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Efficient mobilization of E1-deleted adenovirus type 5 vectors by wild-type adenoviruses of other serotypes

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Mobilization of replication-deficient adenovirus vectors can lead to spread and shedding of the vector. Here we show that in cultured HepG2 cells wild-type (*wt*) adenoviruses of subgroup A (Ad12), B (Ad7, 11 and 16), C (Ad1, 2 and 5) and E (Ad4) can efficiently mobilize Ad5CMV*luc*, a Δ*E1*Δ*E3*-Ad5 vector carrying the firefly luciferase gene as reporter. In addition, we show that Ad5CMV*luc* can be propagated on Ad12E1-transformed human embryonic retinoblasts. This provides evidence that expression of the E1 region of Ad12 is sufficient for mobilizing Δ*E1*-Ad5-derived vectors. Thus, in therapeutic applications of replication-defective Ad vectors any active Ad infection is of potential concern, independent of the serotype involved. To prevent vector mobilization by *wt* Ads, new vectors should be developed in which essential functions such as the initiation of DNA replication and genome packaging are restricted.

Adenoviruses (Ad) are a diverse family of viruses. In humans at least 51 different serotypes have been described, which have been divided into six subgroups (formerly species) based on haemagglutination properties with red blood cells, oncogenic potential and G + C content of their DNA (Horwitz, 1995). Replication-defective derivatives of serotypes 5 (Ad5) and 2 (Ad2), generated by deletion of sequences of the viral E1, E2 and E4 regions, have been widely used as gene-transfer vehicles in experimental gene therapy (Benihoud *et al.*, 1999). Therapeutic application of recombinant Ad vectors is complicated by the presence of replication-competent adenoviruses (RCA). The presence of RCA in the vector batches may lead to inflammation and tissue damage in the recipient (Fallaux *et al.*, 1999; Hermens & Verhaagen, 1997). In addition, RCA can mobilize the Ad vector (Imler *et al.*, 1995), and this could lead increased vector shedding. The development and implementation of improved helper cells (Fallaux *et al.*, 1998) and vectors (Hehir *et al.*, 1996), as well as stringent quality-control measures (Roitsch *et al.*, 2001; Fallaux *et al.*, 1999), can virtually eliminate the problem of RCA in the vector batches.

However, this does not solve the mobilization problem completely. It is conceivable that after receiving gene therapy, patients develop a viraemia with a wild-type (*wt*) Ad. Indeed, administration of *wt* Ad5 into lungs of cotton rats previously exposed to replication-defective Ad5 vectors led to mobilization of the vector (Imler *et al.*, 1995). The risk of a mobilization by *wt* Ad of the same serotype is limited, as most patients will develop immunity against the vector serotype (Harvey *et al.*, 1999). It is not known, however, whether the vectors can be mobilized by other serotypes, viz. serotypes different from the one from which the vector had been derived. To study this possibility, we determined whether *wt* Ads of subgroups A, B, C and E could mobilize the vector Ad5CMV*luc*, a Δ*E1*Δ*E3*-Ad5 vector carrying the firefly luciferase gene as a reporter.

Firstly, we set up a marker-mobilization assay. HepG2 human hepatoma cells were cultured in 24-well plates in DMEM containing 8% FCS. At 90% confluency the cells were infected with an RCA-free stock of Ad5CMV*luc* at an m.o.i. of 10. Twenty-four hours post-infection (p.i.) the cells were washed with PBS and exposed, at an m.o.i. of 5, to stocks of Ads of subgroups A (Ad12), B (Ad7, 11 and 16), C (Ad1, 2 and 5) and E (Ad4) that had been purified twice on CsCl gradients. All Ad titres were determined by plaque assay on 911 cells (Fallaux *et al.*, 1996). At this m.o.i. all *wt* Ads gave complete CPE at 60 to 120 h p.i. (data not shown). As controls, HepG2 cell cultures were infected with the Δ*E1*Δ*E3*-Ad5 vector Ad5CMV*lacZ*, or mock infected. In a parallel experiment, the assay was performed in the presence of 20 μg/ml cytosine arabinoside (ara-C) in the culture medium. This concentration of ara-C effectively inhibits Ad replication in HepG2 cells (data not shown). Three days after the initial infection, CPE was apparent in all cultures. At that time, the cells were harvested with the medium and the virus was released from the cells by three cycles of freeze–thawing. After the last cycle, the lysates were cleared by centrifugation for 2 min at 1600 ***g***. The

