

Organization of the double-stranded RNA-activated protein kinase DAI and virus-associated VA RNA_I in adenovirus-2-infected HeLa cells

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SUMMARY

We have examined the cellular distribution of the double-stranded RNA-activated protein kinase DAI in adenovirus 2 (Ad2) -infected and uninfected HeLa cells. In uninfected cells DAI was found to be concentrated in the cytoplasm. In addition, DAI was localized in the nucleoli and diffusely distributed throughout the nucleoplasm. Cells treated with α -interferon displayed a similar pattern of distribution for DAI. When RNA polymerase I activity was inhibited by the drug actinomycin D, nucleoli segregated and DAI was found to colocalize with the dense fibrillar region of the nucleoli. During mitosis, the distribution of DAI paralleled that of rRNA. In adenovirus-infected cells the localization of DAI was similar to that in uninfected interphase cells. VA RNA_I

was detected in Ad2-infected cells by 10-14 hours post-infection as fine dots in the nucleoplasm. By 18-24 hours post-infection, VA RNA_I appeared in bigger and more abundant dots in the nucleoplasm and the cytoplasm was intensively labeled. Transient expression of the VA RNA_I gene in uninfected cells resulted in a similar localization of the RNA. Our results are consistent with a role for DAI and VA RNA_I in protein synthesis and suggest that DAI may play an early role in ribosome biogenesis in the nucleolus in addition to its cytoplasmic role in translation.

Key words: adenovirus, nucleolus, translation, RNA localization

INTRODUCTION

Adenovirus 2 (Ad2) belongs to subgroup C of adenoviruses, which have the ability to transform cells. The virion is 80 nm in diameter and is composed of a core that includes the viral DNA genome of about 36 kb and four proteins, and a shell that comprises at least 15 different structural proteins organized in a complex capsid (Pettersson, 1984; Akusjärvi et al., 1986; Stewart et al., 1991). Whereas the majority of the viral genome is transcribed by RNA polymerase II (see Ziff, 1980; Tooze, 1982; Flint, 1986; Akusjärvi et al., 1986), the two virus-associated (VA) RNAs, VA RNA_I and VA RNA_{II} (Reich et al., 1966) are synthesized by RNA polymerase III (Price and Penman, 1972; Mathews, 1975; Akusjärvi et al., 1980; Akusjärvi et al., 1986). These two small, noncoding RNAs are about 160 nucleotides long (Akusjärvi et al., 1980) and are transcribed from the r-strand at about 30 map units in the genome (Mathews, 1975; Akusjärvi et al., 1980; Akusjärvi et al., 1986). Although the genes for these RNAs are arranged in tandem and separated by a short DNA spacer (Mathews, 1975; Mathews and Pettersson, 1978; Akusjärvi et al., 1980) they are differentially expressed. Both RNAs are produced early in infection but VA RNA_I synthesis increases rapidly in the late phase and becomes the most abundant RNA in the cytoplasm of infected cells (Söderlund et al., 1976).

Although regulation of Ad2 mRNA formation late in infection takes place extensively in the cell nucleus, including control of transcription and post-transcriptional events such as methylation, splicing, polyadenylation and transport (see McGrogan and Raskas, 1977; Nevins and Darnell, 1978; Philipson 1979; Ziff, 1980; Tooze, 1982; Darnell, 1982; Sharp, 1984; Flint, 1986), once in the cytoplasm, a complex series of events regulate the expression of the Ad2 genome at the level of protein synthesis.

During the late phase of infection, cellular protein synthesis is inhibited while viral protein production is enhanced (Beltz and Flint, 1979). The mechanism whereby the cellular translational apparatus is selectively engaged in viral protein synthesis is not fully understood despite considerable effort expended to try to understand the different aspects of this phenomenon (Mathews, 1990; Zhang and Schneider, 1993). Translational control in adenovirus infected cells involves the participation of the cellular kinase DAI (double-stranded RNA-activated inhibitor of translation) and the small viral VA RNA_I (see Mathews, 1990; Mathews et al., 1990; Mathews and Shenk, 1991).

DAI, also known as the interferon-induced protein kinase (p68) is present in a latent form in most cells (Kostura and Mathews, 1989; Mathews and Shenk, 1991). It is activated by low concentrations of double-stranded (ds) RNA, probably generated during viral infection by symmetrical tran-

scription of the genome (Maran and Mathews, 1988), but high concentrations of dsRNA prevent such an activation (Mathews et al., 1990; Mathews and Shenk, 1991). Upon binding of dsRNA, the protein displays two kinase activities. First, the enzyme autophosphorylates, resulting in unmasking of its second activity, which is to phosphorylate the β -subunit of the eukaryotic translation initiation factor-2 (eIF-2). In uninfected cells, DAI remains in a latent, inactive form and does not phosphorylate eIF-2, allowing this factor to engage in multiple rounds of initiation of protein synthesis (Schneider et al., 1984, 1985; Reichel et al., 1985; Mathews, 1990; Mathews et al., 1990; Mathews and Shenk, 1991).

A mutant virus lacking the gene for VA RNA_I grows poorly, and the defect was ascribed to a lesion in protein synthesis (Thimmappaya et al., 1982; Mathews, 1990; Mathews and Shenk, 1991). The sequence of VA RNA_I is known (Akusjärvi et al., 1980; Pe'ery et al., 1993). It forms extensive secondary structure (Furtado et al., 1989; Mellits and Mathews, 1988; Pe'ery et al., 1993) and functions to prevent DAI activation. In fact, VA RNA_I can bind to DAI (Katze et al., 1987; Galabru et al., 1989; Mellits et al., 1990a) mediated principally by its duplexed apical stem (Mellits et al., 1990a; Ghadge et al., 1991; Clarke et al., unpublished), while another structural feature, the central domain, is responsible for the blocking function (Mellits and Mathews, 1988; Furtado et al., 1989; Mellits et al., 1990b).

During adenovirus infection, VA RNA_I prevents the activation of DAI by viral dsRNA (Maran and Mathews, 1988), thereby maintaining the rate of protein synthesis in the cell (Mathews, 1990; Mathews et al., 1990; Mathews and Shenk, 1991). In addition, it has been proposed that the VA RNA_I-DAI system contributes to the preferential translation of viral mRNAs late in infection (O'Malley et al., 1989; Svensson and Akusjärvi, 1985).

In the present study we have used polyclonal and monoclonal antibodies to DAI and a DNA probe to Ad2 VA RNA_I to localize these molecules in the cell by immunofluorescence microscopy and in situ hybridization. Single- and double-label (data not shown) experiments have shown that DAI and VA RNA_I localize diffusely in the cytoplasm of infected cells late in infection. Furthermore, DAI was found in the nucleolus and nucleoplasm of uninfected and infected cells. VA RNA_I was localized as discrete and abundant small round bodies in the nuclei of infected cells or uninfected cells transfected with the VA RNA_I gene. Our results provide cytochemical evidence to support the role of DAI and VA RNA_I in protein synthesis late in adenovirus infected cells, and also suggest a possible role for DAI in the early steps of ribosome biogenesis within the nucleolus.

MATERIALS AND METHODS

Cells, virus and infection

HeLa cells were grown on glass coverslips for 1-2 days in Dulbecco's modified Eagle's medium (Gibco) containing 10% fetal bovine serum. Cells were infected with wild type adenovirus-2 (Ad2) at a multiplicity of infection (moi) of 30 plaque forming units (pfu)/cell. After 1 hour of adsorption, the monolayers were rinsed with PBS and fresh medium was added. Further incubation for 7, 10, 14, 18 or 24 hours was carried out before in situ hybridization or immunocytochemistry.

