JOURNAL OF VIROLOGY, Nov. 1988, p. 4153–4166
0022-538X/88/114153-14$02.00/0
Copyright © 1988, American Society for Microbiology
Vol. 62, No. 11

Differential Distribution of the Adenovirus E1A Proteins and Colocalization of E1A with the 70-Kilodalton Cellular Heat Shock Protein in Infected Cells

EILEEN WHITE,* DAVID SPECTOR, AND WILLIAM WELCH
Cold Spring Harbor Laboratory, P.O. Box 100, Cold Spring Harbor, New York 11724

Received 21 April 1988/Accepted 1 August 1988

Five distinct localization patterns were observed for the adenovirus E1A proteins in the nuclei of infected HeLa cells: diffuse, reticular, nucleolar, punctate, and peripheral. The variable distribution of E1A was correlated with the time postinfection and the cell cycle stage of the host cell at the time of infection. All staining patterns, with the exception of peripheral E1A localization, were associated with the early phase of infection since only the diffuse, reticular, nucleolar, and punctate staining patterns were observed in the presence of hydroxyurea. Because the E1A proteins (12S and 13S) stimulate the expression of the cellular heat shock 70-kilodalton protein (hsp70), we examined the intracellular distribution of hsp70 in the adenovirus-infected cells. Whereas hsp70 was predominantly cytoplasmic in the cells before infection, after adenovirus infection most of the protein was now found within the nucleus. Specifically, hsp70 was found within the nucleoli as well as exhibiting reticular, diffuse, and punctate nuclear staining patterns, analogous to those observed for the E1A proteins. Double-label indirect immunofluorescence of E1A and hsp70 in infected cells demonstrated a colocalization of these proteins in the nucleus. Translocation of hsp70 to the nucleus was dependent upon both adenovirus infection and expression of the E1A proteins. The localization of hsp70 was unaltered by infection with an E1A 9S cDNA virus which does not synthesize a functional E1A gene product. Moreover, the discrete nuclear localization patterns of E1A and the colocalization of E1A with hsp70 were not observed in adenovirus-transformed 293 cells which constitutively express E1A and E1B. E1A displayed exclusively diffuse nuclear staining in 293 cells; however, localization of E1A into the discrete nuclear patterns occurred after adenovirus infection of 293 cells. Immunoprecipitation of labeled infected-cell extracts with a monoclonal antibody directed against the E1A proteins resulted in precipitation of small amounts of hsp70 along with E1A. These data indicate that the adenovirus E1A proteins colocalize with, and possibly form a physical complex with, cellular hsp70 in infected cells. The relevance of this association, with respect to the function of these proteins during infection and the association of other oncoproteins with hsp70, is discussed.

The adenovirus E1A proteins play an essential role in both productive infection and transformation by adenovirus. The primary function of the E1A proteins in productive infection is to stimulate viral gene transcription (reviewed in reference 1), a requirement for efficient viral gene expression and the subsequent production of virus. Stimulation of transcription by E1A is, however, not restricted to viral genes. Transcription of certain cellular genes also increases in cells infected with virus or when the E1A proteins are expressed after DNA transfection. In addition to transactivating transcription, the E1A proteins also appear capable of repressing enhancer-dependent transcription. Thus, the E1A proteins are capable of regulating gene expression in both a positive and negative fashion.

In addition to modulating transcriptional events, the E1A proteins stimulate cellular DNA synthesis (20, 40), a process thought to be central to the role of E1A in transformation (reviewed in references 2 and 9). Alone, the E1A proteins are capable of causing primary rodent cells to grow indefinitely in culture. However, properties associated with complete transformation, such as growth to high saturation density and morphological transformation, require expression of another oncogene such as c-Ha-*ras* or the adenovirus E1B gene, along with E1A (17, 36).

Two main transcripts are produced from the E1A transcription unit at early times postinfection and in transformed cells (reviewed in references 1 and 33). These have been designated the 13S and 12S mRNAs in adenovirus type 2 (Ad2) and Ad5 serotypes. The protein products of these mRNAs are 289 and 243 amino acids in length, respectively. The 289- and 243-amino-acid proteins have identical amino acid sequences, with the exception that the 243-amino-acid protein is missing 46 internal amino acids encoded by the large E1A protein, due to the use of an alternative splice site. Although expression of either protein conveys the capacity to produce transformed foci in culture, only the 13S product is capable of stimulating transcription to a high degree. A 9S mRNA is produced from the E1A transcription unit at late times in infection, but as yet no known function in either the regulation of transcription or the transformation process has been associated with the 9S gene product (12, 57).

Both the 289- and 243-amino-acid E1A proteins are posttranslationally modified by phosphorylation (10, 14, 30, 43, 55) and are rapidly transported to the nucleus (8, 26). Utilizing polyclonal antibodies specific for the E1A 13S gene product, Feldman and Nevins (7) reported that the 289-amino-acid protein is predominantly found in the nucleus of infected cells, with a portion being tightly associated with the insoluble nuclear matrix. With the availability of antibodies capable of recognizing both the 13S and 12S E1A gene products, as well as adenovirus mutants which express individual cDNA genes for the 13S and 12S mRNAs, it was found that both forms of E1A are predominantly localized within the nucleus of infected cells (14, 37). It is the 13S and

* Corresponding author.

not the 12S product, however, that appears to be nuclear matrix associated in infected and transformed cells (37).

How the E1A proteins usurp the cellular transcription and replication machinery to modulate transcription events and cause cellular proliferation is still not clearly known. However, the E1A proteins themselves bind weakly to DNA (4), and there is evidence that they stimulate transcription by increasing the activity or amount of cellular transcription factors (28, 56). Interestingly, one of the cellular genes that is transcriptionally stimulated by the adenovirus E1A proteins is that encoding the 70-kilodalton (kDa) cellular heat shock protein hsp70 (21, 34, 52). The stimulated expression of hsp70 is primarily a function of the 13S E1A product, although stimulation has been observed with the 12S product to a lesser degree (38). The reason for enhanced expression of hsp70 in the presence of E1A is not known. However, preexisting levels of hsp70 have been correlated with the permissivity of human cells for adenovirus infection (18).

The expression of hsp70 has also been shown to increase in human cells upon transfection with either the simian virus 40 (SV40) large T antigen or the c-*myc* genes (23–25). Interestingly, in HeLa cells the constitutive expression of hsp70 appears to be a cell cycle-related event occurring very near the G_1-S boundary (31). Although not clearly understood, hsp70 has been found in rather stable complexes with mutant forms of either the polyomavirus medium T antigen or the cellular p53 protein (16, 35, 41, 44). Cellular p53 is thought to be involved in the transformation process, perhaps as a competence factor in the regulation of cellular DNA synthesis. In this respect, it is worth noting that the bacterial homolog of hsp70, the DnaK protein, is required for bacterial DNA and RNA synthesis at high temperatures and is necessary for bacteriophage lambda replication (6, 19, 53). Hence it is tempting to speculate that the constitutive expression of hsp70 is necessary for normal cell proliferation and that its association with proteins like p53 plays a role in the maintenance of the transformed phenotype.

In the present study we have carefully examined the subcellular distribution of the adenovirus E1A protein in productively infected HeLa cells, using indirect immunofluorescence and immunoelectron microscopy. We show that during infection the E1A proteins exhibit a number of different distribution patterns within the cell nucleus. Specifically, the staining patterns observed included (i) diffuse nuclear, (ii) reticular, (iii) punctate, (iv) nucleolar, and (v) peripheral. Two important factors account for the heterogeneity of E1A staining: the particular phase of infection (early versus late) and the cell cycle stage of the cell at the time of infection. Since this localization of the E1A proteins resembled the localization of hsp70 after heat-shock treatment of uninfected cells, and because hsp70 levels increase in cells upon adenovirus infection, we also examined the intracellular staining patterns of hsp70 in infected cells. In some but not all of the infected cells, a colocalization of hsp70 and E1A was observed. This colocalization was most apparent in those cells displaying both nucleolar and reticular E1A staining patterns. Immunoprecipitation studies using monoclonal antibodies to E1A demonstrated an apparently stable association of E1A with the hsp70 proteins.

MATERIALS AND METHODS

Cells and viruses. Human HeLa cells were grown in monolayer culture with Dulbecco modified Eagle medium with 10% fetal bovine serum. Human 293 cells (11), which constitutively express adenovirus E1A and E1B gene products, were grown in Dulbecco modified Eagle medium with 10% calf serum. The wild-type Ad2 was grown in infected HeLa cells and band purified by $CsCl_2$ density gradient sedimentation as previously described (29, 50). Adenovirus mutants containing the E1A 13S, 12S, and 9S cDNA genes in place of genomic E1A sequences (32, 57) were kindly provided by E. Moran and B. Zerler (Cold Spring Harbor Laboratory).

Indirect immunofluorescence techniques. Cells were fixed in 2% formaldehyde in phosphate-buffered saline (PBS) (pH 7.3) for 15 min at 25°C. Cells were then washed (four changes, 15 min each) in PBS (pH 7.3) and incubated in PBS–0.2% Triton X-100–0.5% normal goat serum for 5 min at 4°C. Samples were then washed for 30 min in several changes of PBS (pH 7.3) containing 0.5% normal goat serum and incubated with the primary antibody (E1A, hsp70) at the appropriate dilution for 1 h at 37°C. Cells were then washed in PBS (pH 7.3) (four changes of 15 min each) and incubated in fluorescein isothiocyanate-conjugated (goat anti-mouse) or rhodamine-conjugated (goat anti-rabbit) secondary antibody (Cappel Laboratories) at a dilution of 1:20 for 1 h at 37°C. Samples were then washed in PBS (pH 7.3) (four changes, 15 min each) and mounted in 90% glycerol–10% PBS with 4% *n*-propyl gallate. Samples prepared for immunofluorescence were photographed on a Zeiss epifluorescence microscope equipped with a Nikon UFX automatic camera or a Zeiss Photomicroscope III.

In some experiments cells were fixed in 100% methanol at −20°C for 10 min before staining, as previously described (48). Localization of the E1A proteins in Ad2-infected HeLa cells was identical with either the methanol or formaldehyde fixation procedures. Improved preservation of E1A staining was obtained with formaldehyde fixation of 293 cells and HeLa cells infected with the 12S virus.

Mouse monoclonal antibodies (M73) directed against both the 243- and 289-amino-acid E1A proteins (14) were obtained from E. Harlow (Cold Spring Harbor Laboratory), as was a monoclonal antibody directed against cellular p53 (13). Rabbit polyclonal antibodies directed against the E1A proteins (39) were obtained from K. Spindler and A. Berk (Department of Microbiology and Molecular Biology Institute, University of California, Los Angeles). Mouse monoclonal antibodies directed against hsp70 (C92) were prepared and characterized as described previously (47). A mouse monoclonal antibody specific for the E1B 55-kDa protein (6A8) was also used (B. Stillman, R. McKay, and E. White, unpublished data).

