[Frontiers in Bioscience 10, 1106-1117, May 1, 2005]

FUNCTIONS OF THE ADENOVIRUS E4 PROTEINS AND THEIR IMPACT ON VIRAL VECTORS

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1. ABSTRACT

Viral vectors based on recombinant adenoviruses (rAd) are used extensively as gene delivery systems for investigations of gene function and gene therapy applications. In both cases, the desire is for efficient expression of a transgene without substantial toxicity to the target cell. The first generation of rAd vectors were deleted of early region E1 and sometimes additionally early region E3. Evaluation of these vectors in preclinical animal models revealed transient gene expression, accompanied by cytotoxicity and immune responses. Expression of remaining viral genes, even in the absence of the E1 region, contributes to these effects and therefore it is important to have an appreciation of the functions of these viral genes. In particular, the early region E4 has been shown to affect transgene persistence, vector toxicity and immunogenicity. Proteins from the E4 genes can modulate transcription, the cell-cycle, cell signaling and DNA repair. In addition, some of these proteins also cause oncogenic transformation. Therefore interactions of these viral genes with key cell regulators should be taken into account when engineering rAd vectors. This review will summarize our knowledge of E4 functions and the implications of recent findings on the development of rAd vectors.

2. INTRODUCTION

The first generation of rAd vectors consist of the E1 region replaced with the transgene to be expressed (reviewed in Refs. 1, 2). Although efficient for gene delivery, these vectors demonstrate only transient gene expression *in vivo*. Lack of persistence from adenoviral vectors is due in part to activation of cellular immune responses to expression of viral and transgene products, leading to death and clearance of transduced cells. The limitations of these vectors prompted efforts to cripple the virus further, by deletion of additional early regions such as E4. Subsequent comparison of first generation vectors to those that additionally lack the E4 region has demonstrated that E4 gene products can affect persistence of transgene expression, cytotoxicity and immune responses. Therefore, it is important to have an understanding of the effects of the remaining viral proteins on cellular processes, in order to minimize negative effects of vectors and maximize transgene persistence.

A full appreciation of the functions of gene products encoded by the viral genes that remain in viral vectors will allow rational design of safer and more efficient delivery systems. Transcription from the early E4 region can be observed for the first generation of E1-deleted vectors (3) and E4 gene products have a significant effect on host cell metabolism. Proteins expressed from the E4 region have been implicated in the regulation of a diverse set of functions during productive infection including transcription, viral DNA replication, RNA splicing and processing, late protein synthesis and the transition from the early to late stages of infection. Recent work has also suggested that E4 gene products target cellular regulators of cell signaling and DNA repair and that this contributes to cell transformation and

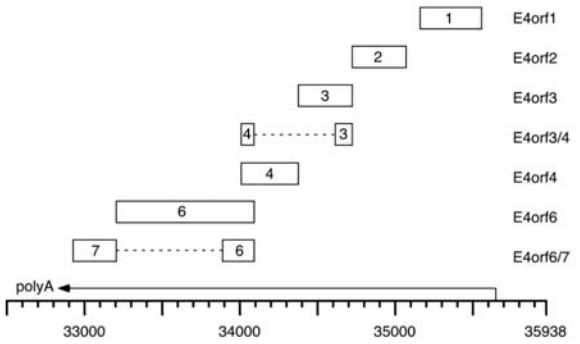

Figure 1. The gene products of the E4 region from Ad5. The E4 region is located at the right hand end of the Ad5 genome. A single promoter and polyadenylation signal (polyA), generate a primary transcript of approximately 2800 nucleotides (depicted as an leftwards pointing arrow). Splicing produces distinct mRNAs that encode the seven predicted polypeptides shown as open boxes. Their relative position in the Ad5 genome is indicated by the nucleotide sequence at the bottom. Dashed lines indicate fusion proteins.

oncogenicity. The bulk of our knowledge of E4 gene products and their functions has come from studies of the closely-related Ad2 and Ad5 serotypes. This review will discuss the current status of our knowledge about the functions of the various E4 proteins, focusing predominantly on those from Ad5 as they are the best studied, and most of the adenovirus vectors being used are based on this serotype. Further details on the E4 gene products and their role in viral replication can be found in a number of excellent reviews that focus on the E4 region (4-6).

