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Functional Interaction of Nuclear Transport-Defective Simian Virus 40 Large T Antigen with Chromatin and Nuclear Matrix

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We analyzed the subcellular distribution of nuclear transport-defective simian virus 40 Lys-128-mutant (cT-3 [R. E. Lanford and J. S. Butel, Cell 37:801–813, 1984] and d10 [D. Kalderon, W. D. Richardson, A. F. Markham, and A. E. Smith, Nature (London) 311:33–38, 1984]) large T antigens in various Lys-128-mutant-transformed rodent cells and in Lys-128-mutant d10-infected TC7 cells. Small but significant amounts of the mutant large T antigens were found in association with nuclear substructures, both in mutant-transformed and in mutant-infected cells. Experiments with TC7 cells made incompetent for cell division by ^{60}Co irradiation supported the assumption that Lys-128-mutant large T antigen did not associate with nuclear components during mitosis but most likely was transported into the nucleus because the Lys-128 mutation was leaky for nuclear transport. Low-level simian virus 40 DNA replication and production of infectious mutant virus progeny in TC7 cells indicated that the association of Lys-128-mutant large T antigen with nuclear substructures is functional.

The multitude of tasks performed by the simian virus 40 (SV40) large tumor antigen (large T) in viral replication and in cellular transformation requires its functional interaction with a variety of cellular targets. At the structural level, these targets are associated with different structural systems of the cell, leading to an unusual subcellular distribution of large T in SV40-infected and -transformed cells: approximately 2% of total large T in SV40-transformed cells is specifically associated with the plasma membrane, with about 10% of plasma membrane-associated large T specifically exposed on the cell surface (19, 44). Some further 10% of total large T is associated with intracellular membranes (44), but the vast majority of large T is found in the cell nucleus. Nuclear large T is further subcompartmentalized, as it is found in the nucleoplasm, associated with the chromatin, and tightly bound to the nuclear matrix (41). Although it is reasonable to assume that the various cellular subclasses of large T perform different functions in viral replication and transformation, definite proof for this hypothesis still is lacking. On the other hand, understanding the functions of large T performed in conjunction with distinct cellular structures would help to elucidate the complex pattern of regulatory actions exerted by large T in infected and transformed cells.

Such a picture is beginning to emerge for the nuclear subclasses of large T. Recent publications from our laboratory provided evidence that the association of large T with different structural systems of the nucleus subcompartmentalizes large T subclasses exhibiting different biochemical activities in vitro and exerting different functions in viral infection and in cellular transformation in vivo. In infected cells, nuclear subclasses of large T differ in their DNA-binding and ATPase activities (38), and their expression follows a defined pattern during the course of infection (37). In SV40-transformed cells, the chromatin seems to be an important target for large T in maintaining the transformed phenotype, as chromatin association of large T is rapidly lost in cells of *tsA* N-type (i.e., temperature-sensitive) transformants of rat F111 cells after a shift to the nonpermissive growth temperature. In contrast, this association is preserved in cells of *tsA* A-type (i.e., temperature-insensitive) transformants under the same growth conditions, indicating a stabilization of the mutant large T in these cells in a functionally active conformation (W. Richter and W. Deppert, submitted for publication). Furthermore, retention of chromatin association in cells of *tsA* A-type transformants correlated with the expression of a metabolically stabilized p53 in these cells, suggesting that metabolic stabilization of p53 is one of the functions of chromatin-associated large T in SV40-transformed cells necessary for maintaining the transformed phenotype (7, 9).

Transport of large T into the cell nucleus is mediated by a very efficient nuclear translocation signal. This signal comprises large T amino acid residues 126 or 127 to 132 or 133 and thus consists of the six to eight predominantly basic amino acid residues PKKKRKV (17). This sequence had been identified independently by Lanford and Butel (23) and Kalderon and Smith (18), who found that a nonconservative exchange of amino acid residue Lys-128 resulted in a mutated large T defective in nuclear transport. Such Lys-128-mutant large T had been described as being defective in directing viral replication (17, 23, 24). The mutant large T, however, still was able to transform immortalized mouse and rat cells with wild-type efficiency but was defective in immortalizing primary cells (17, 26). In addition, Lys-128-mutant large T, like wild-type large T, was able to form tumors in transgenic mice (30), suggesting an unrestricted potential of the mutant large T in tumor formation in vivo. Since Lys-128-mutant large T is predominantly found in the cytoplasm of transformed cells and accumulates at elevated levels on the cell surface (22, 36), it has been proposed that nuclear large T is dispensable for maintaining the transformed phenotype and for initiation of tumors in vivo but is required for large T immortalization functions (reviewed in reference 2). This conclusion, however, is strictly dependent on the complete absence of the mutant large T from the nucleus, since retention of the transformed phenotype at the nonpermissive growth temperature in temperature-insensi-

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tive (A-type) *tsA* mutant-transformed cells correlated with only about 15 to 20% of the mutant large T staying associated with the cellular chromatin and the nuclear matrix (Richter and Deppert, in press). So even small amounts of nuclear large T seem to be able to maintain the transformed phenotype. Such small amounts might not have been detected in previous analyses of Lys-128-mutant-transformed cells by immunofluorescence microscopy only.

To reconcile these seemingly contradictory views on the roles of nuclear large T in cellular transformation, two explanations may be envisioned. (i) Since the subcellular location of Lys-128-mutant large T in transformed cells so far has been analyzed by immunofluorescence microscopy of whole cells only, the predominant cytoplasmic fluorescence in these cells might have obscured minor but functionally relevant amounts of mutant large T present in the cell nucleus; (ii) alternatively, it seems equally plausible that Lys-128-mutant large T indeed does not enter the cell nucleus and might use a different route for establishing cellular transformation than wild-type or *tsA* mutant large T.

