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The genes coding for the cytoskeletal proteins actin and vimentin in warm-blooded vertebrates

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Recombinant plasmids were made containing cDNAs synthesized on hamster mRNAs coding for cytoskeletal (β- or γ-) actins and for vimentin. Hybridization of the actin probe on restriction digests of one avian and five mammalian DNAs yielded multiple bands; the vimentin probe revealed only one band (accompanied by 2–3 faint bands in some DNAs). The results obtained with the vimentin probe indicate that the corresponding coding sequences: (a) are highly conserved in warm-blooded vertebrates like the actin sequences; (b) have strongly diverged from those coding for other intermediate filament proteins, since hybridization of the vimentin probe does not lead to a diagnostic multiband pattern; and (c) most likely contribute a single gene, in contrast to the sequences coding for other cytoskeletal proteins. Hybridization of the probes on mRNAs from the different sources used showed that the non-coding sequences of both vimentin and actin genes are conserved in length.

Key words: cytoskeleton/recombinant DNA/multigene families

Introduction

Intermediate-sized filaments (IF), microfilaments and microtubules are essential structural constituents of the cytoskeleton of eukaryotic cells (Lazarides, 1980). IF can be visualized by electron microscopy as 7–11 nm thick fibrils (Lazarides, 1981). Gel electrophoresis and immunofluorescence studies can distinguish between different types of IF, and five major classes have thus been defined: (1) vimentin filaments, found in a variety of cells of mesenchymal origin or in cultured cells of any origin; (2) keratin filaments, found in cells of epithelial origin; (3) desmin filaments, found essentially in muscle cells; (4) neurofilaments, found in neurones; and (5) glial filaments, which are specific to astroglia.

Protein sequences and immunological cross-reactivities have shown that actins and tubulins, the major constituents of microfilaments and microtubules, respectively, and, to a lesser extent, IF proteins, are conserved throughout evolution (Lazarides, 1980; Lazarides, 1981; Vandekerckhove and Weber, 1979; Franke *et al.*, 1979; Geisler and Weber, 1981). The organization of the genes coding for actins and tubulins has been mainly investigated using plasmids carrying specific cDNAs (for review, see Firtel, 1981); such studies have shown that these proteins are encoded by multigene families, that their biosynthesis is developmentally regulated (McKeown and Firtel, 1981), and that the corresponding genes are conserved in sequence in different species (Cleveland *et al.*, 1980). In mammals, two-dimensional gel analysis and direct protein sequencing have revealed three different forms of actin; α-actin, found in skeletal and cardiac muscle cells, and encoded by at least four genes, and β- and γ-actin, found in microfilaments of non-muscle cells (Vandekerckhove and Weber, 1979). It has been assumed that there are two β-actin genes per haploid human genome (Vandekerckhove *et al.*, 1980), and at least four human β-actin genes have been found by Kedes (personal communication). Similarly, both α- and β-tubulin are encoded by multiple genes (Cleveland *et al.*, 1981). No results on these points have yet been reported in the case of IF proteins. Protein sequences have shown, however, considerable homology between desmin, vimentin and, to a certain extent, wool keratin and tropomyosin; this suggests that IF proteins are encoded by a multigene family (Geisler and Weber, 1981). In this connection, it is of interest that intermediate filaments of BHK cells and bovine epidermal keratinocytes appear to fit with a general model composed of a similar three-chain unit which contains regions of coiled α-helix interspersed with regions of non-α-helix, the former having the same size, the latter different sizes (Steinert *et al.*, 1980).