3. THE E4 PROMOTER AND REGULATION OF GENE EXPRESSION

The E4 region of Ad5 and gene products of its open reading frames (orfs) are shown schematically in Figure 1. E4 is located at the right hand end of the genome between map units 91.3 to 99.1. A single E4 promoter generates a primary transcript that is subject to a complex program of alternative splicing to produce at least 18 distinct mRNAs (7, 8). Seven different polypeptide products are predicted to be encoded from these mRNAs and six of these have been demonstrated to exist in infected cells. The E4 promoter is activated very early after infection by the E1a transcriptional activator protein and transcription continues into the late phase of infection (9-11). At later times transcription declines due to repression by the E2a gene product (12, 13) and feedback inhibition of E1a-mediated transactivation by the E4orf4 protein (14). Additionally, production of E4 proteins is temporally regulated at the post-transcriptional level by alternative splicing (8, 15).

4. FUNCTIONS OF THE E4 GENE PRODUCTS

Many of the E4 orfs produce multifunctional viral regulators. Growth of a mutant virus with deletion of the entire E4 region is severely impaired (16, 17), but mutations within individual orfs have minimal or modest effects. Deletion of the E4 region results in defects in viral DNA replication, accumulation of late viral messages and proteins, virus particle assembly and shut-off of host protein synthesis (16-20). Genetic studies have revealed functional redundancy between E4orf3 and E4orf6 (21, 22). The other E4 gene products are dispensable for lytic growth of the virus in cell culture. There is no functional information about the gene products of E4orf2 and E4orf3/4, although the former has been detected as a soluble cytoplasmic component in infected cells (23). The E4 region has been characterized from some other serotypes and these have a similar genomic organization, although E4orf1 is not conserved in Ad40 (24). In the non-human adenoviruses the degree of E4 conservation with Ad5 can vary, with bovine Ad2 showing conservation of four orfs (25), murine Ad1 demonstrating only limited homology with E4orf6 (26) and the chicken adenovirus CELO possessing no discernable E4 region (27).

4.1. E4orf1

The E4orf1 sequence is fairly conserved across all human adenoviruses so far sequenced. The mRNA for E4orf1 of Ad5 accumulates late after infection and its cytoplasmic localization is dependent on the E1b55K/E4orf6 complex, suggesting that E4orf1 may supply a function(s) in the late stage of the lytic life-cycle (15). Expression of an E4orf1 protein has only been detected in cells infected with subgroup D viruses Ad9 and Ad26 (28, 29). Mutations in Ad5 E4orf1 have only minimal effects on viral growth and little is known about the functions of the protein in viral infection. The sequences of E4orf1 from all characterized human serotypes display significant similarity to other viral and animal dUTP pyrophosphatase (dUTPase) enzymes (30). Although the E4orf1 proteins do not possess detectable enzymatic activity, this observation suggests that they may have evolved from an ancestral dUTPase. The E4orf1 proteins also interact with a similar set of cellular factors via a functional PDZ domain-binding motif in the C-terminus (31). PDZ domains are modular units of approximately 80 amino acids that mediate protein-protein interactions and proteins with PDZ domains are often involved in signal transduction. Among the PDZ domain-containing proteins that interact with E4orf1 are DLG, MUPP1 and MAGI-1 (31-33). These interactions may be important for E4orf1 functions and contribute towards the oncogenicity of Ad9 E4orf1 (see below).

4.2. E4orf3

The first E4 gene product to be identified was the 11 kDa protein E4orf3 (34). It is highly conserved both immunologically and at the amino acid sequence level. E4orf3 is functionally redundant with the E4orf6 product during lytic growth, and each are independently sufficient for efficient viral DNA replication, late viral protein synthesis, shut-off of host protein synthesis and virus production (16, 21, 22). The E4orf3 protein is synthesized early in infection and has been shown to be tightly associated with the nuclear matrix (34). E4orf3 expression induces the reorganization of nuclear bodies called PML

oncogenic domains (PODs) or ND10. These large nuclear structures contain a growing number of proteins and have been implicated in multiple cellular functions including transformation, genomic stability, DNA repair, transcriptional control, apoptosis and the interferon response (reviewed in Ref. 35). E4orf3 expression induces rearrangement of PODs into track-like structures in transfected and virus infected cells (36, 37). The mechanism by which E4orf3 accomplishes these drastic changes in the architecture of nuclear structures and the cellular consequence of POD disruption remain unclear. As many viruses target the PODs and in some cases rearrange their components, it has been suggested that reorganization is advantageous for viral replication (38, 39). E4orf3 may play a role in initiation of viral replication during the early stages of the life-cycle, while other viral proteins that interact with the PODs may be able to compensate for its absence during high multiplicities of infection (36). The E4orf3 polypeptide can physically associate with the viral E1b55K protein, causing its relocalization to the nucleus and recruitment into the discrete track-like structures (40).