Antibodies and probes

Polyclonal antibodies were prepared as described (Green and Mathews, 1992). Monoclonal antibody to DAI (Laurent et al., 1985) was a gift from A. G. Hovanessian (Institut Pasteur). Human autoantibody to fibrillarin was provided by R. L. Ochs (Scripps Research Institute). Approximately 3 μ g of DNA encoding VA RNA_I (plasmid pT7VA/Ad2I; Mellits et al., 1990b) was labeled by nick translation in the presence of 50 μ M of bio-11-dUTP (Sigma), dATP, dCTP and dGTP. The size of the resulting fragments was monitored by electrophoresis in a 1% agarose gel. Nick translation reactions were stopped when a fragment size of about 200 base pairs was obtained. The probe was purified through a Sephadex G-50 (Boehringer Mannheim) column and stored at -20°C until use.

Immunofluorescence

After fixation for 15 minutes with 2% paraformaldehyde or for 3 minutes at -20°C with 100% methanol, immunofluorescence was performed as described (Spector and Smith, 1986) using polyclonal antibodies to DAI at a dilution of 1:30 or monoclonal antibodies to DAI at a dilution of 1:5 in Tris buffered saline (TBS: 20 mM Tris-HCl, pH 7.6, 150 mM NaCl), with 20 mM Na₃N and 1% Tween-20. As controls, we performed immunofluorescence with preimmune serum for the polyclonal antibody or with antibodies to DAI preabsorbed with immunoaffinity purified (Galabru and Hovanessian, 1987) or glycerol gradient fractionated (Kostura and Mathews, 1989) DAI. Incubation times with the first antibody were either 1 hour at room temperature or 16 hours at 4°C . Texas Red-conjugated horse anti-mouse, Texas Red-conjugated goat anti-rabbit (Vector, Burlingame California) or FITC-conjugated goat anti-mouse (Organon-Teknika, West Chester, PA) secondary antibodies were used at a dilution of 1:20 in TBS plus 5% bovine serum albumin (BSA) and 5% normal goat serum (NGS). Coverslips were mounted in anti-fade medium (90% glycerol, 10% PBS plus 1 mg/ml of paraphenylenediamine). The final pH was adjusted to 8.0 with 0.5 M carbonate-bicarbonate buffer, pH 9.0. Cells were examined with a Nikon (Nikon Inc., Melville, New York) FXA epifluorescence microscope equipped with a $\times 60$, 1.4 NA objective lens.

In situ hybridization and detection

Cells were fixed with freshly made 2% or 4% paraformaldehyde in PBS, pH 7.3, for 15 minutes at room temperature at 0, 7, 10, 14, 18 and 24 hours post-infection (hpi). After rinsing in PBS (3 \times 10 minutes each), cells were permeabilized with 0.2% Triton X-100/0.5% NGS for 5 minutes at 4°C , then rinsed in PBS (3 \times 10 minutes each) and in 2 \times SSC (5 minutes). For each coverslip, 750 ng of nick translated DNA encoding VA RNA_I and 20 μ g of carrier *E. coli* tRNA were dried down in a Speed Vac (Savant). The probe and tRNA were denatured by heating in 10 μ l of deionized formamide for 10 minutes at 70°C . The probe was chilled on ice immediately and hybridization buffer containing 4 μ l of 5% BSA, 4 μ l of 50% dextran sulfate, and 2 μ l of 20 \times SSC was added to each tube so that the final concentration in the hybridization mixture was about 40 ng/ μ l of the VA RNA_I probe, 1 μ g/ μ l of *E. coli* tRNA, 4 \times SSC, 1% BSA and 10% dextran sulfate. Then, 20 μ l of hybridization mixture was placed onto each coverslip. Coverslips were inverted onto a slide, sealed with rubber cement, and incubated in a humidified chamber for 16 hours at 42°C . After rinsing in 2 \times SSC, 50% formamide at 37°C , 2 \times SSC and 1 \times SSC at room temperature for 30 minutes each, cells were incubated in 4 \times SSC, 0.25% BSA, and 2 μ g/ μ l avidin-FITC (Vector, Burlingame, California) for 90 minutes at room temperature in a humidified chamber in the dark. Coverslips were then rinsed in 4 \times SSC for 30 minutes at room temperature, 4 \times SSC, 0.1% Triton X-100 for 10 minutes and 4 \times SSC three times