To analyze these possibilities, we first determined the subcellular location of Lys-128-mutant large T in a variety of transformed cells, using an in situ cell fractionation procedure previously developed in our laboratory (41, 42). We demonstrated that a small but significant portion of the mutant large T can be found in the nucleus and is associated with nuclear substructures. To test the biological relevance of nuclear Lys-128-mutant large T, we analyzed the nuclear location and replicative functions of Lys-128-mutant large T expressed in permissive monkey TC7 cells after infection with Lys-128-mutant virus. We showed that the subcellular location of the mutant large T changes during the course of infection from strictly cytoplasmic at 24 h postinfection (p.i.) to predominantly nuclear at 96 h p.i. This change in subcellular distribution seems to be due to the Lys-128 mutation being leaky for nuclear transport. TC7 cells transfected with SV40 DNA encoding Lys-128-mutant virus replicated SV40 DNA and produced mutant virus, albeit at very low efficiency, indicating that the association of the mutant large T with nuclear substructures is functional.

MATERIALS AND METHODS

Cells and viruses. The following cell lines were used: TC7 cells (35), Cos-1 cells (12), SV40-transformed mouse embryo fibroblasts (MEF/SV-1), MEF transformed by SV40 cT-3 (MEF/cT-3), 3T3 cells transformed by SV40 cT-3 (3T3/cT-3), and rat-1 cells transformed by Lys-128-mutant ×12 (Px12 cells). MEF/SV-1 cells, MEF/cT-3 cells, and 3T3/cT-3 cells (26) were kindly provided by R. E. Lanford. Px12 cells (17) were kindly provided by A. E. Smith. The cells were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. SV40 strain 776 was propagated in TC7 cells. SV40 Lys-128-mutant virus was obtained by transfection of plasmid pXd10 (see below) into Cos-1 cells and freeze-thawing 5 to 8 days posttransfection.

Plasmids. Plasmids were cloned in *Escherichia coli* DH-1 and purified by CsCl density centrifugation, followed by sucrose density centrifugation. Plasmid pSV was kindly provided by E. Fanning (10). Plasmid pXd10, which contains SV40 Lys-128-mutant virus (Lys-128 changed to Thr) was obtained by inserting a *Pvu*II fragment of plasmid pd10 (kindly provided by A. E. Smith [17]) containing the Lys-128 mutation into plasmid pSVX-1 (*Bam*HI-restricted SV40 wild-type virus cloned into the *Bam*HI site of plasmid pXF3 [27]).

Transfection. TC7 cells (10^6) or Cos-1 cells (10^6) were transfected with 5 μg of purified *Bam*HI-digested plasmid DNA by electroporation (300 to 350 V/25 μFd) with a Bio-Rad Gene Pulser.

Labeling of cells. Cells were labeled with [^{35}S]methionine (100 μCi per plate per ml) before fractionation as described previously (41).

Irradiation of cells. TC7 cells were grown to 30% confluence on 5-cm tissue culture dishes and then were irradiated with a ^{60}Co radiator for 9.5 min (1,800 rem). At 24 h after this treatment, tissue culture medium was changed to remove dead cells (about 5%). The remaining cells stopped dividing around 36 h after irradiation at 80% confluence, and no further mitosis and cell growth were observed. Protein synthesis of irradiated cells was not significantly altered, whereas DNA synthesis was slightly increased (data not shown). Cells treated this way remained attached to the substratum for 6 to 8 days at 80% confluence and could be used for transfections and infections. After infection with SV40 wild-type virus at 48 h postirradiation, irradiated cells were able to produce infectious virus progeny in regular amounts (data not shown).

In situ cell fractionation. A detailed description of the cell fractionation procedure as well as the characterization of extracts and structures has been given elsewhere (42). Conditions of extraction were slightly modified (14, 37). Briefly, monolayer cultures of TC7 cells were washed with KM buffer (pH 6.8; 10 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 5 mM dithiothreitol, 10% glycerol, 10 mM morpholinepropanesulfonic acid [MOPS]) and were lysed for 30 min at 2°C in KM buffer containing 1% Nonidet P-40 (NP-40) and 20% immunoglobulin-free fetal calf serum (NP-40 extract). Nuclear structures still attached to the substratum were incubated with 100 μg of DNase I (30 min, 32°C) and then with KM buffer adjusted to 2 M NaCl (30 min, 2°C) (DNase–high-salt extract). Finally, the residual protein skeleton was solubilized in a buffer containing 1% Empigen BB (60 min, 2°C) (Empigen BB extract).

Immunoprecipitation. All extracts were immediately adjusted to pH 9.0, 200 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, and 1% NP-40, were cleared by centrifugation (20,000 × *g*, 30 min, 2°C), and were immunoprecipitated for large T with 10 μl of ascites fluid of monoclonal antibody PAb 108 (13) and 200 μl of settled protein A-Sepharose (4 h, 2°C). Immune complexes were washed extensively, and large T was eluted from protein A-Sepharose and then subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis.

SDS-polyacrylamide gel electrophoresis and Western immunoblotting. Viral proteins were analyzed by gel electrophoresis on SDS-10% polyacrylamide slab gels by the system of Laemmli (20).

Western blotting (1) of large T was performed with rabbit anti-T serum and [^{3}H]protein A as described previously (14, 37).

***Dpn*I DNA replication assay.** TC7 cells were transfected with *Bam*HI-digested plasmid DNA as described above, and viral DNA was extracted by the method of Hirt (15) at different times posttransfection. To differentiate DNAs replicated in TC7 cells from bacterial input DNAs, one-half of the Hirt extracts was digested with *Dpn*I, whereas the other half remained untreated. Adenine residues of DNAs cloned in *E. coli* DH-1 are methylated at the cleavage site of restriction enzyme *Dpn*I and therefore will be recognized by *Dpn*I. When there is no methylated recognition site, as in DNAs of eucaryotic origin, no *Dpn*I cleavage occurs. The

