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RAPID COMMUNICATION

Identification and Elimination of an Aberrant Splice Product from cDNAs Encoding the Human Adenovirus Type 5 E4orf6 Protein

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Growing awareness of the central role of the E4orf6 protein in regulating the infectious cycle of human adenoviruses has led to greatly intensified efforts to define its functions and mechanisms of action. Many workers employ cDNAs to generate plasmid or viral vectors to express E4orf6 in the absence of other viral products. In addition to the normal 34-kDa product, such vectors consistently produce a polypeptide of about 8 kDa. In the present report we show that this protein is produced by an aberrant mRNA utilizing the 5′ splice donor site used normally by the virus to produce the E4orf6/7 product, which shares 58 residues with E4orf6. This amino terminal coding sequence is linked to a 3′ sequence via a novel splice acceptor site in an alternative reading frame of the E4orf6 cDNA. The 5′ donor site was altered by PCR-directed mutagenesis to yield a construct that produces high levels of E4orf6 in the absence of the 8-kDa polypeptide. This construct should eliminate some of the problems encountered previously using the wild-type E4orf6 coding sequence.

The early region 4 (E4) transcription unit of human adenoviruses produces at least seven products through extensive RNA splicing (*1*). The 34-kDa polypeptide of E4 open reading frame 6 (E4orf6) performs several functions critical for viral replication. Another transcript encodes a related product, E4orf6/7, which shares 58 amino terminal residues with E4orf6 and functions to bind to and facilitate the activity of transcription factor E2F (*2–4*). E4orf6 is a nuclear protein that contains both a nuclear localization signal (NLS) and an amphipathic arginine-rich α-helical nuclear retention signal (NRS) believed to target E4orf6 to the nucleus (*5, 6*). In addition, a nuclear export signal (NES) is present that has homology to the HIV Rev protein NES (*7*) and is believed to mediate nuclear-cytoplasmic shuttling of E4orf6 (*5*). E4orf6 was recently shown to be a zinc-binding protein, and mutation of several of the conserved cysteine and histidine residues inactivate E4orf6 functions (*8*). Although E4orf6 can interact directly with the cellular p53 tumor suppressor (*9, 10*) and block its ability to transactivate some target promoters (*9*), it derives much of its biological activity when complexed with the early region 1B 55-kDa (E1B-55K) protein (*11–13*). E4orf6/E1B-55K complexes are also involved in the selective transport of viral mRNAs to the cytoplasm late in the infectious cycle (*14–17*). This complex also enhances viral mRNA translation (*18, 19*), at least in part by this selective transport function. Finally, E4orf6/E1B55K complexes induce the degradation of the p53 tumor suppressor (*10, 20–22*). This function is critical, as expression of the early region 1A (E1A) protein activates and stabilizes p53 (*23, 24*), an effect that could lead to premature apoptosis of the infected cell. Inhibition and degradation of p53 probably at least partially underlie the ability of E4orf6 to enhance transformation by E1A and other oncogenes (*20, 25, 26*). E4orf6 and E4orf6/7 were recently found to associate with DNA-PK and inhibit double-strand break repair (*27*), thus preventing the formation of adenovirus genome concatamers during replication (*28*). Several of these functions may involve complex formation by E4orf6 with a series of cellular proteins of as yet unidentified cellular proteins (*29*).

A number of laboratories study the functions of E4orf6 in the absence of other adenovirus proteins using either plasmid expression vectors (*19*) or an adenovirus vector (*10*) containing E4orf6 cDNAs. We have noticed that, in addition to the 34-kDa E4orf6 product, a major protein of about 8 kDa is produced from these cDNAs. Expression of this species could complicate interpretation of studies on E4orf6 if this protein possesses biological activity. In the present studies we have used a series of E4orf6 deletion mutants to identify the origin of the 8-kDa species and have generated a new construct that produces high levels of E4orf6 in the absence of this polypeptide.

