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# Mode of transgene expression after fusion to early or late viral genes of a conditionally replicating adenovirus via an optimized internal ribosome entry site in vitro and in vivo

Angel A. Rivera,<sup>a</sup> Minghui Wang,<sup>a</sup> Kaori Suzuki,<sup>a</sup> Taco G. Uil,<sup>a,1</sup> Victor Krasnykh,<sup>a,2</sup>  
David T. Curiel,<sup>a</sup> and Dirk M. Nettelbeck<sup>a,b,\*</sup>

<sup>a</sup>Division of Human Gene Therapy, Departments of Medicine, Pathology, and Surgery, and the Gene Therapy Center,  
University of Alabama at Birmingham, Birmingham, AL, 35294 USA

<sup>b</sup>Department of Dermatology, University Medical Center Erlangen, 91052 Erlangen, Germany

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## Abstract

The expression of therapeutic genes by oncolytic viruses is a promising strategy to improve viral oncolysis, to augment gene transfer compared with a nonreplicating adenoviral vector, or to combine virotherapy and gene therapy. Both the mode of transgene expression and the locale of transgene insertion into the virus genome critically determine the efficacy of this approach. We report here on the properties of oncolytic adenoviruses which contain the luciferase cDNA fused via an optimized internal ribosome entry site (IRES) to the immediate early adenoviral gene E1A (AdΔE1AIL), the early gene E2B (AdΔE2BIL), or the late fiber gene (AdΔfiberIL). These viruses showed distinct kinetics of transgene expression and luciferase activity. Early after infection, luciferase activities were lower for these viruses, especially for AdΔE2BIL, compared with nonreplicating AdTL, which contained the luciferase gene expressed from the strong CMV promoter. However, 6 days after infection, luciferase activities were approximately four (AdΔE1AIL) to six (AdΔfiberIL) orders of magnitude higher than for AdTL, reflecting virus replication and efficient transgene expression. Similar results were obtained in vivo after intratumoral injection of AdΔE2BIL, AdΔfiberIL, and AdTL. AdΔfiberIL and the parental virus, Ad5-Δ24, resulted in similar cytotoxicity, but AdΔE2BIL and AdΔE1AIL were slightly attenuated. Disruption of the expression of neighboring viral genes by insertion of the transgene was minimal for AdΔE2BIL and AdΔfiberIL, but substantial for AdΔE1AIL. Our observations suggest that insertion of IRES-transgene cassettes into viral transcription units is an attractive strategy for the development of armed oncolytic adenoviruses with defined kinetics and strength of transgene expression.

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**Keywords:** Viral vector; Viral oncolysis; Conditionally replicative adenovirus; Gene therapy; Armed oncolytic adenovirus; Early and late transgene expression; IRES; Timing of transgene expression

Viral oncolysis, or virotherapy, is a novel and promising cancer treatment modality that implements viruses as therapeutic agents in an effort to exploit their highly evolved

qualities to efficiently infect human cells, replicate, kill the host cell to release the progeny virions, and spread (Kim and McCormick, 1996; Ring, 2002). Thereby, this new strategy establishes a therapeutic mechanism distinct from conventional therapies to which advanced cancers are often resistant. Recent advances in molecular virology and recombinant DNA technology have been a major impetus for this field because they facilitate the engineering of advanced virotherapeutics.

Adenoviruses possess the critical basic properties that are required for viral oncolysis (Alemany et al., 2000; Curiel, 2000; Zhang, 1999). These include a lytic replication cycle, a highly evolved gene transfer machinery,

\* Corresponding author. Department of Dermatology, University Medical Center Erlangen, Hartmannstrasse 14, D-91052 Erlangen, Germany. Fax: +49-9131-853-6417.

E-mail address: [dirk.nettelbeck@derma.imed.uni-erlangen.de](mailto:dirk.nettelbeck@derma.imed.uni-erlangen.de) (D.M. Nettelbeck).

<sup>1</sup> Present address: Department of Molecular Cell Biology, Leiden University Medical Center, Leiden, The Netherlands.

<sup>2</sup> Present address: Department of Experimental Diagnostic Imaging, MD Anderson Cancer Center, University of Texas, Houston, TX.

stability of virus particles, the ease of virus production at high titers, and a favorable safety profile because of their low pathogenicity, nonintegrating genome, and genetic stability. Importantly, the advanced knowledge of adenovirus structure, genome organization, and life cycle allows for molecular modifications that are required to derive effective therapeutics. This knowledge has been essential for the engineering of adenoviruses with tumor-restricted replication capacity and is also required for the incorporation of transgenes into the genome of oncolytic adenoviruses.

Conditionally replicative adenoviruses (CRAds) implement tumor-specific viral replication and cell killing, a fundamental requirement for the concept of viral oncolysis (Alemany et al., 2000; Curiel, 2000). Most approaches for the development of CRAds have focused on the genetic engineering of E1 genes, the first viral genes expressed after infection of the host cell and key regulators of adenoviral replication. Within CRAds, E1 genes have been genetically engineered by two means: by partial or complete gene deletions to ablate functions which are required for virus replication in normal cells but dispensable in cancer cells; or by replacement of viral promoters with tumor-specific promoters. An example for the former strategy is Ad5- $\Delta$ 24, also called *d1922-947*, a virus that contains a 24-bp deletion within the conserved region 2 of the E1A gene (Fueyo et al., 2000; Heise et al., 2000). The resulting E1A mutant is not able to bind and inactivate pRb, as required for adenoviral replication in normal cells. However, pRb binding of E1A in cancer cells is not necessary, because pRb is inactivated in the majority of tumors and in proliferating cells per se, resulting in tumor-selective replication capacity of Ad5- $\Delta$ 24.

Initial clinical studies demonstrated that CRAd injections were well tolerated (Reid et al., 2002). Moreover, they validated the concept of adenoviral oncolysis in vivo by demonstrating tumor-specific viral replication and tumor cell killing. However, durable responses have been rare for oncolytic adenoviruses indicating that current CRAds are not potent enough as a single agent. Interestingly, the clinical observation of synergistic effects of adenoviral oncolysis and chemotherapy or radiotherapy resulting in tumor reductions in individual patients revealed that virotherapy lacks cross-resistance with various conventional therapies and suggests high potential for combination therapies.

The co-expression of transgenes by CRAds (reviewed in Hermiston and Kuhn, 2002) is a new avenue for improving various aspects of viral oncolysis and is primarily aimed at the monitoring of CRAd replication and spread, augmentation of viral release, or combination therapy. These applications require the incorporation of different “genetic payloads”. For example, transgenes that encode fluorescent proteins, receptors for radiotracers, or secretory peptides (Peng et al., 2002; Vassaux and Groot-Wassink, 2003) have potential for monitoring and tracking

of CRAds within the patient. Moreover, apoptosis-inducing genes can be exploited for enhancing release of adenovirus particles (Sauthoff et al., 2002; van Beusechem et al., 2002). Finally, the incorporation of therapeutic genes can result in a combination therapy mediated by a single agent, an “armed CRAd”. Candidate therapeutic genes include those encoding prodrug-converting enzymes for molecular chemotherapy (Akbulut et al., 2003; Bernt et al., 2002; Freytag et al., 1998; Hawkins and Hermiston, 2001b; Lambright et al., 2001; Lee et al., 2001; Nanda et al., 2001; Rogulski et al., 2000; Wildner et al., 1999a, 1999b), angiogenesis inhibitors, or cytokines (Bristol et al., 2003; Hawkins and Hermiston, 2001a, 2001b; Hawkins et al., 2001).

These various strategies have different requirements for quantity and kinetics of transgene expression. They have to consider the potential interference of transgene expression or activity of the encoded protein with adenoviral replication in a given infected cell and likewise, adverse effects of shutdown of host cell protein synthesis with quantitative transgene expression. For example, the monitoring of CRAd-infected cells by imaging genes might require early transgene expression before onset of host cell shut-off and beginning cell lysis. In contrast, enhanced viral release or certain combination therapies might necessitate late expression of apoptosis-inducing or cell-killing therapeutic genes to avoid interference with productive virus replication. In addition, restriction of transgene expression by CRAds until after replication of the viral genome prevents transgene function in healthy cells, where replication of the CRAd genome is blocked. Thus, a late mode of transgene expression by CRAds is tumor-specific.

