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Involvement of the consensus sequence motif at coil 2b in the assembly and stability of vimentin filaments

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Summary

Nearly all intermediate filament (IF) proteins share two sequence motifs located at the N- and the C-terminal ends of their helical rod domain ('coil 1a' and 'coil 2b', respectively). To examine the structural role of the coil 2b motif, we have performed in vitro assembly studies and in vivo microinjection experiments employing two site-specific reagents: (a) a 20-residue synthetic peptide (C-2) representing the conserved motif itself and (b) a monoclonal antibody (anti-IFA) that recognises an epitope within the conserved coil 2b sequence. We demonstrate here that vimentin protofilaments, when induced to assemble in the presence of C-2 or anti-IFA, show a lower propensity to polymerise and yield various abberant structures. The few filaments that are formed under these conditions appear much shorter than normal IFs and are unravelled or aggregated. Furthermore, when preformed vimentin filaments are exposed to C-2 or anti-IFA, most of the normal IFs are converted into shorter filamentous forms that possess an abberant morphology. None of these effects is seen when vimentin subunits are coincubated with control peptides. Microinjection of anti-IFA into the cytoplasm of interphasic 3T3 cells provokes collapse of vimentin IFs into a juxtanuclear mass and formation of numerous amorphous aggregates distributed throughout the cytoplasm. These two effects are not seen when the anti-IFA is microinjected into the cell nucleus. Our results provide experimental evidence supporting previous suggestions for a role for the conserved coil 2b sequence in filament assembly. We propose that this region is interacting with other sites along the vimentin molecule and that these interactions are essential for proper protofilament-protofilament alignment and filament stability.

Key words: vimentin, anti-IFA, microinjection, filament assembly, intermediate filaments.

Introduction

IF proteins share a common molecular organisation (Hanukoglu and Fuchs, 1982, 1983; Fisher et al., 1986; Geisler and Weber, 1982; McKeon et al., 1986; Steinert et al., 1983; for recent reviews see Steinert and Roop, 1988; Stewart, 1990). Each subunit molecule possesses a highly conserved alpha-helical domain of 310-350 residues ('rod'), flanked by two variable and non-helical end-domains ('head' and 'tail' domains, respectively). The sequences of the alpha-helical domains conform to a heptad-repeat pattern that provides a physical basis for assembling two-chain coiled-coils, the fundamental building blocks of IFs (Geisler and Weber, 1982). Despite the sequence variability among different IF proteins, two positions along the IF subunit molecules are very much conserved (Conway and Parry, 1988): one is found near the N-terminal end of the helical domain (coil 1a) and the other near the C-terminal end of the helical domain (coil 2b). The remarkable conservation in these two sites suggests that the corresponding sequences may play a crucial structural role during assembly and filament formation.

This postulate is supported by previous in vivo and in vitro studies. Deletion mutagenesis and transfection experiments have revealed that cytokeratin forms lacking the tail domain and part of the conserved coil 2b region, although they are readily incorporated into the endogenous cytokeratin networks of tissue culture cells, disrupt native IFs (Albers and Fuchs, 1987, 1989; Coulombe et al., 1990). Similar results have been obtained in transfection experiments with mutagenised neurofilament triplet proteins (Wong and Cleveland, 1990; Gill et al., 1990). In vitro, cytokeratins point-mutagenised in the coil 2b consensus motif exhibit very aberrant polymerisation properties at the standard salt concentrations used for assembly of wild-type subunits (Hatzfeld and Weber, 1991). On the other hand, purified cytokeratins missing the tail domain and the coil 2b motif assemble (in the presence of a normal cytokeratin partner) into nearly normal IFs at lower salt (Coulombe et al., 1990).

