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Adenovirus DNA-Binding Protein in Cells Infected with Wild-Type 5 Adenovirus and Two DNA-Minus, Temperature-Sensitive Mutants, H5ts125 and H5ts149

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Studies have been done to characterize further H5ts125, an adenovirus type 5 conditionally lethal, temperature-sensitive (ts) mutant defective in initiation of DNA synthesis and to investigate whether the single-strand-specific DNA-binding (72,000 molecular weight) protein is coded by the mutated viral gene. When H5ts125-infected cells were labeled with [^{35}S]methionine at 32°C and then incubated without isotope at 39.5°C, the mutant's nonpermissive temperature, the 72,000 molecular weight polypeptide was progressively degraded. Immunofluorescence examination of cells infected with wild-type virus, H5ts125, and H5ts149 (a second, unique DNA-minus mutant) showed that immunologically reactive DNA-binding protein was barely detectable in H5ts125-infected cells at 39.5°C, whereas this protein was present in wild-type- and H5ts149-infected cells, that the protein made at 32°C in H5ts125-infected cells lost its ability to bind specific DNA-binding protein antibody when the infected cells were shifted to 39.5°C, and that if H5ts125-infected cells were shifted from the restrictive temperature to 32°C, even in the presence of cycloheximide to stop protein synthesis, immunologically reactive DNA-binding protein reappeared.

Early after adenovirus infection a single-strand-specific, DNA-binding protein appears (15, 17, 18), which varies in molecular weight from 60,000 for type 12 (14, 15) to about 72,000 (72K) for adenovirus types 2 and 5 (14, 18). The function of this protein is unknown, but its similarity to the gene 32 protein of bacteriophage T4 (5) suggested that the adenovirus DNA-binding protein may also be associated with replication of the viral DNA (17, 18). This possibility is supported by the finding that H5ts125, a temperature-sensitive (ts) mutant defective in initiation of viral DNA replication (6, 9), appears to produce an altered DNA-binding protein (19). The relationship of the H5ts125 mutation to the viral DNA-binding protein (for convenience, also called the 72K protein) is based upon the findings that the 72K protein is present in diminished amounts in H5ts125-infected cells cultured at 39.5°C (the nonpermissive temperature) or after a shift from 32°C (the permissive temperature) to 39.5°C and that the 72K protein from cells infected with H5ts125 at 32°C dissociates from DNA-cellulose at a lower temperature than the 72K protein made by wild-type (WT) virus (19).

The experiments to be reported in this communication were developed to test further the possibility that the adenovirus DNA-binding protein is a product of the gene mutated in H5ts125. Evidence will be presented showing that the 72K protein made at 39.5°C is degraded, that only minute amounts of immunologically reactive protein are produced at 39.5°C, and that immunologically active DNA-binding protein synthesized at 32°C becomes nonreactive after a shift of the infected culture to 39.5°C.

MATERIALS AND METHODS

Viruses and cell cultures. Suspension and monolayer cultures of KB cells were grown in Eagle minimum essential medium supplemented with 10% human or calf serum, as previously described (2, 12). Viral stocks of plaque-purified WT adenovirus type 5 were prepared in suspension cultures incubated at 36°C for 35 h; stocks of two ts mutants, H5ts125 and H5ts149 (6, 9), were made in suspension cultures incubated at 32°C for 60 h. Plaque (6, 12) and fluorescent-focus (7) assays were used for viral quantitation.

Polyacrylamide gel electrophoresis. Infected cell pellets were washed with 0.15 M NaCl buffered at pH 7.2 with 0.01 M phosphate (PBS) and then resuspended in 0.4 ml of gel sample buffer (1% sodium dodecyl sulfate, 1% 2-β-mercaptoethanol, 50 mM Tris [pH 6.8], and 10% glycerol). Ten percent poly-

acrylamide gels were prepared in a slab gel apparatus with a 4% stacking gel, using the buffer system described by Laemmli (11). The acrylamide-bisacrylamide ratio was 30:0.8. The samples, which contained no more than 10 μg of protein per well, were electrophoresed at 70 V until the dye front reached the bottom of the gel. Gels were stained with Coomassie brilliant blue (Colob) and dried under vacuum, and radioautography was performed, using Kodak Royal X-omat X-ray film.

Immunofluorescence. KB cells grown as monolayers on glass cover slips were infected with 0.1 to 0.5 PFU of mutant or WT virus per cell and incubated under varying conditions of temperature and time. At completion of the incubation period, the cover slip cultures were washed with PBS and air-dried for 1 h at 37°C. The cells were then fixed in absolute acetone at room temperature for 10 min and air-dried for an additional 30 min. Indirect immunofluorescence was carried out as previously described (3): the initial reaction was done with a 1:120 dilution of unlabeled antiserum from rabbits immunized with purified adenovirus-specific DNA-binding protein (kindly supplied by L. Philipson, University of Uppsala, Uppsala, Sweden); the final reaction was made with a 1:20 dilution of goat anti-rabbit immunoglobulin G conjugated with fluorescein isothiocyanate. Cover slips were washed, inverted in a drop of glycerol, mounted on a microscope slide, and observed with ultraviolet illumination (3).