We have constructed plasmids containing cDNAs synthesized on hamster mRNAs coding for vimentin and β- or γ-actin. Hybridization of our actin probe on restriction digests of hamster, mouse, rat, human, calf, and chicken DNAs yielded multiple bands in accord with previous results (Cleveland *et al.*, 1980). However, the vimentin probe yielded only a single major band with most DNA sources and additional faint bands with some species. These results have several interesting implications: first, the sequences coding for vimentin are highly conserved in warm-blooded vertebrates, resembling those sequences coding for actins, which are also highly conserved in evolution; second, the sequences coding for other IF proteins have diverged considerably from those coding for vimentin, as shown by the absence of the typical multiband hybridization patterns, like that obtained with the actin probe; third, the sequences coding for vimentin very likely correspond to a single gene. It has also been shown that the non-coding sequences of both vimentin and actin mRNAs are conserved in length.

Results

Construction and characterization of actin and vimentin cDNA plasmids

As a convenient source of mRNAs coding for cytoskeletal proteins, we chose an SV40-transformed epithelial cell line from hamster lens that grows in suspension; amongst the major proteins synthesized by these cells are vimentin and the cytoskeletal actins; lens crystallins are not synthesized in detectable amounts (Bloemendal *et al.*, 1980). Poly A$^+$ cytoplasmic RNA was denatured with 10 mM methylmercury hydroxide and fractionated on an isokinetic sucrose density gradient (Figure 1A). Aliquots from gradient fractions were directly assayed by *in vitro* protein synthesis, and the translation products were analyzed by SDS-polyacrylamide gel elec-

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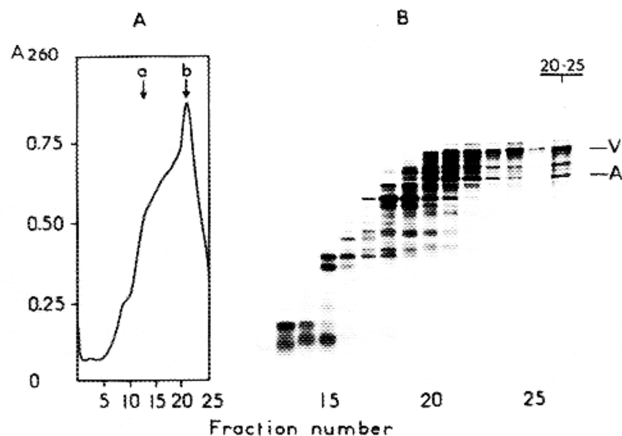

Fig. 1. Fractionation of poly A$^+$ RNA from a hamster lens epithelial cell line. Poly A$^+$ RNA (160 μg) was denatured with 10 mM methylmercury hydroxide, centrifuged on a sucrose density gradient, and collected in 0.4 ml fractions. **(A)** Absorption profile at 260 nm. The positions of rabbit globin mRNA (a), and of rat 18S rRNA (b) run on parallel gradients are indicated; 1 μl aliquots from fractions 8–25 were directly assayed for *in vitro* translation and products were run on a 13% SDS-polyacrylamide gel. **(B)** Fluorogram of the gel. Actin (A) and vimentin (V) are indicated. Pooled fractions 20–25, enriched in actin and vimentin mRNAs, were used as templates for cDNA synthesis.

trophoresis (Figure 1B). mRNAs directing the synthesis of polypeptides having the same molecular weight as actin and vimentin were present in fractions 20–25; these were pooled and used as templates for cDNA synthesis. Recombinant cDNA plasmids were constructed as described in Materials and methods.

cDNA plasmids were screened for those containing actin and vimentin specific sequences using two different methods. Since *in vitro* translation of mRNA preparations from the hamster cells had shown that actin and vimentin were major products (Bloemendal *et al.*, 1980), it was conceivable that the corresponding mRNAs were highly represented in the total mRNA population. Therefore, a preliminary colony hybridization with a ^{32}P-labeled single-stranded cDNA probe made from the original mRNAs (fractions 20–25) was performed to select for cDNA clones corresponding to abundant mRNA transcripts. Forty clones giving strong colony hybridization signals were selected and mRNAs hybridizing to each one of these plasmids were translated *in vitro*. Among these, the mRNAs species hybridizing to one plasmid, pAct-1, directed the synthesis of a protein comigrating with actin (Figure 2A, lane 3), whereas mRNAs hybridizing to other plasmids directed the synthesis of relatively abundant proteins, but not of vimentin. In particular, eight plasmids specific for a 45-kd protein, one of the major translation products, were detected.