One function shared by E4orf3 and E4orf6 is the ability to prevent concatemer formation upon viral infection. During infections with wild-type Ad the double-stranded DNA genome exists almost exclusively as linear monomers. However, deletion of the E4 region leads to concatemerization of the viral genome (41, 42). This end-joining of viral genomes requires cellular proteins involved in non-homologous recombination, such as ligase IV and DNA-PK (42, 43). In addition there is a requirement for the Mre11 complex, a conserved multi-functional protein complex that is composed of the cellular proteins Mre11, Rad50, and NBS1 (43). Adenovirus has evolved two mechanisms by which to inactivate the Mre11 complex and prevent concatemer formation. In uninfected cells the Mre11/Rad50/NBS1 proteins appear throughout the nucleoplasm, but are also concentrated at sites of replication and at PODs (44, 45). Early after Ad infection the Mre11/Rad50/NBS1 proteins are detected in nuclear speckles, partially co-localized with PML tracks and the E4orf3 protein (43; unpublished data). The E4orf3 protein is sufficient to disrupt PODs, and reorganize both PML and the Mre11 complex (43). The E4orf3 protein can rescue an E4-deleted virus by preventing concatemerization, but a mutant E4orf3 that does not disrupt PODs cannot. This suggests that disruption of PODs and/or relocalization of the Mre11 complex is required to prevent genome concatemerization during the early stages of adenoviral replication.

4.3. E4orf4

In Ad5 the E4orf4 protein is 114 amino acids, and the sequence is highly conserved across all sequenced adenoviruses (46). The protein is highly stable and remains at a constant steady-state level even late into infection. The E4orf4 product is not essential for virus growth and its deletion has minimal effect (16). However, a virus lacking E4orf4 was more cytotoxic than wild-type virus in non-permissive rodent cells (47). A mutant that expressed E4orf4 in the absence of all other E4 genes was highly defective for DNA synthesis, suggesting a role for the viral protein in regulating replication (48). The E4orf4 product has been shown to regulate protein phosphorylation during infection through its ability to bind to protein phosphatase 2A (PP2A), one of the major serine/threonine-specific phosphatases in the cell (49). Expression of the adenovirus E1a protein induces activity of the transcription factor AP-1 via upregulation of JunB and c-Fos proteins, and both E1a and c-Fos become hyperphosphorylated (47). E4orf4 regulates these phosphorylation events through recruitment of PP2A and in this way inhibits transactivation (49). Dephosphorylation of viral/cellular proteins is also involved in the ability of E4orf4 to down-regulate expression from the E4 promoter (14).

The E4orf4/PP2A complex is also thought to regulate splicing of late adenoviral RNAs. E4orf4 induces the dephosphorylation of cellular SR proteins (50), a family of serine/arginine-rich splicing factors that regulate alternative splicing. The E4orf4 protein interacts specifically with a subset of SR proteins and binds preferentially to hyperphosphorylated forms of SF2/ASF and SRp30c (51). It has been proposed that recruitment of PP2A phosphatase by E4orf4 leads to dephosphorylation of the SR proteins, which in turn relieves their repressive effects on viral pre-mRNA splicing (51).

E4orf4 inhibits colony formation by inducing apoptosis of transformed cells in a p53-independent manner (52-54) that requires interaction with PP2A (46, 55). E4orf4 expression also induces PP2A-dependent growth arrest in *Saccharomyces cerevisiae* at the G2/M phase of the cell-cycle (56, 57). The exact mechanism for apoptosis in mammalian cells is not known, but E4orf4 has been linked to dysregulation of Src family kinases, which could lead to rearrangement of the actin cytoskeleton and loss of survival signals (58). It has been suggested that apoptosis induced by E4orf4 may play a role at the end of the infectious life cycle by killing cells and thus enabling viral spread (59).

4.4. E4orf6

The ability of E4orf6 to provide sufficient functions for viral replication in the absence of all other E4 proteins has made it the most studied of the E4 gene products. The E4orf6 sequence produces a 34 kDa product that is highly conserved across a variety of adenovirus serotypes (60). There is a conserved arrangement of several cysteine and histidine residues in a central cysteine-rich section that forms a functional zinc-binding domain (60). There is also a conserved arginine-rich region at the C-terminus that forms an amphipathic a-helix and is important for E4orf6 function (61, 62).

