Nucleic Acid-Binding Activities of the Intermediate Filament Subunit Proteins Desmin and Glial Fibrillary Acidic Protein

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In analogy to experimental results previously obtained with vimentin and neurofilament triplet proteins, the intermediate filament (IF) proteins desmin and glial fibrillary acidic protein (GFAP) were also found to have high capacities to associate with nucleic acids. Employing a collection of native and heat-denatured pro- and eukaryotic DNAs, a series of naturally occurring single-stranded (ss)RNAs and a variety of synthetic polynucleotides of the RNA and DNA type, both proteins could be shown to bind preferentially to single-stranded polynucleotides. In the case of ssDNA and synthetic polyribonucleotides, a clear dependency of the binding activity on the G-content of the nucleic acids was demonstrated. The interaction of desmin with ssDNA and tRNA was characterized by strong cooperativity. When a mixture of desmin and vimentin was offered to excess ssDNA, the cooperativity effect brought about segregation of both protein species into two distinct populations of deoxyribonucleoprotein particles with substantially different sedimentation rates; this segregation is in sharp contrast to the ability of desmin and vimentin to form heteropolymers in filament assembly. In general, desmin and GFAP were found to be similar to vimentin and neurofilament proteins in their nucleic acid-binding properties. However, there were also striking differences between individual non-epithelial IF proteins at this level.

Introduction

Our previous studies on the interaction *in vitro* of IF subunit proteins with other cellular constituents have shown that the mesenchyme-specific protein vimentin has a strong and specific affinity for single-stranded nucleic acids [1–4]. Under physiological ionic conditions, it binds preferentially to ssDNA [3] but complexes with G-rich RNA are stable as well [2]. We have extended these investigations to mammalian neurofilament proteins and demonstrated that all three triplet proteins also have high capacities to associate with single-stranded polynucleotides [4]. While the 68 kD and 145 kD subunits preferentially interact with ssDNA, the 200 kD neurofilament protein appears to have a higher affinity for ssRNA (*e.g.* rRNA). These results were complemented by our recent observation that in competition experiments employing supercoiled, relaxed and linearized plasmid DNA all non-epithelial IF proteins specifically select the superhelical form of DNA as a binding component (unpublished observations). In addition, they form salt-stable complexes with core histones, particularly with the arginine-rich histones H3 and H4 (unpublished observations).

Because DNA and histones almost exclusively occur in the nucleus of eukaryotic cells, the strong and specific affinities of IF subunits and their derivatives for these compounds led us to speculate that IF proteins might eventually have a nuclear function [5–7]. Based on the additional facts that intermediate filaments are associated with Ca^{2+}-sequestering membrane systems (for a review, see [7]) and their subunits are highly vulnerable to Ca^{2+}-dependent, posttranslational modification [8–14], we suggested that IF proteins are involved in the transmission of intra- and extracellular signals from peripheral sites of the cell to the nucleus and, thus, in processes such as DNA replication (mitogenesis), transcription (gene expression) and processing and transport of nuclear RNA [5–7].

With the present investigation, we complete the screening of non-epithelial IF subunit proteins and demonstrate that also in regard to their nucleic acid-

Abbreviations: GFAP, glial fibrillary acidic protein; IF, intermediate filament; NFP, neurofilament protein; EAT, Ehrlich ascites tumor; ss, single-stranded; ds, double-stranded; SDS, sodium dodecyl sulphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; TPCK, N-tosyl-L-phenylalanine chloromethyl ketone.

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binding properties desmin and GFAP are closely related to vimentin and neurofilament proteins.

Materials and Methods

Materials

All synthetic polynucleotides of the RNA and DNA type, salmon testis DNA, chicken erythrocyte DNA and human placenta DNA were purchased from Pharmacia P-L Biochemicals, Uppsala, Sweden. *Bacillus subtilis* DNA was from Calbiochem, San Diego, CA, USA, *Micrococcus luteus* DNA from Serva, Heidelberg, FRG, calf thymus DNA, *Escherichia coli* DNA and *Clostridium perfringens* DNA from Sigma, St. Louis, MO, USA, and *E. coli* 5S rRNA, coliphage MS2 RNA, calf liver tRNA and *E. coli* tRNA from Boehringer Mannheim, Mannheim, FRG. Since the DNAs from *B. subtilis*, chicken erythrocytes, *C. perfringens*, human placenta and *M. luteus* were heavily contaminated with RNAses, they were treated with proteinase K (Serva) and 5 times phenolized in the presence of SDS. All DNA solutions were sonicated at 0 °C to reduce the viscosity and, for denaturation, heated at 100 °C for 10 min and quickly cooled in ice water. The digestion of native calf thymus DNA with nuclease S1 (Bethesda Research Laboratories) was carried out as described previously [3], except that a proteinase K treatment of the digest before phenolization was included. The preparation of polynucleosomal DNA from Ehrlich ascites tumor (EAT) cells followed the methods described by Lawson and Cole [15] and Renz *et al.* [16], including phenolization of proteinase K-treated, sucrose gradient-purified polynucleosomes. Total rRNA was isolated from EAT cell ribosomes by phenolization as described previously [1] and 18S and 28S rRNA were separated by sucrose gradient centrifugation in 10 mM Tris acetate, pH 7.6, 3 mM EDTA. *E. coli* 16S rRNA was obtained from *E. coli* Q13 ribosomes in the same way [17]. Desmin was prepared from porcine stomach smooth muscle [18], GFAP from bovine brain white matter [19] and vimentin from cultured EAT cells [20, 21]. DEAE-Sepharose CL-6B was supplied by Pharmacia, Uppsala, Sweden, Soluene 350 by Packard Instrument International S.A., Zürich, Switzerland, and tritiated amino acid mixture (TRK 440) by Amersham-Buchler, Braunschweig, FRG. All other chemicals were of reagent grade and obtained from Merck, Darmstadt, FRG, Roth, Karlsruhe, FRG and Serva, Heidelberg, FRG.

Cells

EAT cells were propagated in suspension culture in minimum essential medium (MEM) supplemented with 5% heat-inactivated calf serum (HCS) as described previously [22]. The cells were harvested at densities between 1 and 1.5×10^6 cells/ml.