Isotopes and chemicals. [^{35}S]methionine (464 Ci/mmol) was purchased from New England Nuclear Corp. Fluorescein isothiocyanate-conjugated goat anti-rabbit antiserum was obtained from Hyland Laboratories.

RESULTS

Stability of the DNA-binding (72K) protein in H5ts125-infected cells. Van der Vliet et al. (19) have shown that the 72K protein is extracted in greatly reduced amounts from cells infected with H5ts125 and incubated at 39.5°C. These workers also demonstrated a decrease in the putative viral gene product when H5ts125-infected cells were shifted from 32 to 39.5°C (19). Since viral DNA replication is rapidly reduced when H5ts125-infected cultures are changed from 32 to 39.5°C, but only slowly decreased when cells infected with another DNA-minus mutant, H5ts149, are similarly treated (5), it was important to determine whether the decrease in DNA synthesis was correlated with a degradation of the 72K DNA-binding protein. Accordingly, cells were infected with 100 PFU of H5ts125, H5ts149, and WT virus per cell and incubated at 32°C for 24 h, when the rate of DNA replication was maximum (unpublished data). The infected cells were labeled with [^{35}S]methionine (50 μCi/ml) for 30 min, washed, resuspended in medium containing excess methionine (10 mM), cultures at 32°C for an additional 30 min, and shifted to 39.5°C. The cultures were sampled at the time of shift-up and at increasing times after the temperature change. The stability of the DNA-binding protein was then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography of the dried gel.

The autoradiogram and densitometer tracings of a representative experiment (Fig. 1 and 2) clearly show the significant decrease of the 72K DNA-binding protein in H5ts125-infected cells: at 2 h after the shift from 32 to 39.5°C, the 72K polypeptide had diminished about 60%, and only about 20% of the original labeled protein was detectable 6 h after the change to the restrictive temperature. In sharp contrast, the 72K protein was remarkably stable in the cells infected by H5ts149, another DNA-minus ts mutant that is also defective in initiation of DNA synthesis, but is in a unique complementation group (9). The DNA-binding protein of H5ts149-infected cells was as stable as the 72K protein in cells infected with WT virus. It should also be noted (Fig. 1) that during the chase period, the 72K protein developed a detectably slower electrophoretic mobility, probably owing to a post-translation modification of the protein, such as phosphorylation (16).

Immunofluorescence characteristics of adenovirus DNA-binding proteins in WT-, H5ts125-, and H5ts149-infected cells. The immunofluorescence technique was used to localize the adenovirus-induced DNA-binding protein's site of action, to note the appearance of the "DNA factories" in which it probably functions, and to estimate its immunological reactivity in cells infected with the DNA-minus ts mutants and WT virus.

In cells infected with WT virus and incubated at 32°C for 24 h or 39.5°C for 16 h, the DNA-binding protein was noted in discrete, ball-like foci within the nucleus (Fig. 3). With time, the foci usually coalesced into large, brightly fluorescent masses. Cells infected with H5ts125 and incubated at 32°C were closely similar to WT-infected cells (Fig. 4a). When, however, H5ts125-infected cells were incubated at 36°C, a semipermissive temperature, the fluorescent masses were markedly reduced in size and number (Fig. 4b), and when the H5ts125-infected cells were incubated at 39.5°C, the DNA-binding protein was barely detectable (Fig. 4c).

Experiments were done to determine whether the rapid cessation of DNA synthesis, which occurs when H5ts125-infected cells are shifted from a permissive to a restrictive temperature (5), is reflected in a comparable reduc-