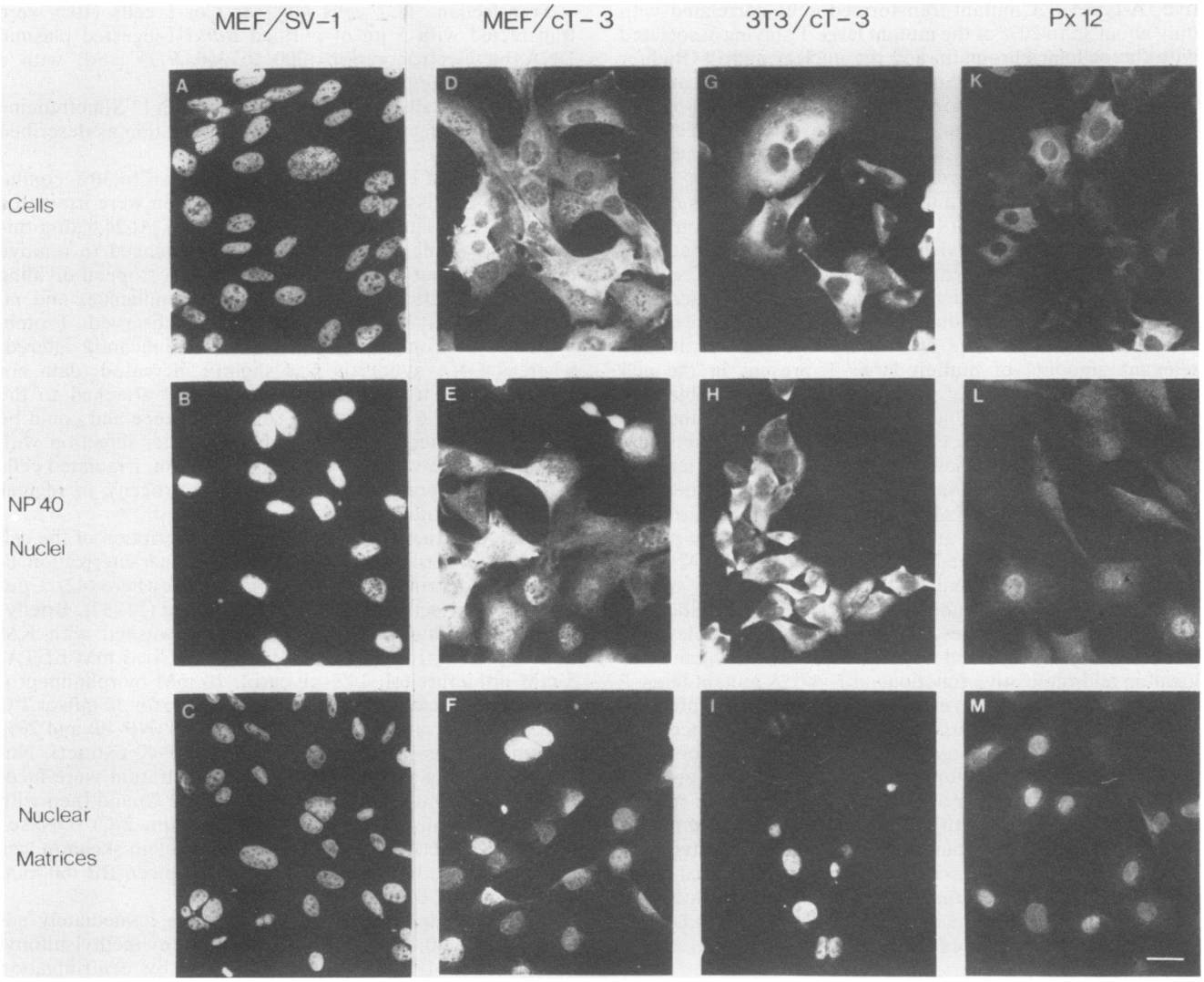

FIG. 1. Analysis of subcellular locations of large T in SV40 wild type (MEF/SV-1)- and in SV40 Lys-128-mutant (MEF/cT-3, 3T3/cT-3, and Px12)-transformed cells. Cells grown on cover slips were subfractionated in situ and analyzed by immunofluorescence microscopy for large T as described in Materials and Methods. Row 1, Large T fluorescence of unfractionated cells (Cells); row 2, cells after NP-40 extraction (NP40 Nuclei); row 3, cellular structures after DNase–high-salt extraction (Nuclear Matrices). Bar represents 15 μm.

two aliquots then were analyzed in parallel by agarose gel electrophoresis, followed by Southern analysis (39) after transfer onto Gene Screen Plus hybridization transfer membrane and hybridization with *Bam*HI-digested ^{32}P-end-labeled (27) SV40 DNA. This way, input DNA grown in bacteria could be distinguished from viral DNA replicated in the cells.

Immunofluorescence microscopy. Cells grown on cover slips (12 mm) were subfractionated as described above. Cells and nuclear structures were fixed in methanol and acetone, and immunofluorescence analysis was performed as described previously (31, 41). Fluorescence was viewed with a Zeiss photomicroscope 2.

RESULTS

Association of Lys-128-mutant large T with nuclear substructures. To determine the exact subcellular location of Lys-128-mutant large T in Lys-128-mutant-transformed cells, we analyzed cell lines MEF/cT-3 and 3T3/cT-3 (MEF and mouse 3T3 cells transformed by the Lys-128-mutant cT-3 [26]) and Px12 (rat-1 cells transformed by the Lys-128-mutant x12 [17]) by an in situ cell fractionation procedure previously developed and characterized in our laboratory (41, 42). This method provides the advantage that cellular structures remain attached to the substratum during all steps of preparation, preventing collapse of individual structural systems on top of each other. Therefore, cytoplasmic areas of the cells remain separated from nuclear areas and can still be visualized after complete extraction of the cells (42).