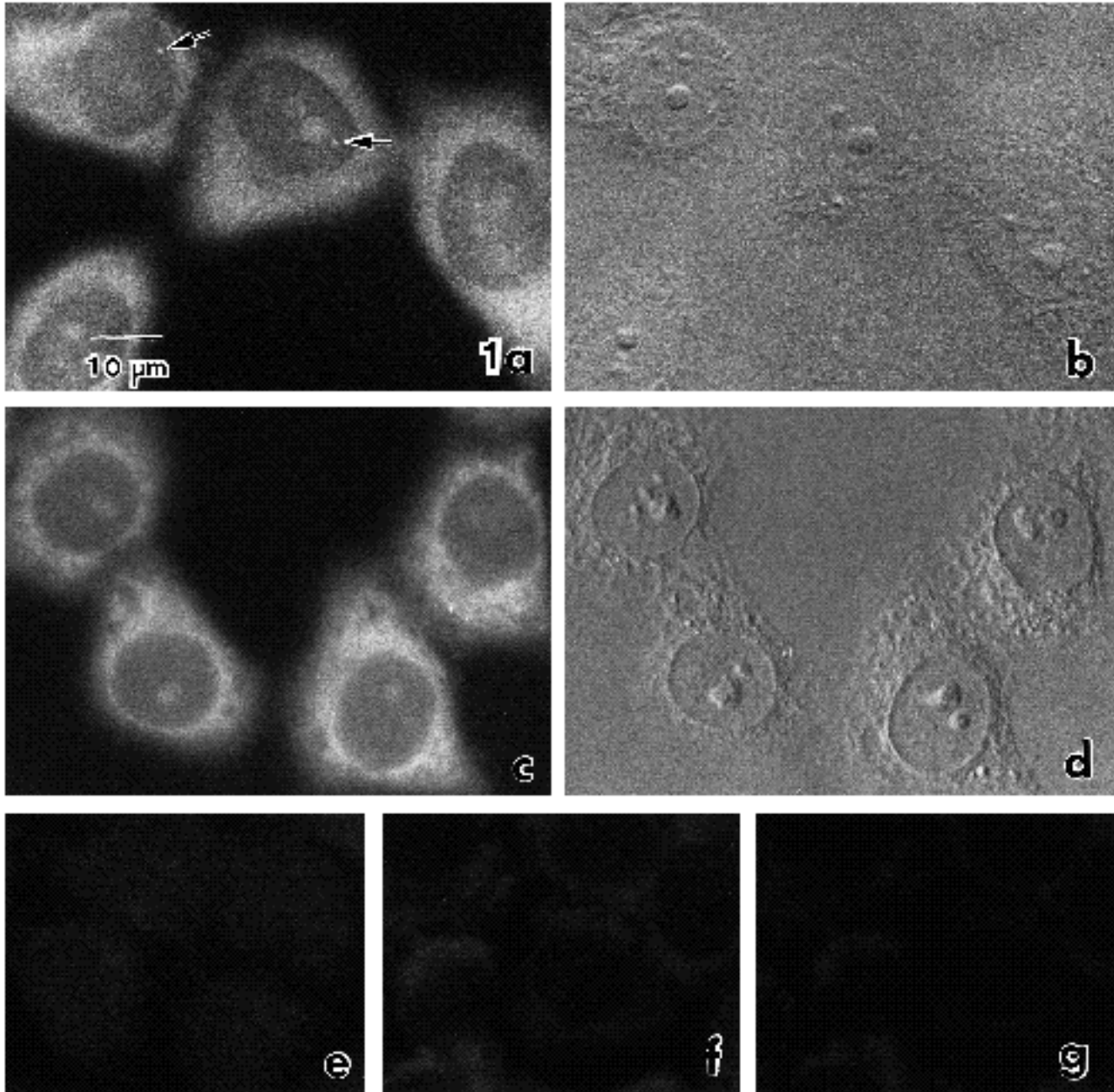


Fig. 1. Localization of DAI in uninfected HeLa cells. Immunofluorescence using polyclonal (a) or monoclonal (c) antibodies, and corresponding differential interference contrast images (b, d) of HeLa cells shows the presence of DAI in the cytoplasm, and nucleoli. The nucleoplasm is also slightly labeled. Coiled bodies were also labeled with polyclonal antibodies to DAI (a, arrows). Cells incubated with preimmune serum (e) or monoclonal antibodies to DAI preabsorbed with immunoaffinity purified (Galabru and Hovanessian, 1987) (f) or glycerol gradient fractionated DAI (Kostura and Mathews, 1989) (g) used as primary antibodies, showed no signal.

for 10 minutes each. Coverslips were mounted in 90% glycerol, 10% PBS, containing 1 mg/ml paraphenylenediamine as an antioxidant to prevent bleaching. Cells were examined with a Nikon FXA epifluorescence microscope.

In situ hybridization-immunofluorescence combined protocols

Immunofluorescence following in situ hybridization was conducted as described (Huang and Spector, 1991) with the follow-

ing modifications. Briefly, cells were hybridized as above and after incubation in FITC-avidin to detect the hybrids, and washes with 4× SSC, cells were placed in PBS for 10 minutes and incubated with monoclonal antibody to DAI as described above. The inverse procedure, antibody labeling followed by in situ hybridization, was also carried out and produced similar results.

Confocal laser scanning microscopy

A Zeiss (Carl Zeiss Inc., Thornwood, New York) confocal laser

scanning microscope, equipped with a $\times 63/1.4$ NA oil immersion lens and an argon ion laser (wavelength, 488 nm) to excite FITC-fluorescence was used for optical sectioning. Data sets of optical sections were collected at 250 nm intervals through each nucleus. Single optical sections and left/right stereo pairs of labeled nuclei reconstructed from serial optical sections were generated using the Zeiss Confocal Laser Scanning software version 2.03. Images were recorded on a Sony color video printer (Mavigraph) and on Kodak T-Max 100 film or Kodak Ektachrome 100 HC film using a Matrix Multicolor image recording system.

Nuclease digestions

Paraformaldehyde fixed cells were treated with either DNase I or RNase A at a concentration of 100 $\mu\text{g/ml}$ for 2 hours at room temperature. After extensive washes cells were prepared for in situ hybridization as described above.

Post-embedding immunoelectron microscopy

Monolayers of HeLa cells were fixed in 4% paraformaldehyde plus 0.05% glutaraldehyde in PBS, pH 7.3, dehydrated in ethanol and embedded in Lowicryl K4M resin. Thin sections (80 nm) were cut with a Reichert-Jung Ultracut E ultramicrotome using a Diatome diamond knife and were mounted on gold grids. The grids were floated in TBS for 60 minutes at room temperature and incubated with monoclonal antibody to DAI at a dilution of 1:2 in TBS for 16 h at 4°C. Then 15 nm colloidal gold-conjugated goat anti-mouse IgG (Amersham) (diluted 1:20 in TBS) was used as secondary antibody for 60 minutes at room temperature. After rinsing with TBS for 10 minutes, grids were washed with distilled water and stained with uranyl acetate and lead citrate. The samples were observed with a Hitachi H-7000 transmission electron microscope (Hitachi Ltd., Rockville, MD) operating at 75 kV.

Drug studies

HeLa cells growing on coverslips were exposed to 0.2 $\mu\text{g/ml}$ of actinomycin D for 4 hours at 37°C (Ochs et al., 1985a). After drug treatment, cells were immunolabeled as described above with a monoclonal antibody to DAI or human autoantibody against fibrillarin at a dilution of 1:200. Detection was carried out with secondary antibodies coupled to FITC or Texas Red, respectively.

Transfection

HeLa cells growing on coverslips were transfected with 2.5 $\mu\text{g/cover slip}$ of plasmid pMHVA (Mellits and Mathews, 1988) for 6 hours, using a modified calcium phosphate technique (Wigler et al., 1978). Cells were then fixed and processed for in situ hybridization using the pT7VA/Ad2I probe 24, 48 and 60 hours post-transfection. Control cells were transfected with 2.5 μg of plasmid pUC119.

Interferon treatment

HeLa cells growing on coverslips were treated with β -interferon (obtained from Paul Trotta, Schering Corp., Bloomfield, New Jersey) at a concentration of 800 units/ml for 18 hours at 37°C. After the treatment, cells were immunolabeled with monoclonal antibody to DAI.

RESULTS

DAI localizes in the cytoplasm and nucleoli of uninfected and Ad2 infected HeLa cells

We have localized DAI in the cytoplasm and nucleoli of uninfected HeLa cells with either polyclonal (Fig. 1a) or monoclonal (Fig. 1c) antibodies directed against the pro-

tein. Intense and almost homogeneous signal was observed in the cytoplasm. In addition, a weaker fluorescence was detected in the nucleoli and also a diffuse labeling of the nucleoplasm was observed. The labeling with polyclonal and monoclonal antibodies was similar but with the polyclonal antibody we consistently observed the staining of additional nuclear bodies that we identified as coiled bodies (Fig. 1a). The significance of the presence of DAI in the coiled bodies is unclear. Coiled bodies are ribonucleoprotein structures that contain factors related to pre-rRNA and pre-mRNA metabolism (Brasch and Ochs, 1992). However, they are not present in all cells and they appear to be characteristic of transformed cells (Spector et al., 1992).

The specificity of the antibodies used in this study was demonstrated by performing immunofluorescence with a preimmune serum (Fig. 1e) and with anti-DAI antibody, which was preabsorbed with purified DAI protein (Fig. 1f-g). In both cases, no signal was observed in the cytoplasm, nucleoplasm or nucleolus. The specificity of the monoclonal antibody was previously demonstrated by immunoblot and immunoprecipitation (Laurent et al., 1985). This antibody

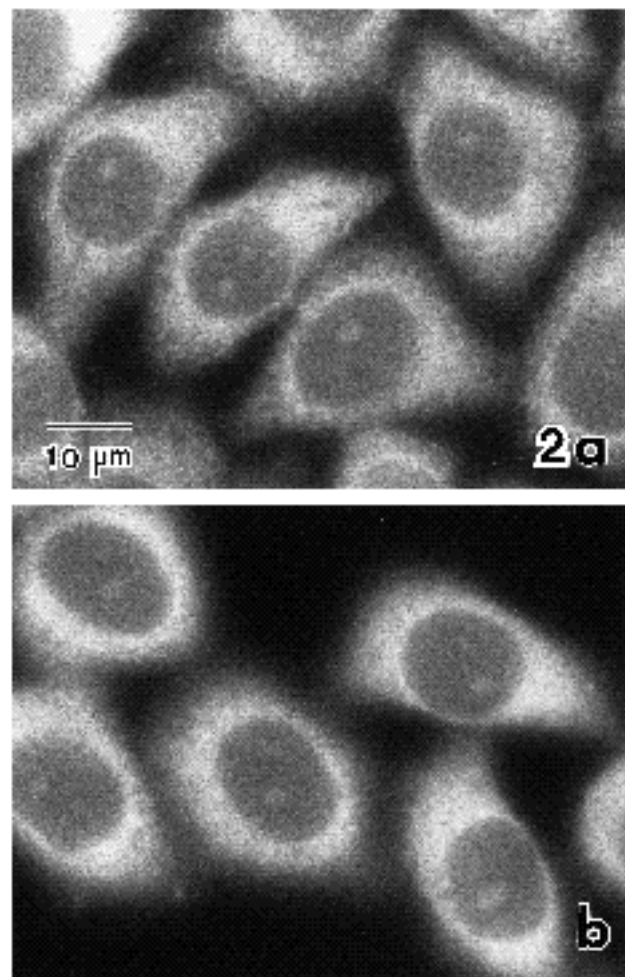


Fig. 2. Immunofluorescent localization of DAI in uninfected β -interferon treated HeLa cells. Cells untreated (a) or treated with β -interferon (b) both display cytoplasmic and nucleolar localization of DAI.

