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REVIEW ARTICLE

E4 gene function in adenovirus, adenovirus vector and adeno-associated virus infections

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Introduction

Over the past three years, there have been several interesting advances in our understanding of the functions encoded by the adenovirus (Ad) E4 gene. In this review, I hope to consider these recent findings in the context of earlier work. Most studies of E4 have focussed on the closely related human serotypes Ad2 and Ad5; however, specific features of other serotypes are mentioned when appropriate. Within the limits of space available, I have not been able to cite all the older relevant papers and apologize to my colleagues for any omissions; other reviews give a fuller coverage of this material (Bridge & Pettersson, 1995; Imperiale *et al.*, 1995; Cress & Nevins, 1996). For an overview of Ad replication, see Shenk (1996).

The Ad E4 gene

Human Ads have characteristic linear double-stranded DNA genomes 34–36 kbp in length. Several genomes have been completely sequenced, and partial sequence is available for other types. To date, all these viruses have a gene that is clearly related to the E4 gene identified in the first Ad to be sequenced, human Ad2. In the distantly related avian Ad, chicken embryo lethal orphan virus (CELO), which has a nearly 44 kbp genome, there is no recognizable E4 region (Chiocca *et al.*, 1996), although at least one human Ad E4 product has a structural analogue in a similar location in CELO (Weiss *et al.*, 1997). A schematic representation of the Ad5 genome and a detail of the E4 region are shown in Fig. 1. Primary transcripts from the E4 promoter are subject to alternative splicing events that produce an array of at least 18 distinct mRNAs (Virtanen *et al.*, 1984). These are predicted to encode seven different polypeptides, all but one of which (Orf3/4) have been demonstrated to exist in infected cells. The other human Ad E4 genes characterized so far have a similar sequence organization although the Orf1 reading frame is not conserved in Ad40 (Davison *et al.*, 1993). Non-human mammalian Ads have E4 regions with homology to Ad5 E4 which varies in extent from considerable identity across four reading frames in bovine Ad2 (Fitzgerald *et al.*, 1997) to limited homology only with Orf6 in murine Ad1 (Ball *et al.*, 1991) and ovine Ad (Vrati *et al.*, 1996); there may be two Orf6 homologues in the ovine virus.

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Genetic analysis of E4 function

The E4 region encodes one or more functions which are required for lytic growth in standard cell culture systems, as demonstrated by the grossly defective phenotype of virus in which the entire E4 gene is deleted. However, initial attempts to define the functions of individual polypeptides by mutation of each E4 reading frame met with limited success since only an E4 Orf6 mutation showed any effect on growth and this effect was modest in comparison with the effect of a total E4 deletion (Halbert *et al.*, 1985). Subsequently, this paradox was resolved by the discovery that two of the E4 products, Orf3 and Orf6, could partially or totally compensate for deficiencies in each other (Bridge & Ketner, 1989; Huang & Hearing, 1989*a*). Much attention has since been focussed on understanding the functions of Orf3 and Orf6. Despite the minimal effect on virus

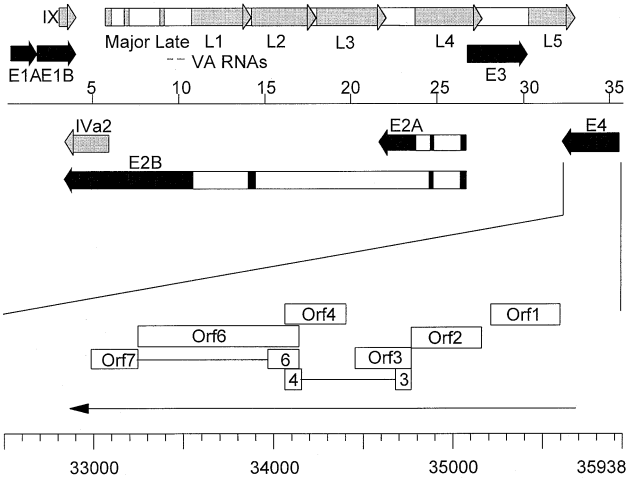

Fig. 1. The genome organization of human Ad5. At the top of the figure the genome is represented as a line with lengths marked in kbp from the conventional left end. Thick arrows represent early and late transcription units (black and grey, respectively). Open boxes represent the major introns. The E4 gene is enlarged as a line scale with lengths in bp. The primary transcript is shown as a black arrow in a 5′ to 3′ direction and each of the potential encoded proteins is shown as an open box; proteins whose coding regions are split by intron sequences are shown as boxes linked by a line. Overlapping proteins have different polypeptide sequences unless they share the same number.