E4orf6 forms a multi-functional complex with the viral E1b55K protein (63) that is involved in viral DNA replication, RNA processing, nucleo-cytoplasmic transport of late viral mRNA, and the shut-off of host protein synthesis. This complex somehow enables export of late viral mRNAs but prevents transport of cellular RNAs (16, 22, 64). The complex can shuttle between the nucleus and the cytoplasm and nuclear export signals (NESs) have been reported for both E1b55K (65) and E4orf6 (66) that might directly mediate

transport of late viral mRNAs. The role of the E4orf6 NES in RNA transport and late gene expression remains controversial (67, 68). The E4orf6 and E1b55K proteins can both independently inhibit the transcriptional activity of p53; E1b55K binds to the transactivation domain at the N-terminus (69, 70) and E4orf6 binds at the C-terminus (61, 71). Infection of cells with mutant viruses that do not express either E1b55K or E4orf6 gene products results in stabilization of p53 protein levels (72, 73), at least in part due to the viral E1a polypeptides (74). The E1b55K/E4orf6 complex promotes proteasomal degradation of p53 during viral infections (72, 73, 75), as well as in transfection assays (61, 73, 76, 77) and in a 293 cell line that inducibly expresses E4orf6 (78). Targeted degradation of p53 requires association with cellular factors including Cullin-5, Elongin B and Elongin C (79). This complex can act as a ubiquitin ligase and mediates polyubiquitinylation of the p53 protein *in vitro* (79, 80).

As described above for E4orf3, the E4orf6 protein is also involved in preventing concatemerization of the viral genome during infection (41). This is achieved by the E1b55K/E4orf6 complex that targets the Mre11/Rad50/NBS1 proteins for proteasome-mediated degradation (43). This observation suggests that members of the Mre11 complex may also be substrates for modification by the ubiquitin ligase that is recruited by E4orf6 (79). By degrading the Mre11 complex, adenovirus not only blocks joining of viral genomes but also prevents the cell from mounting a DNA damage response to double-strand breaks (43); unpublished data).

4.5. E4orf6/7

E4orf6/7 is a fusion between open reading frames 6 and 7. In the case of Ad5 the protein consists of 58 residues from the amino-terminus of E4orf6, fused to the 92 amino acids of E4orf7. The E4orf6/7 protein modulates transcriptional activity through its interaction with the cellular E2F/DP family of cellular transcription factors. The E4orf6/7 protein forms a dimer that links two E2F molecules and stabilizes their binding to two E2F sites present as an inverted repeat in the Ad E2 early promoter (81, 82). The stabilized binding of E2F correlates with E4-induced transactivation of the E2 promoter (81, 83, 84). The E2F family of transcription factors are important regulators of cellular proliferation and are involved in activating cellular genes involved in cell cycle and DNA synthesis (reviewed in Ref. 85). During virus infection the E1a protein binds to the cellular retinoblastoma (Rb) family members and dissociates these repressors from a complex with E2F proteins. Free E2F is then bound by E4orf6/7, which leads to transactivation of the viral E2 promoter, as well cellular promoters that contain appropriately placed binding sites, such as the E2F-1 promoter itself (86). Activation of cellular E2F target proteins may be crucial for the viral life-cycle and may even be the predominant function of E4orf6/7. The E4orf6/7 protein can also functionally compensate for lack of E1a expression in Ad infection, by directly displacing Rb protein family members from E2F and activating the E2 promoter (87).

5. E4 AND ONCOGENESIS

Adenoviruses have not been reported to be associated with any human cancers. However, for a long time human adenoviruses have been known to possess the ability to transform cultured rodent cells (88). A subset of adenovirus serotypes can also induce tumor formation directly in rats and hamsters. The observation that human adenovirus type 12 induced malignant tumors in newborn hamsters was one of the first experimental examples of oncogenesis by a human virus. Despite the lack of epidemiological evidence for a direct link to human cancer, adenoviruses and their gene products have been useful tools to probe the mechanisms of transformation.