Vimentin-specific plasmids were isolated by hybridizing mRNAs to 288 plasmids distributed in 24 groups of 12 clones each; mRNAs hybridizing to two groups directed the synthesis of a product comigrating with vimentin. The two plasmids responsible for this hybridization, pVim-1 (Figure 2A, lane 4) and pVim-2 (not shown), were then isolated.

The identity of the polypeptides synthesized by mRNAs hybridizing to pAct-1 and pVim-1 was established by two-dimensional gel electrophoresis (Figure 2B, C, D). The translation products of the mRNA selected by pAct-1 comigrated with both unlabeled β- and γ-actin run on the same gel, suggesting that this plasmid is able to hybridize to both corresponding mRNAs. The major translation product ob-

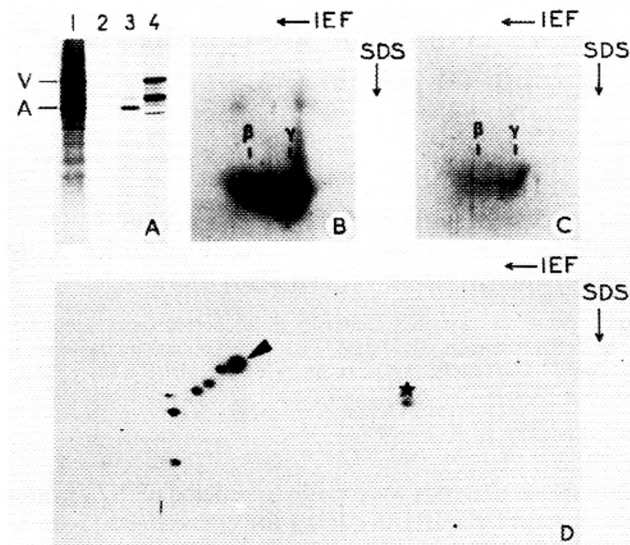

Fig. 2. Identification of *in vitro* translation products of mRNAs hybridizing to recombinant plasmids. Plasmid DNAs from individual clones were subjected to positive hybridization-translation assays as detailed in Materials and methods. **(A)** *In vitro* translation products: from the initial size-fractionated mRNAs **(lane 1)**; from mRNA hybridized to pBR322 **(lane 2)**; from mRNA hybridized to pAct-1 **(lane 3)**; from mRNA hybridized to pVim-1 **(lane 4)** (this includes characteristic proteolytic cleavage products of vimentin). Actin (A) and vimentin (V) are indicated. The common band present in the four lanes is endogenous to the reticulocyte lysate; differences in its intensity are due to differences in loads. **(B)** and **(C)** correspond to Coomassie Blue staining of unlabeled bovine brain actin **(B)** and fluorography of translation products synthesized by mRNAs hybridizing to pAct-1 **(C)**, run on the same gel. **(D)** Autoradiograph of translation products derived from mRNA hybridizing to pVim-1. Vimentin was identified by comigration on the same gel with unlabeled vimentin extracted from hamster lens "epithelial" cells, and is indicated here by an arrowhead; a ladder of specific breakdown products can be seen. The spot marked with an asterisk is endogenous to the reticulocyte lysate. IEF and SDS indicate the direction of electrophoretic migration under isoelectrofocusing and in the presence of SDS, respectively.

