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Nuclear matrix localization and SUMO-1 modification of adenovirus type 5 E1b 55K protein are controlled by E4 Orf6 protein

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Human adenovirus serotype 5 encodes three proteins, E1b 55K, E4 Orf3 and E4 Orf6, which interact with each other and with components of the nucleus to regulate mRNA processing and export, viral DNA replication and p53-dependent apoptosis. Previous studies have shown that, during wild-type infection, 55K associates initially with structures termed ND10, which are sites of localization of the promyelocytic leukaemia protein, and then moves, dependent upon its interaction with Orf6, to the establishing virus replication centres. Absence of either Orf3 or Orf6 affects the localization of 55K and so may affect its function. In this study, the influence of Orf3 and Orf6 expression on the association of 55K with the insoluble matrix fraction of the nucleus and with ND10 particularly was examined. Overexpression of Orf6 was sufficient to block the association of 55K with this fraction, irrespective of the presence of Orf3. This effect depended upon the two proteins being able to interact. However, the association of 55K with ND10, which persists throughout infection in the absence of Orf6, required Orf3 to be present, thus distinguishing two subsets of matrix-associated 55K. A modified form of 55K, formation of which was blocked by mutating the known site of SUMO-1 attachment, was more abundant in the absence of Orf6 but unaffected by the absence of Orf3. Thus, this modification is favoured when 55K remains associated with the matrix but does not correlate with its stable association with ND10, many components of which are modified by SUMO-1.

INTRODUCTION

Human adenovirus serotype 5 (Ad5) early genes encode a series of non-structural proteins that serve collectively to adapt the intracellular environment to facilitate progeny particle production. Among these proteins are the 55 kDa protein from the E1b gene (55K) and the protein products of the E4 open reading frames 3 and 6 (Orf3 and Orf6). Early studies of mutants unable to express one or more of these three proteins indicated that they perform complex, partially redundant and interdependent functions during

infection of standard cell cultures, with apparent roles in regulating viral RNA splicing, mRNA export from the nucleus and, in the case of Orf6, DNA replication (reviewed by Imperiale *et al.*, 1995; Leppard, 1998). Recently, a role for these proteins in regulating double-strand DNA break repair has been demonstrated (Stracker *et al.*, 2002).

The idea that 55K and Orf6 were functionally related was first suggested by the observation of a physical complex between them by co-immunoprecipitation (Sarnow *et al.*, 1984). More recently, an interaction between 55K and Orf3 has also been shown (Leppard & Everett, 1999). Similarly, an association occurs between 55K and/or Orf6 and the cellular tumour suppressor protein p53, and these interactions lead to the inactivation and targeted degradation of p53, so blocking p53-dependent apoptosis (Sarnow *et al.*, 1982a; Kao *et al.*, 1990; Yew & Berk, 1992; Dobner *et al.*, 1996). Orf3 may antagonise this effect on p53 through its interaction with 55K (König *et al.*, 1999).

The subcellular localization of these proteins has also been studied extensively. In infected cells, 55K is substantially localized to the nucleus. In the early phase of infection it associates with host cell nuclear structures termed ND10 or PODs (Doucas *et al.*, 1996). Later, it becomes associated with the periphery of virus replication centres, dependent on the presence of Orf6 (Ornelles & Shenk, 1991). When expressed by transfection in uninfected cells, 55K is unable to reach the nucleus without the co-expression of either Orf3 or Orf6 (Goodrum *et al.*, 1996; König *et al.*, 1999). In the absence of both these E4 proteins, it localizes to cytoplasmic bodies. In contrast, both E4 proteins, when expressed alone, enter the nucleus, Orf6 showing a diffuse localization (Goodrum *et al.*, 1996), while Orf3 localizes to, and causes the reorganization of, the same ND10 structures that are initial sites of 55K localization (Carvalho *et al.*, 1995; Doucas *et al.*, 1996). During infection, Orf3 shows essentially the same localization as during transfection, while Orf6 forms a reticular network in the nucleus that partially overlaps with the distribution of 55K in replication centres (Gonzalez & Flint, 2002). Absence of either Orf3 or Orf6 during infection significantly alters the nuclear localization pattern of 55K (Leppard & Everett, 1999). Finally, both Orf6 and 55K can shuttle independently between nucleus and cytoplasm (Dobbelstein *et al.*, 1997; Dosch *et al.*, 2001). Thus, one factor in the complex phenotypes observed when one or more of these proteins is absent from an infection may be the interdependence of these proteins for correct localization within the cell.

The association of both 55K and Orf3 with ND10 is likely to be functionally significant. ND10 are dynamic nuclear substructures that are defined by the presence of the promyelocytic leukaemia (PML) protein but which also contain a large number of other components, many of which are recruited into these structures only under specific physiological conditions (reviewed by Negorev & Maul, 2001). The function(s) of ND10 in uninfected cells remains unclear, with suggested roles in regulating gene

expression, the cell cycle and apoptosis. Since a number of unrelated viruses target these structures in various ways (reviewed by Everett, 2001; Regad & Chelbi-Alix, 2001), it is probable that viruses either need to utilize cell functions that are provided by ND10 or their components and/or that they need to disrupt such a function that would otherwise be adverse to the infectious outcome. The fact that ND10 components are induced by the natural antiviral component interferon (Lavau *et al.*, 1995), coupled with the observation that overexpression of PML inhibits the growth of two interferon-sensitive viruses (Chelbi-Alix *et al.*, 1998), supports the latter idea.

The experiments reported here were designed to examine further the interplay between 55K, Orf3 and Orf6. In particular, they address the role of Orf3 and Orf6 in determining the localization of 55K to the insoluble nuclear substructure termed the nuclear matrix, of which ND10 form a part.

METHODS

Cells, viruses and antibodies. Cell lines and their maintenance were as described (Leppard & Everett, 1999). The viruses used in this study are listed in Table 1. Virus stocks were grown and titred in 293 cells (viruses *wt300*, *dl309*, *dl338*, A143, H224, H354 and S380) or W162 cells (all other viruses). Titres determined in W162 cells were adjusted to '293-equivalent' titres as described previously (Leppard & Everett, 1999).