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growth of interrupting the other E4 reading frames, functions for two further proteins, Orf4 and Orf6/7, have since been defined, as has a role for the Orf1 protein in mammary tumorigenesis induced by Ad9.

Functions of E4 Orf3 and Orf6

The roles of Orf3 and Orf6 in promoting viral gene expression and replication

The understanding of how E4 Orf3 and Orf6 operate was advanced by molecular analysis of the infectious cycles of appropriate mutants. Much of this information has been reviewed previously (Imperiale *et al.*, 1995). Both proteins increase viral late protein production by facilitating the cytoplasmic accumulation of the relevant mRNAs at a post-transcriptional level. Orf6 operates as a complex with the 55 kDa ('55K') product of the Ad E1B gene to increase the rate of export of these mRNAs from the nucleus (RNA transport). This complex also inhibits the export of most cellular mRNAs. Additionally, both Orf3 and Orf6, operating independently, can improve the stability of unprocessed late RNA in the nucleus and hence increase the pool of RNA available for maturation and transport. Thus, although mutations in either of these two E4 proteins can be complemented to an extent by the other protein, they do not affect identical functions.

The E4 Orf6–E1B 55K complex does not alter the pattern or extent of splicing of late viral RNAs and it is thought to act at an early point in the pathway of RNA transport (Leppard & Shenk, 1989; Bridge & Ketner, 1990). In contrast, the parallel and redundant functions of Orf3 and Orf6 in nuclear RNA stabilization may be linked directly to RNA splicing. Both proteins have been shown to affect RNA splicing patterns (Öhman *et al.*, 1993; Nordqvist *et al.*, 1994); Orf3 promotes exon inclusion in both Ad major late (ML)-derived and non-viral transcripts while Orf6 promotes exon exclusion. The splicing of ML transcripts is complex and these two proteins are required for the proper completion of this process, failing which the RNAs show an enhanced rate of degradation. The fact that the nuclear RNA stabilization effect common to Orf3 and Orf6 is restricted to RNAs from the ML promoter fits with this interpretation (Bridge *et al.*, 1991). However, it is not clear from these results how two proteins with opposite activities in RNA splicing can complement for each other's absence during an infection.

Both Orf3 and Orf6 also play a role in the replication of Ad DNA. Mutant viruses unable to make one or other protein show a modest reduction in DNA synthesis; mutants unable to make either protein are substantially deficient in replication (Bridge & Ketner, 1989; Huang & Hearing, 1989*a*). Although the replication defects of mutants unable to make any E4 proteins and those specifically impaired in both Orf3 and Orf6 appeared from these studies to be identical at moderate multiplicities of infection, it was later shown that Orf4 modulates these effects (Bridge *et al.*, 1993*a*). Viruses which retain Orf4 in the absence of Orf3 and Orf6 are severely defective in replication at multiplicities and times post-infection when total E4-deletion mutants produce close to normal levels of DNA; supplying Orf4 either *in cis* or *in trans* to these latter infections inhibits replication. Since Orf4 has since been shown to down-regulate both E1A activity and the activity of various cell transcription factors (discussed below), its effect on DNA replication may be attributed to reduced expression of both viral and cellular proteins necessary for replication, consequent upon these transcriptional effects. This, however, has not been formally demonstrated.