Double-label indirect immunofluorescence was performed with Ad2-infected HeLa cells by staining with the E1A rabbit polyclonal antibody and the anti-hsp70 mouse monoclonal antibody, followed by rhodamine-conjugated goat anti-rabbit secondary antibody and fluorescein isothiocyanate-conjugated goat anti-mouse secondary antibody. No cross-reactivity was observed with the goat anti-rabbit antibodies and mouse immunoglobulin G or with the goat anti-mouse antibodies and rabbit immunoglobulin G.

Immunoelectron microscopy. Cells were fixed in 2% formaldehyde plus 0.01% glutaraldehyde in PBS (pH 7.3) for 15 min at 25°C. Fixed cells were then washed three times for 15 min each in sodium borohydride (0.5 mg/ml) and incubated in 0.2% Triton X-100–0.5% normal goat serum in PBS (pH 7.3) for 5 min at 4°C. Samples were then washed for 30 min in several changes of PBS that contained 0.5% normal goat serum and were incubated in E1A monoclonal antibody (1:20) for 1 h at 37°C. Samples were washed overnight in PBS at 4°C to remove unbound antibody and were then incubated

in peroxidase-conjugated secondary antibody at a dilution of 1:20 for 1 h at 37°C. After washing in PBS (pH 7.3) for 15 min and 0.05 M Tris (pH 7.6) for 30 min, samples were incubated in 0.05% 3,3'-diaminobenzidine in 0.05 M Tris (pH 7.6) for 30 min. Samples were then suspended in 0.05% 3,3'-diaminobenzidine supplemented with 0.01% H_2O_2 for 5 min, washed in 0.05 M Tris (pH 7.6) for 30 min, and fixed in 2% OsO_4 in 0.1 M sodium cacodylate buffer (pH 7.3) for 1 h. Subsequently, samples were rinsed in distilled water, dehydrated in a graded series of ethanol, and infiltrated and embedded in Poly/Bed 812 (Polysciences Inc., Warrington, Pa.). Polymerization was carried out at 60°C for 72 h. Samples were sectioned with a diamond knife using a Reichert Ultracut 4 ultramicrotome and examined with a Hitachi H-7000 transmission electron microscope operated at 75 kV.

Flow cytometry. Cells (10^6) growing in monolayer culture were harvested, washed, and suspended in 0.25 ml of PBS, and fixed by gradual addition of 4 ml of 100% ethanol while vortexing. After fixation overnight, the cells were washed twice and suspended in 0.25 ml of PBS containing 1% bovine serum albumin. The fixed cells were then treated with RNase A and 25 μl of 50-mg/ml propidium iodide solution in sodium citrate. Fluorescence intensities were determined by quantitative flow cytometry using an Epics C System (Coulter Electronics).

Metabolic labeling and immunoprecipitation. HeLa cells growing in monolayer culture were infected with Ad2 (multiplicity of infection, 100 PFU/cell) in either the presence or absence of hydroxyurea (10 mM). Infected cells were labeled with [^{35}S]methionine (100 μCi/ml) from 8 to 20 h postinfection and then harvested for either two-dimensional gel electrophoresis or "native" immunoprecipitation analysis. For the former, the cells were lysed in Laemmli sample buffer (27) containing 1% sodium dodecyl sulfate (SDS), the extracts were heated at 100°C for 5 min, and nuclease was added. The samples were then analyzed by isoelectric focusing (pH 5–7 gradient gels) in the first dimension, followed by SDS-polyacrylamide gel electrophoresis (12.5% polyacrylamide gels) in the second dimension as described previously (45).

For immunoprecipitation studies the cells were lysed in RIPA + SDS (PBS containing 1% Triton X-100, 1% deoxycholate, and 0.1% SDS). After sonication, the cell extracts were clarified in an Eppendorf centrifuge for 20 min at 4°C. A 60-μl volume of a 50% solution of protein A-Sepharose (Pharmacia) in RIPA + SDS was added to clarified extracts, and the mixture was incubated for 30 min at 4°C. After removal of the protein A beads by centrifugation, the appropriate antibodies (see the legend to Fig. 8) were added and the mixtures were incubated on a rotary shaker for 4 h at 4°C. In some cases rabbit anti-mouse (heavy- and light-chain-specific) antibodies were added, and in all cases the immune complexes were captured by the addition of 60 μl of a 50% (vol/vol) solution of protein A-Sepharose in RIPA + SDS. After 30 min of incubation the immune complexes were washed five times with RIPA + SDS and then released from the beads by boiling in Laemmli sample buffer. Resultant immune complexes were analyzed by both one- and two-dimensional gel electrophoresis.

RESULTS

Differential localization of the E1A proteins in infected HeLa cells. The subnuclear distribution of adenovirus E1A proteins was examined in situ by immunofluorescence of HeLa cells infected with wild-type Ad2 at a multiplicity of infection of 100 PFU/cell at 24 h postinfection. Populations of cells examined (1×10^3 to 3×10^3) exhibited five different localization patterns of the E1A proteins (Fig. 1 and Table 1). Approximately one-half (52%) of the cells exhibited diffuse nuclear staining which excluded the nucleoli (Fig. 1a and b). This pattern of staining has previously been observed using antibodies specific for DNA and histones and resembles the previously reported staining pattern of the E1A proteins in infected cells (7, 14, 37). The second most abundant pattern was a reticular distribution (Fig. 1c and d) observed in 25% of the infected cells. When focusing through the nucleus, this pattern was observed to represent a well-defined branched network apparently contiguous throughout the cell nucleus. Punctate nuclear staining was observed in 15% of the infected cells (Fig. 1e and f). A similar pattern of staining has previously been reported for the small nuclear ribonucleoprotein particles; however, when examined by double-label immunofluorescence the punctate staining observed for E1A did not appear coincident with the small nuclear ribonucleoprotein particle staining (data not shown). Nucleolar specific staining of E1A was observed in 6% of the infected cells (Fig. 1g and h). Finally, 2% of the cells exhibited staining near the periphery of the nucleus, adjacent to the nuclear lamina-pore complex (Fig. 1i and j).

We have similarly examined infected cells by indirect immunofluorescence for the intracellular distribution of the other adenovirus nuclear antigens (the E2 72-kDa DNA-binding protein and the E1B 55- and 19 kDa tumor antigens, as well as the late proteins hexon and the L4 100-kDa proteins). These adenovirus-encoded proteins exhibited intranuclear distributions very different from that of E1A (data not shown), indicating that the localization domain of E1A within the nucleus is not shared by these other viral nuclear proteins.

Immunoelectron microscopy was used in an attempt to gain more insight into the localization of the E1A proteins. The diffuse (Fig. 2a). and punctate (Fig. 2c) staining patterns appeared identical to that which was observed by immunofluorescence (Fig. 1a and c). From the immunoelectron microscopy data (and examination of serial sections) it was evident that the reticular staining pattern exhibited continuity with the nuclear periphery and clearly showed a "networklike" distribution which further distinguished it from the other patterns (Fig. 2b). Upon closer examination of the nucleolar staining pattern with immunoelectron microscopy, localization of the E1A proteins was found to be restricted to the granular region of the nucleolus (Fig. 2d), the site of preribosome assembly. In the least predominant staining pattern (approximately 2% of the infected cells), E1A localized at the nuclear periphery (Fig. 2e) adjacent to the nuclear lamina-pore complex. Numerous nuclear pore profiles were observed in the region of peripheral immunostaining (Fig. 2e and f).

In addition to the five distinct patterns of immunostaining, overlapping patterns such as diffuse plus nucleolar, or punctate plus nucleolar, were also observed, suggesting that the localization of E1A during infection may be in a dynamic state and perhaps involves a progression from one staining distribution to another. Therefore, we investigated whether this variation in the distribution of the E1A proteins in infected cells might change during the course of infection. The distribution of the E1A proteins in infected cells was examined by indirect immunofluorescence at different times postinfection. At early times (6 to 12 h), the infected cells displayed either the diffuse nuclear staining pattern or no

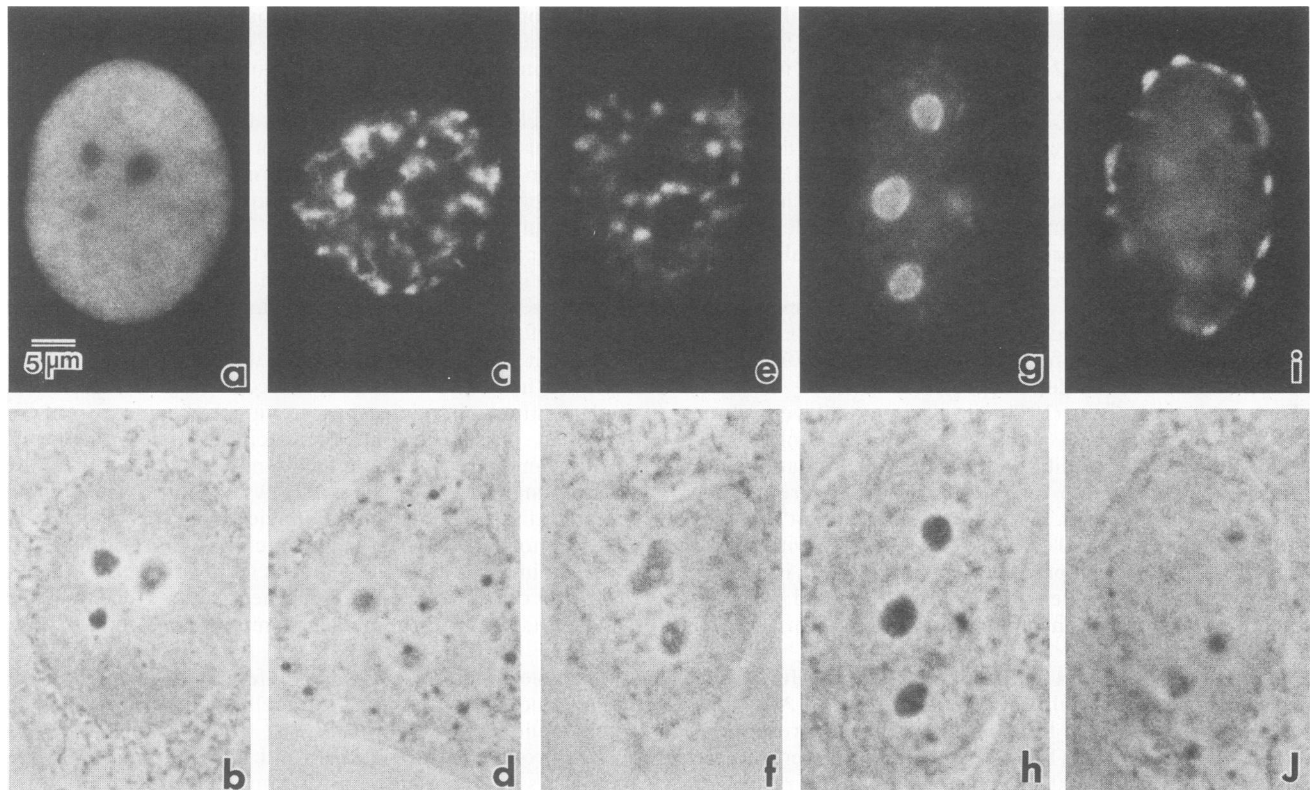

FIG. 1. Indirect immunofluorescent staining of infected cells with E1A monoclonal antibodies. HeLa cells growing on glass cover slips were infected with Ad2 (multiplicity of infection of 100 PFU/cell), and at 24 h postinfection the cells were fixed with formaldehyde and analyzed for the distribution of the E1A protein either (top panels) by indirect immunofluorescence using monoclonal antibodies directed against the E1A proteins or (bottom panels) by phase-contrast microscopy. The five major nuclear immunostaining patterns of E1A are shown: (a and b) diffuse nonnucleolar; (c and d) reticular; (e and f) speckled; (g and h) nucleolar; or (i and j) peripheral.

fluorescence at all (data not shown). By 12 to 24 h postinfection, there occurred an increased number of cells where E1A exhibited reticular, nucleolar, or punctate staining patterns. Finally, at late times postinfection (24 to 48 h), there was an accumulation of cells displaying either peripheral nuclear staining or no staining at all. From these results it is clear that the localization of the E1A proteins differs during the course of infection, perhaps corresponding to the different events which occur in the early and late phases of infection.