Specific antibodies used to detect antigens on Western blots and by immunofluorescence were: E1b 55K – mouse monoclonal antibody (mAb) 2A6 (Sarnow *et al.*, 1982b); E1b 55K – rat mAb 9C10 (Zantema *et al.*, 1985); E4 Orf2 – rabbit monospecific serum (Dix & Leppard, 1995); E4 Orf3 – rat mAb 6A11 (Nevels *et al.*, 1999); E4 Orf4 – rabbit monospecific serum (Shtrichman & Kleinberger, 1998); and E4 Orf6 and Orf6/7 – mouse mAb M45 (Obert *et al.*, 1994). Secondary antibodies conjugated to HRP for Western blot detection were goat anti-mouse IgG and goat anti-rat IgG (Chemicon), and donkey anti-rabbit IgG (Amersham). Secondary antibodies conjugated to fluorophores were Alexa 488–goat anti-mouse IgG and Alexa 594–goat anti-rat IgG (Molecular Probes).

Cell infection, transfection, fractionation and analysis. Cells were plated at 50 % confluence for infection 24 h later with 10 p.f.u. per cell of virus. For plasmid transfection, cells were plated at 25 % confluence and transfected 24 h later using Lipofectin and Plus Reagent (Invitrogen), according to the manufacturer's protocols. To produce total extracts, cell monolayers were washed once in PBS and then lysed in sample buffer [200 µl per 10⁶ cells of 2 % (w/v) SDS, 10 % (v/v) glycerol, 25 mM Tris/HCl, pH 6.8, and 0.1 M DTT]. For nuclear matrix preparations, cells were fractionated as described (Leppard & Shenk, 1989; Leppard & Everett, 1999) and the nuclear matrix pellet solubilized in 50 µl per 10⁶ cells of

sample buffer containing 0.1 M DTT. All samples were incubated at 100 °C for 5–10 min to denature the proteins and to degrade the DNA, prior to loading on a 10 % SDS-polyacrylamide gel (15 % for analysis of the smaller E4 proteins). After blotting, filters were blocked in PBST (0.05 % Tween 20 in PBS) containing 5 % w/v non-fat dried milk overnight at 4 °C. Antigens were detected by incubating the filter with primary antibody in PBST/milk for 1 h, washing in PBST for 30–60 min with four buffer changes, then similarly incubating with an appropriate secondary antibody conjugated to HRP. Following washing, bound HRP was detected using Western Lightning Detection reagent (Perkin-Elmer), according to the manufacturer's instructions, with exposures taken on HyperECL film (Amersham). Films were scanned using an HP Scanjet 6100C/T and figures assembled in Microsoft Photodraw.

Table 1. Viruses used in this study

Virus	Genotype	Reference
<i>wt300</i>	Wild-type	Jones & Shenk (1978)
<i>dl309</i>	E3 partial deletion (pseudo-wild-type)	Jones & Shenk (1979)
<i>dl324</i>	E3 partial deletion (pseudo-wild-type)	Thimmappaya <i>et al.</i> (1982)
<i>dl338</i>	E1b 55K frame-shift deletion (<i>dl309</i> background)	Pilder <i>et al.</i> (1986)
<i>in351</i>	E4 Orf1 frame-shift insertion (<i>dl324</i> background)	Halbert <i>et al.</i> (1985)
<i>in352</i>	E4 Orf2 frame-shift insertion (<i>dl324</i> background)	Halbert <i>et al.</i> (1985)
<i>inOrf3</i>	E4 Orf3 frame-shift insertion (<i>wt300</i> background)	Huang & Hearing (1989)
<i>dl358</i>	E4 Orf4 frame-shift deletion (<i>dl324</i> background)	Halbert <i>et al.</i> (1985)
<i>dl355</i>	E4 Orf6 frame-shift deletion (<i>dl324</i> background)	Halbert <i>et al.</i> (1985)
<i>dl366*</i>	E4 total deletion (<i>wt300</i> background)	Huang & Hearing (1989)
<i>dl366*+3</i>	As <i>dl366*</i> with Orf3 restored	Huang & Hearing (1989)
<i>dl366*+4</i>	As <i>dl366*</i> with Orf4 restored	Huang & Hearing (1989)
<i>dl367</i>	Combines <i>dl338</i> and <i>dl355</i> lesions (<i>dl324</i> background)	Cutt <i>et al.</i> (1987)
<i>dl1-3</i>	E4 deletion of Orf1, Orf2 and Orf3 (<i>wt300</i> background)	Huang & Hearing (1989)
<i>dl1-4</i>	E4 deletion of Orf1, Orf2, Orf3 and Orf4 (<i>wt300</i> background)	Huang & Hearing (1989)
A143†	E1b 55K in-frame 4 aa insertion (<i>dl309</i> background)	Yew <i>et al.</i> (1990)
H224†	E1b 55K in-frame 4 aa insertion (<i>dl309</i> background)	Yew <i>et al.</i> (1990)
H354†	E1b 55K in-frame 4 aa insertion (plus one alteration) (<i>dl309</i> background)	Yew <i>et al.</i> (1990)
S380†	E1b 55K in-frame 4 aa insertion (plus one alteration) (<i>dl309</i> background)	Yew <i>et al.</i> (1990)

† Viruses A143, H224, H354 and S380 carry E1 sequences from Ad2 in an otherwise Ad5 background.

For immunofluorescence analysis, cells were grown on glass coverslips, fixed and antigens detected as described previously (Leppard & Everett, 1999). Nuclear matrix samples were centrifuged onto coverslips prior to fixation. Images were collected on a Leica SP2 confocal system using a 63× objective and assembled in Microsoft Photodraw without further manipulation.

