPO_4$ precipitation method (14). Forty-eight hours after transfection the cells were passaged into medium containing hygromycin at 100 μg/ml. Individual hygromycin-resistant colonies were isolated and expanded.

Analysis of Genomic DNA. Genomic DNA from LP-293 cells and the hygromycin-resistant cell lines were harvested using standard protocols (14), and 200 ng of DNA from each cell line was analyzed by PCR in a solution containing primers p602a and p2158c at 2 ng/ml, 10 mM Tris·HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, and 0.001% gelatin. The forward primer p602a (5′-TTCATTTTATGTTTCAGGTTCAGGG-3′) is located in the simian virus 40 polyadenylylation sequence. The reverse primer p2158c (5′-TTACCGCCACACTCGCAGGG-3′) is Ad-sequence specific with the 5′ nucleotide located at position 3394 of the Ad5 genome (see figure 1 in ref. 10). PCR was performed with a Perkin–Elmer model 9600 thermocycler using the following cycling parameters: initial denaturation at 94°C for 3 min, three cycles of denaturation at 94°C for 30 sec, annealing at 50°C for 30 sec, and extension at 72°C for 60 sec, followed by another 27 cycles with an increased annealing temperature at 56°C, with a final extension at 72°C for 10 min.

Analysis of Cell Line RNA. Total RNA was extracted from each of the cell lines via the RNAzol method (15). Fifteen micrograms of RNA from each cell line was electrophoresed on a 0.8% agarose-formaldehyde gel and transferred to a Nytran membrane by blotting. The Nytran filter was UV crosslinked and analyzed by probing with the two ^{32}P-labeled 1-kb *Sca* I subfragments of Ad (see Fig. 1).

Replication-Complementation Assay of H5ts36. LP-293 cells or the hygromycin-resistant cell lines were seeded onto

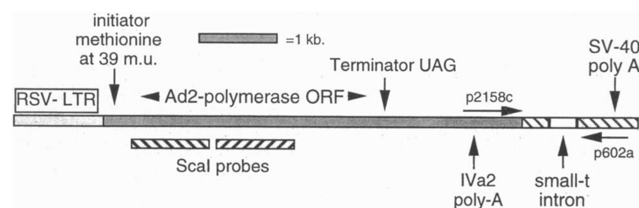

FIG. 1. Schematic representation of the Ad-pol expression plasmid pRSV-pol (8). pRSV-pol includes the initiator methionine and amino-terminal peptides encoded by the exon at map units (m.u.) 39 of the Ad genome. The locations of the PCR primers p602a and p2158c, the two *Sca* I 1-kb probes used for Northern analyses, the pol terminator codon, and the polyadenylylation site of the IVa2 gene (at 11.2 m.u. of the Ad genome) are indicated. ORF, open reading frame; small-t, small tumor antigen.

60-mm dishes at densities of 2.5–3.0 × 10^6 cells per dish, infected at a multiplicity of infection (MOI) of 10, and incubated for 24 hr at 38.5°C or for 48 hr at 32°C. Total DNA was harvested from each plate; then 2 μg of each sample were digested with *Hin*dIII and assayed by Southern analysis using ^{32}P-labeled H5ts36 virion DNA as a probe. Densitometric analysis of the 8010-bp *Hin*dIII fragment in each lane was done on a Molecular Dynamics PhophoroImager using the IPLAB GEL image processing system, Version 1.5.

Transfection Assays. Parental 293 cells, as well as hygromycin-resistant cell lines, were grown to near confluency on 60-mm dishes and transfected with either 3 μg of purified H5ts36 virion DNA or with 3.5 μg of plasmid pFG140, using the cationic lipid Lipofectamine (GIBCO/BRL). Cells that received the H5ts36 virion DNA were incubated at 32°C for 14 days or at 38.5°C for 10 days. The pFG140-transfected cells were incubated at 37.5°C for 10 days. All plates were then stained with the neutral red agar overlay, and plaques were counted the next day.