Previous studies indicated that the 8-kDa species was

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detected using rabbit polyclonal serum specific for the amino terminus of E4orf6, but not with a serum prepared against the carboxy terminal region (*29*). These results suggested that the 8-kDa species contains amino terminal E4orf6 sequences. Our group has made a large series of small in-frame E4orf6 deletion mutants to undertake detailed functional mapping of E4orf6. Construction of such mutants involved both restriction enzyme digestion and PCR-mediated mutagenesis and has been described in detail elsewhere (E. Querido, S. W.-L. Thirlwell, M. R. Morisson, H. Chu-Pham-Dang, D. Boivin, and P. E. Branton, submitted for publication). Briefly, the cDNA for human adenovirus type 5 (Ad5) E4orf6 was cloned into pcDNA3 vector DNA in which expression of E4orf6 occurs from the cytomegalovirus (CMV) promoter. All mutants were present in this vector. The internal deletion mutants were prepared by a four-oligonucleotide PCR method (*30*). The E4orf6sf mutant was also prepared by this method using appropriate oligonucleotides. The G198A, R240E/R241E, and E255A/E256A point mutants were made by three-oligonucleotide PCR amplification and dl1-13 by a two-oligonucleotide PCR amplification. Mutant dl275–294 was obtained by first engineering stop codons in each of the three reading frames at the carboxy terminus of E4orf6, followed by digestion with the unique cutter, *BSS*HII, and then blunt end ligation. All E4orf6 mutants were verified by sequencing. Point mutants affecting the NES (L90A/I92A) and the NRS (R248E), as well as an NLS/NRS double mutant (L90A/I92A/R248E), were generously provided by Matthias Dobbelstein (*5*).

Plasmid DNA encoding wild-type and mutant forms of E4orf6 were introduced into H1299 cells by lipofection and, following separation by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and transfer to nitrocellulose filters, E4orf6-related products were detected by Western immunoblotting using E4orf6-N serum, which recognizes the amino terminus of E4orf6 (*29*). Figure 1 shows the results of such an experiment. Clearly all cDNAs expressed high levels of E4orf6 protein. In addition to full-length E4orf6, two different cDNAs containing wild-type E4orf6 also expressed the 8-kDa species (lanes 19 and 24). Extracts from cells lipofected with empty pcDNA3 vector contained neither product (lane 23). The 8-kDa species was also detected with cDNAs expressing E4orf6 containing various point mutations (lanes 16–18 and 20–22), including those affecting the NRS (R240E/R241E, lane 17; R248E, lane 21) and the NES (L90A/I92A, lane 20; L90A/I92A/R248E, lane 22). The deletion mutants were more informative as to the origin of the 8-kDa species. It was detected with dl65–82 (lane 6) and all deletion mutants toward the carboxy terminus (dl83–94, etc., lanes 6–14), except dl249–274 (lane 15, see below); however, it was absent with deletion mutants dl1–13, dl13–23, dl23–34, dl35–43, and dl49–69 (lanes 1–5, respectively). These results indicated that the 8-kDa species must contain a maximum of 69 amino terminal residues of E4orf6.

