Adenovirus core protein V interacts with p32 – a protein which is associated with both the mitochondria and the nucleus

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Introduction

Adenoviruses have a complex capsid structure enclosing the virus core which itself consists of the adenovirus genome and its associated proteins. The major core protein VII is very basic in character and is tightly associated with the virus DNA. Two other proteins are also in close proximity to the DNA – µ protein and the terminal protein, the latter being covalently linked to the 5′ termini of the DNA. The remaining core protein, V, appears to be in a bridging location being associated both with protein VII and with polypeptide VI – a polypeptide situated at the apices of the icosahedral capsid [Matthews & Russell, 1994, 1995, 1998 (accompanying paper)].

Entry of the virus into the cytoplasm of the host cell appears to be effected by interaction of the virus capsomeres with cellular receptors and integrins although the integrity of the virus capsid also seems to be important (for a review see Russell & Kemp, 1995). However, the processes governing the entry of the virus genome into the cell nucleus where virus transcription and replication occurs are not understood. We have shown that the virus capsid appears to be stripped off during the primary events in the infection of the host cell and that the core proteins enter the nucleus along with virus DNA (Matthews & Russell, 1998). In the light of these observations we surmised that core protein V may play a role in localizing the virus genome to the nucleus, particularly as the polypeptide harbours a number of putative nuclear location signals (NLSs) including a bipartite NLS (Dingwall & Laskey, 1991). We therefore employed the yeast ‘two-hybrid’ technique using polypeptide V as the ‘bait’ along with a human cDNA library. This procedure indicated that polypeptide V interacted with a cellular protein, designated p32 protein, previously established as being in close association with human splicing factor ASF/SF2 (Krainer et al., 1991). A variety of in vitro and in vivo assays confirmed the interaction and by utilizing an antiserum prepared against the purified p32 we have, by immunofluorescence and confocal microscopy, been able to demonstrate that it has a cytoplasmic location in close association with mitochondria as well as being present in the nucleus as granules and tubules and that it seems to play a role in infection. We postulate that p32 is a component of a shuttle system that operates between the cytoplasm and the nucleus.

Methods

† Cells and viruses. HeLa and A549 cells cultured in minimal Eagle's medium (MEM) were used to propagate human wild-type 2 adenovirus (Ad2 wt) and the temperature sensitive mutant ts1 (see Weber, 1995). Cells were infected and viruses purified as described previously (Matthews & Russell, 1994).
Western and west-Western blotting. Samples of infected cell extracts, purified virus and other proteins were prepared, subjected to SDS-PAGE and transferred to nitrocellulose sheets. Western and west-Western blotting were then performed – all as described more fully by Matthews & Russell (1994).

Cloning and expression of recombinant protein V in E. coli. Amplification of DNA using the PCR technique, cloning, sequencing of recombinant clones (to ensure the correct reading frame) and expression were as previously described (Matthews & Russell, 1994, 1995).

Primers were also designed so that the amplified protein V gene could be cloned in-frame into a yeast ‘two-hybrid’ vector (pGBT9; Clontech) to examine whether protein V interacted with cellular proteins in a manner which could be detected by the yeast two-hybrid assay (see below).

Screening for cellular proteins that interact with adenovirus protein V using the yeast two-hybrid system. The gene for protein V (see above) was inserted into plasmid pGBT9 as the bait to select a partner from a cDNA human lymphocyte library which was inserted proximal to the GAL4 activating domain in a plasmid kindly supplied by S. J. Elledge (Durfee et al., 1993). The Clontech protocols for transfection of HPTC yeast cells (requiring histidine) and selection in the absence of histidine and on the basis of production of β-galactosidase were followed. In the critical experiment approximately 50,000 cDNA clones were tested providing 20 clones in the initial screen. These yeast cultures were then submitted to a ‘curing’ procedure involving growth in media containing histidine and tryptophan and colonies finally replica-plated onto solid media lacking or containing tryptophan. Colonies which would not grow in the absence of tryptophan (i.e. which contain only the cDNA fusion plasmid and not the ‘bait’ plasmid) were selected. Only two colonies survived this procedure and after transformation of the extracted yeast DNA into competent V.LI BLUE E. coli the plasmids were sequenced using standard (Clontech) primers and a Sequenase kit (USB).