The role of the adenoviral E1 genes in transformation has been well studied and documented (reviewed in ref. 88). The E1a and E1b genes are necessary and sufficient for transformation of primary rodent cells in tissue culture for most adenoviruses, except for Ad9 (see below). However there is also evidence that E4 gene products have transformation potential and can contribute to the transformed phenotype produced by E1 genes. The E1 gene products alone are not sufficient to induce tumors in new-born hamsters, as demonstrated by experiments with a chimeric virus containing the E1 region from the highly oncogenic Ad12 and the remainder of the viral genome from Ad5 (89). In addition, rat cells transfected with the E1 region of Ad12 are morphologically transformed but do not form colonies in soft agar (90). However, co-transfection with the E4 region generates a completely transformed phenotype with colonies that grow in soft agar and induce tumors when transplanted into rats. Similarly the morphology and growth of rat fibroblasts transformed by E1 from Ad2 was altered by addition of the E4 region (91). In contrast, Ad9 induces estrogen-dependent mammary tumors in female rats independently of the E1-region, and the major oncogenic determinant resides in the E4 region (92, 93). These studies implicate the E4 region in oncogenicity and recent work has revealed roles for individual gene products.

5.1. Serotype 5

The E4orf3 and E4orf6 proteins of Ad5 have both been shown to enhance transformation of rodent cells in conjunction with the E1a and E1b proteins (94-96). They lead to alterations in morphology, and E4orf6 enhances tumor growth of cells transplanted into rodents. In the case of E4orf6, its ability to inactivate transactivation by the p53 tumor suppressor protein and to cooperate with E1b55K in p53 degradation may contribute to its oncogencity (96). There also appears to be a distinct oncogenic activity that is independent of p53 stability, and maps to a C-terminal segment of E4orf6 that has been termed the "oncodomain" (97). The transforming properties of E4orf3 are not associated with regulation of p53, but may be linked to its affects on the PODs (95).

The E4orf3 and E4orf6 proteins have also been shown to cooperate with E1a to transform rodent cells in a "hit-and-run" fashion (98). The absence of viral genes or proteins in the majority of these tumors suggests that viral gene expression is needed only transiently to induce the mutations leading to transformation, but is not required to maintain the transformed phenotype. The mechanism by which the E4 proteins cause mutations is unknown but

could be accomplished by introducing genomic instability through the targeting of cellular proteins that normally function to recognize and correct damaged DNA.

Our recent discovery that both E4orf3 and E4orf6 target the Mre11 complex identifies a potential cellular factor involved in their transforming potential (43). The Mre11 complex is important for DNA double-strand break repair (DSBR), and has been implicated in homologous recombination, non-homologous end-joining, meiotic recombination and telomere maintenance (99, 100). This complex is thought to be a central player in the human DNA-damage response and may be responsible for linking DNA damage detection to cell-cycle checkpoints and DNA repair. DSBR acts to maintain genomic stability and hence defects in cellular responses to double-strand breaks (DSBs) predispose to malignancy. This can be seen in the human diseases Nijmegen breakage syndrome and AT-like disorder (ATLD), where the NBS1 and Mre11 genes are respectively mutated (101). The Mre11 complex members are components of the PODs, and by altering their nuclear localization the E4orf3 protein may affect their functioning and thus compromise genomic integrity. Disruption of PODs may also result in mis-localization and inactivation of other cellular proteins involved in maintaining genome stability, such as the BLM helicase that is also a constituent of these nuclear bodies and has been linked to DNA repair. Understanding the mechanism of E4orf3 function could provide insight into the functions of PODs and the Mre11 complex in genome stability and transformation.

The E1b55K/E4orf6 proteins target the cellular Mre11 complex for proteasome-mediated degradation (43). This degradation is required to prevent concatemerization of the viral genome during lytic infection but may also have dire consequences for the cell. The E4 proteins can also inhibit DSBs generated by the yeast HO endonuclease in a rAd vector system (102), suggesting that in addition to preventing concatemerization of viral genomes, the E4 proteins will also prevent repair of DSBs in the genome of the infected cell. Removing the Mre11 complex also renders the cell unable to activate the appropriate signal transduction responses to DNA damage (unpublished data). This will affect cell-cycle damage checkpoints and may ultimately lead to the accumulation of DNA damage that could contribute to oncogenicity. Finally, the observed association of both E4orf3 and E4orf6 with the catalytic subunit of the cellular DNA-dependent protein kinase (DNA-PKcs) may affect its function in repair of DNA damage (42).