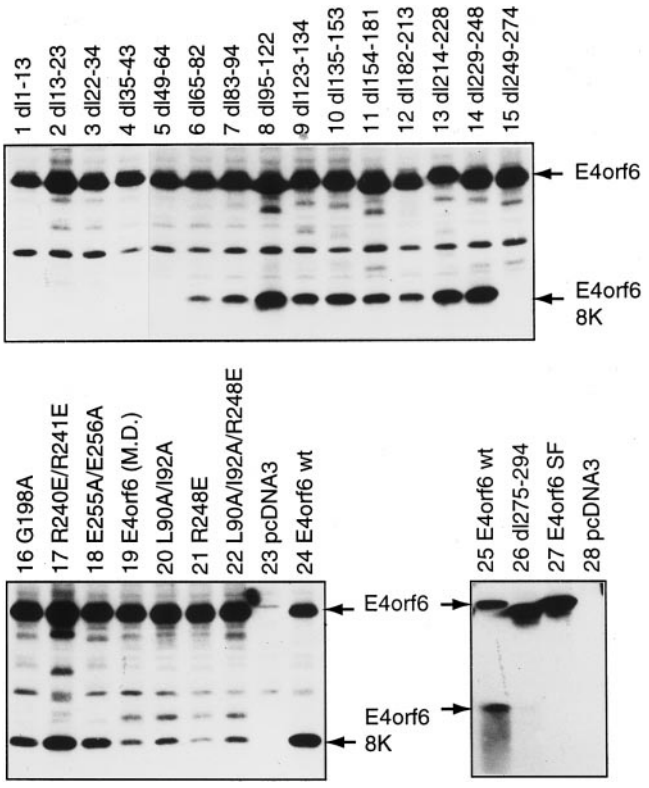

FIG. 1. Analysis of the synthesis of E4orf6-related proteins using cDNAs expressing wild-type and mutant E4orf6 products. H1299 cells were lipofected according to the Gibco/BRL lipofectin protocol. Whole cell extracts were prepared and 40 μg of protein was analyzed by SDS–PAGE using gels containing either 14 or 15% polyacrylamide. After transfer to nitrocellulose, samples were immunobloted using 1808 E4orf6 N-terminal serum, as described previously (29).

The most likely explanation for the appearance of the 8-kDa species was that it is created from the strong splice donor site for the mRNA encoding E4orf6/7, which shares 58 amino terminal residues with E4orf6. Figure 2A shows the amino and carboxy terminal amino acid and nucleotide sequences for E4orf6. E4orf6 contains a perfect C/A-A-G-G-T-A/G-A-G-T donor site starting at nucleotide A172, in which the splice occurs following nucleotide 174 to yield a 58-residue amino terminal region. Analysis of the E4orf6 sequence indicated that one likely splice acceptor exists that conforms to the consensus sequence T/C → 11bp–N-C/T-A-G-G, located at C792-A793-G794-G795 (see Fig. 2A). This site also seemed the most probable acceptor because mutant dl249–274, which lacks this region, does not produce the 8-kDa polypeptide (Fig. 1, lane 15). This region includes nucleotides 792–795, which are components of the codons for residues Cys-264 and Arg-265 within the 249–274 deletion (see Fig. 2A). Use of such a splice acceptor would encode an additional 24 residues in an alternate reading frame from that of E4orf6 before terminating at a TGA at