Baby hamster kidney (BHK-21/C13/$A_3$281) cells (kindly provided by Dr. S. H. Revell, Institute for Cancer Research, London, England) were grown in Dulbecco's modified MEM (DMEM) supplemented with 10% fetal calf serum (FCS) in standard-sized roller bottles with 5% CO_2 in the atmosphere [23]. For radioactive labeling, nearly confluent monolayers were first incubated for 10 min with DMEM lacking amino acids (except tryptophan, cysteine and glutamine) and containing 10% FCS dialysed against 0.15 M NaCl (wash medium). Incubation was then continued for 3 h with 10 ml/roller bottle of the same medium containing 0.5 mCi [^{3}H] amino acid mixture. Thereafter, the cultures received another 10 ml portion of wash medium followed by 30 ml of normal DMEM supplemented with 10% FCS after another 3 h interval. After continued culturing for 20 h, the cells were washed at 0 °C with 10 mM Tris acetate, pH 7.6, 0.15 M NaCl (Tris-saline) containing 1 mM PMSF, 0.5 mM TPCK and 1 mM EGTA as proteinase inhibitors. The cells were scraped off the glass wall with a rubber policeman, pelleted by centrifugation at $600 \times g_{av}$ for 10 min in a glass Dounce homogenizer and washed once with Tris-saline containing proteinase inhibitors.

Preparation of [^{3}H] desmin and [^{3}H] vimentin from BHK-21/C13 cells

5.2 g of packed BHK-21/C13 cells grown in the presence of [^{3}H] amino acid mixture were extracted 5 times with a total of 100 ml of 10 mM Tris acetate, pH 7.6, 1 mM EGTA, 4 mM $MgCl_2$, 6 mM 2-mercaptoethanol, 0.5% (w/v) Triton X-100 containing 1 mM PMSF and 0.5 mM TPCK in a tightly-fitting all-glass Dounce homogenizer by 10 strokes each time and interval centrifugation at $600 \times g_{av}$ for 10 min. The combined supernatants were recentrifuged at $30{,}000 \times g_{av}$ for 5 min; the pellet was combined with the major fraction of detergent-resistant residual cell structures. The cell residues were dissolved in 10 mM

Tris acetate, pH 7.6, 3 mM EDTA, 6 mM 2-mercaptoethanol, 6 M urea. The high viscosity due to release of DNA was reduced by shearing of the solution with an Ultra-Turrax (Janke and Kunkel, Staufen, FRG). DEAE-Sepharose CL-6B previously equilibrated with Tris/EDTA/urea buffer was stirred into the solution. The slurry was gently shaken for 1 h and filled into a column on top of a short cushion of equilibrated DEAE-Sepharose CL-6B. The column was briefly washed with Tris/EDTA/urea buffer and bound material eluted with a linear 0 to 500 mM KCl gradient in Tris/EDTA/urea buffer. The gradient volume was at least 10 times the bed volume of the column. The elution of [^{3}H] desmin and [^{3}H] vimentin was followed by SDS-polyacrylamide gel electrophoresis. Desmin- and vimentin-containing fractions were combined and dialysed against Tris/EDTA/urea buffer. The mixture of both filament proteins was further purified by affinity chromatography on arginine methylester-Sepharose 4B [24]. Finally, desmin was separated from vimentin by ssDNA-cellulose chromatography in Tris/EDTA/urea buffer [18]. 4 mg [^{3}H] desmin and 9.1 mg [^{3}H] vimentin with specific activities of 2.45×10^6 cpm/mg and 2.2×10^6 cpm/mg, respectively, were obtained from 5.2 g wet cell pellet. Both proteins were dissolved in 10 mM Tris acetate, pH 7.6, 6 mM 2-mercaptoethanol, frozen in liquid N_2 and stored at −80 °C.

Reactions of desmin and GFAP with nucleic acids and sucrose gradient analysis of the reaction products

650 μg of total EAT cell rRNA was allowed to react with 10 μg of [^{3}H] desmin in 400 μl of 10 mM Tris acetate, pH 7.6, 3 mM EDTA, 6 mM 2-mercaptoethanol (standard reaction buffer) for 30 min at 0 °C. In competition experiments using ds- and ssDNA from calf thymus, additional 500 μg of each competing nucleic acid was included in the reaction mixture. The reaction products were loaded on top of 12.4 ml linear 10 to 30% (w/w) sucrose gradients in standard reaction buffer and centrifuged at $200{,}000 \times g_{av}$ and 2 °C for 14 h in the SW 40 Ti rotor of the Beckman L2 65B centrifuge. 5-drop fractions were collected directly into scintillation vials and mixed with 0.5 ml of Soluene 350 and 10 ml of a toluene-based scintillation cocktail. After radioactivity measurement in a Packard Tri-Carb 460 CD liquid scintillation spectrometer, the radioactivity distributions of the gradients were normalized. During fractionation, the gradients were monitored at a wavelength of 260 nm.

To determine the relative desmin-binding activities of a variety of naturally occurring and synthetic RNAs and DNAs, a constant amount of 155 μg of 28S rRNA was mixed with 125, 250 and 375 μg of the nucleic acid to be examined and 10 μg of [^{3}H] desmin. In the case of some very expensive synthetic polynucleotides, the amounts of the competing nucleic acids were reduced to one half the usual quantities. After standing at 0 °C for 30 min, the reaction products were analysed by sucrose gradient centrifugation as described above. The radioactivities detected in the 28S rRNA peak were normalized and expressed as percentages of the control which was obtained by reaction of [^{3}H] desmin with 28S rRNA alone.

The cooperative binding of desmin to calf thymus ssDNA was analysed using unlabeled protein. 540 μg of desmin was reacted with 200 and 400 μg portions of ssDNA in 500 μl of standard reaction buffer. The binding of 360 μg of desmin to 225 and 450 μg portions of coliphage MS2 RNA was carried out under the same conditions. The reaction mixtures containing ssDNA were centrifuged for 28 h on standard sucrose gradients at $200{,}000 \times g_{av}$ and 2 °C, those containing MS2 RNA for 10 h. When unlabeled desmin and vimentin were allowed to react simultaneously with calf thymus ssDNA, a mixture of 540 μg of desmin and 480 μg of vimentin was added to 1 mg of ssDNA in 600 μl of the standard reaction buffer. Sucrose gradient centrifugation under standard conditions was for 28 h at $200{,}000 \times g_{av}$. Protein distributions were determined by SDS-polyacrylamide gel electrophoresis and scanning of the Coomassie Brilliant Blue-stained gels at a wavelength of 590 nm.

The titration of 375 μg portions of calf liver tRNA with 10 to 800 μg portions of unlabeled desmin was carried out in 400 μl standard reaction mixtures. For the reaction of vimentin and GFAP with 375 μg of calf liver tRNA, 800 μg of each protein was used. Sucrose gradient centrifugation was at $200{,}000 \times g_{av}$ and 2 °C for 40 h.