The E4orf6/7 product of Ad5 has also been tested for its effect on transformation. It can promote morphological transformation of primary rat cells in combination with an E1a mutant that does not bind pRb and release E2F (103). However, in the case of wild-type E1a or E1a plus E1b, inclusion of E4orf6/7 reduced the transformation frequency (96). In other assays it suppressed the growth of untransformed rat cells, possibly in a p53-dependent manner, and could induce apotosis (103). Mutants suggest that both the positive and negative effects of E4orf6/7 on transformation may be linked to its ability to interact with E2F-1 (103).

5.2. Other serotypes

In addition to Ad5, many other Ad serotypes are being developed into rAd vectors, and in many cases the functions of their E4 proteins are unknown. The potential oncogencity of E4 proteins from other serotypes has not been examined extensively, except for the Ad9 serotype. It is interesting to note that while the E4orf3 gene is highly conserved between different serotypes of human adenovirus, not all of the functions are shared. For example, we have found that while all E4orf3 proteins tested were able to disrupt the PML protein into track-like structures, not all led to redistribution of the Mre11 complex (unpublished data). To our knowledge the transforming potential of E4orf3 proteins from serotypes other than Ad5 has not yet been tested but may reveal the importance of specific cellular targets. The oncogenic potential of E4orf6 proteins from other serotypes has not been reported but E4orf6 proteins from Ad4, Ad9 and Ad12 can each target the Mre11 complex for degradation (unpublished data), suggesting that they will also inhibit DSBR.

Ad9 is distinct from the other human Ads in that it elicits only estrogen-dependent mammary tumors in animals (104). The oncogenic determinant maps solely to the E4 region and the product of E4orf1 (28, 93). Expression of Ad9 E4orf1 is sufficient to induce a transformed phenotype and enhance oncogenicity *in vivo* (105). Although the E4orf1 sequence is well conserved and the homologous protein from other serotypes can transform human TE85 cells in culture, only E4orf1 from Ad9 can promote tumors in animals (28, 30). The C-terminal PDZ-binding domain of Ad9 E4orf1 is essential for transformation and mediates interactions with cellular proteins containing PDZ domains (106, 107). It is interesting to note that other viral oncoproteins also bind to PDZ domain proteins (32, 33). Some of these PDZ proteins are common targets of the non-tumorigenic E4orf1 proteins, but the cellular tumor suppressor protein ZO-2 interacts exclusively with E4orf1 of Ad9 and may thus contribute to its oncogenicity (108). Interactions with PDZ proteins results in their sequestration in the cytoplasm, and these interactions are thought to be important for selective stimulation of phosphatidylinositol 3-kinase at the plasma membrane (32, 33). The interaction of Ad9 E4orf1 with PDZ proteins and the subsequent altering of signaling complexes probably contribute to its oncogenic potential.

6. E4 IN ADENOVIRUS VECTORS

The initial impetus to develop rAd vectors deleted of the E4 region came from a desire to accommodate larger transgenes and also in an attempt to decrease the destructive host immune responses to first generation rAd vectors. Production of deleted viruses was achieved either by the use of complementing cell lines that provided essential E4 functions, or by retaining individual orfs that enable replication.

6.1. E4-deleted vectors

The two essential but redundant gene products from E4 are the E4orf3 and E4orf6 proteins. By retaining either E4orf3 or E4orf6 (109), vectors can be generated that can replicate in 293 cells without the need for a specialized complementing cell line. However, a full deletion of the E4 region requires vector growth and propagation in cell lines that complement the defect. To bypass the toxic effects of E4 gene expression, one approach was to generate 293-based cell lines that express only the E4orf6 gene product under the control of an inducible promoter. This was achieved with promoters such as the metal-inducible metallothionein (110, 111), a tetracycline regulated promoter (112) or the glucocorticoid-inducible MMTV (110, 113). Other cells lines were made with the entire E4 region under an inducible promoter (110, 114). These cell lines allowed generation and growth of viruses deleted for both the E1 and E4 coding regions. When tested *in vivo* these viruses produced less late viral proteins than the first generation E1-deleted vectors, and this was accompanied by reduced apoptosis, toxicity, and blunted host immune responses, as well as increased duration of transgene expression (110, 115). However some other groups have reported conflicting results, with no difference in immune responses or persistence of transgene expression and vector DNA (112). Vectors with E4 deletions also displayed diminished humoral immunity in both murine and non-human primate lung, with decreased levels of neutralizing antibodies and reduced IL-6 and IL-8 induction (116). These results suggest that deleting the E4 region can be beneficial for rAd vectors by reducing cytotoxicity and immune responses.