To specifically distinguish which distributions of E1A were associated with the early versus the late phase of infection, the localization of E1A was determined in cells infected in the presence of hydroxyurea. Under these conditions, viral DNA synthesis is inhibited, and thus the infected cells remain blocked in the early phase of infection (42). When infected cells were maintained in hydroxyurea throughout the infection, the E1A proteins exhibited the diffuse, reticular, punctate, and nucleolar staining patterns (Table 1). Therefore, these heterogeneous nuclear distributions of E1A occur in the early phase of adenovirus infection and are apparently independent of the gross changes which occur in nuclear structure accompanying the late phase of viral infection (58). In contrast, the peripheral nuclear dis-

TABLE 1. Frequency of the distribution patterns of E1A after infection[a]

Expt	Frequency (%) of pattern:					
	No staining	Diffuse	Reticular	Nucleolar	Punctate	Peripheral
1						
Ad2 infected; 24 hpi	0	52	25	6	15	2
2						
Ad2 infected; 18 hpi	68	28		2		2
Ad2 infected; 18 hpi + HU	59	20		21		0
Pretreat HU 24 h; Ad2 infected; 18 hpi + HU	27	72		1		0
Pretreat HU 24 h; Ad2 infected; 42 hpi + HU	0	97		3		0
Pretreat HU 24 h; Ad2 infected; 48 hpi + HU	0	90		10		0

[a] Infected cells were stained by indirect immunofluorescence and scored for appearance of specific E1A localization patterns at 24 h postinfection (hpi) (experiment 1). Experiment 2 data represent cells in the same experiment evaluated under different conditions, with the reticular, nucleolar, and punctate E1A distributions collectively represented as a single percentage. Cells in experiment 2 were either untreated and infected and fixed at 18 h postinfection, infected in the presence of hydroxyurea (HU) and fixed at 18 h postinfection, or pretreated with hydroxyurea for 24 h and infected and maintained in the drug until fixation at 18, 42, or 48 h postinfection.

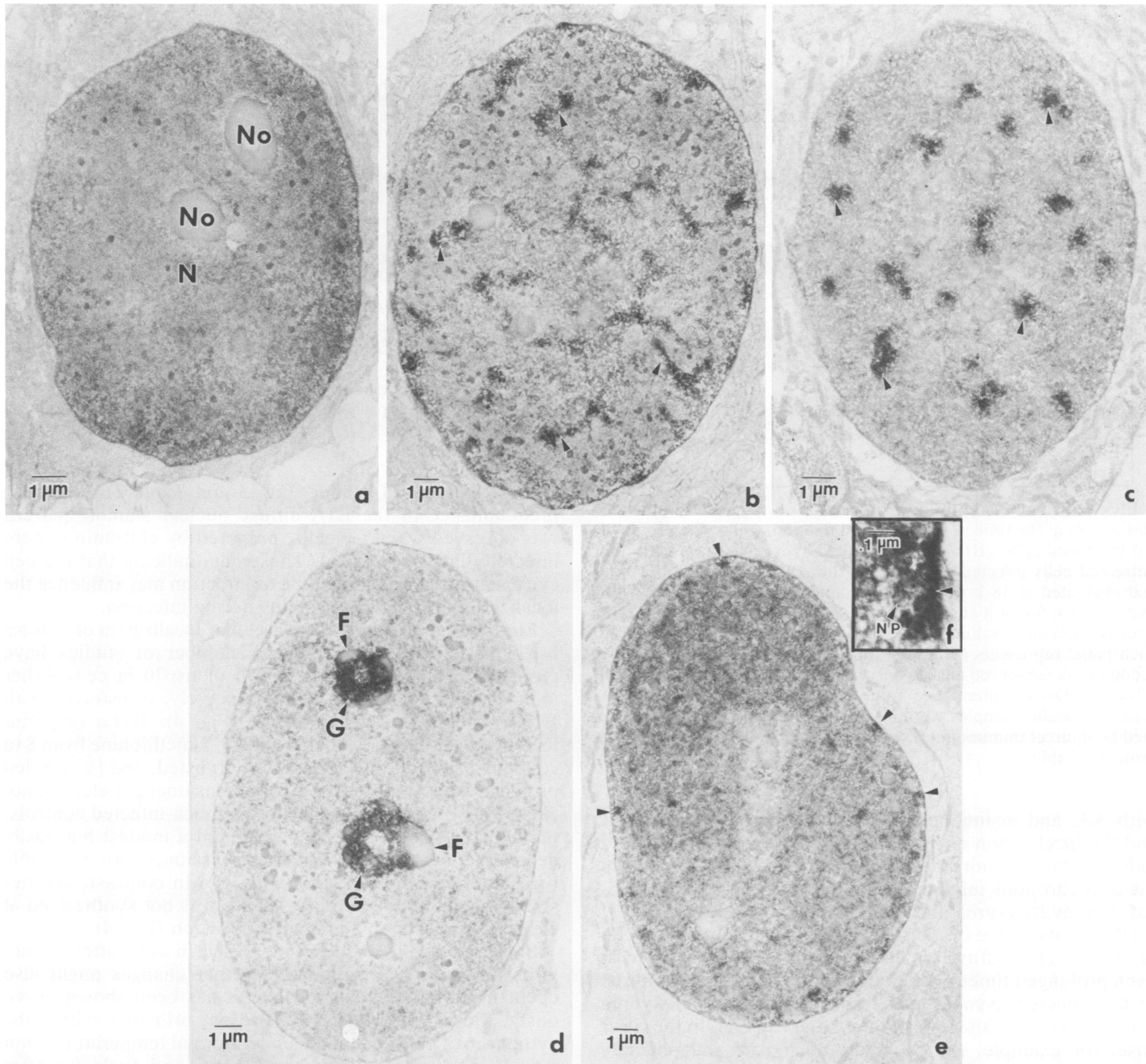

FIG. 2. Localization of the E1A proteins in infected cells at the electron microscopic level. HeLa cells were infected with Ad2, and 24 h later the cells were prepared for immunoelectron microscopy using E1A monoclonal antibodies as described in Materials and Methods. Shown are representative examples of the (a) diffuse, (b) reticular, (c) speckled, (d) nucleolar, and (e and f) peripheral staining patterns of E1A. The nucleolar distribution of E1A is limited to the granular region (G) of the nucleolus (panel d). The nucleolar fibrillar regions (F) are devoid of immunoreactivity (panel d). Numerous nuclear pore (NP) profiles can be observed in the regions of peripheral immunoreactivity (panels e and f). N, Nucleus; No, nucleolus.

tribution of E1A was not observed in the cells infected in the presence of hydroxyurea, and therefore this distribution of the protein may be associated with the late phase of infection (Table 1).

Although the distribution of E1A in infected cells changed over the course of infection, there was still considerable variation in the localization at any given time postinfection. The two most likely explanations for this heterogeneity in staining patterns are that (i) the course of infection is asynchronous or (ii) the differential localization of E1A may be a function of the cell cycle. We believe the former possibility to be unlikely since the cells were infected with 100 PFU of Ad2 per cell. To test the second possibility, HeLa cells were pretreated with hydroxyurea for 24 h to perturb the cell cycle. The cells were then infected with the virus, and the localization of the E1A proteins was determined by indirect immunofluorescence. The DNA content (and the cell cycle stage) of the cells was determined at the time of infection as well as after infection by flow cytometry. The prior treatment of the cells with hydroxyurea for 24 h resulted in an accumulation of cells with a G_1 and S DNA content and a diminution of cells with a G_2 DNA content, due to the inhibition of cellular DNA synthesis (Fig. 3 and Table 2). The hydroxyurea-treated cells were then infected

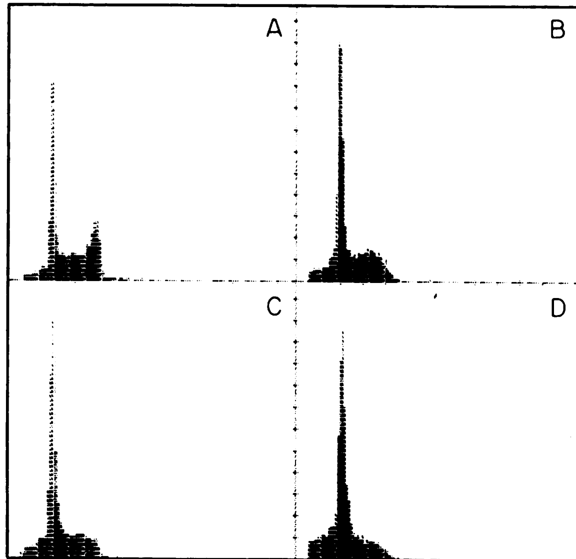

FIG. 3. Comparison by flow cytometry of cell cycle profiles of HeLa cells at the time of infection and fixation. Cell cycle profile of (A) untreated cells, (B) cells treated with hydroxyurea for 24 h, (C) untreated cells infected with Ad2 in the presence of hydroxyurea and evaluated at 18 h postinfection, and (D) cells pretreated with hydroxyurea for 24 h and then infected with Ad2 in the presence of hydroxyurea and evaluated at 18 h post infection. The first peak in each panel represents cells with a G_1 (2N) DNA content, and the second peak (observed clearly only in panel A) represents cells with a G_2 (4N) DNA content. See Table 2 for quantitation of cell cycle profiles. Parallel samples were infected with adenovirus and examined by indirect immunofluorescence for the localization of the E1A proteins (Table 1).

with Ad2 and maintained in the presence of hydroxyurea, and the localization of the E1A proteins was determined by indirect immunofluorescence. Unlike the results obtained in the asynchronous infected cells (e.g., Fig. 1), cells pretreated with hydroxyurea and then infected resulted in a nearly total predominance of a diffuse nuclear distribution of E1A (Table 1). This diffuse nuclear staining pattern persisted at even prolonged times postinfection (42 and 48 h), in contrast to the infected asynchronized cells in which hydroxyurea was added only at the time of infection (Table 1). In the latter case, for example, the reticular, nucleolar, and punctate distributions were well represented. It should be noted that it is the cell cycle stage at the time of infection that is significant, since addition of hydroxyurea only at the time of infection still permitted accumulation of G_1 and S cells at the time of fixation 18 h later (Fig. 3 and Table 2). A similar predominance of the E1A diffuse nuclear staining pattern was observed when G_1 cells, prepared by elutriation, were infected (data not shown). These data indicate that the cell cycle stage of a cell at the time of infection may influence the localization of the E1A proteins during infection.

