Expression of adenovirus type 5 E4 Orf2 protein during lytic infection

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The human adenovirus type 5 E4 transcription unit has the potential to encode at least seven distinct polypeptides from reading frames accessed by differential splicing of a single primary transcript. Only some of these polypeptides have yet been detected during viral infection of cultured cells. Mutational inactivation of the reading frames whose products have not been described has no apparent effect on the growth of virus in standard cultured human cell lines, indicating that these proteins, if they exist, have only a subtle, non-essential role in the replication cycle. We have raised an antiserum to one of these undefined products, E4 Orf2, expressed in bacteria. Using this reagent, it was possible to show that Orf2 was expressed during the lytic cycle in HeLa cells, being a soluble cytoplasmic component appearing with early kinetics. No association of Orf2 protein with other infected cell components was detected.

The E4 region of adenovirus type 5 (Ad5) lies at the right-hand end of the genome. Its promoter is active at early times post-infection (p.i.), with activity continuing into the late phase. The E4 primary transcript is subject to a complex pattern of differential splicing giving rise to a number of distinct mRNAs (Freyer et al., 1984; Tigges & Raskas, 1984; Virtanen et al., 1984). The pattern of differential splicing changes over the course of infection so that certain of these mRNAs appear only during the late phase of infection (Tigges & Raskas, 1984; Ross & Ziff, 1992; Dix & Leppard, 1993). The E4 gene contains a number of open reading frames (ORFs), predicted from the DNA sequence (Chroboczek et al., 1992; Dix & Leppard, 1992). Based upon the observed pattern of E4 mRNA splicing, these ORFs were predicted to encode seven distinct polypeptides, designated Orf1, 2, 3, 3/4, 4, 6 and 6/7 (Fig. 1). To date, four of these (Orf3, 4, 6 and 6/7) have been detected in Ad5-infected cells and have had functions ascribed to them (Sarnow et al., 1982, 1984; Downey et al., 1983; Cutt et al., 1987; Bridge et al., 1993). One of the other three predicted proteins, Orf1, has been described recently in studies of the distantly related adenovirus type 9 (Javier, 1994). The results reported here relate to one of the remaining two predicted E4 proteins, Orf2.

Mutant strains of Ad5 that are unable to make Orf2 grow to virtually wild-type levels in HeLa cells (Halbert et al., 1985). More recently, complete deletion of this reading frame has also been shown to have minimal impact on growth phenotype (Bridge & Ketner, 1989; Huang & Hearing, 1989a). Therefore, this protein apparently does not play an essential role in the growth of Ad5, at least under standard culture conditions. Nevertheless, the conservation of the Orf2 sequence between adenovirus serotypes (Dix & Leppard, 1992; Javier et al., 1992; Davison et al., 1993; Sprengel et al., 1994) suggests that it does encode a protein that has a significant role in the infectious process under certain circumstances.

To examine further the significance of the Orf2 region of Ad5 E4, a polyclonal antiserum was raised to Orf2 protein expressed in bacteria. A cDNA fragment suitable for the expression of E4 Orf2 was obtained by PCR amplification of Ad5 genomic DNA using the enzyme manufacturer's recommended buffer conditions and two primers: 5' TGATGGATCCGCAGACATGTTTGAGA 3' and 5' AGGTGAATTCCCTCAAGCAGAATC 3'. The DNA obtained was cloned after cutting at the BamHI and EcoRI sites incorporated at the 5' ends of the PCR primers. Individual clones of the amplified DNA fragment were subjected to complete DNA sequence analysis to confirm the integrity of the reading frame. These sequences were as expected (data not shown). The Orf2 cDNA fragment was cloned in frame with the glutathione S-transferase (GST) gene in pGEX2T (Pharmacia). GST-Orf2 fusion protein was expressed at significant levels upon induction with IPTG and was present at least partly in the soluble fraction from broken cells (Fig. 2 lanes 1 to 4) although the majority of the protein was insoluble (lanes 5 to 8). The

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Fig. 1. Ad5 early region 4 showing the predicted translated regions. The arrow represents the primary E4 transcript with 5'-to-3' polarity. The E4 proteins predicted to be encoded by the alternatively spliced mRNAs produced from the primary transcript are indicated by open boxes (Virtanen et al., 1984). The genome scale indicates the distance in base pairs from the left-hand end of the genome (Chroboczek et al., 1992; Dix & Leppard, 1992).

