DNA-mediated transport of the intermediate filament protein vimentin into the nucleus of cultured cells

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SUMMARY

A number of characteristic properties of intermediate filament (IF) proteins, such as nucleic acid-binding activity, affinity for histones and structural relatedness to transcription factors and nuclear matrix proteins, in conjunction with the tight association of IFs with the nucleus, suggest that these proteins might also fulfill nuclear functions in addition to their structure-organizing and -stabilizing activities in the cytoplasm. Yet, cytoplasmic IF proteins do not possess nuclear localization signals. In a search for carriers capable of transporting the IF protein vimentin into the nucleus, complexes of FITC-vimentin with various DNAs were microinjected into the cytoplasm of cultured cells and the intracellular distribution of the protein was followed by confocal laser scanning microscopy. The single-stranded oligodeoxyribonucleotides oligo(dG)$_{25}$, oligo[d(GT)$_{12}$G] and oligo[d(G$_3$T$_2$A)$_4$G] proved to be excellent nuclear carriers for vimentin. However, in fibroblasts, fluorescence-labeled vimentin taken up by the nuclei remained undetectable with affinity-purified, polyclonal anti-vimentin antibody, whereas it was readily identifiable in the nuclei of microinjected epithelial cells in this way. Moreover, when FITC-vimentin was preinjected into fibroblasts and allowed to assemble into the endogenous vimentin filament system, it was still transferred into the nucleus by post-injected oligo(dG)$_{25}$, although to a lesser extent. Superhelical circular DNAs, like pBR322, SV40 and mitochondrial DNA, were also characterized by considerable capacities for nuclear vimentin transport; these transport potentials were totally destroyed by relaxation or linearization of the DNA molecules. Nevertheless, certain linear double-stranded DNA molecules with a high affinity for vimentin IFs, such as repetitive telomere and centromere or mobile long interspersed repeat (LINE) DNA, could carry FITC-vimentin into the nucleus. This was also true for a 375 bp extrachromosomal linear DNA fragment which occurs in the cytoplasm of mouse tumor cells and which is capable of immortalizing human lymphocytes. On the basis of these results, it appears very likely that cellular and viral products of reverse transcription as well as other extrachromosomal DNAs, which are circular, superhelical and apparently shuttling between the cytoplasm and the nucleus (eccDNA), are constantly loaded with vimentin in vimentin-positive cells. Since such DNAs are considered as markers of genomic instability, it is conceivable that vimentin directly participates as an architectural, chromatin-modifying protein in recombinatorial processes set off by these DNAs in the nucleus.

Key words: Intermediate filament, Vimentin, Extrachromosomal DNA, DNA-vimentin complex, Nuclear import

INTRODUCTION

Although intermediate filaments (IFs) as one of the major components of the eukaryotic cytoskeleton are widely spread in nature and have been known for more than a quarter of a century, their biological functions have until recently been largely unknown (for reviews on IFs, see Fuchs and Weber, 1994; Klymkowsky et al., 1989; Quinlan et al., 1995; Skalli and Goldman, 1991; Steinert and Roop, 1988). During the last decade, studies on natural and experimentally induced pathological conditions of epithelia (for reviews see Albers, 1996; Fuchs, 1997; Lane, 1995; Steinert, 1996), the nerve system (reviewed by Houseweart and Cleveland, 1998; Julien, 1997) and muscle (reviewed by Capetanaki et al., 1997; Galou

et al., 1997) in development and adulthood have revealed serious effects of genetic disorders of IFs and their associated proteins on the structural integrity of the respective cells and tissues. These effects manifest themselves as dramatic changes in cell shape and sensitivity of the cells to mechanical stress with manifold adverse influences on normal physiological tissue performance. The techniques of gene knockout and overexpression of IF proteins in transgenic mice have made important contributions to the unraveling of the cytoskeletal function(s) of cytoplasmic(c)IFs (for references, see Galou et al., 1997, and the above reviews).

Because of the developmentally regulated expression and tissue-specific distribution of the multiple subunit proteins of IFs (in vertebrates, there are more than 40 different IF protein

species), these are thought to play roles in processes of cellular differentiation and organismal development. It is a generally held view that the differential functionality of the various IF types is based on the evolutionarily conserved multi-domain structure of their subunit proteins which determines the assembly of a class of filaments of, on the one side, constant gross morphology but, on the other, different surface properties. While the well-conserved α-helical rod domains of the various IF proteins constitute the filament body proper, the structurally highly variable, non-α-helical, N-terminal head and C-terminal tail regions are largely exposed on the surface of the filament core, thus being available for interactions with other cellular substructures characteristic of differentiated cells (Steinert et al., 1985). It is in this way that cIFs are presumed to act as structure-organizing and -stabilizing cytoskeletal elements, as mechanical integrators of cellular space (Lazarides, 1980).

On the other hand, cIF proteins exhibit considerable in vitro affinities for nuclear constituents, such as DNA, RNA and histones, and they are structurally related to nuclear matrix proteins and transcription factors, suggesting their participation in DNA-based nuclear events, like DNA-replication, -transcription, -recombination and -repair (for a review, see Traub and Shoeman, 1994a). In this respect, cIFs may be considered as part of a structural-functional continuum of protein filaments extending between the plasma membrane and the interior of the nucleus where it consists of the IFs of the nuclear lamina and the 10 nm core filaments morphologically indistinguishable from cIFs (He et al., 1990; Jackson and Cook, 1988). Since, however, cIF proteins do not possess canonical nuclear localization signals (NLSs), it is difficult to imagine how they could exert intranuclear functions. It was speculated that due to the amphiphilic, lipid bilayer-perturbing character of the N-terminal head domains exposed in large number on the filament surface nucleus-associated IFs might directly penetrate the double nuclear membrane in order to establish contact with nuclear matrix/chromatin complexes at the nuclear periphery just underneath the inner nuclear membrane (Traub and Shoeman, 1994a). In any event, after treatment of eukaryotic cells with nonionic detergents, cIFs remain tightly associated with the nucleus (Carmo-Fonseca and David-Ferreira, 1990; Fey et al., 1984; French et al., 1989). Because of the preference of cIF proteins for guanine(G)-rich DNAs and the periodic arrangement of the N-terminal, nucleic acid-binding head domains on the filament surface, it was assumed that IFs entering the nuclear interior interact with chromatin regions containing G-rich and repetitive DNA sequence elements (Traub and Shoeman, 1994a). Indeed, from a mixture of mouse genomic, double-stranded DNA fragments, vimentin IFs reconstituted in vitro selected G-rich, highly repetitive DNA sequences, like micro- and minisatellite DNAs, telomere DNA, mobile DNA sequences and, surprisingly (because of its relatively high A/T content), centromeric γ-satellite DNA (Wang et al., 1996, and unpublished results).

cIF proteins could gain access to the nuclear interior in other ways during mitosis or via karyophilic carrier substances. In mitosis, the nuclear envelope is destroyed and the nuclear and cytoplasmic matrices are largely disassembled or rearranged by post-translational modification of their constituent proteins (Chou et al., 1990; Eriksson et al., 1992; Evans, 1989; Marugg,

1992; Nigg, 1992) so that these, now in an activated form, can interact with each other. cIF proteins could thus be incorporated via complexes with karyoskeletal elements into the structure of the newly forming daughter nuclei. With regard to the second possibility, the transport of transcription factors lacking classical NLSs from the cytoplasm into the nuclear interior by nuclear carrier proteins may serve as a model (for references see Traub and Shoeman, 1994b).

However, not only carrier proteins possessing NLSs could mediate the transport of cIF proteins into the interphase nucleus, but also any other component that has affinity for the filament proteins and that is actively channelled through the nuclear pore complexes (NPCs). Recently, it has been demonstrated that biotinylated oligo(dG)$_n$ with a minimum length of 15 nucleotides or other biotinylated G-rich, single-stranded oligodeoxyribonucleotides are able to transport fluorescence-labeled or gold-conjugated streptavidin complexes lacking NLSs through the nuclear pores from the cytoplasm into the nuclear interior (Hartig et al., 1998). That is, such single-stranded oligodeoxyribonucleotides can act as NLSs as well.