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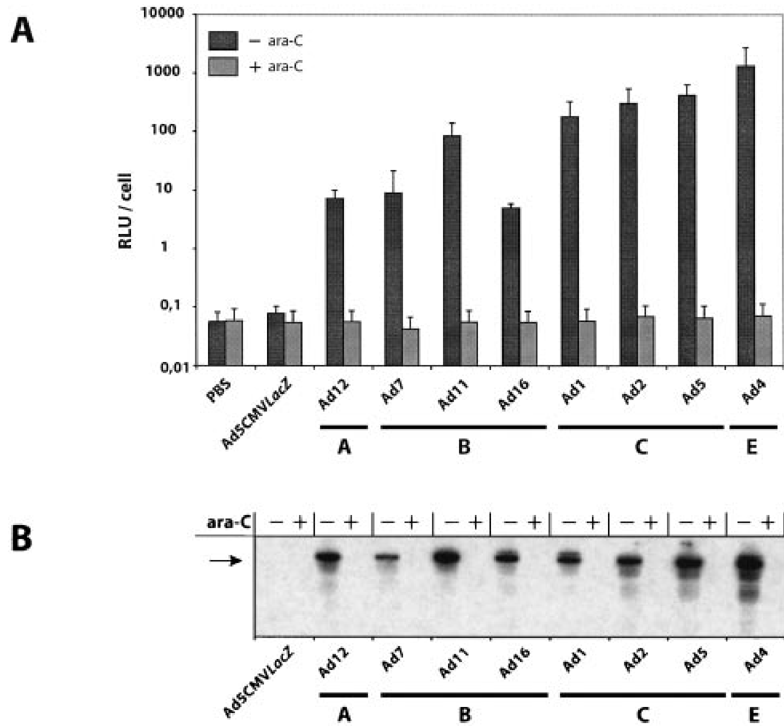

Fig. 1. Luciferase expression in HepG2 cells infected with mobilized Ad5CMV*Luc*. HepG2 cells were infected with Ad5CMV*Luc* (m.o.i. = 10) and 24 h p.i. were co-infected with *wt* Ads (m.o.i. = 5) in the presence or absence of 20 mg/ml ara-C. As negative controls, Ad5CMV*luc*-infected cells were co-infected with Ad5CMV*LacZ* (m.o.i. = 5) or mock-infected, either with or without ara-C. Cells were harvested 48 h co-post-infection and freeze–thawed three times to release the virus. Dilutions of the virus were used to infect HepG2 cells. Eighteen hours after infection cells were lysed and assayed for luciferase activity (A). The graph depicts the means (± 1 SD) of three experiments. In parallel, low molecular mass DNA was extracted from infected cells according to the Hirt method and digested with *Pvu*I. Ad5CMV*Luc* DNA was detected by a radiolabelled *Luc*-specific probe in Southern analysis (B). The arrow indicates the hybridizing 6 kb luciferase-gene fragment.

amount of mobilized Ad5CMV*luc* could be determined by measuring the amount of luciferase virus present in the lysate. To quantify the luciferase virus, HepG2 cells were infected with Ad derived from the HepG2 lysates and luciferase activity was determined 18 h p.i. To inhibit replication of the *wt* Ads present in the lysates, which may affect the luciferase activity, 20 µg/ml ara-C was added to the medium of the indicator cells. The results of the mobilization assays are depicted in Fig. 1(A). Luciferase expression in HepG2 cells infected with virus derived from the Ad5CMV*Luc*- and Ad5CMV*lacZ*-coinfected cells was as low as in HepG2 cells infected with virus derived from Ad5CMV*Luc* and in PBS-treated cells. So, no significant mobilization had occurred either in the mock-infected cells or in the Ad5CMV*lacZ*-infected cells. In contrast, luciferase activity was readily detectable in all assays where *wt* Ads had been added, suggesting that all serotypes could mobilize Ad5CMV*luc*. Similar results were obtained when the cells were harvested 5 days p.i. with Ad5CMV*luc* (data not shown). The amount of Ad5CMV*luc* produced upon coinfection by wt Ad5 was only 10-fold lower than the yield of Ad5CMV*luc* in the E1-complementing cell line 911 (compare the luciferase activity per cell in the wt Ad5 co-infection in Fig. 1A with the levels in 911 cells in Fig. 2A).