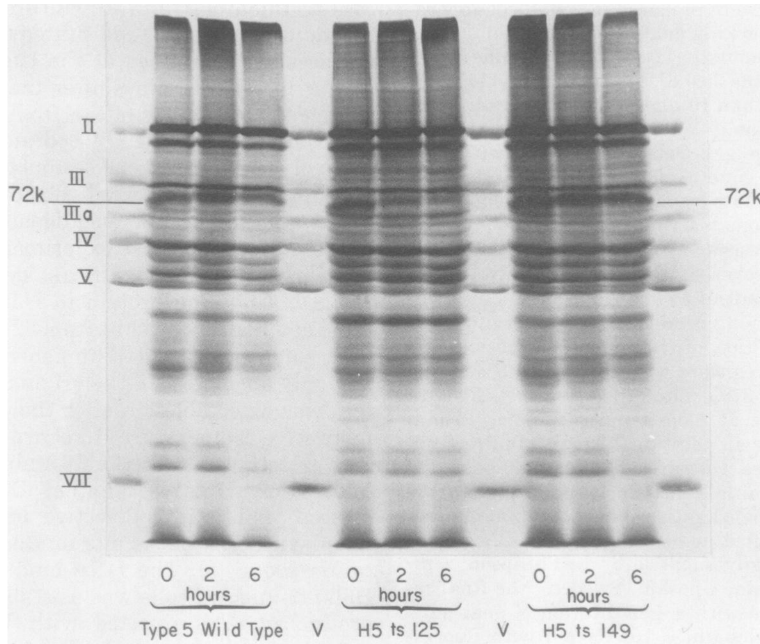


FIG. 1. *Stability of the viral DNA-binding protein (the 72K protein) at 39.5°C in cells infected with H5ts125, H5ts149, and WT 5 adenovirus. KB cells in suspension culture were infected with 100 PFU/cell and incubated at 32°C for 24 h. Infected cells were labeled with [^{35}S]methionine (50 μCi/ml) for 30 min at 32°C, cultured in medium containing a 10-fold (10 mM) excess of unlabeled methionine for 30 min, and then shifted to 39.5°C. Samples were taken from cultures at 0, 2, and 6 h after the shift to 39.5°C, cell extracts containing approximately equal numbers of counts were electrophoresed in a 10% polyacrylamide slab gel (see text), and an autoradiogram of the dried gel was developed. Purified, labeled virus was disrupted and electrophoresed to provide standard markers (labeled "V"; some of virion polypeptides were artificially accentuated to make bands more prominent for photography).*

tion in antigenicity of the DNA-binding protein. Infected cultures that had been incubated at 32°C for 24 h were shifted to 39.5°C and examined after 2 and 6 h. The immunofluorescence was sharply reduced 2 h after the shift (Fig. 5a) and was almost absent 6 h after the change to the restrictive temperature (Fig. 5b). These data support the postulates that functional DNA-binding protein is present in a reduced amount in cells infected with H5ts125 at 39.5°C and that the protein synthesized at 32°C becomes nonfunctional and is slowly degraded (Fig. 1 and 2) when the temperature is raised to a restrictive condition.

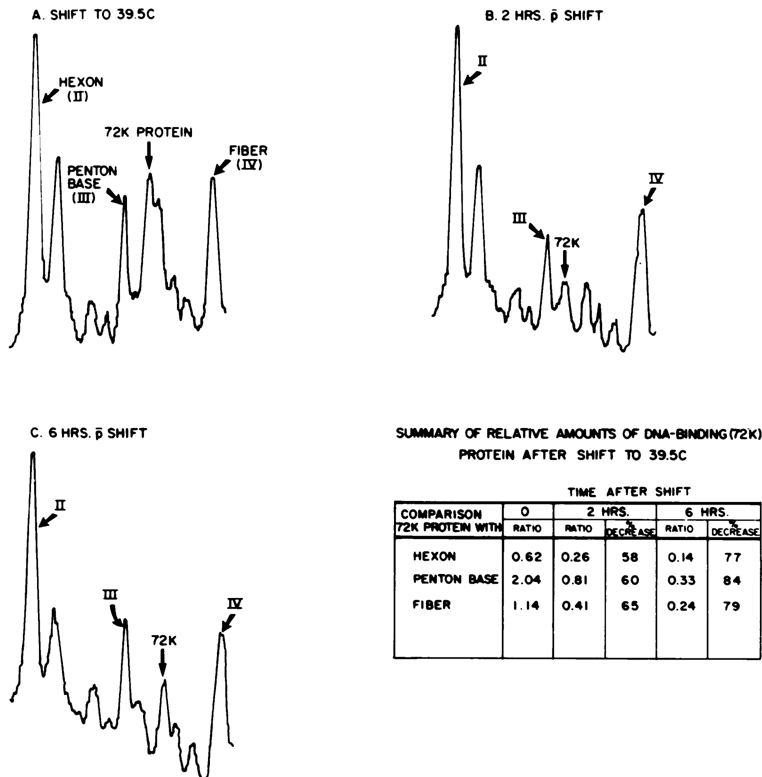
Since biosynthesis of viral DNA begins without delay, albeit at a slow rate, after the incubation temperature of H5ts125-infected cells is lowered from 39.5 to 32°C (T. Schutzbank and H. S. Ginsberg, manuscript in preparation), it was postulated that immunologically reactive DNA-binding protein would also appear when the temperature was reduced from 39.5 to 32°C. The results of an experiment to test this prediction (Fig. 6a and b) show that the appearance of immunologically reactive DNA-binding protein appeared as early as 1 h after the shift-down (data not shown), and by 6 h after the change the quantity of DNA-binding protein was indistinguishable from that in H5ts125-infected cells incubated at 32°C (Fig. 4a) or WT-infected cells at 32 to 39.5°C (Fig. 3). Indeed, when 10 μg of cycloheximide per ml was added to cultures to stop protein synthesis 30 min before reducing the temperature incubation, immunologically reactive DNA-binding protein still appeared within 1 h (data not shown). The amount of DNA-binding protein, however, was not as great as in the absence of the protein inhibitor. Hence, the 72K DNA-binding protein that was not degraded at 39.5°C could appropriately fold upon shift to 32°C so that the protein became immunologically active and biochemically functional.