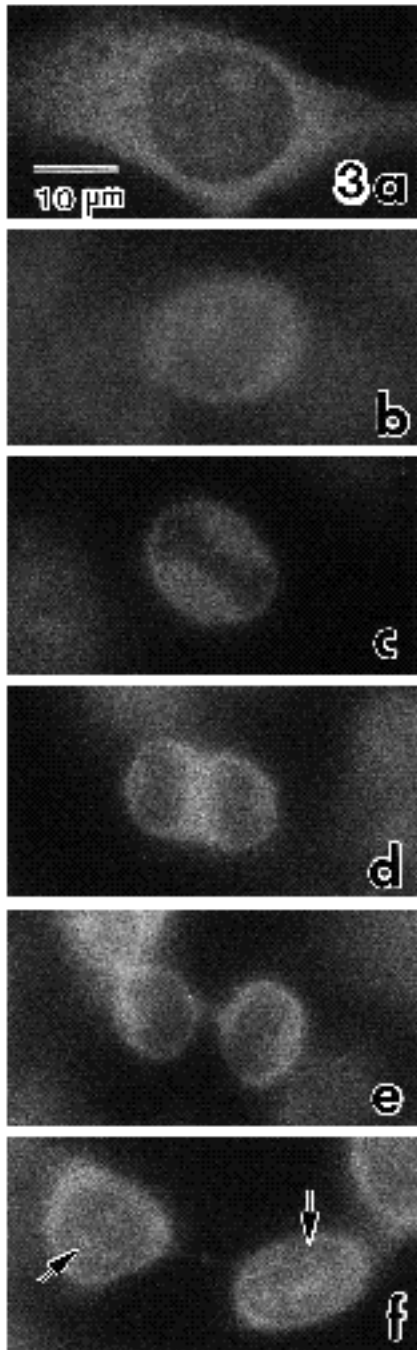


Fig. 3. Immunofluorescent localization of DAI in uninfected HeLa cells during mitosis. Interphase cells (a) showed cytoplasmic staining as well as faint labeling in nucleoli and nucleoplasm. During prophase (b), nucleolar DAI is diffusely distributed as the nucleolus breaks down. In metaphase (c), anaphase (d) and telophase (e) the chromosomes are not labeled. By early G₁ (f) DAI appears in the newly forming nucleoli (arrows).

was used for all subsequent immunolocalization experiments. Since DAI is an interferon-induced protein we determined the localization of this kinase after α -interferon treatment. Interferons (IFNs) are cytokines with antiviral, cell growth regulatory and immunomodulatory activities (Pestka et al., 1987). Binding of IFNs to the cell surface

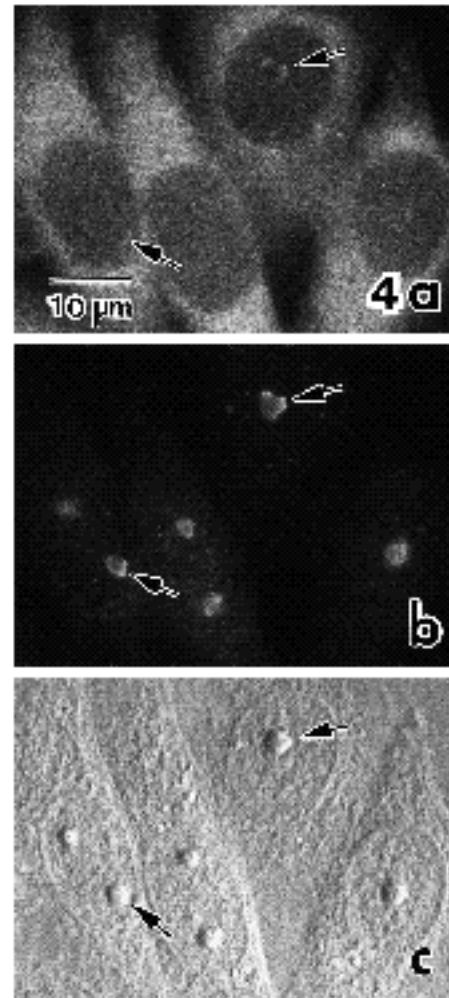


Fig. 4. Distribution of DAI in uninfected actinomycin D-treated HeLa cells. Double-label immunofluorescence of cells with anti-DAI (a) and anti-fibrillarin (b) antibodies after treatment with 0.2 μ g/ml of actinomycin D for 4 hours shows cytoplasmic staining for DAI (a). Colocalization of DAI and fibrillarin is observed in the segregated nucleoli (a-c, arrows). (c) Corresponding differential interference contrast image.

induces the synthesis of several proteins, including DAI, that are localized to the cytoplasm and nuclei. Treatment of HeLa cells with 800 units/ml of α -interferon did not alter the distribution of DAI, although the signal appeared brighter (Fig. 2b) than in non-treated cells (Fig. 2a). Other proteins such as the Mx family (Pavlovic and Staehli, 1991), and some isoenzymes from the (2'-5')oligoadenylate synthetase family have been localized to the nucleus (Chebath et al., 1987). Recently, the murine IFN-induced 72 kDa protein encoded by the Ifi 204 gene was also found in the nucleolus (Choubey and Lengyel, 1992). These observations and our results suggest that nuclear localization may be a common characteristic of IFN-induced proteins.

To correlate the distribution of DAI with other cytoplasmic and nucleolar components we monitored the behavior of DAI during mitosis (Fig. 3). Label remained distributed in the cytoplasm during the different phases of mitosis