Replication of Orf3/Orf6-deficient and total E4-deletion mutants is qualitatively as well as quantitatively impaired. These viruses produce concatemeric Ad DNA which includes a variety of DNA junctions between the two genome ends; this is not seen in infections by wild-type or other types of E4-mutant virus (Weiden & Ginsberg, 1994). Such concatemers are not thought to be an essential feature of the Ad DNA replication mechanism. It is possible that they do arise at low levels during a wild-type infection and that an E4 product or another factor which is dependent upon E4 for its activity normally resolves them into linear molecules. However, since the concatemers detected by Weiden & Ginsberg did not contain perfect end-to-end joins, their resolution could not easily give rise to intact linear genomes.

What then is the role of the Orf3 and Orf6 proteins in Ad DNA synthesis? Perhaps the almost total failure of late gene expression in these infections, whereby the proteins which complex with DNA to form new viral cores are not made, leaves the DNA produced vulnerable to degradation, recombination and/or ligation and thus the extent of productive replication is diminished. This might also explain the production of concatemers by Orf3/Orf6 mutants. Alternatively, absence of both proteins may have effects on early gene expression which directly affect the capacity for DNA synthesis; early protein synthesis by such mutants has not been characterized in detail. Finally, the absence of Orf3 and Orf6 may delay changes in the population of host cell factors or structures in the nucleus that are necessary to support optimal viral DNA synthesis.

Orf3 and Orf6: interactions with host cell components

The various stages of Ad gene expression and replication in the nucleus occur in the context of a precise spatial organization which develops during the course of infection (reviewed by Bridge & Pettersson, 1995, 1996). Given the complex effects of the Orf3 and Orf6 proteins on gene expression and replication, there is considerable interest in understanding the interactions which these proteins make with host cell components during this process.

Orf6, which is known to associate with E1B 55K, is needed for the latter protein to associate with the peripheral zones of viral inclusion bodies which form in the nucleus during

infection (Ornelles & Shenk, 1991); these are regions where late viral transcription and RNA processing occur (Pombo *et al.*, 1994). In the absence of Orf6, E1B 55K instead remains associated with discrete granular or fibrillar structures in the nucleus (Ornelles & Shenk, 1991). These data led to the suggestion that E1B 55K–Orf6 associates with and causes the redistribution within the cell of some cell factors necessary for RNA biogenesis. More recently, the effect of Orf6 on E1B 55K localization has been examined by transfection (Goodrum *et al.*, 1996). In this system, Orf6 expressed in human and other primate cells is absolutely required for the E1B protein to localize to the nucleus; in its absence, E1B 55K adopts a cytoplasmic localization that is not seen during infection. Surprisingly though, Orf6 is not able to move E1B 55K to the nucleus in non-permissive rodent cells. These findings clearly implicate a cell type-specific factor in the normal localization of these proteins, although there must also be an Orf6-independent mechanism whereby E1B 55K can reach the nucleus during infection.

Recent data suggest that E4 Orf3 directly affects the distribution of a group of essential transcription/replication factors in the nucleus. Structures known as PODs have been defined in the nucleus using a variety of specific antisera (Dyck *et al.*, 1994). Ad infection rapidly promotes a reorganization of these structures, with their protein components appearing first in tracks and some then moving to peripheral regions of the virus replication zones (Carvalho *et al.*, 1995; Doucas *et al.*, 1996). Using viruses with a variety of mutations, this POD reorganization function was assigned to E4 Orf3, perhaps with a subsidiary role for Orf6. Orf3 protein co-localizes with POD components, both before and after reorganization; the reorganized POD components also associate transiently with the E1B 55K protein before it moves to the periphery of the replication centres. Orf3 alone can induce POD reorganization in the absence of infection.

The failure of POD reorganization in E4-negative infections is overcome to an extent by a high multiplicity of infection, suggesting a link between this physical manifestation of E4 function and the multiplicity-dependent defect in DNA replication by these mutants. Ad infection also results in a progressive redistribution of splicing factors in the nucleus (Bridge *et al.*, 1993*b*, 1995). The relationship of this reorganization to the effect on PODs is not certain. Carvalho *et al.* (1995) reported that the POD component PML did not co-localize with splicing factors, but this result was recorded at 16 h post-infection, a time when splicing factors have been reported already to be localized to the peripheral zones of the replication centres and those POD components which relocate to the replication centres (PML is not one of these) have already reached their target (Bridge *et al.*, 1993*b*; Doucas *et al.*, 1996); it is possible therefore that splicing factor and POD component re-distribution may be aspects of a single process.