RESULTS

Isolation of Cell Lines that Support the Growth of H5ts36 at 38.5°C. LP-293 cells were cotransfected with the plasmids pRSV-pol and pCEP4 using a molar ratio of 10:1, respectively, and the transfected cells were selected in hygromycin-containing medium. Twenty hygromycin-resistant cell lines were expanded and screened for the ability to express the Ad pol protein. Initially, the individual cell lines were assayed for the ability to support growth of the viral pol mutant H5ts36, an Ad5-derived virus defective for viral replication at the nonpermissive temperature (12). We speculated that constitutive expression of the wild-type Ad pol protein in a clonal population of 293 cells should allow the growth of H5ts36 at 38.5°C. However, it was unclear whether constitutive expression of the Ad pol would be toxic when coexpressed with the E1 proteins in 293 cells. Similar toxicity problems have been seen with the Ad ssDBP and the pTP (16, 17). Of the 20 hygromycin-resistant cell lines isolated, seven were able to allow plaque formation with H5ts36 at the nonpermissive temperature, unlike the parental LP-293 cells (see Table 1). Approximately 3000 pfu of H5ts36 were used per infection;

Table 1. Plaquing ability of H5ts36 at the nonpermissive temperature using 293 Ad-pol-expressing cell lines

Cell line	Passage number	Plaques, no.	
		32.0°C	38.5°C
293	—	>500	0
B-6	9	>500	>500
	20	>500	>500
B-9	5	>500	>500
	14	90	18
C-1	5	>500	>500
	13	>500	>500
C-4	5	>500	>500
	14	>500	>500
C-7	5	>500	>500
	14	>500	>500
C-13	5	>500	>500
	27	120	4
C-14	5	>500	>500
	27	>500	370

Dishes (60 mm) of near-confluent cells of each cell line were infected with the same dilution of H5ts36 (≈3000 pfu per dish) at the temperature indicated, overlaid with agar media, and stained for plaques as outlined. Passage number refers to the number of serial passages after initial transfection with plasmid pRSV-pol.

therefore all cell lines were capable of producing at least 500-fold more plaques at the nonpermissive temperature (at earlier passage numbers) than the parental 293 cells. The cell line B-6 produced plaques 1 day earlier than the other cell lines, which may reflect increased pol expression (see below). Cell line B-9 demonstrated an increased doubling time, whereas each of the other cell lines displayed no growth disadvantages relative to the parental LP-293 cells. As shown in Table 1, even after multiple passages (in some instances up to 4 mo of serial passaging), the cells could still produce large numbers of H5ts36 plaques at the nonpermissive temperature, indicating that the RSV-LTR/promoter remained active for extended periods. However, cell lines B-9 and C-13 displayed a decreased ability to plaque the virus at 32°C, as well as at 38.5°C, suggesting that a global viral complementation defect had occurred in these cell lines after extended passaging. The remaining cell lines screened at later passages demonstrated no such defect, even after 20 passages (e.g., cell line B-6, Table 1).

Genomic Analysis of Ad Pol Expressing Cell Lines. Genomic DNA from LP-293 cells and each of the seven cell lines able to complement H5ts36 at 38.5°C were analyzed by PCR for the presence of pRSV-pol-derived sequences. As shown in Fig. 2, all cell lines capable of H5ts36 plaque formation at 38.5°C contained the Ad pol DNA sequences, whereas the LP-293 cells did not yield any amplification product with these primers. This result shows that each of the selected cell lines stably cointegrated not only the hygromycin-resistance plasmid pCEP4, but also pRSV-pol.