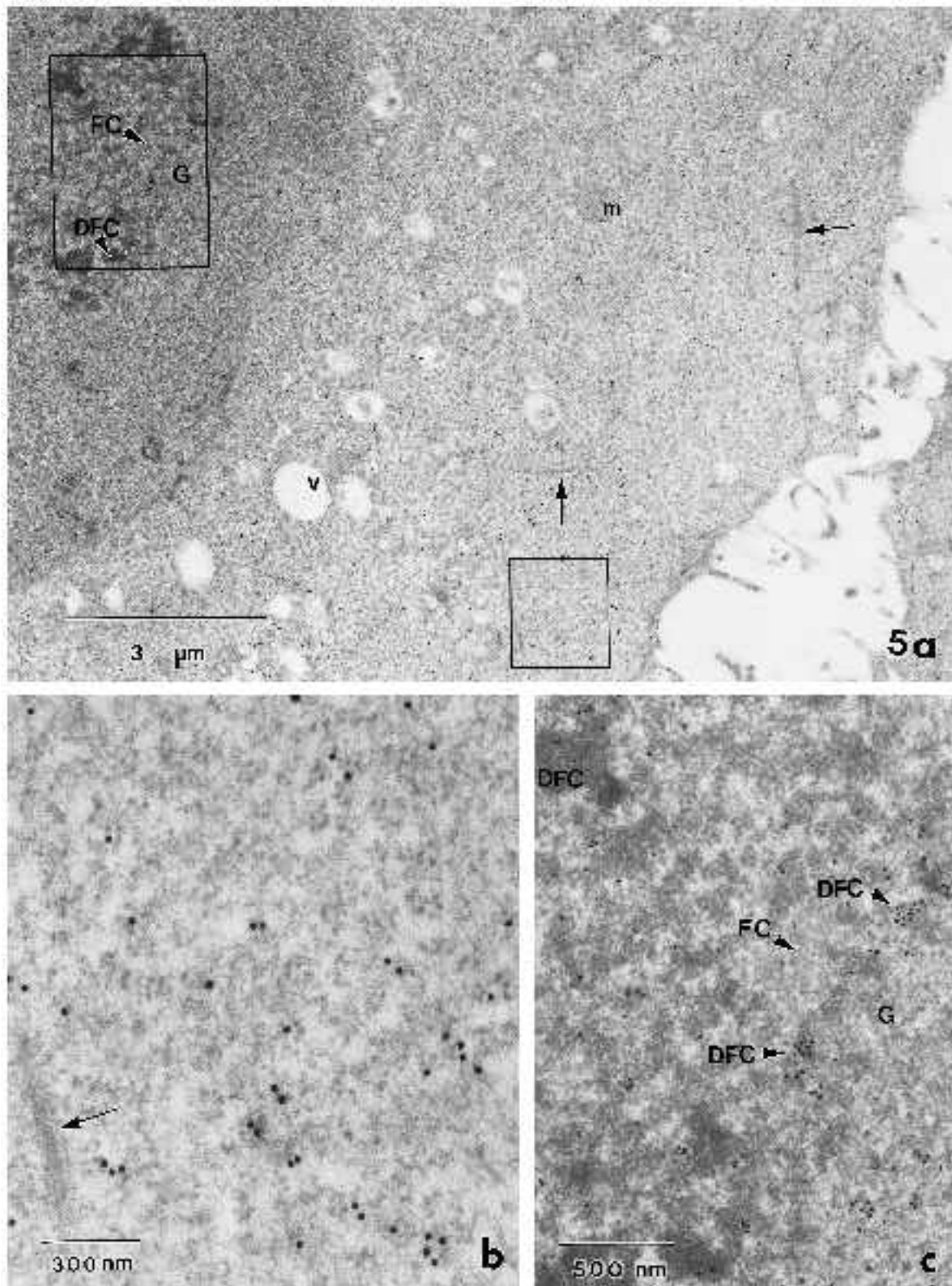


Fig. 5. Ultrastructural localization of DAI in uninfected HeLa cells using immunoelectron microscopy. DAI is present in ribosome enriched areas of the cytoplasm (a,b), where cytoskeletal elements are observed (arrows) but are not immunolabeled. DAI is observed to a lesser extent in the nucleoplasm and the fibrillar portion of the nucleolus (a,c). Higher magnification of the cytoplasm (small square area in (a)) is shown in (b). Higher magnification of the nucleolus (large rectangular area in (a)) is shown in (c). m, mitochondrion; v, vacuole; FC, fibrillar center; DFC, dense fibrillar component; G, granular component.

(Fig. 3a-f), but was excluded from the chromosomes. By late telophase and early G₁ phase, DAI appeared in the newly forming nucleoli in every daughter cell (Fig. 3f, arrows). The distribution of DAI during interphase and mitosis parallels the distribution of rRNA as visualized by in situ hybridization (Jiménez-García et al., unpublished). Because of the nucleolar localization and its behavior during mitosis, we studied the distribution of DAI in actinomycin D (ActD)-treated cells (Fig. 4). ActD inhibits transcription by RNA polymerase I producing well defined morphological changes in the nucleolus (Ochs et al., 1985a). Upon ActD treatment, the three different components of the nucleolus spatially segregate in such a way that they are easily distinguishable from each other when observed using different staining techniques. This pattern is characterized by the presence of a large nucleolar body that is composed of the granular portion, and small buds that are constituted by the fibrillar portion, i.e. the dense fibrillar component and the fibrillar centers. When HeLa cells were treated in this manner, and then double labeled with anti-DAI and anti-fibrillar antibodies, DAI and fibrillar (which is a marker for the dense fibrillar component of the nucleolus; Ochs et al., 1985a), were segregated and colocalized to the fibrillar portion of the nucleolus (Fig. 4a,b).

The distribution of DAI in the cell was determined at high resolution by conducting immunoelectron microscopy on HeLa cell sections with monoclonal antibodies to DAI (Fig. 5). Label was observed in the ribosome-enriched areas of the cytoplasm, where cytoskeletal elements (arrows) were also visible but were not immunolabeled (Fig. 5a,b). Vacuoles and other organelles such as mitochondria, were unlabeled. The nucleoplasm was diffusely labeled (Fig. 5a). In the nucleolus, label was observed mainly in the dense fibrillar component although little label was visualized in the granular compartment and the fibrillar centers (Fig. 5a,c). To examine the distribution of DAI in Ad2-infected cells, infected HeLa cells were immunolabeled with monoclonal antibodies to DAI after hybridization to a total Ad2 probe to identify the infected cells and to determine the stage of infection that they had reached. At early and late stages after infection (visualized as cells with label as fine dots or abundant inclusions in the nucleus, respectively), DAI was observed both in the cytoplasm and in the nucleolus (Fig. 6a, arrows). Some diffuse label was also present in the nucleoplasm. Cytoplasmic viral RNA and DAI (Fig. 6b) were both diffusely distributed late in infection (heavily stained cell in Fig. 6b).

VA RNA_I is present in the cytoplasm and nucleus of Ad2-infected HeLa cells late in infection

VA RNA_I is produced abundantly during the late phase of adenovirus infection (Soderlund et al., 1976). We performed in situ hybridization in HeLa cells infected with Ad2 for 24 hours to determine the cellular distribution of this RNA (Fig. 7). Hybridization signal was intense and diffusely distributed in the cytoplasm of infected cells (Fig. 7a). In the nucleus, VA RNA_I was found in the form of small very intense bright round bodies, which were abundant and distributed throughout the nucleoplasm (Fig. 7a, arrowheads). In addition, VA RNA_I was also detected in more elaborate ring-shaped viral inclusions. The nucleo-