To confirm replication of Ad5CMV*luc* DNA after infection of the cells with *wt* Ads, low-molecular mass DNA was extracted by Hirt isolation (Hirt, 1967) from HepG2/Ad5CMV*luc* cells infected with the various *wt* Ads. Southern analysis of *Pvu*I-digested Hirt DNA with a luciferase gene-specific probe demonstrated replication of the Ad5CMV*luc* vector (Fig. 1B). Replication (Fig. 1B), as well as the mobilization (Fig. 1A), was sensitive to 20 µg/ml ara-C, indicating that replication is essential for mobilization of the vector. The data suggest that all Ad serotypes tested can mobilize the Ad5CMV*luc* vector.

Two mechanisms could contribute to the replication and mobilization of the Ad5CMV*luc* vector. Firstly, the E1 region of the *wt* virus could functionally complement the E1 deletion of the vector, and activate expression of the Ad5 genes of the vector. In the 'E1 complementation' model the vector would be replicated by and packaged in its homologous proteins. Thus, the presence of the E1 proteins of the *wt* Ad would be sufficient to mobilize the vector. Alternatively, in the 'facilitated replication and packaging' model, the *wt* Ad's E1 region alone is unable to activate expression of the vector genome. Hence, mobilization can only occur if the *wt* Ad-derived proteins other than, or in addition to, E1 facilitate replication and packaging of the vector DNA.

To test whether expression of E1 is sufficient to replicate the Ad5CMV*luc* vector we used a panel of human embryonic retinoblasts (HER) cell lines transformed with the E1 region of Ad12 and, as controls, cells transformed with the SV40 early region (van den Heuvel *et al*., 1992). As negative and positive controls we used cell lines HepG2 and 911 (Fallaux *et al*., 1996), an HER line transformed with the E1 region of Ad5, respectively. These cell lines were infected with the Ad5CMV*luc* at an m.o.i. of 10. At 3 days p.i., the cells and media were harvested, freeze–thawed (three cycles), and assayed for the