Cloning and expression of protein p32 in E. coli. Analysis of the original cDNA clone of p32 by restriction and sequencing indicated that the cDNA contained the codons for the gene from just after the start codon for the preprotein for p32 and extended well beyond the stop codon for this gene. The gene sequence for mature p32 was amplified from the cDNA library clone and inserted into another activation domain vector (pGAD 424; Clontech) ensuring that the gene was in-frame. This new clone (pGADp32) also interacted with protein V in the yeast two-hybrid system using the procedures described above.

For the cloning and expression of protein p32 two primers were used:
Primer 1: 5′ GCCGATCCGAATTCCATGACACCGACGGAGAC 3′
Primer A: 5′ CTTTGACGGATTCGTCGCTCCTACTGGC 3′

Primers 1 and A amplify between nucleotides 281 and 946 of the p32 gene (using the nucleotide numbering of Honore et al. (1993)). The p32 protein is derived from a larger precursor by proteolytic processing between amino acids 73 and 74 exposing an N-terminal leucine (codon CTG); this codon was altered to a methionine codon in primer 1 (double underlined) as part of an NdeI restriction enzyme site. Using either the BamHII site or the NdeI site in primer 2 the gene was cloned into pRSETA respectively either with (clone rP32) or without (clone rMp32) the ‘six histidine’ nickel-binding motif being present at the N terminus of the expressed recombinant protein. The EcoRI site in primer 1 allows in-frame cloning into the pGAD 424 vector (pGADp32) in order to study further the interaction of protein V and p32 in the yeast two-hybrid system (see above).

Expression and purification of rP32 from E. coli were carried out using Ni–agarose as for protein V. For the purification of rMp32, growth, induction and harvesting of the bacterial pellet were as described by Matthews & Russell (1994). Once harvested, the bacteria were lysed, genomic DNA enzymatically degraded and the lysate cleared before applying to a Mono Q ion exchange column (Pharmacia) using FPLC. A continuous gradient from low salt (10 mM Tris–HCl pH 8.0) to high salt (10 mM Tris–HCl pH 8.0; 1 M KC1) was applied to the column and the eluates collected in 0.5 ml fractions. Fractions were assayed for rMp32 content by SDS–PAGE followed by staining with Coomassie brilliant blue. Appropriate fractions were pooled and dialysed against PBS.

Preparation of rabbit polyclonal antibodies against p32 protein. A young adult rabbit was given an intramuscular injection of 25 µg rP32 in PBS purified in the presence of 6 M guanidine.HCl (with Freund’s complete adjuvant). This was followed 10 days later by a second intramuscular injection of rMp32 (400 µg protein which had been dialysed against 6 M guanidine.HCl and then back into PBS and rP32 (20 µg initially purified in the presence of 6 M guanidine.HCl and a further 20 µg purified without exposure to 6 M guanidine.HCl) mixed as before with Freund’s complete adjuvant. Ten days and 17 days later the rabbit received intravenous injections of rMp32 (each injection comprising 600 µg total protein of which half had been previously exposed to 6 M guanidine.HCl). Fourteen days after the second intravenous injection a final intravenous injection was administered (400 µg rMp32 protein which had not been exposed to 6 M guanidine.HCl). Ten days after the last injection the rabbit was bled out as test bleeds had indicated serum antibodies had been raised against p32 protein. Preimmune serum was obtained prior to any injections. All animals were kept and used in accordance with the appropriate UK Home Office guidelines and regulations.

Coupling of rMp32 to activated Sepharose. rMp32 (1 mg (500 µl)) of was coupled to 0.5 g of CNBr-activated Sepharose (Sigma) using standard protocols and the final p32 matrix was suspended in approximately 4 ml PBS, 0.1% sodium azide.

