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Enhanced Cytotoxicity without Internuclear Spread of Adenovirus upon Cell Fusion by Measles Virus Glycoproteins

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The efficiency of viruses in cancer therapy is enhanced by proteins that mediate the fusion of infected cells with their neighbors. It was reported that replication-competent adenovirus particles can spread between nuclei within fusion-generated syncytia. To assess this conjecture, we generated fusogenic adenoviruses that express a balanced ratio of the F and H glycoproteins of measles virus. The viruses displayed enhanced cytotoxicity but largely unchanged replication efficiencies compared to a nonfusogenic virus. Most notably, the virus genomes did not spread through fusion-generated multinuclear cells. Hence, adenovirus replication in syncytia remains largely restricted to initially transduced nuclei.

As a tool for cancer therapy, adenoviruses are being engineered to replicate in tumor cells, thus enhancing their therapeutic effect by multiple rounds of infection (2, 4, 8). An attractive strategy for obtaining "armed" therapeutic viruses (18–20) consists of the expression of fusogenic proteins that lead to the fusion of infected cells with adjacent cells, thereby increasing the toxicity of the viruses (3, 22). Fusogenic proteins are typically derived from enveloped viruses that use them to fuse membranes and penetrate cells. Such proteins from human immunodeficiency virus type 1 or from measles virus have been inserted into the adenovirus genome (1, 14, 24, 27). Fusogenic recombinant adenoviruses obtained in this way spread more efficiently through tumor xenografts. This raises the possibility that viral DNAs can spread between nuclei within syncytia formed by cell fusion. In particular, one report documented the existence of viral particles in multiple nuclei and suggested that DNA replication can occur in many nuclei of a syncytium (24). Potentially, this internuclear adenoviral DNA spread might strongly enhance infection efficiency. However, it is not clear how adenoviral DNA can efficiently enter nuclei if the newly synthesized particles do not leave their host cell and enter another cell to undergo standard maturation steps (36, 37). This raises the following fundamental question concerning the biology of adenovirus replication. Are virus release and reuptake necessary for the delivery of viral DNA to the nucleus and for its subsequent replication?

Fusogenic adenovirus. To create a fusogenic recombinant adenovirus, we inserted a bicistronic expression cassette for measles virus glycoproteins F and H to replace the E1 gene region within the plasmid pAdEasy1 (21), which contains the other essential regions of the adenovirus genome (Fig. 1A). To drive the expression of F and H, we used either the major immediate early promoter of cytomegalovirus (CMV promoter) or the adenovirus major late promoter (MLP). The mRNA consisted of the coding region for H followed by the encephalomyocarditis virus internal ribosomal entry site (IRES) and the F coding region. All viruses were rescued by use of a plasmid-based AdEasy system (21), and Ad CMV βGal, which was used as a control, was described previously (23). Our cloning strategies are available upon request. When transfected into E1-complementing HER911 cells (12), the plasmid containing the CMV F&H cassette induced extensive cell fusion, which was visualized by the use of green fluorescent protein (GFP) expressed by the same plasmid (Fig. 1B, a). The addition of fusion inhibitory peptide (FIP) virtually abolished cell fusion (Fig. 1B, b), in agreement with results obtained in the context of measles virus infection (29, 33). When F and H expression was put under the control of the adenovirus MLP, the transfected cells fused much less efficiently, if at all, regardless of the presence or absence of FIP (Fig. 1B, c and d). This was expected, since the MLP reaches its full activity only at the late stage of adenovirus infection (25, 36). Ten days after transfection in the presence or absence of FIP, the cells were harvested and the virus yield was determined. When fusion was allowed, the amount of rescued virus was about 10-fold lower than that when it was inhibited by FIP (Fig. 1C), possibly explaining previous difficulties in the construction of fusogenic adenoviruses (14). Thus, the use of a fusion inhibitory peptide or the expression of fusogenic gene products only in the late phase can each increase the efficacy of virus rescue.