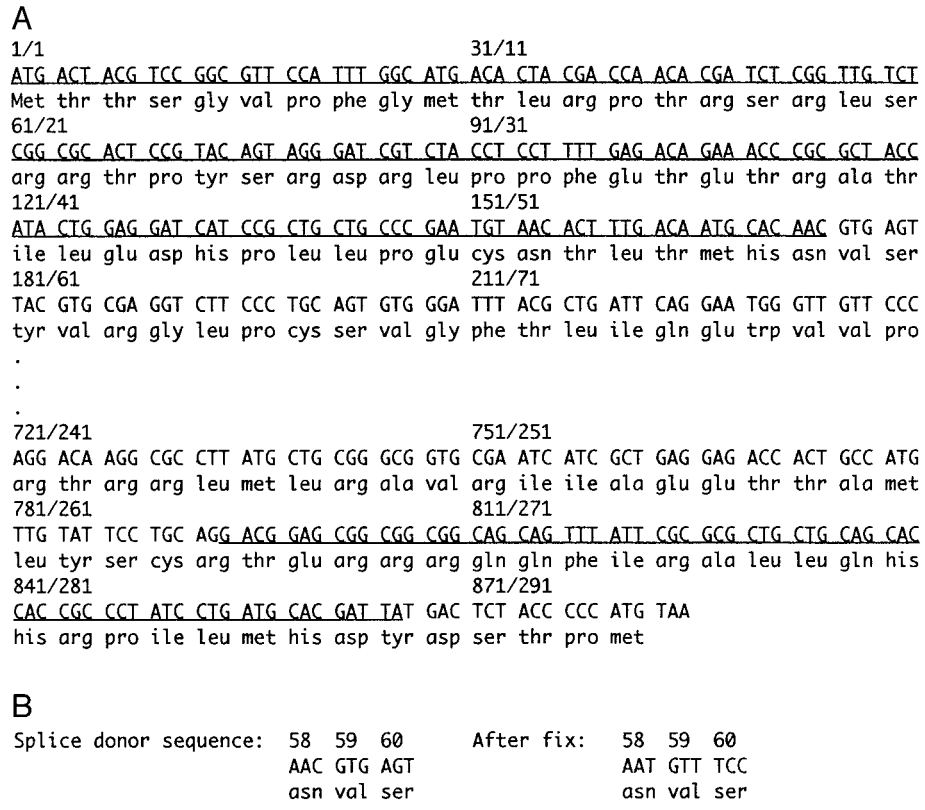

FIG. 2. Analysis of splice sites in Ad5 E4orf6 cDNA. (A) Ad5 E4orf6 DNA and protein sequence. The 5′ amino and 3′ carboxy sequences of Ad5 E4orf6 have been included and the central region omitted. The underlined base pairs are those that encode the 8-kDa product. Nucleotide/amino acid positions are indicated above the sequence. (B) Mutation of the 5′ splice donor sequence. Bases changed in E4orf6sf "splice-fix" cDNA sequence to abolish production of the 8-kDa product have been illustrated.

nucleotides 868–870. The molecular mass of this hypothetical 82-residue protein would be about 8 kDa.

To determine whether this process was indeed the origin of the 8-kDa protein, a series of point mutations was introduced into the third nucleotide of the codons for Asn-58 and Val-59, and the entire codon for Ser-60 was altered to an alternative codon sequence (see Fig. 2B). Plasmid cDNAs encoding wild-type E4orf6, dl275–294, and the E4orf6 "splice-fix" mutant (E4orf6sf) were lipofected into cells and E4orf6-related polypeptides again detected by Western blotting using E4orf6-N serum. Figure 1 shows that the 8-kDa species was present with wild-type E4orf6 cDNA (lane 25) but absent with the E4orf6sf (SF) plasmid (lane 27). Thus it was likely that the polypeptide predicted in Fig. 2 represents the 8-kDa species. E4orf6 mutant dl275–294 (lane 26) produces a 7.5-kDa species at very low levels (not evident in the exposure shown in Fig. 1). This 72-amino-acid polypeptide would terminate at an alternative reading frame stop codon present in this mutant. In addition, Fig. 1 (compare lanes 25 and 27) shows that higher amounts of full-length E4orf6 are produced with E4orf6sf.

The results from this study have determined the origin of the 8-kDa species that is synthesized from E4orf6 cDNAs. It should be noted that this 8-kDa species is not produced at all from adenoviruses containing genomic viral DNA (*29*), and thus it appears that the normal 3′ splice acceptor site used for E4orf6/7 mRNA is highly preferred. Such would be predicted as this site contains a more or less perfect acceptor sequence, whereas that at nucleotides 792–795 is less than optimal. Use of the altered E4orf6sf cDNA sequence would eliminate synthesis of this unnatural E4orf6-related product and eliminate possible confusion in analyses of E4orf6 function in studies employing E4orf6 cDNAs. Both *in vitro* and *in vivo* binding studies have shown that E4orf6/7, which contains the 58 amino terminal residues of E4orf6, is able to bind E1B-55K (*13*); however, we have recently found that the minimal region of E4orf6 required to bind E1B-55K is located between residues 39 and 107 (Querido *et al.,* submitted). These results leave open the possibility that the 8-kDa E4orf6-related product could interact with E1B-55K. In addition, the interaction of E4orf6 with several other proteins, including p53 (*9*), DNA-PK (*27*), and an additional multiprotein complex (*29*), could be affected. Thus it would be preferable to conduct future functional studies with cDNAs that express higher levels of only the normal E4orf6 product.