6.2. Retaining individual E4 orfs

Retaining expression of some of the E4 gene products may also have a beneficial effect on transduction from rAd vectors. In some cases it was noted that deletion of the E4 region led to a significant reduction in the efficiency and persistence of transgene expression from E1-deleted vectors (117-119). Long-term extra-chromosomal persistence of the vector DNA suggested that the decrease in expression was due to transcriptional silencing (117-119). The implication from these studies was that expression of an E4 product(s) acted in *trans* to prevent down-regulation of the transgene promoter. A systematic analysis of transgene expression in different vector backbones identified a role for the E4orf3 protein in long-term persistence (120). The effect can be rescued by subsequent infection with a vector that contains E4 genes, suggesting that the E4 product(s) activates transcription from the silenced vector (119). Expression from the CMV promoter was shut-down in vectors that lacked E4, but retention of E4orf3 resulted in longevity of expression in murine lung and liver (120, 121). Silencing appears dependent upon the promoter and tissue type of the targeted cells, and in some organs there may also be a contribution from other E4 gene products (121, 122). The exact mechanism by which E4orf3 regulates activity from heterologous promoters remains unclear, although a contribution may come from increased rates of transcription (121). Vectors that retain a functional E4orf3 may thus provide long-term gene expression combined with the desire for low toxicity and inflammatory response (123).

7. CONCLUSIONS

The effects of E4 on vector biology are dependent upon the components of the transgene cassette, the cell and tissue type being transduced, and the relative immunogenicity of the transgene. Removing the E4 genes from rAd vectors provides clear benefits in some cases, but retaining parts of the E4 region may also enhance vector performance in certain circumstances. Given the potentially hazardous interactions between E4 gene products and cellular regulators, careful consideration should be given as to whether E4 coding regions should be included in rAd vectors.

Including the E4orf3 sequence in rAd vector leads to transcriptional activation and persistence of transgene expression. This is consistent with the observation that E4orf3 expression induces redistribution of the CREB binding protein (CBP), a transcription co-factor that accumulates at PODs, and that this is accompanied by transcriptional activation for at least some promoters (124). It may be possible to include a mutant of E4orf3 that has lost its ability to disrupt PODs and bind DNA-PKcs but still retains its effects on transgene expression. The differences that we have observed between the E4orf3 proteins from different serotypes suggests that targeting of the Mre11 complex may not be shared amongst all of them (unpublished data). Inclusion of the E4orf3 gene from a serotype other than Ad5, or a carefully designed mutant, may supply all the desired effects without the chances of oncogenicity. An interesting experiment in which the ICP0 protein from herpes simplex virus was put under control of the E4 promoter in an E1/E4-deleted virus demonstrated that the promoter was active at a low level, and also suggested that although the POD structures were reorganized there was not a significant perturbation of cellular metabolic function (125). The cellular effects of E4orf3 expression and its disruption of PODs remain to be determined.

Vectors that contain E4 show significantly more induction of hepatotoxicity than vectors that lack E4, but the reason for this is unclear. Toxicity may be due to the effects of E4 proteins on the expression or functioning of endogenous cellular genes. Given the known effects of multiple E4 orfs on the regulation of transcription, splicing and phosphorylation events, this may not be so surprising. Endogenous genes activated by E1-deleted rAd vectors have been analyzed by differential screening of a subtracted library after infection of human endothelial cells (3). Among the activated genes were those that coded for proteins involved in intracellular signaling, growth regulation, and the cytoskeleton. None of these endogenous endothelial genes were activated when cells were infected with a vector deleted of E4 in addition to E1 (3). The role of individual E4 orfs in modulating cellular gene expression after vector transduction of target cells remains to be determined, but the ability of E4orf6/7 to activate genes through E2F (87) may be a contributing

factor. These alterations may also be cell type specific (3) and their consequences will thus vary depending of the target tissue, producing toxicity in liver but perhaps not in other tissues.

Transduction by standard E1-deleted rAd vectors can also perturb normal cell-cycle progression. For example, infection of primary human airway epithelial cells *in vitro* by an E1-deleted virus slowed cell proliferation due to increased apoptotic death and delayed S-phase (126). Infection with rAd vectors may affect multiple cell-cycle checkpoints. Transduction of epithelial cell lines in culture has been shown to cause G2-M arrest of the cell-cycle, which was associated with an increase in the protein levels of cyclin A, cyclin B1, cyclin D and cyclin-dependent kinase (127) . There was also evidence of polyploidy in infected cells. These effects were all absent in infection with a rAd that lacked all E4 orfs except E4orf6 (127), suggesting that E4 gene products in vectors can affect cell-cycle progression.