The interaction of GFAP with nucleic acids was also studied employing unlabeled protein. 650 μg of total EAT cell rRNA was reacted with 425 μg of GFAP either alone or in competition with 500 μg of calf thymus ds- and ssDNA, respectively, in 650 μl of standard reaction buffer. In competition experiments

performed with calf liver tRNA, a mixture of 375 μg of tRNA and 650 μg of rRNA was incubated with 800 μg of GFAP for 30 min at 0 °C. Sucrose gradient centrifugation of the reaction products was at 200,000 × g_{av} and 2 °C for 14 h.

Other methods

Protein concentrations were determined with the Bio-Rad reagent as described by Bradford [25] with bovine serum albumin as a standard. SDS-polyacrylamide slab gel electrophoresis on 9 to 15% gradient gels was carried out as described previously [22].

Results

The nucleic acid-binding properties of desmin were determined employing the same strategy as used for the respective analysis of vimentin [2, 3]. Radioactively labeled desmin isolated from BHK-21 cells was offered to a mixture of 28S rRNA and the polynucleotide to be examined and the distribution of the filament protein among both nucleic acids was determined. Prerequisite for the applicability of this procedure was, firstly, that free desmin sedimented homogenously on sucrose gradients at a relatively slow rate (8 to 9S) and, secondly, that all desmin associated with rRNA in the absence of competing polynucleotides. Both conditions were fulfilled (data not shown). Since desmin shows the strong tendency to aggregate at higher salt concentrations with the formation of fast sedimenting complexes, the competition experiments had to be conducted at low ionic strength and in the absence of di- and polyvalent cations. In this context, however, it is pertinent to refer to the observation that the salt stability of adducts produced from the closely related IF protein vimentin and polynucleotides goes parallel with the affinity the reactants show for each other at low ionic strength [2, 3].

Binding of [^{3}H] desmin to naturally occurring RNAs

We first analysed the association of [^{3}H] desmin with a small collection of naturally occurring RNAs (Fig. 1). It included prokaryotic as well as eukaryotic RNAs, mostly constituents of the protein-synthesizing machinery such as rRNAs and tRNAs. All RNAs tested turned out to be efficient desmin binders but, surprisingly, the tRNA mixtures showed the highest desmin-binding potentials. Calf liver tRNA was

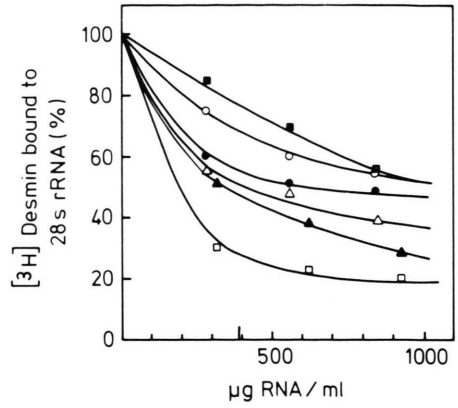

Fig. 1. Competition by EAT cell 28S rRNA and various naturally occurring RNAs for [^{3}H] desmin.
Calf liver tRNA (□——□), EAT cell 18S rRNA (●——●), *E. coli* 16S rRNA (△——△), sonicated coliphage MS2 RNA (○——○), *E. coli* 5S rRNA (■——■), *E. coli* tRNA (▲——▲). The bar on the abscissa indicates the amount of 28S rRNA used in relation to the amounts of the other RNAs. For experimental details, see Materials and Methods.

about twice as effective in interacting with desmin as 28S rRNA. This is in clear contrast to the relatively low capacity of tRNA to associate with vimentin [3] (see also Fig. 9a). Except in the case of tRNA (see Fig. 9b), the sucrose gradient profiles did not reveal any cooperativity of desmin binding (data not shown).

Binding of [^{3}H] desmin to synthetic homopolyribonucleotides

To obtain some information on to what extent the interaction of RNAs with [^{3}H] desmin is dependent on their base compositions, a series of synthetic homopolyribonucleotides was tested for their capability to associate with the filament protein. As depicted in Fig. 2, poly(rA), poly(rC) and poly(rU) were totally incompetent in competing with 28S rRNA for desmin. On the other hand, homopolyribonucleotides like poly(rG), poly(rX) and poly(rI) turned out to be extremely efficient competitors. Furthermore, although poly(rU) was only a weak desmin binder, the substitution of oxygen in position C-4 of uracil by sulfur brought about an enormous increase in the desmin-binding capacity of the respective homopolymer. From these results it can be generalized that, with the exception of uracil, nuclear bases with an oxygen or sulfur atom in posi-

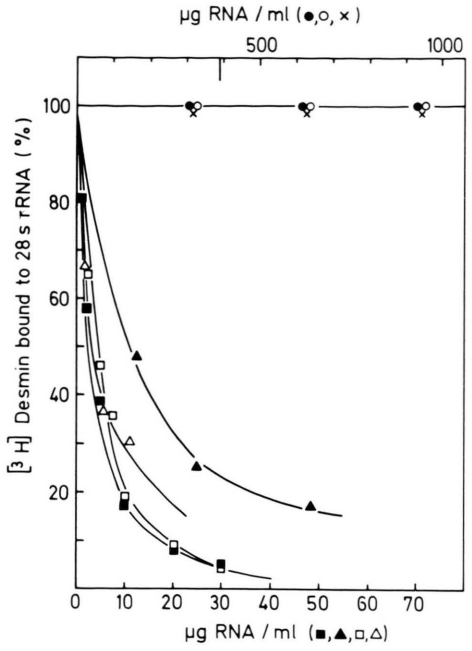

Fig. 2. Competition by EAT cell 28S rRNA and various synthetic RNA homopolymers for [^{3}H] desmin. Poly(rA) (●——●), poly(rC) (○——○), poly(rU) (×——×), poly(rI) (▲——▲), poly(rS^{4}U) (△——△), poly(rG) (■——■), poly(rX) (□——□). The bar on the upper abscissa indicates the amount of 28S rRNA used in competition with the amounts of the various RNA homopolymers. For experimental details, see Materials and Methods.