Complementation of the Replication Defect of H5ts36 by Ad-pol-expressing Cell Lines. The C → T transition at nt 7623 of the H5ts36 genome alters the DNA-binding affinity of the Ad pol protein, rendering it defective for viral replication at nonpermissive temperatures (7, 12, 18). To analyze the functional activity of the Ad pol protein expressed by each of the packaging cell lines, a viral replication-complementation assay was done. The cell lines were infected at an MOI of 10 with H5ts36 and incubated at either 32°C or 38.5°C for 48 hr or 24 hr, respectively. Total DNA was then isolated, digested with *Hin*dIII, transferred to nylon membranes, and hybridized with ^{32}P-labeled H5ts36 genomic DNA. As displayed in Fig. 3, H5ts36 had a diminished ability to replicate in LP-293 cells at the nonpermissive temperature. In contrast, all seven of the previously selected cell lines were able to support replication of H5ts36 virion DNA at 38.5°C to levels approaching those occurring in LP-293 cells at 32°C.

A densitometric analysis of the amount of H5ts36 viral DNA replicated in each of the cell lines at permissive and nonpermissive temperatures is presented in Table 2. For this assay, the relative amounts of the 8010-bp *Hin*dIII fragment were compared. Levels of replication at the permissive temperature were all within 4-fold of each other, regardless of which cell line was analyzed, but at the nonpermissive temperature LP-293 cells reveal the H5ts36 replication defect. The viral bands present in the LP-293 DNA sample at 38.5°C represented input virion DNA as well as low-level replication of H5ts36 DNA, which is generated due to the leakiness of the ts mutation at the high MOI used in this experiment (data not shown and ref. 12). The Ad-pol-expressing cell lines were all found to be capable of augmenting H5ts36 genome replication at 38.5°C. Although one cell line (B-9) allowed H5ts36 replication to levels only 16-fold greater than LP-293 cells, this was the same cell line that was observed to display poor growth properties. Each of the remaining Ad-pol-expressing cell lines allowed substantially greater replication of H5ts36 at nonpermissive temperatures, compared to LP-293 cells (see Table 2). An enhancement of replication up to 75-fold above that of LP-293 cells was observed with the cell line C-7 at 38.5°C. We also observed a substantially more rapid onset of viral cytopathic effect in Ad-pol-expressing cell lines at either temperature. These estimates of H5ts36 replication-complementation are conservative because they have not been adjusted for the low-level replication of H5ts36 at 38.5°C (12). The leakiness of the H5ts36 mutation could potentially be overcome with the use of a virus deleted for the Ad-pol gene.

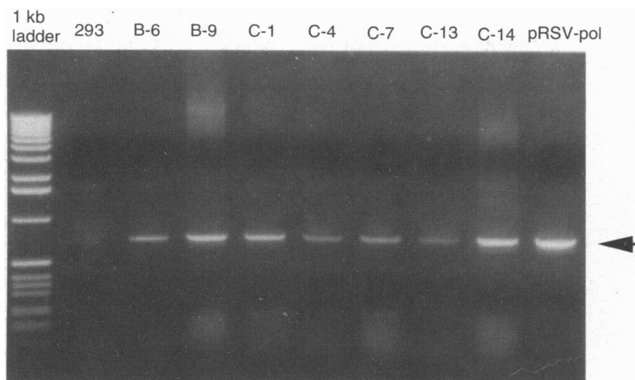

FIG. 2. PCR analysis of the hygromycin-resistant Ad-pol-expressing cell lines. The primers p602a and p2158c were used to amplify the sequences contained in the expression plasmid pRSV-pol. PCR products from the indicated cell lines were separated on a 1.0% agarose gel and visualized with ethidium bromide staining. A 1-kb ladder (GIBCO/BRL) was used as a size marker, and the plasmid pRSV-pol was used as a positive control. The ≈750-bp PCR products are indicated by an arrow.