A control matrix was prepared using the same protocol except that 5 mg of purified glucose oxidase (Sigma) was used for coupling to the Sepharose.

Affinity purification of p32 polyclonal antiserum. Approximately 20 mg of rMp32–Sepharose beads was incubated with 0.6 ml of rabbit anti-p32 serum at 4 °C on an end-over-end shaker for 3 h. The beads were washed with 10 × 1 ml aliquots of PBS, each time pelleting the beads by low-speed centrifugation (2000 g, 2 min). After the final wash 100 µl 0.2 M glycine (pH 2.0) was added and the beads were vortexed before pelleting, removing the supernatant and adding it to 20 µl 1 M Tris–HCl pH 9. A further four aliquots were collected in this manner, pooled and stored at −20 °C. The matched pre-immune serum was treated in the same manner except that protein A–Sepharose (Sigma) beads were used. The affinity-purified antibodies were assayed for total protein content using Naphthalene Black, indicating a concentration of 0.1 mg/ml for anti-p32 serum and 0.4 mg/ml for the preimmune serum.

Immunoprecipitation. Approximately 3 × 10⁷ A549 cells were infected or mock infected with Ad2 at an m.o.i. of 10 p.f.u. per cell and at 18 h the cells were harvested. After washing, the cells were suspended in 100 µl of lysis buffer (20 mM Tris–HCl pH 8.0, 0.15 M NaCl, 5 mM EDTA, 0.5% NP40, 0.01% PMSF) and sonicated (MSE Soniprep 150) on ice. The extracts were then centrifuged (15,000 g, 15 min) and supernatants utilized for immunoprecipitation. Before adding antibodies the extracts were incubated with formalin-fixed Staphylococcus protein A in PBS at room temperature for 15 min and centrifuged (15,000 g, 10 min). Aliquots (25 µl) of the supernatants were then incubated with 10 µl rabbit antibodies at the appropriate dilutions at room temperature for 60 min and then 20 µl 10% Staphylococcus A in PBS was added and
after a further 15 min submitted to centrifugation (10000 g, 5 min). The pellets were resuspended in washing buffer (20 mM Tris–HCl pH 8.0, 0.25 M NaCl, 5 mM EDTA, 0.5% NP40, 0.001% PMSF), centrifuged and washed three times by centrifugation and resuspension. The final washed pellets were resuspended in disruption buffer and after boiling were submitted to SDS–PAGE, the resultant gel blotted onto nitrocellulose and Western blotting performed as described previously using a 1:1 mixture of rabbit antiserum against p32 and protein V at dilutions of 1:20. Horseradish peroxidase linked to protein A (Amersham) was utilized prior to development by ECL.