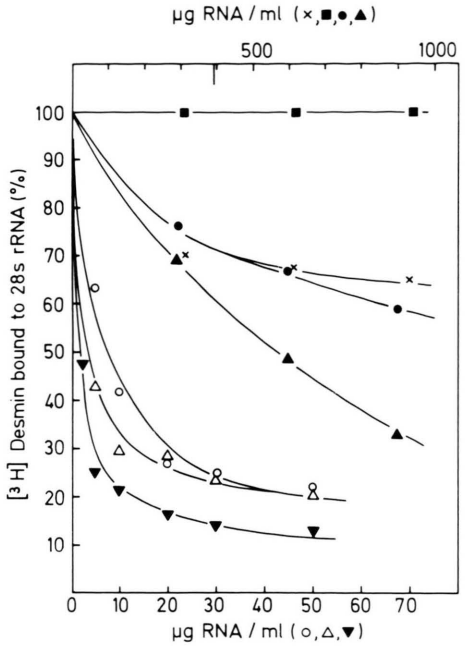

Fig. 3. Competition by EAT cell 28S rRNA and various RNA heteropolymers and RNA duplexes for [^{3}H] desmin. Poly(rA)·poly(rU) (■——■), poly(rI)·poly(rC) (×——×), poly(rIC) (●——●), poly(rAU) (▲——▲), poly(rAG) (○——○), poly(rUG) (△——△), poly(rAUG) (▼——▼). The bar on the upper abscissa indicates the amount of 28S rRNA used in relation to the amounts of the various synthetic RNAs. For experimental details, see Materials and Methods.

tion C-4 of pyrimidines and position C-6 of purines render polyribonucleotides particularly potent in desmin binding.

Binding of [^{3}H] desmin to synthetic RNA duplexes and heteropolyribonucleotides

The examination of the binding of [^{3}H] desmin to synthetic RNAs possessing variable degrees of double-strandedness promised to throw some light on the influence of the secondary structure of RNAs on the binding reaction. As expected on the basis of data previously obtained with vimentin [2], poly(rA)·poly(rU) was totally inactive in the standard competition assay, whereas poly(rI)·poly(rC) was moderately active (Fig. 3). This result shows that the desmin-binding capacity of a highly active homopolyribonucleotide (*e.g.* poly(rI)) is drastically reduced when the polymer is incorporated into a double-stranded RNA duplex.

Concerning the desmin-binding activities of RNA heteropolymers, no general rules could be derived from the results obtained (Fig. 3). Whereas poly(rI) was dramatically reduced in its desmin-binding potential when rI was partially substituted by rC (poly(rIC)), poly(rAU) had a significantly higher desmin-binding capacity than could be expected from the activities of its corresponding homopolyribonucleotides poly(rA) and poly(rU) (Fig. 2). However, the copolymerization of rG with rA and/or rU resulted in the formation of heteropolymers showing only little difference in their desmin-binding activities (Fig. 3) to poly(rG) (Fig. 2).

Interaction of [^{3}H] desmin with naturally occurring DNAs

Fig. 4 shows the distribution of [^{3}H] desmin among total EAT cell rRNA and native and heat-denatured calf thymus DNA, respectively. It is clear from the

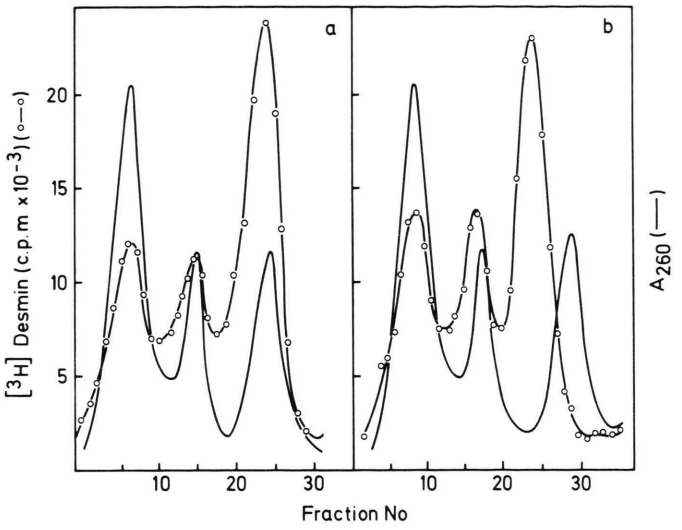

Fig. 4. Competition by total EAT cell rRNA and double-stranded (panel a) and single-stranded (panel b) calf thymus DNA for [^{3}H] desmin. For experimental details, see Materials and Methods.

distribution profiles presented that both forms of DNA bound a substantial fraction of the added desmin. This finding is in distinct contrast to the situation seen with vimentin which was shown to have a preference for ssDNA [3]. Moreover, it is striking that the association products of desmin with ssDNA sedimented far ahead of the bulk of free ssDNA, indicating cooperative interaction of both components (Fig. 4b); no such effect was observed with dsDNA (Fig. 4a).

To throw light on this differential behavior of desmin and vimentin and to explore whether the unexpectedly high affinity of desmin for native calf thymus DNA is also seen with other DNAs, a variety of pro- and eukaryotic DNAs were examined. From the results summarized in Fig. 5, it appears that native

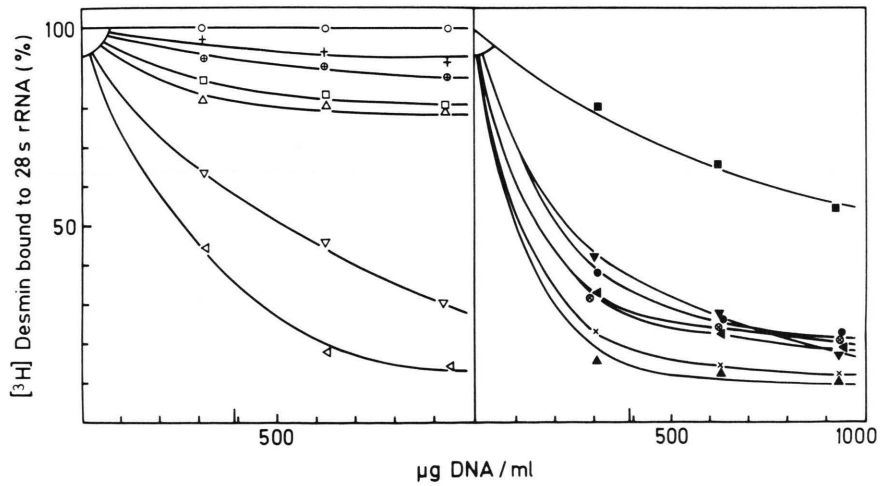

Fig. 5. Competition by EAT cell 28S rRNA and various naturally occurring DNAs in their native (panel a) and heat-denatured (panel b) forms for [^{3}H] desmin.
Salmon sperm DNA: native (○——○), denatured (●——●); EAT cell polynucleosomal DNA: native (+——+), denatured (×——×); chicken erythrocyte DNA: native (⊕——⊕), denatured (⊗——⊗); Clostridium perfringens DNA: native (□——□), denatured (■——■); *Micrococcus luteus* DNA: native (△——△), denatured (▲——▲); human placenta DNA: native (▽——▽), denatured (▼——▼); calf thymus DNA: native (◁——◁), denatured (◀——◀). The bar on the abscissa indicates the amount of 28S rRNA used in competition with the amounts of the various DNAs. For experimental details, see Materials and Methods.