Many of the functions of E4orf6 are intimately linked to its association with the E1b55K, and as this is removed from all E1-deleted rAd vectors the functions of the E1b55K/E4orf6 complex will be lost. However, E4orf6 by itself still has an effect on cellular proteins. For example, even though E4orf6 can only degrade the p53 tumor suppressor when associated with E1b55K, alone it can still bind and inhibit p53 function (61, 71). The Mre11 complex is only degraded in the presence of both E1b55K and E4orf6 and it appears that it is the E1b55K protein that is involved in substrate recognition (unpublished data). However, it is possible that E4orf6 may also interact with this complex in the absence of E1b55K. E4orf6 has also been reported to bind to the DNA-PKcs protein, although there is no evidence that the kinase activity of this cellular regulator is inhibited by the interaction (42). The association of E4orf6 with these key cellular regulators and its potential oncogenicity, suggest that it would be advisable to delete it from viral vectors unless there is some clearly defined benefit for its inclusion.

Lessons learned from studying the functions of the E4 gene products may also suggest applications outside of the context of rAd vectors. For example, the ability of the E4orf3 protein to increase the duration of transgene expression from the CMV promoter has been utilized in a non-viral vector to enhance expression from a plasmid vector in the absence of any other adenoviral genes (128). The E4orf4 protein preferentially kills transformed cells by inducing apoptosis that is p53-independent (55). It has been reported that a large number of human cancer cell lines are susceptible to E4orf4 killing, while there is minimal effect on primary cells (59). This suggests that therapeutic benefit could be derived from the E4orf4 protein in cancer gene therapy applications. Additionally, degradation of the Mre11 complex by the E4orf6/E1b55K proteins prevents the cell from mounting a DNA damage response (unpublished data) and could thus be used as part of a gene therapy approach to sensitize cancer cells to radiation and chemotherapeutic drugs for cancer treatments. Inactivation of cellular DNA damage responses by E4 proteins could be a contributing factor in the observed synergy between chemotherapy and oncolytic adenovirus viruses for cancer therapy (129), and therefore retaining the E4 region may be beneficial for these vectors.

In conclusion, there appear to be valid arguments both for inclusion and deletion of the E4 region from rAd vectors. Removing E4 orfs reduces cytotoxicity and inflammation *in vivo*, as well as increasing the coding capacity for the transgene. Deleting E4 in addition to E1 decreases viral gene expression and directly eliminates several viral gene products that interact and antagonize important cellular targets. It is clear that the choice of promoter is critical for sustained transgene expression *in vivo* from E4-deleted vectors. Most of the studies so far described with E4-deleted rAd vectors have been performed in mice and so extrapolation of the results to larger animals and humans remains to be determined. However, there may be some sacrifices that are made when E4 genes are deleted, such as the effect of E4orf3 on transgene persistence *in vivo*. Ongoing studies to separate the functions of E4orf3 may provide mutants that retain the effects on transgene persistence but have lost the potential for oncogenicity. It has also been suggested that the E4 region imparts some protection against lysis by Ad-specific cytotoxic T lymphocytes in the transduced target cell (130). Although transformation by adenovirus proteins has not been reported for primary human cells (all studies have been done in rodent cells), the interactions with DNA repair factors (p53, DNA-PKcs and the Mre11 complex) have all been observed in human cells. The potential negative effects of E4 gene expression from vectors on genome integrity in the target cell remains to be determined but given recent findings it should be examined. In cases where long-term gene expression is required, such as with gene therapy of inherited diseases in humans, there should be prudent selection of the most appropriate vector in order to minimize potential safety concerns. Care should also be taken in interpretation of gene function studies when using rAd vectors that still contain the E4 region. Further studies that probe the functions of this interesting collection of proteins expressed from the E4 region will contribute to our understanding of how they affect cell processes and will help to determine whether they should be deleted or retained in rAd vectors.

8. ACKNOWLEDGEMENTS

I am grateful to past and present members of my lab for fruitful discussions on the functions of the E4 proteins. Our work on E4 has been supported by grants from the National Institutes of Health (AI43341 and CA97093). I apologize to those whose primary work was not cited due to space constraints.

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Key Words: Adenovirus, Gene Therapy, Recombinant Viral Vectors, Virus Transformation, E4 Proteins, Review

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