Cytotoxicity. When HER911 cells were infected with Ad CMV F&H at a low multiplicity of infection (MOI), large syncytia formed around infected cells (Fig. 2A), whereas no such phenomenon was observed when we used a virus expressing beta-galactosidase instead of the measles virus glycoproteins. At a high MOI, cells infected with the fusogenic adenovirus formed large spheric syncytia that detached from the

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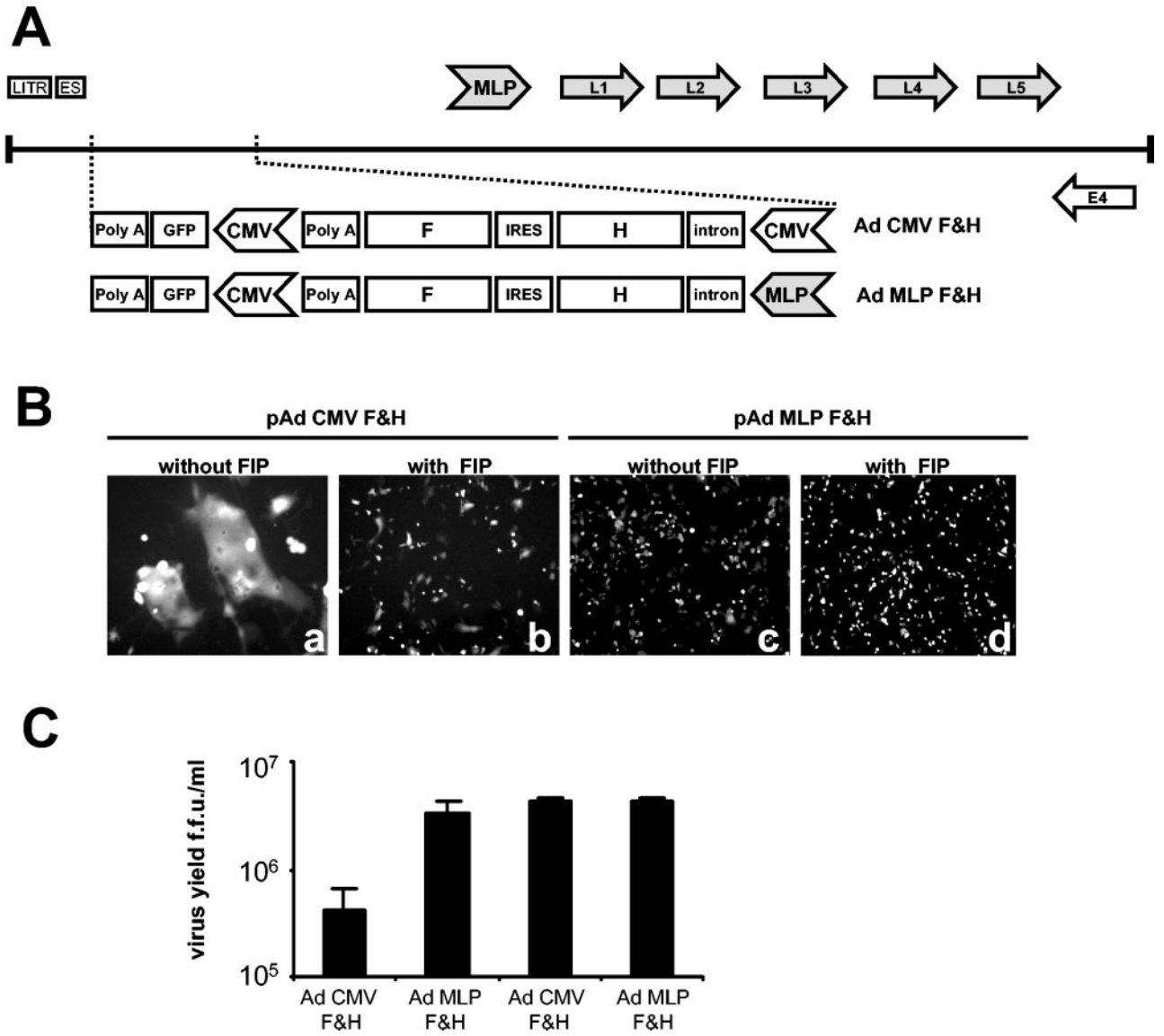