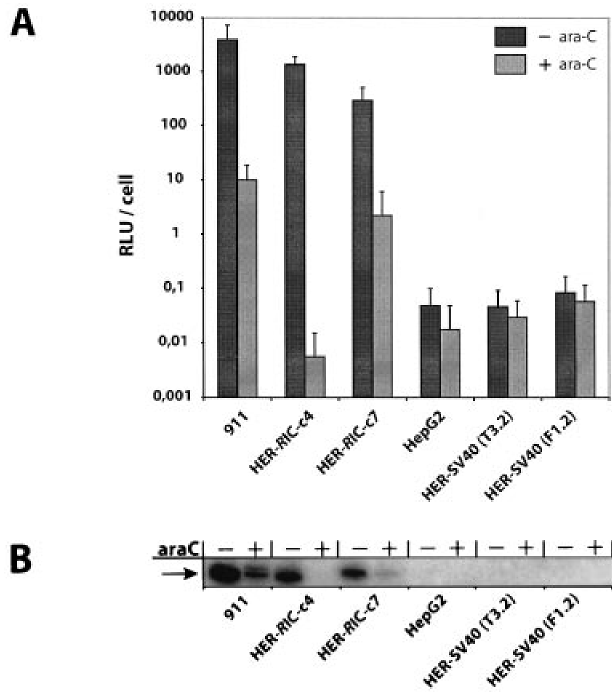

Fig. 2. Mobilization of rAd5Luc by *trans*-complementation of Ad12E1. HepG2, 911, HER-*R*IC-c4, HER-*R*IC-c7, HER-SV40(T3·2) and HER-SV40(F1·2) cells were exposed to Ad5CMV*luc* (m.o.i. = 10) in the presence or absence of 20 mg/ml ara-C. Forty-eight hours p.i., cells were harvested and the virus was released by three cycles of freeze–thawing. Subsequently, HepG2 cells were exposed to dilutions of the sample to quantify the amount of luciferase virus. Eighteen hours p.i., cells were lysed and assayed for luciferase expression (A). The graph depicts the means ($\pm$1 SD) of three experiments. In parallel, low molecular weight DNA was extracted from these cells according to the Hirt method. DNA was digested with *Pvu*I. Ad5CMV*Luc* DNA was detected by a radiolabelled *Luc* fragment in Southern analysis (B). The arrow indicates the hybridizing 6 kb luciferase-gene fragment.

presence of the Ad5CMV*luc* vector by infecting HepG2 cells with Ad derived from the HepG2 lysates followed by luciferase assay (Fig. 2A). Ad5CMV*luc* propagation was detectable neither in the HepG2 cells nor in the SV40-transformed HER-SV40(T3.2) and HER-SV40(F1.2) cells. In contrast, the HER-*R*IC-c4 and HER-*R*IC-c7 cells produced readily detectable amounts of reporter virus. The amplification factor, that is the ratio of the Ad5CMV*luc* production in the cultures divided by the Ad5CMV*luc* production in the ara-C-treated cultures, was only moderately lower than the amplification factor obtained in the Ad5E1-transformed 911 cells. Virus replication was sensitive to ara-C. Similar results were obtained if the viruses were harvested 5 days p.i. (data not shown). Southern analysis confirmed replication of the vector in the Ad5E1- and Ad12E1-transformed HER lines, and not in the SV40-transformed HER lines (Fig. 2B). Taken together, these data show that *wt* Ads of subgroups A, B, C and E can mobilize Δ*E1*Δ*E3*-Ad5 vectors and that for Ad12 at least, *trans*-complementation by E1 is sufficient for mobilization at least for Ad12E1.

Our data confirm and extend the earlier observation that *Bam*HI-digested wt Ad12 (subgroup A) and Ad7 (subgroup B) could efficiently complement an E1 deletion of Ad2 (subgroup C) (Brusca & Chinnadurai, 1981). Also, hybrid Ad5 and Ad12 viruses in which the E1 region had been replaced (in part) by the corresponding region of Ad12 and Ad5, respectively, were demonstrated to be viable (Bernards *et al.*, 1983). However, another study showed that a defect in the E1 region of Ad2 could only be complemented poorly by Ad9 (subgroup D) (Jannun & Chinnadurai, 1987). In addition, we show here that Ad12E1-transformed HER cell lines complement the E1 deletion in Ad5 and allow the Ad5 vector to be propagated.

It remains to be established if the 'facilitated replication and packaging' model really contributes to the mobilization of Δ*E1*Δ*E3*-Ad5 vectors. It has been shown that Ad5-derived helper-dependent Ad vectors can be amplified and packaged into Ad2-derived capsids (Morral *et al.*, 1999), demonstrating the feasibility of the model. It should be borne in mind, however, that that Ad2 and Ad5 are highly similar 'twin' serotypes and have origins of DNA replication and packaging signals that are identical in sequence. Indeed, it has been shown recently that Ad12 (subgroup A), Ad7 (subgroup B) and Ad 17 (subgroup D) cannot complement the defect of Ad5 mutant *pm8001* (Zhang *et al.*, 2001). This mutant cannot package its DNA due to a mutation in the L1 52/55 kDa gene. This indicates that the DNA packaging system of different serotypes cannot productively interact with Ad5 DNA. Thus, mobilization by E1 complementation appears to be the more general mechanism.

Our data demonstrate that Ad5-derived vectors can be mobilized not only by *wt* viruses of the homologous serotype, but also by different serotypes. Thus, in therapeutic applications of replication-defective Ad vectors any active Ad infection is of potential concern, independent of the serotype involved. To prevent vector mobilization by *wt* Ads, new vectors should be developed in which essential functions such as the initiation of DNA replication the genome packaging of DNA (Imler *et al.*, 1995) are further restricted.

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