The interaction in vitro of the intermediate filament protein vimentin with naturally occurring RNAs and DNAs

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The binding of the intermediate filament protein vimentin to a variety of naturally occurring RNAs and DNAs was studied. The relative capacities of the various nucleic acids to associate with pure [3H]vimentin were determined in competition experiments with 28S rRNA from Ehrlich ascites tumor cells. The reaction products were analyzed by sucrose gradient centrifugation at low ionic strength and in the presence of EDTA. Under these ionic conditions, vimentin reacted preferentially with single-stranded nucleic acids, particularly with those of high (G + C) content. The vimentin binding potentials of single-stranded RNAs and DNAs were largely comparable. However, when the concentrations of mono- and divalent cations were raised to physiological and higher values, only single-stranded DNA retained its vimentin binding capacity. With increasing KCl concentrations at 0 to 1 mM Mg2+, increasing amounts of vimentin were detected in complexes which sedimented considerably faster than the bulk of the DNA, suggesting cooperative binding of vimentin. The salt optimum of this cooperativity was at 200 mM KCl. Thus, the capability of vimentin to discriminate between single-stranded RNA and DNA under physiological ionic conditions points to specificity of the interaction of vimentin with nucleic acids.

Since the first observations of intermediate filaments in vertebrate cells about 20 years ago (see, for instance, Refs. 1 and 2), it has been postulated that they play some, as yet, undefined role in the construction of the cytoskeleton. This assumption has been based on the insolubility of their subunit proteins under physiological conditions and their characteristic three-dimensional distribution in the cytoplasm (for reviews see Refs. 3-5). As a result of electron and immunofluorescence microscopic examinations of intact cells and detergent-resistant cytoskeletal frameworks, it has been postulated that intermediate filaments are, in conjunction with microfilaments, microtubules, and microtubular, responsible for such functions as the anchorage of the nucleus in the cell (6-9), maintenance of cell shape (7, 10, 11), spatial organization of cytoplasmic organelles (12-17), and cell division (18-21). In this connection, it is assumed that the ordered spatial arrangement of cell organelles is a prerequisite for the coordinated sequence of cellular events.

However, to date, there is little biochemical or functional evidence to support this hypothesis. In contrast, evidence has been presented recently which makes it increasingly doubtful that intermediate filaments fulfill cytoskeletal roles as their major function. For instance, during very early stages of embryonic development, cells were found to be totally free of intermediate filaments as judged by indirect immunofluorescence microscopy and polyacrylamide gel electrophoresis (22, 23). Moreover, in various tissues of mature vertebrates, no or only minute amounts of certain types of intermediate filaments could be detected. The intermediate filament systems developed only when these cells were taken into cell culture (1, 24-27). On the other hand, tissues, in which even in the presence of proteinase inhibitors neither vimentin nor desmin could be detected, possess high activities of a vimentin- and desmin-specific, Ca2+-activated proteinase (28). This may indicate that vimentin and desmin are synthesized and rapidly turned over but not used for the construction of intermediate filaments. The most convincing criticism directed against the cytoskeletal function of intermediate filaments, at least in in vitro grown cells, has come quite recently from studies on the effect of microinjection of antibodies raised against intermediate filament subunit proteins into living cells (29-31). Employing indirect immunofluorescence microscopy, it was shown that the intermediate filament meshwork, which was originally distributed throughout the cytoplasm, had collapsed around the nucleus. However, the cells stayed alive, showing that the normal morphology and activities of the cells such as adherence to the substratum, locomotion, mitosis, cytokinesis, and saltatory movement of intracellular vesicles, granules, and mitochondria were independent of the distribution of intermediate filaments.

Since vimentin and its corresponding Ca2+-activated proteinase were highly conserved during evolution (28, 32) and this is very likely also true for other intermediate filament subunit-proteinase combinations—this system must play an important role in the life cycle of vertebrate cells. In order to shed light on its involvement in cellular processes, it is important now to investigate its function at the biochemical and molecular biological level rather than at the morphological level.

We have recently shown that the extraction of Ehrlich ascites tumor cells with Triton X-100 at low ionic strength and in the presence of Mg2+ results in the formation of Triton X-100-resistant residual cell structures in which virtually all vimentin and a substantial fraction of the polyribosomes are retained. Lowering of the Mg2+ concentration causes dissociation of the detergent-resistant cell residues into their individual constituents which then can be separated by differential and sucrose gradient centrifugation. Whereas in the presence of Mg2+ ribosomes and vimentin sediment separately on sucrose gradients (33), after chelation of Mg2+ by EDTA there is a close association of vimentin with the unfolded ribosomal subunits (34, 35).
Further experimentation has revealed that vimentin binds to the RNA moiety of the ribosomes with high affinity, provided that the ionic strength and the concentrations of divalent and polyvalent cations in the reaction medium are low. Certain characteristics of the reaction between partially purified vimentin and isolated ribosomes and rRNA, respectively, in the presence and absence of urea and ethidium bromide, point to an as yet undefined role of the secondary structure of the rRNA in this binding reaction. To further substantiate this indication, we tested the vimentin-binding properties of a variety of naturally occurring single- and double-stranded ribo- and deoxyribonucleic acids. The results of this investigation, which are reported here, fully support our previous findings obtained with rRNA. They show that vimentin is a nucleic acid-binding protein which preferentially interacts with single-stranded nucleic acids and, under physiological ionic conditions, discriminates between single-stranded RNA and DNA in favor of DNA.