human placenta DNA also had a desmin-binding capacity above average. One possibility which might explain this unusually high potency of the two native DNAs was that they either were contaminated with ssRNA or sonication, for the purpose of viscosity reduction, caused their partial denaturation with the production of single-stranded regions. However, pancreatic RNAse and nuclease S1 treatment of sonicated native calf thymus DNA did not reduce its strong desmin-binding activity (data not shown), thus leaving the above problem unsolved. The remaining DNAs examined had low to moderate desmin-binding activities when tested in their native configurations (Fig. 5a) but moderate to high binding potentials in their heat-denatured forms (Fig. 5b). Heat-denatured *M. luteus* DNA with a (G + C) content of approximately 70% had a desmin-binding potential at least one order of magnitude higher than that of heat-denatured *C. perfringens* DNA with a (G + C) content of about 30%. This indicates that, as in the case of vimentin [3], the desmin-binding capacity of single-stranded, naturally occurring DNAs is a function of their (G + C) content.

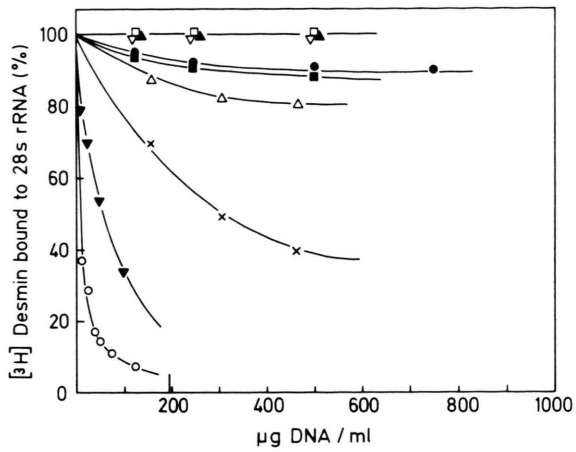

Fig. 6. Competition by EAT cell 28S rRNA and various DNA homopolymers, DNA heteropolymers, DNA duplexes and alternating copolymeric DNAs for [^{3}H] desmin. Poly(dA) (□——□), poly(dT) (■——■), poly(dC) (●——●), poly(dG) (○——○); poly(dG,dC) (▼——▼); poly(dA)·poly(dT) (▲——▲), poly(dG)·poly(dC) (×——×); poly(dA−dT)·poly(dA−dT) (▽——▽), poly(dG−dC)·poly(dG−dC) (△——△). The bar on the abscissa indicates the amount of 28S rRNA used in competition with the amounts of the other polydeoxyribonucleotides. For experimental details, see Materials and Methods.

Interaction of [^{3}H] desmin with synthetic polydeoxyribonucleotides

With the use of some homo- and heteropolydeoxyribonucleotides, DNA duplexes and strictly alternating double-stranded copolymers as reactants in the standard competition assay (Fig. 6), the binding properties of desmin were found to be very similar to those previously reported for vimentin [2]. Among the homopolydeoxyribonucleotides, poly(dG) showed an extremely high desmin-binding activity, whereas the other homopolymers proved to be only weak competitors. This observation readily explains the dependency of the desmin-binding potentials of heat-denatured, naturally occurring DNAs on their (G + C) contents (Fig. 5b). However, when poly(dG) was annealed to poly(dC), the resulting duplex showed a substantially diminished activity, in contrast to the heteropolymer poly(dG,dC) which was still a potent desmin-binder. The strictly alternating copolymer poly(dG−dC)·poly(dG−dC), on the other hand, exhibited a comparably low desmin-binding activity. The almost complete suspension of the interaction of polydeoxyribonucleotides with desmin by annealing them to their complementary polynucleotide strands could also be demonstrated for the poly(dT) → poly(dA)·poly(dT) transition.

Cooperative interaction of desmin and vimentin with ssDNA

The result of the competition experiment described in Fig. 4b indicated that desmin associated only with a limited number of DNA molecules in a cooperative manner when offered to a large excess of ssDNA. To further examine this, unlabeled desmin was reacted with heat-denatured calf thymus DNA in the absence of any competing nucleic acid. Figs. 7b and c show that the appearance of a distinct, faster sedimenting population of deoxyribonucleoprotein particles was accompanied by a reduction in the amount of free ssDNA. Within certain concentration limits, the quantity and sucrose gradient position of the faster sedimenting deoxyribonucleoprotein particles were independent of the original amount of ssDNA having been reacted with a constant amount of desmin. In addition, keeping the amount of ssDNA constant and varying the concentration of desmin, virtually all ssDNA could be transferred into

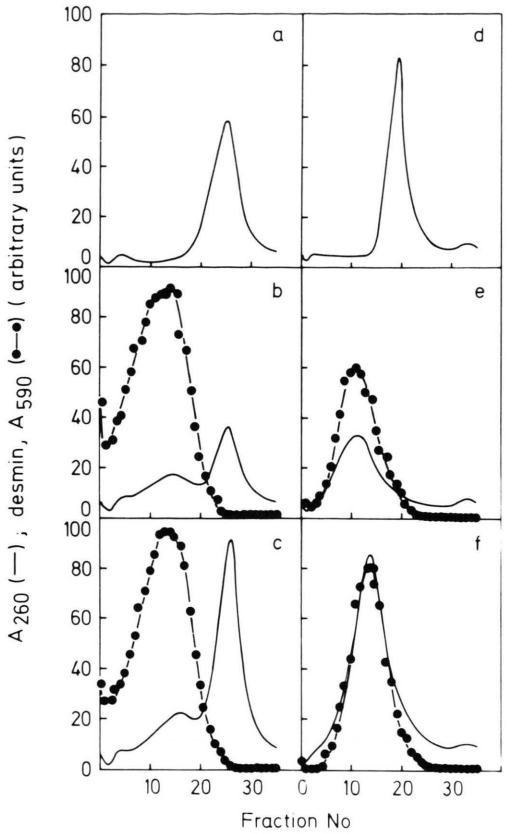

Fig. 7. Cooperative interaction of desmin with calf thymus single-stranded DNA but not with coliphage MS2 RNA. Sucrose gradient analyses of the reaction products.
Panel a: control profile of heat-denatured calf thymus (ss)DNA; panel b: sedimentation profile of the ssDNA-desmin association products; panel c: the same as panel a but with twice the amount of ssDNA in the reaction mixture; panels d to f: parallel experiment performed with single-stranded coliphage MS2 RNA and a somewhat reduced amount of desmin. The distribution of unlabeled desmin was determined by SDS-polyacrylamide gel electrophoresis and scanning of the Coomassie Brilliant Blue-stained gels at 590 nm. For experimental details, see Materials and Methods.

the nucleoprotein particles (data not shown). Interestingly, this cooperativity effect was not observed with single-stranded coliphage MS2 RNA (Fig. 7d to f) or 18S and 28S rRNA (data not shown). However, the sedimentation rates of all these RNAs were steadily and uniformly increased by enhancing amounts of the filament protein.