The prime objective of our study was to evaluate a system for co-expression of transgenes by CRAds that is based on fusion of a transgene to immediate early, early, or late viral genes via an internal ribosome entry site (IRES). This system exploits endogenous viral regulatory elements such as promoters, splicing signals, leader sequences, or polyadenylation signals for efficient and regulated expression of the transgene within a polycistronic message. We generated recombinant oncolytic adenoviruses derived from Ad5- $\Delta$ 24 (Fueyo et al., 2000; Suzuki et al., 2001) by fusion of the luciferase cDNA to the viral E1A, E2B, and fiber genes via an improved IRES. These viruses were analyzed for strength and kinetics of transgene expression and activity, for the effect of transgene incorporation on the expression of surrounding viral genes, and for cytolytic activity in monolayer cultures. Strength and kinetics of luciferase activity were also determined after intratumoral injection into subcutaneous tumors. Our results have important implications for co-expression strategies in adenoviral oncolysis and can be combined with the various described strategies to restrict Ad replication to tumor cells.

## Results

### *Construction of CRAd genomes for early and late co-expression of transgenes fused to different viral genes via an optimized IRES sequence*

To establish a strategy for exploitation of the adenoviral gene expression machinery for transgene expression, we pursued the strategy depicted in Fig. 1A. The luciferase reporter gene was fused via an internal ribosome entry site (IRES; Martinez-Salas, 1999) to viral genes E1A, E2B, or fiber of CRAd Ad5- $\Delta$ 24 (Fueyo et al., 2000; Suzuki et al., 2001) in the order viral gene–IRES–luciferase to generate Ad $\Delta$ E1AIL, Ad $\Delta$ E2BIL, and Ad $\Delta$ fiberIL, respectively (IRESLuc-CRAds). This strategy aimed at the efficient transgene expression at immediate early, early, or late stages of the viral life cycle, respectively. As strong IRES activity is critical for this strategy, we initially sought to optimize the IRES starting from the encephalomyocarditis virus (EMCV) IRES which was previously reported to result in superior transgene expression of various tested sequences (Harries et al., 2000). In this regard, positioning of the start ATG codon (Rees et al., 1996) and the spacing between stop codon of the upstream gene to the IRES (Attal et al., 1999; Kobayashi et al., 2001) were reported to influence transgene expression from the IRES. Three plasmids with the luciferase gene fused with distinct versions of the EMCV IRES sequence to the adenoviral E1A gene were generated (Fig. 1C). The first construct, pSE1A $\Delta$ IRESLuc, contains the IRES as described previously (Fuerst et al., 1986), with the start codon used by EMCV mutated and the start codon of the transgene inserted downstream. In the second plasmid, pSE1A $\Delta$ IRESLucATG, the start ATG of the luciferase gene was placed in the original start ATG position of EMCV. In a third construct, pSE1A $\Delta$ spIRESLucATG, a 54-bp spacer derived from the 3'NTR of the human CAT gene was placed between E1A gene and IRES. These constructs and pGL3SV40p, which contains the strong SV40 promoter driving luciferase expression, were transiently transfected into A549 cells (Fig. 1C). The construct with spacer and corrected ATG position resulted in the strongest luciferase activity, which was approximately 10-fold higher compared to the construct without spacer and without original ATG position and approximately 30% stronger compared with the construct without spacer, but with original ATG position. Furthermore, this construct resulted in 10-fold higher luciferase activity than pGL3SV40p, indicating a high level of transgene expression from E1A-IRES. We thus exploited the EMCV IRES with the original ATG position and upstream spacer for designing of the IRESLuc-CRAds. For this strategy, we had to take the endogenous viral polyadenylation and promoter sequences into consideration (Fig. 1B). For the generation of Ad $\Delta$ E1AIL, a spacer-IRES-Luc cassette was inserted between the E1A

translation stop signal and the E1A polyadenylation sequence, thus 94 bp upstream of the E1B TATA box. For Ad $\Delta$ E2BIL and Ad $\Delta$ fiberIL, cloning sites were generated downstream of the E2B or fiber ORFs, respectively. Furthermore, polyadenylation signals that overlap with the respective translation stop codons were mutated (see Materials and methods) to prevent transcription termination upstream of the inserted IRES. Consequently, a polyadenylation signal was incorporated downstream of the inserted spacer-IRES-Luc cassettes for these viruses. For an overview of the adenoviruses used in this study see Table 1.

### *Cytolytic activity of CRAds that contain IRES-transgene cassettes in different loci*

For analysis of the cytolytic activity of IRESLuc-CRAds, we infected permissive A549 cells with Ad $\Delta$ E1AIL, Ad $\Delta$ E2BIL, Ad $\Delta$ fiberIL, the parental virus Ad5- $\Delta$ 24 or with nonreplicating AdCMVLuc at multiplicities of infection (MOI) (pfu/cell) 10, 1, 0.1, and 0.01. Ten days after infection, surviving cells were fixed and stained with crystal violet (Fig. 2). Ad $\Delta$ fiberIL resulted in similar cytotoxicity as Ad5- $\Delta$ 24, whereas Ad $\Delta$ E2BIL and Ad $\Delta$ E1AIL were attenuated by less than one order of magnitude or by approximately one order of magnitude, respectively (that is less than one order of magnitude or approximately one order of magnitude higher virus titers were required to achieve similar cytotoxicity). Thus, expression of luciferase by CRAds per se did not attenuate adenoviral cytotoxicity; however, insertion of the IRES-transgene cassette at distinct loci, in this case after the E1A or E2B genes, can reduce cytolytic activity of CRAds.

### *Efficacy and kinetics of transgene activity of IRESLuc-CRAds compared with a first generation adenoviral vector*

Next, we sought to analyze the magnitude and timing of transgene expression by IRESLuc-CRAds and to compare it to transgene expression directed by the strong CMV promoter in a first generation, nonreplicative Ad vector, AdTL (Seki et al., 2002). For this purpose we infected A549 cells with Ad $\Delta$ E1AIL, Ad $\Delta$ E2BIL, Ad $\Delta$ fiberIL, or AdTL and determined luciferase activity 6, 10, 14, 18, 22, and 26 h after virus infection (Fig. 3, left panels). At 6 h postinfection, luciferase activities were 25-fold (Ad $\Delta$ fiberIL), 70-fold (Ad $\Delta$ E1AIL), or 1443-fold (Ad $\Delta$ E2BIL) lower for the IRESLuc-CRAds relative to AdTL (Fig. 3A). However, at 26 h postinfection, luciferase activity of Ad $\Delta$ E1AIL was similar to AdTL, and those of Ad $\Delta$ E2BIL and Ad $\Delta$ fiberIL were 7-fold or 27-fold higher, respectively. Thus, transgene expression by IRESLuc-CRAds was weak early after adenovirus infection, especially for Ad $\Delta$ E2BIL, but increased dramatically