**EXPERIMENTAL PROCEDURES**

**Materials**

\(^{3}H\)-labeled vimentin was prepared as described previously (36). The protein concentration was 1.5 mg/ml, 10 mM Tris/acetate, pH 7.6, 1 mM EDTA. 6 mM 2-mercaptoethanol, the specific activity 8 x 10\(^{5}\) cpm/mg. rRNA was isolated from Ehrlich ascites tumor cell ribosomes by phenolization as described previously (35) and 18 S and 28 S RNA were separated by sucrose gradient centrifugation in 10 mM Tris/acetate, pH 7.6, 3 mM EDTA. Calf liver rRNA, Escherichia coli \(\lambda\) S RNA, and coliphage MS2 RNA were purchased from Boehringer Münchenn, Germany. Heterogeneous nuclear RNA was isolated from exponentially growing EAT cells (7) by phenolizing ribonucleoprotein particles which had been released from purified nuclei by hypotonic shock in distilled H\(_2\)O. Escherichia coli 16 S RNA was a gift of Dr. R. Lührmann, Max-Planck-Institut für Molekulare Genetik, Berlin (Germany). Sulfolobus solfataricus DSM 1616 DNA, Halobacterium halobium R1 DNA, coliphage T4 DNA, and coliphage T5 DNA of Dr. W. Zillig and Dr. H. Schnabel, Max-Planck-Institut für Biochemie, München, Germany, coliphage \(\lambda\) DNA and coliphage T3 DNA of Dr. M. Schweiger, Universität Innsbruck, Austria. Calf thymus DNA, Escherichia coli DNA, Clostridium perfringens DNA, and EGTA were from Sigma, Bovine subtilis DNA from Calbiochem, Micrococcus lysodeikticus DNA from Miles Laboratories, salmon sperm DNA from Serva, and nuclease S1 from Bethesda Research Laboratories, Inc. Because the DNAs from E. coli, C. perfringens, M. lysodeikticus, B. subtilis, and salmon sperm were highly contaminated by ribonucleases, they had to be purified by 5-fold phenolization in the presence of sodium dodecyl sulfate. All DNA solutions were sonicated (Branson sonifier) at 0 °C to reduce the viscosity and, for denaturation, heated at 100 °C for 10 min and quickly cooled in ice water. For the isolation of single-stranded fd DNA, fd bacteriophages were multiplied in E. coli KB35 and subjected to phenolization; for a detailed description of the methods employed, see Ref. 38. Sucrose DAB-7 was supplied by Suddeutsche Zucker Aktiengesellschaft (Mannheim, Germany) and Soluene 350 by Packard Instrument International S.A. (Zürich, Switzerland). All other chemicals were of reagent grade and purchased from Merck AG (Darmstadt, Germany).

**Digestion of Native Calf Thymus DNA with Nuclease S1**

Forty mg of calf thymus DNA was dissolved in 8 ml of 30 mM Na-acetate, pH 4.6, 50 mM NaCl, 1 mM ZnSO\(_4\), 5% glycerol and sonicated (Branson sonifier) at 0 °C to reduce the viscosity. The solution was divided into halves. One-half was mixed with 0.5 mg of bovine serum albumin and 3000 units of nuclease S1. Both solutions were incubated at 37 °C for 15 min and then phenolized 5 times with 5-ml portions of an H\(_2\)O-saturated phenol/chloroform (4:1) mixture in the presence of 15% sodium dodecyl sulfate and 20 mM EDTA. The DNAs were precipitated by the addition of 15 ml of cold ethanol, collected by low speed centrifugation, dissolved in 2.5 ml of 10 mM Tris/acetate, pH 7.6, 3 mM EDTA, 6 mM 2-mercaptoethanol (Buffer A), and dialyzed against 1 liter of Buffer A overnight. The yields were 255 Anm units of control DNA and 208 Anm units of nuclease S1-treated DNA.

**Reactions of \(^{3}H\)-Vimentin with Nucleic Acids and Analysis of the Reaction Products by Sucrose Gradient Centrifugation**

Competition by EAT Cell 28 S rRNA and Nucleic Acids for Vimentin at Low Salt Concentrations—37.5 pg of \(^{3}H\)-vimentin (30,000 cpm) was incubated with mixtures of 154 pg of 28 S rRNA and 4 different amounts of various nucleic acids as specified in the figures under “Results” at 2 °C for 1 h in 400 μl of Buffer A. The reaction mixtures were layered on top of 12.4-ml linear 10 to 30% (w/w) sucrose gradients in Buffer A and centrifuged at 40,000 rpm (200,000 × g), and 2 °C for 14 h in the SW 40 Ti rotor of the Beckman L2 65B centrifuge. During fractionation, the gradients were monitored at a wavelength of 260 nm using a 1 mm LKB flow-through cell mounted in a Gilford 2400 S spectrophotometer. Five drops/fraction were collected into scintillation vials and mixed with 0.5 ml of Soluene 350 and 10 ml of a toluene-based scintillation mixture. The radioactivity of each fraction was measured in a Packard Tri-Carb 3330 liquid scintillation spectrometer. The radioactivity profiles of all gradients were normalized and the radioactivity detected in the 28 S RNA peaks was expressed as a percentage of the control which was obtained by reaction of \(^{3}H\)-vimentin with 28 S RNA.

**Effect of the Ionic Conditions on the Binding of Vimentin to EAT Cell tRNA and rRNA, and fd DNA—Forty-eight pg of \(^{3}H\)-vimentin was incubated with either 750 pg of total EAT cell rRNA, 250 pg of bacteriophage MS2 rRNA, or 100 pg of single-stranded bacteriophage fd DNA in a final volume of 400 μl for 1 h at 0 °C. The basal reaction buffer was 10 mM Tris/acetate, pH 7.6, 6 mM 2-mercaptoethanol. When the effect of the KCI concentration alone on the binding reaction was tested, the incubation mixtures contained, additionally, 3 mM EDTA and increasing amounts of KCl as specified in the figures. If the binding was tested at different Mg\(^{2+}\) concentrations or at various combinations of Mg\(^{2+}\) and KCl, EDTA was omitted from the reaction medium. The reaction mixtures were layered on top of 12.4-ml linear 10 to 30% (w/w) sucrose gradients in buffers whose ionic compositions were identical with those of the reaction mixtures and which also contained 6 mM 2-mercaptoethanol. The gradients were centrifuged at 40,000 rpm (290,000 × g), and 2 °C for time periods specified in the legends to the figures using an SW 40 Ti rotor of the Beckman L2 65B centrifuge. They were analyzed for nucleic acid and vimentin distribution as described above. In order to determine the radioactivity sedimented to the bottom of the centriufuge tubes, the invisible pellets were dissolved in 0.4 ml of Buffer A by manual pipetting. The samples were normally processed for radioactivity measurement. After quench correction, the radioactivity profiles of all gradients were normalized and the radioactivity detected in the nucleic acid peaks as well as in free vimentin (which stayed close to the top of the gradients) was expressed as percentages of the control of each reaction series. As a further control, the nucleic acids and \(^{3}H\)-vimentin were analyzed separately under various ionic conditions employed.