Since oligo(dG)$_n$ and other G-rich nucleic acids exhibit a high affinity for cIF proteins (Traub and Nelson, 1983), it seems possible that they are able to complex the latter directly without the biotin-streptavidin linkage and to carry them through the NPCs into the nuclear interior. In the present investigation, fluorescence-labeled vimentin was directly complexed with oligo(dG)$_{25}$ and other, naturally occurring DNAs and microinjected into the cytoplasm of various cultured monolayer cells and its intracellular distribution was followed by confocal laser scanning (CLS) microscopy. The observation that all these DNAs can function as efficient transporter molecules for vimentin implies that cIF proteins can and do indeed enter the nucleus via a piggyback mechanism. It opens the way for a more considered evaluation of the hypothesis concerning the potential role of cIF proteins as mediators of nuclear genome activities (Traub and Shoeman, 1994a).

MATERIALS AND METHODS

Cells

Human GMO 0970C fibroblasts, human breast carcinoma MCF-7 cells and marsupial kidney PtK$_2$ cells were grown as described (Hartig et al., 1997, 1998). Embryo fibroblasts from mouse, guinea pig, hamster, Tupaja, cat and chicken (own productions) were grown in minimum essential Eagle medium (MEM; ICN Biomedicals GmbH, Meckenheim, Germany) supplemented with 10% fetal calf serum (FCS; Gibco-BRL, Life Technologies, Eggenheim, Germany). HeLa cells (CCL 2, The American Type Culture Collection (ATCC)) were maintained in Dulbecco's modified MEM (DMEM; ICN Biomedicals GmbH) containing 10% FCS. Cells of the subclone F8 of the human adrenal cortex carcinoma-derived cell line SW13 (ATCC CCL 105) which is devoid of endogenous, cytoplasmic IF proteins were grown in Leibovitz's L-15 medium (ICN Biomedicals GmbH) supplemented with 10% FCS. For microinjection, all cells were grown on Eppendorf Cellocate glass coverslips (Nr. 5245 954001; Eppendorf, Hamburg, Germany) to subconfluency in a humid atmosphere containing 5% CO$_2$.

Proteins

Vimentin was prepared from Ehrlich ascites tumor cells as previously described (Nelson et al., 1982). It was labeled with fluorescein by

reaction with 5(6)-carboxyfluorescein-*N*-hydroxysuccinimide ester (FLUOS; Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's recommendations at various molar ratios of protein to FLUOS, ranging from 1:2 to 1:62. This was done to test for optimal labeling density representing the best tradeoff between an intense fluorescence signal (highly sensitive detection), retention of native properties of vimentin (polymerization into IFs) and generation of artificial nuclear localization signals (proteins extensively labeled with fluorescein enter the nucleus independent of classical NLS signals; data not shown). Fluorescein-labeled vimentin (FITC-vimentin) was additionally purified from unreacted FLUOS reagent by extensive diafiltration on Amicon Centricon-30 or -50 Microconcentrators (Millipore, Eschborn, Germany) in 10 mM MOPS, 0.1 mM EDTA, pH 7.4. The labeling yield was determined spectrophotometrically using Boehringer Mannheim's equation to be 1.2, 3.6 or 8 fluoresceins per vimentin tetramer, at protein:FLUOS molar ratios of 1:2, 1:10 and 1:50. A moderate increase in DNA-mediated transportability was seen with FITC-vimentin preparations carrying 2-3 fluorescein molecules per protein molecule. FITC-vimentin labeled with FLUOS at a ratio of 1:22 was polymerization-competent in SW13 vimentin$^-$ cells, whereas FITC-vimentin labeled with FLUOS at a ratio of 1:62 was not (data not shown). Vimentin was also labeled with tetramethylrhodamine isothiocyanate (TRITC; Sigma, Deisenhofen, Germany) and purified essentially as described for labeling with FLUOS; approximately 2.1 moles of tetramethylrhodamine were incorporated per mole of vimentin. In addition, vimentin was labeled with biotin: ε-caproylamidobiotin-*N*-hydroxysuccinimide ester (Gibco-BRL; 0.1 mg in 5 μl anhydrous dimethylformamide) was added to 95 μl of 100 mM sodium borate buffer, pH 8.5, containing 1 mg vimentin, and incubated at room temperature for 1 hour. The biotinylated vimentin was purified by column chromatography and diafiltration as described for FITC-vimentin. FITC-ExtrAvidin was purchased from Sigma, FITC-donkey anti-goat IgG and TRITC-rabbit anti-goat IgG antibodies from Dianova (Hamburg, Germany) and Sigma, respectively.

Nucleic acids

Unless otherwise noted, all nucleic acid manipulations were as described in standard protocols (Ausubel et al., 1994; Sambrook et al., 1989). Nucleic acid concentrations were determined spectrophotometrically, using a constant factor of 1 mg/ml = 20 OD_{260nm} for all nucleic acids (Shoeman et al., 1988). Porcine mitochondrial DNA was purified from pig liver mitochondria, essentially as described for other species (Andersson et al., 1985; Tapper et al., 1983). Oligonucleotides and plasmid DNAs were prepared as previously described (Hartig et al., 1997; Maxon et al., 1989; Shoeman et al., 1997). The vertebrate telomere repeat oligonucleotide 25mer had the sequence 5′-d(G_3T_2A)$_4$G-3′. Superhelical SV 40 DNA (Form I) (5,243 bp) was purchased from Gibco-BRL. Superhelical DNAs were converted to their relaxed forms with calf thymus topoisomerase I (Gibco-BRL), using reaction conditions supplied by the manufacturer, or to their linear forms by cleavage with an appropriate, single-cutting restriction endonuclease. A 375 bp extrachromosomal linear DNA fragment, corresponding to the mouse genomic DNA insert of plasmid pEFC38 (Abken et al., 1993), was produced by digesting pEFC38 with restriction endonuclease *Bam*HI and the single-stranded ends were filled in with T4 DNA polymerase. Linear DNAs containing mouse telomere repeats (1 kbp), a centromeric γ-satellite sequence (450 bp) (Wang et al., 1996) and a LINE sequence (631 bp) (unpublished) were produced by preparative PCR from plasmids and primers.

Other procedures

Nucleic acid/FITC-vimentin complexes were formed by mixing equal masses of each component (generally at about 1 mg/ml in 10 mM MOPS, 0.1 mM EDTA, pH 7.4) and incubating for 10 or more minutes on ice before the filling of the microinjection needles and the beginning of the microinjection procedure. Oligo (dG)$_{25}$/FITC-vimentin complexes were incubated for about 60 minutes at room temperature (18-20°C) before microinjection. Microinjection into intact cells was performed as described (Hartig et al., 1997). When wheat germ agglutinin (WGA; Sigma) (Dabauvalle et al., 1988; Finlay et al., 1987) was coinjected, it was used at a concentration of 1.25 mg/ml. FITC-dextrans (4,300 and 71,300 Da; Sigma), microinjected alone or together with oligo(dG)$_{25}$ (1 mg/ml), were used at a concentration of 5 mg/ml. Following microinjection, cells were fixed and processed for fluorescence microscopy at room temperature, unless otherwise stated. For direct visualization of FITC-vimentin, cells were fixed for 10 minutes in 100 mM Na-phosphate, pH 7.4, containing 4% paraformaldehyde and 0.05% glutaraldehyde; thereafter, the cells were washed in phosphate-buffered saline (PBS), treated with RNase A (10 units/ml), washed and mounted in antifade reagents and propidium iodide as described (Giese et al., 1997). For immunofluorescence detection of unlabeled and FITC- or TRITC-conjugated vimentin, cells were fixed (as described above), permeabilized by treatment with 0.2% (v/v) Triton X-100 in PBS for either 10 minutes (MCF-7 cells) or 5 minutes (all other cell types), blocked with 1% (w/v) bovine serum albumin (BSA) in PBS for 10 minutes, washed in PBS and incubated for 1 hour at 37°C with an affinity-purified goat anti-vimentin antibody (1:400 dilution; Hartig et al., 1997). The specimens were washed with PBS, blocked for 10 minutes with BSA in PBS and incubated for 1 hour at 37°C with a FITC- or TRITC-labeled anti-goat IgG antibody, depending on the fluorescence label of vimentin. After washing in PBS, the cells were fixed and processed as described above. The processing of cells microinjected with complexes of oligo(dG)$_{25}$ and biotinylated vimentin was similar to that described for the antibody labeling of vimentin, except that FITC-ExtrAvidin (diluted 1:50 in 1% BSA in PBS; Sigma) was substituted for the antibodies. Conventional fluorescence and CLS microscopic analysis of cell specimens were performed as previously described (Hartig et al., 1997).