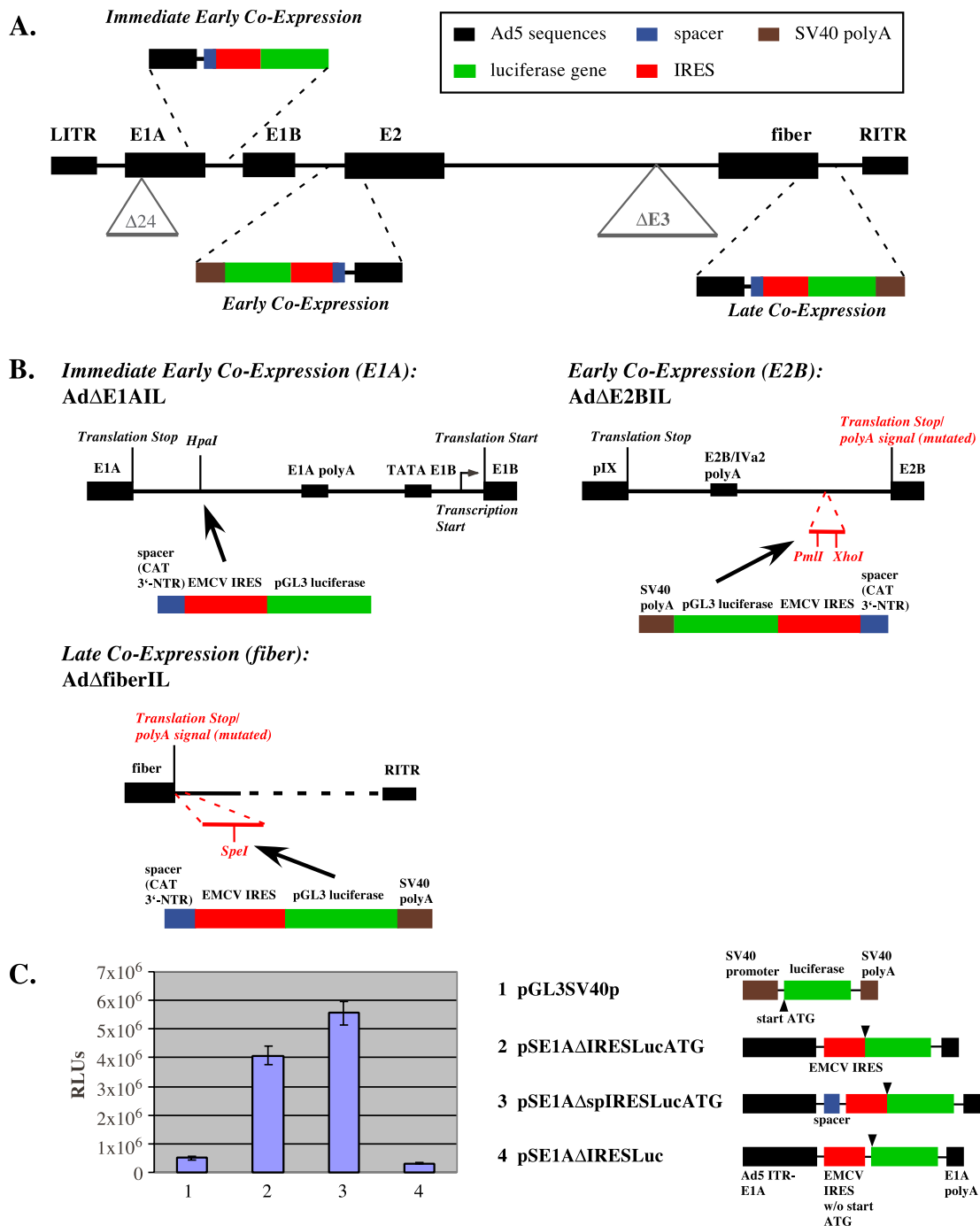


Fig. 1. Strategy for transgene expression from an optimized IRES in CRAds. (A) Locales for insertion of IRES-Luc cassettes into the genome of Ad5-Δ24. Deletion of 24 nucleotides of conserved region 2 of E1A (Δ24) restricts viral replication to tumor cells; the E3 region is deleted (ΔE3). (B) Individual insertion sites for IRES-luciferase cassettes into the viral genome. Modifications to the ad genome for insertion of cloning sites and mutation of polyadenylation signals are shown in red. (C) Analysis of IRES activity. Three plasmids with different E1A-IRESLuc constructs (schematic representation on right) and a plasmid with SV40 promoter-driven luciferase were transfected into A549 cells. Experiments were performed in triplicates. Mean RLUs and standard deviations (error bars) are shown on the left.

within 26 h with AdΔfiberIL resulting in the highest luciferase activity at that time point. The increase in luciferase activity from 6 to 26 h was 47-fold,  $3.7 \times 10^3$ -fold,  $4.9 \times 10^5$ -fold, and  $3.1 \times 10^4$ -fold for AdTL, AdΔE1AIL, AdΔE2BIL, and AdΔfiberIL, respectively. To

further demonstrate the kinetics of transgene expression of individual IRESLuc-CRAds with consideration given to different absolute expression levels, relative luciferase activities were plotted as fractions of readings at 26 h for each virus individually (Fig. 3B, logarithmic; Fig.



3C, linear). This analysis confirmed the substantially delayed onset of transgene expression from IRESLuc-CRAds relative to AdTL. Relative expression from Ad $\Delta$ E1AIL increased earlier during virus infection than from Ad $\Delta$ E2BIL and Ad $\Delta$ fiberIL. Surprisingly, luciferase expression from the fiber fusion construct was higher relative to the E2B fusion construct at 6 h postinfection not only in absolute readings (Fig. 3A), but also after normalization (Fig. 3B). However, relative transgene expression from Ad $\Delta$ E2BIL surpassed relative expression from Ad $\Delta$ fiberIL between 10 and 14 h, whereas the increase was steeper for Ad $\Delta$ fiberIL at later time points. Overall, these patterns—with the exception of Ad $\Delta$ fiberIL early after infection—resembled the timing of transgene expression expected from the kinetics of E1A, E2B, and fiber expression in wild-type adenovirus. However, there was only a minimal time interval between the onset of transgene expression from Ad $\Delta$ E2BIL and Ad $\Delta$ fiberIL (Fig. 3C).

Next, we evaluated luciferase activity over a more extended time interval of 6 days after infection of A549 cells with IRESLuc-CRAds and AdTL at 100-fold lower virus dose (Fig. 3D). On day 1 relative luciferase activities of all viruses matched those at 26 h of the previous experiment, however absolute values were near three orders of magnitude lower, resulting from the lower MOI used for infection. Luciferase activities for the nonreplicating AdTL were constant until day 5 and decreased on day 6. In contrast, luciferase activities for all IRESLuc-CRAds increased continuously over time with the sharpest increase from day 1 to day 2. On day 6, luciferase activities of Ad $\Delta$ E1AIL, Ad $\Delta$ E2BIL, and Ad $\Delta$ fiberIL were about four, five, or six orders of magnitude higher, respectively, than luciferase activity of AdTL.

#### *Kinetics of mRNA expression from the transgene and from neighboring viral genes of IRESLuc-CRAds*

To analyze the kinetics of expression of luciferase and viral genes of IRESLuc-CRAds, we quantified the mRNA expression 5, 10, 15, 20, and 25 h after infection

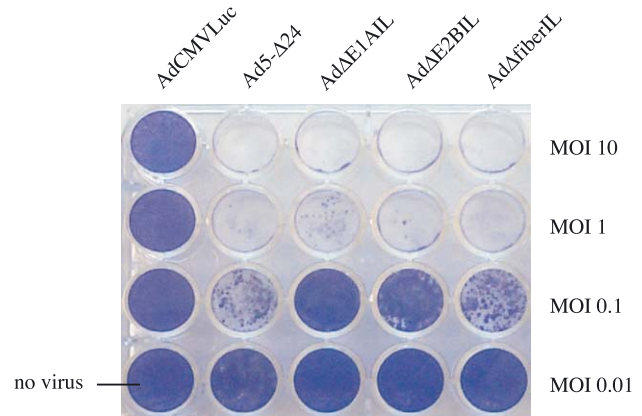


Fig. 2. Cytotoxicity of IRESLuc-CRAds and the parental virus, Ad5- $\Delta$ 24. A549 cells were infected with indicated recombinant adenoviruses at indicated titers. Adherent cells were stained 10 days after infection with crystal violet. Dead cells detach and were washed off.

of A549 cells by real-time PCR. First, we compared mRNA copy numbers of luciferase with those of the fused viral genes E1A, E2B, or fiber after infection with Ad $\Delta$ E1AIL, Ad $\Delta$ E2BIL, or Ad $\Delta$ fiberIL, respectively. We determined similar copy numbers for luciferase and E1A, or luciferase and E2B of Ad $\Delta$ E1AIL or Ad $\Delta$ E2BIL, respectively, as expected for messages of a polycistronic mRNA (Fig. 4A). For Ad $\Delta$ fiberIL, copy numbers for luciferase message were approximately 25% of those of fiber message over the whole time interval evaluated in the experiment. This observation might indicate early transcription termination for a fraction of synthesized mRNAs.