TABLE 2. Quantitation of cell cycle profiles[a]

Time of sampling	Treatment of cells	DNA content (% of total) in:		
		G_1	S	G_2
Time of infection	Untreated	50	20	30
	Pretreat 24 h + HU	63	22	15
Time of fixation	Ad2 infected; 18 hpi + HU	69	17	12
	Pretreat HU 24 h; Ad2 infected; 18 hpi + HU	73	27	10

[a] See Fig. 3 for abbreviations, see Table 1, footnote *a*.

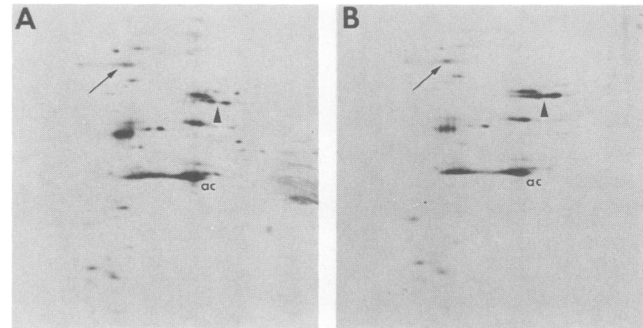

FIG. 4. Induction of hsp70 by As2 infection. HeLa cells growing on 10-cm dishes were (A) mock infected or (B) infected with Ad2. After labeling with [^{35}S]methionine from 8 to 20 h postinfection, the cells were collected and the labeled proteins were analyzed by two-dimensional gel electrophoresis. The position of hsp70 (hsp72) is indicated by the arrowhead. The position of hsp90 is indicated by the arrow. ac, Actin.

Elevated expression and intracellular localization of cellular hsp70 in infected HeLa cells. A number of studies have reported the increased expression of hsp70 in cells either infected with adenovirus or, alternatively, transfected with E1A-encoding DNA (21, 34, 52) (Fig. 4). HeLa cells, infected with Ad2, were labeled with [^{35}S]methionine from 8 to 20 h postinfection, the cells were harvested, and the labeled proteins were analyzed by two-dimensional gel electrophoresis (Fig. 4). As compared with the mock-infected controls, the adenovirus-infected cells exhibited a modest but easily detectable increase in hsp70 expression, both inducible hsp72 and constitutive hsp73 (Fig. 4). In contrast, another stress-inducible polypeptide, hsp90, was not synthesized at elevated levels upon adenovirus infection (Fig. 4).

Since the levels of hsp70 increased in cells after adenovirus infection, we examined whether changes might also occur in its intracellular locale. As has been shown previously, the hsp70 proteins are present primarily within the cytoplasm of cells incubated under normal temperatures, but quickly redistribute within the nucleus and nucleolus after heat shock treatment (46). The distribution of hsp70 was determined in Ad2-infected HeLa cells by using a monoclonal antibody specific to the highly heat-inducible form of hsp70 (47). Similar to what has been described before for HeLa cells grown at 37°C, the staining of hsp70 in the mock-infected cells was primarily diffuse cytoplasmic (see, for example, Fig. 9). After infection with Ad2, hsp70 staining appeared more intense, consistent with its increased synthesis as shown in Fig. 4. In the infected cells much of the hsp70 staining was observed within both the cytoplasm and the nucleus. Moreover, the nuclear staining appeared heterogeneous, with many of the cells displaying nucleolar, reticular, punctate, and diffuse staining patterns (Fig. 5). These changes in hsp70 intracellular locale appeared dependent upon the time at which we analyzed the cells after the infection. At earlier times postinfection (<12 h) much of the hsp70 staining was cytoplasmic. As the infection progressed (>12 to 24 h), there occurred an increased frequency of cells displaying these various intranuclear distributions of the

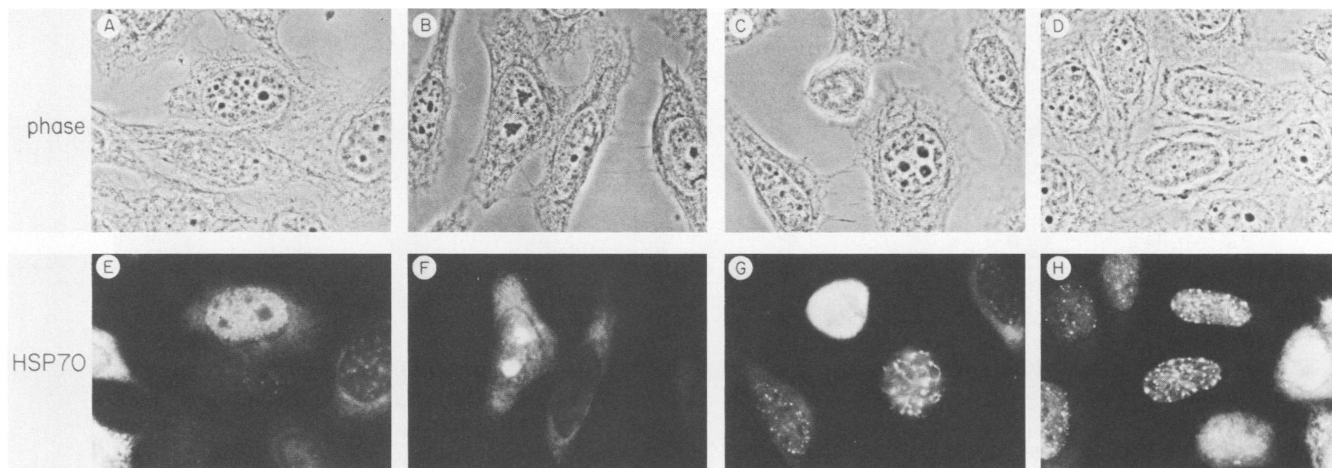

FIG. 5. Analysis of the distribution of hsp70 in HeLa cells infected with Ad2. HeLa cells growing on glass cover slips were infected at a multiplicity of infection of 100, and 24 h postinfection the cells were analyzed for the distribution of hsp70 via indirect immunofluorescence as described in the text. Several different staining patterns of hsp70 were observed: nuclear speckles, nucleolar, reticular, and diffuse nuclear. Shown are selected fields of cells which displayed altered localization of hsp70 (roughly 25 to 50% of the total population in this experiment). Mock-infected HeLa cells showed faint diffuse cytoplasmic staining of hsp70 (see, for example, Fig. 6 and 9).

hsp70 protein, particularly that of the reticular and nucleolar staining patterns. At the later times postinfection (24 to 48 h), most of the cells exhibited a diffuse or punctate nuclear distribution of the hsp70 protein (data not shown). Thus, in addition to elevating its expression, adenovirus infection results in distinct changes in the intracellular localization of hsp70. Moreover, these changes in hsp70 expression and intracellular distribution appear dependent upon functional E1A and are not simply a consequence of viral infection. For example, cells infected with an adenovirus mutant capable of expressing only the nonfunctional 9S E1A cDNA gene product showed neither an increase in hsp70 expression nor changes in its intracellular locale, even at high multiplicities of infection and prolonged times postinfection (24 and 48 h) (Fig. 6). A 9S virus, which would normally be defective for growth at low multiplicities of infection, is capable of expressing both early and late viral gene products, synthesizing viral DNA, and producing virus and therefore progresses to the late phase of infection at this high multiplicity (500 PFU/cell) and time postinfection (49, 51). This result clearly demonstrates that the altered localization of hsp70 after infection is dependent upon expression of the E1A proteins and is not simply a manifestation of a productive infection.

Colocalization of cellular hsp70 with the E1A proteins in infected HeLa cells. The nuclear staining patterns of hsp70 after adenovirus infection closely resembled the staining patterns of the E1A proteins in infected cells. We therefore investigated the possibility that hsp70 and E1A may colocalize after adenovirus infection. Adenovirus-infected cells were stained by double-label direct immunofluorescence using a rabbit polyclonal antibody directed against E1A (39) and a mouse monoclonal antibody directed against hsp70. At early times postinfection when the localization of E1A was predominantly diffuse-nuclear, hsp70 was found to be localized primarily in the cytoplasm, as in the mock-infected cells (data not shown). At intermediate times postinfection (12 to 24 h), at the time when both E1A and hsp70 displayed intranuclear distributions, their staining patterns in most cases appeared coincident (Fig. 7). In the subset of cells where E1A was found associated with nucleoli, in most cases (greater than 50%) hsp70 also localized to the nucleolus in the same cell (Fig. 7A, E, and I). The colocalization of hsp70 and E1A was most obvious in those cells displaying nucleolar and reticular staining patterns (Fig. 7). The number of cells where E1A localized in the nucleolar, reticular, and punctate staining patterns varied from experiment to experiment but usually maximally represented 20 to 50% of the total cell population. Of those, at least half (10 to 25%) displayed colocalization of E1A and hsp70 at these sites within the nucleus. Therefore, the E1A-hsp70 colocalization occurred in a subset of infected cells, but in a significant proportion.

The colocalization of E1A and hsp70 was also observed, in cells infected and incubated in the presence of hydroxyurea, to prevent passage of the infected cells into the late phase of infection (data not shown). This indicates that the colocalization of the two proteins occurs as an early event during infection and is independent of viral DNA replication.