All Lys-128-mutant-transformed cells exhibited a bright cytoplasmic fluorescence before extraction, whereas a strictly nuclear fluorescence was seen in SV40 wild type-transformed control cells (MEF transformed with SV40 wild-type DNA [26]) (Fig. 1). Close analysis, however, revealed that at least in MEF/cT-3 cells some nuclear

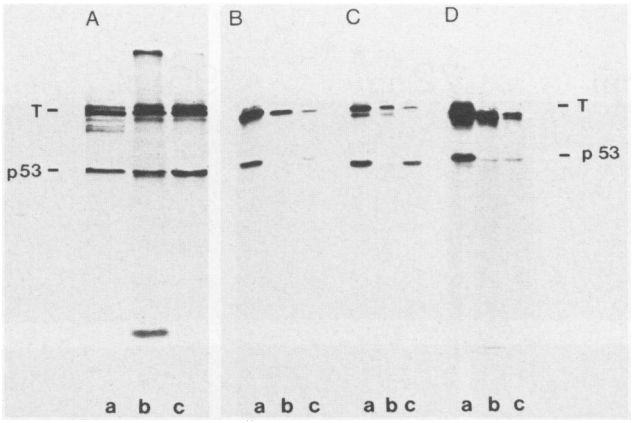

FIG. 2. Subcellular distribution of SV40 wild-type large T and SV40 Lys-128-mutant large T in subcellular fractions of transformed cells. MEF/SV-1 cells (A), MEF/cT-3 cells (B), 3T3/cT-3 cells (C), and Px12 cells (D) were labeled for 2 h with [^{35}S]methionine and subfractionated in situ. Extracts were immunoprecipitated with anti-SDS T serum, and immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis followed by fluorography. Lanes: a, NP-40 extracts; b, DNase–high-salt extracts; c, Empigen BB extracts.

staining was visible. Extraction of NP-40-soluble cytoplasmic, nucleoplasmic, and membrane constituents in the first extraction step (41, 42) did not grossly alter the fluorescence pattern in either SV40 wild type- or Lys-128-mutant-transformed cells, indicating that the cytoplasmic large T in Lys-128-mutant-transformed cells is bound to some cytoplasmic structure in the cells. However, in all mutant-transformed cells, nuclear staining was clearly visible. Since it previously was shown that this first extraction step quantitatively solubilizes nucleoplasmic large T (41), the nuclear fluorescence observed in NP-40-treated nuclei already indicated that some Lys-128-mutant large T in these cells was associated with structural systems of the nucleus. This became especially evident after DNase–high-salt treatment of the NP-40-treated nuclei, when a bright nuclear fluorescence was observed in wild type- as well as in all mutant-transformed cells, reflecting the association of large T with the nuclear matrix.

The association of Lys-128-mutant large T with structural systems of the nucleus was further confirmed by biochemical analysis of nuclear subfractions of [^{35}S]methionine-labeled cells. The DNase–high-salt fraction (Fig. 2, lanes b) as well as the nuclear matrix fraction (Fig. 2, lanes c) of all mutant-transformed cells contained large T, although considerably less than those fractions from SV40 wild type-transformed cells.

Association of Lys-128-mutant large T with nuclear substructures of infected cells. The data provided so far demonstrated that Lys-128-mutant large T can be found in association with nuclear substructures of cells transformed by this mutant. However, they do not indicate whether this large T is functional or explain how the mutant large T was able to reach the nucleus. Analysis of these questions is difficult, if not impossible, in mutant-transformed cells, since no easily testable function is known for large T in association with nuclear substructures of transformed cells. Furthermore, even the presence of nuclear Lys-128-mutant large T in transformed cells does not imply that the mutant protein as such has the ability to migrate into the nucleus, since transformation by SV40 in general is very inefficient and depends on a strong selection process (34). One therefore might also envision that the nuclear location of Lys-128-mutant large T in transformed cells is the result of such a selection process, as had already been suggested for the generation of Lys-128-mutant-transformed cells from MEF (26).

To circumvent these difficulties, we analyzed the subcellular distribution of Lys-128-mutant large T expressed in monkey TC7 cells after infection with SV40 Lys-128-mutant virus. This system offers the advantage that the subcellular distribution of the mutant large T can be observed during the course of infection, avoiding cellular selection processes. Furthermore, testable functions of nuclear subclasses are defined either by their ability to replicate SV40 DNA in vivo or by exhibiting different biochemical activities in vitro (for reviews, see references 6 and 40).

TC7 cells were infected with SV40 Lys-128-mutant virus grown in Cos-1 cells (see Materials and Methods) at a multiplicity of infection of 1. The subcellular location of the mutant large T during the course of infection was analyzed by in situ cell fractionation, followed by immunofluorescence microscopy analysis of the structures prepared, as well as by immunoprecipitation of large T from the respective subcellular fractions and evaluation by Western blotting. Before these experiments, the SV40 Lys-128-mutant virus stocks were tested for possible contamination with wild-type virus which might have been generated by recombination with the SV40 DNA integrated in Cos-1 cells. With all stocks used in these experiments, however, less than 0.1% of the infected cells exhibited a nuclear T-antigen fluorescence indicating the presence of wild-type virus.

Figure 3 shows the result of the immunofluorescence analysis of TC7 cells infected with SV40 Lys-128-mutant virus 24, 48, 72, and 96 h p.i. At 24 h p.i., Lys-128-mutant large T was strictly found in cytoplasmic areas of the infected cells and even after cell fractionation was not detectable at nuclear structures. At 48 h p.i., mutant large T still was found predominantly in the cytoplasm, but, after subfractionation, could also be detected in association with nuclear substructures. Association of the mutant large T with nuclear substructures became more prominent at 72 h p.i. At 96 h p.i., the apparent subcellular distribution of the mutant large T was drastically different from that at the beginning of the infection, since now most cells exhibited a nuclear fluorescence. This change in subcellular distribution of the mutant large T was quantitatively evaluated by Western blotting of large T immunoprecipitates from the various subcellular fractions obtained after in situ cell fractionation of SV40 Lys-128-mutant virus-infected TC7 cells 24, 48, 72, and 96 h p.i. Infections with wild-type virus were analyzed in parallel. For comparison, Fig. 4A first demonstrates the subnuclear distribution of wild-type large T in infected cells, with its prominent shift between early (24 h) and late (48 h) times p.i. (37). Analysis of cellular extracts of SV40 Lys-128-mutant-infected cells (Fig. 4B) confirmed that at 24 h p.i. the mutant large T was not associated with the structural system of the nucleus to any significant extent but could be recovered almost quantitatively from the NP-40 extract. At 48 h p.i., a small fraction of the mutant large T was found also in the DNase–high-salt extract as well as in the nuclear matrix fraction. At around 72 h p.i., the total amount of mutant large T in the infected cells had reached its steady-state level. Nevertheless, the proportion of large T present in the DNase–high-salt fraction and, at 96 h p.i., particularly in the nuclear matrix fraction had still increased. Thus, these data indicate that Lys-128-mutant large T is able