Plasmid mutagenesis. The K¹⁰⁴→R mutation in 55K was constructed by two-step PCR mutagenesis using as template a subclone containing the Ad5 E1b 55K coding region. Primers corresponded to 0001-8820 © 2003 SGM

positions 2021–2040, 2319–2339, 2339–2319 and 2666–2646 (all 5′→3′, nucleotide positions from the Ad5 complete genome sequence) with the two central primers carrying sequence alterations specifying an A→G substitution at position 2329, converting codon 104 from AAG (lysine) to AGG (arginine). The second-round PCR product was cleaved at the *KpnI* (2048) and *PstI* (2502) sites in E1b and used to replace the equivalent fragment in the subclone. A *KpnI* (2048)–*HindIII* (2804) fragment from this clone was then used to replace the equivalent segment of pwt*XhoI*-C (containing the Ad5 genomic DNA fragment 1–5788). The *KpnI*–*PstI* region of this clone was sequenced to verify both the intended mutation and the absence of any other sequence alterations.

RESULTS

Nuclear matrix association of E1b 55K does not depend on E4 Orf3 status

Previous work has shown that the association of the E1b 55K protein with the nuclear matrix during infection is dependent on the E4 status of the virus (Leppard & Everett, 1999). Specifically, absence of E4 Orf6 significantly enhanced 55K matrix association, where it co-localized with Orf3 in reorganized ND10, while a mutant that lacked expression of Orf1, Orf2 and Orf3 was completely deficient in this association. Since 55K was also shown to interact with Orf3 in cell extracts, these results suggested that Orf3 was required for, and Orf6 disrupted, the association of 55K with these nuclear foci, although a role for Orf1 and/or Orf2 could not be excluded.

To examine further the effects of Orf3 and Orf6 expression on the localization of 55K, infections by an expanded panel of mutant viruses (Table 1) were studied using a Western blot assay. As shown in Fig. 1(a), each of the viruses expressed 55K to a similar level when total cell extracts were analysed. However, nuclear matrix preparations produced in parallel showed marked differences between the viruses in the amounts of 55K found in this subcellular fraction (Fig. 1b). As expected from previous immunofluorescence analysis, *wt300* and *dl355*, which lack Orf6 expression, showed strong association of 55K with this fraction, whilst the E4 Orf1/2/3-deficient virus, *dl1-3*, showed minimal 55K in the matrix fraction (Leppard & Everett, 1999). However, results from other viruses did not support the idea that Orf3 was required for efficient association of 55K with the matrix, since *inOrf3*, lacking only Orf3, and *dl366**, lacking all of E4, showed high levels of 55K in the matrix fraction. Moreover, three other viruses showed minimal levels of 55K in the matrix, similar to *dl1-3*, only one of which, *dl1-4*, was expected to lack Orf3 expression.

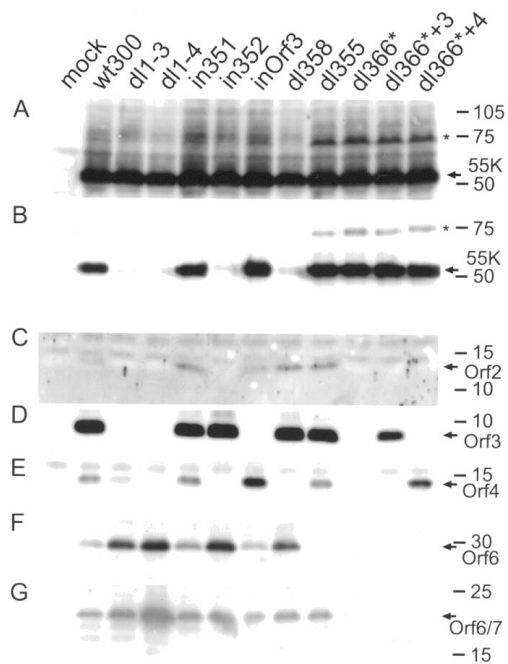


Fig. 1. Dependence of E1b 55K nuclear matrix association on E4 genotype. Two sets of HeLa cell cultures were infected in parallel with the viruses indicated at the top of the figure. At 16 h p.i., one set of cultures was harvested directly into SDS gel sample buffer, the other was fractionated to provide a nuclear matrix preparation that was then solubilized in SDS gel sample buffer. Samples were analysed by SDS-PAGE and antigens detected by Western blotting. Loadings per lane were approximately 8×10^4 cell equivalents (total cell extracts, a, c–g) and approximately 3×10^5 cell equivalents (nuclear matrix, b). 55K detection in (a, b) was performed in parallel with antibody 2A6; the results shown are from the same exposure time. (c–g) Gels were probed for E4 Orf2, Orf3, Orf4, Orf6 and Orf6/7, respectively. The migration positions of relevant molecular mass markers are shown at the right of each panel. * – an approximately 75 kDa molecular mass form of E1b 55K.

Given these results, total extracts from these infections were assessed for individual E4 proteins by Western blotting to confirm that each of the viruses used had the expected expression profile (no antiserum was available for Orf1). Qualitatively, with the exception of *in352*, each of the viruses expressed the proteins expected from knowledge of its specific lesion (Fig. 1c–g and Table 1). Thus, *inOrf3* expressed Orf2, Orf4, Orf6 and Orf6/7 but lacked Orf3, while *dl366** lacked expression of all of the E4 proteins tested. Therefore, the strong matrix-association of 55K seen for these two viruses was occurring in the absence of Orf3 protein and was not due to the viruses having incorrect genetic characteristics in E4.

It was possible that the nuclear matrix-associated 55K in these various infections was not equivalently localized in each case. Therefore, nuclear matrix was prepared and analysed for 55K by immunofluorescence (Fig. 2). Among the infections with high levels of nuclear matrix 55K in Fig. 1(b), 55K was found in very similar distributions in both the presence (*wt300*, *dl355* and *dl366*+3*) and absence (*inOrf3* and *dl366**) of Orf3. In contrast, infections with low amounts of matrix 55K showed only a weak diffuse staining (*dl358*, an Orf3-positive example, is shown). Thus the association of 55K with the nuclear matrix appears to be independent of Orf3 status.