DISCUSSION

The conditionally lethal, temperature-sensitive mutant H5ts125 is an "early" mutant defective in the initiation of viral DNA replication

(1, 6, 9). The aggregate of evidence gathered indicates that this mutant's affected gene codes for the single-strand-specific DNA-binding protein (72K protein) synthesized in adenovirus-infected cells (18, 19). This evidence may be summarized as follows. (i) The 72K protein is present in greatly reduced amounts in H5ts125-infected cells cultured at the nonpermissive temperature and in cells shifted from the permissive to the restrictive conditions (19). (ii) The 72K protein is synthesized but is soon degraded in H5ts125-infected cells incubated at 39.5°C or when infected cells are shifted from 32 to 39.5°C (Fig. 1 and 2). (iii) The 72K protein, although synthesized in H5ts125-infected cells at 39.5°C, ineffectively forms an immunologically reactive ligand (Fig. 4c). (iv) The immunologically active DNA-binding protein made at 32°C in H5ts125-infected cells loses its antigenic reactivity when the temperature is increased to the restrictive temperature. (v) Gene 125 (i.e., the H5ts125 mutated gene), by heterotypic recombination-endonuclease restriction mapping (Grodzicker and Sambrook, unpublished data), is located within 0.59 and 0.71 map units from the left end of the genome, which is approximately the same position as H5ts125 on the genetic recombination map (10) and the locus of the 72K protein on the transcription-translation map of adenovirus type 2 (13).

Biochemical evidence indicates that the adenovirus DNA-binding protein is an "early" viral protein (8, 17, 18) and that it can be isolated in association with replicating viral DNA (17, 20). The circumscribed, virus-specific masses noted by immunofluorescence are probably the intranuclear "factories" for viral DNA synthesis, and they appear to correspond to the distinctive, small, rounded nuclear inclusions, which are initially eosinophilic during early stages of infection, but soon become basophilic and Feulgen positive (4). It should be further emphasized that replication of viral DNA is correlated with the accumulation or degradation of the 72K DNA-binding protein in H5ts125-infected cells. At 32°C DNA synthesis



SUMMARY OF RELATIVE AMOUNTS OF DNA-BINDING (72K) PROTEIN AFTER SHIFT TO 39.5C

TIME AFTER SHIFT

COMPARISON 72K PROTEIN WITH	0	2 HRS.		6 HRS.	
	RATIO	RATIO	% DECREASE	RATIO	% DECREASE
HEXON	0.62	0.26	58	0.14	77
PENTON BASE	2.04	0.81	60	0.33	84
FIBER	1.14	0.41	65	0.24	79

FIG. 2. *Densitometer tracings were made of the polyacrylamide gels developed with extracts of H5ts125-infected cells shown in Fig. 1. The areas under the curves for the 72K, hexon (II), penton base (III), and fiber (IV) protein were estimated by weighing. The ratios of the 72K protein to the three major capsid proteins were determined for each time to estimate quantitatively the degradation of the 72K protein at 39.5°C.*