In previous experiments, it was noticed that vimentin also binds cooperatively to ssDNA [3], but the sedimentation coefficient of the resulting adducts was substantially lower than that of the corresponding desmin-ssDNA aggregates. It was of interest, therefore, to investigate the sedimentation behavior of ssDNA as well as of desmin and vimentin when both proteins were allowed to react simultaneously with excess ssDNA. As shown in Fig. 8, the changes in the sedimentation pattern of ssDNA induced by desmin (Fig. 8a) and vimentin (Fig. 8c) individually were exactly maintained when both proteins reacted at the same time with heat-denatured calf thymus DNA (Fig. 8b). It is clear from this result that the interaction of ssDNA with a mixture of desmin and

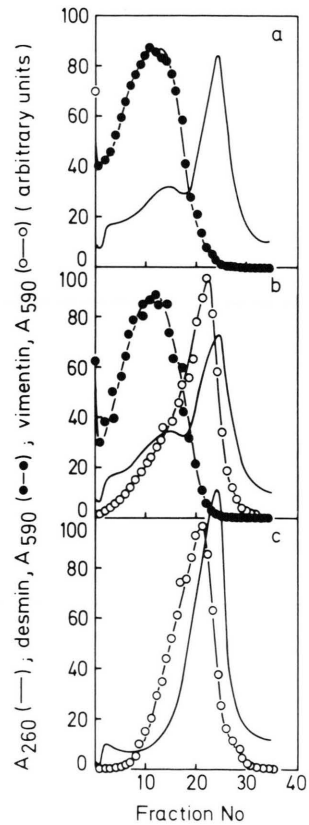

Fig. 8. Segregation of desmin and vimentin into two distinct populations of deoxyribonucleoprotein particles upon reaction with calf thymus single-stranded DNA. Sucrose gradient analyses of the reaction products.
Panel a: reaction of calf thymus ssDNA with desmin; panel b: reaction of calf thymus ssDNA with a 1:1 mixture of desmin and vimentin; panel c: reaction of calf thymus ssDNA with vimentin. The distributions of the IF proteins in the sucrose gradients were determined by SDS-polyacrylamide gel electrophoresis and scanning of the Coomassie Brilliant Blue-stained gels at 590 nm. For experimental details, see Materials and Methods.

vimentin leads to segregation of both proteins into two distinct populations of deoxyribonucleoprotein particles with considerably different sedimentation rates.

Cooperative interaction of desmin with calf liver tRNA

Thus far, our studies on the interaction of vimentin [3] and neurofilament proteins [4] with nucleic acids have limited the above cooperativity effect to ssDNA. Since vimentin and desmin are structurally closely related molecules [26–30], it came as a surprise that desmin also showed cooperative interaction with tRNA. When a constant amount of calf liver tRNA was titrated with unlabeled desmin, progressively increasing quantities of association products with a distinct sedimentation coefficient were formed at the expense of free tRNA; all desmin was incorporated into the aggregates which on sucrose gradients sedimented ahead of free tRNA (Fig. 9b). By contrast, vimentin was only partially complexed by tRNA with the production of aggregates which cosedimented with free tRNA; the larger part of vimentin sedimented in its free form at 8 to 9S (Fig. 9a).

Interaction of GFAP with nucleic acids

In default of radioactively labeled protein, the binding experiments were carried out employing unlabeled GFAP from bovine brain white matter. In sucrose gradient centrifugation at low ionic strength, the protein could be shown to be homogenous and to have a sedimentation coefficient of 8 to 9S (Fig. 10a). As illustrated in Fig. 10b, it underwent a dramatic redistribution when it was reacted with total EAT cell rRNA; the protein was quantitatively bound to both 18S and 28S rRNA species. In competition experiments employing total rRNA and heat-denatured and native calf thymus DNA, respectively, GFAP showed a low but significant reactivity with ssDNA (Fig. 10d) but only very little interaction with dsDNA (Fig. 10f).

Another characteristic feature distinguishing GFAP from vimentin was the high capacity of the glial filament protein to bind to calf liver tRNA. In competition with total EAT cell rRNA, tRNA was the more efficient GFAP-binding component (data not shown). In this respect, GFAP was more closely related to desmin than to vimentin which showed only a very low tRNA-binding activity (Fig. 9a) [3]. These relationships also became evident when the reactions of vimentin, desmin and GFAP with tRNA were performed in the absence of competing rRNA. The IF protein distribution profiles of Fig. 9 demonstrate that only desmin and GFAP quantitatively associated with tRNA and that the reaction in both cases was characterized by cooperativity; although with respect to the sedimentation rates of the result-

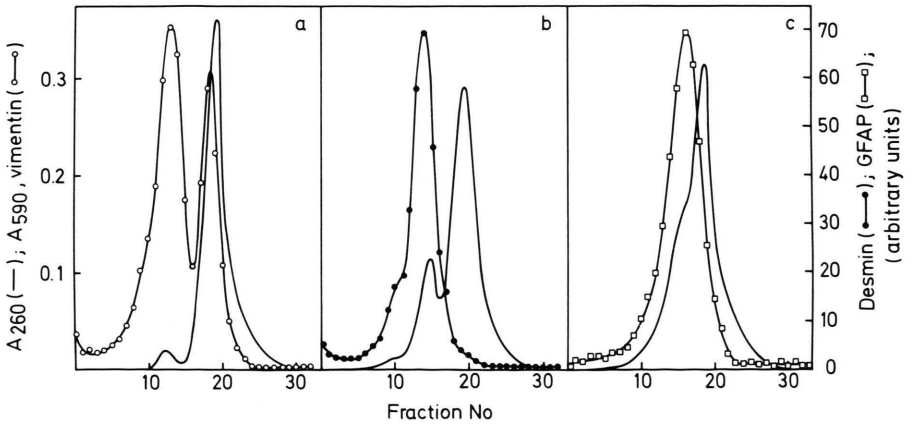

Fig. 9. Binding of vimentin (panel a), desmin (panel b) and GFAP (panel c) to calf liver tRNA and sucrose gradient analyses of the reaction products.
The distributions of the IF proteins in the sucrose gradients were determined by SDS-polyacrylamide gel electrophoresis and scanning of the Coomassie Brilliant Blue-stained gels at 590 nm. Note that in panel a most of the vimentin sedimented in its free form with a sedimentation coefficient of 8 to 9S. For experimental details, see Materials and Methods.

