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

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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.

Key words: 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,
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-Δ24, also called Δ922-947, 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-Δ24.

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-Δ24 (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-Δ24 (Fueyo et al., 2000; Suzuki et al., 2001) in the order viral gene–IRES–luciferase to generate AdΔE1AIL, AdΔE2BIL, and AdΔfiberIL, respectively (IRESLuc-CRAds). This strategy aimed at the efficient transgene expression at immediate, 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, pSE1AΔIRESLuc, 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, pSE1AΔspIRESLucATG, the start ATG of the luciferase gene was placed in the original start ATG position of EMCV. In a third construct, pSE1AΔspIRIRESLucATG, 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 AdΔE1AIL, 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 AdΔE2BIL and AdΔfiberIL, 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 AdΔE1AIL, AdΔE2BIL, AdΔfiberIL, the parental virus Ad5-Δ24 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). AdΔfiberIL resulted in similar cytotoxicity as Ad5-Δ24, whereas AdΔE2BIL and AdE1AIL 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 AdΔE1AIL, AdΔE2BIL, AdΔfiberIL, 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 (AdΔfiberIL), 70-fold (AdΔE1AIL), or 1443-fold (AdΔE2BIL) lower for the IRESLuc-CRAds relative to AdTL (Fig. 3A). However, at 26 h postinfection, luciferase activity of AdΔE1AIL was similar to AdTL, and those of AdΔE2BIL and AdΔfiberIL were 7-fold or 27-fold higher, respectively. Thus, transgene expression by IRESLuc-CRAds was weak early after adenovirus infection, especially for AdΔE2BIL, but increased dramatically...
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 × 10^3-fold, 4.9 × 10^5-fold, and 3.1 × 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.
transgene expression from Ad was only a minimal time interval between the onset of fiber expression in wild-type adenovirus. However, there expression expected from the kinetics of E1A, E2B, and early after infection—resembled the timing of transgene expression of magnitude lower, resulting from the lower MOI used for infection. Overall, these patterns—with the exception of AdΔfiberIL 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 AdΔE2BIL and AdΔfiberIL (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 AdΔE1AIL, AdΔE2BIL, and AdΔfiberIL 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 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 AdΔE1AIL, AdΔE2BIL, or AdΔfiberIL, respectively. We determined similar copy numbers for luciferase and E1A, or luciferase and E2B of AdΔE1AIL or AdΔE2BIL, respectively, as expected for messages of a polycistronic mRNA (Fig. 4A). For AdΔfiberIL, 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-Δ24. Moreover, we analyzed mRNA copy numbers of the fiber gene, which, representing late expression, reflect replication efficiency. For AdΔE1AIL, expression of E1A mRNA was severely attenuated compared with the parental virus, Ad5-Δ24 (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 AdΔE2BIL, expression of E2B was slightly decreased at 10–25 h compared with the parental virus. Protein IX mRNA copy numbers were similar for AdΔE2BIL and Ad5-Δ24 until 10 h postinfection, but were lower at later time points. However, expression of fiber mRNA was almost identical for AdΔE2BIL and Ad5-Δ24. Compared with the parental
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.
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 AdΔfiberIL > AdΔE1AIL > AdΔE2BIL at 5 or 6 h postinfection and eventually were AdΔfiberIL > AdΔE2BIL > AdΔE1AIL. AdΔE2BIL surpassed AdΔE1AIL 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 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, several 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 AdΔfiberIL. This virus resulted in approximately one or two orders of magnitude higher luciferase activity than AdΔE2BIL or AdΔE1AIL, 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 AdΔfiberIL, varied only minimally from expression of the same genes by the parental virus Ad5-Δ24. Accordingly, cytotoxicity of AdΔfiberIL and Ad5-Δ24 to tumor cells was identical. Interestingly, copy numbers of luciferase mRNA were about one-fourth of fiber mRNA copy numbers for AdΔfiberIL 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 AdΔE1AIL (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 AdΔE2BIL compared with AdΔfiberIL early after infection of A549 cells (Fig. 3A). Even after normalization with luciferase readings at 26 h, relative luciferase activity was higher for AdΔfiberIL than for AdΔE2BIL at 6 h postinfection (Fig. 3B). Correspondingly, we observed relatively high luciferase and fiber mRNA copy numbers for AdΔfiberIL at 5 h postinfection (Fig. 5). However, luciferase mRNA expression and enzyme activity increased more for AdΔE2BIL than for AdΔfiberIL fiberIL from 5 to 10 h or 6 to 14 h, respectively, but more for AdΔfiberIL 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 AdΔfiberIL than fiber mRNA copy numbers for Ad5-Δ24 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 AdΔfiberIL. This interference with endogenous viral gene regulation might be due to enhancer activity of the inserted fragment or results from the modification of mRNA procession or stability. Such effects might depend on the nucleotide sequence of the transgene and thus need to be analyzed for each transgene individually. For AdΔE2BIL, 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 AdΔE1AIL (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 AdΔE2BIL, in contrast to AdΔfiberIL, copy numbers of luciferase and fused viral messages were nearly identical, indicating that mutation of the E2B transcription termination signal in AdΔE2BIL was successful and no premature transcription termination occurred. Cytotoxicity of AdΔE2BIL to A549 cells was somewhat attenuated relative to Ad5-Δ24, 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.