Next, we assessed whether insertion of heterologous sequences into the adenoviral genome interferes with expression of neighboring genes, which is dependent on the corresponding promoter activity, mRNA stability, and splicing. Therefore, we compared mRNA expression of viral genes adjacent to the inserted IRESLuc cassette to mRNA expression of the same genes in the parental virus, Ad5- $\Delta$ 24. Moreover, we analyzed mRNA copy numbers of the fiber gene, which, representing late expression, reflect replication efficiency. For Ad $\Delta$ E1AIL, expression of E1A mRNA was severely attenuated compared with the parental virus, Ad5- $\Delta$ 24 (Fig. 4B). This observation implies that insertion of the IRESLuc cassette, or of heterologous sequences per se, downstream of the E1A reading frame reduces transcription of E1A or stability of E1A mRNA. Also, mRNA copy numbers of E2B during early infection (until 15 h) and of fiber were reduced for this virus. For Ad $\Delta$ E2BIL, expression of E2B was slightly decreased at 10–25 h compared with the parental virus. Protein IX mRNA copy numbers were similar for Ad $\Delta$ E2BIL and Ad5- $\Delta$ 24 until 10 h postinfection, but were lower at later time points. However, expression of fiber mRNA was almost identical for Ad $\Delta$ E2BIL and Ad5- $\Delta$ 24. Compared with the parental

Table 1

| Virus               | Features   |
|---------------------|--|
| AdCMVLuc            | Nonreplicating ad, CMV-luciferase (Reynolds et al., 2001)      |
| AdTL                | Nonreplicating ad, CMV-luciferase (Seki et al., 2002)          |
| Ad5- $\Delta$ 24    | CRAd, E1A CR2 mutant (Fueyo et al., 2000; Suzuki et al., 2001) |
| Ad $\Delta$ E1AIL   | Ad5- $\Delta$ 24-derived, IRES-Luc fused downstream to E1A     |
| Ad $\Delta$ E2BIL   | Ad5- $\Delta$ 24-derived, IRES-Luc fused downstream to E2B     |
| Ad $\Delta$ fiberIL | Ad5- $\Delta$ 24-derived, IRES-Luc fused downstream to fiber   |

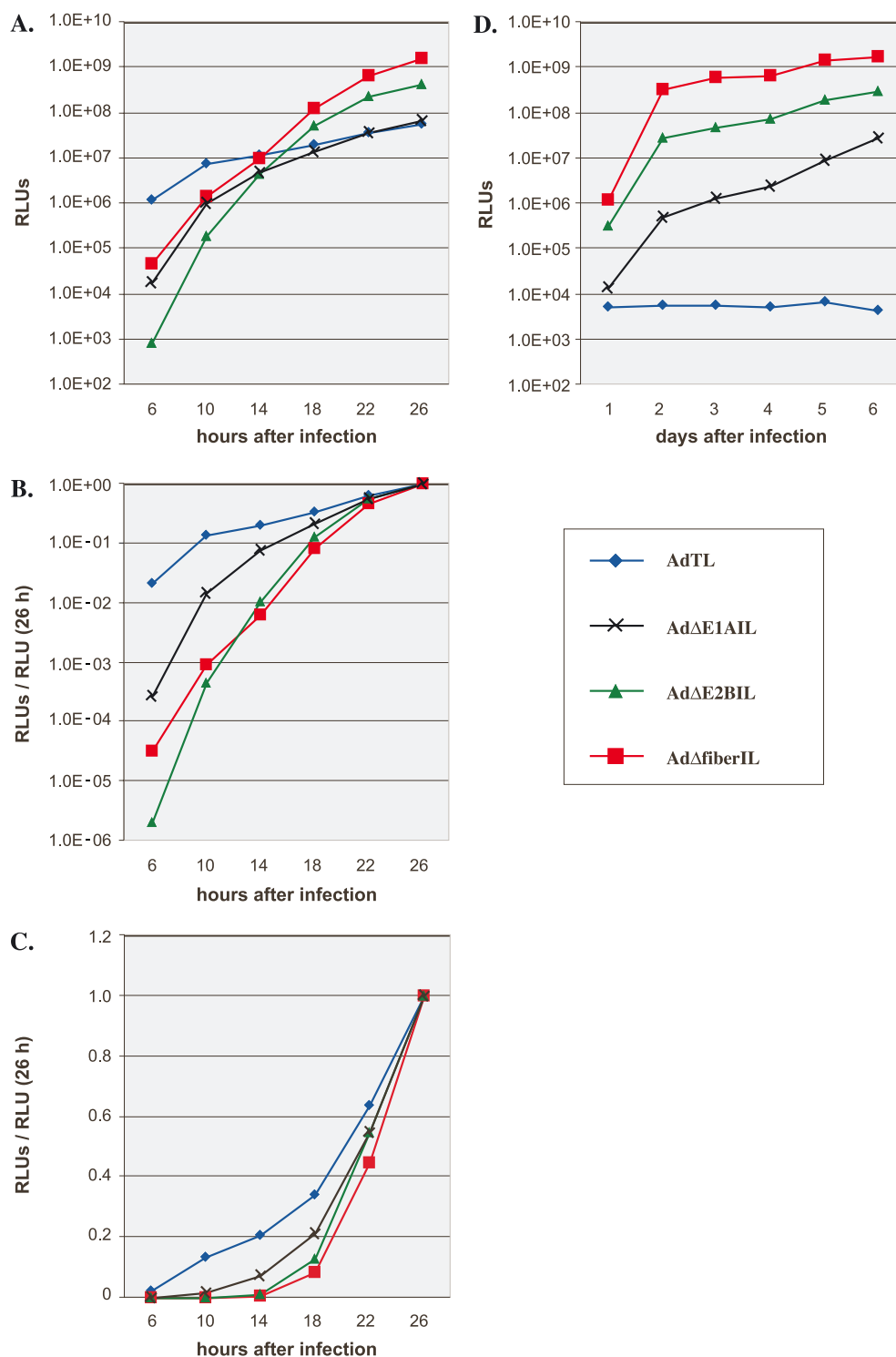


Fig. 3. Kinetics and efficiency of transgene expression by IRESLuc-CRAds versus nonreplicating AdTL: Luciferase assay. A549 cells were infected with AdΔE1AIL, AdΔE2BIL, AdΔfiberIL, and AdTL at MOI 5 (A–C) or 0.05 (D) and luciferase activities were determined at indicated time points. Experiments were performed in triplicates. Mean RLUs are shown in A and D. Standard deviations were within the marks. B and C show relative RLUs after normalization with readings at 26 h for each virus individually. Note the logarithmic scale in diagrams A, B, and D, but linear scale in diagram C.

virus, expression of fiber and E4 messages by AdΔfiberIL was increased early after adenoviral infection but was similar at later time points. Thus, for AdΔE2BIL and

AdΔfiberIL, expression of neighboring viral genes was minimally modified without effect on accumulation of fiber message.