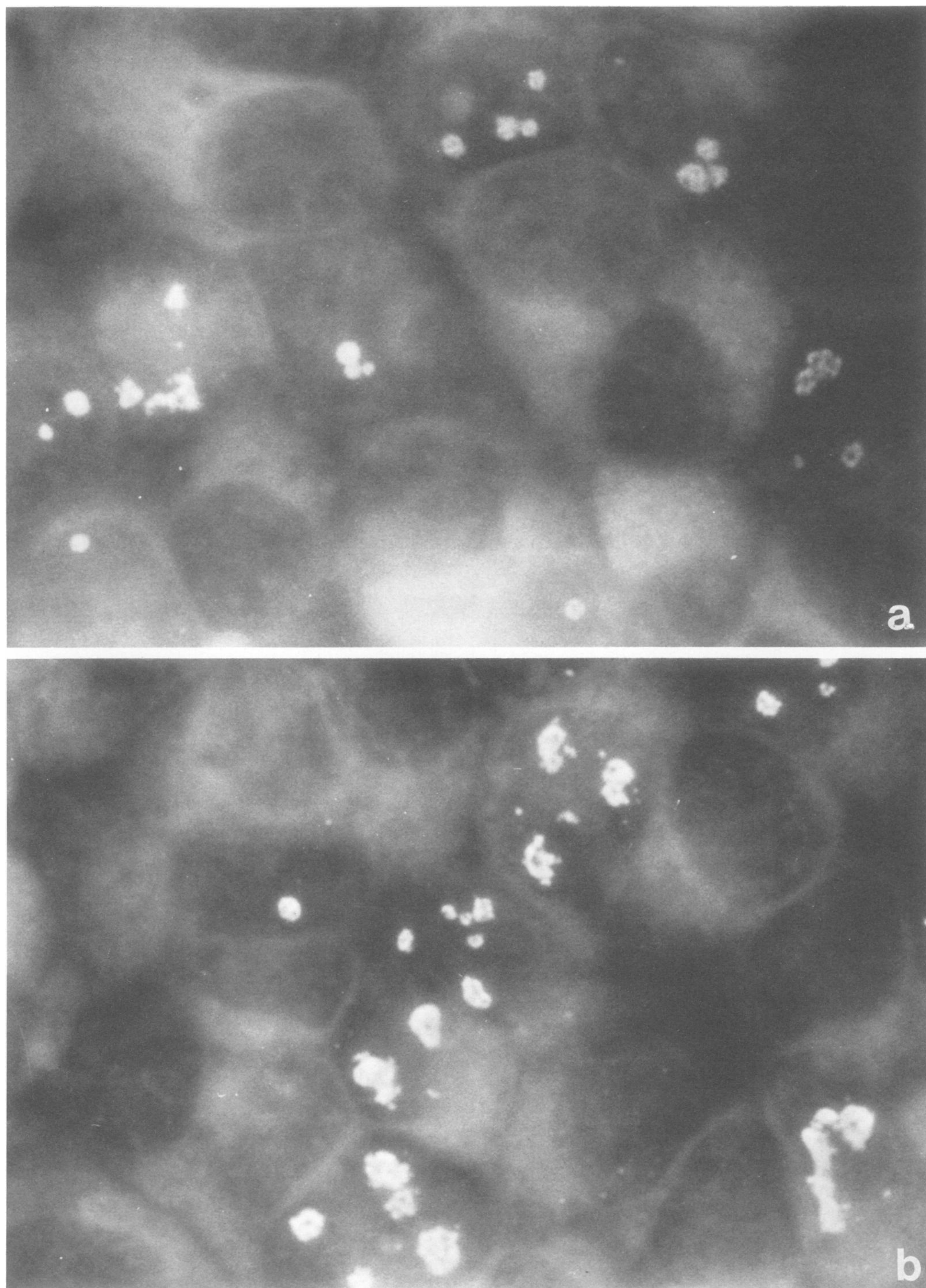


FIG. 3. *Immunofluorescent appearance of adenovirus type 5 DNA-binding protein. Cover slip cultures of KB cells were infected with 0.1 PFU of WT virus per cell and incubated at 32°C for 24 h (a) or 39.5°C for 16 h (b). Indirect immunofluorescence was developed with acetone-fixed cover slips; the initial reaction consisted of a 1:120 dilution of unlabeled rabbit antiserum directed against the adenovirus-specific DNA-binding protein; the final reaction was carried out with a 1:20 dilution of goat anti-rabbit immunoglobulin G conjugated with fluorescein isothiocyanate mixed with rhodamine conjugated to bovine serum albumin. ×309.*