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REFERENCES

1. Cutt, J. R., Shenk, T., and Hearing, P. (1987). Analysis of adenovirus early region 4-encoded polypeptides synthesized in productively infected cells. *J. Virol.* **61,** 543–552.
2. Hardy, S., Engel, D. A., and Shenk, T. (1989). An adenovirus early region 4 gene product is required for induction of the infection-specific form of cellular E2F activity. *Genes Dev.* **3,** 1062–1074.
3. Huang, M. M., and Hearing, P. (1989). The adenovirus early region 4 open reading frame 6/7 protein regulates the DNA binding activity of the cellular transcription factor, E2F, through a direct complex. *Genes Dev.* **3,** 1699–1710.
4. Reichel, R., Neill, S. D., Kovesdi, I., Simon, M. C., Raychaudhuri, P., and Nevins, J. R. (1989). The adenovirus E4 gene, in addition to the E1A gene, is important for trans-activation of E2 transcription and for E2F activation. *J. Virol.* **63,** 3643–3650.
5. Dobbelstein, M., Roth, J., Kimberly, W. T., Levine, A. J., and Shenk, T. (1997). Nuclear export of the E1B–55kDa and E4 34-kDa adenoviral oncoproteins mediated by a rev-like signal sequence. *EMBO J.* **16,** 4276–4282.
6. Orlando, J. S., and Ornelles, D. A. (1999). An arginine-faced amphipathic alpha helix is required for adenovirus type 5 E4orf6 protein function. *J. Virol.* **73,** 4600–4610.
7. Fischer, U., Huber, J., Boelens, W. C., Mattaj, I. W., and Luhrmann, R. (1995). The HIV-1 Rev activation domain is a nuclear export signal that accesses and export pathway used by specific cellular RNAs. *Cell* **82,** 475–483.
8. Boyer, J. L., and Ketner, G. (2000). Genetic analysis of a potential zinc-binding domain of the adenovirus E4 34K protein. *J. Biol. Chem.* **275,** 14969–14978.
9. Dobner, T., Horikoshi, N., Rubenwolf, S., and Shenk, T. (1996). Blockage by adenovirus E4orf6 of transcriptional activation by the p53 tumor suppressor. *Science* **272,** 1470–1473.
10. Querido, E., Marcellus, R. C., Lai, A., Charbonneau, R., Teodoro, J. G., Ketner, G., and Branton, P. E. (1997). Regulation of p53 levels by the E1B 55-kilodalton protein and E4orf6 in adenovirus-infected cells. *J. Virol.* **71,** 3788–3798.
11. Sarnow, P., Hearing, P., Anderson, C. W., Halbert, D. N., Shenk, T., and Levine, A. J. (1984). Adenovirus early region 1B 58,000-dalton tumor antigen is physically associated with an early region 4 25,000-dalton protein in productively infected cells. *J. Virol.* **49,** 692–700.
12. Goodrum, F. D., Shenk, T., and Ornelles, D. A. (1996). Adenovirus early region 4 34-kilodalton protein directs the nuclear localization of the early region 1B 55-kilodalton protein in primate cells. *J. Virol.* **70,** 6323–6335.
13. Rubenwolf, S., Schütt, H., Nevels, M., Wolf, H., and Dobner, T. (1997). Structural analysis of the adenovirus type 5 E1B 55-kilodalton-E4orf6 protein complex. *J. Virol.* **71,** 1115–1123.
14. Babiss, L. E., Ginsberg, H. S., and Darnell, J. J. (1985). Adenovirus E1B proteins are required for accumulation of late viral mRNA and for effects on cellular mRNA translation and transport. *Mol. Cell. Biol.* **5,** 2552–2558.