When the influence of increasing vimentin concentrations on the formation of association products with fd DNA was tested, 48 pg of \(^{3}H\)-vimentin was mixed with up to 1 μg of unlabeled vimentin. The reaction mixtures were processed as described above.

**RESULTS**

The study of the interaction of vimentin with various naturally occurring ribo- and deoxyribonucleic acids was greatly facilitated by the availability of a radioactively labeled vimentin preparation. It was obtained from Triton X-100-resistant residual cell structures of EAT cells grown in the presence of \(^{3}H\)-amino-acids. (NH\(_4\))\(_2\)SO\(_4\) fractionation of a low ionic strength extract of the detergent-resistant cell residues followed by delipidation and ion exchange chromatography of the material precipitating between 0 and 25% (NH\(_4\))\(_2\)SO\(_4\) saturation yielded pure vimentin (36). It sedimented with approximately 8 S upon sucrose gradient centrifugation in 10 mM Tris/acetate, pH 7.6, 3 mM EDTA, 6 mM 2-mercaptoethanol (standard ionic conditions) (Fig. 1a). As illustrated in Fig. 1b, vimentin reacted with free total rRNA from EAT cells
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Figure 1. Binding of [3H]vimentin to total EAT cell rRNA. a, for control, 680 μg of total EAT cell rRNA and 37.5 μg of [3H]vimentin were centrifuged separately on 10 to 30% (w/w) sucrose gradients in 10 mM Tris/acetate, pH 7.6, 3 mM EDTA, 6 mM 2-mercaptoethanol. b, sedimentation profile of the association products obtained by reaction of 680 μg of total EAT cell rRNA and 37.5 μg of [3H]vimentin. The radioactivity distribution profiles of both panels are normalized. For experimental details see "Experimental Procedures."

with the formation of complexes which sedimented, because only a small amount of radioactive vimentin was used, with the same rates as the original free rRNA species. In conformation of our previous results, which were obtained with an only partially purified vimentin preparation and by employing polyacrylamide gradient slab gel electrophoresis as an analytical means (35), the pure, radioactive vimentin exhibited a higher affinity for 18 S than for 28 S rRNA. With respect to the interpretation of the results of the competition assays described below, it was important that all vimentin added to the reaction mixture was bound to rRNA. Even a 50-fold amount of vimentin did not saturate the nucleic acids.

The relative affinities of vimentin for various naturally occurring nucleic acids were measured by performing competition experiments with rRNA from EAT cells as the constant vimentin binding component. The strategy of the experiments is outlined in Fig. 2 with native and heat-denatured calf thymus DNA as the competing vimentin binding components. The radioactivity detected in the 18 S and 28 S rRNA peaks decreased in response to the addition of the competing nucleic acids. Compared with the control (Fig. 1b), both rRNA species were relatively equally depleted of vimentin which now appeared associated with the competing nucleic acids. It is clear from Fig. 2 that vimentin had a considerably higher affinity for heat-denatured, partially single-stranded calf thymus DNA than for native, double-stranded DNA. It is interesting to note that the fraction of vimentin associated with heat-denatured DNA sedimented well ahead of the bulk of DNA, indicating cooperativity of binding.

Although most nucleic acids tested in this investigation sedimented more slowly than 18 S rRNA on sucrose gradients, in many cases it was difficult to achieve complete separation of the competing nucleic acids. This complicated the evaluation of the vimentin distribution profiles. Therefore, all following comparative measurements were performed with purified 28 S rRNA from EAT cells as the constant vimentin binding component. After sucrose gradient centrifugation of the reaction products, the radioactivity ([3H]vimentin) distribution was determined and normalized and the amount of radioactivity sedimenting in association with 28 S rRNA was expressed as a percentage of the total radioactivity recovered from the sucrose gradients.

In a first set of experiments, we determined the relative affinities of vimentin for various naturally occurring, single-stranded RNAs. The data presented in Fig. 3 demonstrate that eukaryotic as well as prokaryotic RNAs were able to bind vimentin. However, there were considerable differences in the binding affinities. Whereas 5 S rRNA from E. coli and tRNA from calf liver competed only weakly with EAT cell 28 S rRNA, E. coli 16S rRNA, and EAT cell hnRNA were very good competitors. The affinity of vimentin for hnRNA was approximately 3 times higher than that for EAT cell 28 S rRNA.

The relative affinities of vimentin for a series of naturally occurring DNAs in their native and denatured form were determined in the identical manner. In order to reduce the sedimentation coefficients of the DNAs, the DNA solutions were sonicated in the cold. In addition, a part of each solution was heated at 100 °C for 10 min, rapidly cooled in ice water, and immediately used for competition experiments. A first comparison of the affinities of vimentin for native (Fig. 4a) and denatured (Fig. 4b) DNAs clearly shows a much stronger
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**FIG. 2.** Competition by total EAT cell rRNA and native and heat-denatured calf thymus DNA, respectively, for \[^{3}H\]vimentin. The reaction conditions described under "Experimental Procedures" were used. 

a, sucrose gradient analysis of the association products obtained by reaction of 37.5 µg of \[^{3}H\]vimentin with a mixture of 680 µg of total EAT cell rRNA and 250 µg of sonicated, native calf thymus DNA. b, as a, except that 250 µg of sonicated, heat-denatured calf thymus DNA was used for competition with 680 µg of total EAT cell rRNA. The radioactivity distribution profiles of both panels are normalized.