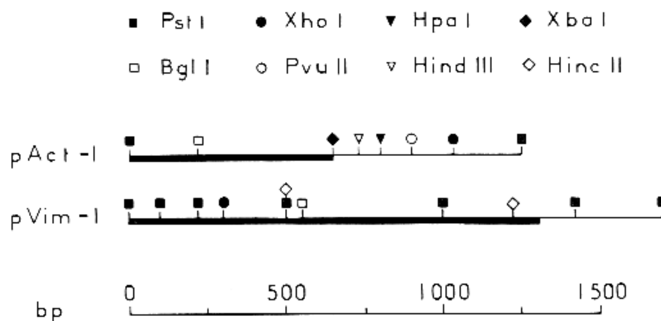

Fig. 3. Restriction maps of pAct-1 and pVim-1 inserts. Regions coding for the C-terminal sections of actin and vimentin were identified on the basis of the correspondence between restriction sites and amino acid sequences (see text); they are indicated by thick lines. The 3' non-coding regions are indicated by thin lines. The *Eco*RI site of pBR322 is located 750 bp to the left of the left end of the map.

tained from pVim-1 comigrates with unlabeled vimentin run on the same gel. In addition, there is a ladder of specific breakdown products similar to that found in Triton-KCl extracts of chicken myoblasts (Gard and Lazarides, 1980).

From the length of the actin polypeptide chain, 375 amino acids (Vandekerckhove and Weber, 1979), and the molecular weight (mol. wt.) of vimentin 57 000 (Franke *et al.*, 1979), the lengths of the coding regions in the corresponding mRNAs can be calculated as ~1100 and 1450 bases, respectively;

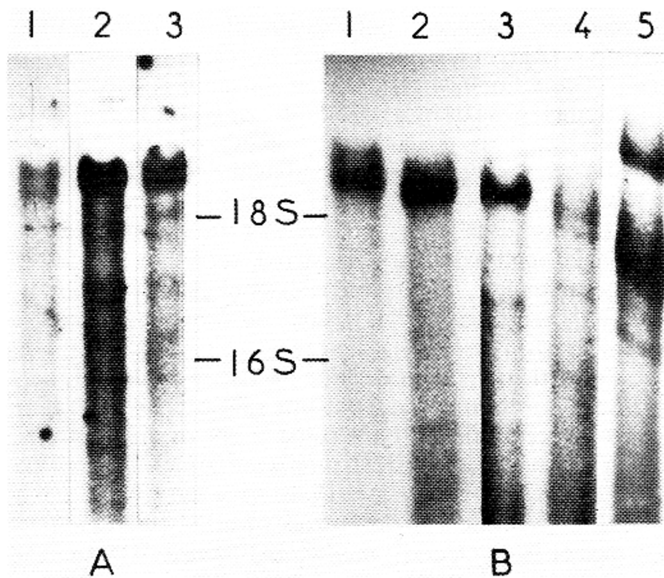

Fig. 4. RNA blot hybridization with the actin and vimentin probes. Polyribosomal poly A$^+$ RNA (20 μg) from calf lenses (1), the hamster cell line (2), rat (3), duck (4), and chicken (5) lenses, were fractionated by electrophoresis on a 1.5% agarose gel in the presence of 10 mM methylmercury hydroxide, transferred to nitrocellulose filters, and hybridized with the probes. **(A)** Hybridization with the pAct-1 probe. **(B)** Hybridization with the pVim-1 probe. Markers run on the gel were rat 28S rRNA (5000 bases), *E. coli* 23S rRNA (2940 bases), rat 18S rRNA (1940 bases), *E. coli* 16S rRNA (1570 bases) (McMaster and Carmichael, 1977).