RNA Analysis of Ad-pol-expressing Cell Lines. Total RNA was extracted from each of the cell lines, electrophoretically separated and transferred to nylon membranes, and then probed with the two 1-kb ^{32}P-labeled *Sca* I subfragments of the Ad genome that are complimentary to the 5′ end of the Ad-pol mRNA. Fig. 4 reveals that the RNA derived from cell lines B-6 and C-7 contained two species of RNA, estimated to be ≈4800 and ≈7000 nt in length, whereas LP-293-derived RNA had no detectable hybridization signal. The presence of two pol RNA species suggests that the polyadenylylation signal of the Ad IVa2 gene (present in the Ad-pol construct, see Fig. 1) is being used by the cell RNA-processing machinery, in addition to the simian virus 40 polyadenylylation signal. Similar analysis of RNA derived from the cell lines C-1, C-4, C-13, and C-14 also detected the same two transcripts as those detected in the RNA of cell lines B-6 and C-7, but at decreased levels (data not shown), suggesting that even low levels of Ad-pol mRNA expression can allow for the efficient replication of polymerase mutants such as H5ts36. The cell line B-6 expressed high levels of pol transcript and can plaque H5ts36 1 day earlier than the other cell lines at 38.5°C, suggesting a causal relationship. It is interesting to note that the two polymerase transcripts are also detected in RNA isolated from cell line B-9, but substantial amounts of a larger RNA transcript (size > 10 kb) is also present (see Fig. 4). The high-level production of the aberrant message may be related to the increased doubling time previously noted in this cell line.

Transfectability of Ad Pol-Expressing Cell Lines. Some of the cell lines were also analyzed for the ability to support production of H5ts36 virions after transfection with H5ts36 genome DNA. Table 3 demonstrates that transfection of H5ts36 DNA at the nonpermissive temperature allows for ample plaque production in all of the Ad pol-expressing cell lines tested, unlike the parental LP-293 cells. Cell line C-13 was at passage number 29 and demonstrated a somewhat decreased ability to generate plaques at this extended passage number. These same cell lines are also capable of producing plaques when transfected with the plasmid pFG140, a plasmid capable of producing infectious, E1-dependent Ad upon transfection of the parental 293 cells (data not shown; ref. 19). These observations suggest that the Ad-pol-expressing cell lines should be useful for the production of second-generation Ad vectors deleted not only for the *E1* genes, but also for the pol gene.

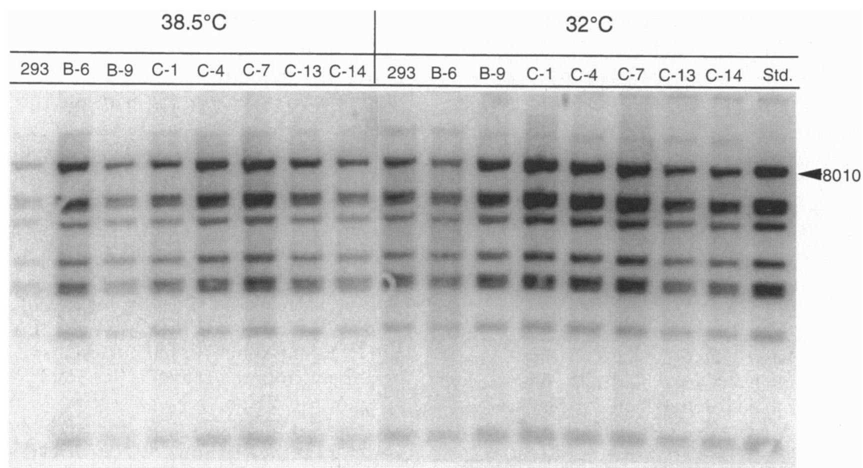

FIG. 3. Replication of H5ts36 at permissive and nonpermissive temperatures. Each of the indicated cell lines was infected with H5ts36 at an MOI of 10 and incubated at the following temperatures: 24 hr at 38.5°C or 48 hr at 32.0°C. Total DNA was extracted, digested with *Hin*dIII, separated on a 1.0% agarose gel, transferred to a nylon membrane, and hybridized with ^{32}P-labeled H5ts36 virion DNA. The standard (Std.) is 1 μg of *Hin*dIII-digested H5ts36 virion DNA. The 8010-bp *Hin*dIII fragments analyzed by densitometry are indicated by an arrow.