RESULTS

The capacity of a variety of DNA species to carry the IF protein vimentin into the nucleus of cultured cells was determined by complexing DNA with FITC-conjugated vimentin in vitro, microinjecting the adducts into the cytoplasm and following the intracellular distribution of the fluorescence-labeled protein by CLS microscopy. As shown in Fig. 1A, FITC-vimentin by itself was unable to enter the nucleus of human fibroblasts during an incubation time of 1 hour and longer; it was deposited in the form of unstructured aggregates in the perinuclear region, which, dependent on the time post-injection, slowly merged into IFs. However, when FITC-vimentin was bound to oligo(dG)$_{25}$ and then injected, substantial amounts of the protein were found as numerous, differently sized speckles in the nuclear interior (Fig. 1B). It should explicitly be pointed out that throughout this investigation the microinjected cells were subjected to optical sectioning in order to demonstrate that the fluorescent speckles seen inside the nucleus (normally visualized with propidium iodide) have a limited extension perpendicular to the section plane, and thus to exclude the possibility that they represent nuclear invaginations or channels occupied by cytoplasmic vimentin. This precaution seemed necessary since recently cytoplasmic channels have been observed to run through the nucleus of cultured cells (Fricker et al., 1997; Lui et al., 1998). Moreover, all micrographs shown depict sections along the

Fig. 1. Confocal laser scanning microscopy of human fibroblasts microinjected with FITC-vimentin and its complexes with oligo(dG)$_{25}$. Microinjection of (A) FITC-vimentin alone and (B) oligo(dG)$_{25}$/FITC-vimentin complexes and intracellular distribution of the fluorescent filament protein 1 hour postinjection (p.i.). (C) Microinjection of FITC-vimentin and its assembly into the endogenous vimentin IF system during a 20 hour incubation period. (D) Microinjection of oligo(dG)$_{25}$ 20 hours after the injection of FITC-vimentin (see C) and fluorescence microscopic analysis of the cells 1 hour later. (E,F) Microinjection of oligo(dG)$_{25}$/FITC-vimentin complexes in the absence (E) and presence (F) of wheat germ agglutinin and fluorescence microscopic analysis of the cells 30 minutes p.i. (G) Microinjection of complexes consisting of oligo(dG)$_{25}$ and unlabeled vimentin and immunofluorescence microscopic analysis of the cells with a polyclonal anti-vimentin and a FITC-conjugated secondary antibody 1 hour p.i. (H,I) Microinjection of oligo(dG)$_{25}$/FITC-vimentin complexes and attempt to additionally visualize the fluorescent protein transported into the nucleus (H) with a polyclonal anti-vimentin

and a TRITC-conjugated secondary antibody (I) 1 hour p.i. (A to G) Nuclei are stained with propidium iodide. All optical sections are through the equatorial plane of the nuclei. Green fluorescence: vimentin (A to H); red fluorescence: propidium iodide (A to G); vimentin (I). Bar, 10 μm.
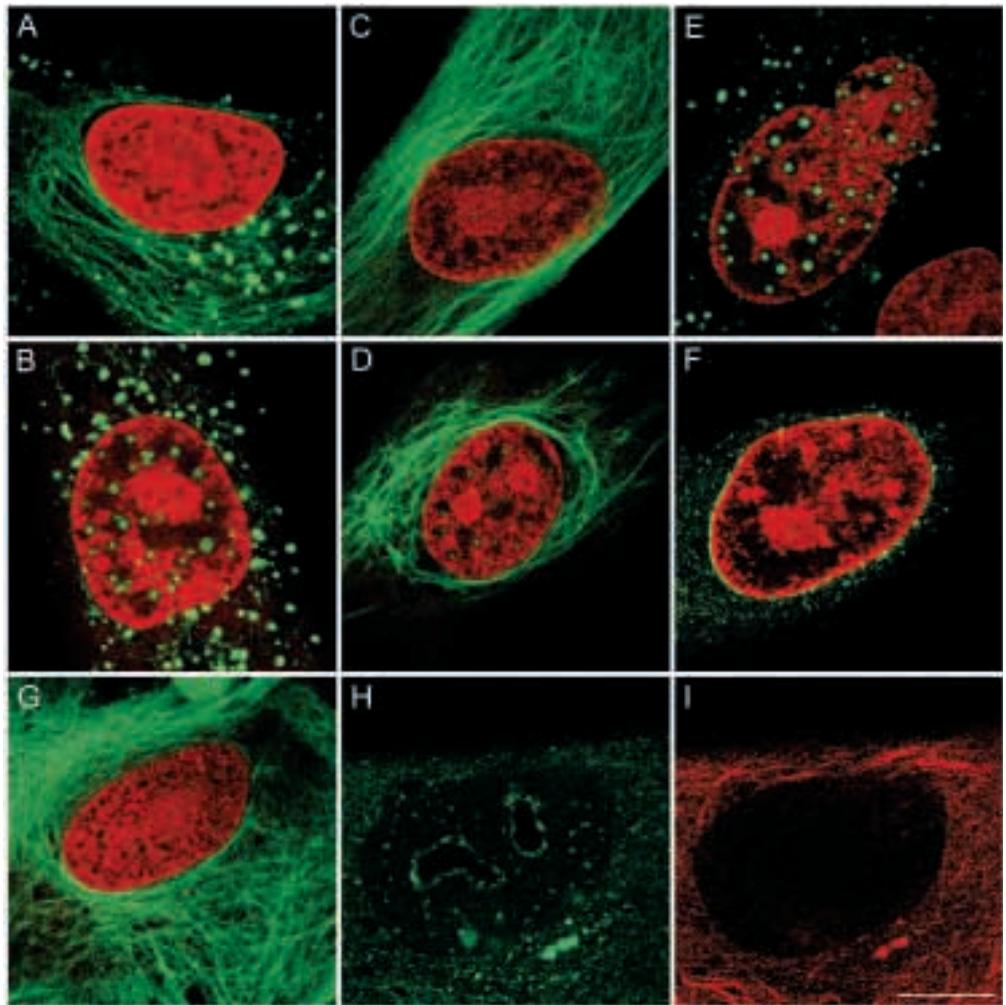
equatorial plane of the nucleus, whose midpoints are at a maximal distance from the cytoplasm.

It was of further interest to find out whether microinjected oligo(dG)$_{25}$ was able to extract vimentin molecules from intact IFs and to transport them into the nucleus. FITC-vimentin was preinjected into human fibroblasts and allowed to incorporate into the endogenous vimentin IF system over a period of 20 hours (Fig. 1C) prior to oligo(dG)$_{25}$ injection. Fig. 1D shows that under such conditions the nucleus still took up considerable quantities of FITC-vimentin, although to a reduced extent, in comparison with the situation seen when the two compounds were introduced into the cell as preformed complexes. Since this microinjection protocol used a relatively large amount of oligonucleotide, damage of the nuclear membrane by the nucleic acid had to be taken into consideration. Yet, coinjection of wheat germ agglutinin together with the complexes totally prevented their nuclear incorporation within a period of 30 minutes (Fig. 1E,F). This demonstrated that the transport process is an active one and mediated by the NPCs of the nuclear envelope.

Additional evidence against permeabilization or damage of the nuclear membrane by large quantities of oligo(dG)$_{25}$ was

provided by analysis of the intracellular distribution of FITC-dextrans which had been microinjected alone and together with the oligonucleotide into the cytoplasm of PtK$_2$ cells or human fibroblasts. One hour after application of low molecular mass FITC-dextran (4,300 Da) to the cells, this was found in substantial amounts in the nuclear interior, in the absence as well as in the presence of oligo(dG)$_{25}$. Yet, FITC-dextran with a molecular mass of 71,300 applied under the same conditions was rejected by the nuclei, no matter whether oligo(dG)$_{25}$ was absent or present (data not shown).