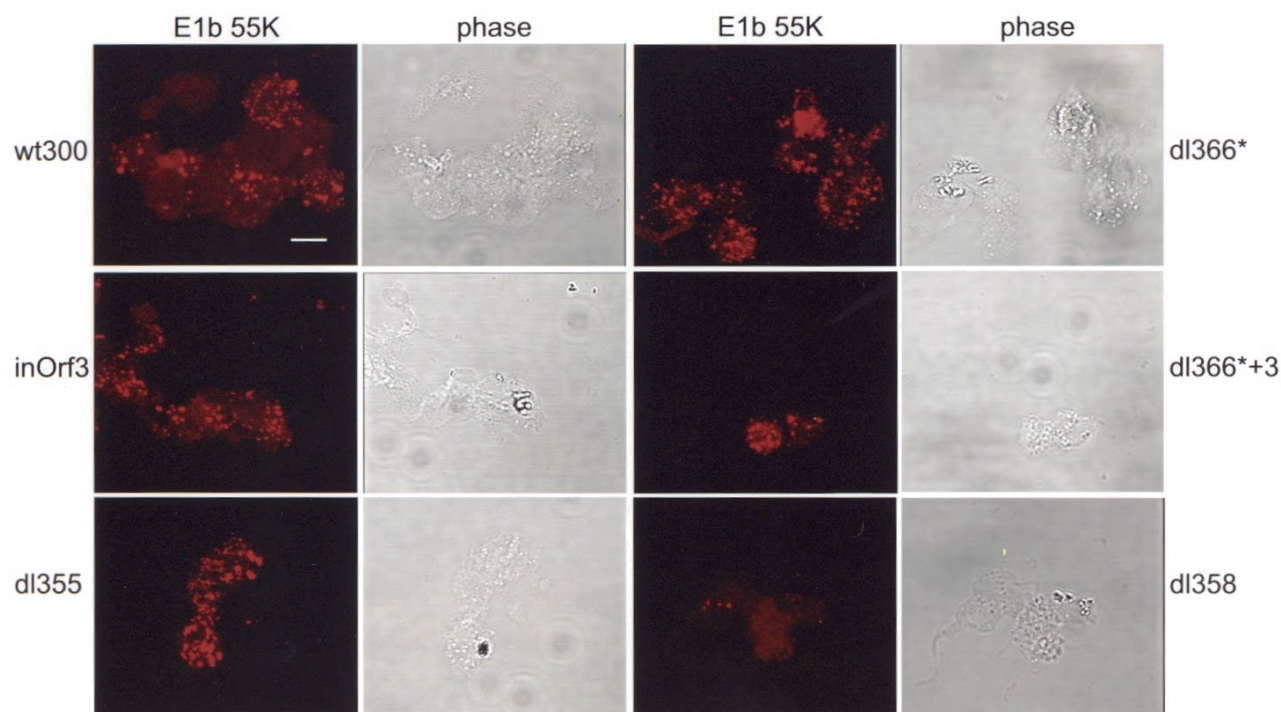


Fig. 2. E1b 55K localization within the nuclear matrix is independent of Orf3 status. Nuclear matrix samples, prepared at 16 h p.i. from HeLa cells infected with virus as indicated, were probed for 55K using mAb 9C10. Fluorescent images shown represent maximum projections of Z-stacks, paired with transmitted light images from single optical sections taken from the centre of each stack. Bar, 8 μ m.

E4 protein expression from mutant *in352*

Mutant *in352* did not express either E4 Orf2 or Orf4 (Fig. 1c, e). This virus was constructed by specific cleavage, DNA repair and blunt-end re-ligation at a *HindIII* site within the E4 Orf2 reading frame in a plasmid clone followed by virus reconstruction by recombination with a viral genomic DNA fragment *in vivo* (Halbert *et al.*, 1985). Thus, it was expected to lack expression of only the Orf2 protein. Since the function of Orf2 is unknown (Dix & Leppard, 1995), a role for this protein in regulating expression of Orf4 was considered; however, the fact that *dl1-3* was able to express Orf4 in the absence of Orf2 discounted this possibility. To test for potential second site mutations in *in352* that might affect E4 Orf4 expression, viral genomic DNA was prepared directly from *in352*-infected cells and sequenced across the Orf4 region. This revealed a single base pair deletion in the Orf4 reading frame (position 34324, data not shown) that is predicted to cause translational frame-shift and termination after only 18 codons, 12 of them missense. The stock of *in352* used for these experiments was grown in 1992 from a seed stock obtained from the originating laboratory in 1988, only 3 years after publication of its isolation. Thus, it is probable that the same additional lesion is present in all stocks of this virus.

Matrix association of 55K is determined by Orf6 expression levels

Although the viruses tested, with the exception of *in352*, expressed the E4 proteins expected, they did not

all do so to the same levels as *wt300*. Viruses *in351*, *dl358* and *dl355* overexpressed Orf2 (Fig. 1c), while *inOrf3* and *dl366*+4* overexpressed, and *dl1-3* underexpressed, Orf4 (Fig. 1e). Orf6/7 was grossly overexpressed by *dl1-4* and to a lesser extent by *dl1-3* and *in352* (Fig. 1g). Most notably, *dl1-3*, *dl1-4*, *in352* and *dl358* all overexpressed Orf6 (Fig. 1f). These viruses were the same ones that showed reduced matrix association of 55K (Fig. 1b).

This correlation suggested that Orf6 overexpression might be the principal cause of loss of 55K matrix localization in infections by viruses such as *dl1-3*. However, since the four viruses overexpressing Orf6 also had severely reduced or absent Orf4 expression, it was possible that lack of Orf4 was a factor in preventing 55K association with the matrix. To test this, a co-infection between *dl1-4* and *dl338*, an E4-wild-type, 55K non-expressing virus was analysed (Fig. 3). This infection provides overexpression of Orf6 against a background of expression of all the E4 proteins, including Orf4. As expected, *dl338* alone showed no matrix-associated 55K, while *dl1-4* showed a minimal level as compared to wild-type. Co-infection by *dl338* and *dl1-4* did not alter the minimal 55K matrix association phenotype of the latter virus. Thus, it is Orf6 overexpression, and not absence of Orf4, that is associated with reduced matrix association of 55K.