DISCUSSION

Human 293 cells, which support the growth of E1-defective Ad, have allowed Ad to be widely utilized as a vector for gene transfer *in vitro* and *in vivo.* Typically, a gene expression cassette is placed into a modified Ad genome deleted for the *E1* region of genes using either intermolecular recombination or intermolecular ligation of restriction enzyme-digested viral DNA. The recombinant Ad vectors are then serially propagated in the E1-producing 293 cells. The E1 gene products are the first proteins expressed after viral infection of a host cell, and one of their major roles is the trans-activation of the other viral early and late promoters. Because Ad vectors are deleted for E1 gene products, they should be replication incompetent, due to the lack of expression of other Ad early genes. However, this block has been overcome *in vitro*, for example, when a high MOI of E1-defective virus was used (20). This and other observations suggest that some cells may have endogenous "E1-like" activities that allow the E1-independent expression of Ad early and late genes (21). Furthermore, *in vivo*, it has become clear that E1-defective viruses are not completely replication defective, as both replicated viral DNA, as well as early and late proteins, can be detected after infection (5). One hypothesis suggests that the E1-independent expression of viral proteins initiates a host immune response directed against virally infected cells and results in the transient expression of Ad-delivered foreign genes (22). For example, in immunologically tolerant or deficient animals, Ad-mediated gene transfer is highly efficient, and sustained foreign gene expression can be demonstrated in the target tissue for extended periods (3, 23). Unfortunately, the same is not true in immunocompetent animals; usually high numbers of infected cells can express the foreign gene transiently, but immune-mediated elimination of the same cells leads to a rapid loss of foreign gene expression (5).

These observations suggest that further deletion of Ad genes from current-generation Ad vectors should result in a diminished immune response and prolonged foreign gene expression. We hypothesize that a mutation that blocks replication of the linear Ad genome would also decrease the expression of other viral proteins. It has been demonstrated that after viral replication a block to the transcription of the Ad late proteins is relieved, allowing for enhanced late-gene expression (24). This is, in part, a cis-mediated activation of the major late promoter. For example, when Ad-infected cells in the late stages of viral infection were superinfected with a second virus, there was expression of late proteins from the second virus only *after* it had replicated its genome (24). This observation suggests that a modified Ad vector blocked in its ability to

Table 2. Densitometric analysis of H5ts36 replication

Cell line	8010-bp *Hin*dIII fragment generated, ratio	
	32.0°C	38.5°C
LP-293	42.6	1.0
B-6	27.3	57.2
B-9	69.0	15.8
C-1	113.6	34.1
C-4	100.2	65.5
C-7	114.9	75.0
C-13	43.1	42.2
C-14	46.7	27.8

Relative levels of H5ts36 virion DNA replication determined by densitometric analysis of the 8010-bp *Hin*dIII fragment isolated from each of the cell line DNA samples, using the IPLAB GEL Version 1.5 computer program. The surface area of the 8010-bp fragment in 293 cells incubated at 38.5°C is designated as 1 and includes some replicated H5ts36 virion DNA (data not shown). The numbers in each column represent the ratio between the density of the 8010-bp fragment isolated in the indicated cell line and the density of the same band present in LP-293 cells at 38.5°C.

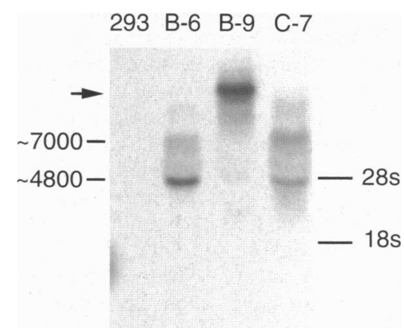

FIG. 4. Analysis of cell line RNA. Fifteen micrograms of total RNA isolated from each of the indicated cell lines was electrophoresed, blotted onto a nylon membrane, and hybridized with the two ^{32}P-labeled 1-kb *Sca* I fragments spanning positions 6095–8105 of the Ad5 genome (see Fig. 1). The locations of the smaller and larger species of Ad-pol mRNA are indicated relative to the 28S and 18S ribosomal RNAs. The aberrant transcript expressed by the B-9 cell line is indicated by an arrow.