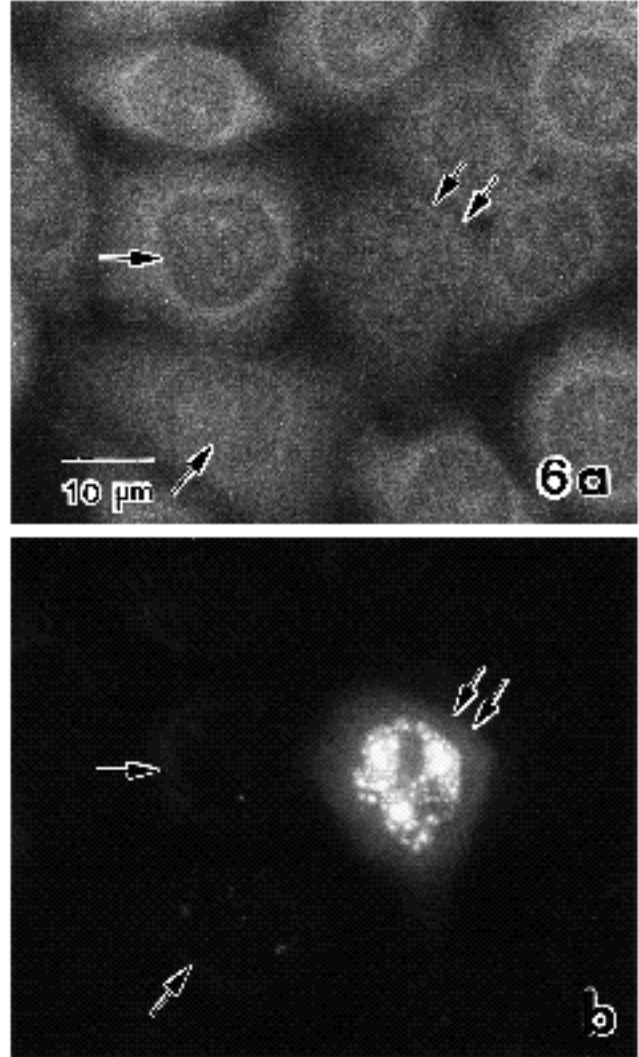


Fig. 6. Localization of DAI in Ad2-infected HeLa cells. In situ hybridization with a total genomic Ad2 DNA probe to identify infected cells (b), followed with anti-DAI (a) in the same cells shows the cytoplasmic distribution of DAI. In addition, the nucleolus and nucleoplasm are labeled for DAI. Cells in an early stage of infection are indicated by an arrow (a,b). Double arrows show a cell late in infection.

lus, on the other hand, was unlabeled. To confirm that the signal observed in the nucleus and cytoplasm was specific for RNA and not for single-stranded viral DNA that is produced during Ad2 DNA replication (Lechner and Kelly, 1977), we performed nuclease digestions prior to the hybridization protocol. Cells incubated in 100 μg/ml of DNase I for 2 hours at room temperature still exhibited hybridization signal in the cytoplasm and the small rounded bodies in the nuclei (Fig. 7c). However, RNase A treatment prior to hybridization eliminated the hybridization signal in the cytoplasm and in the round nuclear inclusions (Fig. 7d). These data show that VA RNA_I is contained within the small round inclusions. Control in situ hybridizations conducted without probe gave no signal (data not shown). To determine the pattern of expression

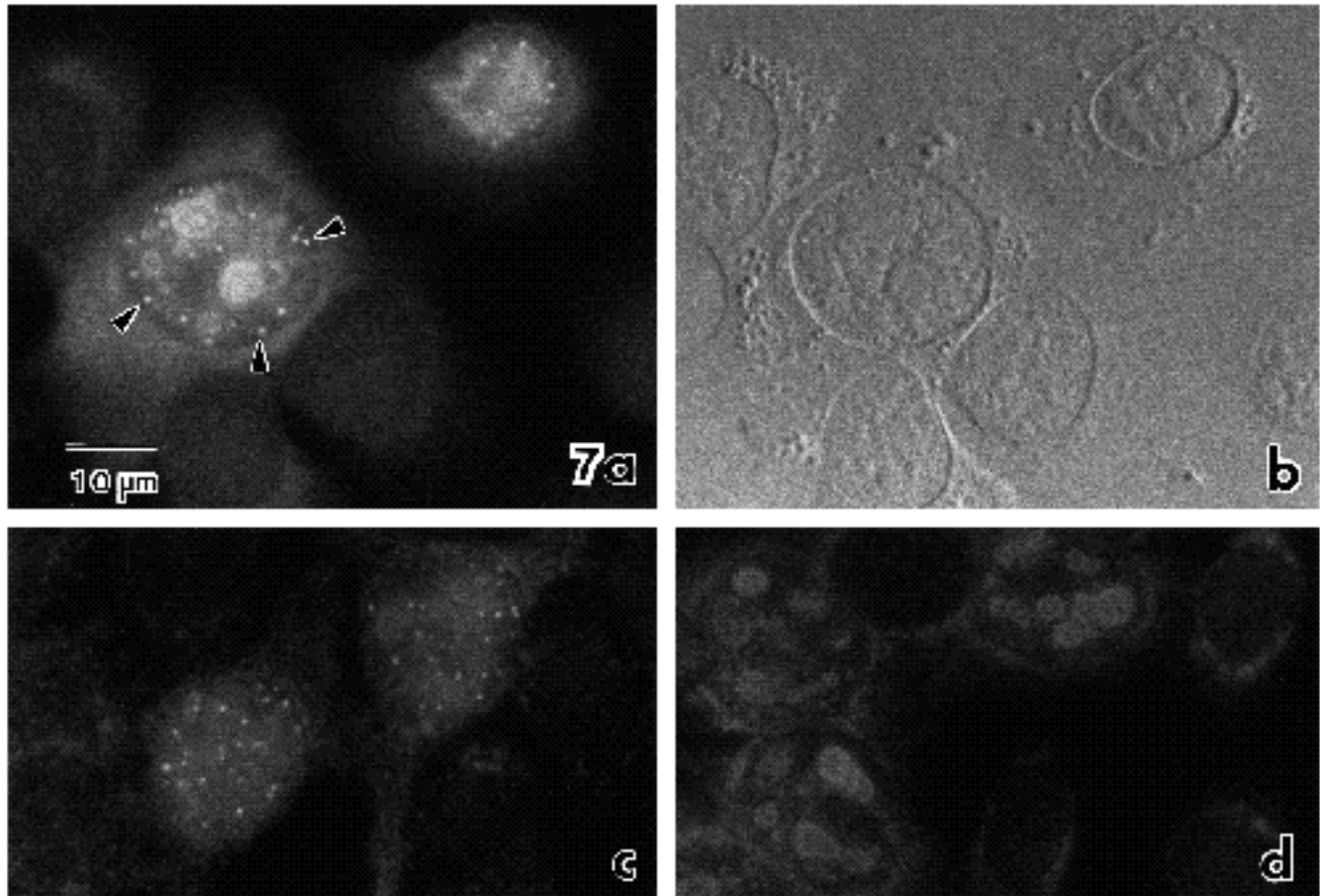


Fig. 7. Localization of VA RNA₁ in HeLa cells infected with Ad2 for 24 hours, using in situ hybridization. (a) VA RNA₁ appears to be localized in abundant small round dots in the nucleus (arrowheads) as well as being diffusely distributed in the cytoplasm. (b) Corresponding differential interference contrast image. The hybridization signal (dots) are still observed after DNase I pre-treatment (c) but are not observed after RNase A pre-treatment (d).

of VA RNA₁ during the course of infection we performed in situ hybridization at 7, 10, 14, 18 and 24 hpi. For comparison, we also used an Ad2 genomic probe to localize total Ad2 RNA (Fig. 8). Total viral RNA was widely distributed and gave a very intense signal at 10–14 hpi (Fig. 8a) and 18–24 hpi (Fig. 8b). VA RNA₁ was first detected by 10–14 hpi as fine dots in the nucleoplasm and diffusely distributed in the cytoplasm of infected cells (Fig. 8c). By 18–24 hpi these fine and rounded bodies increased in size and number while the label in the cytoplasm also became more intense (Fig. 8d). No signal was observed before 10 hpi either in the nucleus or in the cytoplasm (data not shown).

To determine the three-dimensional distribution of the viral inclusions enriched in VA RNA in the infected nuclei, we reconstructed images of infected cells after in situ hybridization using a confocal laser scanning microscope. Fig. 9 shows a stereopair (where different planes of focus are displayed in the same picture) revealing the abundance of these bodies and their distribution at the different depths of the nuclei. The cytoplasm is observed to be homogeneously labeled.