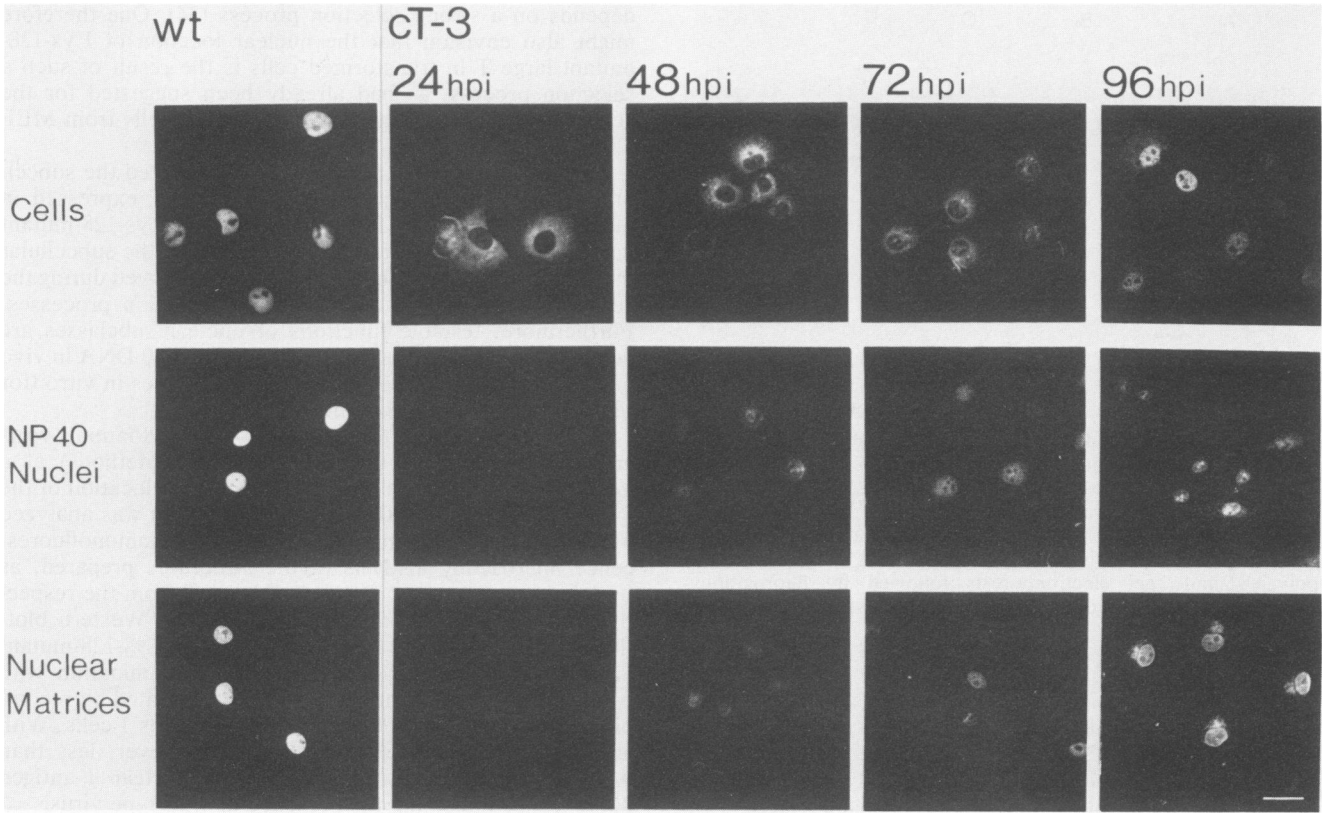

FIG. 3. Immunofluorescence analysis of subcellular locations of large T in SV40 Lys-128-mutant virus-infected TC7 cells during the course of infection. TC7 cells were infected with SV40 Lys-128-mutant virus (multiplicity of infection of 1), subfractionated in situ at 24, 48, 72, and 96 h p.i., and analyzed by immunofluorescence microscopy. SV40 wild type (wt)-infected TC7 cells were analyzed in parallel 24 h p.i. Bar represents 15 μm.

to enter the cell nucleus and then is subcompartmentalized to its functional in vivo locations.

Lys-128 mutation is leaky for nuclear transport. The results presented above raise the question of how Lys-128-mutant large T might be able to reach the cell nucleus. Two possibilities might be envisioned. (i) We previously identified a putative nuclear matrix association domain on large T contained within amino acid residues 320 to 550 (8). Since TC7 cells infected with SV40 Lys-128-mutant virus still grow and divide in a similar fashion as uninfected cells (data not shown), it is possible that the mutant protein associates with nuclear matrix components during mitosis, i.e., at a time when the nuclear envelope is disintegrated, and then stays associated with these components when the nucleus rebuilds. (ii) Alternatively, it is also conceivable that the Lys-128 mutation is somewhat leaky, allowing for a limited transport of the mutated protein into the cell nucleus.

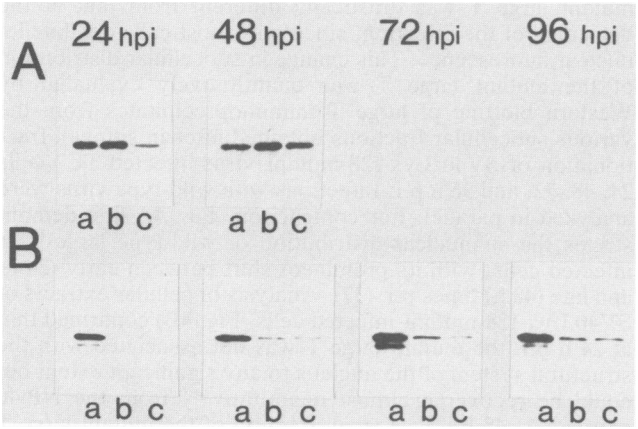

FIG. 4. Subcellular distribution of large T in SV40 wild-type- and SV40 Lys-128-mutant virus-infected TC7 cells during the course of infection. Cells were infected with either SV40 wild-type (A) or SV40 Lys-128-mutant (B) virus (multiplicity of infection of 1) and subfractionated at 24, 48, 72, and 96 h p.i. Large T was immunoprecipitated, analyzed by SDS-polyacrylamide gel electrophoresis and quantitatively evaluated by Western blotting. Lanes: a, NP-40 extracts; b, DNase–high-salt extracts; c, Empigen BB extracts.