Finally, the E4 Orf6 protein has been reported recently to bind to p53 (Dobner *et al.*, 1996). p53 is a critical regulator of the cell cycle, responding to DNA damage by arresting the cycle or committing the cell to apoptosis (reviewed by Ko & Prives, 1996) and it is well established that the E1B 55K protein interacts with p53 to block its transcription activation function (Kao *et al.*, 1990; Yew & Berk, 1992). Orf6 also binds to p53 in a manner that is independent of the presence of E1B 55K, and this interaction is as effective as the binding of E1B 55K in blocking p53 activity (Dobner *et al.*, 1996). Furthermore, Orf6 alone can block p53-induced apoptosis and can cooperate with E1A to transform rodent cells; it can also enhance transformation by E1A plus E1B 55K (Moore *et al.*, 1996). The sequences of Orf6 and E1B 55K needed for their reciprocal interaction have recently been mapped (Rubenwolf *et al.*, 1997). Whilst the binding site for Orf6 on E1B 55K is very similar to that of p53 (Kao *et al.*, 1990), there is no requirement for p53 in order to observe the direct association of Orf6 with E1B 55K. Since Orf6 and E1B 55K bind to one another and their mapped binding sites on p53 do not overlap it is possible that they may both bind to p53 simultaneously to form a ternary complex.

Why Ad apparently provides two independent functions for p53 inactivation is unclear. Given the observation that E1B 55K and Orf6 are additive in their cooperation with E1A in transformation assays, perhaps neither of them alone is able to inactivate p53 completely under normal intracellular conditions. Such inactivation is believed to be crucial for the success of infection by Ad and other DNA viruses and there is a precedent for the redundant provision of important functions in Ad with the presence of three mechanisms for counteracting the effect of tumour necrosis factor on infected cells (reviewed by Wold *et al.*, 1995).

Function of E4 Orf1 in lytic infection and oncogenesis

The oncogenic properties of Ads, in particular tumour formation in rodents mediated by Ad12 and cell transformation by Ad2, Ad5 or Ad12, have been widely studied and attributed to cooperation between E1A and E1B gene products. However Ad9, which causes mammary tumours in susceptible strains of rat, presents an exception. Studies of recombinants between Ad9 and the closely related but non-tumorigenic Ad26 showed that Ad9 E4, in the presence of E1A and E1B from either serotype, was required to confer the mammary tumorigenesis phenotype (Javier *et al.*, 1992) and that disruption of the Orf1 reading frame eliminated this property (Javier, 1994). Thus, the E4 Orf1 protein confers on Ad9 its specific mammary tumorigenesis and transforming properties.

Ad9 E4 Orf1, expressed from a cytomegalovirus (CMV) promoter, is sufficient for CREF cell transformation but, despite the Orf1 proteins being relatively well conserved among human Ad of different subgroups, other Orf1 proteins were inactive in this assay (Javier, 1994). However, in an independent study, Öhman *et al.* (1995) showed that Ad2 E4

Orf1 could increase the size and alter the morphology of Ad2 E1-induced CREF cell foci; recently, the apparent absence of transforming activity in Orf1 from viruses other than Ad9 has been shown to be due to extremely low levels of expression of these Orf1 proteins in CREF cells (Weiss *et al.*, 1997). In contrast, levels of expression of Orf1 from all serotypes tested were much closer to that of Ad9 Orf1 when transfected into human TE85 cells, and in these cells all the Orf1 constructs gave equivalent levels of transformation. It appears then that E4 Orf1 is a transforming protein in a variety of human Ad serotypes but that this activity is manifest only when expression reaches a sufficient level.

