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Efficient Dual Transcomplementation of Adenovirus E1 and E4 Regions from a 293-Derived Cell Line Expressing a Minimal E4 Functional Unit

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Transgene expression after the administration of recombinant adenovirus with E1 deleted is constantly transient. It is admitted that E1A-substituting activities of cellular or viral origin allow viral antigen synthesis and trigger cytotoxic lymphocyte-mediated clearance of the recipient cells. Our approach to solving this problem relies on the additional deletion of the E4 region from the vector backbone as this region upregulates viral gene expression at both transcriptional and posttranscriptional levels. As a prerequisite to the construction of E1 E4 doubly defective adenoviruses, we investigated the possibility of transcomplementing both functions within a single cell. In particular, the distal ORF6+ORF7 segment from the E4 locus of adenovirus type 5 was cloned under the control of the dexamethasone-inducible mouse mammary tumor virus long terminal repeat. Following transfection into 293 cells, clone IGRP2 was retained and characterized as it can rescue the growth defect of all E1$^+$ E4$^-$ adenoviral deletants tested. DNA and RNA analysis experiments verified that the mouse mammary tumor virus promoter drives the expression of the ORF6+ORF7 unit and permits its bona fide alternative splicing, generating ORF6/7 mRNA in addition to the ORF6-expressing primary transcript. Importantly, IGRP2 cells sustain cell confluence for a period longer than that of 293 parental cells and allow the plaque purification of E1$^-$ or E4$^-$ defective viruses. The dual expression of E1 and E4 regulatory genes within IGRP2 cells is demonstrated by the construction, plaque purification, and helper-free propagation of recombinant *lacZ*-encoding doubly defective adenoviruses harboring different E4 deletions. In addition, the emergence, if any, of replicative particles during viral propagation in this novel packaging cell line will be drastically impaired as only a limited segment of E4 has been integrated.

Contrary to the other viral vectors being evaluated in gene transfer clinical protocols, E1-deleted adenoviruses retain most of the genome and express viral antigens in vivo, as documented for mice, cotton rats, and xenografts of human bronchial epithelia (8, 9, 43). Because E1a encodes transcription transactivators that target most viral promoters, residual viral gene expression is a likely consequence of cellular transactivators (referred to hereafter as E1A-like factors). For example, E1A-like activity has been documented for undifferentiated F9 embryonal carcinoma cells (18). c-MYC and an interleukin 6 (a major inflammatory cytokine)-regulated cellular factor are candidates to mediate this transactivating function, as previously proposed (26, 32, 33). As do others, we believe that dose-dependent inflammation after the administration of E1-deleted adenoviruses is actually mediated by cytotoxic lymphocytes specifically recognizing viral antigens on the surfaces of infected cells (42, 43).

Doubly crippled (second-generation) adenoviral vectors have been designed by the additional introduction of a thermosensitive mutation (*ts*125) into the E2a gene of E1-deleted vectors. At 39°C, this point mutation completely inactivates the 72-kDa DNA-binding protein, a protein critical for not only viral DNA replication (37) but also efficient transactivation of the viral late transcription unit, at least in vitro (6). Importantly, the administration of a *lacZ*-encoding second-generation virus improved transgene persistence and decreased the inflammatory response after transfer to mouse livers (10). However, the *ts*125 mutation is subject to frequent reversion (37a), and the mutated DNA-binding protein is partially functional at 37°C (10). Furthermore, E4 mRNAs are significantly increased in H5ts125-infected cells at the nonpermissive temperature (1). Upregulation of E4, a natural target of DNA-binding protein repression (23), is therefore possible after in vivo transfer of second-generation adenovectors, at least in those cells which exhibit E1A-substituting activities.

The only E2a-transcomplementing cell lines previously described have been derived from HeLa cells and as such do not transcomplement the E1 region (4, 19). Recently, a 293-derived cell line capable of transcomplementing the pTP adenoviral protein has been reported; it might be useful for the construction of adenoviruses with double deletions of E1 and pTP (31). Our approach to silence viral gene expression relies on deletion of the E4 regulatory genes together with deletion of E1. This regulatory region was targeted because it is required for viral propagation as a result of its broad involvement in viral gene expression (transcriptional and posttranscriptional). First, maximal transactivation of the E2a promoter requires the simultaneous activity of E1 and E4 proteins (for a review, see reference 20). Second, growth-defective E4 mutants exhibit multiple defects, including delays in viral DNA replication and the accumulation of DNA concatemers (14, 39), together with defects for both viral late nuclear RNA and late protein synthesis (5, 14, 15, 30, 41), virus particle assembly (11), and host cell protein shutoff (14, 45). In fact, E4 somehow is involved in the overall process of viral late protein synthesis,

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perhaps by controlling or allowing alternative polyadenylation and/or splicing of the 28-kb late transcription unit (24, 25) from which most virion structural proteins are generated. Third, E4 is mostly expressed during the early phase of infection (i.e., prior to viral DNA replication), so efficient transcomplementation from a limited number of integrated copies is achievable. In fact, the W162 cell line, a Vero-derived ($E1^-$) cell line, stably expresses E4 from its own promoter and allows the growth of ($E1^+$) E4-defective adenoviruses (40), further demonstrating that E4 is amenable to deletion and transcomplementation from a cell line. Fourth, large E4 deletions exist. Genetic analyses have actually demonstrated that E4 deletants are defective for viral growth when the deletion simultaneously inactivates two E4 gene products (ORF3 and ORF6) (2, 17), as these redundant proteins are required for the overall process of late protein synthesis during infection (5, 14, 15, 41). This redundancy is obviously the molecular basis of the absence of known thermosensitive mutations in E4. Finally, no cellular E4-substituting activity has ever been reported. It should also be remembered that E4 is somehow involved in host cell protein synthesis shutoff, apparently through an unknown late product (45). It is therefore conceivable that even low-level expression of the E4 region could be directly cytotoxic to the recipient cell in vivo. In that respect, the deletion of E4 from the vector backbone is also certainly desired.