In order to obtain information on the binding state of vimentin transported by DNA into the nucleus, human fibroblasts microinjected with complexes consisting of unlabeled vimentin and oligo(dG)$_{25}$ were examined by immunofluorescence microscopy with affinity-purified polyclonal anti-vimentin and FITC-conjugated secondary antibodies. As seen in Fig. 1G, the antibodies did not recognize any antigenic structure in the nuclear interior but only vimentin IFs in the cytoplasm. This failure could have only two explanations: either the DNA-mediated transport of FITC-vimentin into the nucleus (Fig. 1B) was facilitated by the fluorescence label of the filament protein or the protein was

Fig. 2. Confocal laser scanning microscopy of epithelial PtK$_2$ cells after microinjection of FITC- or biotinyl-vimentin and its complexes with oligo(dG)$_{25}$. Microinjection of (A) FITC-vimentin alone and (B) oligo(dG)$_{25}$/FITC-vimentin complexes and intracellular distribution of the fluorescent filament protein 1 hour p.i. (C) Preinjection of FITC-vimentin and its assembly into the endogenous vimentin IF system during a 20 hour incubation period, followed by injection of oligo(dG)$_{25}$ and fluorescence microscopic analysis of the cells 1 hour later. (D) Microinjection of complexes consisting of oligo(dG)$_{25}$ and unlabeled vimentin and immunofluorescence microscopic analysis of the cells with a polyclonal anti-vimentin and a FITC-conjugated secondary antibody 1 hour p.i. (E) Microinjection of biotinylated vimentin and its detection with FITC-ExtrAvidin 1 hour p.i. (F) Microinjection of oligo(dG)$_{25}$/biotinyl-vimentin and analysis of the cells with FITC-ExtrAvidin 1 hour p.i. (A to F) Nuclei are stained with propidium iodide. All optical sections are through the equatorial plane of the nuclei. Green fluorescence: vimentin; red fluorescence: propidium iodide. Bar, 10 μm.
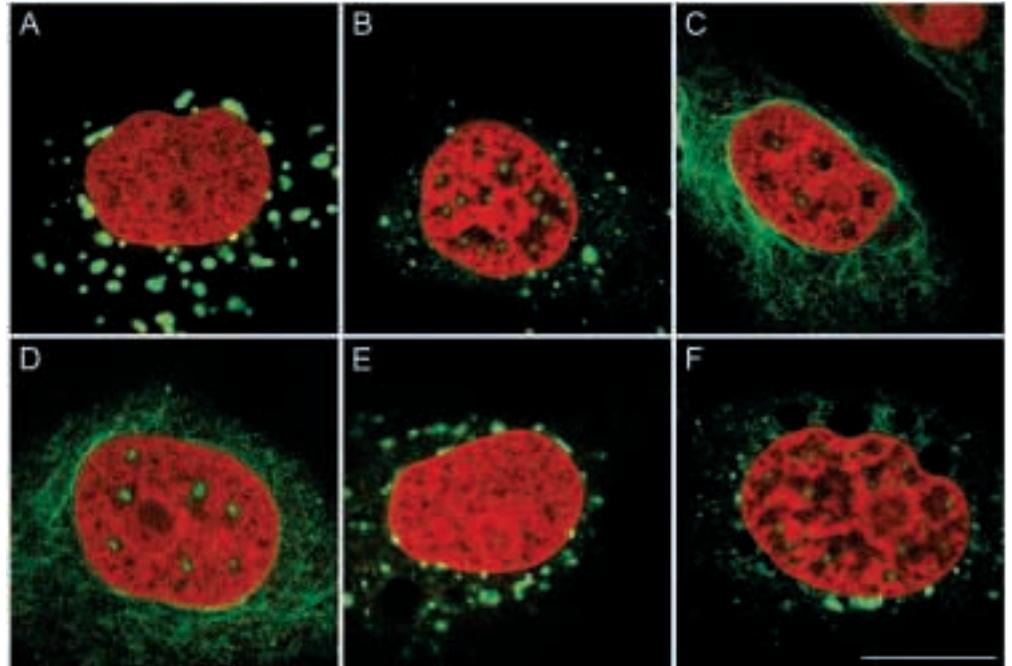
immunologically undetectable because of its intimate interaction with intranuclear structures. The second possibility could be verified since FITC-conjugated vimentin imported into the nucleus by oligo(dG)$_{25}$ and appearing as distinct fluorescent deposits around the nucleoli and in the nucleoplasm (Fig. 1H) did not produce any fluorescent signal with polyclonal anti-vimentin and TRITC-labeled secondary antibodies (Fig. 1I). The same result was obtained with TRITC-conjugated vimentin and polyclonal anti-vimentin and FITC-labeled secondary antibodies (data not shown). That the masking effect was not unique to human fibroblasts was demonstrated by the occurrence of the same phenomenon in

Fig. 3. Kinetic behavior of FITC-vimentin transported via oligo(dG)$_{25}$ into the nuclei of human fibroblasts and segregation of the protein and oligonucleotide moieties of the complexes after nuclear import. (A to E) Confocal fluorescence microscopic analysis of human fibroblasts microinjected with oligo(dG)$_{25}$/FITC-vimentin complexes after 1 (A), 2 (B), 4 (C), 6 (D) and 8 (E) hours p.i. Note the dispersion and slow disappearance of the FITC-vimentin speckles from the nuclei. (F) Partial segregation of the protein and nucleic acid moieties of TRITC-oligo(dG)$_{25}$/FITC-vimentin complexes having entered the nucleus of a human fibroblast cell during a 1 hour incubation period. Access of TRITC-oligo(dG)$_{25}$ to the nucleolar interior and deposition of FITC-vimentin on the surface of the nucleoli are apparent. (A to E) Nuclei are stained with propidium iodide. All optical sections are through the equatorial plane of the nuclei. Green fluorescence: vimentin (A to F); red fluorescence: vimentin (A to E); TRITC-oligo(dG)$_{25}$ (F). Bar, 10 μm.
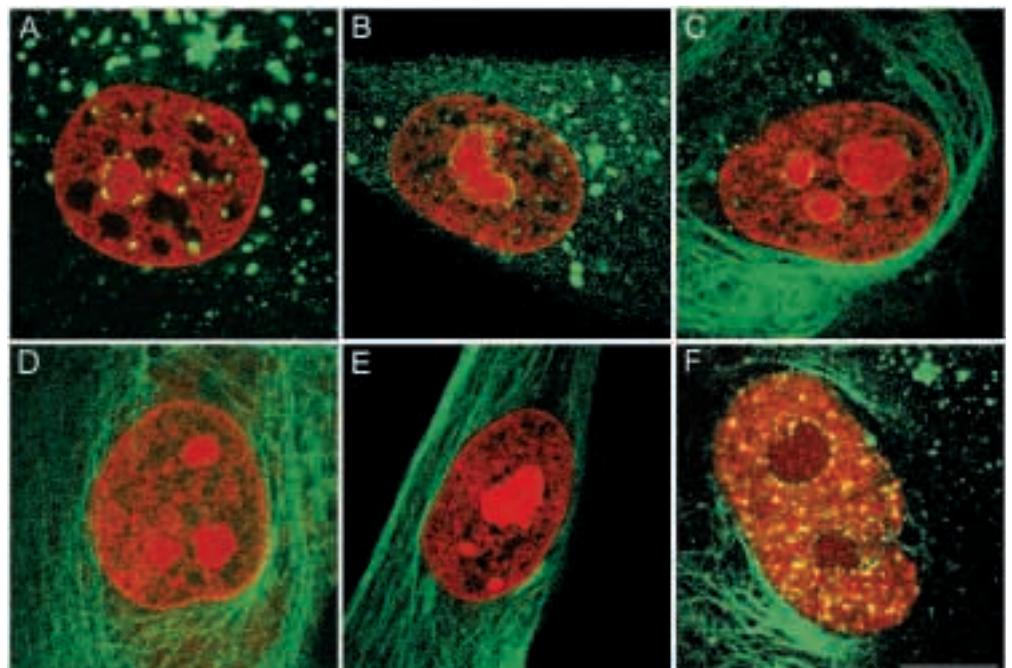
fibroblasts from guinea pig, hamster, Tupaja, cat and chicken (data not shown).

Another way to disprove an active participation of the fluorescence label in oligo(dG)$_{25}$-mediated translocation of vimentin into the nucleus was to conjugate vimentin with a biotinyl group and to detect this with FITC-ExtrAvidin. However, treatment of control human fibroblasts and fibroblasts microinjected with oligo(dG)$_{25}$/biotinyl-vimentin with FITC-ExtrAvidin produced a very high background staining, making an unambiguous detection of vimentin in the nucleus impossible (data not shown).