Evidence of an association between E1A and hsp70 in infected cells. Having observed both an increase in hsp70 expression and a colocalization of a portion of E1A and hsp70 in the infected cells, we next examined whether we could isolate the two proteins as a complex from the infected cells. For these experiments HeLa cells were either (i) mock infected, (ii) infected with Ad2, (iii) infected with Ad2 in the presence of hydroxyurea, or (iv) mock infected in the presence of hydroxyurea. After labeling with [^{35}S]methionine, the cells were harvested and prepared for "native" immunoprecipitation studies as described in Materials and Methods. Briefly, this procedure, which involves a rather gentle solubilization of the cells in Triton X-100 and sodium deoxycholate, is routinely used to examine for possible protein-protein associations within an immune complex. The antibodies used in these studies included monoclonal antibody C92, specific for the stress-inducible form of hsp70; monoclonal antibody M73, directed against both the 12S and 13S forms of E1A; monoclonal antibody 6A8, specific for the adenovirus E1B 55-kDa tumor antigen; and monoclonal antibody PAb421, specific for the cellular p53 protein. In those cells mock infected and analyzed with these various antibodies, only the anti-hsp70 antibody showed a positive reaction (Fig. 8A, lane 2). In addition to hsp70, approximately four other proteins were present in the immune complex, one of which is the hsp90 protein. As expected, the

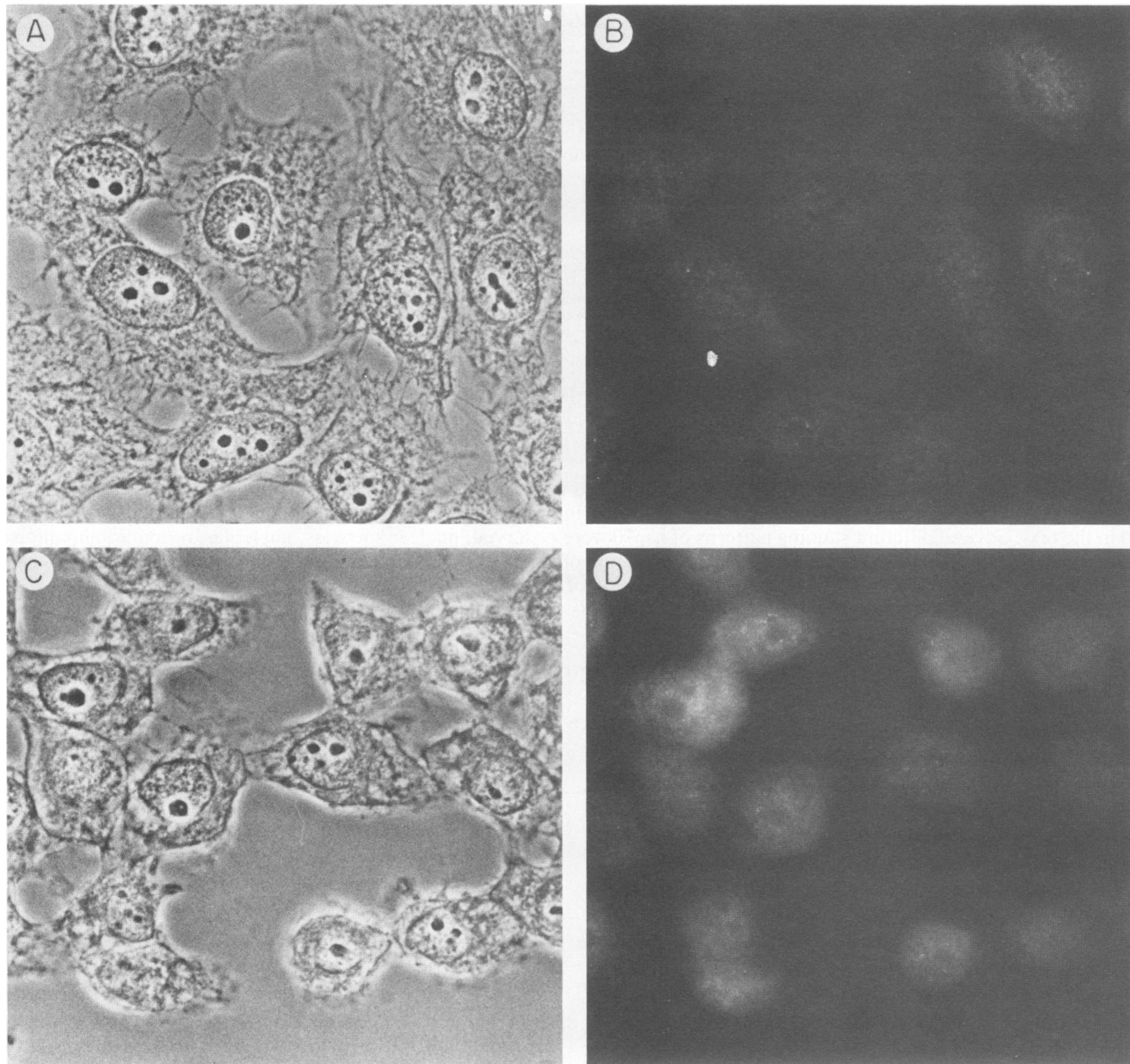

FIG. 6. Localization of hsp70 in HeLa cells infected with the E1A 9S cDNA virus. HeLa cells growing on glass cover slips were either (A and B) mock infected or (C and D) infected with the 9S virus at a multiplicity of infection of 500. Cells were fixed with methanol at 24 h postinfection and examined by indirect immunofluorescence (B and D) with anti-hsp70 monoclonal antibodies. (A and C) Phase-contrast images of the same fields in panels B and D. The slightly brighter appearance of the 9S-infected cells simply reflects the more founded morphology of the cells, a consequence of the adenovirus-induced cytopathic effect.

anti-E1A or -E1B antibodies did not immunoprecipitate any proteins from the uninfected cells. In addition, because there is little or no expression of p53 in the HeLa cells, no reaction product was observed using the p53 monoclonal antibody. In the case of the infected cells, the situation was quite different. First, a limited number of proteins were observed in the immunoprecipitate in which no antibody was added. These proteins (Fig. 8B, lane 1) represent background proteins which apparently bind to the protein A-Sepharose beads in a nonspecific manner. All of the immunoprecipitates contained these same proteins in approximately the same amounts (Fig. 8B). Immunoprecipitation using the hsp70 antibody resulted in a considerable precipitation of the hsp70 proteins as well as the same proteins precipitated in the mock-infected cells (Fig. 8B, lane 2). In addition, several proteins of approximately 90 to 100 kDa and of approximately 45 kDa were also observed. Immunoprecipitation with the E1A monoclonal antibody resulted in the precipitation of E1A, a 100-kDa protein, and discernible levels of hsp70 (seen as a doublet in Fig. 8B, lane 3). Finally, no reaction product, other than the background proteins, was observed with the p53 antibodies. In the case of the E1B antibodies, large amounts of E1B as well as some very high-molecular-weight proteins were immunoprecipitated (Fig. 8B, lanes 4 and 5, respectively). Note that only in those precipitations using the E1A antibodies, as compared to the no-antibody, p53, or E1B precipitations, was there an obvious coprecipitation of hsp70. In addition, it can be seen that only a fraction of the total hsp70 coprecipitates with E1A in the infected cells. Confirmation of this 70-kDa doublet in the E1A immunoprecipitations as hsp70 was accomplished by analysis of the immunoprecipitations on two-dimensional gels and comigration with authentic purified hsp70 (data not shown).

An analysis of the mock-infected or Ad2-infected cells incubated in the presence of hydroxyurea (to prevent passage into the late phase of infection) yielded similar, albeit slightly different, results. In the case of the mock-infected

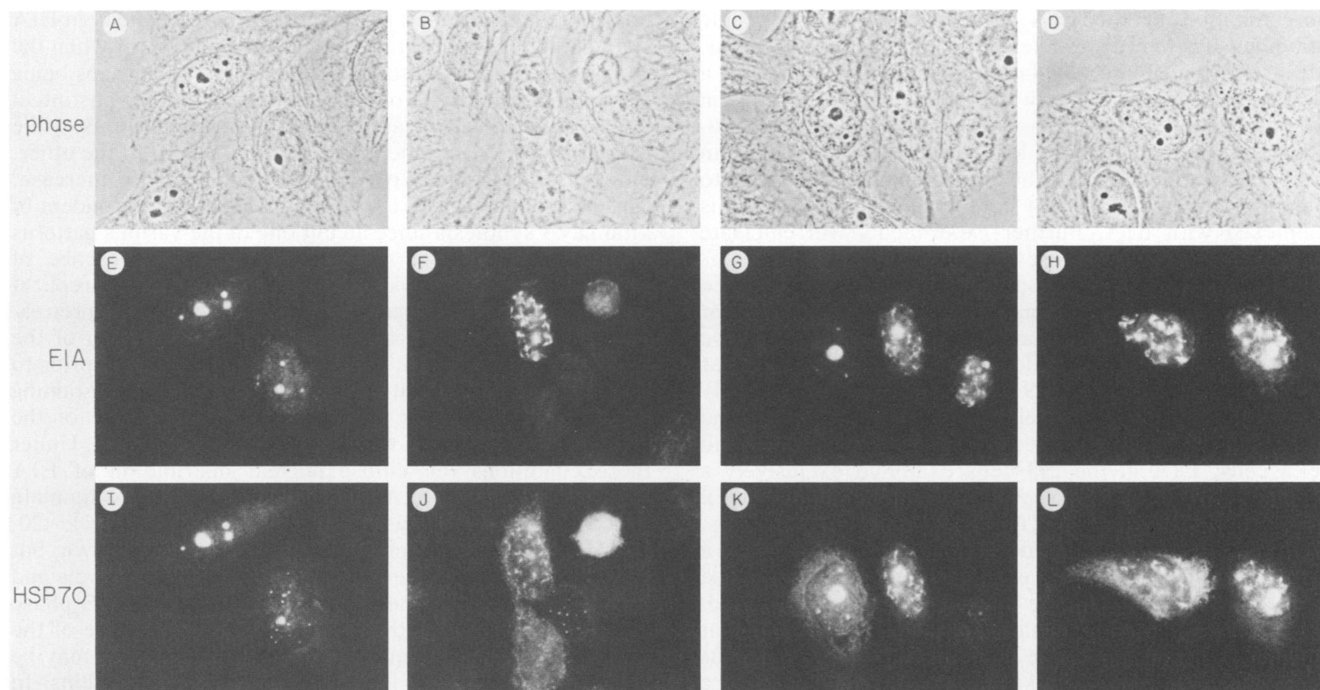

FIG. 7. E1A and hsp70 colocalize in Ad2-infected HeLa cells. HeLa cells growing on glass cover slips were infected with Ad2 (multiplicity of infection, 100), and 24 h later the cells were fixed and simultaneously analyzed for the distribution of both hsp70 and E1A using double-label indirect immunofluorescence. Detection of the rabbit anti-E1A antibody and of the mouse anti-hsp70 antibody was done by subsequent incubation with rhodamine-conjugated goat anti-rabbit and fluorescein-conjugated goat anti-mouse antibodies, respectively. Shown are selected fields of cells which displayed colocalization of E1A and hsp70 (roughly 10 to 25% of the total population in this experiment). Note that the same cells are shown in each vertical column.

cells, only the hsp70 antibodies resulted in an immunoprecipitation product (Fig. 8C). Note, however, that the relative recovery of hsp70 appeared less, and there was a corresponding decrease in both the number and amount of the other proteins (e.g. hsp90) which coprecipitated. In the case of Ad2-infected cells maintained in the presence of hydroxyurea, few or no background proteins were precipitated in the absence of the first antibody (e.g., the control immunoprecipitation; Fig. 8D, lane 1). Therefore, the background proteins observed earlier (i.e., Fig. 8B, lane 1) presumably represent proteins produced late in the adenovirus infection (and most likely include adenovirus structural proteins such as hexon). Immunoprecipitation from the infected cells with the hsp70 antibody resulted in the precipitation of considerable amounts of the hsp70 proteins and a number of other proteins, as observed before. Finally, large amounts of E1A as well as the hsp70 doublet were observed in those immunoprecipitates utilizing the E1A antibody. In fact, the relative amounts of both proteins (E1A and hsp70) were higher in the infected cells treated with hydroxyurea than in the cells infected without the drug. Other proteins coprecipitating with the E1A proteins, most notably a species migrating with a molecular size of approximately 100 kDa, likely represent other cellular proteins of unknown function that have been observed to coprecipitate with E1A (15, 54). Finally, we should emphasize that these studies indicate that hsp70 may also be interacting with other adenovirus-encoded proteins. A few viral late polypeptides were observed to coprecipitate with hsp70 (Fig. 8B, lane 2), and a very small amount of hsp70 was observed coprecipitating with the E1B 55-kDa protein (Fig. 8D, lane 5).