E1 and E4 encode regulatory functions which interact with common cellular targets during a productive viral cycle. One example is E2F, a crucial transcription factor for cell cycle control and progression which is also involved in cooperative binding to a unique cognate target DNA sequence, the adenoviral E2a promoter (for a review, see reference 20). E1 and E4 cross talk is also evidenced by the E1B 55-kDa interaction with the p53 cellular protein and the E4 ORF6 gene product, leading to the following outcomes: inhibition of p53-mediated transcriptional activation (44) and selective cytoplasmic accumulation of viral late mRNAs (28). Finally the E1/E4 interplay is further documented by the effect of the E4 ORF4 gene product on the phosphorylation status of cellular and viral proteins, including E1A (22). As many of the E4 proteins antagonize and/or interact with E1A and E1B proteins, we assumed that a cell line transcomplementing both functions would certainly not express these regulatory genes to high constitutive levels. As some of the E4 gene products are dispensable for viral growth (15, 17), we constructed and characterized a 293-derived cell line expressing a limited portion of the E4 locus from a dexamethasone (DEX)-inducible promoter.

MATERIALS AND METHODS

Construction of plasmid pORF6Gen. The adenovirus type 5 (Ad5) 1.6-kb *Bgl*II restriction fragment (positions 34115 to 32490) encodes a 1.3-kb fragment (position 34115 to the E4 polyadenylation signal at position 32821) corresponding to the 3′ half (ORF6+ORF7) of the E4 region (nucleotide numbers refer to the sequence submitted under GenBank accession no. M73260). This *Bgl*II fragment was cloned between the *Bgl*II and *Bam*HI sites of plasmid pIC20H (21). The resulting construct thus carries the ORF6+ORF7 unit within a *Xho*I-*Sph*I fragment. This restriction fragment was cloned between the *Sal*I and *Sph*I sites of plasmid pPY4, a pIC20H derivative containing a 1.4-kb *Avr*II-*Sal*I restriction fragment corresponding to the mouse mammary tumor virus (MMTV) long terminal repeat (LTR) of plasmid pMSG (Pharmacia). Therefore, the resulting plasmid (pPY15) carries the ORF6+ORF7 unit cloned under the control of the MMTV LTR. In this expression cassette, the first translation initiation codon is that of ORF6 (position 34077 of the Ad5 genome); it is separated from the CAP site of the MMTV LTR-derived transcript by 325 nucleotides (nt). Plasmid pORF6Gen is derived from plasmid pPY15 and contains the *Xho*I fragment of plasmid pKIXX (Pharmacia) which confers geneticin resistance in eukaryotic cells. In plasmid pORF6Gen, the geneticin resistance gene and the E4 ORF6+ORF7 unit are transcribed in the same direction.

Cells and viruses. The 293 cell line used in this report was from the European Collection of Animal Cell Cultures. In our hands, the viral productivity of a first-generation adenovirus grown in these cells was approximately 100 PFU per cell. 293 cells were transfected as previously described (13). Geneticin selection was performed at 400 μg/ml. H2dl808 ($ORF1^+$) (5, 41), H5dl1004 ($ORF1^+$), H5dl1007, H5dl1014 ($ORF4^+$) (2), and H5dl1011 (3) are defective ($E1^+$) adenoviruses carrying large deletions of the E4 region (see Fig. 1A). Viral stocks (10^8 to 10^9 PFU) of these deletants were prepared, and the titers of virus were determined on W162 cells (40), as they do not replicate in 293 cells. For each virus, the extent of the E4 deletion was confirmed by DNA sequencing. In particular, the deletion in H5dl1014 ($ORF4^+$) extends from the *Sma*I site at position 33093 to position 33695 and from the *Ssp*I site at position 34634 to the *Sma*I site at position 35355. AdRSVβGal is a first-generation recombinant virus expressing a nucleus-targeted β-galactosidase from the Rous sarcoma virus LTR (35). All viral stocks were purified by step and isopycnic CsCl gradient centrifugations, with subsequent dialysis at 15°C. They were aliquoted and stored at −70°C in 10% glycerol.

PCR analysis of genomic DNA. Genomic DNAs were prepared from 293 and IGRP2 cells as previously described (29). Two micrograms was used as the template for PCR with *Taq* polymerase from Appligene (Strasbourg, France) and oligonucleotide 1 (5′-AAGCAGCCAAGGGGTTGTTT-3′ [nt 52 to 71 of the MMTV LTR from plasmid pMSG]) and oligonucleotide 2 (5′-ACCCTAG TATTCAACCTGCC-3′ [positions 32921 to 32940 of the Ad5 genome]). These primers amplify a fragment of 2,617 bp from plasmid pORF6Gen. PCR was performed with an initial denaturation step of 94°C for 5 min, followed by 30 cycles consisting of denaturation at 94°C for 1 min, annealing at 60°C for 2 min, and extension at 70°C for 3 min, with extension in the last cycle lasting for 10 min. The PCR products were analyzed on a 1% agarose gel by electrophoresis and identified by Southern blotting and hybridization with a radiolabeled probe spanning either the 1.6-kb ORF6+ORF7 region or the 1.4-kb MMTV LTR. [α-^{32}P]dCTP was incorporated by random priming and Klenow DNA polymerase incubation.