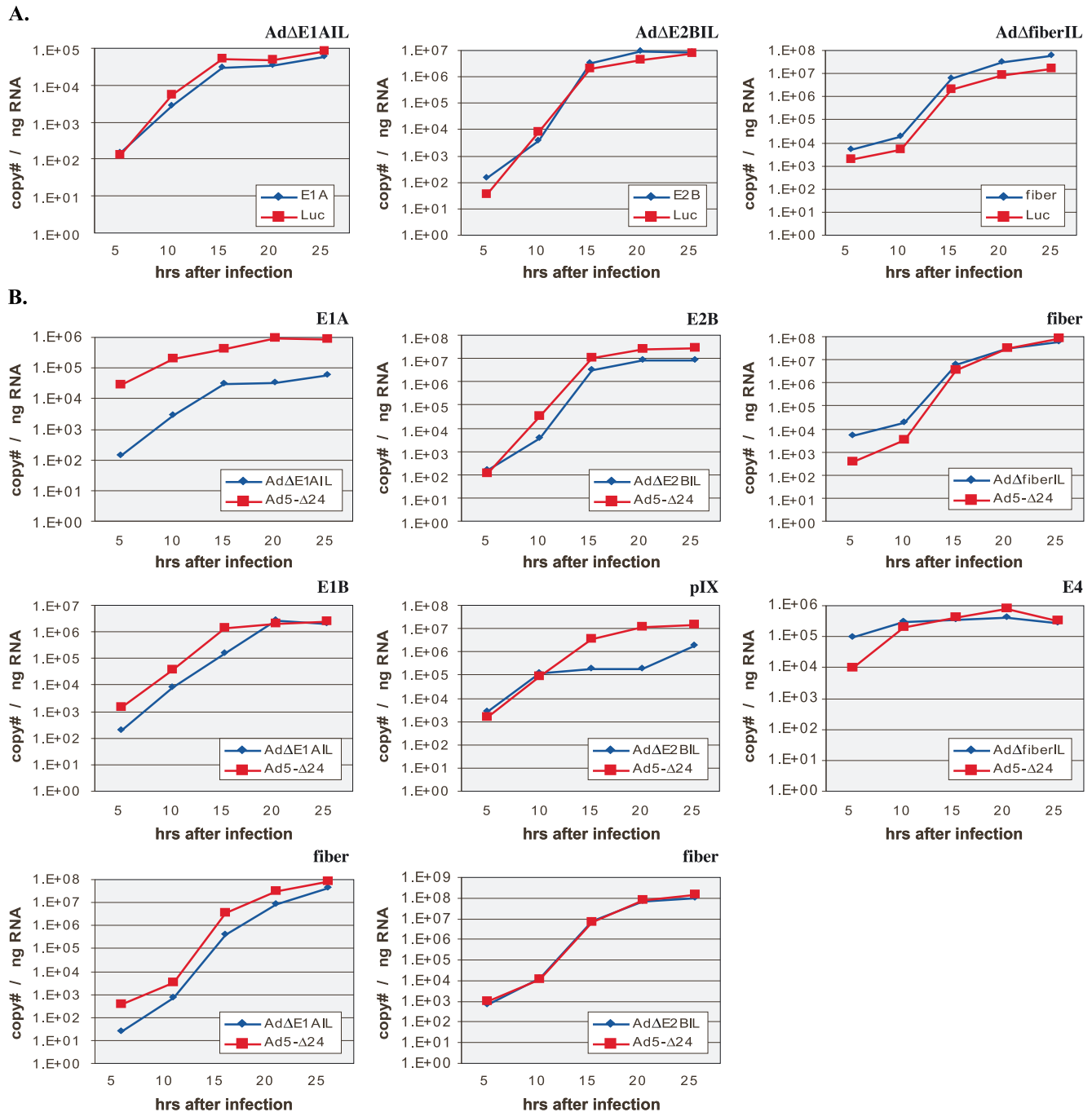


Fig. 4. Kinetics and efficiency of mRNA expression from the transgene and from viral genes of IRESLuc-CRAds and of parental Ad5- $\Delta$ 24. A549 cells were infected with Ad $\Delta$ E1AIL, Ad $\Delta$ E2BIL, Ad $\Delta$ fiberIL, and Ad5- $\Delta$ 24. Messenger RNA copy numbers were determined for the indicated genes at 5, 10, 15, 20, and 25 h after infection. (A) Copy numbers of luciferase mRNA and of mRNA of the viral gene to which luciferase was fused via an IRES (as indicated in the legend) were plotted for the IRESLuc-CRAds (title). (B) Messenger RNA copy numbers of viral genes adjacent to the inserted IRESLuc cassette and of the fiber gene (as indicated in the title) were plotted for IRESLuc-CRAds versus the parental virus, Ad5- $\Delta$ 24 (legend).

#### Correlation of luciferase mRNA expression and protein activity

Luciferase activities after infection of A549 with IRESLuc-CRAds correlated with copy numbers of luciferase mRNA if a delay between mRNA expression and protein activity was taken into account (Figs. 5 and 3A and 3D).

Both luciferase mRNA levels and protein activities initiated with Ad $\Delta$ fiberIL > Ad $\Delta$ E1AIL > Ad $\Delta$ E2BIL at 5 or 6 h postinfection and eventually were Ad $\Delta$ fiberIL > Ad $\Delta$ E2BIL > Ad $\Delta$ E1AIL. Ad $\Delta$ E2BIL surpassed Ad $\Delta$ E1AIL for luciferase mRNA copy numbers at day 10 postinfection and for luciferase enzyme activity on day 18. A relatively high luciferase mRNA copy number for



AdΔfiberIL at 5 h after virus infection is in accord with the surprisingly high luciferase activity early during virus infection (Figs. 3A and 3B). However, mRNA copy numbers increased dramatically for AdΔE2BIL, but not for AdΔfiberIL from 5 to 10 h postinfection, whereas the increase was more pronounced for AdΔfiberIL after 10 h, again resembling luciferase activities (Fig. 3B). For AdTL, mRNA levels correlated with enzyme activity late during adenovirus replication (compare mRNA copy numbers at 25 h with enzyme activity at day 2). At early time points, luciferase activity was stronger for AdTL than for IRESLuc-CRAds until 14 h postinfection, whereas luciferase mRNA levels were already higher for all IRESLuc-CRAds at 10 h postinfection, reflecting the delay between mRNA expression and protein activity.

*Efficacy and kinetics of luciferase activity of IRESLuc-CRAds versus a first generation adenoviral vector in vivo*

Next, we evaluated the activity of IRESLuc-CRAds in vivo. For this purpose, sc A549 xenografts established in nude mice were injected intratumorally with the IRESLuc-CRAds AdΔE2BIL and AdΔfiberIL, which showed the most promising results in vitro, or with nonreplicating AdTL. Tumors were harvested at 1, 2, 5, or 8 days postinfection and luciferase activity of tumor lysates was determined (Fig. 6). Overall, the in vivo results resembled our in vitro observations described above: (i) luciferase activities strongly increased for both IRESLuc-CRAds, resulting in

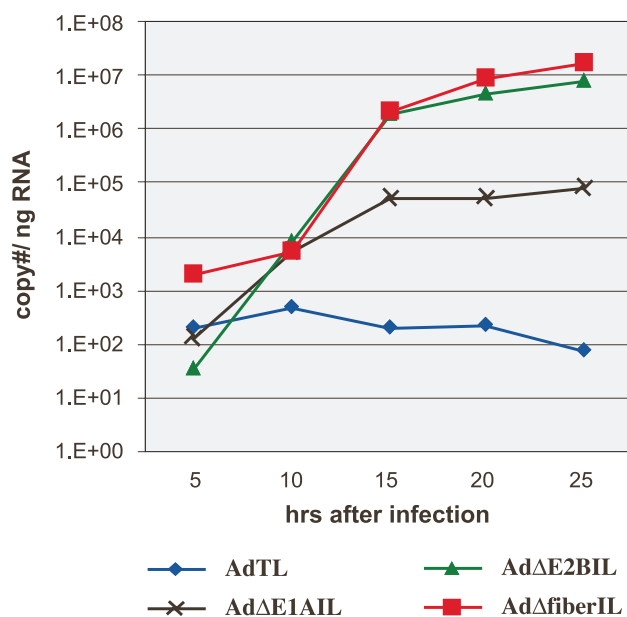


Fig. 5. Luciferase mRNA copy numbers for IRESLuc-CRAds and AdTL. Luciferase mRNA copy numbers are shown at indicated time points after infection of A549 cells with AdΔE1AIL, AdΔE2BIL, AdΔfiberIL, and AdTL.

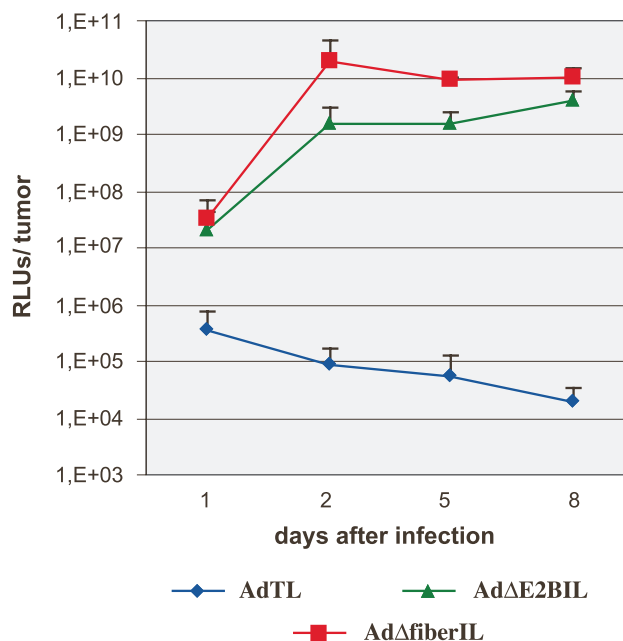


Fig. 6. Luciferase activity in A549 tumors after intratumoral injection of AdΔE2BIL, AdΔfiberIL, and AdTL. Viruses were injected into sc A549 xenografts and luciferase activity was determined at indicated days after infection. Experiments were performed in groups of four mice per virus and day (3 for AdTL, day 8). Mean RLUs and standard deviations (error bars) are shown.

reporter activities more than five orders of magnitude higher than for AdTL on day 8; (ii) the strongest increase in luciferase activities for AdΔE2BIL and AdΔfiberIL was observed from day 1 to day 2; and (iii) AdΔfiberIL resulted in the highest luciferase activities. In contrast to the in vitro results, luciferase activities for AdTL decreased from day 1 to day 8. From day 2 to day 8, luciferase activity did not increase for AdΔfiberIL and increased only slightly for AdΔE2BIL.

## Discussion

Transgene expression by oncolytic viruses is a promising strategy to improve viral oncolysis, for example, by combination therapy, apoptosis induction for enhanced viral release, or monitoring of viral spread. Next to the selection of the candidate transgene, the appropriate mode of transgene expression and a suitable locale of transgene insertion into the virus genome are pivotal for this approach. Our study shows that fusion of transgenes via an optimized IRES to different viral genes of replicating adenoviruses results in dramatic increases of transgene expression after infection of tumor cells, both in vitro and in vivo, compared with a nonreplicating adenovirus which expresses the same transgene from the powerful CMV promoter and enhancer. Augmentation of transgene activity was up to six orders of magnitude. For this strategy, we exploited the EMCV

IRES and could show that the position of the post-IRES start ATG is critical for efficient transgene expression. Moreover, insertion of a spacer sequence between the viral gene and IRES further increased transgene expression. These observations are in accord with previous reports (Attal et al., 1999; Kobayashi et al., 2001; Rees et al., 1996).