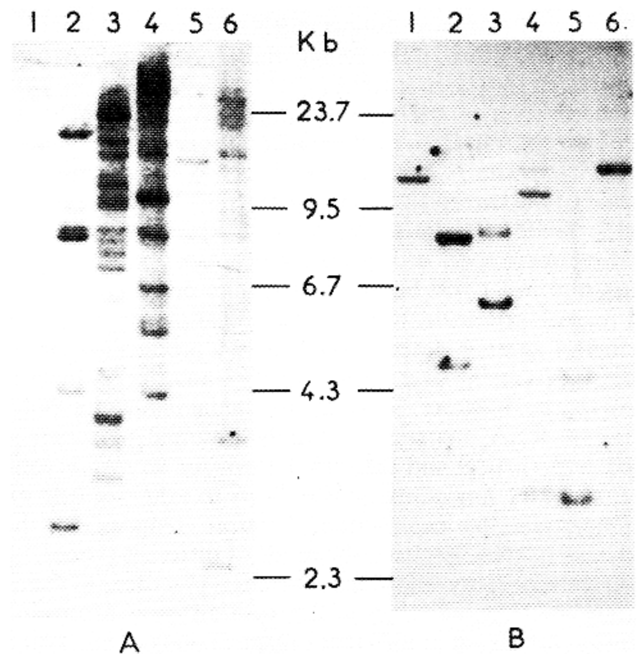

Fig. 5. DNA blot hybridization with the actin and vimentin probes. DNA from chicken erythrocytes (1), hamster liver (2), mouse liver (3), rat liver (4), calf thymus (5), and human liver (6), were degraded by *Eco*RI, fractionated on 0.6% agarose gels, transferred to nitrocellulose filters, and hybridized with **(A)** pAct-1, and **(B)** pVim-1. Loads were 8 μg of DNA in all cases, except for chicken where load was only 4 μg. *Hin*dIII digests of λ DNA were used as mol. wt. markers.

since both mRNAs are ~2100 bases long in hamster cells (see below), non-coding regions, including the poly A tract, must be 1000 bases and 600 bases long, for actin and vimentin mRNAs, respectively. The cDNA inserts in pAct-1 (1250 bp) and pVim-1 (1700 bp) contain, therefore, a substantial portion of the coding sequence of actin and most of that of vimentin. In fact, a comparison of the restriction maps of the cDNA inserts of pAct-1 and pVim-1 with predictions based on the protein sequences established for bovine β- and γ-actin (Vandekerckhove and Weber, 1979) and for the 141 amino acids from the C-terminal section of porcine vimentin (Geisler and Weber, 1981) allow us tentatively to locate the coding and non-coding regions present in the inserts (Figure 3). The comparison was made using a computer program (Roizès and Pelaquier, 1980) and was possible because of the conserved amino acid sequence of these proteins in mammals. This analysis also suggests that the cDNA inserts correspond to the region coding for the C-terminal section of the proteins and to the 3′ non-coding regions. Finally, it should be mentioned that the restriction map of pVim-2 (whose insert length was 1200 bp) overlapped with that of pVim-1 (data not shown).

Size determination of actin and vimentin mRNAs

Nick-translated, ^{32}P-labeled pAct-1 and pVim-1 were hybridized to poly A$^+$ RNA from the hamster lens epithelial cell line and from rat, calf, chicken, and duck lenses. Both probes cross-hybridized to the RNAs from these five different sources. Hybridization of pAct-1 to mRNAs from three mammals revealed a band, corresponding to 2100 bases; in the case of calf, a distinct additional band of 2000 bases could be detected (Figure 4A). Since our probe hybridizes to both β- and γ-actin mRNAs (Figure 2C), this result suggests that both mRNAs have virtually the same size in hamster and rat, but not in calf. With the vimentin probe, an mRNA species 2070 bases long could be revealed in all cases; in addition, the two avian mRNAs showed a strongly hybridizing band corresponding to 2400 bases and several weaker bands of lower mol. wts. (Figure 4B).

Detection of actin and vimentin sequences in genomic DNAs

*Eco*RI restriction digests of human, calf, rat, hamster, mouse, and chicken DNAs were hybridized with nick-translated ^{32}P-labeled actin and vimentin plasmids. With the actin probe, all DNAs displayed multiple bands of widely different intensities (Figure 5A). Calf and chicken DNAs exhibit simpler multiband patterns of lower intensity than the other DNAs; hamster DNA displayed a rather simple pattern with strong bands. The vimentin probe, by contrast, hybridized to only one strong band in chicken, rat, calf, and human DNAs or to two strong bands in hamster and mouse DNAs; one or two additional faint bands appeared in rat, calf, and human DNAs, but not in chicken, hamster, or mouse DNAs (Figure 5B). One or two strong hybridization bands were also obtained with restriction digests of mouse, human, rat, calf, and hamster DNAs produced with *Bam*HI, *Hin*dIII, *Xho*I, *Sal*I, and *Cla*I (data not shown). Interestingly, only one strong hybridization band was found in the *Bam*HI digest of hamster DNA, and in the *Xho*I digest of mouse DNA. Therefore, all six DNAs showed a single hybridization band with at least one restriction digest.