RT-PCR. Total mRNAs were prepared from subconfluent IGRP2 cell monolayers essentially as previously described (34), and poly(A)$^+$ RNAs were isolated by oligo(dT) selection (29). All reagents for the reverse transcription (RT) step were obtained through Life Technologies. Poly(A)$^+$ preparations (500 ng) were treated with 0.5 U of DNase I and reverse transcribed with an oligo(dT) primer. One-tenth of the first-strand cDNA preparation was used as a template for PCR with oligonucleotides 2 and 3 (5′-ATCATCACAAGAGCGGAACG-3′ [nt 227 to 246 relative to the MMTV LTR CAP site; see Fig. 2D]). These primers were designed to amplify a fragment of 1,255 bp from the unspliced ORF6 mRNA and 545 bp from the spliced ORF6/7 mRNA (12, 36, 38). PCR amplification products were analysed on a 1% agarose gel by electrophoresis and identified by Southern blotting and hybridization to the 1.6-kb radiolabeled ORF6+ORF7 probe.

Protein analysis. Cells infected at 20 PFU per cell were collected late (48 or 72 h postinfection) and washed twice with phosphate-buffered saline (PBS). They were lysed in 1× sodium dodecyl sulfate (SDS) gel loading buffer (29), and boiled samples were submitted to 7% polyacrylamide–0.1% SDS gel electrophoresis. Proteins were transferred from the gel onto Hybond-C membranes (Amersham) and probed with fiber-specific antibodies (a polyclonal serum obtained from P. Boulanger, Montpellier, France, or a monoclonal antibody obtained from J. Chroboczek, Grenoble, France). Bound antibodies were detected with secondary horseradish peroxidase-conjugated antibodies according to the instructions of the manufacturer (ECL detection kit; Amersham).

Clonal construction of virus AdβGal/dl1014. AdβGal/dl1014 was constructed in IGRP2 cells by homologous recombination between three electropurified fragments. (i) Fragment I (6.8 kb) is a *lacZ*-encoding *Nar*I fragment from the left end of the AdRSVβGal genome; contamination with the uncut (replicative) AdRSVβGal genome is very unlikely because complete *Nar*I digestion generates this fragment among 25 other fragments ranging from 26 nt to 3.8 kb. (ii) Fragment II is a 9.4-kb *Dra*I fragment derived from the AdRSVβGal genome which overlaps fragment I by 1,509 nt. It was purified from 10 additional fragments ranging from 494 nt to 4.8 kb after complete restriction with *Dra*I and *Afl*II. (iii) Fragment III is a 21.3-kb *Nsi*I fragment from the H5dl1014 genome which overlaps fragment II by 1,652 nt. It was purified after complete *Nsi*I digestion, producing seven other fragments ranging from 178 nt to 4 kb. The purified fragments were cotransfected in IGRP2 cells as described above for 293 cells, except that 1 μM DEX was added to the culture medium every 4 days for 3 weeks. At this stage, cells were harvested, frozen and thawed three times in a dry ice-ethanol bath, and centrifuged at 3,000 × *g* for 20 min. The cell lysate was then added to fresh IGRP2 cells in the presence of DEX (1 μM), and a cytopathic effect (CPE) was observed after 5 days.

Viral stock preparation and titration of AdβGal/dl1014. Viral stocks were obtained after progressive amplification of a single β-galactosidase-expressing plaque of the virus with both deletions on fresh IGRP2 cells grown in the presence of 1 μM DEX. After CsCl gradient purifications and dialysis, the titers of the virus were determined on IGRP2-derived monolayers or as *lacZ*-transducing units (TU) after the infection of nonpermissive cells. The titration of AdβGal/dl1014 on IGRP2 cells was performed by plaque assay, as described above for AdRSVβGal on 293 cells, with an agar overlay containing 1 μM DEX. Fresh DEX-containing agar overlay was added every 6 days, and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) staining was performed at 3 weeks postinfection. For the determination of viral titers expressed in TU/ml, subconfluent monolayers of W162 cells or primary rabbit vascular smooth muscle

cells (a gift from D. Branellec, Rhône-Poulenc Rorer, Vitry, France) were infected on six-well plates with serial dilutions of the virus in PBS (0.4 ml) for 1 h at 37°C. After the removal of the inoculum, culture medium containing 0.5% fetal calf serum was added, and cells were incubated for 48 h prior to overnight X-Gal staining. Positive cells were counted at a magnification of ×240 on at least 10 fields (with a minimum of 100 positive cells counted), and the titer was calculated from the mean value by using a ratio of 1/1,225 between the surface of the observation field and that of the culture well.

Molecular characterization of AdβGal/dl1014. The viral DNA was prepared from one stock as previously described (5). DNA samples (2 μg) were submitted to restriction analysis and run on a 1% agarose gel. The fragments were transferred to Hybond-N membranes (Amersham) and hybridized with a radiolabeled probe corresponding to the adenovirus inverted terminal repeat (ITR) to specifically detect the fragments located at both ends of the viral genome (i.e., E1 and E4). For PCR amplification of the E4 region, viral DNA was first extracted from a purified viral stock. DNA (0.1 fg to 1 μg) was used as the template for PCR with oligonucleotide 5′-TTTCATTCAGTAGTATAGCCC-3′ and oligonucleotide 5′-CTGGTAAGGCTGACTGTTAGGC-3′. These primers amplify fragments of 2,692 and 1,368 bp from the RSVβGal ($E4^+$) and *dl*1014 viral genomes, respectively. For PCR amplification of the E1 region, oligonucleotide 5′-AGTAGTACGGATAGCTGTGACT-3′ and oligonucleotide 5′-TCTTGT GTCTCACAACCGCTCT-3′, which amplify a 750-bp fragment from the *dl*1014 ($E1^+$) viral genome, were used. The PCR products were run on a 1% agarose gel by electrophoresis, and their identities were verified by Southern blotting and hybridization with an E1- or E4-specific radiolabeled probe.