**FIG. 3.** Competition by EAT cell 28 S rRNA and various naturally occurring RNAs for \[^{3}H\]vimentin. 37.5 µg of \[^{3}H\]vimentin was reacted with mixtures of 154 µg of 28 S rRNA and three different amounts of various RNAs. The reaction products were

Binding of vimentin to partially single-stranded DNAs. Concerning the binding to native DNAs, varying degrees of competition with 28 S rRNA were observed (Fig. 4a). Whereas, in general, native DNAs from a series of bacteriophages were very weak competitors, those of eukaryotic origin bound vimentin more efficiently. With the exception of DNA from *M. lysodeikticus*, which among the native DNAs tested was the most active vimentin binding component, the DNAs from bacterial cells interacted with vimentin only moderately. In the case of denatured DNAs, the preference of vimentin for GC-rich DNAs was particularly striking. When plotted versus the GC contents of the various DNAs tested, the relative vimentin binding capacities fell on a hyperbolic curve (Fig. 5). Only the value for bacteriophage T4 DNA deviated from this relationship and this was probably due to glycosylation of the hydroxymethyl cytosine bases of the DNA. Denatured DNA from *M. lysodeikticus*, with a GC content of 72.3%, bound vimentin approximately 11 times stronger than EAT cell 28 S rRNA, whereas the corresponding value for denatured DNA from *C. perfringens*, with a GC content of 30.9%, was 3 times lower in comparison with 28 S rRNA.

In order to show that the heat-induced enhancement of the analyzed by sucrose gradient centrifugation in 10 mM Tris/acetate, pH 7.6, 3 mM EDTA, 6 mM 2-mercaptoethanol. After normalization of the radioactivity distribution profiles, the radioactivity bound to 28 S rRNA was expressed as a percentage of the control. For control, \[^{3}H\]vimentin was reacted with 28 S rRNA only. EAT cell 18 S rRNA, •; EAT cell hnRNA, ▲; calf liver tRNA, □; E. coli 16 S rRNA, △; E. coli 5 S rRNA, ●; coliphage MS2 RNA, ○. The bar on the abscissa indicates the amount of 28 S rRNA used in relation to the amounts of the other RNAs.
vimentin binding capacity of DNA was indeed the result of DNA melting and not due to some other unknown temperature effect, we partially reannealed the separated DNA strands by slow cooling of the heated DNA solution. Although it was impossible to achieve complete renaturation, Fig. 6 shows that the heat-denatured DNA had partially lost its high vimentin binding capacity as a result of supercoil-helix transformation.

It was surprising to find that native calf thymus DNA had the same vimentin binding capacity as M. lysodeikticus DNA, although both nucleic acids have considerably different GC contents. Since it was possible that native calf thymus DNA was particularly sensitive to local denaturation by sonication, the sonicated DNA was digested with nuclease S1 to remove single-stranded regions. However, as shown in Fig. 7, this treatment caused only a slight reduction of the binding of vimentin in the standard competition assay.

Thus far, our experimental results show that at low salt concentration and in the absence of divalent cations naturally occurring, partially single-stranded nucleic acids are efficient vimentin binding components and that, in the case of DNAs, vimentin prefers GC-rich base sequences. However, in order to demonstrate that this interaction also occurs in vivo, it was necessary to show that the association products are resistant to physiological ionic conditions. For this purpose, soluble, radioactively labeled vimentin was first reacted with total rRNA from EAT cells in solution of increasing KCl concentration and in the presence of EDTA. The reaction products were analyzed by sucrose gradient centrifugation under ionic conditions identical with those of the reaction mixtures. As illustrated in Fig. 8, at 100 mM KCl total rRNA bound approximately 90% of the input vimentin but at 150 mM KCl almost no vimentin was bound. Fig. 8 also shows that the vimentin which was not bound by 28 S rRNA between 50 and 100 mM KCl associated with 18 S rRNA, thus giving rise to a transient binding maximum of the small rRNA at 100 mM KCl. Then, a steep decrease of the vimentin binding capacity of 18 S rRNA with almost no binding at 150 mM KCl was observed. This result shows that the association products consisting of vimentin and 18 S rRNA are more resistant to dissociation by KCl than those derived from 28 S rRNA. It is paralleled by our previous finding that at low ionic strength 18 S rRNA has a higher vimentin binding potential than 28 S rRNA (35, 39) (Fig. 1b).

Thus, our experimental results show that at low salt concentration and in the absence of divalent cations naturally occurring, partially single-stranded nucleic acids are efficient vimentin binding components and that, in the case of DNAs, vimentin prefers GC-rich base sequences. However, in order to demonstrate that this interaction also occurs in vivo, it was necessary to show that the association products are resistant to physiological ionic conditions. For this purpose, soluble, radioactively labeled vimentin was first reacted with total rRNA from EAT cells in solution of increasing KCl concentration and in the presence of EDTA. The reaction products were analyzed by sucrose gradient centrifugation under ionic conditions identical with those of the reaction mixtures. As illustrated in Fig. 8, at 100 mM KCl total rRNA bound approximately 90% of the input vimentin but at 150 mM KCl almost no vimentin was bound. Fig. 8 also shows that the vimentin which was not bound by 28 S rRNA between 50 and 100 mM KCl associated with 18 S rRNA, thus giving rise to a transient binding maximum of the small rRNA at 100 mM KCl. Then, a steep decrease of the vimentin binding capacity of 18 S rRNA with almost no binding at 150 mM KCl was observed. This result shows that the association products consisting of vimentin and 18 S rRNA are more resistant to dissociation by KCl than those derived from 28 S rRNA. It is paralleled by our previous finding that at low ionic strength 18 S rRNA has a higher vimentin binding potential than 28 S rRNA (35, 39) (Fig. 1b). It should be noted that on the control sucrose gradients, where rRNA and vimentin were analyzed separately, the major part of the protein sedimented with the same rate at high ionic strength as at low salt concentration, with the exception that, with increasing ionic strength, a slight shoulder emerged on the leading edge of the protein peak. Almost identical binding results were obtained with bacteriophage MS2 RNA (Fig. 10a).