■ Fluorescent antibody procedures. HeLa or A549 cells (free of mycoplasma as checked by the DAPI technique (Russell et al. 1975)) were grown as monolayers in circular coverslips in the wells of a 24-well plastic plate. Each well was infected (or mock infected) with 10 µl of cold (4 °C) Ad2 virus seed (using virus partially purified by fluorocarbon extraction (Winters & Russell, 1971)) in 50 µl 1% newborn calf serum in MEM (NBC-MEM) to give an added m.o.i. of about 100 p.f.u. per cell. In those experiments examining cells later in infection the multiplicity was reduced to 1 p.f.u. per cell. After incubation at 37 °C in an atmosphere of 5% CO₂ for 30 min, 150 µl of warm NBC-MEM was added to each well and all the medium removed and replaced with 1 ml of fresh warm NBC-MEM. After incubation for the appropriate time the coverslips were removed, washed once in PBS and then added to a solution of 4% paraformaldehyde in buffer A (100 mM NaCl, 150 mM sucrose, 5 mM HEPES, 1.5 mM MgCl₂, 0.5 mM PMSF, 0.5 mM EDTA at pH 6.8) on ice for 5 min. The coverslips were then washed in PBS and transferred to the permeation buffer (0.5% Triton X-100 in buffer A) for 5 min at room temperature. In some experiments where greater penetration of the nucleus was desirable the cells on the coverslips were fixed by placing in methanol–acetone (1:1) at −20 °C for 10 min and then air dried. After a wash in PBS the fixed cells on coverslips were then spotted with 10 µl of the appropriate antibody dilutions in PBS and incubated in a humidified box at 37 °C for 20 min. Further washing in PBS was followed by spotting the coverslips with 10 µl of a mixture of Texas Red- or Rhodamine-conjugated anti-rabbit and Fluorescent Green anti-mouse IgG (Jackson Immuno Research Laboratories, Inc. and Centricon International) in PBS at the optimum dilutions. Incubation at 37 °C for 20 min was followed by washing in PBS and the coverslips were mounted in Citifluor NF1 (University of Kent) and examined in a Bio-Rad Confocal Laser microscope. Antibodies utilized in addition to the ones described elsewhere were: a mouse monoclonal antibody against adenovirus DNA-binding protein (DBP) prepared using DBP purified as described previously (Russell et al., 1989); a mouse monoclonal antibody from Jane Flint prepared using purified V protein from disrupted virus (Lunt et al., 1988); and a mouse monoclonal antibody (3G5-F7-G3 (Molecular Probes)) to subunit Vlc of cytochrome oxidase.

To validate the specificity of the p32 antiserum, 10 µl of affinity-purified serum (0.1 mg/ml) was incubated with 20 µl of purified rMp32 (2 mg/ml) and 30 µl PBS at room temperature in an end-over-end shaker for 90 min. This was followed by centrifugation (15000 g, 5 min) and the supernatant utilized for fluorescent staining and compared to a control supernatant obtained after parallel incubation and centrifugation of the p32 antiserum with BSA (2 mg/ml).

Results

A yeast two-hybrid assay selected the cellular protein p32

The gene for protein V was inserted into the shuttle plasmid pGBT9 fused to the DNA-binding domain of GAL4 (Clontech). The plasmid was transformed into the yeast strain HP7C (requiring histidine) and the resulting yeast cells were utilized to prepare competent yeast cells into which was transfected a plasmid (kindly supplied by S. Elledge) containing a cDNA library from human lymphocytes fused to the gene encoding the GAL4-activating domain. A standard yeast two-hybrid selection procedure (Clontech) was carried out (see Methods) yielding two colonies which fulfilled the criteria for interaction of the gene products. Checking the sequences obtained against the GenEMBL database revealed that one of the plasmids contained 120 bases of the gene for the ‘splicing factor associated protein p32’ as originally described by Krainer et al. (1991). The p32 protein was subsequently shown to be synthesized as a precursor protein of 282 amino acids which is processed in human cells by removal of the N-terminal 73 residues to form the mature p32 protein (Honore et al., 1993).

Primers were designed and utilized to facilitate PCR amplification of the mature p32 using the cDNA library clone and to insert the gene into the pRSET A expression plasmid (i.e. fused with the Ni-binding sequences) (rp32) and also for insertion in the plasmid without fusion to the histidine residues (rMp32). In all these manipulations, the N-terminal leucine of the mature p32 was altered to methionine (see Methods). The p32 gene was also inserted into the plasmid pGAD 424 – containing the GAL4-transactivating domain protein. The latter plasmid was then used as a partner to the p4BT9 plasmid containing the V gene to confirm that co-transfection of yeast resulted in colonies with a phenotype indicative of interaction between the two gene products (data not shown).