Despite the observation that hsp70 coprecipitated with E1A-specific antibodies, we were unable to observe obvious

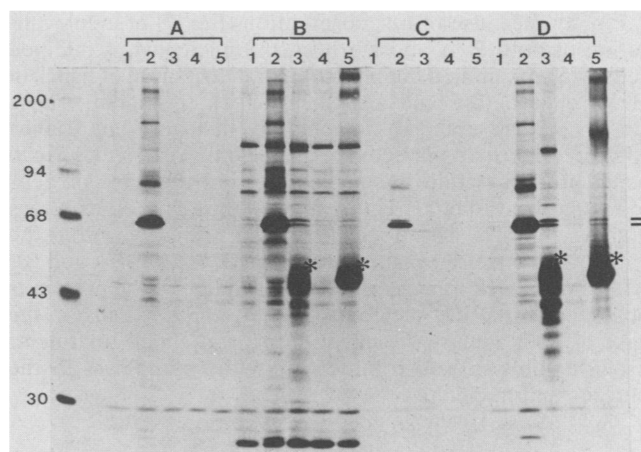

FIG. 8. A coassociation of hsp70 and E1A as demonstrated by immunoprecipitation analysis from Ad2-infected cells. HeLa cells growing in 10-cm dishes were (A) mock infected, (B) infected with Ad2, (C) mock infected in the presence of hydroxyurea, or (D) infected with Ad2 in the presence of hydroxyurea (multiplicity of infection, 100). The cells were labeled with [^{35}S]methionine, and at 20 h postinfection the cells were harvested in RIPA + SDS. The cell lysates were sonicated, clarified, and used for immunoprecipitation analysis with antibodies specific for hsp70, E1A, p53, or E1B 55K proteins as described in the text. The resultant immunoprecipitations were analyzed in a 10% polyacrylamide gel. Molecular mass markers are indicated on the left, the hsp70 doublet on the right, and the position of either E1A or E1B protein by an asterisk. Within each panel: lane 1, no antibody; lane 2, anti-hsp70; lane 3, anti-E1A; lane 4, anti-p53; and lane 5, anti-E1B.

coprecipitation of the E1A proteins with hsp70-specific antibodies. There are two likely explanations for this. First, only a fraction of the cellular hsp70 is complexed with a fraction of the E1A protein in the infected cell. E1A may in fact coprecipitate with the hsp70 using hsp70-specific antibodies, but the level may be below the level of detection in these experiments. Second, the monoclonal antibody used to immunoprecipitate hsp70 may not recognize hsp70 when it is complexed with E1A. Further experiments will elucidate whether one or both of these possibilities are correct.

Localization of E1A and hsp70 in cells stably transformed by E1A and E1B. To determine whether the five intranuclear localization patterns of E1A are dependent upon productive infection, or occurred in adenovirus-transformed cells as well, we examined human 293 cells. These cells are stably transformed and constitutively express both the E1A and E1B gene products (11). In contrast to adenovirus-infected HeLa cells, E1A in the 293 cells exhibited exclusively a diffuse nuclear, nonnucleolar distribution (Fig. 9E and F). However, upon infection of the 293 cells with adenovirus, E1A was observed now to localize within the nucleus in a reticularlike fashion (Fig. 9G and H). In some of the infected 293 cells a nucleolar locale of E1A was also observed. Hence, it would appear that the heterogeneous nuclear staining patterns of E1A are a consequence of adenovirus infection. We have also observed that infection of HeLa cells with an E1A 13S cDNA virus results in the appearance of heterogeneous E1A localization indistinguishable from wild-type virus-infected cells. In contrast, infection with an E1A 12S cDNA virus produces homogeneous diffuse nuclear staining, similar to that of 293 cells (data not shown). Perhaps a function encoded by the E1A 13S unique region, which primarily functions to stimulate adenovirus gene transcription, causes differential localization of E1A in infected cells.

Finally, because of our observation (Fig. 7) of a colocalization of both E1A and hsp70 in the nucleolus of infected cells, we reexamined the intracellular distribution of hsp70 in HeLa cells before and after heat shock treatment. These studies are presented to demonstrate that until our studies with the adenovirus-infected cells, the only instance of hsp70 being present within the nucleolus is in the case of cells following heat shock treatment or exposed to other stress regimes (46, 47). Hsp70 is distributed primarily within the cytoplasm of HeLa cells maintained at 37°C (Fig. 9A and B). After heat shock treatment, however, much of the protein is observed within the nucleolus (Fig. 9C and D). Thus, in the case of both adenovirus infection or heat shock treatment, hsp70 exhibits a redistribution from the cytoplasm to the nucleus and nucleolus.

DISCUSSION

The E1A proteins appear to function in modifying important aspects of eucaryotic gene transcription, DNA replication, and cell cycle control. They are also essential for mediating oncogenic transformation by adenovirus. The E1A proteins must function, either directly or indirectly, by interacting with the cellular transcription and replication machineries. Identifying cellular proteins that interact or physically associate with the E1A proteins is obviously a first step towards understanding the mechanism by which alterations in these cellular events occur.

By both direct immunofluorescence and immunoelectron microscopy, five discrete nuclear distributions of E1A were observed in the infected cell: diffuse, reticular, punctate, nucleolar, and peripheral. Much of this heterogeneity in E1A staining patterns appeared to be a consequence of when the cells were examined after infection as well as perhaps being cell cycle dependent. For example, at early times postinfection, E1A staining appeared diffuse within the nucleus, while at later times, the number of cells which exhibited the other, more discrete staining patterns of E1A began to increase. This heterogeneity of E1A staining appeared independent of viral DNA synthesis since all but one of the various patterns were observed in the cells infected in the presence of hydroxyurea, an agent known to inhibit viral DNA replication. Moreover, experiments in which the cells were pretreated with hydroxyurea, to result in an accumulation of the cells in both the late G_1 and S phases of cell cycle prior to infection, indicated that the heterogeneity in E1A staining patterns observed was most likely a consequence of the point in the cell cycle at which the cells were infected. Under these conditions the diffuse nuclear distribution of E1A predominated. The E1A proteins are known to stimulate host cell DNA synthesis and to perturb the cell cycle (20, 40). How the E1A proteins accomplish this is unknown, but conceivably, the requirements for altering the cell cycle and stimulating DNA synthesis in cells in late G_1 and S phase may be different from those of cells in the G_2 phase of the cell cycle. Different requirements for E1A function may be reflected in differential localization of the E1A proteins. In addition, because expression of E1A may be cell cycle regulated (22), this could affect the levels of E1A expression, its differential intranuclear locale, and perhaps its function during infection.

Our studies have shown that in addition to elevating the synthesis of hsp70, adenovirus infection of HeLa cells also results in a redistribution of the intracellular localization of hsp70 in the infected cell, and in some cases an apparent association of the two proteins. Examination of the localization of the E1A proteins in adenovirus-infected cells has led us to provide new evidence for an association between the E1A proteins and cellular hsp70 in the infected cell. Using double-label indirect immunofluorescence, we observed the two proteins to colocalize during the early phase of infection. The codistribution of E1A and hsp70 was most evident in those cells displaying either a reticular or nucleolar locale of E1A. Both the colocalization of hsp70 with E1A and its nuclear and nucleolar distribution were somewhat unexpected. Previous studies in our laboratory have shown that the majority of hsp70 resides in the cytoplasm of HeLa cells incubated under normal conditions. After heat shock treatment of the cells, both preexisting hsp70 and the newly synthesized protein rapidly redistribute into the nucleus, with a considerable portion of the protein present within the granular region of the nucleolus (46a, 47). During recovery from the heat shock treatment, the hsp70 proteins slowly return to the cytoplasm. In no other instance besides cellular stress have we ever detected hsp70 within the nucleolus. Therefore, expression of the E1A proteins during productive infection is either directly or indirectly responsible for not only stimulating the increased expression of hsp70 but also redirecting its intracellular localization in a manner similar (but not identical) to that which occurs during cellular stress.

Thus, considering that hsp70 was observed within the nucleus and nucleolus of cells after adenovirus infection, and that E1A and hsp70 colocalize (as shown by indirect immunofluorescence) and may coassociate (as indicated by their coimmunoprecipitation), the obvious question arises as to whether the association of these two proteins is relevant with respect to virus lifestyle or morphogenesis. Because we