Similar binding experiments were performed at various Mg$^{2+}$ concentrations. As shown in Fig. 9, between 0.35 and 0.85 mM Mg$^{2+}$, the affinity of vimentin for total rRNA de-
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Fig. 5. [3H]Vimentin binding capacities of various heat-denatured DNAs as a function of their (G + C) contents. The percentages of the total [3H]vimentin bound to 28 S rRNA in competition with the same amounts of various heat-denatured DNAs are plotted versus the (G + C) contents of these DNAs. The percentages were taken from Fig. 4b. To show that this relationship is a hyperbolic function, in the inset the percentages are plotted versus the reciprocals of the (G + C) contents after these had been reduced by 20%. Calf thymus DNA, ○; salmon sperm DNA, □; E. coli DNA, □; C. perfringens DNA, +; M. lysodeikticus DNA, ▪; S. solfatarius DNA, ▲; B. subtilis DNA, ◄; H. halobium DNA, ▼; coliphage T3 DNA, ●; coliphage T4 DNA, △; coliphage T5 DNA, ▲.

Fig. 6. The effect of denaturation/renaturation of coliphage T3 DNA on its [3H]vimentin binding capacity. Sonicated, native coliphage T3 DNA (○) was denatured by heating at 100 °C for 10 min. One-half of the solution was cooled slowly to allow renanening of the separated DNA strands (□), the other half was quickly cooled in ice water (●). The different DNA solutions were mixed, in different ratios, with a constant amount of 28 S rRNA and [3H]vimentin and the reaction products were analyzed by sucrose gradient centrifugation as described in detail under “Experimental Procedures” and briefly outlined in the legend to Fig. 3. The bar on the abscissa denotes the amount of 28 S rRNA used in relation to the amounts of the various DNAs.

Fig. 7. The effect of nuclease S1 digestion of sonicated, native calf thymus DNA on its [3H]vimentin binding capacity. Sonicated, native calf thymus DNA was digested with nuclease S1 (□). A control sample was treated in the same way, except that the nuclease was omitted (○). After phenolization, a part of each solution was heat-denatured (−S1, △; +S1, ▲). The various DNAs were mixed, in different ratios, with a constant amount of 28 S rRNA and [3H]vimentin and the reaction products were analyzed by sucrose gradient centrifugation as described in detail under “Experimental Procedures” and briefly outlined in the legend to Fig. 3. The bar on the abscissa denotes the amount of 28 S rRNA used in relation to the amounts of the various DNAs.

Fig. 8. Effect of the KCl concentration on the interaction of [3H]vimentin with 18 S, 28 S, and total rRNA from EAT cells. 750-μg portions of total rRNA from EAT cells were incubated with 48-μg portions of [3H]vimentin in 10 mM Tris/acetate, pH 7.6, 3 mM EDTA, 6 mM 2-mercaptoethanol at varying KCl concentrations. The reaction products were analyzed on 10 to 30% (w/w) sucrose gradients which had the same ionic compositions as the reaction mixtures. The gradients were centrifuged at 10 to 50° (w/w) sucrose gradients which had the same ionic compositions as the reaction mixtures. The gradients were centrifuged at 10 to 50° (w/w) sucrose gradients which had the same ionic compositions as the reaction mixtures. The gradients were centrifuged at 40,000 rpm and 2°C for 12 h in the SW 40 Ti rotor of the Beckman L2 65B centrifuge. The radioactivity distributions of all gradients were normalized and the radioactivities computed for the 18 S and 28 S rRNA peaks were expressed as percentages of the control which was run in the absence of KCl. For experimental details, see “Experimental Procedures.” ●, 18 S rRNA; □, 28 S rRNA; ◄, total rRNA. Increased rapidly. In agreement with our previous results obtained with partially purified vimentin (35), there was virtually no binding of the protein at 1.5 mM Mg++. Concerning the vimentin binding capacities of the individual rRNA species, as in the case of increasing KCl concentrations, a differential effect of Mg++ was observed. Due to competition between both rRNA species for vimentin and the higher resistance of the 18 S rRNA-vimentin complexes to dissociation, the binding of vimentin to the small rRNA passed through a transient maximum (150%) at 0.35 mM Mg++. At this Mg++ concentra-
run in the absence of Mg"++. For experimental details see "Experimental Procedures." 18 S rRNA; 0, 28 S rRNA; □, total rRNA; ■, fd DNA.

In order to find out whether KCl and Mg"++ exert their negative effects on RNA-vimentin complexes additively or whether there might be a mutually supporting positive effect of both cations, the binding reactions and the sucrose gradient analyses of the reaction products were performed in the presence of different concentrations of both KCl and Mg"++. The results point to an additive, negative effect of mono- and divalent cations (data not shown). Since it is possible that in the presence of KCl and Mg"++ the RNA molecules attained configurations which, due to steric hindrance, were unable to interact with vimentin, rRNA-vimentin complexes were preformed in solution of low ionic strength and in the absence of divalent cations and then analyzed on sucrose gradients containing KCl and Mg"++ at different concentrations. However, the same results were obtained as when the reaction was allowed to proceed in the presence of both salts (data not shown).