As has been recently argued (Stewart, 1990), a potential limitation in studies with recombinant proteins can be the problem of deletion-induced conformational changes. On the one hand, when truncated IF proteins fail to assemble, one cannot easily discriminate between specific effects caused by the excision of a functional site and non-specific effects due to global or regional misfolding of the mutagenised polypeptide chain. On the other hand, extensive deletions may mask the effects of a mutation by removing regions involved in opposing interactions. To bypass such caveats, we have reinvestigated the role of the consensus coil 2b sequence employing purified vimentin subunits, a synthetic analogue of the conserved coil 2b motif (C-2), and a monoclonal antibody recognizing this site (anti-IFA; see Pruss et al., 1981). Similar approaches have been employed in the past to probe the role of specific sites along IF proteins in filament assembly (Birkenberger and Ip, 1990; Kouklis et al., 1991).

The rationale for choosing these reagents is based on the hypothesis that the coil 2b region in one vimentin molecule may specifically associate with distinct sites in neighbouring subunits. If this assumption were correct, one would expect a synthetic analogue of the coil 2b motif to be capable of competing with the natural coil

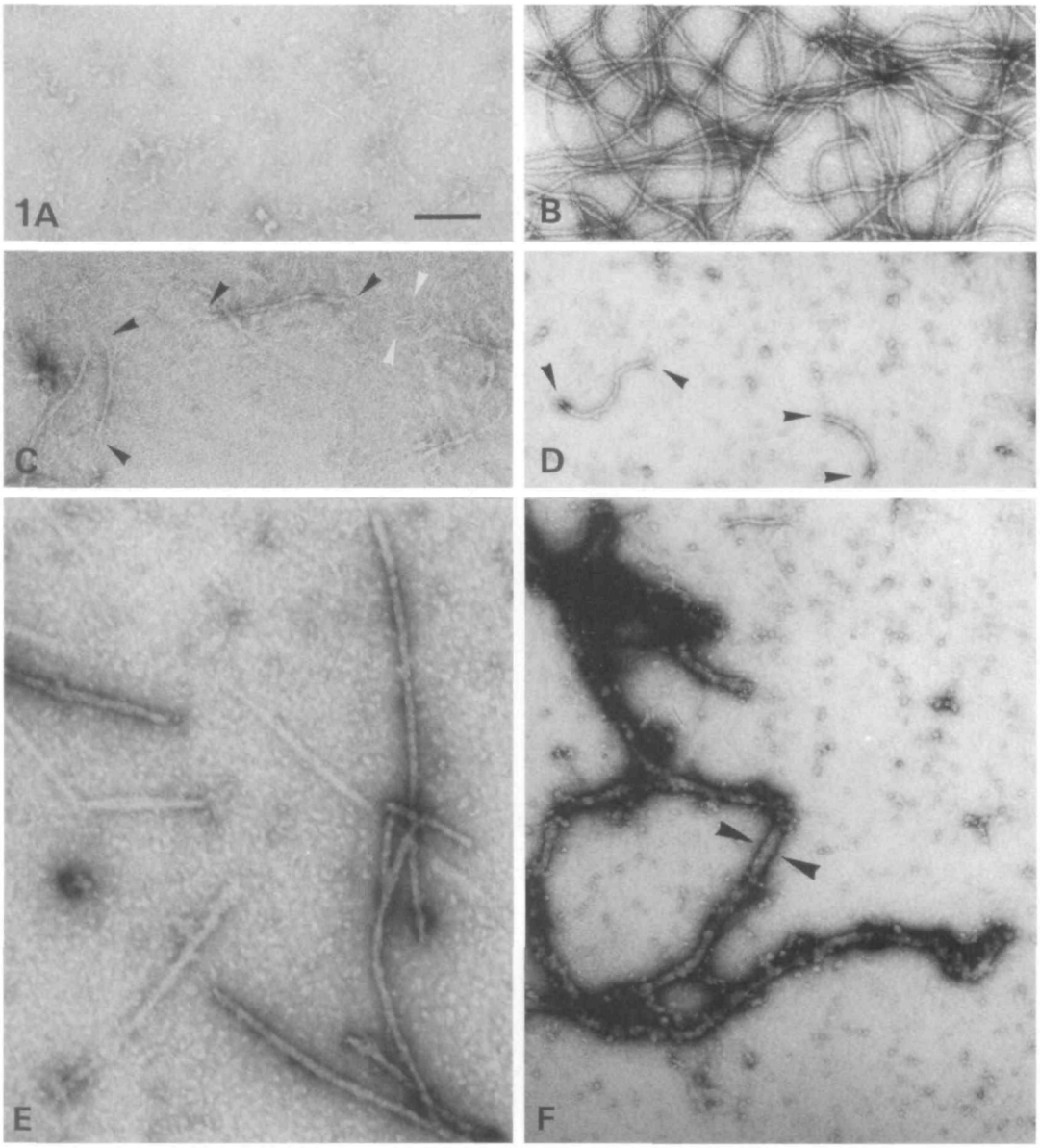


Fig. 1. Dose-dependence of the peptide C-2 effects on vimentin assembly. Vimentin protofilaments (A) were preincubated (60 min, room temperature) either with buffer alone (B), or with a 50-fold (C, E), or 70-fold (D, F) molar excess of C-2. After this preincubation, the samples were adjusted to 150 mM KCl and further incubated for 60 min at 37°C. The polymers formed were visualised by negative staining and electron microscopy. All reaction mixtures contained 150 μg/ml of vimentin. Note the scarcity of filaments, the short filaments (thin dark arrowheads), the unravelled filaments in the background (light arrowheads) and the thick filamentous structures (thick arrowheads) in the samples pre-incubated with C-2. Bar: A, B, C, D, F 100 nm; E, 73.5 nm.