We next wanted to determine whether the pattern of expression for VA RNA₁ reflects the intrinsic distribution

characteristics of this molecule, or whether it is influenced by events occurring during viral infection. To this end, uninfected HeLa cells were transfected with the DNA encoding VA RNA₁ and were hybridized in situ after 24, 48 and 60 hours (Fig. 10). Cells transfected with the plasmid pUC119 containing no VA insert were not labeled (Fig. 10a). By 24 hours after transfection with a plasmid containing the VA RNA gene, VA RNA₁ started to appear as fine dots in the nucleoplasm (Fig. 10b). By 48 hours after transfection, VA RNA₁ was present as bigger dots in the nucleus and as a faint cytoplasmic distribution (Fig. 10c). By 60 hours post-transfection, the cytoplasm was even more heavily labeled than at 48 hours, and the nuclei were filled with bigger and more abundant round bodies, that were not present in the nucleolus (Fig. 10d). Therefore, the pattern of expression of VA RNA₁ in transfected and Ad2 infected cells was similar.

DISCUSSION

We document here the localization of DAI in the cytoplasm and nucleolus of both uninfected and Ad2-infected HeLa cells; this pattern of distribution remained unchanged after

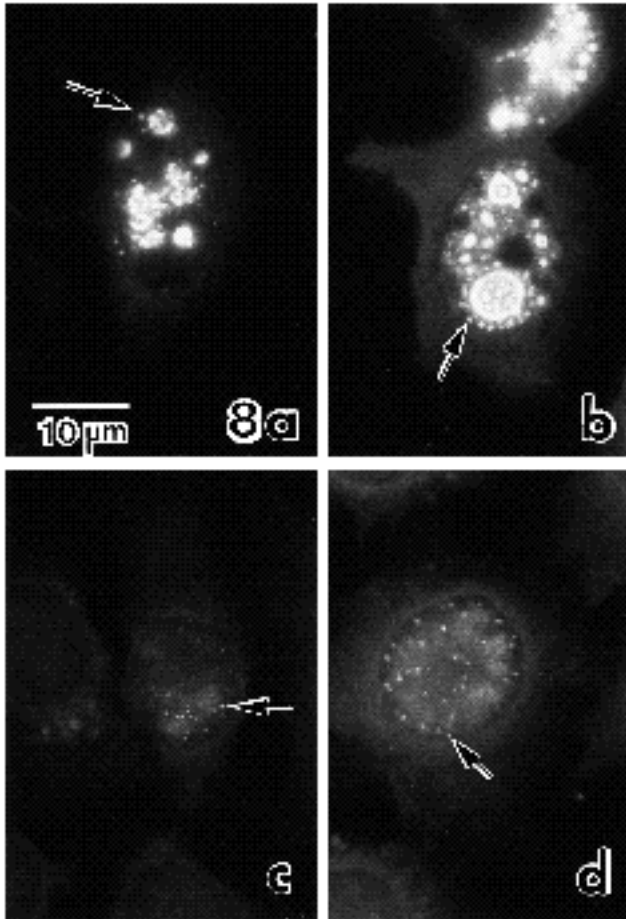


Fig. 8. Localization of VA RNA_I at different time points after Ad2 infection by in situ hybridization. The first detectable VA RNA_I signal appeared at about 10 hpi (c) as very fine dots in the nucleoplasm, in association with the growing viral inclusions that contain ssDNA and RNA (a). Some diffuse cytoplasmic signal was also observed. By 24 hpi VA RNA_I was present as larger dots in the nucleus and diffusely in the cytoplasm (d). Total Ad2 RNA at 10 hpi (a) or 24 hpi (b) is shown for comparison.

treatment of uninfected cells with γ -IFN, although IFN treatment elevates the amount of DAI protein in the cell by a factor of 5 to 10 (Hovanessian, 1989). Considering the role DAI has in the initiation of translation it was antici-

patated that this protein would be present in the cytoplasm. It has been shown previously that many of the factors implicated in protein synthesis are compartmentalized in the cytoplasm. Active polysomes are associated with the cytoskeleton (Lenk et al., 1977) and translational initiation factors are associated with ribosomes and the cytoskeleton (Howe and Hershey, 1984). Our results confirm that at least one site of action for DAI is in the cytoplasm. The presence of DAI in the nucleolus and to a lesser extent in the nucleoplasm was unexpected. Interestingly, it was reported recently that eIF-4E is also partially located in the nucleus (Lejbkiewicz et al., 1992) suggesting that many of the proteins participating in translation may also have roles in nuclear function. One may speculate as to the possible role of DAI in the nucleus. Since the nucleolus is known to be the site of pre-rRNA synthesis and ribosome assembly (Scheer and Benavente, 1990), the nucleolar localization of DAI may indicate that it has a role in the early stages of ribosome biogenesis. The nucleoplasmic staining may represent DAI migrating from the nucleolus to the cytoplasm. Alternatively, the nucleoplasmic presence of DAI in uninfected cells may be correlated with its ability to phosphorylate histones (Hovanessian, 1989) or other nuclear proteins (Ghosh and Baltimore, 1990).

Many nucleolar proteins are known to be present around the mitotic chromosomes and organized as prenucleolar bodies (PNB) that fuse to the nucleolar organizer (NOR), contributing to the newly formed interphase nucleolus at telophase (De la Torre and Giménez-Martín, 1982; Ochs et al., 1985b; Jiménez-García et al., 1989). During mitosis DAI was not found in PNB at telophase. DAI was first detected after mitosis during early G₁. At this stage, the nucleoli are already active in transcription (De la Torre and Giménez-Martín, 1982), suggesting an association with the early phases of ribosome biogenesis. Since DAI has a strong cytoplasmic localization in addition to its nucleolar distribution, we considered the possibility that DAI may be associated with ribosomal subunits. Therefore, we treated cells with actinomycin D which inhibits ribosomal RNA synthesis and results in a segregation of nucleolar components. The behavior of nucleolar DAI upon actinomycin D treatment was the same as that of the nucleolar protein fibrillar. It is known that fibrillar is present in PNBs and it is the major nucleolar protein found in the dense fibrillar component of the nucleolus (Ochs et al., 1985a). Interest-

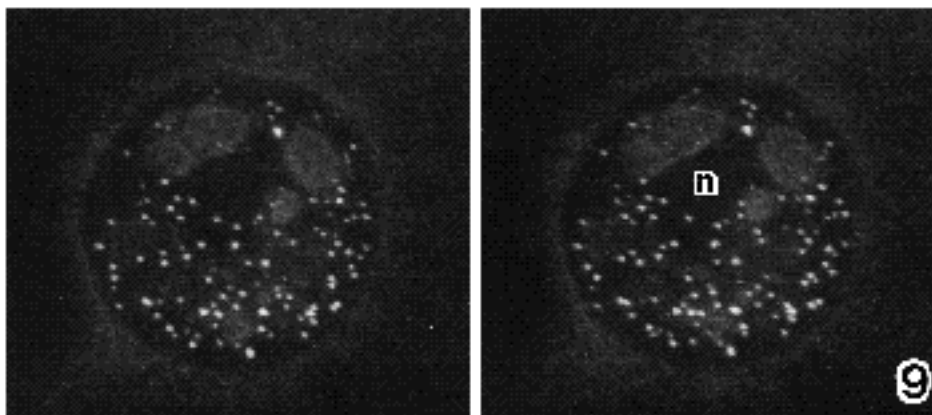


Fig. 9. Stereo images of VA RNA_I localization in Ad2 infected HeLa cells. The three-dimensional organization of VA RNA_I is visualized as small, round bodies in the nucleoplasm. The cytoplasmic portion of VA RNA_I is diffusely distributed.