Fig. 2. The expression of the GST-Orf2 fusion protein from pID22 at 30 °C and 37 °C. Bacterial cells harbouring the plasmid were induced with IPTG (0.1 mM) and harvested after a further 0 (lanes 1 and 5), 2 (lanes 2 and 6), 4 (lanes 3 and 7) or 6 (lanes 4 and 8) h at 37 °C or 20 h at 30 °C (lanes 10 and 11). Cells were broken by sonication in PBS and the crude lysates fractionated into soluble (lanes 1 to 4 and 10) and particulate (lanes 5 to 8 and 11) fractions. GST-Orf2 protein was purified by affinity chromatography on glutathione-Sepharose from the 6 h post-induction soluble fraction (lane 9). All samples are the equivalent of 0.1 ml of culture, except the purified fraction which is equivalent to 2 ml of culture. Proteins were visualized by Coomassie blue staining. The positions of protein markers and their molecular masses (kDa) are indicated on the left.

The proportion of the fusion protein found in the soluble fraction was increased by reducing the growth temperature to 30 °C (lanes 10 and 11). The GST-Orf2 fusion protein was purified from the soluble fraction by affinity chromatography on glutathione-Sepharose (lane 9) and used for subcutaneous immunization of a half-lop rabbit. Protein (200 µg) in PBS was emulsified in Freund’s complete adjuvant and divided between four sites of injection. Immunization was repeated every 3 weeks using a similar dose of antigen in Freund’s incomplete adjuvant. Sera from sequential bleeds showed high titres in an ELISA which employed the fusion protein as immobilized antigen; titres increased to a plateau after three immunizations and were maintained thereafter (data not shown).

The Orf2 antiserum was next used to determine whether or not Orf2 was expressed during viral infection. Western blot analysis (Towbin et al., 1979) was performed using Hybond-C filters (Amersham), with detection by biotinylated secondary antibody/streptavidin–horseradish peroxidase complex (Amersham) and chloronaphthol substrate (Bio-Rad). Wild-type Ad5 virus (wt300)-infected HeLa cells, cultured in Dulbecco’s modified Eagle’s medium supple-
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Fig. 3. The expression of Orf2 protein in adenovirus-infected HeLa cells. (a) HeLa cells (2.5 x 10⁵), infected with either wt300 (lanes 1 to 5 and 9) or dll-3 (lanes 6 and 7) at an m.o.i. of 10, were lysed in RIPA buffer (1% w/v sodium deoxycholate, 1% v/v NP40, 0.1% w/v SDS, 50 mm-Tris–HCl pH 8.0, 150 mm-NaCl) at 5 (lane 1), 9 (lanes 2 and 6), 16 (lane 3), 24 (lanes 4, 7 and 9) or 32 (lane 5) h p.i. and assayed for the presence of the Orf2 protein by western blotting using either the anti-GST–Orf2 fusion antiserum (lanes 1 to 8) or preimmune serum (lane 9). Lane 8 contains purified, bacterially expressed, Orf2 protein, cleaved from GST–Orf2 fusion protein with thrombin. (b) HeLa cells (1 x 10⁷), infected with either wt300 (lanes 10 to 13) or dll352 (lanes 14 to 17) at an m.o.i. of 10 were radiolabelled (100 μCi [³⁵S]methionine; 1000 Ci/mmol, Amersham; per 1 x 10⁷ cells) for 1 h prior to lysis in 1 ml of RIPA buffer at 8 (lanes 10 and 14), 16 (lanes 11 and 15), 24 (lanes 12 and 16) or 32 (lanes 13 and 17) h p.i. All samples were adsorbed with preimmune serum and immunoprecipitated for 30 min, clarified by centrifugation, and then immunoprecipitated with 2 μl of anti-GST–Orf2 antiserum before separation by SDS-PAGE. The positions of protein markers and their molecular masses (kDa) are indicated on the left.

Fig. 4. The subcellular localization of the Orf2 protein within the infected cell. Ad5 wt300-infected (lanes 1 to 4) and dll-3-infected (lanes 5 to 8) HeLa cells, labelled for 4 h (100 μCi [³⁵S]methionine per 1 x 10⁷ cells) and harvested at 18 h p.i., were lysed by Dounce homogenization in hypotonic buffer (10 mm-Tris–HCl pH 7.5, 10 mm-NaCl, 1.5 mm-MgCl₂, 0.01 mg/ml PMSF). Lysates were fractionated by sequential centrifugation for 5 min at 500 g, 20 min at 50000 g, and 60 min at 200000 g to produce P₁₀₀ (lanes 1 and 5), P₉₀ (lanes 2 and 6), P₇₀ (lanes 3 and 7), and S₉₀₀ (lanes 4 and 8) fractions which were then immunoprecipitated as for Fig. 3(b). The positions of protein markers and their molecular masses (kDa) are indicated on the left.