Table 3. Transfection efficiency of Ad-pol-expressing cell lines

Cell line	Plaques, no.	
	32.0°C	38.5°C
LP-293	>500	0
B-6	>500	>500
B-9	ND	ND
C-1	ND	>500
C-4	ND	>500
C-7	ND	>500
C-13	ND	100
C-14	ND	>500

Dishes (60 mm) of near-confluent cells of each cell line were transfected with 3 μg of H5ts36 virion DNA, incubated at the indicated temperature, overlaid with agar media, and stained for plaques as outlined. ND, not determined.

replicate should have decreased expression of late proteins, resulting in a diminished immune response.

This hypothesis has been partially addressed with the use of a modified "second-generation" Ad vector that encodes a ts mutation in the Ad ssDBP (22). Encouragingly, use of the ts Ad vector resulted in a diminished inflammatory response, as well as an extended period of foreign gene expression in some of the animal tissues analyzed (5, 25). Unfortunately, due to the leakiness of the ts mutation (not only because of a core body temperature of 37°C, but also due to the high MOIs), ssDBP protein expression and hexon mRNA expression (a viral late protein) were still detectable (5). A more desirable vector would include a deletion within the coding region of the ssDBP. Unfortunately, such a vector would require the coexpression of adequate amounts of the ssDBP protein in the presence of the E1 proteins, a situation previously demonstrated to be toxic (16). Therefore, we opted to express the viral pol protein (which is also required for replication of the linear Ad genome) in 293 cells. This expression has been achieved, demonstrating the potential for construction of Ad vectors deleted for a substantial portion of the pol gene, as well as the *E1* genes.

The Ad pol-expressing cells isolated in this report demonstrate that constitutive coexpression of the *E1* and pol genes is not toxic. Recently, the isolation of 293 cells that also express the viral pTP has been reported (17). The theoretical possibility exists that a 293 cell line could be isolated that expresses both the pol protein as well as the pTP. Such a cell line could support the growth of an Ad vector deleted for both of these genes, introducing a further block to viral replication in host tissues and potentially an additional decrease in the immune recognition of virally infected cells. Such a vector would have a significantly greater carrying capacity, allowing for the construction of recombinant vectors carrying larger recombinant genes or for the use of larger, tissue-specific enhancer/promoter elements regulating the expression of transferred genes. Increased cloning capacity will be required for efficient viral delivery of large genes, such as the 14-kb dystrophin cDNA (26).

This is the first report, to our knowledge, of the constitutive expression of the Ad-pol protein in 293 cells and only the second report of two distinct Ad genome-encoded proteins being constitutively coexpressed with the E1 gene products already present in 293 cells (17). In the field of Ad biology, a number of cell lines have been generated [E1 region-expressing 293 cells (27), E4 region-expressing Vero cells (28), and ssDBP-expressing HeLa cells (29)], which have enabled the isolation of viral mutants deleted for the respective gene functions as well as allowing for the detailed functional analysis of the respective subregions. Isolation of Ad-pol-expressing cell lines will permit further study of the viral genome encoding the pol protein. This subregion contains a number of important elements, including portions of the major late promoter and the tripartite leader sequences. Functional analysis of the major late promoter region, as well as the role of the tripartite leader sequences in their normal viral chromosomal locations, can now be addressed by direct deletion analysis without concern about disrupting the pol-coding region.

Note Added in Proof. We have now isolated 293 cells that constitutively coexpress the Ad-pol *and* the Ad-pTP proteins, so that the construction of vectors deleted for both gene functions is now possible.

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