Discussion

This work provides information mainly bearing on the mRNAs for cytoskeletal actins and for vimentin, and on the corresponding genes in some warm-blooded vertebrates.

Hybridization of the actin probe pAct-1 with mRNAs from hamster, rat, and calf revealed an actin mRNA species 2100 bases long, in agreement with results previously reported for both mammals and birds (Cleveland *et al.*, 1980; Hunter and Garrels, 1977; Katcoff *et al.*, 1980; Minty *et al.*, 1981). Only

in the case of calf, two distinct mRNA species, differing in length by 100 bases, were found; these presumably correspond to β- and γ-actin mRNAs.

Hybridization of the vimentin probe pVim-1 to mRNAs from hamster, rat, calf, chicken, and duck revealed a vimentin mRNA species 2070 bases long. This was accompanied, in the case of the two avian RNAs, by several additional mRNA species. Some of these species were of lower mol. wt. and could represent specific degradation products, but one had a higher mol. wt. (2400 bases). This species might correspond to a nuclear precursor, to a polyribosomal precursor, to the product of another vimentin gene, or to a product arising by different processing of a single primary transcript. A nuclear precursor is, however, unlikely to contaminate the polyribosomal RNA preparation; a polyribosomal precursor is purely hypothetical and thus not known for any eukaryotic gene; a second vimentin gene is unlikely to exist (see below). The most plausible explanation, therefore, remains that the 2400-base mRNA species is the result of different processing of the same primary transcript. There is a precedent in the α-amylase mRNAs, although in this case different processing pathways are found in different organs (Young *et al.*, 1981).

The lengths of the mRNAs coding for cytoskeletal actins and vimentin are essentially the same in all mammals tested. Since the coding regions are conserved in length (Vandekerckhove and Weber, 1979; Franke *et al.*, 1979), this implies that the lengths of the non-coding regions, 1000 and 600 bases, respectively, are also conserved. In addition, the 5′ and 3′ non-coding regions should be individually conserved in length, unless differences in the two regions compensate each other. This length conservation, while not precluding a sequence divergence, which was in fact observed (Shani *et al.*, 1981), is an unexpected finding which may suggest a "structural" role for the non-coding regions.

The hybridization results obtained with the pAct-1 and pVim-1 probes on DNAs from warm-blooded vertebrates indicate a conservation of the sequences of the corresponding actin and vimentin genes. Such a conservation has been found for the genes of other structural proteins (Cleveland *et al.*, 1980) and is not necessarily limited to warm-blooded vertebrates. In fact, both our probes hybridize to other eukaryotic DNAs, but less strongly (data not shown). It should be pointed out, however, that the hybridization bands obtained with pAct-1 are much stronger with rodent DNAs than with DNAs of the other species, whereas those obtained with pVim-1 are much closer in intensity with all the different DNAs. This suggests greater sequence conservation of the vimentin gene than the actin genes.

As far as the gene copy number is concerned, the situation appears to be different for actin and for vimentin. With the actin probe, all DNAs display multiple bands, indicating that the actin genes form a multigene family (which includes, in fact, the muscle α-actin genes as well), in agreement with previous reports (Firtel, 1981; Cleveland *et al.*, 1980; Engel *et al.*, 1981). Rather surprisingly, the hamster probe revealed many more hybridization bands in mouse and rat than in hamster DNA, suggesting that the copy number of actin is different in these different rodents. A high number of hybridization bands was also found in human DNA, whereas this number was smaller in chicken and calf. In this case, however, the sequence divergence might be responsible for the difference seen.