Next, we determined the salt stability of complexes consisting of vimentin and single-stranded DNA. In order to separate the nucleic acid and its vimentin complexes as far as possible from the freely sedimenting vimentin on sucrose gradients, a well-defined, fast sedimenting, single-stranded DNA was chosen as the vimentin binding component, bacteriophage fd DNA (22 S). Fig. 10a shows that, as expected, the complexes derived from fd DNA were much more resistant to KCl than the corresponding reaction products obtained from rRNA (Fig. 8) or single-stranded bacteriophage MS2 RNA. At 150 mM KC1 vimentin associated quantitatively with fd DNA, whereas the affinity of vimentin for MS2 RNA was already completely abolished at 125 mM KCl. Also, at higher KCl concentrations, there was still substantial binding of vimentin to fd DNA with a 50% efficiency at 220 mM KCl. Even at 300 mM KCl, approximately 25% of the input vimentin tightly associated with the DNA. The sucrose gradient analysis of the reaction products further revealed that, at KCl concentrations higher than 100 mM, increasing amounts of vimentin sedimented well ahead of the bulk of the DNA. A considerable part of vimentin was recovered from the bottom of the centrifuge tubes. In control gradients on which vimentin and fd DNA were centrifuged separately at various KCl concentrations, neither vimentin nor fd DNA could be detected at positions where the fast sedimenting reaction products had been localized. It must be concluded, therefore, that the fast sedimenting material did not result from nonspecific aggregation of the individual components but that vimentin interacted cooperatively with the DNA. In Fig. 10a, that part of the input vimentin which was detected in particles sedimenting faster than the bulk of the DNA was added to that determined for the DNA peak. It was also expressed as a percentage of the normalization standard and plotted as per cent cooperativity (□). b, fd DNA was incubated with [3H]vimentin as described under a, except that the incubation mixtures and the 10 to 30% (w/w) sucrose gradients contained, in addition to varying amounts of KCl, 0.5 mM (■) and 1 mM (□) Mg"++, respectively. The centrifugation time was 5 h at 40,000 rpm and 2°C in the SW 40 Ti rotor; □, per cent cooperativity in the presence of 0.5 mM Mg"++; □, per cent cooperativity in the presence of 1 mM Mg"++. For experimental details, see "Experimental Procedures."
Identical experiments were conducted at various Mg\(^{2+}\) concentrations. The results depicted in Fig. 9 clearly show that complexes consisting of fd DNA and vimentin were substantially more evident in the reaction mixtures than the corresponding products derived from rRNA. At 1 mM Mg\(^{2+}\), almost 60% of the total vimentin was still associated with fd DNA and the affinity of vimentin for fd DNA diminished only slowly with further increasing Mg\(^{2+}\) concentration.

When the affinity of vimentin for single-stranded fdDNA was tested in the presence of both KCl and Mg\(^{2+}\), a strong, binding-promoting effect of the divalent cations at KCl concentrations higher than 150 mM KCl was observed. However, there were no significant differences between the effects exerted by 0.5 and 1 mM Mg\(^{2+}\). In both cases, even at 300 mM KCl, approximately 80% of the input vimentin was tightly bound to fd DNA in contrast to only 25% in the absence of Mg\(^{2+}\). If we take into account the thorough washing of the DNA-protein complexes in sucrose gradients with ionic compositions identical with those of the reaction mixtures, this figure represents a remarkably high stability of the complexes. It is in striking contrast to the complete instability of RNA-vimentin complexes under the same ionic conditions. Also, the amount of cooperatively bound vimentin, with its maximum close to 200 mM KCl, was considerably diminished in the presence of Mg\(^{2+}\). At 300 mM KCl, virtually all vimentin complexed with fd DNA was bound in a cooperative manner. In comparison with the results obtained in the absence of Mg\(^{2+}\), there was also substantial cooperative interaction between 0 and 100 mM KCl. It should be noted that in the absence of KCl the input vimentin was efficiently but not quantitatively bound to the DNA (Fig. 10, a and b). Finally, under all ionic conditions tested, vimentin and fd DNA stayed quantitatively in the upper parts of the sucrose gradients when the reactants were centrifuged separately.

**DISCUSSION**

With the present investigation we have extended the characterization of the binding of the intermediate filament protein vimentin to nucleic acids. It would have been advantageous to perform the competition experiments described in the first section under "Results," as well as the analysis of the effect of the ionic environment on the binding reaction by employing nucleic acids covalently bound to cellulose or agarose. However, we observed strong secondary interactions of vimentin with the carbohydrate matrices. Therefore, we had to analyze the reaction products by sucrose gradient centrifugation. This, in turn, required the employment of a physically well defined, fast sedimenting polynucleotide of intermediate vimentin binding capacity which is available in large quantities. 28 S rRNA from EAT cells turned out to be the best competitor in this respect. Since, however, the binding of vimentin to rRNA is sensitive to higher concentrations of mono- and divalent cations (see Figs. 8 and 9), the relative affinities of the filament protein for the various nucleic acids had to be determined at low ionic strength and in the absence of divalent cations.

Although these conditions are not physiological, the results obtained, nevertheless, permit a first insight into the principles governing the interaction of vimentin with nucleic acids. In conjunction with the results of the screening of a large variety of synthetic single- and double-stranded polyribo- and polydeoxynucleotides for their vimentin binding potentials,¹ they provide the basis for further experimentation, especially concerning the search for possible, distinct vimentin binding sites on nucleic acids. It is conceivable that a small fraction of the total vimentin is tightly bound to specific acceptor sites of the nucleic acids but that this highly specific association is covered up by the less tight and specific binding of the remaining major fraction of the protein. This possibility has to be considered since we were unable to saturate EAT cell rRNA, bacteriophage MS2 RNA, and also various DNAs with large amounts of purified vimentin.¹ With respect to stability and specificity of the binding, the microheterogeneity of the vimentin population as a result of phosphorylation (40-44) also has to be taken into consideration. Finally, highly specific and salt-stable binding of vimentin to nucleic acids might be dependent on the presence of distinct auxiliary proteins. Thus, the requirement for unphysiological ionic conditions observed in many cases (for instance, in those of RNAs) in vitro does not exclude the interaction of vimentin or its derivatives with nucleic acids at higher concentrations of mono-, di-, and polyvalent cations in vitro.

At low ionic strength and in the absence of divalent cations, the competition experiments revealed the preferential binding of vimentin to single-stranded nucleic acids, particularly to those of high GC content. This is in perfect agreement with the exceptionally high vimentin binding potential of poly(dG) and poly(rG).² The vimentin binding properties of naturally occurring RNAs were largely comparable with those of heat-denatured, partially single-stranded DNAs, with the exception of calf thymus rRNA and E. coli 5 S rRNA which in their activities were comparable with native DNA. It should be pointed out that among the naturally occurring RNAs hnRNA from EAT cells was the most potent competitor for vimentin in the competition assay.