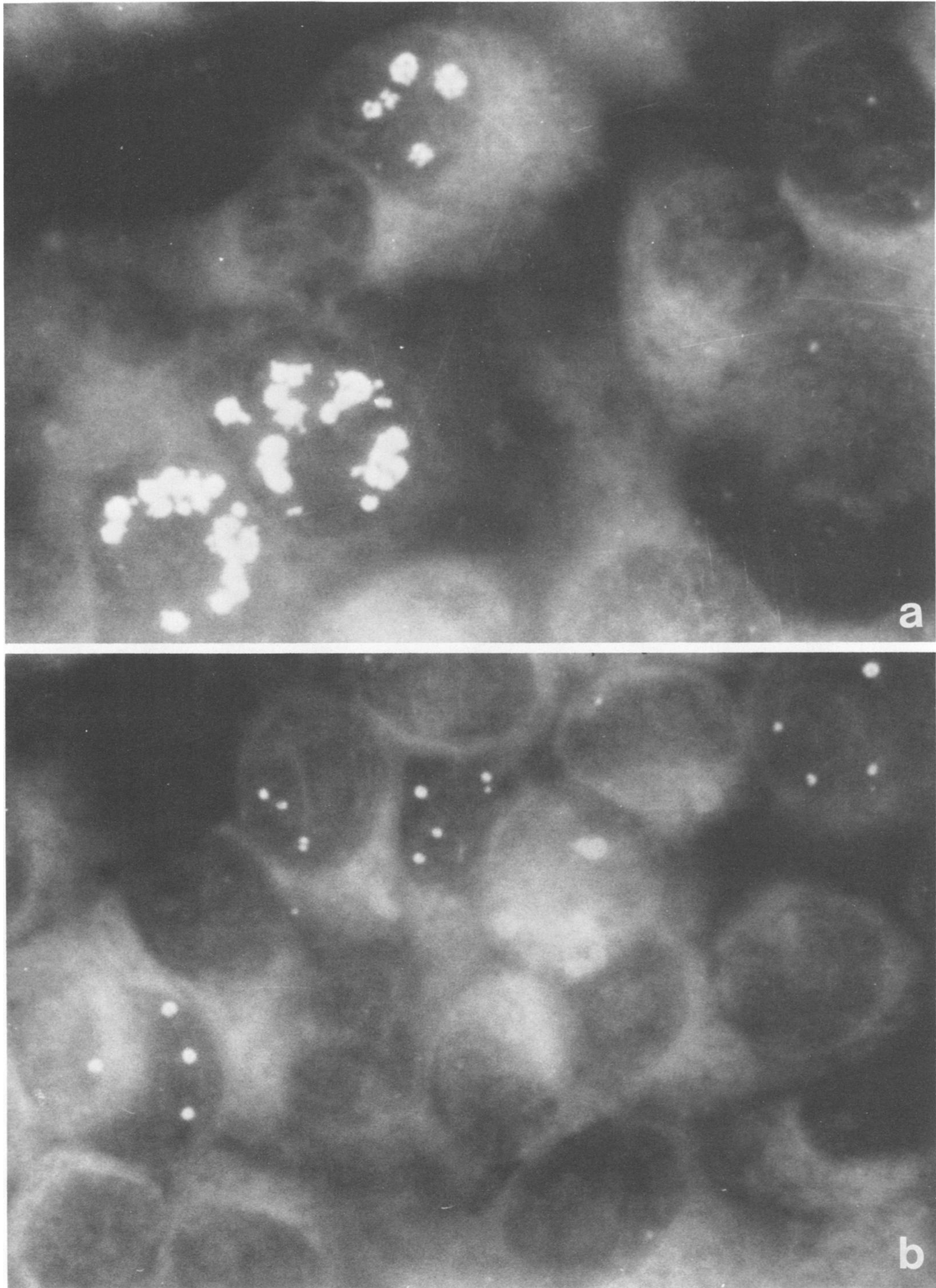


FIG. 4. a & b.

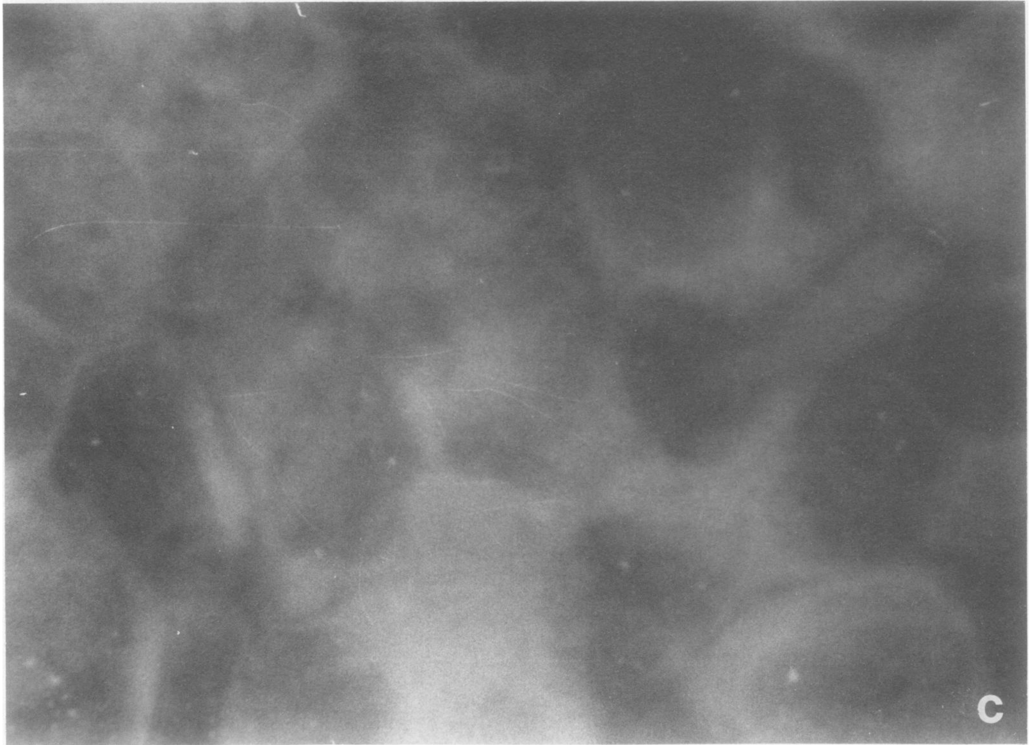


FIG. 4. *Immunological measurement of the adenovirus DNA-binding protein in cells infected with H5ts125 (0.1 PFU per cell) and incubated at 32°C for 24 h (a), 36°C for 18 h (b), or 39.5°C for 16 h (c). The immunofluorescent reaction was carried out as described in the legend to Fig. 3. ×309.*