The biochemical function of Ad9 Orf1 is not yet understood. Orf1 sequences are related to those of dUTPase enzymes from various sources. However, the human Ad Orf1 sequences all lack an essential conserved dUTPase motif and are inactive in dUTPase assays (Weiss *et al.*, 1997). In contrast, the avian Ad CELO has a putative dUTPase gene in a location analogous to E4 Orf1 (Chiocca *et al.*, 1996); this gene retains all essential dUTPase motifs and is active in the enzyme assay when tested as a GST fusion protein (Weiss *et al.*, 1997). Thus, the human Ad Orf1 genes appear to have evolved from an ancestral dUTPase gene and to have acquired transforming activity in the process.

What might be the role of E4 Orf1 in lytic infection? Ad9 Orf1 is detectable during infection of human A549 cells (Javier, 1994) and is located in the cytoplasm of transformed CREF cells (Weiss *et al.*, 1996) but expression has not so far been demonstrated for other virus types. As already noted, Orf1-deficient Ad5 shows no growth defect in HeLa cells. Given the transforming activity of Orf1, its role in lytic infection may be to stimulate quiescent cells and so it may be required only in certain cell types. However, this would be likely to be an early function and mRNA capable of encoding Orf1 accumulates relatively late during Ad5 infection of HeLa cells (Dix & Leppard, 1993). Alternatively, since Orf1 protein expression can apparently vary with cell type, expression in those cells where Orf1 mutants have been tested phenotypically may be insufficient for a difference between wild-type and mutant to have been observed.

Functions of the remaining E4 proteins in lytic infection

The role of Orf6/7

E4 Orf 6/7 modulates the activity of the cellular transcription factor E2F. It facilitates the cooperative binding of E2F to DNA at two E2F sites which are present as an inverted repeat in the Ad E2 early promoter by forming a direct complex with E2F and stabilizing the DNA-bound form (Huang & Hearing, 1989 *b*). Orf6/7 dimerizes to link two E2F molecules and thereby stabilizes their binding to the E2 promoter (Cress & Nevins, 1994; Obert *et al.*, 1994). This induced, stabilized binding of E2F is suggested to correlate with the ability of E4 to transactivate the E2 early promoter (Babiss, 1989; Hardy *et al.*, 1989; Reichel *et al.*, 1989), although the timing of appearance of this enhanced binding form of E2F does not always coincide with the activation of the E2 promoter; this may reflect differences in the sensitivity of these different types of assay.

E2F activity comprises a variety of heterodimers between E2F polypeptide family members and a related molecule, DP-1. The activity of these is controlled during the cell cycle by their binding to the retinoblastoma protein p105 and to other members of this family (reviewed by Cress & Nevins, 1996). The sequences in E2F-1 and DP-1 that mediate association with E4 Orf6/7 have been characterized (Cress & Nevins, 1994; Helin & Harlow, 1994; O'Connor & Hearing, 1994). These sequences overlap with the binding site for proteins of the p105 family. Phosphorylation of E2F-1 also regulates these interactions (Fagan *et al.*, 1994). Phosphorylation of specific residues blocks binding to p105 but is essential for binding Orf6/7. Thus, binding of E2F to E4 Orf6/7 and to p105 is mutually exclusive. Although it retains specific affinity for E2F sites, the complex E2F–p105 complex is thought to be transcriptionally inactive; however, the complex with E4 Orf6/7 is, as already discussed, an activator via E2F sites.

Although much of the evidence for the activity of E4 Orf6/7 comes from studying its effect on the E2 promoter, this activation may not be the crucial effect of E4 Orf6/7 since the E2 promoter is still activated sufficiently to support normal growth in virus which lacks Orf6/7 expression (Halbert *et al.*, 1985). Rather, the critical targets of Orf6/7 activation via E2F may be cellular genes whose products are important for the S phase of the cell cycle. Several of these, including the *E2F-1* gene (Johnson *et al.*, 1994), have E2F sites in their promoters that are arranged similarly to those in Ad E2 and activation of these genes may be the role for which Orf6/7 has evolved.