FIG. 1. Improving the yield of recombinant fusogenic adenovirus by use of FIP or by late expression of fusogenic proteins. (A) Schematic presentation of fusogenic adenoviruses (linear genomes). Vector genomes are depicted and indicate the assembly of the transgene. The virus genome starts with the left inverted terminal repeat region (LITR) and the encapsidation signal (ES). The positions of some of the early (E) and late (L) genes are indicated. The promoters used to express F and H were either the cytomegalovirus major immediate early promoter (CMV) or the adenovirus major late promoter (MLP). The genes encoding the H and F genes of measles virus (strain Edmonston) were separated by the IRES of encephalomyocarditis virus. A polyadenylation signal (polyA) was then followed by a CMV promoter-driven expression cassette for GFP. (B) Cell morphology upon transfection of GFP-expressing virus genomes. HER911 cells were transfected (Lipofectamine Plus; Invitrogen) with linearized plasmids containing the virus genomes shown in panel A. FIP was added as indicated. After 48 h, green fluorescence was monitored by microscopy. (C) Virus yield 10 days after transfection, given in fluorescence-forming units (f.f.u.) per ml of cell lysate. HER911 cells were transfected and treated as described for panel B. After 10 days, the emerged virus was harvested and quantified on a fresh monolayer of HER911 cells as described previously (23, 40). The average titers of at least three independent experiments are shown along with standard errors. The left two columns reflect results obtained in the absence of FIP; the right two columns show the titers obtained in the presence of FIP.

solid support, an effect that was inhibited by the addition of FIP (data not shown). When F and H cDNAs were expressed from the adenovirus major late promoter, fusion was delayed but eventually occurred at a similar extent as that with Ad CMV F&H (Fig. 2A). The overall cytotoxicity of fusogenic adenoviruses was assessed by infecting HER911 cells. At various time points, detached cells were washed off, and adherent cells were stained with crystal violet. Ad CMV F&H as well as Ad MLP F&H displayed cytotoxicity at earlier time points than did a nonfusogenic virus (Fig. 2B). The addition of FIP decreased the cytotoxicity (data not shown). We concluded that the expression of fusogenic proteins can enhance the cytotoxicity of a replicating adenovirus.

Virus replication. HER911 cells were infected with Ad CMV F&H or an otherwise identical virus that expressed beta-galactosidase (Ad CMV βGal), followed by determinations of