mented with 10% newborn calf serum, expressed a protein of approximately 14000 Da that reacted specifically with the anti-Orf2 antiserum (Fig. 3a, lanes 2 to 5) and not with the preimmune serum (lane 9), and which co-migrated with bacterially expressed, purified Orf2 protein cleaved from GST–Orf2 fusion protein with thrombin (lane 8). This protein was not detected in extracts of cells infected by the mutant virus dllOrf1–3 which lacks the E4 Orf1, 2 and 3 reading frames (Huang & Hearing, 1989a; Fig. 3a, lanes 6 and 7), or in cells infected by the Orf2-specific mutant dll352 (Halbert et al., 1985; Fig. 3b, lanes 14 to 17). Analysis of the synthesis of Orf2 by pulse radiolabelling and immunoprecipitation showed an increasing rate from 8 h p.i. to a plateau at 16 h and 24 h p.i. (Fig. 3b, lanes 10 to 12). This temporal pattern exactly parallels that of E4 mRNA C levels (encoding Orf2) reported previously (Dix & Leppard, 1993).

To determine which compartments of the cell contained Orf2 protein, indirect immunofluorescence was
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Fig. 5. Molecular mass of the Orf2 protein in infected cell extracts, determined by sucrose gradient analysis. Ad5 wt300-infected HeLa cells were labelled for 3 h (100 μCi [35S]methionine per 1 x 10⁷ cells) and harvested at 16 h p.i. The cells were lysed by Dounce homogenization as for Fig. 4 and a crude cytoplasmic fraction (S₀.₅) was prepared by centrifugation at 500 g for 5 min. The S₀.₅ fraction from 1 x 10⁷ cells was loaded onto a 5 to 20% (w/w), 5 ml sucrose gradient formed in the lysis buffer and centrifuged at 240000 g for 6 h. The gradient was then collected in 0.5 ml fractions (1 to 10) and analysed for the presence of the Orf2 protein by immunoprecipitation with 2 μl of GST–Orf2 antiserum. The positions of protein markers and their molecular masses (kDa) are indicated on the left.

Some Orf2 was detected in the nuclear and membrane fractions (lanes 1 and 2), but this could represent either residual unbroken cells or soluble cytoplasmic contamination of these fractions rather than functional compartmentation. A control fractionation of Orf2 mutant virus-infected cells showed no Orf2 protein (lanes 5 to 8).

To determine whether Orf2 was part of a large complex of proteins when released from cells, extracts of labelled cells were subjected to sucrose gradient centrifugation analysis. The gradient was then fractionated and fractions were divided into two portions for immunoprecipitation with either anti-Orf2 antiserum (Fig. 5) or preimmune serum (data not shown). The Orf2 protein was detected principally in fractions 8 and 9, which were derived from near the top of the gradient, indicating that Orf2 was present in the extract in a low molecular weight form.

It was of interest to determine whether the E4 Orf2 gene product made any detectable interactions with...
other infected cell components. Inspection of repeated immunoprecipitation analyses revealed no specifically co-precipitated, labelled protein species that might have indicated molecular interactions of Orf2 with other proteins (for example, Fig. 3b). These analyses were extended to include the use of less stringent lysis and immunoprecipitation buffers than the standard RIPA conditions. Similarly, no cosedimentation of labelled species with Orf2 was observed during sucrose gradient analysis (Fig. 5). If Orf2 interacts with other infected cell components, these interactions must be either transient, so that only a small fraction of Orf2 is participating at any given time, or of low affinity so as to prevent detection by the methods employed.

With this description of Orf2 biosynthesis, all but two of the presently predicted E4 protein products have been detected in Ad5-infected cells (Cutt et al., 1987; Downey et al., 1983; Sarnow et al., 1982, 1984; Bridge et al., 1993). One of the remaining undetected proteins, Orf1, has been found in Ad9-infected cells (Javier, 1994). The previously described Ad5 E4 products have been ascribed various functions in the control of gene expression (Bridge & Ketner, 1989; Halbert et al., 1985; Huang & Hearing, 1989a, b; Müller et al., 1992). The data presented here do not allow a biochemical description of the role of the E4 Orf2 protein during Ad5 infection. However, given that the infectious yield of a virus deficient in Orf2 expression when grown in HeLa cells is undiminished in comparison with wild-type virus (Halbert et al., 1985), this role is likely to be a subtle one, perhaps important only in certain cell types or under certain growth conditions. To examine the possibility that a phenotype might be manifested when virus was grown in normal cells, growth in human WI-38 non-transformed fibroblasts was tested; however, Orf2-mutant virus grew to the same final titre as wild-type virus; related functions are grouped together at various levels is perhaps an indication of the likely role of Orf2 protein; related functions are grouped together in other Ad5 transcription units. Further experiments will be needed to define the activities associated with this protein.

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References


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