Because of these difficulties, the experiments described above were repeated with other cell lines. In epithelial PtK$_2$ cells, FITC-vimentin microinjected alone did not enter the nucleus (Fig. 2A), whereas complexes of the fluorescent protein with oligo(dG)$_{25}$ were readily taken up (Fig. 2B). As in human fibroblasts, oligo(dG)$_{25}$ injected 20 hours after application of FITC-vimentin and its incorporation into endogenous vimentin IFs was able to pick up the fluorescent protein from the filaments and to transport it into the nucleus (Fig. 2C). However, a clear difference between both cell lines was noticed when the oligo(dG)$_{25}$-mediated nuclear import of unlabeled vimentin was followed with polyclonal anti-vimentin and FITC-conjugated secondary antibodies. In PtK$_2$ cells, this procedure revealed an intranuclear vimentin distribution (Fig. 2D) virtually indistinguishable from that seen with FITC-vimentin (Fig. 2B). An identical, positive nuclear response to immunolabeling was noticed in human breast carcinoma MCF-7 cells microinjected with oligo(dG)$_{25}$/vimentin complexes (data not shown). Because of low background staining (Fig. 2E), a positive nuclear reaction was also observed when biotinylated vimentin was microinjected together with oligo(dG)$_{25}$ into PtK$_2$ cells and this was localized with FITC-ExtrAvidin (Fig. 2F). These data proved that the oligo(dG)$_{25}$-dependent transportability of vimentin into the nucleus is a property of the filament protein per se and not due to its conjugation with a fluorescence label.

FITC-vimentin, which via oligo(dG)$_{25}$-mediated transport had accumulated as large aggregates in the nuclei of microinjected human fibroblasts during a 1 hour incubation (Fig. 3A), again left the nuclear interior over time, probably to be incorporated into IFs together with FITC-vimentin that had remained in the cytoplasm (Fig. 3A to E). During this process, the fluorescent vimentin speckles became progressively dispersed and then largely disappeared from the nucleoplasm after longer incubation times (Fig. 3E). This process occurred during an apparently intact state of the microinjected cells, which were structurally indistinguishable from control-injected cells during the first 24 hours, but detached from the substratum and were lost to analysis 1.5 to 2 days postinjection. Separate injection of FITC-vimentin and oligo(dG)$_{25}$ showed that the oligonucleotide was responsible for the limited survival and/or recovery of the microinjected cells (data not shown).

Concerning the fate of vimentin imported by nuclei, it could additionally be demonstrated that the oligo(dG)$_{25}$/FITC-vimentin complexes taken up dissociated to a considerable extent and that the released vimentin either formed aggregates or associated with intranuclear structures. This segregation process was clearly visible when complexes consisting of TRITC-conjugated oligo(dG)$_{25}$ and FITC-labeled vimentin

were used for microinjection into human fibroblasts and the routine staining of nuclei with propidium iodide was omitted. Due to superimposition of red and green fluorescence, the complexes appeared as yellow speckles in the nuclear interior whereas the dissociated TRITC-oligo(dG)$_{25}$ and FITC-vimentin components were recognizable by their inherent red and green fluorescence, respectively (Fig. 3F). It also became apparent that, while TRITC-oligo(dG)$_{25}$ had access to the interior of nucleoli, FITC-vimentin was retained at the nucleolar border.

Among other single-stranded oligodeoxyribonucleotides tested, only oligo[d(GT)$_{12}$G] and telomeric oligo [d(G$_3$T$_2$A)$_4$G] proved to be efficient carrier molecules for the transport of vimentin into the nucleus of human fibroblasts. Oligo [d(GA)$_{12}$G], also rich in G, exhibited a substantially weaker transport activity, depositing only small amounts of FITC-vimentin as fine aggregates on the outer surface of the nucleoli. Oligo(dT)$_{25}$ and oligo(dC)$_{25}$ were rather inactive as vimentin carrier molecules and oligo(dA)$_{25}$ did not exhibit any vimentin transport activity whatsoever (data not shown). Similar results were obtained with a variety of other cell lines (see Materials and Methods), but not with HeLa cells. HeLa cells exhibited only a limited capacity to transport FITC-oligo(dG)$_{25}$ and no transport of oligo(dG)$_{25}$/FITC-vimentin complexes was seen.

Physiologically more relevant DNAs capable of nuclear transfer of vimentin in cultured cells were supercoiled plasmid DNAs. Fig. 4A illustrates the accumulation of substantial quantities of FITC-vimentin in the interior of human fibroblast nuclei when the protein was complexed with superhelical pBR322 DNA and then microinjected into the cytoplasm. Previous relaxation of pBR322 DNA by single strand scission (or its linearization; data not shown) totally abolished its nuclear transport capacity for vimentin (Fig. 4B). Since plasmids, like pBR322, represent prokaryotic DNAs, it was searched for superhelical DNAs of eukaryotic origin exhibiting a similarly marked nuclear vimentin transport potential. Such material was found in the form of SV40 DNA which, under superhelical tension, effected the intranuclear deposition of FITC-vimentin in distinct speckles in the nucleoplasm and around nucleoli in human fibroblast cells (Fig. 4C), but was completely inactive when relaxed (Fig. 4D) or linearized (data not shown). Even mitochondrial DNA of much larger molecular size (16.5 kbp) was capable of carrying FITC-vimentin into the nuclei of human fibroblasts when it was employed in its circular, superhelical form (Fig. 4E).

All of the transport-competent DNAs described so far are distinguished by high affinity for vimentin as one of two prerequisites for efficient transfer of the filament protein from the cytoplasm into the nucleus. The other requirement for nuclear transport is, of course, a high karyophilicity of the carrier DNA. On the basis of previously obtained results showing that linear telomere DNA also possesses a reasonably high vimentin-binding potential, a double-stranded mouse telomere fragment of 1 kbp length was loaded with FITC-vimentin and injected into the cytoplasm of human fibroblast cells, even though its karyophilicity was unknown. Fig. 4F shows nuclear uptake of significant quantities of the complexes. Similar circumstances apply to mouse centromere (γ-satellite) and LINE (long interspersed nuclear element) DNA and, indeed, 450 bp and 631 bp fragments, respectively,

of the repetitive and mobile DNAs also transferred considerable amounts of FITC-vimentin into the nucleus of human fibroblasts (Fig. 4G and H, respectively). Furthermore, a 375 bp extrachromosomal, linear and double-stranded DNA fragment, which occurs in the cytoplasm of Ehrlich ascites tumor cells and which is capable of immortalizing human lymphocytes, also turned out to be an active nuclear vimentin transporter that deposited FITC-vimentin aggregates in the nucleoplasm and on the surface of nucleoli, respectively.

During this investigation, it was repeatedly observed that the transportability of vimentin into the nucleus of cultured cells by transport-competent DNAs is somewhat increased if the IF protein carries FITC groups in a number distinctly exceeding a labeling density of 1. This is demonstrated in Fig. 5 for cIF-deficient human SW13 adenocarcinoma cells and a series of DNAs with a high affinity for vimentin. Fig. 5A shows a deeply invaginated nucleus which had incorporated large quantities of vimentin carrying several (~2 to 3) FITC groups per protein molecule following coinjection of the protein with oligo(dG)$_{25}$ into the cytoplasm. Substantial quantities of this vimentin preparation were also incorporated into the nuclei of SW13 cells when supercoiled pBR322 DNA was used as a carrier molecule (Fig. 5B). As previously demonstrated for human fibroblasts (Fig. 4B) and PtK$_2$ cells (data not shown), this incorporation was reduced to a low level when the plasmid DNA was relaxed prior to complexation with FITC-vimentin (Fig. 5C). The stimulatory effect of stronger protein labeling with FITC on the DNA-mediated translocation of vimentin into the nucleus was particularly striking in the case of supercoiled SV40 DNA (Fig. 5D). The higher labeling density also seemed to permit the protein to partially enter the nucleoli although it was to a great extent retained at the nucleoplasmic-nucleolar border. Enzymatic relaxation (Fig. 5E) or linearization (data not shown) of the superhelical DNA again largely destroyed its nuclear carrier potential for vimentin. In SW13 cells, the 375 bp extrachromosomal DNA fragment from Ehrlich ascites tumor cells also efficiently transferred the more densely labeled filament protein into the nucleus (Fig. 5F), whereas single-stranded bacteriophage fd DNA was rather inactive as a carrier (Fig. 5G). When the FITC-conjugated vimentin was injected alone into the cytoplasm of SW13 cells, it quantitatively stayed in the cytoplasm despite its heavier labeling (Fig. 5H). Finally, while in mouse skin fibroblasts superhelical SV40 DNA-mediated incorporation of FITC-vimentin of low labeling density into the nucleus was generally modest when the fluorescent protein was preinjected and allowed to be incorporated into the endogenous IF system prior to DNA application, with FITC-vimentin of higher labeling density an efficient nuclear incorporation of the protein under the latter conditions was achieved (Fig. 5I). The resulting vimentin distribution provides another demonstrative example for the preferential deposition of the filament protein on the nucleolar surface.