Hybridization of the vimentin probe shows a pattern quite different from that obtained with the actin probe since, in all six genomes examined, only single, strong hybridization bands, 3 – 12 kb in size, were found, in at least one restriction digest. These findings suggest that vimentin is encoded by either a single gene or by a tight cluster of genes. In calf DNA the band size, 3 kb, precludes the existence of more than one vimentin gene, since the corresponding mRNA is already 2.1 kb in size (1.7 kb of which are represented in the probe). This being the case, it is highly unlikely that the vimentin gene is present in multiple tightly clustered copies in the other genomes examined. A detailed analysis of genomic clones is, however, needed to provide a final confirmation of such a general conclusion.

As far as the faint hybridization bands are concerned, they may be due to small gene fragments split off the main gene segment by restriction enzyme cut(s) in one or more introns, and/or to other genes of the IF multigene family. The first explanation appears to be more likely since the second one is difficult to reconcile with the absence of such bands in restriction digests from chicken, mouse, and hamster DNAs. The latter case is particularly striking since a hamster probe was used and this should have more homology with the other IF genes of hamster than with those of other species.

An implication of this interpretation is that genes from the IF multigene family have diverged enough so as not to give any evident hybridization with pVim-1, at least under the rather stringent conditions used in this work. It should be noted that the large sequence divergence of the genes of the IF family is accompanied by a high sequence conservation in the corresponding proteins, as vimentin shares a 64% homology over the last 141 C-terminal amino acids with another IF protein, desmin; in particular, a stretch of 37 amino acids in this region is common to the two proteins with only one amino acid change (Geisler and Weber, 1981). Such a situation is not a new one, since it has already been found in the case of other structural proteins.

Our conclusion that, in all likelihood, there is just one vimentin gene in the species examined is at variance with indications to the contrary based on preliminary characterization of the chicken vimentin gene (Zehner and Paterson, 1981). However, after the work described here was completed, a report appeared (Zehner *et al.*, 1981) confirming our finding of only one vimentin gene and two vimentin mRNA species in chicken.

Materials and methods

Isolation and translation of mRNA

Polyribosomes from an SV40-transformed hamster lens epithelial cell line (Bloemendal *et al.*, 1980) and from whole lenses from rat, chicken, and duck, were prepared according to Palmiter (1974); polyribosomes from calf lenses were prepared according to Bloemendal *et al.* (1966). Poly A^+ RNA was purified by oligo(dT)-cellulose chromatography, and fractionated in sucrose gradients after disaggregation in 10 mM methylmercury hydroxide (Dodemont *et al.*, 1981); this denaturing agent, used to date only in gel electrophoresis, was found to produce much better separations than formamide or dimethyl sulfoxide. Fractions containing actin and vimentin mRNAs were identified by assaying 1 μl aliquots directly in a nuclease-treated rabbit reticulocyte lysate (Pelham and Jackson, 1976). Translation products were analyzed by one- and two-dimensional electrophoresis (Laemmli, 1970; O'Farrell, 1975), and revealed by fluorography (Bonner and Laskey, 1974).

Construction of recombinant plasmids

Double-stranded cDNAs were synthesized using a one-step procedure derived from Wickens *et al.* (1978). Reaction mixtures for first strand synthesis (20 μl) consisted of 50 mM Tris, pH 8.3, 8 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 1 mM of each dNTP (including trace [^{32}P]dCTP; Radiochemical Center Amersham, UK), 100 μg/ml oligo$(dT)_{12-18}$ (Collaborative Research, Waltham, MA), 50 μg/ml mRNA and 1000 units/ml reverse