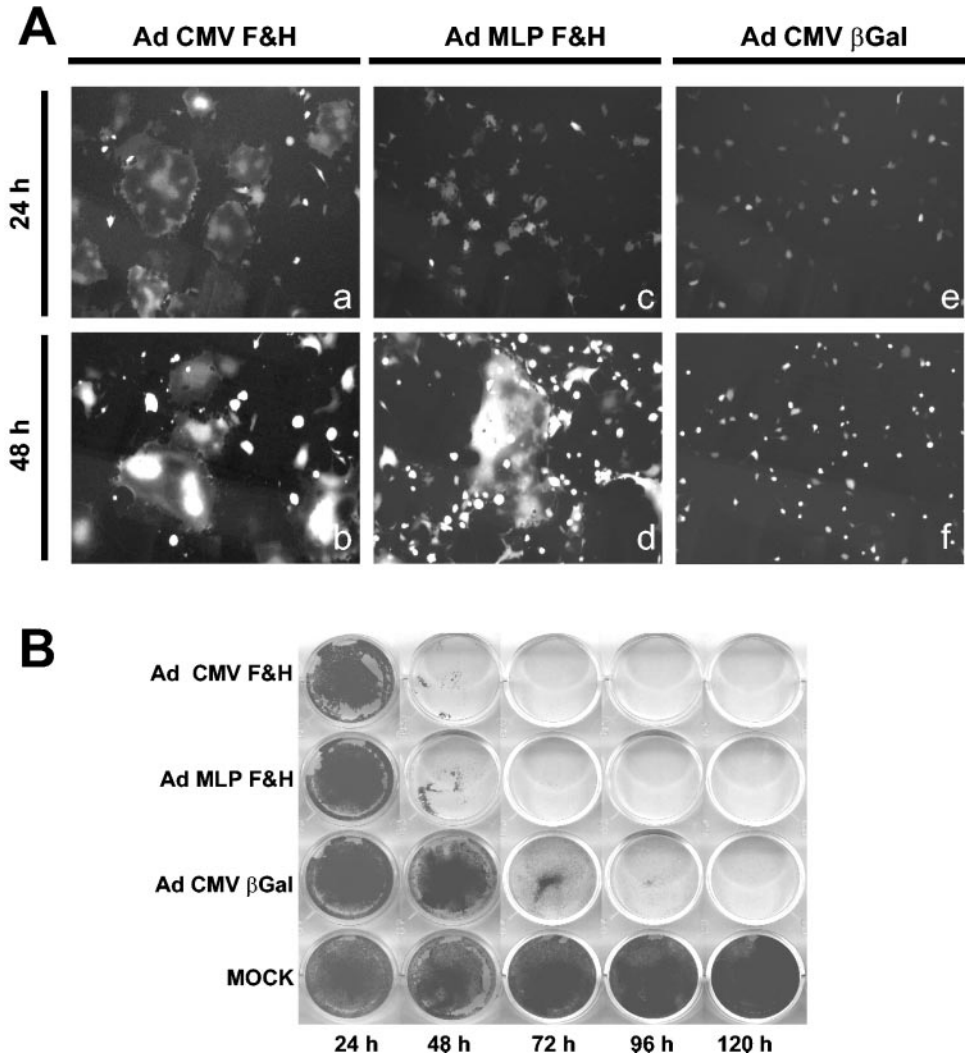
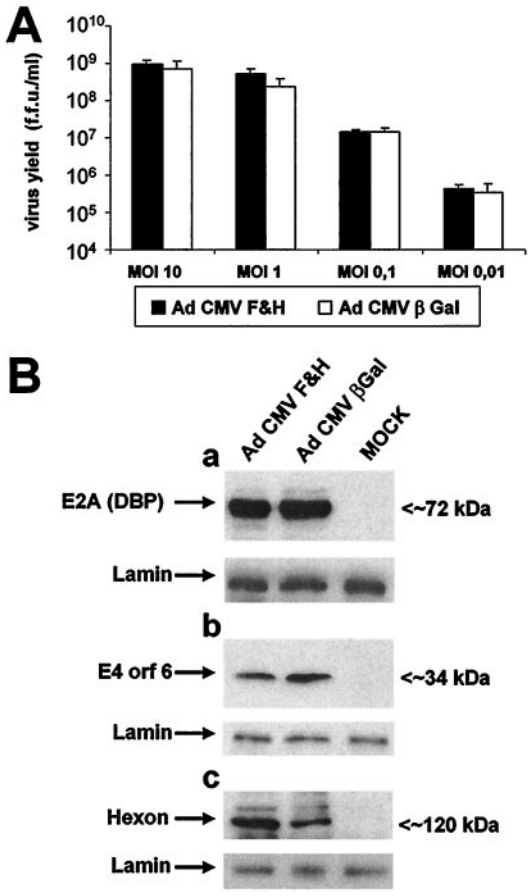