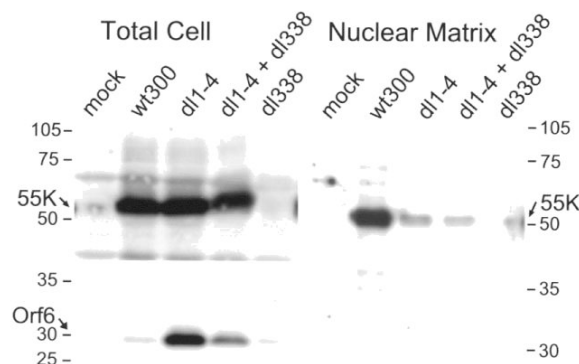


Fig. 3. E4 Orf6 overexpression prevents E1b 55K nuclear matrix association. The experiment was performed and samples were analysed for 55K as described for Fig. 1. The blot of total cell proteins was subsequently stripped and reprobbed for Orf6.

55K association with Orf6 is required to disrupt 55K matrix association

55K and Orf6 interact to form a protein complex (Sarnow *et al.*, 1984) and the Orf6 interaction properties of a series of 55K mutants have been characterized (Yew *et al.*, 1990; Rubenwolf *et al.*, 1997). To test the hypothesis that the 55K–Orf6 interaction was necessary for 55K to be displaced from the matrix, infections by 55K mutants that either retain (H354 and S380), lack (A143) or have substantially impaired (H224) Orf6 interaction ability (Rubenwolf *et al.*, 1997) were analysed (Fig. 4). Although all four viruses showed very similar levels of 55K and Orf6 expression, the two 55K mutants impaired in Orf6 binding showed substantially more matrix 55K than their functional counterparts, with the completely interaction-deficient A143 showing the most. Thus, the ability of Orf6 to associate with 55K correlates with

displacement of 55K from the nuclear matrix fraction. There was considerably more matrix-associated 55K in *wt300* infection than for either of the Orf6-binding mutants. This may reflect the fact that the four mutants are Ad2/Ad5 recombinants. As such, the relative affinities of the 55K–Orf6 and 55K–Orf3 interactions might be altered, so affecting the balance of 55K distribution.

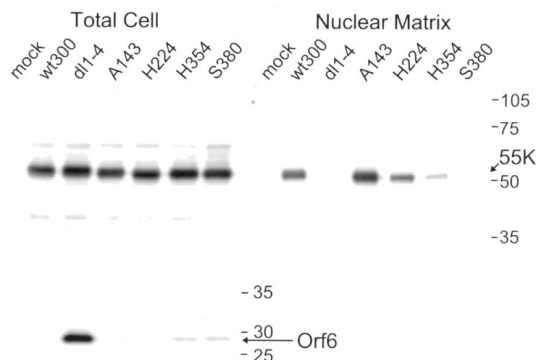


Fig. 4. E1b 55K interaction with E4 Orf6 is required to displace it from the nuclear matrix. Viruses were selected for analysis in comparison with wild-type that encode E1b 55K mutant proteins that either do (H354 and S380), do not (A143) or only minimally (H224) interact with E4 Orf6. The experiment was performed and samples were analysed for 55K or Orf6 as described for Fig. 1.

Matrix association of 55K during the course of infection

It has been reported that, during Ad5 infection, 55K associates first with Orf3 and then later with Orf6, so altering its localization (König *et al.*, 1999). To determine how matrix association of 55K varied with time post-infection (p.i.) under differing levels of Orf6 expression or interaction, infections were performed and samples harvested for analysis at times from 8 to 24 h p.i. (Fig. 5; data for 24 h p.i. were very similar to those for 20 h, not shown). Viruses either unable to express Orf6 (*dl355*) or whose 55K was deficient in Orf6 interaction (A143) showed significantly increased association of 55K with the matrix fraction when compared to wild-type at earlier times p.i. and this effect persisted throughout the time-course for *dl355*. In contrast, a virus that overexpresses Orf6 (*dl1-4*) showed only barely detectable association of 55K with the matrix at any time (longer exposures showed association at a low level from 8 h as for *wt300*, data not shown). Therefore, the amount of Orf6 in an infected cell and the potential for it to interact with 55K influence the matrix localization of 55K from the early stages of infection and not just in the later stages.

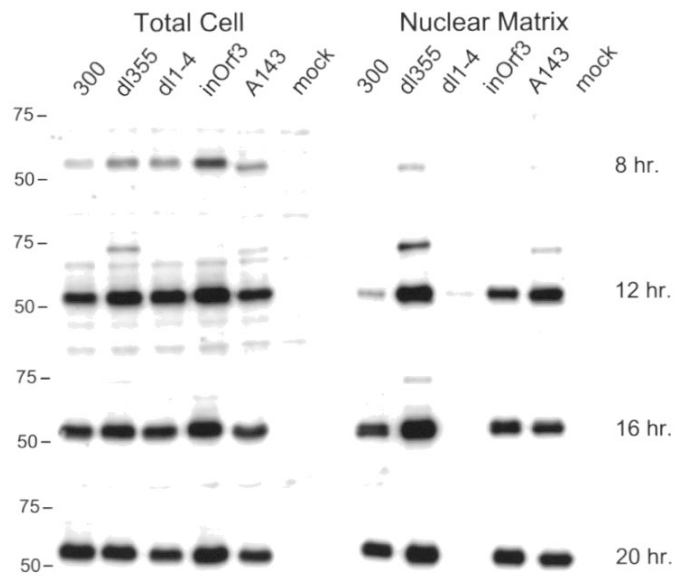


Fig. 5. Time-course analysis of E1b 55K nuclear matrix association in the presence or absence of E4 Orf6. Viruses were selected for analysis in comparison with wild-type that either do not produce Orf3 (*inOrf3*), do not produce Orf6 (*dl355*), overproduce Orf6 (*dl1-4*) or produce 55K that is deficient in Orf6 interaction (*A143*). A series of replicate HeLa cell cultures was infected and harvested at the times indicated. To generate corresponding total and nuclear matrix samples, cells were harvested in PBS and 20 % of the suspension taken as a total sample, the remainder being processed for nuclear matrix. Samples were analysed by SDS-PAGE and 55K detected by Western blotting with antibody 2A6. Loadings per lane were approximately 2×10^4 cell equivalents (total cell extracts) and approximately 1.5×10^5 cell equivalents (nuclear matrix extracts). Blot processing and exposure for corresponding total and nuclear matrix samples at a time-point was carried out in parallel and the panels shown are the same exposure time in each case. The 20 h p.i. panels are exposed for 20 % of the time for the other panels. The migration positions of relevant molecular mass markers are shown at the left of each panel.