AdΔE1AIL 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-Δ24. 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 AdΔE2BIL. For AdΔE1AIL, 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, AdΔfiberIL and AdΔE2BIL 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 AdΔfiberIL and AdΔE2BIL 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 (Abkulut 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 TNFα or GM-CSF (Bristol et al., 2003; Hawkins and Hermiston, 2001a, 2001b; Hawkins et al., 2001), or with prodrug-activating enzymes (Abkulut 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 prodruk 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 AdΔfiberIL. 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 AdΔfiberIL 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% CO2.

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 GGT TTT CAA AGG AA) and with pIRES-EGFP (Clontech, Palo Alto, CA) as template. The resulting fragment was digested with BglII and NcoI 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 inserted into the HpaI site of pShuttleΔ24. Oligonucleotides E2BMfe5′ (5′-GATC CAA TTG GAT TCT TTG ACC CGG GAA C) and CATspacer bs (CAT spacer ts (by inserting into the pGL3IRESLuc and pSE1AΔ24IRESLucATG were constructed by inserting a fragment containing the IRES and luciferase gene from pGL3IRESLuc or from pGL3IRESLucATG, respectively, into the HpaI 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 HpaI 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), E2BXPfor (5′-CAC GTG AATC CTC GAG TTA CTG AGG GGT TTT GCG CGC GCG GTA G), and E2BBstXI3′ (GATC CCA TTA TGG ACG AAT GCA TGG) were used to introduce cloning sites downstream of the E2B/1va2 translational stop site by PCR cloning (inserted restriction sites for XhoI and PmlI in italics, MfeI and BsrXI 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 cloned site of this plasmid to generate pΔ24E2BIL. Plasmid pfiberIL was generated as follows: restriction sites were incorporated into the SnaBI site of plasmid pNEB.PK.SnaBI (Wu et al., 2002) with annealed, 5′-phosphorylated oligonucleotides Fiberlinker ts (5′-ACT TTG TCA TAC ATT GCC CAA GAA TGA CTC GAG TAGA ACT AGT AGA) and Fiberlinker bs (5′-CTC ACT AGT TCTA CTC GAG TCA TTC TTT GCG AAT GTA TGA AAA AGT), simultaneously mutating the fiber polyadenylation signal (inserted XhoI and SpeI sites in italics). Then, spacer, IRES, luciferase gene, and polyA sequences from pGL3spIRESLucATG were inserted into the SpeI (blunt) site. Subsequently, a SpeI (blunt)/Ndel fragment of pAdEasy1 (spanning the E3 deletion; He et al., 1998) was inserted into PacI (blunt) and Ndel 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 pAdEasy1. 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 PacI-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 digestion. Physical particle concentration (viral particles (vp)/ml) was determined by OD260 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 × 10⁴ A549 cells were seeded per well in a 12-well plate. Cells were transfected with 0.7 μg 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 × 10⁴ tumor cells were seeded in 24-well plates and infected with adenoviruses in 200 μl 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-Δ24 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 × 10⁴ A549 cells were seeded per well in a 24-well plate. The next day, cells were infected with AdΔE1AIL, AdΔE2BIL, AdΔfiberIL, 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 × 10⁵ 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 TTT CCG TGA GAG TTG; E1A reverse: 5’CTC GTT AGG CAA GAT CTC GAT ACA; E1A probe: 6FAM-CAC AGC CTG GCG ACC CCC A -TAMRA; E1B forward: 5’TTT CTG GCC ATG CAT CTG TG; E1B reverse: 5’GCC GAC GGA AGA CAA CAG TAG; E1B probe: 6FAM-AGG CGA TTC TTG TGT CTC ACA ACC GCT-TAMRA; pIX forward: 5’CGG 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’GCC ATC TCG ATC ATC GAG CAT ATC; E2B reverse: 5’CCG TGG AAA GAC ATG ACC CT; E2B probe: 6FAM- TGG ACG ACC ACC GAC TAC TGC CGT-TAMRA; E4 forward: 5’GGA GTG CGC CGA GCA AAC; E4 reverse: 5’ACT ACG GCC GGT CCA T; E4 probe: 6FAM- TGG CAT GAC ACT ACG ACC ACC AGC ATC T-TAMRA; fiber forward: 5’TGA TGT TTG ACG CTA CAG CCA TA; fiber reverse: 5’GAT TGT TTG GTG ATG 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 GGA CCA CCT GAT AGC CT-TAMRA. Human glyceraldehyde-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 TCC 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 μl per reaction containing 1× TaqMan EZ RT-PCR 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 (10⁸, 10⁶, 10⁴, and 10² copies/μl) 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/μl) was amplified to generate a standard curve for determination of the RNA concentration of samples. Total RNA (1 μl) sample was added to 9 μl of PCR mixture in each reaction capillary. No template control received 1 μl 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 × 10^3 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 °C for determination of luciferase expression. Tumor specimens 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/s 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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