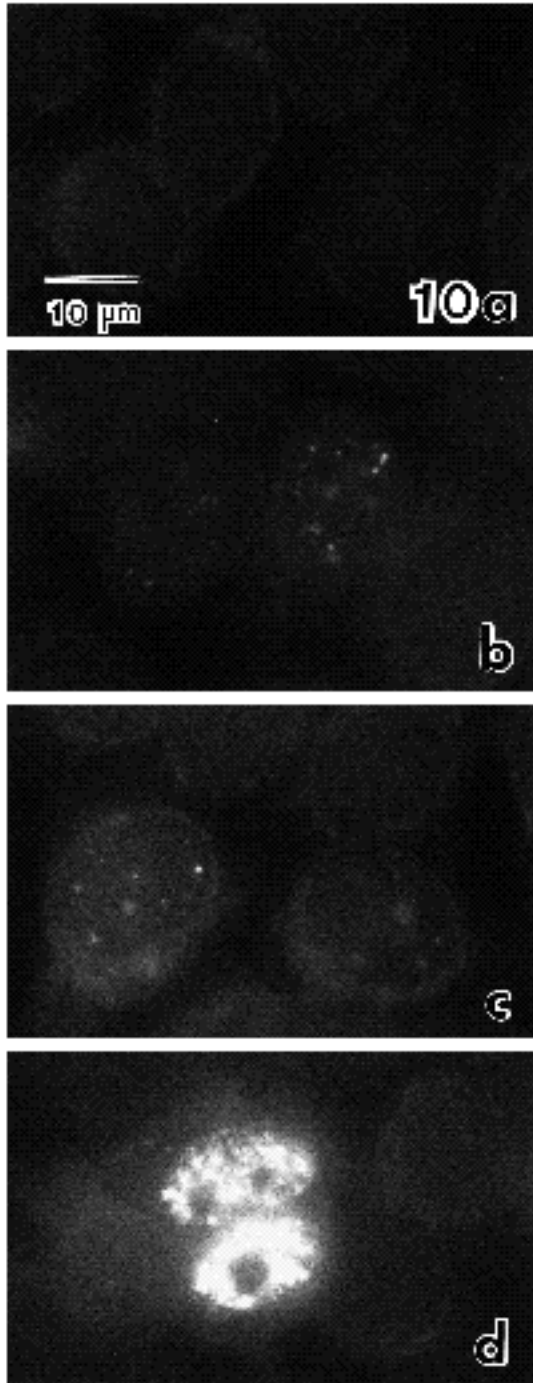


Fig. 10. Localization of VA RNA₁ in transfected HeLa cells. The morphological pattern of expression for VA RNA₁ is similar to that of Ad2 infected HeLa cells. No signal is observed in cells transfected with plasmid pUC119 (a). By 24 hours (b) after transfection with pUCVA, VA RNA₁ appears as fine dots in the nucleoplasm. At 48 hours (c) the label is more abundant in the nucleus and some VA RNA₁ is observed in the cytoplasm. By 60 hours after transfection (d) the nucleus is filled with VA RNA₁ excluding the nucleoli. The cytoplasm at this time is heavily labeled.

ingly, fibrillarin is present in the nucleolar U3 snRNP (Lischwe et al., 1985; Ochs et al., 1985a; Reimer et al., 1987; Reddy and Busch, 1988). U3 snRNA has been implicated in the first steps of pre-rRNA processing (Kass et al., 1990; Tollervey et al., 1991). Therefore, the localization of DAI with fibrillarin may be of functional significance. Moreover, by immunoelectron microscopy, we found DAI to be present in the dense fibrillar component of the nucleolus, although a small amount of label was also found in the fibrillar centers and in the granular component of the nucleolus. A possible role of DAI in the first steps of ribosome biogenesis, indicated by its presence in the dense fibrillar component of the nucleolus, remains to be demonstrated.

The presence of VA RNA₁ in the cytoplasm of Ad2-infected HeLa cells was observed after about 10 hpi and became increasingly evident at later times. Our inability to detect transcripts before 10 hpi is presumably due to the low abundance of VA RNA₁ during the early phase of infection (Söderlund et al., 1976) and the insensitivity of the *in situ* hybridization technique used. Unlike DAI, the observation of this RNA in the nucleus was not unexpected since adenovirus transcripts are produced in the nucleus of the host cell during the lytic cycle (Akusjärvi et al., 1986). Surprisingly, highly organized structures containing VA RNA₁ appear by 10-14 hpi as spherical bodies in the nucleoplasm, becoming larger and more numerous as the infectious cycle progresses. These bodies represent a subset of the total adenovirus RNA (Jiménez-García and Spector, 1993) and suggest a mechanism of accumulation and migration for VA RNA₁ in the nucleus. Because VA RNA₁ is transcribed by RNA polymerase III (Price and Penman, 1972) while the rest of the viral genome is transcribed by RNA polymerase II (see Akusjärvi et al., 1986), the presence of these bodies containing VA RNA₁ may be related to a different site of synthesis and/or transport pathway for transcripts produced by RNA polymerase III. Supporting this contention is the finding that transfected cells displayed the same morphological pattern of localization of this RNA as infected cells, namely the progressive appearance of dots, suggesting that the transcription and/or transport to the cytoplasm of RNA polymerase III transcripts may occur via highly defined nuclear structures. These viral inclusions containing VA RNA₁ may represent the viral RNA-rich compact rings observed by ultrastructural *in situ* hybridization in Ad5-infected cells late in infection (Puvion-Dutilleul et al., 1992). However, it is unlikely that these structures are regions of RNA processing since the VA RNA₁ is not post-transcriptionally processed.

VA RNA₁ was expressed in two steps during the late phase of infection. First, nuclear RNA accumulated as small round bodies that became numerous. Subsequently, the RNA accumulated diffusely in the cytoplasm. Indeed, VA RNA₁ was visualized in the cytoplasm late in infection, suggesting a final destination corresponding to its function in the cytoplasm during protein synthesis. These experiments may suggest a specific signal for VA RNA₁ to be present in the cytoplasm. That signal may have something to do with the molecular structure of the RNA or with the presence of latent DAI in the cytoplasm.

DAI (Zilberstein et al., 1976) and VA RNA₁ (Schneider et al., 1985) have been found in association with ribosomes.

It has been proposed that VA RNA_I and Ad2 mRNAs are associated in infected cells in a manner that results in a functional compartmentalization (O'Malley et al., 1989). The presence of the VA RNA_I protects the translation of Ad2 mRNAs from DAI activation by virally generated dsRNA, while cellular mRNAs, which do not bind VA RNA_I are not protected and therefore not translated. The colocalization of DAI with VA RNA_I in the cytoplasm may represent such a compartmentalization. Furthermore, the presence of both DAI and VA RNA_I in the nucleus, though predominantly not colocalized, suggests the possibility that the association between Ad2 mRNAs, VA RNA_I and DAI may originate in the nucleus prior to export into the cytoplasm. Future experiments will examine the organization of other translational elements in the cytoplasm of Ad2-infected cells.

In summary, we have shown that DAI is present in the cytoplasm, in the nucleoplasm, and in the nucleolus of uninfected and Ad2-infected HeLa cells. In the nucleolus, DAI was found predominantly, although not exclusively, in the dense fibrillar component. On the other hand, VA RNA_I was also present in the cytoplasm and in the nucleus of Ad2-infected HeLa cells. In the nucleus, VA RNA_I was observed to be present in abundant, highly organized, small, round structures distributed in the nucleoplasm, but was excluded from the nucleolus. Both DAI and VA RNA_I were diffusely localized in the cytoplasm.

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