Of note, various features of the developed IRESLuc-CRAds depended on the viral gene to which the transgene was fused. These features were (i) the quantity and timing of transgene expression, (ii) the influence of transgene insertion on the activity of adjacent viral genes, and (iii) the oncolytic efficacy. Highest luciferase activity *in vitro* and *in vivo* was achieved by fusion of the luciferase gene to the viral fiber gene within Ad $\Delta$ fiberIL. This virus resulted in approximately one or two orders of magnitude higher luciferase activity than Ad $\Delta$ E2BIL or Ad $\Delta$ E1AIL, respectively. This was expected because the fiber gene is strongly expressed at late stages of viral infection. Messenger RNA expression from the fiber and E4 genes, which are adjacent to the transgene in the genome of Ad $\Delta$ fiberIL, varied only minimally from expression of the same genes by the parental virus Ad5- $\Delta$ 24. Accordingly, cytotoxicity of Ad $\Delta$ fiberIL and Ad5- $\Delta$ 24 to tumor cells was identical. Interestingly, copy numbers of luciferase mRNA were about one-fourth of fiber mRNA copy numbers for Ad $\Delta$ fiberIL after infection of A549 cells. This indicates premature transcription termination, even though the native polyadenylation signal of the fiber gene, which overlaps with the stop codon, was modified. Thus, there might still be opportunities to further increase transgene expression from fiber-IRES, for example, by analysis of different polyadenylation signal mutants. As expression of the fiber transcription unit is dependent on viral replication (Sauthoff et al., 2002), transgene expression from fiber-IRES, in contrast to transgene expression from early viral transcription units, should be tumor-specific in tumor-targeted CRAds.

For expression of some transgenes by CRAds, for example, for pro-apoptotic or cytotoxic genes, restriction of gene expression to late stages of virus replication will be more important than obtaining high levels of gene expression. Expression from E1A-IRES is not feasible for this purpose because of the early onset of transgene expression as observed for Ad $\Delta$ E1AIL (Figs. 3A and 3B). Expression of the transgene from E2B-IRES might be superior to the expression from fiber-IRES as suggested by our data. Surprisingly, luciferase activity was nearly two orders of magnitude lower for Ad $\Delta$ E2BIL compared with Ad $\Delta$ fiberIL early after infection of A549 cells (Fig. 3A). Even after normalization with luciferase readings at 26 h, relative luciferase activity was higher for Ad $\Delta$ fiberIL than for Ad $\Delta$ E2BIL at 6 h postinfection (Fig. 3B). Correspondingly, we observed relatively high luciferase and fiber mRNA copy numbers for Ad $\Delta$ fiberIL at 5 h postinfection (Fig. 5). However, luciferase mRNA expression and enzyme activity increased more for Ad $\Delta$ E2BIL than for Ad $\Delta$ fiberIL from 5 to 10 h or 6 to 14 h, respectively, but more

for Ad $\Delta$ fiberIL at later time points, thus reflecting native expression patterns in this regard. Copy numbers of both fiber and luciferase mRNAs were more than one order of magnitude higher for Ad $\Delta$ fiberIL than fiber mRNA copy numbers for Ad5- $\Delta$ 24 at 5 h postinfection. Therefore, modified viral gene regulation after insertion of heterologous DNA must for the surprisingly high transgene expression observed early after virus infection for Ad $\Delta$ fiberIL. This interference with endogenous viral gene regulation might be due to enhancer activity of the inserted fragment or results from the modification of mRNA processing or stability. Such effects might depend on the nucleotide sequence of the transgene and thus need to be analyzed for each transgene individually. For Ad $\Delta$ E2BIL, the IRES-Luc cassette was inserted downstream of the E2B reading frame, between the tail-to-tail-oriented E2B and pIX viral genes. Thus, promoter interference, which might be critical for Ad $\Delta$ E1AIL (see below), was not expected. Nevertheless, expression of E2B and pIX mRNA was reduced after 10 or 15 h, respectively, postinfection. This indicates that insertion of IRES-Luc between E2B and pIX interferes with expression of these genes directly, for example, by affecting mRNA stability, or indirectly. For Ad $\Delta$ E2BIL, in contrast to Ad $\Delta$ fiberIL, copy numbers of luciferase and fused viral messages were nearly identical, indicating that mutation of the E2B transcription termination signal in Ad $\Delta$ E2BIL was successful and no premature transcription termination occurred. Cytotoxicity of Ad $\Delta$ E2BIL to A549 cells was somewhat attenuated relative to Ad5- $\Delta$ 24, even though fiber mRNA copy numbers were almost identical from 5 to 25 h for these viruses. Presumably, reduced viral replication and spread were a consequence of modified expression of E2B and pIX.

Ad $\Delta$ E1AIL showed the strongest interference of the inserted IRES-Luc cassette with viral gene expression and cytotoxicity. Expression of E1A mRNA was strongly reduced relative to the parental virus Ad5- $\Delta$ 24. As the E1A promoter and enhancer remained unchanged, this is possibly a result of modified mRNA stability or processing. E1B mRNA copy numbers were reduced 5–15 h after virus infection, but not at 20 and 25 h. Modified E1B promoter activity could be a reason for this observation because the IRES-Luc cassette was inserted into the E1B promoter (Parks et al., 1988), as required for fusion to the E1A gene. Alternatively, reduced read-through transcription from E1A resulting from the insertion of the heterologous sequence might be responsible for the decrease in early E1B mRNA copy numbers. A similar expression pattern for E1B resulting from insertion of a transcription termination signal after the E1A ORF, as previously reported (Maxfield and Spector, 1997), is supportive of this hypothesis. However, we did not include a transcription termination signal in our inserted heterologous sequence. These considerations suggest that insertion of an IRES-transgene cassette in a context of tail-to-tail-oriented viral genes might be advantageous to conserve viral gene expression patterns. Unfortunately, this was not the case for Ad $\Delta$ E2BIL. For Ad $\Delta$ E1AIL, luciferase

mRNA copy numbers were similar to E1A mRNA copy numbers of the fused viral gene.

For all IRESLuc-CRAdS, luciferase activities increased most dramatically during the first 2 days after virus infection, after which the increase slowed down considerably (Fig. 3D). The steep initial increase in luciferase activity observed early after IRESLuc-CRAd infection is dependent on promoter activity, mRNA stability, and replication of the viral genome. However, the increase in luciferase activity for a given virus at later time points (i.e., after the first round of replication) depends on viral spread. In addition, transgene expression is less synchronized at later time points. Still, because IRESLuc-CRAdS were not deficient for replication in A549 cells (Fig. 2) and at least two rounds of virus replication are expected at the MOI used in the experiment presented in Fig. 3D, the increase in luciferase activity between days 2 and 6 might seem surprisingly low. However, the loss of luciferase-expressing cells by viral cell lysis in IRESLuc-CRAd-infected cultures (but not in AdTL-infected cultures) needs to be considered in this context.

In accord with the *in vitro* results, Ad $\Delta$ fiberIL and Ad $\Delta$ E2BIL resulted in approximately six or five orders of magnitude higher luciferase activity, respectively, after virus injection into sc A549 tumors when compared with AdTL. However, in contrast to the *in vitro* results, luciferase activities for Ad $\Delta$ fiberIL and Ad $\Delta$ E2BIL were stable or increased only minimally from day 2 to day 8. These observations might indicate that virus spread is less efficient *in vivo* than in cell cultures. However, it is also possible that infected cells might be eliminated faster *in vivo* than *in vitro*, irrespective of virus replication, as suggested by the early decline of luciferase activity for AdTL *in vivo* (more than 10-fold from day 1 to day 8). Thus, the stable luciferase activities for IRESLuc-CRAdS observed *in vivo* might still be consistent with virus replication.

IRES sequences have been applied for CRAdS for various purposes, such as expression of multiple viral genes from one heterologous promoter (Li et al., 2001; Yu et al., 1999), or co-expression of transgene and viral gene from the CMV enhancer or promoter (Akbulut et al., 2003; Wildner et al., 1999b). These reports have also demonstrated functional activity of the IRES in replicating adenoviruses. Our study extends the analysis of IRES-mediated expression to transcriptional units not previously investigated. Furthermore, we found that fusion of transgenes via an IRES to the E1A gene can interfere with expression patterns of neighboring genes. In this regard, our data show that insertion of IRES-transgene cassettes into the E2B transcription unit might be advantageous.