RESULTS

Design of a minimal E4 functional unit. The 2.8-kb E4 transcription unit from Ad5 is spliced into at least 18 mRNAs (7, 36, 38). The most abundant mRNAs encode six identified ORF gene products (Fig. 1A). Many of these proteins are dispensable for viral growth because a virus (H5ilE4I) engineered to contain only the 1.3-kb distal segment of E4 ORF6+ORF7, thus with ORF1 to ORF4 deleted, can be efficiently propagated in both 293 and HeLa cells (15). Consequently, this 1.3-kb fragment can be referred to as a functional E4 unit (Fig. 1A), at least when it is expressed within an adenoviral backbone (H5ilE4I), from which it directs the expression of the ORF6 and the ORF6/7 gene products (15).

Plasmid pORF6Gen harbors the MMTV LTR-ORF6+ORF7 expression cassette, together with a geneticin selectable marker, as described in Materials and Methods. Translation of the primary transcript ORF6+ORF7 from this plasmid has been designed to generate the ORF6 gene product, as described in Materials and Methods. On the other hand, translation of the alternatively spliced mRNA (positions 33904 to 33192) should generate the ORF6/7 gene product, as described during infection with H5ilE4I (15).

Isolation of clone IGRP2. Preliminary experiments with the 293 cell line indicated only a slight increase in MMTV-driven expression of a chloramphenicol acetyltransferase reporter gene in the presence of 1 μM DEX, while the additional introduction of plasmid pSG5HGR, a simian virus 40-driven glucocorticoid receptor (16) expression plasmid, resulted in a significant enhancement of chloramphenicol acetyltransferase synthesis, suggesting that glucocorticoid receptor availability may be a limiting factor for optimal induction in 293 cells. Thus, plasmids pORF6Gen and pSG5HGR were cotransfected in a molar ratio of 1:2, respectively, to ensure sufficient levels of the glucocorticoid receptor. At 4 weeks posttransfection and after selection in the presence of 400 μg of geneticin per ml, 24 clones were randomly picked up for subsequent cloning. Thirteen clones disappeared at various stages of the amplification process, and the remaining clones were screened for their potential to propagate virus H2dl808 ($ORF1^+$), a prototypic ($E1^+$) E4 deletant (Fig. 1A). Among them, clone IGRP2 was retained because a CPE appeared quickly (6 days) after infection at a low multiplicity (0.2 PFU per cell) in the presence of 1 μM DEX. The CPE on fresh IGRP2 cells was amplifiable, demonstrating that viral propagation was indeed occurring. Identical results were observed after infection with other E4-defective viruses, including H5dl1004 ($ORF1^+$), H5dl1007, H5dl1011, and H5dl1014 ($ORF4^+$). Therefore, clone IGRP2 transcomplements E4 independently of the extent of the deletion harbored by each defective virus. IGRP2 cells infected with E4 deletant *dl*1014 also exhibited a partial shutoff of host cell protein synthesis (data not shown), a result which also supports E4 transcomplementation in these cells, as this phenomenon is likely mediated by a late gene product (45). We also noticed that IGRP2 cells grown in the absence of DEX divide quickly and are two to three times smaller than parental 293 cells. In contrast, cells lost their regular shapes and exhibited arborescence upon continuous passage under DEX conditions, suggesting that the expression of ORF6 and/or ORF6/7 somehow interferes with cell metabolism.

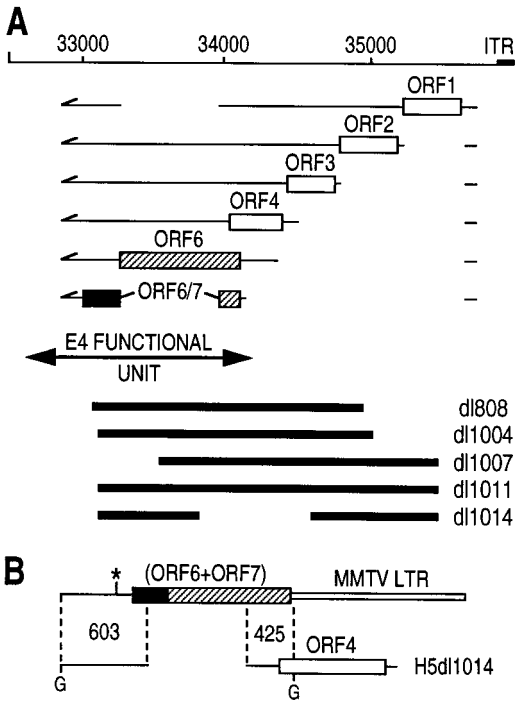

FIG. 1. (A) Genetic organization of the E4 region. The main mRNAs encoding E4 ORF1, ORF2, ORF3, ORF4, ORF6, and ORF6/7 gene products have been previously described (7). H2dl808, H5dl1004, H5dl1007, H5dl1014, and H5dl1011 are defective ($E1^+$) adenoviruses with large E4 deletions (solid bars). Because the viral sequence encompassing ORF1, ORF2, ORF3, and ORF4 can be deleted without dramatic consequences for viral growth (15), the distal part of the E4 region, ORF6+ORF7, constitutes the minimal E4 functional unit (double arrow) expressed from the MMTV LTR promoter in plasmid pORF6Gen. Note the absence of overlapping sequences upstream of E4 ORF6 between the ORF6+ORF7 unit and all E4 deletant viral genomes, with the exception of H5dl1014 (see below). (B) The ORF6+ORF7 unit of plasmid pORF6Gen exhibits two stretches of overlapping sequences (603 and 425 bp) with H5dl1014 DNA (only the relevant part of the genome is represented). G, *Bgl*II site (positions 32490 and 34115; *, E4 polyadenylation site at position 32821.