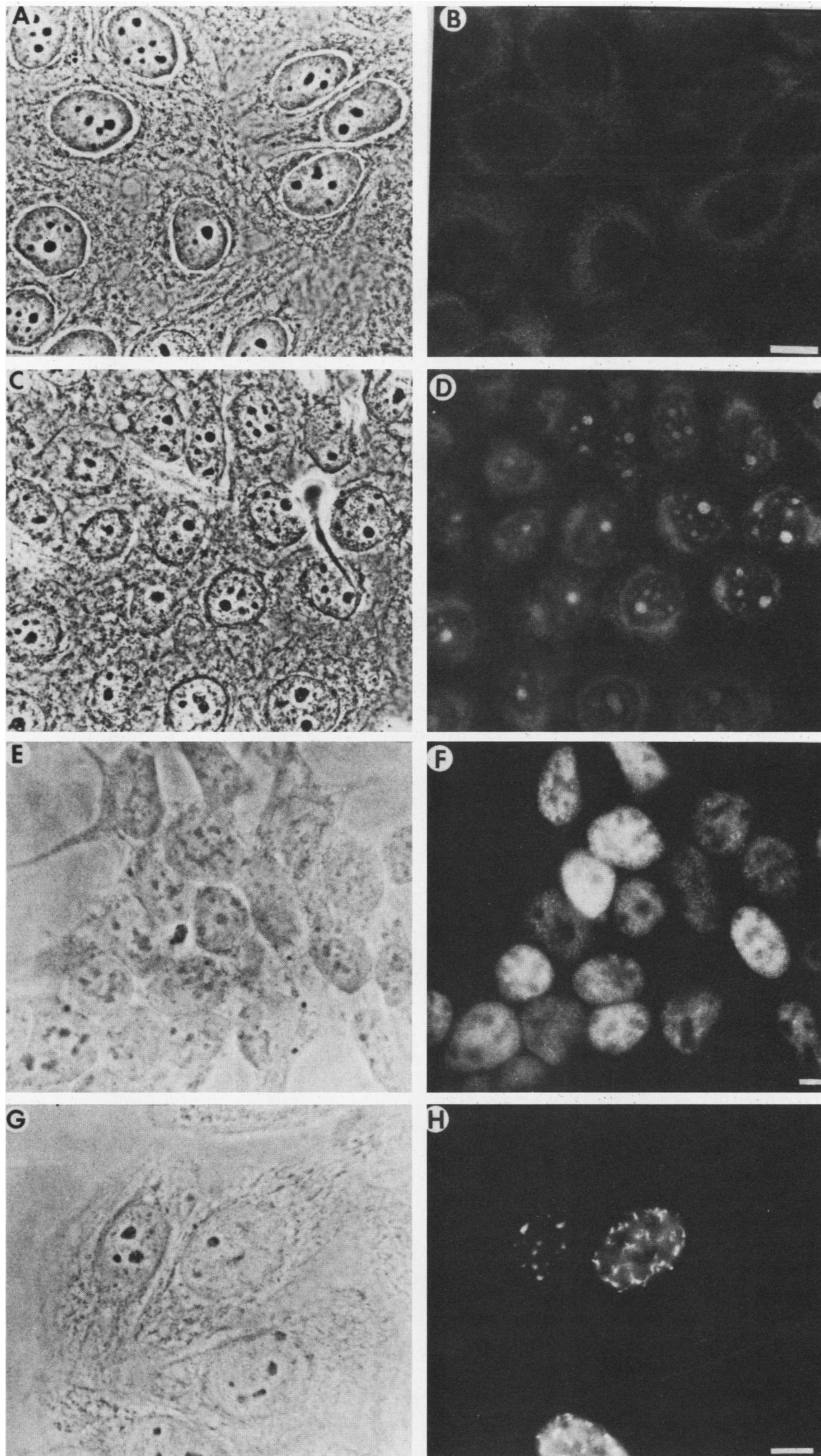

FIG. 9. Localization of hsp70 in normal and heat shock-treated HeLa cells and localization of E1A in normal or Ad2-infected 293 cells. HeLa cells growing on glass cover slips were incubated at either 37 or 42.5°C for 90 min. The cells were then fixed and analyzed for the distribution of hsp70 via indirect immunofluorescence. (A and B) Hsp70 staining in 37°C HeLa cells; (C and D) hsp70 staining in heat shock-treated HeLa cells. 293 cells growing on glass cover slips were either (E and F) mock infected or (G and H) infected with Ad2 (multiplicity of 100), and 24 h postinfection the cells were fixed and analyzed for the distribution of E1A via indirect immunofluorescence. Bars: (B) 10 μm, (F) 5 μm, and (H) 5 μm.

have previously observed a nucleolar localization of hsp70 only in cells experiencing stress (e.g., after heat shock), it might be concluded that adenovirus infection simply represents yet another form of cellular stress. Further support for this idea follows from our observation that in 293 cells, which express E1A, E1B, and hsp70, no obvious colocalization or coassociation of E1A and hsp70 was observed. Instead, the E1A distribution of the 293 cells was primarily diffuse nuclear and that of hsp70 was primarily diffuse cytoplasmic. In contrast, after infection of the 293 cells with Ad2, multiple nuclear staining patterns of E1A similar to those seen in infected HeLa cells were then observed as well as a colocalization of hsp70 with E1A (data not shown). These results indicate that the colocalization of E1A and hsp70 is dependent upon infection of the cells and is not simply a consequence of the cell (i.e., 293 cell) expressing the E1A protein. Moreover, infection of HeLa cells with a construct of adenovirus capable of producing only the nonfunctional 9S form of E1A did not result in an induction of hsp70, nor did it change in its intracellular redistribution even though the cells were infected under conditions which result in a productive viral infection (infection at high multiplicity). These results in sum indicate that the stimulation of hsp70 expression and its colocalization with E1A requires the presence of both functional E1A and a productive virus infection. Productive infection, however, is not simply another form of cellular stress, since a 9S virus infection, when allowed to progress to the late phase, had no effect on hsp70 localization. Furthermore, adenovirus infection did not induce synthesis of another stress protein, hsp90.

Returning to the question of the biological relevance of hsp70 association with E1A in the infected cell, it should be pointed out that both the elevated expression and the coassociation of hsp70 with other so-called oncogenes have been reported in a number of different systems. For example, cells infected with either adenovirus or SV40 exhibit increased levels of hsp70 as compared to the parental noninfected cells (21, 23, 34). In addition, transfection of cells with adenovirus E1A, SV40 virus large T, or c-*myc* cDNA all result in increased expression of hsp70 (24, 38, 52). With respect to complex formation, hsp70 has been observed in association with mutated forms of SV40 large T antigen, polyomavirus middle T antigen, the cellular p53 protein, and, as reported here, the wild-type form of adenovirus E1A (16, 23, 35, 41, 44). Again, the obvious question arises as to whether the association of hsp70 with these various proteins has relevance with respect to their biological function or whether these associations simply reflect a "housekeeping" function of hsp70 in recognizing and/or facilitating the clearing of abnormal or foreign proteins in the cell. Support for the latter idea includes the fact that many of these associations involve mutated forms of the oncogenic proteins which are defective in rendering the transformed phenotype (41, 44). A slightly more complicated situation, however, is emerging with respect to the association of p53 and hsp70. In a number of transformed cell lines rather stable complexes of p53 and hsp70 have been observed. In most of these cases the complexes being observed again represent "mutated" forms of the p53 protein (35, 41). In one case, however, a mutated form of p53, which both binds to hsp70 with high affinity and transforms rat fibroblasts (in cooperation with the *ras* oncogene) with an apparently higher efficiency than does the wild-type form of p53, has been reported by Levine's laboratory (16). Interestingly, in these cells the p53 present in the hsp70-p53 complex is reported to exhibit a longer half-life than that observed for free p53 (5). On the basis of these observations, Levine and co-workers have suggested that the p53-hsp70 complex may in fact be relevant to the activity of p53 and the maintenance of the transformed phenotype. This suggestion is reinforced by the fact that the levels of hsp70, like p53, are elevated in proliferative cells as compared to quiescent cells. In addition, the constitutive expression of hsp70 in HeLa cells has been reported to be cell cycle regulated, with maximal expression occurring near the G_1-S boundary (31). Finally, relevant to the discussion is the fact the the *Escherichia coli* DnaK protein, which shows a 50% homology with the mammalian hsp70 proteins (3), is necessary for the DNA replication of bacteriophage λ as well as for DNA and RNA synthesis of *E. coli* when the organism is grown at high temperatures (6, 19, 53). Thus, it remains possible that in addition to its role in the cell experiencing stress, the constitutive expression of hsp70 may play some role in the proliferative response of cells and that the various complexes of hsp70 with proteins like p53 and E1A may be relevant with respect to the regulation, stabilization, and proper functioning of these so-called cooperating oncogene protein products in the transformed cell.

In discussing the potential role of hsp70 with respect to E1A function and adenovirus infection, results by Imperiale et al. (18) have demonstrated that there is a correlation between the permissivity of cells to adenovirus infection and the preexisting level of hsp70. Specifically, these investigators have shown that cells which contain high levels of constitutive hsp70 appear more permissive for adenovirus infection than do cells which contain low levels of the protein. Moreover, in certain cells, again those which express high levels of hsp70, the E1A gene products apparently are not strictly required for the subsequent induction of early virus gene expression. One interpretation of these results is that certain cells, i.e., those which express high levels of hsp70, already express a protein with an E1A-like function. Such a putative protein then might account for the high constitutive levels of hsp70 observed, analogous to the situation in which E1A itself results in the elevated expression of the hsp70 protein. Hence, it will be interesting to determine whether prior elevation of the preexisting levels of hsp70, via heat shock treatment for example, will render cells more permissive to adenovirus infection. Conversely, using the method of microinjection, we plan to introduce into cells high levels of antibodies specific to hsp70 and determine whether we inhibit viral morphogenesis.

ACKNOWLEDGMENTS

We thank Ed Harlow, Bruce Stillman, and Betty Moran for critically reading the manuscript. We are grateful to Linda Rogers for performing the flow cytometry analysis. The expert technical assistance of Amy Denton, Michael Delannoy, and Ralph Cipriani is greatly appreciated.

This work was supported by Public Health Service grants from the National Cancer Institute (CA13106) and the National Institutes of Health (1 P30 CA45508). W.W. acknowledges the support of a Public Health Service grant (GM33551) from the National Institutes of Health. D.S. acknowledges the support of an American Cancer Society grant (ND-619). The Cold Spring Harbor Electron Microscopy Facility (D.S.) is partially supported by grants from the National Institutes of Health (Public Health Service grants 1 510 RR03430-01 and I P30 CA34408-01), the National Science Foundation (BB5-8604215), and the Fannie H. Rippel Foundation.

LITERATURE CITED

1. **Berk, A. J.** 1986. Adenovirus promoters and E1A transactivation. Annu. Rev. Genet. **20:**45–79.