Several groups have reported on the combination of replicating adenoviruses with the expression of apoptosis-inducing genes, such as p53 (Sauthoff et al., 2002; van Beusechem et al., 2002), with cytokine genes encoding TNF $\alpha$  or GM-CSF (Bristol et al., 2003; Hawkins and Hermiston, 2001a, 2001b; Hawkins et al., 2001), or with prodrug-activating enzymes (Akbulut et al., 2003; Bernt et

al., 2002; Freytag et al., 1998; Hawkins and Hermiston, 2001b; Lambright et al., 2001; Lee et al., 2001; Nanda et al., 2001; Rogulski et al., 2000; Wildner et al., 1999a, 1999b). The latter studies showed that interference of drug-mediated cell killing with virus replication and production is a critical issue for development of armed CRAdS. In addition to the drug of choice, dosing and timing of prodrug application influenced the therapeutic outcome of combination therapy, that is, determined synergy or interference between virus replication and drug-dependent cell killing. However, as virus replication in the tumor is not synchronized over time, timing of enzyme expression during the adenoviral replication cycle in an individual infected cell might be advantageous.

The timing of transgene expression by CRAdS has been recently addressed by different strategies. One approach is to replace an endogenous gene with the transgene of choice. This strategy has been endeavored with various E3 genes and has resulted in transgene expression kinetics similar to those of the replaced gene (Hawkins and Hermiston, 2001a, 2001b; Hawkins et al., 2001; Nanda et al., 2001). Bernt et al. (2002) have reported on a distinct strategy which results in replication-dependent transgene expression implemented by a replication-induced homologous recombination event. This approach, similar to the insertion of transgenes into late transcription units, features late transgene expression that is tumor-specific when adenoviral replication is targeted to cancer cells. In an approach similar to our strategy, Sauthoff et al. have reported on expression of p53 from the fiber transcription unit (Sauthoff et al., 2002). This report shows that p53 expression mimics expression kinetics of the fiber gene, which, overall, is in accord with our data for Ad $\Delta$ fiberIL. Furthermore, viral cytotoxicity and release of virus particles were augmented in a tumor-selective fashion. Optimization of the IRES sequence, as described herein, might further improve the strategy of Sauthoff et al. Also, in those studies, transgene expression was detected by Western blot. This method might be inadequate to detect variations of transgene expression early after virus infection, such as those we detected for Ad $\Delta$ fiberIL with the more sensitive luciferase assay.

We suggest expression of transgenes from distinct viral transcription units exploiting IRES sequences as a means for transgene expression with defined kinetics and expression levels. This strategy does not require the replacement of a viral gene by the transgene and avoids heterologous promoters, which can show an unfavorable timing of expression or whose features might not be conserved in the adenovirus backbone. Various avenues can be pursued to further improve our strategy. First, given that we deleted the E3 region to allow for insertion of the luciferase gene, smaller therapeutic genes would allow for retention of the E3 region, or of individual E3 genes such as ADP for improved virus release and spread (Doronin et al., 2000). However, deletion of ADP in the context of armed CRAdS might also be beneficial as it allows for extended transgene

expression. Secondly, a more detailed analysis of insertion sites, further mutations of transcription termination signals, or viral promoter reconstitution could help to reduce adverse effects on viral gene expression and replication or to improve transgene expression. Thirdly, we are currently analyzing expression of different transgenes by IRES-CRAds. For example, secreted proteins or apoptosis-inducing proteins might require different expression kinetics than cytoplasmatic luciferase. Finally, our approach is compatible with any strategy for replication-selectivity, for example, with promoter-controlled CRAds.

## Materials and methods

### Cell culture

The human tumor cell line A549 (lung adenocarcinoma, ATCC, Manassas, VA) was cultivated in DMEM (Mediatech, Herndon, VA). The human 293 cell line transformed with Ad5 DNA (purchased from Microbix, Toronto, Canada) was grown in DMEM/F12 (50:50; Mediatech). All media were supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 2 mM L-glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin (all Mediatech). Cells were grown at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub>.

### Plasmids and recombinant adenoviruses

For a schematic outline of the cloned constructs and genomes see Fig. 1. Plasmids pGL3IRESLuc, pGL3IRESLucATG, and pGL3spIRESLucATG contained the EMCV IRES sequence linked upstream to the luciferase gene. For pGL3IRESLucATG, the IRES sequence was derived by PCR with oligonucleotides (restriction sites are underlined) IRES 5' (5'-GATC AGA TCT GCC AAT TCC GCC CCT CTC CCT) and IRES 3' (5'-GATC CCA TGG TAT CAT CGT GTT TTT CAA AGG AA) and with pIRES-EGFP (Clontech, Palo Alto, CA) as template. The resulting fragment was digested with *Bgl*III and *Nco*I and cloned into the corresponding restriction sites of pGL3 basic (Promega, Madison, WI). By this strategy, the initiation ATG was reverted to its original position of the EMC virus. Plasmid pGL3IRESLuc was generated by inserting the IRES of plasmid pTM1 (Fuerst et al., 1986) upstream of the luciferase gene of pGL3 basic without placing the start ATG to its original position of the EMC virus. Plasmid pGL3spIRESLucATG is derived from pGL3IRESLucATG by inserting into the *Bgl*III site annealed, 5'-phosphorylated oligonucleotides CATspacer ts (GAT CTA AGG CAG TTA TTG GTG CCC TTA AAC GCC TGG TGC TAC GCC TGA ATA AGT GAT AAA) and CATspacer bs (GATCTT TAT CAC TTA TTC AGG CGT AGC ACC AGG CGT TTA AGG GCA CCA ATA ACT GCC TTA) representing a 54-bp spacer derived from the 3'-NTR of the CAT gene with

flanking nucleotides for insertion into the *Bgl*III site (underlined). The shuttle plasmids pSE1AΔ24IRESLuc and pSE1AΔ24IRESLucATG were constructed by inserting a fragment containing the IRES and luciferase gene from pGL3IRESLuc or from pGL3IRESLucATG, respectively, into the *Hpa*I site of pShuttleΔ24 (that contains a deletion of nucleotides 923–946 corresponding to amino acids LTCHEAGF of E1A; Suzuki et al., 2002). This restriction site is located between stop signal and polyadenylation signal of the E1A gene and 94 nucleotides upstream of the E1B TATA-box. Plasmid pE1AΔ24spIRESLucATG was constructed by inserting a fragment containing spacer, IRES, and luciferase gene from pGL3spIRESLucATG into the *Hpa*I site of pShuttleΔ24. Oligonucleotides E2BMfe5' (5'-GATC CAA TTG GAT TCT TTG ACC CGG GAA C), E2BXPrev (5'-AGC AAG CTC GAG GATT CAC GTG ACA CTT GCT TGA TCC AAA TCC AAA C), E2BXFor (5'-CAC GTG AATC CTC GAG TCA CTT AGG GGT TTT GCG CGC GCG GTA G), and E2BBstXI3' (GATC CCA TCA TTA TGG ACG AAT GCA TGG) were used to introduce cloning sites downstream of the E2B/Iva2 translational stop site by PCR cloning (inserted restriction sites for *Xho*I and *Pml*I in italics, *Mfe*I and *Bst*XI sites for cloning underlined) and to simultaneously mutate the E2B polyadenylation signal in pShuttleΔ24. Subsequently, the spacer, IRES, luciferase gene, and polyA sequences from pGL3spIRESLucATG were inserted into the cloning site of this plasmid to generate pΔ24E2BIL. Plasmid pfiberIL was generated as follows: restriction sites were incorporated into the *Sna*BI site of plasmid pNEB.PK.*Sna*BI (Wu et al., 2002) with annealed, 5'-phosphorylated oligonucleotides Fiberlinker ts (5'-ACT TTT TCA TAC ATT GCC CAA GAA TGA *CTC GAG* TAGA *ACT AGT* AGA) and Fiberlinker bs (5'-TCT *ACT AGT* TCTA *CTC GAG* TCA TTC TTG GGC AAT GTA TGA AAA AGT), simultaneously mutating the fiber polyadenylation signal (inserted *Xho*I and *Spe*I sites in italics). Then, spacer, IRES, luciferase gene, and polyA sequences from pGL3spIRESLucATG were inserted into the *Spe*I (blunt) site. Subsequently, a *Spe*I (blunt)/*Nde*I fragment of pAdEasyI (spanning the E3 deletion; He et al., 1998) was inserted into *Pac*I (blunt) and *Nde*I sites of pfiberIL generating pDE3fiberIL. Plasmids with the genomes of recombinant adenoviruses were generated by homologous recombination in BJ5183 bacteria as described (He et al., 1998). For pAdE1AΔIL or pAdΔE2BIL, pSE1AΔ24spIRESLucATG and pΔE2BIL respectively were recombined with pAdEasyI. For pAdΔfiberIL, pfiberIL was recombined with pVK500 and subsequently with pShuttleΔ24. Plasmids were validated by PCR and restriction digest. Adenovirus particles were produced by transfection of *Pac*I-digested pAd plasmids into A549 cells using Lipofectamine (Life Technologies, Rockville, MD) following the manufacturer's protocol. E1-deleted, nonreplicating viruses that express luciferase, AdTL (Seki et al., 2002), and AdCMVLuc (Reynolds et al., 2001) were amplified in 293 cells. Ad5-Δ24 (Fueyo et