The additional introduction of the E4 unit within 293 cells did not significantly interfere with the initial ability to transcomplement the E1 function, because AdRSVβGal (35) could be grown to roughly normal titers in IGRP2 cells, at least when cells were grown in the absence of DEX (data not shown).

Molecular characterization of IGRP2 cells. Southern blot analysis indicated that clone IGRP2 contains one copy of an MMTV LTR-ORF6+ORF7 cassette within the cellular genome, together with an extra copy of the glucocorticoid-expressing gene derived from plasmid pSG5HGR (data not shown). The integrity of the MMTV LTR-E4 unit in IGRP2

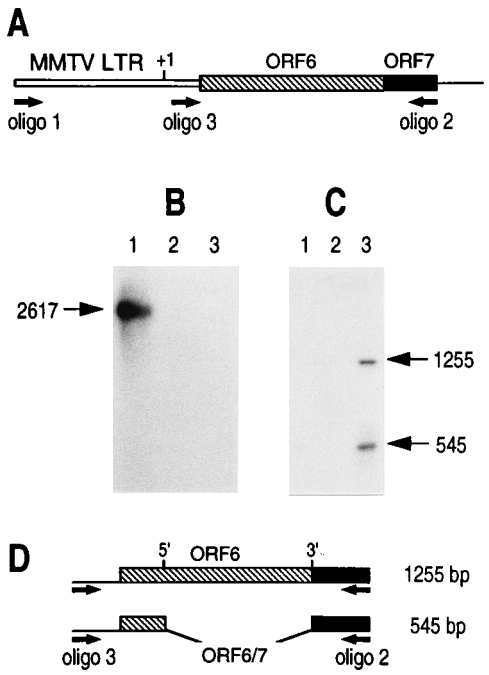

FIG. 2. Molecular characterization of IGRP2. (A) Representation of the MMTV LTR-ORF6+ORF7 cassette. Oligonucleotides 1 and 2 (oligo 1 and oligo 2, respectively) are separated by 2,617 bp and were used for genomic PCR amplification. Oligo 2 and oligo 3 are separated by 1,255 bp and were used for RT-PCR amplification. +1, the transcriptional start site. (B) PCR amplification of 2 μg of genomic DNAs from IGRP2 (lane 1) and 293 (lane 2). Lane 3, same as lane 1, except no DNA was included. The amplified fragment was hybridized with a radiolabeled ORF6+ORF7 probe. (C) Autoradiogram obtained after RT-PCR amplification of 500 ng of poly(A) RNA from 293 cells (lane 1) and IGRP2 cells supplemented with DEX (lane 3). Lane 2, same as lane 3, except no reverse transcriptase was included. Cells were grown for 48 h in 1 μM DEX before the extraction of cellular RNAs. The radiolabeled ORF6+ORF7 probe was used. The amplified products migrate as 1,255- and 545-bp fragments (arrows). (D) Representations of the amplified products from the primary transcript encoding the ORF6 protein (top) and from the ORF6/7 mRNA (bottom). 1255 and 545 bp refer to the distances separating oligo 2 and oligo 3 after RT-PCR amplifications of the primary transcript and spliced ORF6/7 mRNA, respectively.

cells is supported by the observation that PCR amplification of genomic DNA with a 20-mer oligonucleotide (oligonucleotide 1) corresponding to the beginning of the MMTV LTR-ORF6+ORF7 cassette and a 20-mer oligonucleotide (oligonucleotide 2) from the C-terminal part of ORF7 (Fig. 2A) generates a 2.6-kb DNA fragment which is specifically detected by a radiolabeled ORF6+ORF7 or MMTV LTR probe (Fig. 2B and data not shown).

We used Northern (RNA) blot analysis to detect the different mRNAs derived from the ORF6+ORF7 unit when uninfected IGRP2 cells were grown in the presence or absence of DEX. However, no E4-specific signals could be detected, suggesting either a low transcriptional level of expression or rapid degradation of the E4-derived mRNAs synthesized in the absence of additional adenoviral proteins. As previously mentioned, virus H5ilE4I contains an ORF6+ORF7 unit from which a truncated ORF6/7 mRNA is generated after the removal of a 712-nt intron (positions 33904 to 33192) during viral infection (15). To document E4 expression and to investigate if alternative splicing also occurs in clone IGRP2, we used RT-PCR amplification techniques and determined the sizes of the different mRNAs derived from the ORF6+ORF7 unit of clone IGRP2. As shown in Fig. 2C, two main signals are specifically detected with the radiolabeled ORF6+ORF7 probe after RT-PCR amplification of poly(A) RNAs from uninfected IGRP2 cells grown in the presence of DEX. The larger signal is approximately 1.3 kb long, a size which matches the amplification of an unspliced signal derived from the MMTV LTR-ORF6+ORF7 unit. The smaller signal is approximately 0.6 kb long, a size consistent with the removal of the 712-nt intron and the emergence of the ORF6/7 E4 mRNA. Taken together, these results suggest that a bona fide alternative splicing of the ORF6+ORF7 primary transcript derived from the MMTV LTR promoter occurs in IGRP2 cells. Consequently, the ORF6 and ORF6/7 gene products are likely generated in IGRP2 cells after the translation of the primary transcript and spliced ORF6/7 mRNA, respectively.