55K localization during *dl366** infection

The presence of 55K in the nuclear matrix fraction from *dl366**-infected cells was surprising in light of a previous report that 55K was cytoplasmic during *dl366** infection (König *et al.*, 1999). Our previous work had also indicated that 55K co-localized with PML in reorganized ND10 in the absence of Orf6 (Leppard & Everett, 1999) and it was of interest to determine whether Orf3 was necessary for this to occur. Therefore, the localization properties of 55K in the context of various E4 mutations were examined further by immunofluorescence analysis in directly fixed cells (Fig. 6). Hep2 cells were used as PML is more readily detectable in these than in HeLa cells.

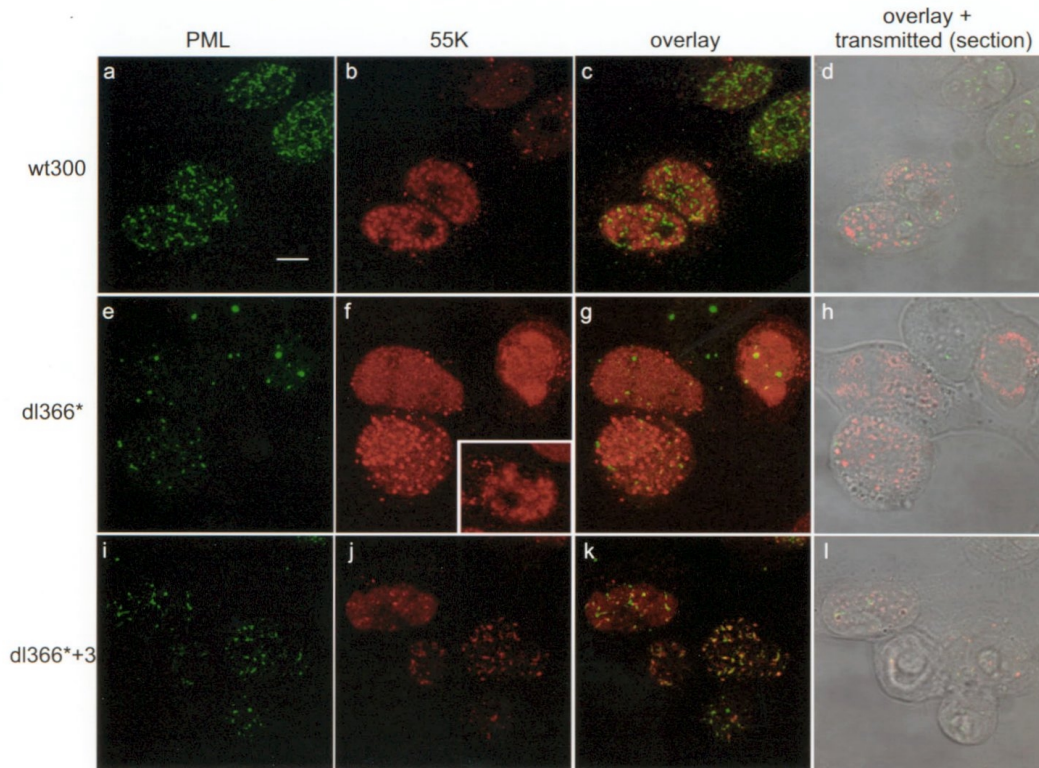


Fig. 6. Localization of E1b 55K to the nucleus in the absence of E4 proteins. Hep2 cells were infected with the viruses indicated and fixed 16 h p.i. Cells were stained sequentially for PML (5E10 – a, e and i) and for 55K (9C10 – b, f and j). Images shown are maximum projections of Z-stacks presented either singly (a–b, e–f and i–j) or overlaid (c, g and k). (d, h and l) Overlays of PML, 55K and transmitted light images are from single optical sections selected from the middle of the relevant Z-stack. The inset in (f) shows a cell from a different field, taken at the same magnification. Bar, 8 μ m.

As expected, *wt300*-infected cells showed reorganized PML. In some cells, 55K partially co-localized with PML, while in others, where infection had progressed further, 55K had moved to the periphery of replication centres (Ornelles & Shenk, 1991) and there was no co-localization (Fig. 6 a–d). 55K in *dl366**-infected cells showed a combination of diffuse and microspeckled nuclear staining (Fig. 6f). In some cells, this pattern resembled the association of 55K with virus replication centres seen in late wild-type infections (Fig. 6f, inset). 55K staining was confirmed to be nuclear by looking at single optical sections overlaid with the equivalent transmitted light image (Fig. 6h). PML was not reorganized by *dl366** infection (i.e. ND10 round in shape, Fig. 6e), as expected given the absence of the Orf3 protein, and when overlaid with the 55K staining pattern no co-localization was detected (Fig. 6g). Neither was co-localization observed in *dl366*+4* infection (data not shown). In contrast, *dl366*+3*, a virus able to express Orf3 but otherwise equivalent to *dl366**, showed reorganized PML (Fig. 6i) that substantially co-localized with 55K (Fig. 6k). Very similar images were obtained for Orf6-deficient, *dl355*-infected, cells (Leppard & Everett, 1999; data not shown). When Orf6 was overexpressed, as in *dl1-3* or *dl1-4* infection, 55K nuclear staining appeared more diffuse than for *wt300*, with little evidence of the staining of

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replication centres (Leppard & Everett, 1999; data not shown). Taken with the Western blotting data, these results show that 55K does reach the nucleus during infection in the absence of all E4 proteins but that its association with PML-containing structures there apparently depends on the presence of Orf3.