DISCUSSION

Previous results from this laboratory have led to the hypothesis that cytoplasmic IF(protein)s not only fulfill cytoskeletal functions in the cytoplasm but also participate in DNA- and RNA-based events taking place in the nuclear

interior (Traub and Shoeman, 1994a). The main objections to this view are, of course, the facts that cytoplasmic IF proteins do not possess classical NLSs which allow them to enter the nuclear interior through the NPCs of the nuclear envelope and the cIF proteins are not routinely seen in the nucleus in conventional immunofluorescence assays. A possible way out of this situation could be the transfer of the filament proteins into the nucleus by karyophilic carrier substances. In order to substantiate this notion, the methods and results of a previous investigation describing oligodeoxyribonucleotide-mediated nuclear import of nonkaryophilic compounds and macromolecular assemblies in digitonin-permeabilized and microinjected mammalian cells (Hartig et al., 1998) were applied to the present study. It was explored whether cultured animal cells have the potential for DNA-mediated translocation of the IF protein vimentin from the cytoplasm into the nucleus via the normal NPC pathway, after DNA had been allowed to either extra- or intracellularly select the protein from a complex mixture of other components before and after microinjection, respectively. A selection of DNAs, ranging from synthetic and recombinant model substances to naturally occurring compounds, were employed to gain information on possible nuclear vimentin carriers in the intact cell and the physiological relevance of DNA-mediated nuclear vimentin import.

Prerequisite for efficient nuclear transport of vimentin by DNAs are a high vimentin-binding activity and a high karyophilicity of the nucleic acids. In accord with previous results (Hartig et al., 1998), these requirements were fulfilled best by single-stranded oligo(dG)$_n$ with a minimal chain length of 15 nucleotides. Among other synthetic single-stranded oligodeoxyribonucleotides tested, oligo[d(GT)$_{12}$G] and oligo[d(G$_3$T$_2$A)$_4$G] turned out to be efficient nuclear carriers for vimentin. However, richness in G does not seem to be an absolute nor a sufficient requirement for high vimentin transport activity since oligo[d(GA)$_{12}$G] was a rather inefficient carrier. Presumably, certain higher order structures of some G-rich oligonucleotides (Ortiz-Lombardia et al., 1995; Shiber et al., 1996) exert a negative influence on their vimentin carrier activity. It was striking that such higher order structures affected both vimentin-binding activity and karyophilicity of the oligonucleotides to approximately the same extent (Shoeman et al., 1997). The very low vimentin transport activity of single-stranded bacteriophage fd DNA, which is an excellent vimentin binder (Traub et al., 1983), might have been due to cleavage of the DNA by cytoplasmic DNases.

Intriguingly, oligo(dG)$_{25}$ was also able to support nuclear import of FITC-vimentin which, after preinjection into the cytoplasm, had been allowed to assemble into the endogenous vimentin IF system of the cell. This capacity may be of particular physiological importance since it would permit cytoplasmic nucleic acids with both a high affinity for vimentin and a high karyophilicity to extract subunit proteins from intact vimentin IFs and to carry them into the nucleus. However, since the nucleic acid-binding site of vimentin is localized in its non-α-helical, N-terminal head domain (Traub et al., 1992) and the latter is required for filament formation and stabilization (Raats and Bloemendal, 1992; Traub and Vorgias, 1983), it costs carrier nucleic acids considerably more effort to transfer previously filamentous vimentin into the nucleus than when they are injected as preformed complexes with protein

Fig. 4. Confocal laser scanning microscopy of human fibroblasts microinjected with complexes consisting of superhelical or linear, double-stranded DNAs and FITC-vimentin. Microinjection of FITC-vimentin complexed with (A) supercoiled plasmid pBR322 DNA, (B) relaxed plasmid pBR322 DNA, (C) supercoiled SV40 DNA, (D) relaxed SV40 DNA, (E) superhelical mitochondrial DNA, (F) 1 kbp linear mouse telomere DNA, (G) 450 bp linear mouse centromeric γ-satellite DNA, (H) 631 bp linear mouse LINE DNA, (I) 375 bp extrachromosomal linear DNA fragment from the cytosol of mouse Ehrlich ascites tumor cells, and intracellular distribution of FITC-vimentin 1 hour p.i. All optical sections are through the equatorial plane of the nuclei which are stained with propidium iodide. Green fluorescence: vimentin; red fluorescence: propidium iodide. Bar, 10 μm.
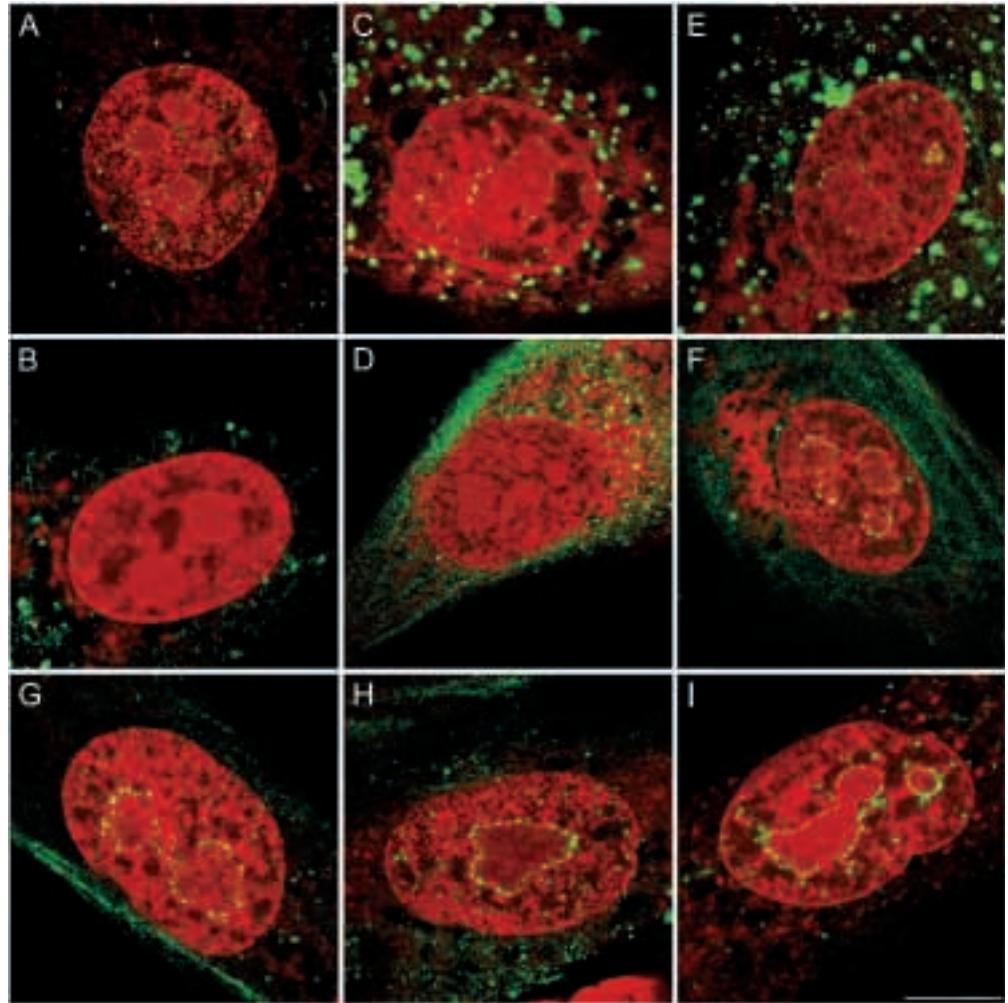
into the cytoplasm. It was attempted to enhance the nuclear incorporation of FITC-vimentin previously assembled into filaments by activating it via phosphorylation. It is known that phosphorylation of the N-terminal head domain of vimentin labilizes IF structure, which may eventually result in complete disassembly of the filaments (for a review see Inagaki et al., 1997). However, coinjection of the catalytic subunit of cAMP-dependent protein kinase (Lamb et al., 1989) with oligo(dG)$_{25}$ into the cytoplasm of human fibroblasts 20 hours after the injection of FITC-vimentin did not lead to an increase in the transport of the labeled protein into the nucleus, although this measure caused the protein to change from a filamentous to a diffuse distribution in the cytoplasm. On the contrary, the amount of FITC-vimentin transferred into the nucleus was substantially reduced in comparison with the control (data not shown). It is conceivable that the protein was overphosphorylated and that this affected its nucleic acid-binding activity, or that the transport activity of the NPCs was impaired by phosphorylation of their protein constituents, or that the nuclear transport-enhancing activation of vimentin requires a protein kinase of different substrate specificity.