FIG. 2. Cytotoxicity induced by fusogenic adenovirus. (A) Timing of fusion. HER911 cells were infected with the indicated virus preparations at an MOI of 0.01 for the indicated times, followed by monitoring of the distribution of GFP by fluorescence microscopy. (B) Time course of cytotoxic effects. HER911 cells were transduced with the indicated viruses (MOI = 1) for the indicated times. Subsequently, detached cells were washed off the plate, and the remaining cell monolayer was stained with crystal violet.

virus yields. As shown in Fig. 3A, virus particle production was not measurably affected by the expression of fusogenic proteins or by the addition of FIP. Similar results were obtained with another cell line, H1299, by the use of combinations of vector and wild-type viruses (data not shown). Upon infection of HER911 cells with fusogenic or nonfusogenic viruses, the levels of viral proteins were assessed by immunoblot analysis as described previously (34). Antibodies were directed against adenovirus E2A (B6-8 [32]) (obtained from J. Flint), the adenovirus hexon protein (goat polyclonal antibody) (Biogenesis), and E4orf6 (M45) (kindly provided by P. Hearing). As shown in Fig. 3B, the expression levels of all proteins were similar between the viruses, regardless of the ability to induce cell fusion. The same was found for viral DNAs, as revealed by semiquantitative PCRs (Fig. 3C). We concluded that cell fusion neither enhances nor reduces the efficiency of virus replication.

Replication centers within syncytia. The failure of cell fusion to enhance the efficiency of virus replication contrasted with the previous suggestion that adenoviruses spread between the nuclei of a syncytium, thereby enhancing replication (24). Since fusogenic adenoviruses provide a unique opportunity to determine whether virus entry and the associated maturation process are essential for the subsequent steps of the viral life cycle, we reexamined the issue of secondary virus replication in nuclei of cells that had fused with an originally infected cell. To this end, we infected HER911 cells with the fusogenic adenovirus at a low MOI. To avoid reinfection, we added a neutralizing antibody (clone 1D6;14, an anti-fiber knob antibody [10]) (kindly provided by J. Douglas) at a neutralizing concentration 3 h after the addition of the virus. At 48 h postinfection, the intracellular localization of the E2A 72-kDa DNA binding protein was determined by immunofluorescence as described previously (34). During adenovirus infection, this protein initially localizes in a diffuse nuclear pattern but then associates with replicating viral DNAs in discrete nuclear foci termed replication centers (30). Syncytia were detected by their characteristic



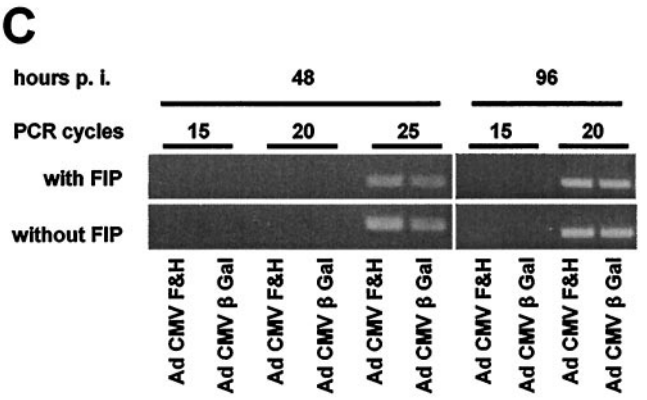


FIG. 3. Replication yield of fusogenic adenovirus. (A) Replication in the presence or absence of fusion. HER911 cells were infected with the indicated virus preparations. At 48 h postinfection, the viruses were harvested and titrated. (B) HER911 cells were infected as indicated (MOI = 0.1). The expression of the viral gene products E2A, E4orf6, and hexon was monitored by immunoblotting 48 h after infection. Lamin was stained as an input control. (C) Upon infection as described for panel B, the amounts of viral DNA were determined at the indicated times postinfection by semiquantitative PCRs with the indicated numbers of PCR cycles in the presence or absence of FIP. The PCR products were visualized by ethidium bromide staining after agarose gel electrophoresis.