A SUMO-modified form of 55K is more abundant in the absence of Orf6

The analysis of 55K from cells infected with the panel of E4 mutants showed the presence of a protein with an approximate molecular mass of 75 kDa (75K) that was immuno-reactive with the anti-55K mAb 2A6 (Fig. 1, *). This protein was present in the nuclear matrix fraction and was possibly enriched in this fraction relative to 55K. It was present in wild-type infections at low levels but at significantly increased levels in cells infected by *dl355*, *dl366**, *dl366*+3* and *dl366*+4*. These four viruses each lack expression of the Orf6 protein and so show strong association of 55K with the nuclear matrix. In contrast, those viruses showing negligible matrix-associated 55K lacked even the low level of this 75K species seen in *wt300* infections. In a time-course analysis, the amount of this protein relative to 55K was maximal at 12 h p.i. and then declined significantly (Fig. 5).

It has been reported recently that a minor proportion of 55K in infected cells is modified at lysine 104 by covalent attachment to SUMO-1, a ubiquitin-like protein (Endter *et al.*, 2001). This modification has been described previously for both PML and another ND10-defining antigen, SP100 (Sternsdorf *et al.*, 1997; Müller *et al.*, 1998), as well as a diverse set of cellular and viral proteins, many of which can associate with ND10 (reviewed by Seeler & Dejean, 2001). Given that the 75K form of 55K was of the size expected for SUMO-modified 55K, the possibility that it might be the SUMO-1-modified form described previously was tested. Codon 104 (lysine) within the E1b 55K reading frame in a clone of the Ad5 genome left-end (1–5788) was changed to arginine by site-directed mutagenesis ($K^{104} \rightarrow R$). This mutation has been shown to abolish SUMO-1 modification of 55K (Endter *et al.*, 2001). The ability of $K^{104} \rightarrow R$ to produce the 75K form of 55K was then tested in transfection assays in comparison with the homologous wild-type plasmid (Fig. 7). The wild-type plasmid gave a prominent band of approximately 75 kDa that co-migrated with the protein 55K* identified previously in *dl355* samples. However, plasmid $K^{104} \rightarrow R$ produced none of this 75K species, although 55K was expressed normally. To further confirm that the 75K species missing from $K^{104} \rightarrow R$ transfections was the same protein as found in Orf6-deficient infections, the effect of Orf6 on expression of this 75K form of 55K from the plasmids was tested. Transfected cells were co-infected with viruses that either did (*dl338*) or did not (*dl367*) express E4 Orf6. Both viruses are 55K-deficient, so any 55K observed must derive from the plasmid. The intensity of the 75K band was significantly reduced by *dl338* co-infection but was unaffected by the control virus, *dl367*. Thus, formation of 55K* depends on having a lysine residue at position 104, strongly suggesting that it is the SUMO-1-modified form of 55K described previously.

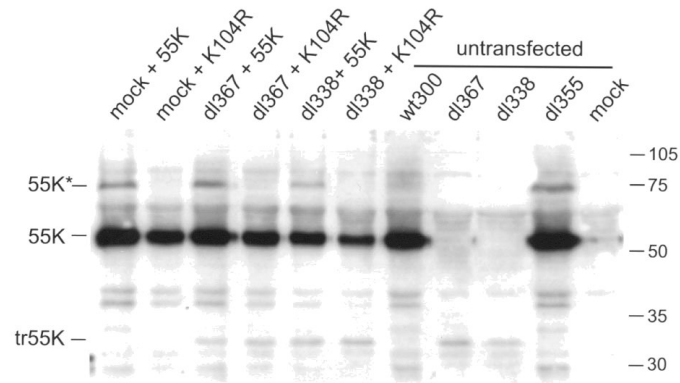


Fig. 7. SUMO-1-modified 55K is more abundant in the absence of Orf6. Replicate cultures containing 3×10^5 HeLa cells were transfected with 400 ng plasmid pwt*XhoI*-C encoding E1b 55K protein (55K) or a substitution mutant derivative ($K^{104} \rightarrow R$) and then superinfected after 8 h with viruses as indicated (control infections were also initiated at this time). Samples were harvested 24 h post-transfection (16 h p.i.). Samples were analysed by SDS-PAGE and 55K antigen detected by Western blotting. Loading per lane was approximately 6×10^4 cell equivalents of extract. The migration positions of relevant molecular mass markers are shown at the right of each panel. tr55K indicates the truncated E1b 55K product expected from the deletion mutation present in *dl338* and *dl367*. For explanation of 55K*, see text.

DISCUSSION

This study has focused on the association of E1b 55K protein with the nuclear matrix, which is defined operationally as the insoluble residue after detergent lysis of the cell and extraction of the nucleus with DNase I and salt and includes structures termed ND10. Infections by a series of E4 mutants showed a subset that had dramatically reduced nuclear matrix 55K. Among the nuclear matrix samples with high levels of 55K by Western blotting, all showed bright punctate foci of 55K. No significant differences in the appearance of these foci were observed that could be correlated with the Orf3 status of the infecting virus. However, significant co-localization of 55K with PML in ND10 in whole cells was seen only in matrix-positive infections that expressed Orf3 in the absence of Orf6. Thus, there are at least two subsets of matrix-associated 55K, one associated with PML in Orf3-modified ND10 and the other not. A small proportion of wild-type infected cells at 16 h p.i. also showed 55K co-localizing with PML. This fits with the observation that, during wild-type infection, the normal temporal pattern of Orf6 expression leads to the progressive loss of this co-localization in favour of an association of 55K with replication centres (Ornelles & Shenk, 1991), as seen in this study in the majority of cells.