Since these experiments performed with oligo(dG)$_{25}$ and related oligonucleotides demonstrated that vimentin can principally be transported into the nucleus by nucleic acids, it was pertinent to search for carrier DNAs of greater physiological relevance. Because of their strong affinity for vimentin and other IF proteins, supercoiled plasmid DNAs offered themselves as promising candidates (Kühn et al., 1987), although their karyophilicity was unknown. The transport process turned out to be absolutely dependent on superhelicity of the DNA molecules since relaxation or linearization of the plasmids completely abolished their carrier capacity for vimentin. As the greatest part of the injected DNA/protein complexes remained in the cytoplasm, only a minor quantity apparently managed to enter the nucleus. Possibly, a substantial fraction of the plasmid DNA was relaxed and thus inactivated as a carrier by mechanical shearing during microinjection or by cytoplasmic enzymes, or the import was limited to a small number of transport-active NPCs.

To refute the argument that nuclear vimentin import mediated by supercoiled plasmid DNAs is still of little physiological relevance because the plasmids represent prokaryotic DNAs which normally do not occur in eukaryotic cells, SV40 DNA and mitochondrial DNA were included in this study as representatives of superhelical DNA from eukaryotic sources. Both DNAs also exhibited considerable nuclear vimentin transport capacity. This carrier potential is remarkable in view of the large size of the DNA molecules, 5,243 bp and 16.5 kbp, respectively. Relaxed and aligned into stretched double-strand structures, this would correspond to

lengths of the resulting rodlets of 1.7 μm and 5.3 μm, respectively. Compared with the height of a NPC, which measures ~70 nm along the pore axis (Kubitscheck et al., 1996), the rodlets are 24 and 75 times longer, respectively. This comparison gives an impression of the work the NPCs have to perform in transferring these long DNA structures into the nuclear interior, particularly since they are loaded with vimentin.

Although SV40 and mitochondrial DNA do not represent normal constituents of the eukaryotic cytosol (the one is characteristic of a pathological condition and the other is sequestered in cytoplasmic organelles), it was possible to show with these DNAs that superhelicity is an apparently important requirement for double-stranded DNA to transfer vimentin into the nucleus and that DNA cannot execute this process in its normal B-configuration. However, this generalization had to be modified when it was discovered that clones of certain short fragments of linear double-stranded DNA occurring in the cytosol of mammalian cells still can mediate nuclear vimentin import. These fragments belong to a pool of extrachromosomal DNA elements which have been identified in established tumor cell lines and biopsies of human tumors but not in cells with normal phenotype and biopsies of normal tissues (Abken,

1995; Abken et al., 1993). The general sequence characteristics of the linear extrachromosomal DNA fragments from mouse are very similar to those of specific mouse genomic DNA fragments selectively bound by vimentin IFs in vitro (Wang et al., 1996, and unpublished results). The 375 bp mouse DNA fragment employed in this study can immortalize primary human lymphocytes, has both gene promoter and silencer activity and is present in transfected lymphoid cells as 1 to 5 genome-integrated copies and 100 episomal copies in the cytoplasmic fraction. Since it has recombination-active ends and stop codons in all possible reading frames, immortalization of lymphocytes has been suggested to result from its insertion into genes involved in cellular senescence or programmed cell death (Abken et al., 1993).

Two other linear double-stranded DNA sequences derived from mouse telomere and centromeric γ-satellite DNA also carried FITC-vimentin into the nucleus. Both repetitive, heterochromatic DNA types bind selectively to in vitro reconstituted vimentin IFs when the latter are offered a mixture of mouse genomic DNA fragments (Wang et al., 1996, and unpublished results). The same behavior was shown by a 631 bp mouse LINE DNA fragment. Presumably, many other linear double-stranded DNA elements isolated by affinity binding to

Fig. 5. Confocal laser scanning microscopy of human adenocarcinoma SW13 cells deficient in cytoplasmic IF proteins after their microinjection with complexes consisting of various DNAs and FITC-vimentin of stronger fluorescence labeling than normal. Microinjection of FITC-vimentin complexed with (A) oligo(dG)$_{25}$, (B) supercoiled plasmid pBR322 DNA, (C) relaxed plasmid pBR322 DNA, (D) supercoiled SV40 DNA, (E) relaxed SV40 DNA, (F) 375 bp extrachromosomal linear DNA fragment from the cytosol of Ehrlich ascites tumor cells, (G) single-stranded bacteriophage fd DNA. (H) Control injection of FITC-vimentin alone. The intracellular vimentin distribution was analysed 1 hour p.i. (I) Preinjection of FITC-vimentin into mouse embryo fibroblasts and its incorporation into the endogenous vimentin IF system during a 20 hour incubation period, followed by injection of supercoiled SV40 DNA and fluorescence microscopic analysis of the intracellular FITC-vimentin distribution after another hour. Note the extraction of a substantial amount of FITC-vimentin from vimentin IFs by supercoiled SV40 DNA and its deposition on the surface of nucleoli. All optical sections are through the equatorial plane of the nuclei which are stained with propidium iodide. Green fluorescence: vimentin; red fluorescence: propidium iodide. Bar, 10 μm.
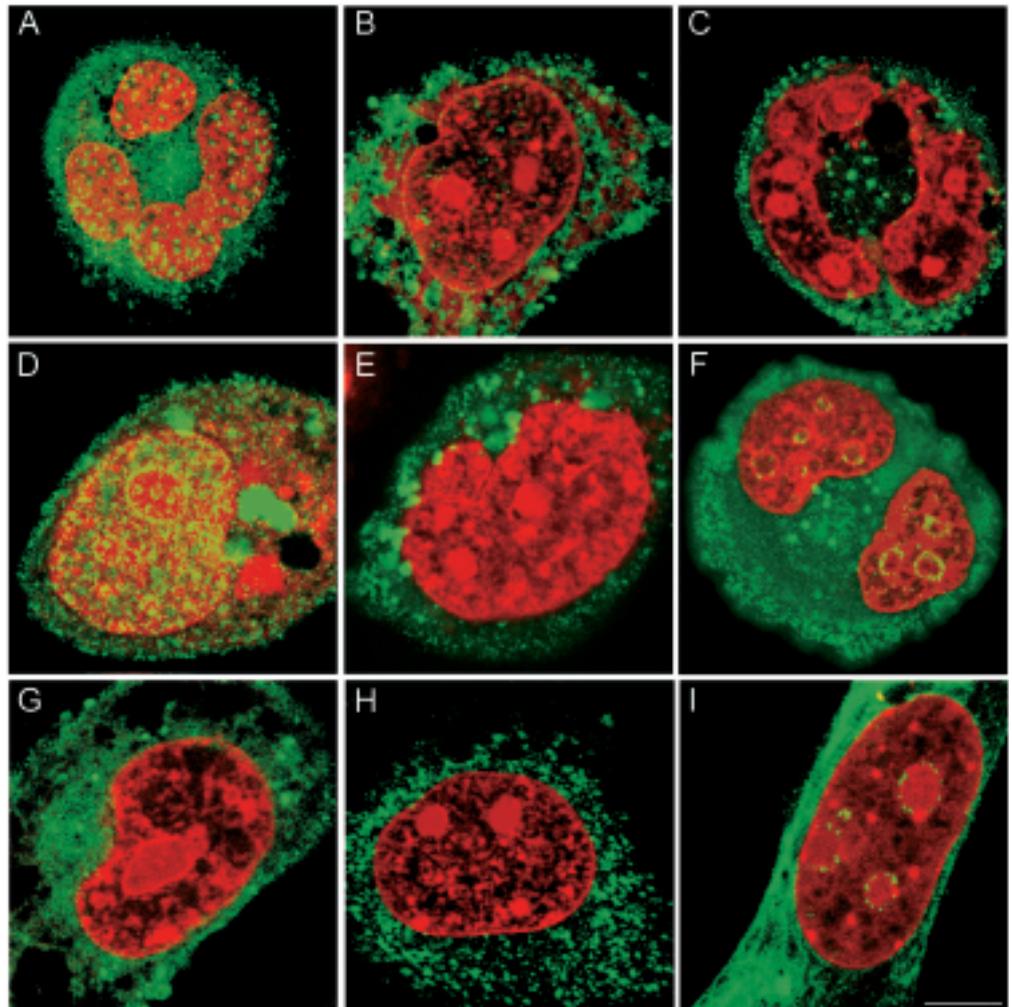
vimentin IFs are capable of mediating nuclear vimentin import. A prerequisite for this activity is that the DNA fragments exhibit a reasonably high karyophilicity to pass the NPCs. This, however, does not seem to generally apply since it has recently been demonstrated that in digitonin-permeabilized cells a fluorescence-labeled 1 kbp linear double-stranded DNA readily migrates into the nucleus in a fashion independent of added cytosolic transport factors but does not accumulate in visible quantities in the nucleus after microinjection into intact cells (Hagstrom et al., 1997). How the double-stranded DNA fragments active in nuclear vimentin transport compare with this DNA in this respect is unknown. Because of their pronounced vimentin carrier activity they either exhibit a substantially higher nuclear migration potential and/or their vimentin-binding capacity is so high that small quantities of DNA are sufficient to transfer detectable amounts of FITC-vimentin into the nucleus. If such linear double-stranded and also relaxed circular DNAs do not have special sequence motives or are not locally folded into certain non-B DNA configurations, they interact with vimentin only very reluctantly and represent therefore very inefficient nuclear vimentin carriers.