To discriminate between these alternatives, we analyzed nuclear transport of Lys-128-mutant large T into TC7 cells which had been ^{60}Co irradiated to abolish their ability to divide before infection with SV40 Lys-128-mutant virus (see Materials and Methods for details). Such treatment should prevent the association of the mutant large T with structural components of the nucleus during mitosis. ^{60}Co-treated cells stopped dividing around 36 h after irradiation but remained viable for approximately 6 to 8 more days. In addition, irradiated cells were able to produce infectious viral progeny upon infection with SV40 wild-type virus (data not shown). Untreated TC7 cells infected with SV40 Lys-128-mutant virus were analyzed in parallel. Also in this experiment, analysis of the subnuclear distribution of wild-type large T in untreated TC7 cells (Fig. 5, panels 1) and in ^{60}Co-irradiated TC7 cells (Fig. 5, panels 2) served as a control. The subnuclear distribution of wild-type large T during the course of infection was not at all affected by ^{60}Co treatment

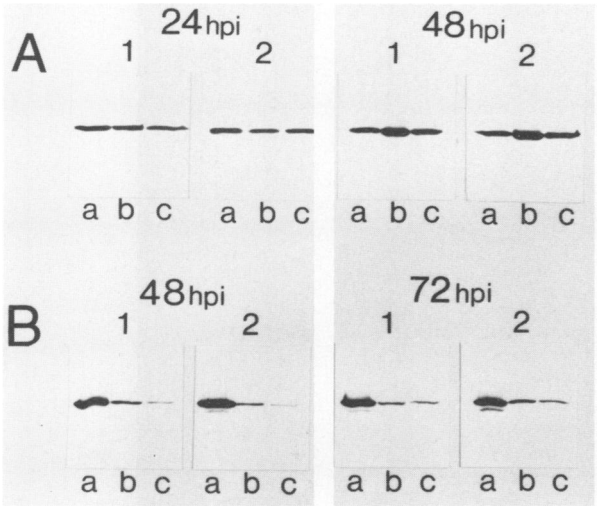

FIG. 5. Subcellular distribution of large T in SV40 wild-type (A) and SV40 Lys-128-mutant (B) virus-infected TC7 cells comparing ^{60}Co-irradiated with nonirradiated cells. ^{60}Co-irradiated and nonirradiated TC7 cells were infected with SV40 wild type or SV40 Lys-128-mutant (multiplicity of infection of 1). The cells were subfractionated at different times p.i., and large T was immunoprecipitated and evaluated by Western blotting. Panels 1, Nonirradiated cells; panels 2, ^{60}Co-irradiated cells. Lanes: a, NP-40 extracts; b, DNase–high-salt extracts; c, Empigen BB extracts.

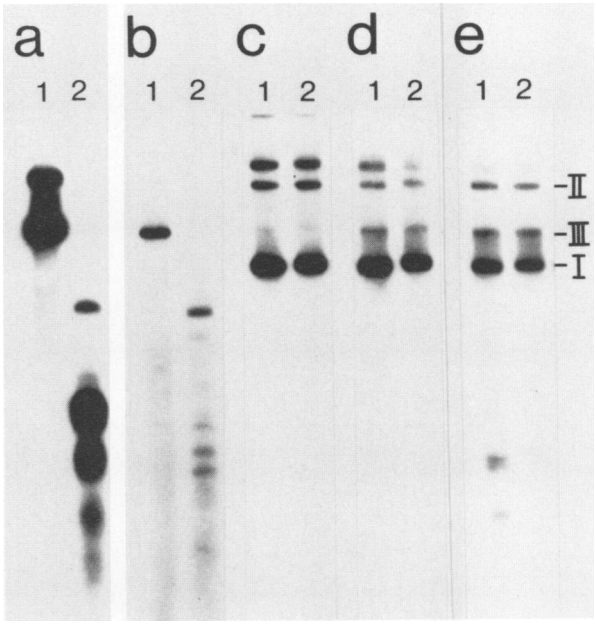

FIG. 6. Southern blot analysis of methylation-sensitive DNA replication assay. TC7 cells were transfected with *Bam*HI-digested plasmids containing SV40 wild-type DNA (pSV) or SV40 Lys-128-mutant DNA (pXd10) by electroporation. Viral DNA was extracted by the method of Hirt (15). To differentiate input DNA from DNA replicated in the cells, one-half of the extracts were *Dpn*I digested, whereas the other half remained untreated. *Dpn*I cleavage sites are methylated in bacterially cloned DNAs and therefore are recognized by the restriction enzyme, whereas DNA of eucaryotic origin is not methylated at this recognition site and remains uncleaved. Undigested Hirt DNAs (lanes 1) and *Dpn*I-digested Hirt DNAs (lanes 2) were analyzed in parallel by agarose gel electrophoresis and Southern blotting. Input DNA of the transfection was analyzed in parallel (a). pSV (wild type)-transfected cells were Hirt extracted 24 h (b), 48 h (c), and 72 h (d) posttransfection. pXd10 (Lys-128- mutant)-transfected cells were extracted 6 days posttransfection (e).

of TC7 cells before infection (Fig. 5A). Similarly, some Lys-128-mutant large T reached the nucleus and was subcompartmentalized during the course of infection, regardless of whether the cells were competent for cell division or not (Fig. 5B). Therefore, we conclude that Lys-128-mutant large T can reach the nucleus by itself, most likely because of some leakiness of the nuclear transport system.