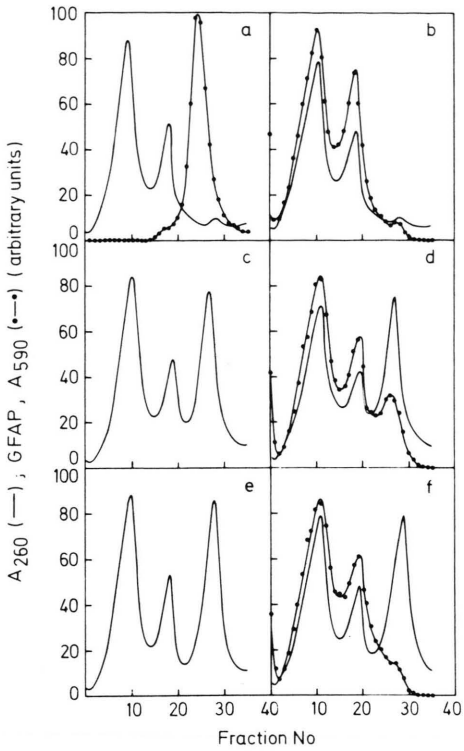

Fig. 10. Sucrose gradient analyses of the association products formed during the reaction of GFAP with total EAT cell rRNA in competition with heat-denatured and native calf thymus DNA.
Panel a: total rRNA and GFAP centrifuged on separate sucrose gradients; panel b: sedimentation profile of the association products formed from GFAP and total rRNA; panel c: optical density profile of a mixture of total rRNA and heat-denatured DNA; panel d: sedimentation profile of the association products formed during the competitive interaction of total rRNA and heat-denatured DNA with GFAP; panel e: optical density profile of a mixture of total rRNA and native DNA; panel f: sedimentation profile of the association products formed during the competitive interaction of total rRNA and native DNA with GFAP. The distribution of GFAP in the sucrose gradients was determined by SDS-polyacrylamide gel electrophoresis and scanning of the Coomassie Brilliant Blue-stained gels at 590 nm. For experimental details, see Materials and Methods.

ing adducts the effect observed with GFAP (Fig. 9c) was less dramatic than with desmin (Fig. 9b).

Discussion

The detailed biochemical analysis of the mesenchyme-specific IF protein vimentin [1–3] and the nerve-specific neurofilament proteins NFP68, NFP145 and NFP200 [4] has identified these polypeptides as nucleic acid-binding proteins with preferential affinities for single-stranded polynucleotides. Since the protein chemical comparison of the various subclasses of IF proteins has demonstrated a close structural relationship between individual polypeptides [31], the muscle-specific IF protein desmin and the glia-specific GFAP were expected to be nucleic acid-binding proteins as well. The results of the present investigation clearly confirm this supposition.

Particularly desmin could be shown to interact preferentially with the single-stranded forms of a collection of synthetic and naturally occurring pro- and eukaryotic nucleic acids. Except for its strong, cooperative interaction with pro- and eukaryotic tRNAs, desmin otherwise proved to be very similar to its close relative vimentin [26–30] in its nucleic acid-binding properties.

First experiments performed to characterize GFAP have shown that this protein also has affinity for RNAs and DNAs. A more comprehensive investigation was impossible because of the unavailability of radioactively labeled GFAP. Such material could be isolated from glioma cells cultured in the presence of [^{3}H] amino acids but it rapidly lost its capacity to interact with nucleic acids upon storage. In any event, the quantitative reaction of unlabeled GFAP with total rRNA, its low but significant affinity for ssDNA and its nearly complete inability to associate with dsDNA in the standard competition assay show that its nucleic acid-binding properties are similar to those of the other non-epithelial IF subunit proteins.

An interesting observation made during the course of these nucleic acid-binding studies relates to the cooperativity of the interaction of desmin with ssDNA. With increasing amounts of desmin bound to excess ssDNA, increasing quantities of faster sedimenting deoxyribonucleoprotein particles appeared on the sucrose gradients concomitant with a reduction in the amount of free ssDNA. This observation might be explained on the assumption that desmin "saturated" a limited number of ssDNA molecules thus rendering this fraction of DNA faster in sedimentation and leaving the sedimentation rate of those DNA molecules unchanged which did not react with the protein. This cooperativity effect very likely is the result of two different binding reactions: basically, the protein molecules interact with ssDNA through their nucleic acid-binding site located in the non-α-helical, N-terminal polypeptide [5] and, secondly, with each other, in lateral alignment, through

domains somehow protruding from the DNA backbone. This interaction might be related to that being functional in the side-by-side association of IF subunit proteins during filament assembly [32–34]. It remains to be seen, however, whether individual protofilament units line up one by one on the ssDNA molecules or whether the discontinuously proceeding formation of faster sedimenting deoxyribonucleoprotein particles is due to the binding of preexisting aggregates of protofilaments. Since under identical reaction conditions the sedimentation rate of nucleoprotein particles obtained from MS2 RNA or rRNA increased steadily and uniformly in the presence of enhancing amounts of desmin, the first mechanism seems to apply. Very likely, during such a titration experiment, G-rich base sequences will preferentially react with desmin and therefore appear first in the population of fast sedimenting nucleoprotein particles; however, this selectivity will be optimally expressed only when the ssDNA molecules are relatively short.

The above cooperativity effect appears allthemore interesting as it was discovered that reaction of ssDNA from calf thymus or other sources with a mixture of desmin and vimentin causes segregation of both protein species into two distinct populations of deoxyribonucleoprotein particles with substantially different sedimentation rates. Obviously, only molecules of the same protein species are capable of laterally associating with one another once they are bound to ssDNA. Molecules of the other protein species are strictly excluded from this association and forced to form their own population of ssDNA-protein adducts. In this context, it is noteworthy that the segregation of desmin and vimentin during interaction with ssDNA is in sharp contrast to the capability of both protein species to form heteropolymers in intermediate filament assembly [35–37]. Both processes, the formation of filaments as well as of association products with ssDNA start out from the tetrameric form of IF subunit proteins. While the tetrameric protofilaments are generally accepted as the actual building blocks of intermediate filaments [32–34], their fate during association with nucleic acids is still obscure. Finally, the observation should be mentioned that not only desmin and vimentin show the above segregation effect with ssDNA but also other pairs of IF subunit proteins such as desmin and GFAP (data not shown) or the neurofilament proteins NFP68 and NFP145 [4].