The high affinity of vimentin IFs for mouse γ-satellite DNA and other centromeric and pericentromeric DNAs (Wang et al., 1996, and unpublished results) may also provide an explanation for the spatial distribution of FITC-vimentin carried into the nucleus by various DNAs. Very often, fluorescent vimentin was seen to mainly localize to the border region between nucleoplasm and nucleoli in addition to its deposition in the form of differently sized speckles throughout the nucleoplasm. It thus differs clearly from a vimentin construct carrying a NLS which, after permanent transfection of cultured cells, accumulated as prominent filamentous aggregates in interconnected interchromosomal nuclear regions, presumably as complexes with specific nuclear RNAs, coiled bodies and PML bodies (Bridger et al., 1998). Moreover, in the case of oligo(dG)$_{25}$ as transporting DNA, there were indications that FITC-vimentin remained partially associated with its carrier but also became segregated from it and handed over to acceptor sites on the nucleolar surface while the carrier DNA moved on to the interior of the nucleoli. These perinucleolar acceptor sites may be identical with centromeric heterochromatin regions which, dependent on cell type, cell cycle phase and differentiation stage, can be arranged around nucleoli or clustered adjacent to the nuclear envelope (Carmo-Fonseca et al., 1996; Haaf and Schmid, 1991). In the course of time, the FITC-vimentin deposits became dispersed throughout the nucleoplasm and eventually the levels of vimentin in the nuclei decreased; it is, however, not quite clear as yet if residual quantities of vimentin remained stably associated with intranuclear structures. Since vimentin also has a high affinity for ribosomal and heterogeneous nuclear RNA (Traub and Nelson, 1982), it is reasonable to assume that the FITC-vimentin transiently bound to centromeric heterochromatin and other intranuclear structures was slowly picked up by preribosomal and pre mRNP particles on their way to the cytoplasm where it joined preformed IFs or gave rise to its own IF network. Alternatively, the FITC-vimentin and its carrier DNA may have been destroyed by intranuclear proteinases and DNases.

Concerning the intranuclear distribution of imported vimentin, it came as a surprise that a potent affinity-purified polyclonal anti-vimentin antibody in combination with a FITC-conjugated secondary antibody failed to detect the filament protein in the nuclei of fibroblasts from a variety of species although it normally was readily identifiable there via its FITC- or TRITC-label. On the other hand, it was immunologically clearly detectable in the nuclei of PtK$_2$ and MCF-7 epithelial cells microinjected with DNA/vimentin complexes. A possible explanation for this discrepancy is that in the nuclei of fibroblast cells the vimentin was masked whereas in the nuclei of epithelial cells it remained accessible to the antibody. The masking phenomenon observed is not unique to nuclear imported vimentin. For instance, catenin, which also acts as a transcriptional activator when bound to a specific nuclear transcription factor at specific DNA sequences, was reported to be identifiable after nuclear import only via fluorescence label but not with specific antibodies (Fagotto et al., 1998). Moreover, in resinless section immunogold electron microscopy of fibroblasts the nuclear lamina became accessible to specific antibodies only after removal of chromatin and ribonucleoproteins with nucleases (Wang and Traub, 1991), a situation which has also been observed for lamins which form part of a diffuse skeleton ramifying throughout the interior of the nucleus (Hozák et al., 1995). The important conclusion to be drawn from these observations is that the cytoplasmic IF protein vimentin could definitely reside in the nucleus although many previous attempts to localize it there immunologically have failed in the past (see, however, Fidlerová et al., 1992). It is noteworthy that vimentin carrying a NLS and deposited in large quantities in the nucleus after permanent transfection of cultured cells readily reacted with primary and fluorescence-labeled secondary antibodies (Bridger et al., 1998), demonstrating that vimentins attain different intranuclear distributions in dependence on their molecular structure and the mechanism of nuclear transport.

Based on the demonstration that a collection of superhelical, repetitive, mobile and/or extrachromosomal DNAs are particularly active in nuclear import of vimentin, the physiological relevance of this transport activity probably has to be sought in connection with the phenomenon of extrachromosomal circular DNA (eccDNA). eccDNAs represent a population of superhelical molecules of chromosomal origin which in dependence on the cell or tissue of origin and various physiological parameters or growth conditions (for reviews see Abken, 1995; Gaubatz, 1990) are heterogeneous in size, sequence content, sequence organization, subcellular localization and abundance. They occur in both the cytoplasm and the nucleus (Kiyama et al., 1989; Stanfield and Helinski, 1984), the larger molecules being preferentially associated with the nuclear compartment and the smaller ones apparently with the cytosol. Sequences thus far identified in cloned eccDNA fragments include satellite DNA, short interspersed (SINE) and long interspersed (LINE) repeat families, retrovirus-like elements, transposable elements and low- and single copy chromosomal sequences (Gaubatz, 1990). Special reference should be made to the overrepresentation of both interspersed and tandemly repetitive sequences in eccDNAs. If these sequence contents are compared with the sequence characteristics of mouse genomic DNA fragments selected by in vitro reconstituted vimentin IFs (Wang et al., 1996, and unpublished results), a most remarkable

correspondence is noticed. eccDNAs can therefore be expected to show a strong tendency to bind vimentin, particularly since they are under superhelical tension, and to take it along into the nucleus where it may stay associated with its carriers or undergo redistribution according to its affinity for different subnuclear structures. eccDNAs may pick up vimentin from cytoplasmic IFs, possibly assisted by post-translational modification and thus activation of the protein subunits in response to intra- and extracellular signals, or recruit it from the small pool of nonfilamentous vimentin which exists in the cytoplasm of vimentin-positive mammalian cells (Blikstad and Lazarides, 1983; Soellner et al., 1985).

Since eccDNAs are thought to be 'the result of normal cellular mechanisms and processes associated with genomic plasticity, which have been retained in evolution to enable efficient responses to selective environmental pressures as well as to the changing requirements of different tissues and cells during development' (Cohen et al., 1997) and senescence (Gaubatz, 1990), it is tempting to speculate that vimentin accompanying eccDNAs actively participates in these processes. In taking advantage of its characteristic nucleic acid- and histone-binding properties and due to its structural relationships to nuclear DNA-binding and nuclear matrix proteins (Traub and Shoeman, 1994a), vimentin may well function as a factor modifying and preparing chromatin for the recombinatory insertion or deletion of genomic DNA elements. In this context, it is noteworthy that, as molecules structurally closely related to vimentin, lamin B (Ludérus et al., 1992) and lamin A and the muscle-specific IF protein desmin (Ludérus et al., 1994) have been shown to bind nuclear matrix attachment regions (MARs) of genomic DNA in vitro. Moreover, in support of the above contention, a large number of SDS-stable crosslinking products between vimentin and interspersed and tandemly repetitive as well as other mobile DNA elements have been isolated, particularly from cells in states of genomic instability (G. Tolstonog and P. Traub, unpublished results). If vimentin should indeed turn out to be involved in genetic processes with long-term phenotypic effects, it would become intelligible that vimentin knockout mice do not show an obvious phenotype at early stages of their lives (Colucci-Guyon et al., 1994) or in the first few generations after their creation.

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