Lys-128-mutant large T is able to direct viral replication in vivo. Our finding that Lys-128-mutant large T can reach the cell nucleus and then is subcompartmentalized already pointed to the possibility that the nuclear fraction of the mutant large T is functionally active in vivo. Since Lys-128-mutant large T exhibits no obvious biochemical defects in vitro, as its possesses DNA-binding as well as ATPase activity (24; unpublished observation), we asked whether Lys-128-mutant large T might still be able to direct viral replication in vivo. This question had already been addressed in previous studies, but viral progeny had not been observed in these analyses (4, 24). However, cells infected with mutant virus had been harvested in parallel with wild-type virus-infected cells. Since our time course for nuclear transport of mutant large T in SV40 Lys-128-mutant virus-infected TC7 cells had shown that mutant large T is transported to the cell nucleus at a very slow rate, it is possible that viral replication had not yet reached a detectable level in mutant virus-infected cells at a time when viral replication already was finished in wild-type virus-infected cells. Two questions were analyzed: first, can Lys-128-mutant large T direct viral DNA replication, and second, does infection of TC7 cells with SV40 Lys-128-mutant virus yield infectious viral progeny?

To analyze the first question, we transfected TC7 cells with cloned and highly purified plasmid DNA by electroporation, with the inserted SV40 Lys-128-mutant DNA excised from the vector with the restriction endonuclease *Bam*HI (for details, see Materials and Methods). Transfections with vectors containing SV40 wild-type DNA were performed in parallel. Since the plasmid DNA had been methylated during growth in bacteria, input vector as well as insert DNA (SV40 wild-type and SV40 Lys-128-mutant DNA, respectively) should be sensitive to digestion with the methylation-specific restriction endonuclease *Dpn*I, whereas viral progeny DNA replicated in TC7 cells should be *Dpn*I insensitive. Viral DNA from SV40 wild-type DNA-transfected cells was harvested 24 h (Fig. 6b), 48 h (Fig. 6c), and 72 h (Fig. 6d) after transfection, and viral DNA from SV40 Lys-128-mutant DNA-transfected cells (Fig. 6e) was harvested 6 days after transfection by the method of Hirt (15). The DNA was then purified. One-half of the DNA remained untreated (Fig. 6, lanes 1), whereas the other half was cut with the appropriate restriction enzyme (*Dpn*I; Fig. 6, lanes 2), and both DNAs were further analyzed by gel electrophoresis followed by Southern analysis (39). Bacterial input DNA was *Dpn*I sensitive, yielding the expected band pattern (Fig. 6a, lane 2). Input SV40 DNA harvested 24 h after transfection still was recovered mainly as linearized form III DNA, indicating that replication had not yet occurred (Fig. 6b, lane 1). In accordance, this DNA was *Dpn*I sensitive (Fig. 6b, lane 2). SV40 DNA extracted both from wild-type (Fig. 6c and d) and from mutant (Fig. 6e) DNA-transfected cells was recovered

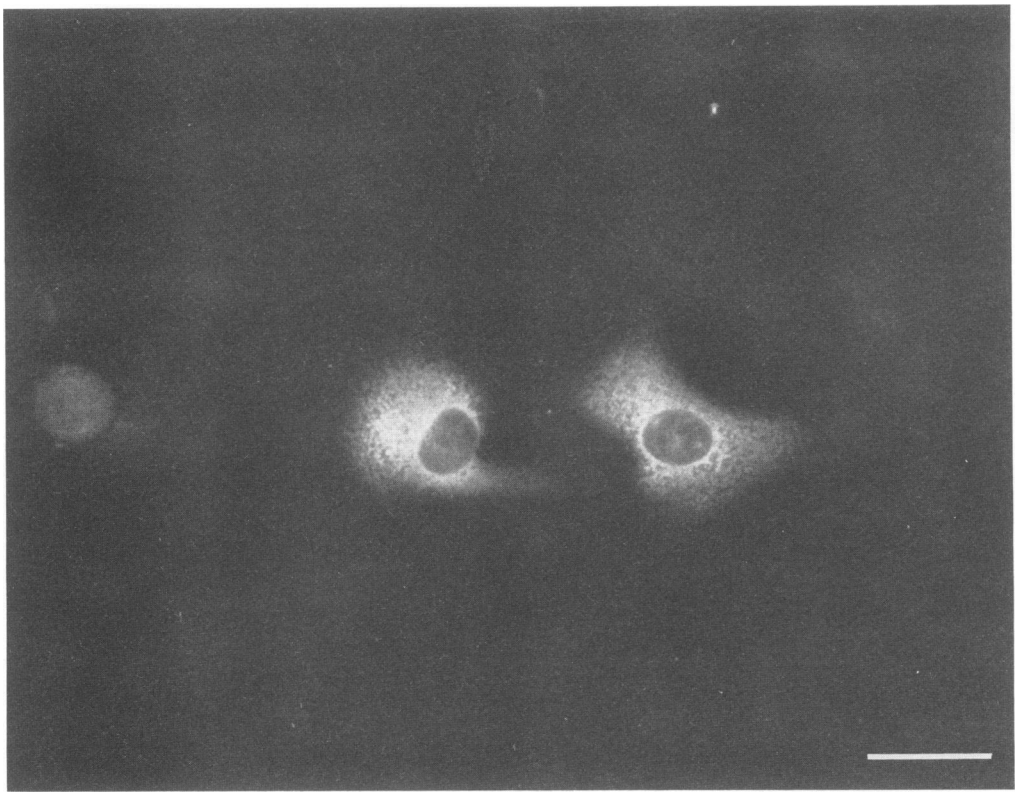

FIG. 7. Immunofluorescence analysis of TC7 cells infected with SV40 Lys-128-mutant virus replicated in TC7 cells. TC7 cells were transfected with SV40 Lys-128-mutant DNA (*Bam*HI-restricted pXd10) by electroporation. Virions were harvested 10 days posttransfection by freeze-thawing, and fresh TC7 cells were infected with the cleared cellular lysate. Infected cells were scored for large T immunofluorescence at 48 h p.i. Bar represents 15 μm.

mainly as SV40 form I DNA, already indicating that viral DNA replication had also occurred in mutant DNA-transfected cells. This conclusion was further confirmed by demonstrating that SV40 DNA extracted from Lys-128-mutant DNA-transfected TC7 cells, like SV40 DNA in SV40 wild-type DNA-transfected cells, had become *Dpn*I insensitive after in vivo replication (Fig. 6e, lane 2).