Expression and purification of p32 protein and preparation of a rabbit polyclonal antibody

Recombinant p32 protein fused to six histidine residues (rp32) was purified by Ni–agarose chromatography as described previously. The rMp32 plasmid also gave good yields of the p32 protein and purification was readily achieved (see Methods) by FPLC using a mono Q column giving a major single band on SDS–PAGE (Fig. 1a). A rabbit antiserum to p32 was raised by inoculation of various preparations of p32 (including one which had been denatured by treatment with guanidine.HCl – see Methods). This protocol was followed to ensure a good response and in the light of previous unsuccessful attempts to obtain an antibody (Simos & Georgatos, 1994). In Western blotting the antiserum reacted well with cellular p32 in HeLa cell extracts but also with a 70 kDa polypeptide (data not shown). However, on purification of the antibody by using purified rMp32 attached to Sepharose beads the antibody reacted in Western blotting only with p32 from HeLa cells (both infected and uninfected) and with the purified rMp32 obtained from E. coli (Fig. 1 bii). Most of the subsequent assays were carried out with this affinity-purified antibody.
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**p32 binds to protein V in infected cells**

A west-Western blot using rV as a probe demonstrated binding to purified rMp32 and probably to p32 with infected cell extract (Fig. 1bii, lanes 3 and 1). It is significant that the interaction could be detected using very low concentrations of rV and even when the p32 was only partially renatured on the blot – suggesting that an efficient recognition mechanism was operating. The binding of rV to other cellular and virus polypeptides could also be observed (Fig. 1bii, lanes 1 and 2) and the significance of some of these events is discussed in the accompanying paper (Matthews & Russell, 1998).

An interaction between p32 and protein V in vivo was also demonstrated by immune precipitation (Fig. 2). In this study extracts of uninfected and infected cells were incubated separately with antisera to p32 and protein V and following addition of fixed Staphylococcus A and centrifugation the washed pellets were submitted to SDS–PAGE followed by Western blotting using a mixture (1:1) of rabbit antisera to p32 and protein V (see Methods). Lane 3 shows that with extracts of infected cells p32 antibody also precipitated V antigen and lane 5 indicates that in the reciprocal reaction V antibody also precipitated the p32 antigen. It should be noted that the V antigen in the infected cell extracts seems to be mostly the degraded 39 kDa species (see Matthews & Russell, 1998).

In another series of experiments (data not shown) extracts of Ad2-infected and uninfected cells were incubated with an rMp32 affinity matrix and it was found that protein V bound to the matrix even after washing with buffer containing 500 mM salt and 0.5% NP40. Other virus proteins (hexon, penton, IIIa, fibre, VI, 23K protease, VII) appeared to elute under the same conditions. No binding of any virus proteins to a matrix of glucose oxidase (with a similar pI to p32) was seen with these elution parameters.

**p32 was detected in the cytoplasm in a pattern consistent with an association with mitochondria and also in the nucleus**

Using the p32 antibody and confocal microscopy, immunofluorescence was readily discernible in the cytoplasm (Fig. 3, a).
Fig. 4. Confocal micrographs of Ad2-infected and mock-infected cells using antibodies labelled with Texas Red or Rhodamine and Fluorescent Green. Cell fixation using paraformaldehyde followed by Triton unless otherwise stated. White bars, 10 µm.

(a) Mock infected A549 cells, fixed in acetone–methanol at −20 °C; rabbit anti-p32 (Texas Red) and mouse monoclonal anti-
(b) (cont. overleaf)
left panel) with a ‘stringy’ appearance very characteristic of mitochondria (see Bereiter-Hahn, 1990, for a review). Confirmation of the association of p32 with mitochondria was obtained by labelling cells with an antibody against cytochrome oxidase (Fig. 3, right panel). By using fixation conditions which allowed more effective permeability of membranes (acetone–methanol, 1:1, at −20 °C) nuclear staining as discrete granules and tubules was also apparent (Fig. 4a). The nuclear location was confirmed by viewing successive sections through the nucleus. With these fixation conditions the characteristic cytoplasmic structures collapsed significantly although considerable congruence between p32 antigen (red) and cytochrome oxidase (green) was retained as indicated by the orange-yellow fluorescence. To ensure that the p32 staining patterns were not due to non-specific binding of the rabbit antibody, immunoglobulin from the pre-immune serum was prepared using Protein A–Sepharose and incubated with cells using approximately 40 times more antibody protein than in the equivalent experiment using p32 antibody. No fluorescence was detected (data not shown). Preincubation of the p32 antibody with an excess of bacterially expressed and purified rMp32 also completely abrogated both the cytoplasmic and nuclear staining (data not shown). The mitochondrial staining was also apparent in a number of other human and monkey cell lines – sometimes with a clumpy cytoplasmic distribution rather than the extended pattern seen in HeLa cells (e.g. in A549 cells – see Fig. 4a).