The role of E4 Orf4

Orf4 regulates protein phosphorylation in the infected cell by binding to protein phosphatase (PP)2A (Müller *et al.*, 1992; Kleinberger & Shenk, 1993). It is not clear whether PP2A is activated by its binding to Orf4 or whether its substrate specificity or localization might be altered. Interaction of PP2A with Orf4 results in the selective hypophosphorylation of some proteins, including Ad E1A and the c-Fos component of the AP1 transcription factor. These hypophosphorylated E1A residues have now been mapped (Whalen *et al.*, 1997); at least one is a target for mitogen-activated protein kinase, which is itself regulated by phosphorylation. Recently, Orf4 was shown to negatively regulate both E4 and E1A transcription during infection, dependent on PP2A activity, and to repress E1A-mediated activation of the E4 promoter in transient assays (Bondesson *et al.*, 1996). Since this repression operated via the E1A CR3 region in a manner that was independent of induced hypophosphorylation of E1A, it is likely that the critical target

for Orf4 here is a cellular factor rather than E1A itself. In contrast, Whalen *et al*. (1997) found that the state of phosphorylation of the relevant residues in E1A was a crucial determinant of E1A-mediated transactivation of E4.

Taken together, these results show that a regulatory loop exists in which E1A proteins first activate E4 transcription, and then E4 Orf4 protein negatively regulates both E1A activity and, directly or indirectly, E4 transcription. The advantage for the virus of this arrangement is perhaps to limit cytotoxic effects during the earlier phases of infection so ensuring maximum productivity. This would fit with the observation of Müller *et al*. (1992) that E4 Orf4 mutants are more effective than wild-type in killing non-permissive rodent cells. These cytotoxic effects may themselves stem from E4 since it has recently been shown to encode a product required for E1A-induced, p53-independent apoptosis (Marcellus *et al*., 1996).

The roles of E4 Orf2 and Orf3/4

There is no functional information about the remaining two proteins from E4. The Orf2 protein is a soluble cytoplasmic component in infected HeLa cells, produced at early times post-infection (Dix & Leppard, 1995). It is not detectably complexed with other viral or cellular proteins. The Orf3/4 protein is predicted to exist, based on analysis of Ad2 mRNA structure in HeLa cells (Virtanen *et al*., 1984), but the protein has not yet been detected in infected cells. Usage of the 5′ splice site required to form the Orf3/4 reading frame was not detected in an Ad5 HeLa cell infection (Dix & Leppard, 1993).

The role of E4 products in AAV infection

Alongside efforts to define the roles of Ad proteins, including E4, in Ad lytic growth and transformation, there has been continuing interest in understanding how Ad functions contribute to the growth of adeno-associated virus (AAV), a parvovirus of the genus *Dependovirus* (for a review of AAV replication, see Berns, 1996). AAV was originally thought to be absolutely dependent on helper functions for its lytic growth, but it is now known that this virus can productively infect without help if the intracellular environment is altered by the application of either genotoxic agents or heat stress. In untreated cells, helper functions can be supplied by either Ad or various herpesviruses, and a variety of Ad genes including E4 have been shown to be required by genetic analysis. The E4 protein implicated in AAV helper function is Orf6. As already discussed, this protein cooperates with an Ad E1B protein to enhance Ad mRNA transport and it is thought to play a similar role in AAV infection; mutant Ad unable to make either the E1B protein or E4 Orf6 are equally deficient in supporting AAV cytoplasmic mRNA accumulation (Samulski & Shenk, 1988).

AAV DNA replication is also severely impaired in the absence of Orf6 (Samulski & Shenk, 1988; Huang & Hearing, 1989*a*). Considering the dependence of AAV gene expression on Ad E4 Orf6 function, this replication defect was thought to be due to a failure to produce the AAV Rep proteins in sufficient quantity. However, recent work has shown that Orf6 has an additional role in promoting formation of a dsDNA molecule from the genomic ssDNA of the incoming virus (Ferrari *et al*., 1996; Fisher *et al*., 1996). In both studies, a recombinant AAV was used in which all viral genes were replaced by a CMV promoter–β-galactosidase (β-gal) gene construct. β-Gal-expressing cells occurred much more frequently in transduced cell populations when Orf6 was supplied *in trans*, either by Ad or an expression plasmid, and this correlated with efficient AAV second-strand synthesis.