Clone IGRP2 expresses a functional ORF6 gene product. Because Western blotting (immunoblotting) with specific monoclonal antibodies did not detect the ORF6 regulatory protein per se within IGRP2 cells, we turned to a functional assay that relies on ORF6-dependent correction of the synthesis defect of a late structural protein. For that purpose, we studied the ability of IGRP2 cells to express a functional ORF6 gene product by specific immunodetection of the fiber protein, a prototypic viral protein derived from the late transcription unit, after infection with defective E4 deletants. We first used a polyclonal serum raised against the fiber protein (Fig. 3). 293 cells infected with H5dl1007 do not express detectable levels of the fiber protein late (72 h) in infection (Fig. 3, lane 7). In contrast, a fiber-specific signal is detected in the extracts from ($E1^-$ $E4^+$) infected W162 cells used as a positive control (Fig. 3, lane 6). The viral fiber protein is also unambiguously detected in infected IGRP2 cells (Fig. 3, lane 4), but not in extracts from uninfected IGRP2 cells (lane 3). A three- to fivefold induction is observed for IGRP2 cells treated with 1 μM DEX at the time of infection (Fig. 3; compare lanes 4 and 5), a result which supports MMTV LTR-driven expression of the ORF6 protein. The fiber synthesis defect is also corrected to the same extent in IGRP2 cells infected with H2dl808 ($ORF1^+$), H5dl1004 ($ORF1^+$), H5dl1011, or H5dl1014 ($ORF4^+$) in the presence of DEX (data not shown). Therefore, clone IGRP2 expresses a functional ORF6 gene product in a DEX-responsive manner after infection with various E4 deletants. Identical conclusions were reached when a fiber-specific monoclonal antibody was used, although in this case the induction effect seemed much more pronounced as the signal was barely detectable without DEX, a direct consequence of the much lower sensitivity of this monoclonal antibody compared with that of the polyclonal serum used (data not shown).

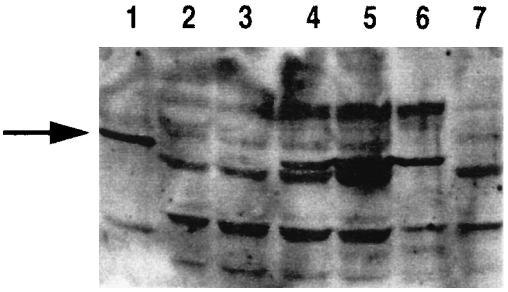

FIG. 3. Correction of the late-protein synthesis defect in IGRP2. Mock- and H5dl1007-infected cells (20 PFU per cell) were collected at 72 h postinfection. Cell extracts were submitted to Western blot analysis with a serum raised against the fiber protein (arrow). Lanes: 1, purified AdRSVβGal virus; 2, extracts from uninfected 293 cells; 3, extracts from uninfected IGRP2 cells; 4 to 7, H5dl1007-infected extracts from IGRP2 cells grown with (lane 5) or without (lane 4) 1 μM DEX or from W162 (lane 6) or 293 (lane 7) cells.

Construction of virus AdβGal/dl1014. Recombinant *lacZ*-encoding adenoviruses with E1 and E4 deletions can be constructed in IGRP2 cells by the conventional strategy that relies on a single homologous recombination event between a plasmid corresponding to the left end of the recombinant genome, coding the Rous sarcoma virus *lacZ* expression cassette inserted in place of the E1 region (35), with an overlapping restricted viral genome corresponding to the long right end of the desired E4 deletant. A major drawback of this approach is that it relies on the presence of a unique *Cla*I restriction site (position 918) in the E4-deleted genomes so that contamination with uncut and/or infectious DNA is almost inevitable and leads to a viral mixture composed of the desired *lacZ*-encoding viruses and the starting E4 deletant. This strategy was indeed time-consuming as many rounds of plaque purification were needed to recover the β-galactosidase-expressing virus. Furthermore, we also wanted to truly demonstrate that clone IGRP2 can propagate a doubly defective $E1^-$ $E4^-$ adenovirus (e.g., virus AdβGal/dl1014) in the absence of any $E1^+$ or $E4^+$ potential helper virus, so we used a clonal construction strategy involving the cotransfection of purified restriction fragments free of any uncut (infectious) viral DNA, as described in Materials and Methods to incorporate the E4 deletion of H5dl1014 into the backbone of a recombinant adenovirus with an E1 deletion. The E4 deletion of H5dl1014 ($ORF4^+$) was chosen because it is associated with a unique viral DNA replication defect (3). In contrast to the other E4 deletants in this study, all of which lack ORF4, H5dl1014 has sequences homologous to both ends of the E4 unit of clone IGRP2 (Fig. 1B). We introduced this particular deletion to investigate the possible emergence of $E4^+$ replicative particles (i.e., ORF4+ORF6+ORF7) by homologous recombination during viral propagation in IGRP2 cells (see below).

Virus AdβGal/dl1014 was constructed in IGRP2 cells after homologous recombinations between three overlapping restriction fragments that combine the E4 deletion from H5dl1014 and the E1 deletion from AdRSVβGal (Fig. 4A). No CPE was evident after the transfection of these fragments in IGRP2 cells supplemented with DEX, a likely consequence of the low frequency with which the two homologous recombination events required to generate a replicative viral particle take place within a single cell. However, a CPE appeared when the cellular content was subsequently added to fresh IGRP2 cells grown in the presence of 1 μM DEX, demonstrating that viral propagation indeed occurred. Restriction analysis of the viral DNA prepared by the Hirt procedure generated the pattern expected of the AdβGal/dl1014 genome, with no obvious contamination with $E1^+$ or $E4^+$ fragments, as judged after ethidium bromide staining of the gel (data not shown). Furthermore, the amplified mixture was plated on IGRP2-derived monolayers in the presence of DEX, and all viral plaques stained positive for β-galactosidase, supporting the absence of contamination with virus H5dl1014.