2. **Branton, P. E., S. T. Bayley, and F. L. Graham.** 1985. Transformation of human adenoviruses. Biochim. Biophys. Acta **780:** 67–94.
3. **Burdwell, J. C. A., and E. A. Craig.** 1984. Major heat shock gene of *Drosophila* and the *Escherichia coli* heat-inducible dna K gene are homologous. Proc. Natl. Acad. Sci. USA **81:**848–852.
4. **Chatterjee, P. K., M. Bruner, S. J. Flint, and M. L. Harter.** 1988. DNA-binding properties of an adenovirus 289R E1A protein. EMBO J. **7:**835–841.
5. **Clarke, C. F., K. Cheng, A. B. Frey, R. Stein, P. W. Hinds, and A. J. Levine.** 1988. Purification of complexes of nuclear oncogene p53 with rat and *Escherichia coli* heat shock proteins: in vitro dissociations of hsc70 and dnaK from murine p53 by ATP. Mol. Cell. Biol. **8:**1206–1215.
6. **Dodson, M., H. Echols, S. Wickner, C. Alfano, K. Memsa-Wilmot, B. Gomes, J. LeBowitz, J. D. Roberts, and R. McMacken.** 1986. Specialized nucleoprotein structures of the origin of replication of bacteriophage λ: localized unwinding of duplex DNA by a six protein reaction. Proc. Natl. Acad. Sci. USA **83:** 7638–7642.
7. **Feldman, L. T., and J. R. Nevins.** 1983. Localization of the adenovirus $E1A_a$ protein, a positive-acting transcriptional factor, in infected cells. Mol. Cell. Biol. **3:**829–838.
8. **Ferguson, B., N. Jones, J. Richler, and M. Rosenberg.** 1984. Adenovirus Ela gene product expressed at high level in *Escherichia coli* is functional. Science **224:**1343–1346.
9. **Flint, S. J.** 1984. Cellular transformation by adenovirus. Pharmacol & Ther. **26:**59–88.
10. **Gaynor, R. B., A. Tsukamoto, C. Montell, and A. J. Berk.** 1982. Enhanced expression of adenovirus transforming proteins. J. Virol. **44:**276–285.
11. **Graham, F. L., J. Smiley, W. C. Russell, and R. Nairn.** 1977. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J. Gen. Virol. **36:**59–77.
12. **Haley, K. P., J. Overhauser, L. E. Babiss, H. S. Ginsberg, and N. C. Jones.** 1984. Transformation properties of type 5 adenovirus mutants that differentially express the E1A gene products. Proc. Natl. Acad. Sci. USA **81:**5734–5738.
13. **Harlow, E., L. V. Crawford, D. C. Pim, and N. M. Williamson.** 1981. Monoclonal antibodies specific for simian virus 40 tumor antigen. J. Virol. **39:**861–869.
14. **Harlow, E., B. Franza, Jr., and C. Schley.** 1985. Monoclonal antibodies specific for adenovirus early region 1A proteins: extensive heterogeneity in early region 1A products. J. Virol. **55:**533–546.
15. **Harlow, E., P. Whyte, B. R. Franza, Jr., and C. Schley.** 1986. Association of adenovirus early region 1A proteins with cellular polypeptides. Mol. Cell. Biol. **6:**1579–1589.
16. **Hinds, P. W., C. A. Finlay, A. B. Frey, and A. J. Levine.** 1987. Immunological evidence for the association of p53 with a heat shock protein, hsc70, in p53-plus-*ras*-transformed cell lines. Mol. Cell. Biol. **7:**2863–2869.
17. **Houweling, A., P. J. van den Elsen, and A. J. van der Eb.** 1980. Partial transformation of primary rat cells by the leftmost 4.5% fragment of adenovirus 5 DNA. Virology **105:**537–550.
18. **Imperiale, M. J., H. Kao, L. T. Feldman, J. R. Nevins, and S. Strickland.** 1984. Common control of the heat shock gene and early adenovirus genes: evidence for a cellular E1A-like activity. Mol. Cell. Biol. **4:**867–874.
19. **Itikawa, H., and J.-I. Ryu.** 1979. Isolation and characterization of a temperature-sensitive *dnaK* mutant of *Escherichia coli* B. J. Bacteriol. **138:**339–344.
20. **Kaczmarek, L., B. Ferguson, M. Rosenberg, and R. Baserga.** 1986. Induction of cellular DNA synthesis by purified adenovirus E1A proteins. Virology **152:**1–10.
21. **Kao, H., and J. R. Nevins.** 1983. Transcriptional activation and subsequent control of the human heat shock gene during adenovirus infection. Mol. Cell. Biol. **3:**2058–2065.
22. **Kao, H.-T., O. Capasso, N. Heintz, and J. R. Nevins.** 1985. Cell cycle control of the human HSP70 gene: implications for the role of a cellular E1A-like function. Mol. Cell. Biol. **5:**628–633.
23. **Khandjian, E. W., and H. Turlen.** 1983. Simian virus 40 and polyoma virus induce synthesis of heat shock proteins in permissive cells. Mol. Cell. Biol. **3:**1–8.
24. **Kingston, R. E., A. S. Baldwin, and P. A. Sharp.** 1984. Regulation of heat shock protein 70 gene expression by c-myc. Nature (London) **312:**280–282.
25. **Kingston, R. E., A. Cowie, R. I. Morimoto, and K. A. Gwinn.** 1986. Binding of polyomavirus large T antigen to the human *hsp70* promoter is not required for *trans* activation. Mol. Cell. Biol. **6:**3180–3190.
26. **Krippl, B., B. Ferguson, N. Jones, M. Rosenberg, and H. Westphal.** 1985. Mapping of functional domains in adenovirus E1A proteins. Proc. Natl. Acad. Sci. USA **82:**7480–7484.
27. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–686.
28. **Leong, K., and A. J. Berk.** 1986. Adenovirus early region 1A protein increases the number of template molecules transcribed in cell-free extracts. Proc. Natl. Acad. Sci. USA **83:**5844–5848.
29. **Lonberg-Holm, K., and L. Philipson.** 1969. Early events of virus-cell interaction in an adenovirus system. J. Virol. **4:**323–338.
30. **Lucher, L. A., P. M. Loewenstein, and M. Green.** 1985. Phosphorylation in vitro of *Escherichia coli*-produced 235R and 266R tumor antigens encoded by human adenovirus type 12 early transformation region 1A. J. Virol. **56:**183–193.
31. **Milarski, K. L., and R. I. Morimoto.** 1986. Expression of human Hsp 70 during the synthetic phase of the cell cycle. Proc. Natl. Acad. Sci. USA **83:**9517–9521.
32. **Moran, E., T. Grodzicker, R. J. Roberts, M. B. Mathews, and B. Zerler.** 1986. Lytic and transforming functions of individual products of the adenovirus E1A gene. J. Virol. **57:**765–775.
33. **Moran, E., and M. B. Mathews.** 1987. Multiple functional domains in the adenovirus E1A gene. Cell **48:**177–178.
34. **Nevins, J. R.** 1982. Induction of the synthesis of a 70,000 dalton mammalian heat shock protein by the adenovirus E1A gene product. Cell **29:**913–919.
35. **Pinhasi-Kimhi, O., D. Michalovitz, A. Ben-Zeev, and M. Oren.** 1986. Specific interaction between the p53 cellular tumour antigen and major heat shock proteins. Nature (London) **320:** 182–185.
36. **Ruley, H. E.** 1983. Adenovirus early region 1A enables viral and cellular transforming genes to transform primary cells in culture. Nature (London) **304:**602–606.
37. **Schmitt, R. C., M. L. Fahnestock, and J. B. Lewis.** 1987. Differential nuclear localization of the major adenovirus type 2 Ela proteins. J. Virol. **61:**247–255.
38. **Simon, M. C., K. Kitchener, H.-T. Kao, E. Hickey, L. Weber, R. Voellmy, N. Heintz, and J. R. Nevins.** 1987. Selective induction of human heat shock gene transcription by the adenovirus E1A gene products, including the 12S E1A product. Mol. Cell. Biol. **7:**2884–2890.
39. **Splindler, K. R., D. S. E. Rosser, and A. J. Berk.** 1984. Analysis of adenovirus transforming proteins from early regions 1A and 1B with antisera to inducible fusion antigens produced in *Escherichia coli* cells. J. Virol. **49:**132–141.
40. **Stabel, S., P. Argos, and L. Philipson.** 1985. The release of growth arrest by microinjection of adenovirus E1A DNA. EMBO J. **4:**2329–2336.
41. **Sturzbecher, H. W., P. Chumakov, W. J. Welch, and J. Jenkins.** 1987. Mutant p53 proteins bind hsp 72/73 cellular heat shock related proteins in SV40-transformed monkey cells. Oncogene **1:**201–211.
42. **Thomas, G. P., and M. B. Mathews.** 1980. DNA replication and the early to late transition in adenovirus infection. Cell **22:**523–533.
43. **Tsukamoto, A. S., A. Ponticelli, A. J. Berk, and R. B. Gaynor.** 1986. Genetic mapping of a major site of phosphorylation in adenovirus type 2 E1A proteins. J. Virol. **59:**14–22.
44. **Walter, G., A. Carbone, and W. J. Welch.** 1987. Medium tumor antigen of polyomavirus transformation-defective mutant NG59 is associated with 73-kilodalton heat shock protein. J. Virol. **61:** 405–410.
45. **Welch, W. J., and J. R. Feramisco.** 1982. Purification of the

major mammalian heat shock proteins. J. Biol. Chem. **257:** 14949–14959.

46. **Welch, W. J., and J. R. Feramisco.** 1984. Nuclear and nucleolar localization of the 72,000 dalton heat shock protein in heat-shocked mammalian cells. J. Biol. Chem. **259:**4501–4510.

46a. **Welch, W. J., and L. A. Mizzen.** 1988. Characterization of the thermotolerant cell. II. Effects on the intracellular distribution of heat shock protein 70, intermediate filaments and small nuclear ribonucleoprotein complexes. J. Cell Biol. **106:**1117–1130.

47. **Welch, W. J., and J. P. Suhan.** 1986. Cellular and biological events in mammalian cells during and after recovery from physiological stress. J. Cell. Biol. **103:**2035–2052.

48. **White, E., S. H. Blose, and B. W. Stillman.** 1984. Nuclear envelope localization of an adenovirus tumor antigen maintains the integrity of cellular DNA. Mol. Cell. Biol. **4:**2865–2875.

49. **White, E., A. Denton, and B. Stillman.** 1988. Role of the adenovirus E1B 19,000-dalton tumor antigen in regulating early gene expression. J. Virol. **62:**3445–3454.

50. **White, E., T. Grodzicker, and B. W. Stillman.** 1984. Mutations in the gene encoding the adenovirus E1B 19K tumor antigen cause degradation of chromosomal DNA. J. Virol. **52:**410–419.

51. **White, E., and B. W. Stillman.** 1987. Expression of adenovirus E1B mutant phenotypes is dependent on the host cell and synthesis of the E1A proteins. J. Virol. **61:**426–435.

52. **Wu, B. J., H. C. Hurst, N. C. Jones, and R. I. Morimoto.** 1986. The E1A 13S product of adenovirus 5 activates transcription of the cellular human HSP70 gene. Mol. Cell. Biol. **6:**2994–2999.

53. **Yamamoto, T., J. McIntyre, S. M. Sell, C. Georgopoulos, D. Skowyra, and M. Zylicz.** 1987. Enzymology of the pre-priming steps in λdv DNA replication in vitro. Proc. Natl. Acad. Sci. USA **84:**7996–7999.

54. **Yee, S.-P., and P. E. Branton.** 1985. Detection of cellular proteins associated with human adenovirus type 5 early region 1A polypeptides. Virology **147:**142–153.

55. **Yee, S.-P., D. T. Rowe, M. L. Tremblay, M. McDermott, and P. E. Branton.** 1983. Identification of human adenovirus early region 1 products by using antisera against synthetic peptides corresponding to the predicted carboxy termini. J. Virol. **46:** 1003–1013.

56. **Yoshinaga, S., N. Dean, M. Han, and A. J. Berk.** 1986. Adenovirus stimulation of transcription by RNA polymerase III: evidence for an E1A-dependent increase in transcription factor IIIC concentration. EMBO J. **5:**343–354.

57. **Zerler, B., E. Moran, K. Maruyama, J. Moomaw, T. Grodzicker, and H. E. Ruley.** 1986. Adenovirus E1A coding sequences that enable *ras* and *pmt* oncogenes to transform cultured primary cells. Mol. Cell. Biol. **6:**887–899.

58. **Zhonghe, Z., J. A. Nickerson, G. Krochmalnic, and S. Penman.** 1987. Alterations in nuclear matrix structure after adenovirus infection. J. Virol. **61:**1007–1018.