The characteristic pattern of p32 staining is perturbed on adenovirus infection

HeLa cells were infected with Ad2 as described in Methods and examined by confocal microscopy using double-labelling with fluorescent antibodies against p32, V and DBP – an ‘early’ non-structural protein essential for virus DNA replication (Fig. 4).

Very early in infection (viz. 45–60 min) protein V was recognized in the cytoplasm of the infected cell accompanied by a marked concomitant alteration and diminution of the p32 antigen distribution. In some cells there was an orange-yellow fluorescence indicative of a close association between p32 (staining red) and protein V (staining green) (Fig. 4b). In contrast, on fixing with acetone–methanol a very striking orange fluorescent pattern as well as orange dots and granules in the nucleus were observed (Fig. 4c). As infection proceeded protein V rapidly entered the nucleus (see accompanying paper: Matthews & Russell, 1998) and the p32 pattern recovered to a great extent (data not shown). At intermediate times, viz. 12–16 h post-infection (p.i.), when early protein synthesis gets under way, the characteristic pattern of p32 antigen can still be observed, particularly in these cells when the early antigen synthesis is just beginning. Fig. 4(d) illustrates this point in relation to the production of the early DBP. However, as late virus protein synthesis takes over the p32 pattern gradually collapses – illustrated in Fig. 4(e, f) in those cells where V antigen production can be detected. At late times of infection very significant changes in p32 pattern and distribution were evident. Thus p32 could be demonstrated in some cells only in the nucleus (Fig. 4g), apparently as the red centre of an orange-yellow ‘doughnut’ of protein V. This nuclear location of p32 was confirmed by viewing successive planes through the nucleus. Other cells at the same time showed protein V atypically in the cytoplasm (Fig. 4f) in all cases the characteristic pattern of p32 was completely disrupted. In the light of these observations we suggest that p32 could be considered as a component of a transport system connecting the mitochondria with the nucleus (see Discussion).

Discussion

These investigations were initiated in an endeavour to throw some light on the events that direct the infecting adenovirus genome to the nucleus after adsorption and entry of the virus. We assumed that this was not a random diffusion process and that it would be more feasible to assume that the virus would hijack some of the normal cellular transport processes, particularly the trafficking to the nucleus appeared to be quite rapid (Dales & Chardonnet, 1973). Using the rationale that the virus core proteins – since they would be exposed on disruption of the capsid – would play a role we investigated protein V, a core protein which was not so tightly bound to the virus DNA as protein VII and appeared to occupy a bridging position between the core and capsid (accompanying paper: Matthews & Russell, 1998). In addition protein V harboured a number of NLS motifs.

Following extensive selection procedures using the ‘yeast two-hybrid’ technique we obtained two clones derived from the human cDNA library which showed the appropriate specific interactions with the V gene. One of these gave a DNA sequence (not shown) which did not have similarity to any sequence in the database. The other clone conformed exactly to a section of the gene product which had previously been described as p32 protein–associated with splicing factor ASF/SF2 (Krainer et al., 1991). Since this protein has been also shown to be associated with a wide range of other proteins (Table 1) it was important to confirm the validity of its binding to protein V by a variety of different techniques. As a first step the mature p32 gene was cloned and expressed, the gene

\( \text{cytochrome oxidase (Fluorescent Green).} \)