al., 2000; Suzuki et al., 2001) and other viruses were amplified in A549 cells. All viruses were purified by two rounds of CsCl equilibrium density gradient ultracentrifugation. Verification of viral genomes and exclusion of wild-type contamination were performed by PCR and restriction digest. Physical particle concentration (viral particles (vp)/ml) was determined by OD<sub>260</sub> reading and biological particle concentration (plaque-forming units (pfu)/ml) was determined by standard plaque assay on 293 cells.

#### *Transfection experiments*

For plasmid transfection,  $6 \times 10^4$  A549 cells were seeded per well in a 12-well plate. Cells were transfected with 0.7  $\mu$ g of plasmid per well using Lipofectamine plus (Life Technologies) according to the manufacturer's instructions. Luciferase activity of cell lysates was determined 2 days after transfection using a luciferase assay system (Promega). Experiments were performed in triplicates; mean values and standard deviations are shown.

#### *Cytotoxicity assay*

For the determination of virus-mediated cytotoxicity,  $1.5 \times 10^4$  tumor cells were seeded in 24-well plates and infected with adenoviruses in 200  $\mu$ l of growth medium containing 2% FBS at indicated multiplicities of infection (MOI = pfu/cell) or mock-infected. The infection medium was replaced with growth medium the next day. When cell lysis was observed for Ad5- $\Delta$ 24 at the lowest titer 10 days after virus infection, cells were fixed and stained with 1% crystal violet in 70% ethanol for 45 min followed by washing with tap water to remove excess color. The plates were dried and images were captured with a Kodak DC260 digital camera (Eastman Kodak, Rochester, NY).

#### *Adenovirus infections for luciferase assay*

For the determination of transgene expression kinetics,  $3 \times 10^4$  A549 cells were seeded per well in a 24-well plate. The next day, cells were infected with Ad $\Delta$ E1AIL, Ad $\Delta$ E2BIL, Ad $\Delta$ fiberIL, or AdTL at indicated MOIs or mock-infected. Luciferase activity of cell lysates was determined at indicated time points after virus infection using a luciferase assay system (Promega). Experiments were performed in triplicates; standard deviations were below 20% and are not visible at logarithmic scale.

#### *RNA quantification by real-time PCR*

For quantification of RNA expression,  $1.5 \times 10^5$  A549 cells were seeded per well in a 6-well plate. The next day, cells were infected with indicated viruses at MOI 20 or mock-infected. Cells were harvested at indicated time points after infection and RNA was purified from the cell lysate with the RNeasy kit including DNase digest (Qiagen,

Valencia, CA) following the manufacturer's instructions. Quantification of RNA copy numbers was performed by real-time PCR as follows. TaqMan primers and probes were designed by the Primer Express 1.0 software and synthesized by Applied Biosystems (Foster City, CA). Oligonucleotide sequences were E1A forward: 5'-AAC CAG TTG CCG TGA GAG TTG; E1A reverse: 5'-CTC GTT AAG CAA GTC CTC GAT ACA; E1A probe: 6FAM-CAC AGC CTG GCG ACG CCC A -TAMRA; E1B forward: 5'-TTT CTG GCC ATG CAT CTG TG; E1B reverse: 5'-GCG GAC GGA AGA CAA CAG TAG; E1B probe: 6FAM-AGG CGA TTC TTG TGT CTC ACA ACC GCT-TAMRA; pIX forward: 5'-CGC GGG ATT GTG ACT GAC T; pIX reverse: 5'-TGA ACG GGA AGC TGC ACT G; pIX probe: 6FAM- TGC TTT CCT GAG CCC GCT TGC A-TAMRA; E2B forward: 5'-GGC ATC TCG ATC CAG CAT ATC; E2B reverse: 5'-CCG TGG AAA GAC ATG ACC CT; E2B probe: 6FAM- TGG ACG AGC ACC GAC TAC TGC CGT-TAMRA; E4 forward: 5'-GGA GTG CGC CGA GAC AAC; E4 reverse: 5'-ACT ACG TCC GGC GTT CCA T; E4 probe: 6FAM- TGG CAT GAC ACT ACG ACC AAC ACG ATC T-TAMRA; fiber forward: 5'-TGA TGT TTG ACG CTA CAG CCA TA; fiber reverse: 5'-GAT TTG TGT TTG GTG CAT TAG GTG; fiber probe: 6FAM-ACC AAA TTC AAG CCC ATC TCC TGC ATT AAT G-TAMRA; luciferase forward: 5'-TGA CCG CCT GAA GTC TCT GA; luciferase reverse: 5'-TGG AGC AAG ATG GAT TCC AAT; and luciferase probe: 6FAM-CAG CGG GAG CCA CCT GAT AGC CT-TAMRA. Human glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was used as house keeping gene for internal control. Oligonucleotide sequences were GAPDH forward: 5'-GGT TTA CAT GTT CCA ATA TGA TTC CA; GAPDH reverse: 5'-ATG GGA TTT CCA TTG ATG ACA AG; and GAPDH probe: 6FAM-CGT TCT CAG CCT TGA CGG TGC CAT-TAMRA. With optimized concentration of primers and probe, the components of real-time PCR mixture were designed to result in a master mix with a final volume of 9  $\mu$ l per reaction containing 1 $\times$  TaqMan EZ RT-PCT Kit (Applied Biosystems), 100 nM forward primer, 100 nM reverse primer, 100 nM probe, and 0.025%BSA. For the assay, a known amount of template DNA of pTG3602 or pGL3 ( $10^8$ ,  $10^6$ ,  $10^4$ , and  $10^2$  copies/ $\mu$ l) was amplified to generate a standard curve for quantification of the copy numbers of unknown samples. Known amount of human total RNA (200, 20, 2 and 0.2 ng/ $\mu$ l) was amplified to generate a standard curve for determination of the RNA concentration of samples. Total RNA (1  $\mu$ l) sample was added to 9  $\mu$ l of PCR mixture in each reaction capillary. No template control received 1  $\mu$ l of water. All capillaries were then sealed and centrifuged using LC Carousel Centrifuge (Roche Molecular Biochemicals, Indianapolis, IN) to facilitate mixing. All PCR was carried out using a LightCycler System (Roche Molecular Biochemicals). Thermal cycling conditions were subjected to 2 min at 50 °C, 30 min at 60 °C, 5 min at 95 °C, and 40 cycles of 20 s at 94 °C and 1 min at 60 °C. Data were



analyzed with LightCycler software and plotted as RNA copy numbers per ng of RNA.

### Animal experiments

Female athymic nude mice (Charles River Laboratories, Inc. Wilmington, MA), 6–8 weeks old, were kept under pathogen-free conditions. Two million A549 cells were inoculated sc into the right flank of each mouse. When tumor nodules reached 5 mm in diameter, a single dose of  $5 \times 10^4$  pfu of AdTL, AdΔE2BIL, or AdΔfiberIL ( $n = 4$  for each virus and time point,  $n = 3$  for AdTL at day 8) was administered intratumorally. At indicated days after virus injection, four mice of each group were sacrificed and tumors were harvested, snap frozen on dry ice–ethanol, and stored at  $-80^\circ\text{C}$  for determination of luciferase expression. Tumor specimen were weighed and subsequently ground to a fine powder using a pestle and mortar cooled in dry ice–ethanol. Tissue powders were lysed in Cell Culture Lysis buffer (Promega). After centrifugation, luciferase activity of the supernatant was determined with the luciferase assay system (Promega). Mean values of RLU per tumor and standard deviations are shown. Animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

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