Growth kinetics and viral productivity. Several stocks of AdβGal/dl1014 were prepared after the infection of 5×10^8 IGRP2 cells supplemented with 1 μM DEX. After CsCl banding, the titers of the purified virus were determined on IGRP2-derived cell monolayers supplemented with DEX and stained with X-Gal at 3 weeks postinfection. This is roughly twice as long as the titration of a virus with E1 deleted on 293-derived monolayers, indicating that the growth of a virus with both deletions in IGRP2 cells is significantly slower. We also noticed that the plaquing efficiency was drastically impaired when DEX was omitted; the number of plaques was reduced by several orders of magnitude and the plaques grew much more slowly when no exogenous DEX was added. This is in good agreement with the results shown in Fig. 3, demonstrating a basal level of E4 rescue in the absence of exogenous DEX addition and its significant induction after the inclusion of 1 μM DEX. Viral stocks ranging from 3×10^9 to 10^{10} PFU were obtained in the presence of DEX and indicated productivity that ranged from at least 7 to 20 PFU per cell. Because the viral titers of adenoviruses with E1 or E1 and E4 deletions are determined on their respective permissive packaging cells (i.e., 293 and IGRP2 cells), one has to be cautious of direct comparisons of viral stocks expressed in PFU. In fact, the viral titers were similar when they were expressed as β-galactosidase-expressing cells (or TU) after the infection of nonpermissive cells for both viruses (e.g., W162). In fact, purified stocks of virus AdRSVβGal of 5×10^{10} ($n = 2$) and 9×10^{10} PFU ($n = 2$) had values of 3.4×10^{10} ($n = 2$) and 4.4×10^{10} TU ($n = 2$), respectively, in W162 cells. In comparison, purified stocks of virus AdβGal/dl1014 of 3×10^9 ($n = 3$) and 5×10^9 PFU ($n = 3$) had values of 5.3×10^{10} ($n = 2$) and 2.1×10^{10} TU ($n = 2$), respectively. When primary rabbit vascular smooth muscle cells were used, the values were 3.2×10^8 ($n = 1$) and 2.5×10^8 TU ($n = 1$) for AdRSVβGal (9×10^{10} PFU) and AdβGal/dl1014 (3×10^9 PFU), respectively. Taken together, these observations indicate that although the growth kinetics of AdβGal/dl1014 in IGRP2 cells is somehow impaired, its viral productivity is indeed very similar to that of an E1-deleted adenovirus grown in 293 cells.

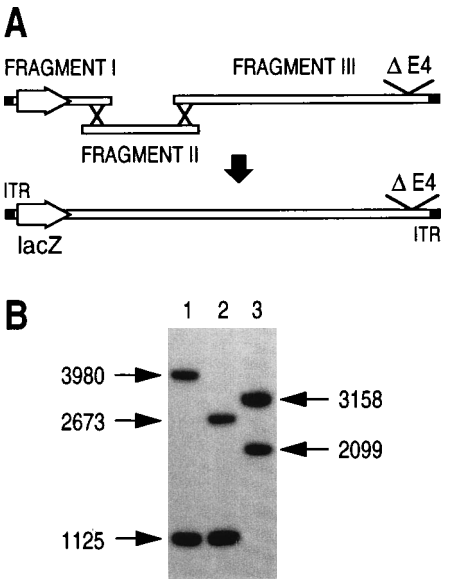

FIG. 4. (A) Construction of virus AdβGal/dl1014 in IGRP2 cells. Fragments I, II, and III are overlapping purified restriction fragments derived from the AdRSVβGal and H5dl1014 genomes, as described in Materials and Methods. The purified fragments were introduced into IGRP2 cells, and DEX (1 μM) was added every 4 days for 3 weeks. The cell lysate was subsequently added to fresh IGRP2 cells in the presence of DEX. The CPE observed after 5 days was amplified, and the viral stock (10^{10} PFU) from which DNA was recovered was prepared. (B) Southern blot analysis of *Stu*I-restricted viral DNA. Two micrograms of *Stu*I-restricted DNA was submitted to Southern analysis with a radiolabeled ITR probe. Lane 1, AdRSVβGal DNA; lane 2, AdβGal/dl1014 DNA; lane 3, H5dl1007 DNA. The sizes (in base pairs) and positions of the $E1^-$ *Stu*I fragment from AdRSVβGal (1125), the $E4^-$ fragments from H5dl1014 and H5dl1007 (2673 and 2099, respectively), an $E1^+$ fragment (3158), and an $E4^+$ fragment (3980) are indicated.

Helper-free propagation of virus AdβGal/dl1014. The absence of potential contaminating $E1^+$ or $E4^+$ virus was assessed by Southern and PCR amplification analyses of the DNA recovered from a purified viral stock. As shown in Fig.

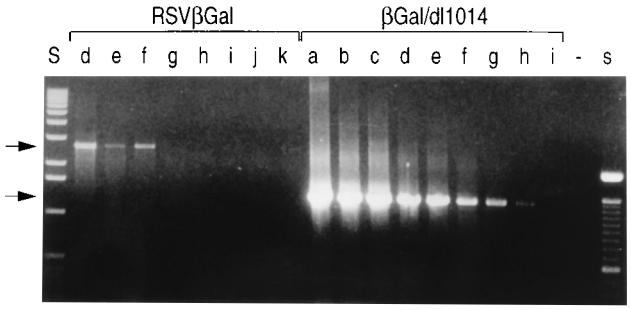

FIG. 5. Comparative PCR analysis of AdRSVβGal and AdβGal/dl1014 DNAs. Viral DNAs were extracted from CsCl-purified viral stocks, and the indicated quantities were used as substrates for PCR amplification: 1 μg (lane a), 100 ng (lane b), 10 ng (lane c), 1 ng (lanes d), 100 pg (lanes e), 10 pg (lanes f), 1 pg (lanes g), 100 fg (lanes h), 10 fg (lanes i), 1 fg (lane j), and 0.1 fg (lane k). Lanes S and s, 1- and 0.1-kb DNA standards, respectively; lane −, the absence of DNA in the amplification mix. The oligonucleotides used amplify a 2.69-kb fragment from an $E4^+$ virus (upper arrow) or a 1.37-kb fragment when the E4 deletion of *dl*1014 is introduced (lower arrow). Southern blot analysis of the gel confirmed no contamination with the 2.69-kb $E4^+$-specific fragment when 1 μg of AdβGal/dl1014 DNA was used for PCR amplification, indicating a sensitivity of at least 10^{-6} as the $E4^+$ fragment is detected when 1 pg of AdRSVβGal DNA is used.