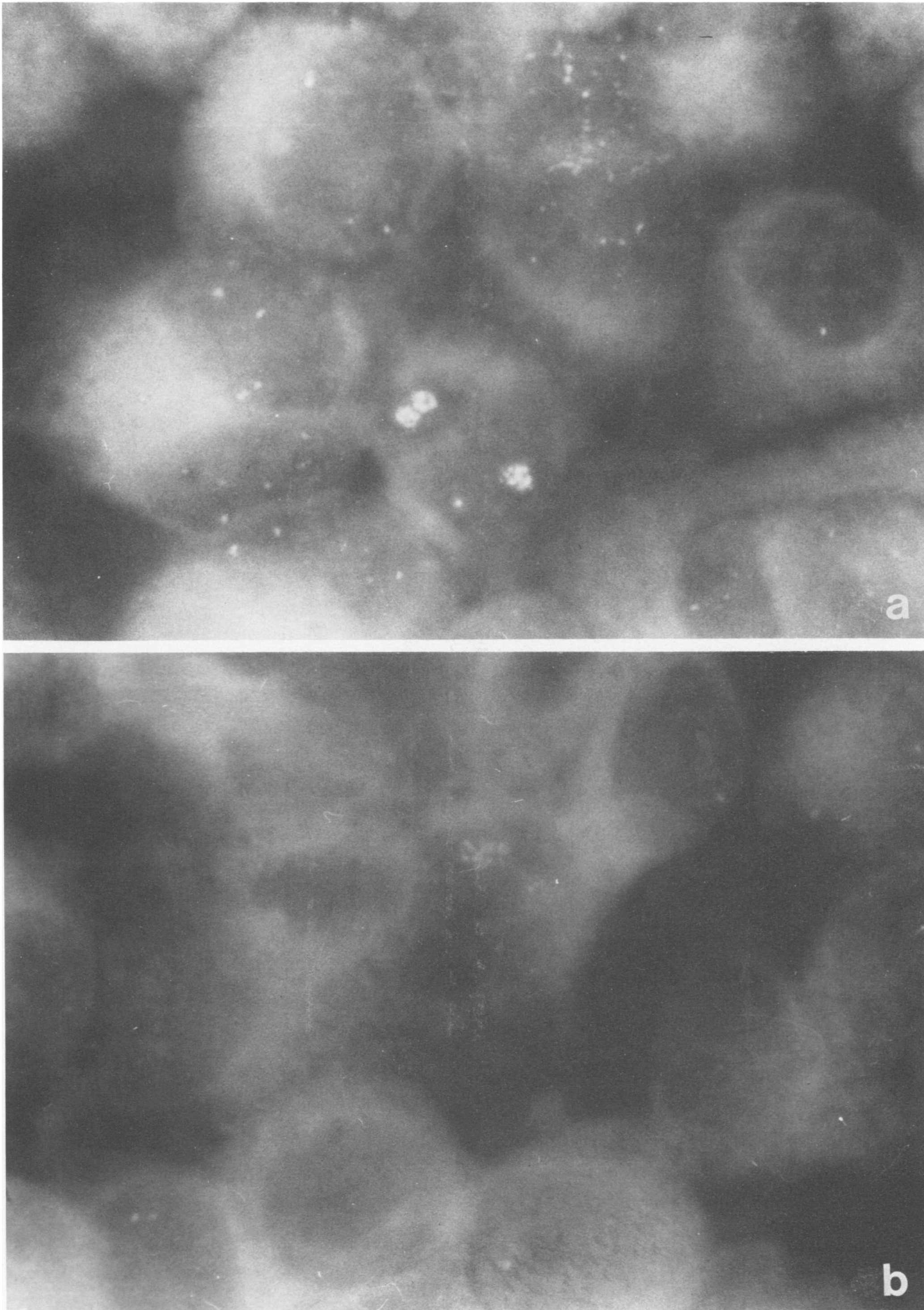


FIG. 5. *Immunological reactivity of the adenovirus DNA-binding protein in H5ts125-infected cells shifted to 39.5°C after an incubation period of 24 h at 32°C: (a) 2 h after change to 39.5°C; (b) 6 h after shift (see legend to Fig. 3 for description of immunofluorescent method employed).* ×309.