- (b) Ad2-infected HeLa cells, 60 min p.i.; rabbit anti-p32 and mouse monoclonal anti-V (green).
- (c) Ad2-infected HeLa cells 45 min p.i.; fixed in acetone–methanol at −20 °C; rabbit anti-p32 (red) and mouse monoclonal anti-V (green).
- (d) Ad2-infected HeLa cells 13 h p.i.; rabbit anti-p32 (red) and mouse monoclonal anti-DBP (green).
- (e, f) Ad2-infected HeLa cells 15 h p.i.; rabbit anti-p32 (red) and mouse monoclonal anti-V (green).
- (g, h) Ad2-infected HeLa cells 22 h p.i.; rabbit anti-p32 (red) and mouse monoclonal anti-V (green).
especially in the nucleus late in infection indicated a close
initial events in the cytoplasm and possibly in the nucleus and
ments. The prevalence of orange-yellow fluorescence in the
results of the double-label fluorescent antibody experi-
Hahn, 1990).
membranes have been described (for a review see Bereiter-
component of a continuous mitochondrial–nuclear network
fixation conditions to promote permeation of the nuclear
against the mitochondrial enzyme cytochrome oxidase (Fig. 3).
was no binding of V to glucose oxidase, which has a similar pI
± 2) to p32. This characteristic has been used to purify protein
Western blotting (Fig. 1) and immunoprecipitation (Fig. 2)
product purified and a specific antibody prepared. ‘West-
Western’ blotting (Fig. 1) and immunoprecipitation (Fig. 2)
clearly demonstrated the close interaction between protein V
and p32 in infected cells. Other experiments showed that this
binding was stable even in the presence of 0.5 M salt and
detergent and was not merely a charge interaction since there
was no binding of V to glucose oxidase, which has a similar pI
(4:2) to p32. This characteristic has been used to purify protein
V from extracts of infected cells (data not shown).
The exploitation of the affinity-purified p32 antibody in
fluorescent antibody studies gave rather surprising results,
particularly in the light of the reports of association of p32 with
proteins that clearly functioned in the nucleus (Table 1). The
remarkable cytoplasmic staining pattern is very characteristic
of mitochondria and this was confirmed by using an antibody
against the mitochondrial enzyme cytochrome oxidase (Fig. 3).
Of even more significance was the finding that by altering the
fixation conditions to promote permeation of the nuclear
membranes distinctive staining of p32 in the nucleus in the
form of granules and tabular structures became evident. These
observations suggested that the p32 antigen could be a
component of a continuous mitochondrial–nuclear network
and indeed structural links between mitochondria and nuclear
membranes have been described (for a review see Bereiter-
Hahn, 1990).
A role for p32 during infection seems likely on considering
the results of the double-label fluorescent antibody experi-
ments. The prevalence of orange-yellow fluorescence in the
initial events in the cytoplasm and possibly in the nucleus and
especially in the nucleus late in infection indicated a close
association of p32 with protein V.
Early in infection (under conditions of high m.o.i.) the
characteristic p32 pattern was significantly perturbed (Fig. 4 h)
but since recovery of the pattern was apparent at about 2 h p.i.
(data not shown) it seems very likely that this phenomenon
could be ascribed to the previously described transient
cytopathic effect from interaction of the host cell with the
pentons in the virus inoculum (Russell et al., 1967). The rapid
trafficking of adenovirus particles towards the nucleus has been
previously noted in the pioneering studies of Dales &
Chardonnnet (1973) who surmised that virions were moving
vectorially along pathways provided by microtubules—an
observation not inconsistent with our findings since micro-
tubules are intimately associated with mitochondria (Bereiter-
Hahn, 1990). At intermediate times of infection the charac-
teristic pattern of p32 antigen gradually collapsed as late
protein synthesis developed (Fig. 4 d, e, f). This finding is
perhaps not surprising and probably reflects the onset of
cytopathicogenicity associated with infection. Late in infection
the association between p32 and V was strikingly demon-
strated by the appearance in the nucleus of orange ‘doughnuts’
with red centres. Since it is well-established that at these times
there is an inhibition of cellular metabolism it seems likely that
the atypical locations of p32 are the consequence of impaired
transport processes. Bearing in mind the close proximity of V
to p32 in both the cytoplasm and nucleus (Fig. 4 b, c, g, h) it is
not unreasonable to suggest that protein V (and the genome)
might normally be delivered to the nucleus along with p32 and
that the latter may then be recycled to the cytoplasm—only
being retained in the nucleus when cellular metabolism is
impaired. If this is the case then it could be hypothesized that
p32 is a component of a normal transport pathway connecting
the mitochondria with the nucleus and that adenoviruses hijack
this to gain entry to the nucleus.