We next analyzed whether infection of TC7 cells with SV40 Lys-128-mutant virus yielded infectious viral progeny. TC7 cells were transfected with purified plasmid DNA encoding complete SV40 Lys-128-mutant virus as described above. Virus was harvested by freeze-thawing 10 days after transfection (for details, see Materials and Methods). The cleared cellular lysates then were used to infect fresh TC7 cells. Infected cells were scored for large T immunofluorescence at 48 h p.i. Figure 7 demonstrates the typical cytoplasmic appearance of Lys-128-mutant large T observed in about 0.1 to 0.2% of the infected cells, providing evidence that TC7 cells transfected with Lys-128-mutant DNA had produced infectious viral particles, albeit with very low efficiency.

DISCUSSION

SV40 large T is a multifunctional protein required for regulation of viral replication in permissive monkey cells as well as for cellular transformation of primary and of established nonpermissive cells from a variety of species (reviewed in references 2, 32, and 43). However, aside from an ATPase-dependent helicase activity and from its ability to interact with regulatory sequences on the SV40 origin of replication in a sequence-specific manner, no further biochemical activities intrinsic to large T have been described so far (reviewed in reference 40). Therefore, many functions attributed to large T must be the result of regulatory interactions of large T with viral and cellular targets. In this regard, large T can influence gene expression by transactivating both viral and cellular sequences (16, 45). In addition, large T might directly modulate functions of cellular regulatory proteins, as it forms tight complexes with the cellular proliferation protein p53 (21, 28) and the gene product of the retinoblastoma gene (5). At yet another level of regulation, large T interacts with various cellular structures, including different structural systems of the nucleus, and with the plasma membrane (reviewed in reference 2).

Whereas molecular functions of large T in lytic infection are understood to some detail (6), very little is known about the biological function(s) of the various cellular subclasses of large T in SV40-induced cellular transformation. In the absence of any testable biochemical function in cellular transformation, one of the best tools to analyze large T functions in this process still is the analysis of large T mutants. Using a matched pair of SV40 *tsA*58 mutant-transformed cells, we previously were able to demonstrate that the cellular chromatin is an important target for large T in maintaining the transformed phenotype (9, 14; Richter and Deppert, in press). The involvement of nuclear large T in the maintenance of cellular transformation, however, contrasts conclusions made following the discovery of nuclear transport-defective Lys-128-mutant large T. This mutant large T

still has the ability to transform established cells with the efficiency of wild-type large T (26) and to form tumors in transgenic mice (30). Since Lys-128-mutant large T-transformed cells overexpress cell surface T (22, 36), it was concluded that nuclear large T is not involved in the maintenance of cellular transformation but rather that this is a function of the cell surface-associated subclass of large T (2).

Since it is a conceptually important question whether maintenance of cellular transformation requires nuclear large T or whether this process can be performed by cell surface T alone, we determined in this study whether small but significant amounts of Lys-128-mutant large T might be able to functionally interact with structural systems of the nucleus. We showed by biochemical cell fractionation that Lys-128-mutant large T expressed in transformed as well as infected cells associates with both the chromatin and the nuclear matrix in functionally significant quantities. The presence of mutant large T in the nuclei of transformed and infected cells most likely is due to the fact that the Lys-128 mutation does not completely abolish nuclear transport. Leakiness of the Lys-128 mutation for nuclear transport first was supported by our finding that Lys-128-mutant large T accumulated at nuclear substructures during the course of infection and then was confirmed by our observation that the subcellular distribution of large T in division-competent and division-incompetent cells infected with either SV40 Lys-128-mutant or SV40 wild-type virus was identical, thus excluding the possibility of a redistribution of large T during mitosis. That a low level of nuclear transport of Lys-128-mutant large T can occur in mutant-infected or -transformed cells is also favored by recent investigations demonstrating that in addition to the large T nuclear transport signal, other factors might influence nuclear transport (3, 11, 25, 29, 33, 46). An alternative interpretation of our experiments, namely, that the small amounts of mutant large T found in DNase–high-salt and Empigen BB extracts might result from an artificial redistribution during cell fractionation, can be excluded. First, Lys-128-mutant large T is strictly cytoplasmic at early times after infection of TC7 cells with Lys-128-mutant virus (Fig. 4). Second, analysis of the subnuclear distribution of large T in cells transformed with a temperature-sensitive SV40 mutant, *tsA58*, demonstrated that the association of the mutant large T with the cellular chromatin and the nuclear matrix in these cells strictly correlated with the phenotype of these cells and was no longer detectable in cells reverting to a normal phenotype at the nonpermissive growth temperature (9, 14).

In contrast to previous reports (4, 24), we were able to demonstrate also that a Lys-128-mutant large T with a nonconservative amino acid exchange (Lys-128 changed to Thr) is functionally active in lytic infections, since TC7 cells transfected with plasmid DNA containing the complete SV40 d10 virus genome not only were able to replicate the mutant genome but also produced infectious virus. The validity of this conclusion, however, strictly depends on the absence of any wild-type large T in these infections, which otherwise might provide a helper function for the Lys-128-mutant large T. This possibility, however, is extremely unlikely, since replicative functions of the mutant large T were analyzed in TC7 cells transfected with purified plasmid DNA encoding Lys-128-mutant large T. Furthermore, the small amount of mutant virus produced in these infections upon reinfection of TC7 cells generated a strictly cytoplasmic large T fluorescence pattern, characteristic for Lys-128-mutant large T. In contrast, any wild-type large T present in these infections would have led to the production of wild-type virus, which would have been easily detected by its strictly nuclear large T fluorescence.

Our finding that Lys-128-mutant large T is replication competent provides the most stringent evidence that its association with nuclear substructures as detected after biochemical cell fractionation is functional in vivo. Therefore, it seems safe to conclude that Lys-128-mutant large T associated with nuclear substructures in transformed cells is also able to exert a transformation-relevant nuclear function(s). This conclusion, however, does not rule out the possibility that in addition to nuclear large T, cell surface T also plays an important role in maintaining SV40-mediated cellular transformation. Further characterization of large T functions in cellular transformation will require that the cellular targets of large T leading to its interaction with different structural systems of the cell be identified at the molecular level.

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