The mechanism by which Orf6 promotes AAV second-strand synthesis is not certain but may relate to the creation in the cell of an environment in which DNA repair synthesis is active. In support of this interpretation, Ferrari *et al*. (1996) showed, using the same assay system, that the genotoxic treatments or stresses which can render cells permissive for AAV growth in the absence of helper can also mimic the effect of E4 Orf6 on β-gal transduction frequency and second-strand synthesis. Since Orf6 is involved in altering the distribution of nuclear components during Ad infection, it may be required by both viruses to make available cellular proteins that are otherwise held in inactive forms in the nucleus. It is interesting to speculate that the AAV replication defect in the absence of Orf6 and the Ad replication defect in the absence of Orf3 and Orf6 discussed earlier may in some way be linked.

E4 and Ad vector development

Much of the recent work on human Ad has been aimed at developing the virus for use as a vector, especially for gene therapy applications. The first generation of Ad vectors lacked E1A, E1B and E3 sequences. To address some of the shortcomings of these vectors, specifically their limited capacity for foreign DNA, their capability for residual replication and their provocation of immune responses, various groups have been working to eliminate more viral genes from them; one target for deletion has been the E4 region. However, recent papers present apparently conflicting data concerning the effect of E4 deletion on transgene persistence *in vivo*.

Gao *et al*. (1996) showed that longevity of β-gal transgene expression from a hybrid CMV enhancer–chicken β-actin promoter in mouse liver was increased by total deletion of E4, provided the animals were immunologically tolerant to β-gal. However Kaplan *et al*. (1997), comparing vectors that were either $E4^+$ or $Orf6^+$ only, found that persistence of expression from CMV-driven β-gal in mouse lung was reduced in $Orf6^+$ vector transduction, again in the absence of immune responses. Finally, Armentano *et al*. (1997) showed that persistence of transgene expression, rather than persistence of the gene itself, could be affected by the E4 status of the vector backbone with the CMV promoter/enhancer being specifically down-regu-

lated in the absence of a wild-type E4 gene; E4 Orf6 alone was not sufficient to overcome this effect.

The basis of these effects of E4 on transgene persistence remains unclear. Armentano *et al.* (1997) considered and discounted the possibility that a low level of Ad replication, dependent on E4, might be necessary for persistence because mutation of E2A, one of the Ad genes directly involved in Ad replication, improves rather than diminishes persistence but they did not rule out non-conventional replication via host functions. This is an interesting suggestion given the effects of E4 deletion on both Ad and AAV replication already discussed. Perhaps non-replicating Ad genomes in the nucleus are vulnerable to DNA damage and must be sustained in a functional state by repair functions which E4 induces. This is clearly an area for further experiment.

From the work discussed above, it seems likely that Ad vectors lacking E4 will not be effective in delivery and long-term retention of transgene expression under all circumstances. It will be important to define carefully the circumstances in which such vectors can work well given the increasing evidence that E4 deletion may be necessary for safety reasons, namely the observations that Orf1 can have transforming activity (Javier, 1994), that Orf6 can block p53 activation (Dobner *et al.*, 1996) and can transform cells in cooperation with E1A (Moore *et al.*, 1996), and that E4 products can mediate E1A-induced apoptosis (Marcellus *et al.*, 1996). It is clear that careful consideration needs to be given to the inclusion or exclusion of specific E4 coding regions from Ad vectors, based on the growing understanding of the functions of their encoded proteins.

Conclusion

Considerable progress has been made recently towards a full understanding of the functions of the Ad E4 proteins. These proteins appear so far to encompass a diverse collection of functions, apparently contrasting with the grouping of related functions into Ad transcription units that has been noted for regions such as E2 and E3. An overall theme for E4 function as it is understood at present could be the modulation of gene expression and replication through interaction with host cell systems. No doubt the rapid flow of new information about these proteins will quickly determine how accurate or durable is this assessment.

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