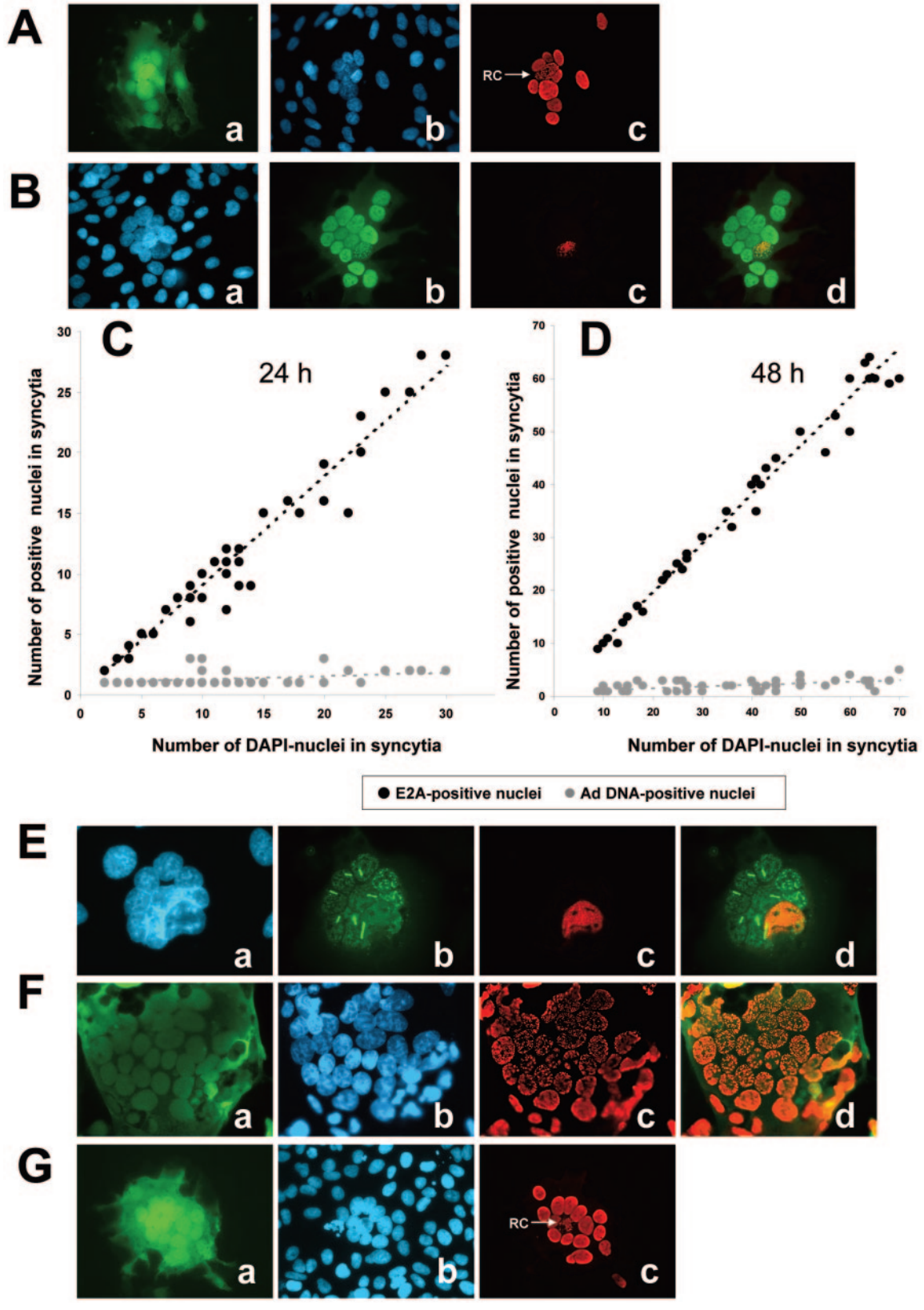
distribution of GFP (Fig. 4A, a). We found that the majority of the nuclei within syncytia (visualized by DAPI staining) (Fig. 4A, b) contained readily detectable E2A 72-kDa protein (Fig. 4A, c). However, only one of these nuclei typically displayed the characteristic distribution of the E2A 72-kDa protein in nuclear foci, suggesting that only one nucleus contained replicating viral DNA (Fig. 4A, c).