The vimentin binding activities of native DNAs varied considerably (Fig. 4a). This might be due, at least in part, to different extents of nicking and partial denaturation of the DNAs in the course of their isolation and by sonication. If we exclude DNA from M. lysodeikticus from the comparison, there seems to be a correlation between the vimentin binding activity and the genomic complexity of the DNAs in the order eukaryotes > prokaryotes > bacteriophages. However, in the case of native calf thymus DNA, the vimentin binding activity could be only slightly reduced by digestion of the DNA with the single-strand-specific nuclease S1 (Fig. 7). This result might indicate that vimentin binds to double-stranded DNAs at their ends or nicks with partial unwinding of the helix, as has been observed in the cases of an adenovirus-specific DNA binding protein (45) and the chick oviduct progesterone receptor (46).

When the concentrations of mono- and divalent cations were raised to physiological and higher values, the single-stranded DNAs turned out to be superior in their vimentin binding activities to single-stranded RNAs. Whereas rRNA from EAT cells and bacteriophage MS2 RNA, for instance, did not form complexes with vimentin at 150 mM KCl, single-stranded bacteriophage fd DNA reacted quantitatively with the protein at this ionic strength. A similar situation was observed when the stability of rRNA- and fd DNA-vimentin complexes was tested in dependence on the Mg\(^{2+}\) concentration. The differences were even better expressed when the binding reactions were performed at physiological concentrations of both KCl and Mg\(^{2+}\). In the presence of 1 mM Mg\(^{2+}\), rRNA did not associate with vimentin within the entire KCl concentration range (0 to 300 mM) tested, with the exception of 13% of the input vimentin being bound at 30 mM KCl (data not shown). However, fd DNA efficiently interacted with the protein under the same conditions.

When the reaction between fd DNA and vimentin was allowed to proceed at low protein concentrations, a substantial amount of vimentin sedimented faster than the bulk of the DNA. We tentatively interpret this observation as being due

¹ P. Traub and W. J. Nelson, manuscript in preparation.

² P. Traub and W. J. Nelson, manuscript in preparation.
to highly cooperative binding of vimentin to a small number of fd DNA molecules. These molecules might be saturated with vimentin and therefore possess a higher sedimentation coefficient. The salt optimum of this cooperativity was at approximately 200 mM KCl at all Mg

° concentrations tested. With decreasing KCl concentration, more and more vimentin was found to be associated with the bulk of the homogeneously sedimenting fd DNA. It is understandable that vimentin molecules lined up on single-stranded DNA molecules, due to protein-protein interaction, are more resistant to dissociation than individually bound vimentin molecules. This effect is also reminiscent of the formation of intermediate filaments from subunit proteins which becomes more efficient with increasing ionic strength.1

However, with enhancing vimentin concentration at 150 mM KCl and 1 mM Mg

°, the absolute amount of protein detected in "saturated" DNA-protein complexes did not increase, in contrast to the absolute amount of protein associated with the bulk of fd DNA which sedimented increasingly faster as the protein concentration went up. Moreover, a considerable part of the vimentin sedimented slower than the bulk of the DNA; it could be always localized in the same position of sucrose gradients (data not shown). Our failure to saturate a larger number of fd DNA molecules with protein might be explained, at least in part, on the basis of the findings described by Schneider and Wetmur (47). These authors reported the direct transfer of cooperative units of E. coli single strand DNA binding protein from donor to recipient DNA strands as the rate-determining step in the protein saturation of individual DNA strands. It is possible that at high vimentin concentrations large cooperative units were formed on all DNA molecules present which were, however, too large for rapid, en bloc transfer. This was actually indicated by the enhancement of the sedimentation coefficient of the majority of the DNA molecules. The fact that at high protein concentrations a considerable part of the vimentin was apparently not bound to DNA was very likely due to the formation of protofilaments in a competing, vimentin-consuming reaction. At subphysiological KCl concentrations and in the absence of Mg

°, also at high protein concentrations, vimentin was quantitatively associated with DNA (data not shown).

The present data on the salt stability of complexes derived from vimentin and natural, single-stranded RNA and DNA, in conjunction with the large variability of the vimentin-binding potentials of a multitude of synthetic nucleic acids, convincingly show that the association of vimentin with nucleic acids is not due to an unspecific, solely electrostatic interaction of these components. In particular, the fact that under physiological ionic conditions vimentin can discriminate between RNA and DNA in favor of DNA points to a high degree of specificity of this association. This is further supported by our finding that, as a result of strict cooperativity, vimentin and desmin segregate into two distinct populations of deoxyribonucleoprotein particles when a mixture of both proteins is reacted with single-stranded DNA.2 So far, we have been unable to demonstrate cooperativity of the binding of vimentin and other intermediate filament subunit proteins to naturally occurring RNA. We regard, therefore, the restriction of the cooperativity to single-stranded DNA as a further proof of the specificity of the interaction of vimentin with nucleic acids. In this context, it should be pointed out that vimentin, as a single-stranded DNA binding protein, very probably does not recognize specific base sequences on DNA molecules; although we have observed the preferential binding of vimentin to G-rich nucleic acids.3 On the basis of these results, we consider the possibility that the association of vimentin with DNA might also occur in vivo. This does not necessarily mean that vimentin itself is the active protein component but that its derivatives might fulfill this function as well, provided that they have preserved their nucleic acid binding sites. However, the unambiguous identification of complexes of nuclear DNA or chromatin with vimentin and/or its derivatives in vivo encounters serious problems. For technical reasons, it is extremely difficult to distinguish between vimentin specifically bound to internal nuclear structures and vimentin persistently adhering to the outer nuclear surface in the form of intermediate filaments or their fragments.