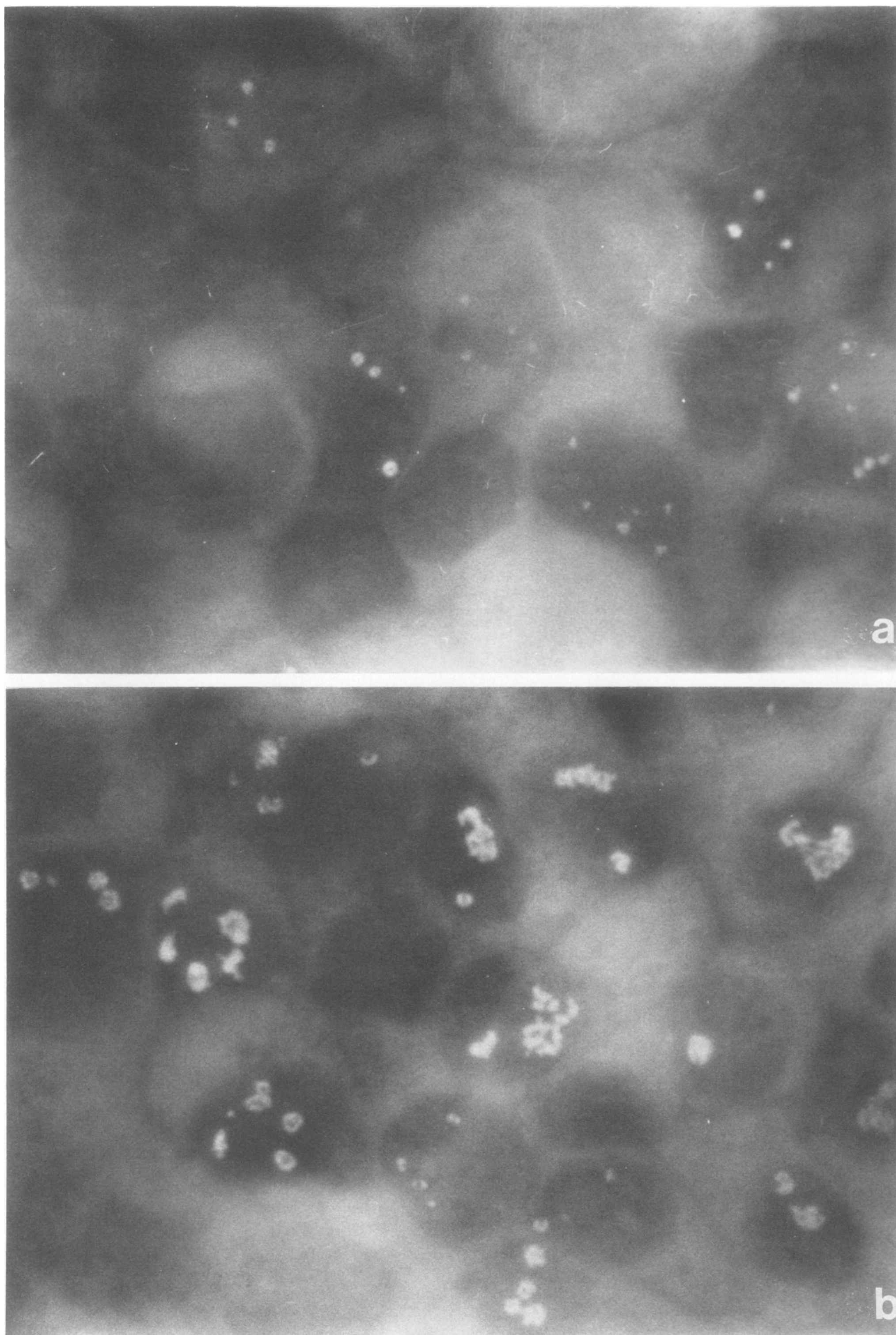


FIG. 6. *Appearance of adenovirus DNA-binding protein after H5ts125-infected cells (0.5 PFU per cell) were changed from incubation at 39.5°C for 16 h to 32°C: (a) 2 h after shift to 32°C; (b) 6 h after shift to 32°C. ×309.*

proceeds unimpeded, as in WT-infected cells, and the 72K protein accumulates; at 39.5°C viral DNA synthesis is blocked and the DNA-binding protein is not immunologically detectable; and when cells are initially incubated at 32°C and shifted to 39.5°C after DNA synthesis has begun, viral DNA replication rapidly declines and the 72K protein slowly becomes immunologically nonreactive (which suggests that the 72K protein loses function more rapidly than immunological reactivity). These data strongly support the findings that the single-strand-specific DNA-binding protein is a nonstructural viral protein essential for the initiation of synthesis, but not for the elongation of the viral DNA strands (9, 19). The actual molecular function of this early viral protein, however, still requires elucidation.

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