After this work was completed a study of p32 by Muta et
al. (1997) asserted that p32 was located in the mitochondrial
matrix and was important in maintaining oxidative
phosphorylation. This latter conclusion was reached by
utilizing the yeast p30 analogue and if accepted then it raises
the possibility that the initial events in adenovirus infection
may modulate the host cell’s energy reservoirs. However,
these workers also suggested that p32 (in contrast to our own
findings) was exclusively located in the cytoplasm and called
for a careful re-examination of the interactions of the many
proteins which have been shown to interact with p32 and are
present in the nucleus (Table 1).

Following our observations as described above we are of
the opinion that although p32 is mainly located in association
with the mitochondria its nuclear presence and possible
involvement in transport processes would provide adequate
explanations for its association with many of the other proteins
listed in Table 1. It is interesting that many of these proteins
are involved in splicing and transcriptional regulation. Thus
HIV Rev appears to be responsible for switching the viral
transcription expression pattern from multiply-spliced viral
RNAs to singly spliced mRNAs in the cytoplasm (Tange et al.,
1996). It is also relevant to note that an earlier publication
(Somasundaran et al., 1994) had shown that HIV RNA

### Table 1. Proteins associated with p32

<table>
<thead>
<tr>
<th>Protein</th>
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<tr>
<td>Adenovirus protein V</td>
<td>This report</td>
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<tr>
<td>ASF/SF2 cell splicing factor</td>
<td>Krainer et al. (1991)</td>
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<tr>
<td>Lamin B receptor protein (p58)</td>
<td>Simos &amp; Georgatus (1994)</td>
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<tr>
<td>(turkey erythrocyte homologue)</td>
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<td>Complement protein C1q</td>
<td>Ghebrehiwet et al. (1994)</td>
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<td>HIV rev mouse homologue (YL2)</td>
<td>Luo et al. (1994)</td>
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<tr>
<td>HIV rev</td>
<td>Tange et al. (1996)</td>
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<td>HIV lat</td>
<td>Yu et al. (1995a)</td>
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<tr>
<td>Herpes simplex virus ORF P</td>
<td>Bruni &amp; Roizman (1996)</td>
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<td>Epstein–Barr virus EBNA-1</td>
<td>Wang et al. (1997)</td>
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<tr>
<td>Transcription factor IIB</td>
<td>Yu et al. (1995b)</td>
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<td>Hyaluronic acid</td>
<td>Deb &amp; Datta (1996)</td>
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<td>Kininogen</td>
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<td>Vitronectin</td>
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trafficked into mitochondria within a short time following virus transcription. Traffic between the nucleus and mitochondria has been described by Chang & Clayton (1987) for an RNA which is necessary for RNA processing and was confirmed later by Li et al. (1994) who utilized both ultrastructural and biochemical approaches to demonstrate the presence of this critical RNA in mitochondria as well as in nucleoli. Thus it may be that the putative p32-associated transport processes involve export of RNAs from the nucleus as well as import of macromolecules into the nucleus.

We also noted that many of the p32-associated proteins have (as protein V) bipartite NLSs (data not shown) and postulated that this motif may provide a ligand perhaps indirectly for p32 association and nuclear import. In an attempt to follow this up and in the light of the reports on the significance of importin as an NLS ligand we carried out a number of experiments using anti-importin α sera (Weis et al., 1995) to ascertain if there was an association between V or p32 and importin but were unable to demonstrate this. Clearly much remains to be uncovered concerning the function of p32 in both infected and uninfected cells.

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