With the present investigation, we have, for the time being, completed the characterization of non-epithelial IF proteins with respect to their nucleic acid-binding activities and it may be appropriate at this point to make some critical and general remarks on the experimental results thus far obtained. Although the binding of IF proteins to nucleic acids rests to a considerable extent on the electrostatic interaction of their arginine-rich N-termini [27, 28, 31, 34, 38–42] with the negatively charged polynucleotide backbones, other more specific binding components must play a role as well. Major support for this contention comes from the observation that IF proteins interact preferentially with single-stranded nucleic acids. In this respect, they are very similar to helix-destabilizing proteins notably of prokaryotic origin, such as *E. coli* single-strand-binding protein, bacteriophage T4 gene 32 protein or bacteriophage T7 ssDNA-binding protein [43, 44]. Despite the accessibility of the phosphate groups in dsDNAs for instance, these show, in general, only a very low reactivity with IF proteins ([2–4] and this investigation). Probably, the free nucleobases of a single-stranded polynucleotide play an important part in its association with IF proteins, a conjecture that appears to be sustained by the influence of the base composition of single-stranded nucleic acids on the binding reaction ([2, 3] and this investigation). It should be noted, however, that in general, ssDNA-binding proteins bind without base or sequence specificity. It was also found in our laboratory that all non-epithelial IF proteins selectively interact with supercoiled DNA when they are reacted with a mixture of superhelical, relaxed and linearized plasmid DNA (unpublished observations). In any event, the fact that different nucleic acids vary in their relative affinities for IF proteins by more than two orders of magnitude ([2, 3] and this investigation) is difficult to explain as the result of a mere electrostatic interaction. The function of the N-terminal arginine residues rather appears to lie in the transitory electrostatic stabilization of the complexes initially formed during collision of the reactants until specific interactions become established.

In contrast to our previous assumption that the N-terminal arginine residues are almost totally dispensable for the association of non-epithelial IF proteins with nucleic acids [5], it is our notion today that they are required at least in part. IF proteins possess such high negative net charges that the specific binding

components of their association products with polynucleotides are probably not strong enough to overcompensate for the coulombic repulsion between both reactants. Our previous contention was based on the known amino acid sequence of vimentin [30, 31] and on the employment of SDS-polyacrylamide gel electrophoresis for the determination of the apparent molecular weights of N-terminal cleavage products of vimentin created by Ca^{2+}-activated proteinase [5]. However, with the knowledge of the precise cleavage sites used by this enzyme in the N-terminus of vimentin [29], a more precise characterization of the resulting breakdown products with respect to the number of N-terminal arginine residues was possible. It thus became clear that N-terminally truncated vimentin molecules need at least 5 out of 11 arginine residues for efficient binding to nucleic acids. In this regard, it might be of great physiological relevance that the proteolytic processing of vimentin in its N-terminal head region can lead to the formation of polypeptides which still bind efficiently to ssDNA but which are totally incapable of assembling into intermediate filaments under physiological ionic conditions [24].

Another characteristic property of IF proteins which is shown by many nucleic acid-binding proteins is their cooperative binding to single-stranded polynucleotides, mostly to ssDNA. This can be considered as further support for the specific interaction of IF proteins with nucleic acids. If such polypeptides established only unspecific, electrostatic relationships with polynucleotides, random aggregation with all nucleic acid molecules of a certain population should be expected. However, even at very low ionic strength, a condition that should "freeze" electrostatic associations once they have formed, excess IF proteins tend to "saturate" a limited number of nucleic acid molecules with the formation of fast sedimenting nucleoprotein particles leaving the remainder of molecules unaffected ([3, 4] and this investigation). Interestingly, the reaction of pairs of different IF subunit proteins with ssDNA leads to segregation of both protein species into two distinct populations of deoxyribonucleoprotein particles ([4] and this investigation). Such an effect should not be demonstrable if the IF proteins reacted with ssDNA in a random, electrostatic fashion. It is clear that the cooperativity of such association reactions is, to a considerable extent, due to specific protein-protein interactions. Since this type of specificity is not observed at the level of intermediate filament formation [35–37], its expression when different IF proteins interact simultaneously with ssDNA might be of considerable physiological significance.

Although the various non-epithelial IF proteins are very similar in their interactions with polynucleotides, they also have nucleic acid-binding properties which are not shared by all of them. The strong tendency of desmin and GFAP to associate with tRNA in a cooperative manner should be particularly stressed (this investigation). Another feature distinguishing GFAP together with the 200 kD neurofilament protein from vimentin [3], the two smaller neurofilament triplet proteins [4] and, though to a lesser extent, also from desmin (this investigation), is their preferential association with ssRNA (this investigation). These differential nucleic acid-binding capacities might be interpreted to indicate that the various IF proteins fulfil different functions, possibly at different sites, in eukaryotic cells. In any case, the functional diversity of non-epithelial IF proteins at the nucleic acid-binding level is in clear contrast to their uniform capacity to assemble into morphologically identical intermediate filaments. Whether these functions are fulfilled in the nucleus or/and in the cytoplasm remains to be elucidated. It is our notion, however, that not the intact IF subunit proteins are charged with specific functions in eukaryotic cells but rather their posttranslational derivatives. Under the ionic conditions of the cytosol, IF proteins can only exist in the form of highly insoluble intermediate filaments and only posttranslational derivatization can transform them into independent, functionally active entities. In this sense, the nucleic acid-binding properties of IF proteins described thus far must be considered as *potential* activities which might be preserved during posttranslational processing.

However, it remains a major question whether or not eukaryotic cells actually make use of the nucleic acid-binding potentials of their IF proteins. As long as the biological role of these proteins is totally unknown, our findings might be helpful in designing experimental approaches to solve this problem. The facts that (1) cytokeratins can be covalently linked to nuclear DNA under *in vivo* conditions [45], (2) other proteins of known nucleic acid-binding activity readily form filaments under certain ionic conditions [46, 47], (3) nuclear lamins with their homologies in primary and secondary structure to intermediate filament proteins [48] associate with chromatin [49, 50],

and (4) IF proteins have high affinities for core histones *in vitro* (our unpublished observations) might make one optimistic in this regard.

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