To assess more rigorously the ability of viral DNA to spread between nuclei, we performed similar infection experiments, followed by the detection of viral DNA by fluorescent in situ hybridization (FISH) as described previously (13). All nuclei within a syncytium were detected by DAPI (4′,6′-diamidino-2-phenylindole) staining (Fig. 4B, a), and E2A-72 kDa was immunostained (Fig. 4B, b). As shown in Fig. 4B, c, only one nucleus within a syncytium typically contained detectable viral DNA.

The presence of viral DNA was evaluated in many syncytia at 24 and 48 h postinfection (Fig. 4C and D). Almost all nuclei within a given syncytium were positive for E2A, whereas only one or a few of them contained viral DNA. We propose that only occasionally, independently infected cells became incorporated into the same syncytium, thereby creating syncytia with more than one nucleus harboring viral DNA. In conclusion, adenovirus DNA does not normally undergo internuclear spreading upon fusion of an infected cell with uninfected neighboring cells.

These findings are in apparent contrast with the detection by electron microscopy of adenovirus particles in virtually all nuclei of a syncytium (24). However, considering that empty

FIG. 4. Formation of replication centers within syncytia. HER911 cells were infected at an MOI of 0.01 with Ad CMV F&H. To avoid extracellular spread, we added an antibody against the fiber knob 3 h after infection. After 48 h, immunostaining of the E2A 72-kDa DNA-binding protein was performed. (A) GFP distribution (a) and DAPI staining (to visualize all nuclei) (b) were monitored, and E2A was visualized by immunofluorescence (c). The typical distribution of E2A in replication centers (RC) within one of the nuclei is indicated by an arrow. (B) The total DNA was stained with DAPI (a), and E2A-72 kDa was visualized by immunofluorescence (b). In addition, viral DNA was stained by FISH (c). Superimposed patterns of E2A and viral DNA are shown in panel d. At least 30 syncytia were evaluated at 24 (C) or 48 (D) h postinfection by FISH and immunostaining. The total number of nuclei within each syncytium was plotted against the number of E2A-positive nuclei (black dots) and against the number of cell nuclei that stained positive for viral DNA (gray dots). (E) To monitor hexon assembly, we transduced HER911 cells as described above and then added an antibody against the fiber knob. After 48 h, immunostaining of the hexon protein was performed (b). The cells were also stained with DAPI to detect all nuclei (a), and FISH was employed to detect viral DNA (c). The patterns of hexon and viral DNA were superimposed (d). (F) To study the formation of replication centers in preformed syncytia, we transfected 911 cells with an expression plasmid for the measles virus F and H proteins. After 24 h, syncytia had formed, and further fusion was stopped by the addition of FIP. The cells were then infected with the wild-type adenovirus dl309, followed by incubation for another 24 h. E2A was then stained as described for panel A. (G) U2OS cells seeded at a low density were coinfected with wild-type adenovirus dl309 and Ad CMV F&H at an MOI of 10. Three hours later, fresh U2OS cells were added to form a confluent monolayer, and an antibody against the fiber knob was added. Forty-eight hours later, the cells were fixed and E2A was stained as described for panel A.


A
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24 h
Number of positive nuclei in syncytia
Number of DAPI-nuclei in syncytia
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Number of positive nuclei in syncytia
Number of DAPI-nuclei in syncytia
E2A-positive nuclei
Ad DNA-positive nuclei
E
a
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particles can hardly be distinguished from DNA-containing viruses by electron microscopy and that the hexon protein is an essential component of both viruses and empty particles, we determined which nuclei contained this protein. Since the hexon protein was detectable in virtually all nuclei of the syncytia (diffusely or in discrete clusters [Fig. 4E]), we suggest that empty particles may have been confused with infectious viruses by electron microscopy.