Orf6 overexpression displaced both subsets of matrix-associated 55K into a more soluble fraction. The fact that Orf3 was necessary for the observation of PML-associated 55K suggests that this association is mediated via the interaction of 55K with Orf3 (Leppard & Everett, 1999), which is known to associate

with ND10 (Carvalho *et al.*, 1995). Alternatively, the presence of Orf3 may alter the kinetics of association of 55K with ND10 so as to permit the observation of the association as a significant fraction of total 55K. The ability of Orf6 to displace 55K from the matrix fraction depended on interaction between the two proteins. A recent study of the localization of 55K from mutant A143, which cannot bind to Orf6, supports this observation (Gonzalez & Flint, 2002). Since Orf3 and Orf6 interactions with 55K define alternative localizations for the protein, it is possible that their interactions are mutually exclusive. However, this remains to be tested.

55K associated with replication centres is probably part of the soluble fraction of 55K rather than matrix-associated 55K, although this remains to be demonstrated directly. There are several lines of evidence for this idea. First, the conditions used to prepare nuclear matrix remove much of the viral DNA-binding protein, which is seen to accumulate predominantly in replication centres (Leppard & Everett, 1999). Second, viruses, such as *dl1-3*, that show little or no nuclear matrix 55K nevertheless replicate normally (Huang & Hearing, 1989). Third, Orf6, which, as shown here, prevents matrix association of 55K, has been shown to be necessary for the movement of 55K into the periphery of replication centres (Ornelles & Shenk, 1991). Thus, the role of nuclear matrix-associated 55K remains uncertain. The Orf3-independent association may simply represent the aberrant fate of the protein in the absence of the full constellation of interacting proteins. However, the Orf3-dependent association is more likely to be significant and may be connected with the reported transient blocking of 55K-mediated p53 inactivation by Orf3 (König *et al.*, 1999).

Overexpression of Orf6 was observed for several viruses in this study resulting from mutations elsewhere in the E4 transcription unit. Such effects are not surprising, although their possible phenotypic significance has not been considered previously. Both *dl1-3* and *dl1-4* lack E4 reading frames and their associated splice acceptor sites (Huang & Hearing, 1989). In their absence, a given amount of transcription from the E4 promoter would be expected to result in proportionally more mRNA for the remaining reading frames. Moreover, the missing splice sites are differentially active at early times during infection, so their absence would be predicted to force the use of acceptor sites normally less active until later in infection (Dix & Leppard, 1993). This can explain the increased expression of Orf6 and Orf6/7 by these viruses, particularly early in infection, but expression from the intact Orf4 reading frame in *dl1-3* is reduced compared to *wt300*. However, the *dl1-3* mutation also removes the splice acceptor normally used to produce Orf4 mRNAs. Thus, the reduced Orf4 expression from this virus likely represents the activity of an alternative or cryptic splice site.

Two other viruses, *in352* and *dl358*, also showed elevated Orf6 expression. Both lack Orf4 expression due to frame-shifting mutations that would not be expected to affect *cis*-acting RNA processing signals

(Halbert *et al.*, 1985; this paper). Orf4 is known to negatively regulate the E4 promoter via its effect on E1a proteins (Bondesson *et al.*, 1996). However, relief of this inhibition in Orf4 mutants should apply equally to expression of all E4 proteins yet the elevation in Orf6 expression appeared to be specific. Alternatively, since the Orf4 reading frame overlaps the N terminus of Orf6 and Orf6/7, elevated Orf6 expression may be due to upregulation of translation initiation at the Orf6 AUG codon in mRNAs that would normally encode Orf4, consequent upon premature termination in Orf4. Finally, the absence of Orf4 may affect the pattern of splicing in E4 so as to increase Orf6 mRNA levels. Orf4 is known to affect splicing patterns during adenovirus infection via its effect on the phosphorylation state of SR proteins (Kanopka *et al.*, 1996).

The absence of Orf6 during infection led to the enhanced production of a minor 55K-immunoreactive species of around 75 kDa (55K*), which was shown to be modified on residue 104, the known site of SUMO-1 modification on 55K (Endter *et al.*, 2001). Such modification is also characteristic of PML and is essential for the protein to form ND10 structures (Müller *et al.*, 1998). In addition, many other ND10 components are SUMO-1-modified, though typically this modification is not necessary for the protein to localize to ND10 (reviewed by Seeler & Dejean, 2001). Given the propensity for SUMO-1-modified proteins to associate with ND10, it seemed likely that this biochemical subset of the protein might be equivalent to the ND10-associated subset seen by immunofluorescence. However, of the four viruses *dl355*, *dl366**, *dl366*+3* and *dl366*+4* that showed increased levels of this modified 55K form, two (*dl366** and *dl366*+4*) clearly did not show increased ND10-associated 55K as compared to *wt300*. Thus, the amount of SUMO-1-modified 55K was not directly related to the steady-state level of 55K associated with ND10. A common feature among these four viruses is that they lack Orf6 expression and so 55K association with the matrix fraction (ND10-associated or not) is maximal throughout infection. This suggests that SUMO modification of 55K is related to this association rather than to ND10 association specifically. As discussed, Orf3 may, through its affinity for both 55K and ND10, alter the kinetics of 55K–ND10 association so that a greater proportion of matrix-associated 55K is seen bound to these structures at steady-state. Perhaps these two components of matrix associated 55K are in dynamic equilibrium. Studies in live cells will be needed to address this question.

Data presented here show that the level of expression of Orf6 during infection is a dominant factor in determining whether or not E1b 55K associates with the nuclear matrix fraction of the cell, while Orf3 expression determines whether or not 55K is found localized with PML in reorganized ND10 within this fraction. Thus, high levels of Orf6 preclude the association of 55K with the matrix, which is a default localization for a significant fraction of 55K in the absence of all E4 proteins. This effect of Orf6 overexpression was dependent on the ability of the 55K protein to form a complex with Orf6. Thus, there are complex effects on 55K localization consequent upon expression of Orf3 and/or Orf6. Moreover,

mutations in E4 outside of the Orf3 and Orf6 coding regions can affect expression of these proteins indirectly. These effects will contribute to the phenotypes observed for such mutants and need to be considered carefully in interpreting data from studies involving such viruses.

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