15. Halbert, D. N., Cutt, J. R., and Shenk, T. (1985). Adenovirus early region 4 encodes functions required for efficient DNA replication, late gene expression, and host cell shutoff. *J. Virol.* **56,** 250–257.
16. Sandler, A. B., and Ketner, G. (1989). Adenovirus early region 4 is essential for normal stability of late nuclear RNAs. *J. Virol.* **63,** 624–630.
17. Bridge, E., and Ketner, G. (1990). Interaction of adenoviral E4 and E1b products in late gene expression. *Virology* **174,** 345–353.
18. Bridge, E., and Ketner, G. (1989). Redundant control of adenovirus late gene expression by early region 4. *J. Virol.* **63,** 631–638.
19. Ohman, K., Nordqvist, K., and Akusjarvi, G. (1993). Two adenovirus proteins with redundant activities in virus growth facilitates tripartite leader mRNA accumulation. *Virology* **194,** 50–58.
20. Moore, M., Horikoshi, N., and Shenk, T. (1996). Oncogenic potential of the adenovirus E4orf6 protein. *Proc. Natl. Acad. Sci. USA* **93,** 11295–11301.
21. Roth, J., Konig, C., Wienzek, S., Weigel, S., Ristea, S., and Dobbelstein, M. (1998). Inactivation of p53 but not p73 by adenovirus type 5 E1B 55-kilodalton and E4 34-kilodalton oncoproteins. *J. Virol.* **72,** 8510–8516.
22. Steegenga, W. T., Riteco, N., Jochemsen, A. G., and Fallaux, F. J. (1998). The large E1B protein together with the E4orf6 protein target p53 for active degradation in adenovirus infected cells. *Oncogene* **16,** 349–357.
23. Lowe, S. W., and Ruley, H. E. (1993). Stabilization of the p53 tumor suppressor is induced by adenovirus 5 E1A and accompanies apoptosis. *Genes Dev.* **7,** 535–545.
24. de Stanchina, E., McCurrach, M. E., Zindy, F., Shieh, S.-Y., Ferbeyre, G., Samuelson, A. V., Prives, C., Roussel, M. F., Sherr, C. J., and Lowe, S. W. (1998). E1A signaling to p53 involves the $p19_{ARF}$ tumor suppressor. *Genes Dev.* **12,** 2434–2442.
25. Nevels, M., Rubenwolf, S., Spruss, T., Wolf, H., and Dobner, T. (1997). The adenovirus E4orf6 protein can promote E1A/E1B-induced focus formation by interfering with p53 tumor suppressor function. *Proc. Natl. Acad. Sci. USA* **94,** 1206–1211.
26. Nevels, M., Spruss, T., Wolf, H., and Dobner, T. (1999). The adenovirus E4orf6 protein contributes to malignant transformation by antagonizing E1A-induced accumulation of the tumor suppressor protein p53. *Oncogene* **18,** 9–17.
27. Boyer, J., Rohleder, K., and Ketner, G. (1999). Adenovirus E4 34K and E4 11K inhibit double strand break repair and are physically associated with the cellular DNA-dependent protein kinase. *Virology* **263,** 307–312.
28. Weiden, M. D., and Ginsberg, H. S. (1994). Deletion of the E4 region of the genome produces adenovirus DNA concatemers. *Proc. Natl. Acad. Sci. USA* **91,** 153–157.
29. Boivin, D., Morrison, M. R., Marcellus, R. C., Querido, E., and Branton, P. E. (1999). Analysis of synthesis, stability, phosphorylation, and interacting polypeptides of the 34-kilodalton product of open reading frame 6 of the early region 4 protein of human adenovirus type 5. *J. Virol.* **73,** 1245–1253.
30. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1999). "Current Protocols in Molecular Biology," Vol. 3. Wiley, NY.