To verify whether the nuclei of a syncytium can support virus replication if they are reached by infectious particles, we infected preformed syncytia with viruses. HER911 cells were transiently transfected with an expression plasmid for F and H. After syncytium formation, fusion progression was blocked by the addition of FIP. The preformed syncytia were then infected with wild-type adenovirus, and the formation of replication centers was monitored by immunostaining of E2A-72 kDa. As shown in Fig. 4F, virtually all nuclei of the preformed syncytia contained replication centers, indicating that the previously observed lack of internuclear spread was not due to a general defect of intrasyncytial nuclei to support the replication of viral DNA.

In the experiments described above, the E1 gene products were provided by integrated E1 regions within the chromosomes of the host cells. To test whether this influenced the internuclear spread of virus replication, we coinfected U2OS cells with a wild-type adenovirus, dl309, and with Ad CMV F&H. These cells were then allowed to fuse with uninfected cells, followed by immunostaining of E2A-72 kDa. As shown in Fig. 4G, the syncytia obtained in this way still contained just one nucleus with replication centers, strongly arguing that virus genomes do not spread, regardless of the origin of E1 gene products.

Fusogenic viruses in cancer therapy. Replication-competent, fusogenic adenoviruses reduce tumor xenografts more efficiently than do their parental viruses (1). Previous results suggested three favorable properties of fusogenic adenoviruses, i.e., a lateral spread of transgenes, a dispersion of viruses to cells that were not initially infected, and a more rapid release of virus (24). The experiments reported here challenge only the second of these points but do not shed doubts on the other two. Thus, even if viral DNA does not efficiently spread between nuclei within a syncytium, cell fusion remains a viable strategy for increasing the oncolytic efficiency of replicating adenoviruses. It is important that the measles virus H protein can be retargeted; different measles virus strains bind the ubiquitously expressed CD46 molecule and the signaling lymphocyte activation molecule (6, 9, 11, 35, 38). However, the H protein residues interacting with natural receptors modified the protein (39) and single-chain antibodies specific for surface proteins preferentially expressed by tumor cells can be added to it, enabling antibody-targeted cell fusion (5, 17, 27, 31).

Virus release and reuptake are essential steps of the replication cycle. The direct detection of viral DNAs by FISH in this study revealed that after being formed in one nucleus within a multinuclear cell, virus particles do not leave it to infect adjacent nuclei, in contrast to a previous suggestion (24). The previous report employed human immunodeficiency virus gp120 and CD4 receptors for fusion with a self-replicating virus, and we cannot formally rule out the possibility that the internuclear spread of genomes might be dependent on the receptors used to induce fusion. However, our results are consistent with the current notion that newly formed virus particles that escape from infected cells (usually by lysis) and start a new infectious cycle must undergo a precisely orchestrated program of dismantling and intracellular transport following cell entry in order to efficiently inject their DNA into the nucleus. Processing occurs within compartments that cannot be reached by a virus without its leaving the cell and entering a new one. Once the virus binds to its receptor molecules, a coated pit is formed below the cell surface before a coated vesicle moves into the cell. During this early phase of infection, the fibers are lost (28). Additional structural changes within the virus particles include the dissociation of the penton base, the degradation of the proteins that connect the DNA to the inside surface of the capsid, and the elimination of the capsid-stabilizing minor proteins (16). A protease activation step in the acidic environment of endosomes is needed for the nuclear uptake of viral DNA (15, 16). These processing steps occur along a predetermined pathway including coated vesicles and the endosome, passing through the cytosol along microtubules, and reaching the nuclear pore complex, where the viral DNA is released into the nucleus (7, 26). Our results indicate that efficient adenovirus replication cannot occur without extracellular particle spread.

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