4B, after a 30-min exposure, *Stu*I digestion of viral DNA generates only two fragments, and their sizes correspond exactly to those of the expected ITR-bearing fragments of the virus with both E1 and E4 deleted (lane 2). One fragment displays mobility which corresponds to that of the 1,125-bp *lacZ*-encoding fragment from AdRSVβGal (Fig. 4B, lane 1), while the second migrates as the 2,673-bp *Stu*I fragment encompassing the E4 deletion of H5dl1014. Prolonged exposure (28 h) of the autoradiogram did not reveal any additional band with a size corresponding to an $E1^+$ (3,158-bp) fragment (Fig. 4B, lane 3) or to the $E4^+$ fragment (3,980 bp) of AdRSVβGal (lane 1). Importantly, neither could a fragment corresponding in size (3,267 bp) to the introduction of the ORF4+ORF6+ORF7 E4 functional unit within the vector backbones be detected, demonstrating that a double recombination event between the cellular E4 unit and the vector backbones did not occur at detectable levels during viral propagation (Fig. 1B). PCR amplification of the viral stock DNA confirmed the absence of detectable contamination with $E1^+$ or $E4^+$ viruses, with sensitivities of 10^{-5} and 10^{-6}, respectively (Fig. 5).

DISCUSSION

Clone IGRP2 is a novel packaging cell line constructed by integrating a minimal E4 functional unit, MMTV LTR-ORF6+ORF7, into the genome of 293 ($E1^+$) cells (Fig. 1). These cells can rescue the growth defect associated with all of the $E1^+$ $E4^-$ adenoviral deletants tested, obviously because they express the ORF6 gene product to levels restoring late viral protein synthesis (Fig. 3). The results of DNA and RNA analysis experiments were consistent with MMTV LTR-driven expression of the ORF6+ORF7 unit and indicated that bona fide alternative splicing generated the ORF6 and ORF6/7 mRNAs (Fig. 2). The concomitant expression of E1 and E4 regulatory genes within IGRP2 cells has been demonstrated by the construction of a recombinant *lacZ*-encoding virus with both deletions that exhibits the E4 deletion of virus H5dl1014. Molecular characterization of the genome from a purified viral stock by Southern and PCR amplification techniques demonstrated the absence of potential helper viruses exhibiting the E1 or E4 region. Neither was an $E4^+$ virus containing the ORF4+ORF6+ORF7 segment detected, although such a virus could emerge as a result of homologous recombination on both sides of the *dl*1014 deletion (Fig. 1B). A fortiori, the emergence of $E4^+$ particles during the propagation of viruses combining the E1 deletion of AdRSVβGal and the E4 deletion of the other defective viruses in this study is not possible in IGRP2 cells because (i) the E4 deletion from H5dl1014 is the only one which exhibits overlapping sequences on both sides of the ORF6+ORF7 unit (Fig. 1) and (ii) a single homologous recombination between the cellular and viral genomes generates a virus lacking its right ITR.

The importance of expressing a minimal E4 functional unit to avoid the emergence of $E4^+$ replicative particles during viral stock preparation is further supported by the observation that a 293-derived cell line engineered to express the complete Ad5 E4 region from the MMTV LTR (clone IGRP4) actually did allow the recovery of β-galactosidase-expressing $E4^+$ viruses (i.e., AdRSVβGal) during the viral growth of AdβGal/dl1004 (6a). Moreover, although this cell line allowed the construction of AdβGal/dl808, AdβGal/dl1004, AdβGal/dl1007, AdβGal/dl1011, and AdβGal/dl1014, these viruses could not be plaque purified on IGRP4-derived monolayers, as the cells could not sustain cell confluence long enough (37b). In contrast, upon inclusion in agar, IGRP2 cells sustain cell confluence for a longer period than do 293 cells in the presence of DEX. We believe that some of the additional E4 ORF expressed in IGRP4 (i.e., ORF1, ORF2, ORF3, or ORF4) is indeed responsible for the IGRP4-associated phenotype. ORF4 is a likely candidate for this because it is expressed in IGRP4 cells (unpublished result) and it targets AP1 (22), an important component of proliferative signal transduction pathways (27). Viruses with both deletions constructed in IGRP4 were in fact plaque purified on IGRP2-derived monolayers (Fig. 6), as these cells are viable for more than 4 weeks in the presence of DEX after they reach cell confluence.

The possibility that the expression of nondeleted viral antigens is a triggering determinant for the immune clearance of recipient cells after adenovirus-mediated gene transfer is certainly attractive. The evaluation of the host inflammatory response and the persistence of β-galactosidase expression after the transfer of viruses with both deletions from this study in relevant animal models will be helpful in understanding the role of the E4 genes in viral antigen synthesis in the absence of E1A.

FIG. 6. AdβGal/dl1011 plaque formation on IGRP2. DEX (1 μM) was added at the time of infection and every 5 days thereafter. The plaques were stained with X-Gal at 22 days postinfection.

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