transcriptase (a generous gift of J. Beard). After incubation at 42°C for 15 min, reaction mixtures were placed in a boiling water bath for 3 min, quickly chilled in ice, and supplemented with an equal volume of the reaction mixture for second strand synthesis; this contained 200 mM Hepes pH 6.9, 12 mM $MgCl_2$, 10 mM DTT, 1 mM each dNTP, and 200 units/ml of the large fragment of *Escherichia coli* DNA polymerase I (Boehringer, Mannheim, FRG). Reaction mixtures were incubated at 15°C for 90 min, heat-inactivated, and filtered on Sephadex G50. cDNAs were precipitated with ethanol in the presence of *E. coli* tRNA carrier, treated with S1 nuclease (BRL, Gaithersburg, MD), and fractionated on 1.5% agarose gels. Fragments 1200 – 2600 bp long were eluted electrophoretically, extracted with phenol, dialyzed against water, and lyophilized. cDNAs and *Pst*I-cleaved pBR322 were tailed with dCTP and dGTP, respectively, using terminal transferase (P.L. Biochemicals, Milwaukee, WI); hybrid molecules were constructed by annealing and used to transform competent *E. coli* HB101 as described (Therwath *et al.*, 1980). Colony hybridization was performed according to Grunstein and Hogness (1975). Plasmid DNAs were extracted according to Birnboim and Doly (1979), or purified by CsCl density gradients in the presence of ethidium bromide. All experiments were performed under the containment conditions recommended by the French Committee on Recombinant DNA.

Positive hybridization-translation assays

2 μg of vector and recombinant plasmid DNAs (in 10 mM Tris, pH 7.5, 1 mM EDTA) were heated at 100°C for 5 min, chilled in ice, brought to a final concentration of 6 x SSC (standard saline citrate; 0.15 M NaCl, 0.015 M trisodium citrate), and spotted on nitrocellulose filters (BA85, Schleicher and Schüll, Dassel, FRG) previously treated with 6 x SSC. Filters were rinsed in 6 x SSC, dried, and heated at 80°C for 2 h *in vacuo*. Prehybridization was performed at 50°C for 2 h in 50% formamide, 10 mM Pipes, pH 6.4, 0.4 M NaCl, 4 mM EDTA, and 100 μg/ml poly (rA). Hybridization was carried out in the same buffer with poly (rA) substituted by 50 – 100 μg/ml mRNA, with the temperature gradually lowered from 55°C to 45°C over a period of 3 h. Filters were washed with the hybridization solution for 30 min at 45°C, followed by five washings, each for 1 min, in 1 x SSC, 0.5% SDS at room temperature, five washings in 0.1 x SSC, 0.1% SDS, at 55°C, and two washings in 10 mM Tris, pH 7.5, 1 mM EDTA, at 55°C. Hybridized mRNAs were extracted by boiling the filters in 100 μl of water for 60 s, and precipitated with ethanol in the presence of 1 μg of *E. coli* tRNA carrier. The precipitates were collected, washed with 75% ethanol, dried, dissolved in 2 μl of water, and assayed for *in vitro* translation (see above).

Electrophoresis of RNA and DNA and blot-hybridization

Electrophoresis of RNA was performed on 1.5% agarose gels containing 10 mM methylmercury hydroxide (Bailey and Davidson, 1976). DNA preparation, digestion with restriction endonucleases, and electrophoresis in horizontal agarose gels were as described (Van der Putten *et al.*, 1979). Transfer of RNA and DNA to nitrocellulose filters was performed according to Thomas (1980), and Southern (1975), respectively. Prehybridization and hybridization conditions with nick-translated plasmid probe ($1 - 5\ 10^8$ c.p.m./μg) was performed as described by Wahl *et al.* (1979), except that dextran sulfate was omitted from the hybridization solution. After hybridization, filters were washed twice with the hybridization solution at 42°C for 1 h, once with 2 x SSC, 0.1% SDS at 55 – 60°C for 15 min and twice with 0.1 x SSC, 0.1% SDS at 55 – 60°C. Autoradiography was for 1 – 5 days at – 70°C with Kodak XR1 film, using intensifying screens.

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