Despite the high specificity of vimentin for single-stranded DNA, its interaction with RNA or ribonucleoprotein particles in a physiological ionic environment is still possible. Such a dual relationship has also been postulated for cytosolic steroid hormone receptors which, as far as their nucleic acid binding properties (46, 48-58) and their sensitivities to specific, Ca2+-dependent thiol proteinases (59-62) are concerned, are strikingly similar to vimentin (35, 63, 64).

In several investigations, steroid hormone receptors have been shown to interact with nuclear and cytosolic ribonucleoprotein particles (65, 66). The nuclear particles from calf uterus have been tentatively identified as ribosomal precursor particles, the cytosolic particles as mature ribosomal subunits (66). In both cases, it was striking that at 0.1 M KCl and in the presence of EDTA the estrogen receptor complex had a lower affinity for the large particles containing 28 S RNA than for the small ones which contained 18 S RNA. This association was very sensitive to high ionic strength, indicating reversibility of the interaction (65, 66). We have made exactly the same observation in the case of the association of vimentin with ribosomal subunits of eukaryotic origin (35, 63) and, as shown in the present communication, with 18 S and 28 S rRNA. In addition, cytosolic RNA and RNase, respectively, had significant effects on the binding of steroid hormone receptors to DNA but only if the salt concentration was kept low and the binding reaction was performed in the absence of divalent cations (55, 58). It was suggested that, under low salt conditions, steroid hormone receptors are associated with RNA. This was also indicated by the observation of a transient binding maximum at 0.1 M KCl when crude cytosolic estrogen receptor was allowed to react with DNA in a solution of increasing KCl concentration (48). The effect was interpreted as being due to either a higher affinity of the receptor for DNA at 0.1 M KCl or a decrease in less specific interactions such as association of the receptor with polyribonucleotides in the cytosol. Our results on the effect of increasing KCl concentration on the interaction of vimentin with a mixture of 18 S and 28 S RNA (Fig. 8) show that the appearance of a transient binding maximum with increasing KCl concentration is the result of both effects: vimentin (and also steroid hormone receptors) is (are) distributed among competing nucleic acids according to its (their) nucleic acid binding potentials at different ionic strengths.

Although in both 18 S and 28 S RNA approximately 65% of the nuclear bases are equally involved in base-pairing and a further 20% of residues take part in chiral structures of RNA, a review, see Ref. 67), the difference in the vimentin (and hormone receptor) binding capacities of both rRNAs is probably due to differences in the thermal stabilities of their double helical regions. Employing the Wallauer-David spreading technique (68), reproducible patterns of (G + C)-rich hairpin loops were found in both pre-rRNA and 28 S rRNA, but no secondary structure in 18 S rRNA (68, 69).

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1 P. Traub, W. J. Nelson, S. Kuhn, and C. E. Vorgias, unpublished observations.
Taking into account that in 28 S rRNA a substantial part of the G-bases are located in double helical regions of high thermal stability and in the loops, it becomes readily understandable that vimentin has a higher affinity for 18 S rRNA than for 28 S rRNA; although mouse 28 S rRNA has a (G + C) content which is, by 7.7%, higher than that of 18 S rRNA (67). We could establish a similar correspondence for E. coli rRNA. When analyzed by the Wellauer-Dawid technique, E. coli pre-rRNA appeared to be completely denatured in formamide/urea (70). On the other hand, when total E. coli rRNA was reacted with vimentin at low ionic strength and in the presence of EDTA and the reaction products were analyzed by sucrose gradient centrifugation, the relative vimentin distribution pattern closely followed the optical density profile of the rRNA (data not shown).

Here it should also be mentioned that the interaction of vimentin with natural RNAs is only stable at low ionic strength and in the absence of divalent cations, the experimental results show that the proteins have at least the potential to react with RNA. Whether this interaction occurs in vivo is uncertain. However, since we have shown that vimentin has strong affinity for poly(rG) and heteropolyribonucleotides of high G content and that the association products are stable in solution of physiological ionic strength, vimentin and/or its derivatives might well interact with RNAs of high G content also in vivo. One candidate, for instance, might be eukaryotic pre-rRNA whose nonconserved spacer regions have a (G + C) content of up to 80% (for a review, see Refs. 67 and 72). Moreover, it has to be considered that the binding experiments in vitro have been performed under unphysiological conditions, in so far as pure or partially purified reactants have been used. Yet, in vivo nucleic acids never occur in their free form but are always associated with proteins. It is possible, therefore, that auxiliary proteins exert a selective and stabilizing effect on the interaction of the proteins of both classes with RNA, as has been demonstrated for the functional relationship between steroid hormone receptors and DNA (73). It remains to be investigated whether changes in vitro in the microenvironment of RNAs or distinct ribonucleotide sequences of these RNAs have an influence on the specificity and stability of the association of vimentin with polyribonucleotides.

On the basis of the present experimental results, we suggest that vimentin and/or its derivatives, in addition to their function as cytoskeletal elements, are involved in gene expression as one of their major cellular functions. The occurrence of substantial amounts of single-stranded DNA in active chromatin (74–77), in conjunction with the preferential and stable binding of vimentin to single-stranded DNA, might point to a possible function of vimentin and/or its derivatives in transcription. In association with RNA, they might also be involved in post-transcriptional control.

Acknowledgments—We greatly appreciate the help of Ulrike Traub and Margot Bialdiga in growing the cells and we thank Anne-marie Scherbarth, Gabi Shoeman, and Brititte Geisel for excellent technical assistance. We also thank Dr. W. Zillig and Dr. H. Schnabel for their generous gifts of Sulfolobus solfataricus DNA, Halobacterium halobium DNA, coliphage T4 DNA, and coliphage T5 DNA, and Dr. M. Schweiger for kindly providing us with samples of coliphage λ DNA and coliphage T3 DNA. Thanks are also due to Dr. R. Lührmann for his provision of Escherichia coli 18 S rRNA.

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J. Biol. Chem. 1983, 258:1456-1466.

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