2b sequence for binding to such 'acceptor' sites during vimentin assembly. Likewise, the anti-IFA antibodies would be expected to destabilise such hypothetical interactions by 'blocking' the coil 2b sites of vimentin protofilaments. Given that point-mutations in the coil 2b motif do not seem to affect the ability of cytokeratin subunits to tetramerise (Hatzfeld and Weber, 1991), we used as a starting material in our assembly studies vimentin *protofilaments* (i.e. vimentin oligomers soluble in low ionic strength buffer solutions), not urea-denatured vimentin monomers. The results of this study strongly support the notion that coil 2b is directly involved in assembly reactions beyond the stage of the oligomeric protofilament.

Materials and methods

Protein chemical procedures

Vimentin was purified from tissue culture cells as previously described (Nelson and Traub, 1982). Urea-denatured material was extensively dialysed against 10 mM Tris-HCl, pH 7.4, at 4°C to assemble protofilaments and kept in aliquots at −70°C. The synthetic peptide C-2, a 20-mer, has been modelled after the conserved coil 2b sequence of mammalian vimentin (see Fig 4A). It includes the nearly invariant sequence T Y R K L L E G, flanked by vimentin-specific residues that vary slightly in other IF proteins. C-2 comprises two complete heptads (I A T Y R K L/L E G E E S R), with a half-heptad at its N-terminal side (A L D I E). The peptide is soluble in water, or isotonic salt, up to ~5 mg/ml. Although bearing a net (negative) charge, it is well buffered by 10 mM Tris-HCl, pH 7.4, at the concentrations used in this study. C-2 and LAT I (a control peptide modelled after the tail domain of chicken lamin A; residues 562-583; Peter et al., 1989) were synthesised at the EMBL protein sequencing and peptide synthesis facility and purified to homogeneity by HPLC. The PI and VI peptides (representing residues 405-434 and 423-446 of the IF proteins peripherin and vimentin, respectively; see Leonard et al., 1988; Quax et al., 1983) were made at the Rockefeller University biopolymer facility (New York, USA). The synthetic peptides were dialysed against 10 mM Tris-HCl, pH 7.4, before each assay using dialysis tubing with a molecular mass cutoff of 1,000 Da. The concentration of the dialysed preparations was determined by amino acid analysis. Anti-IFA (Pruss et al., 1981) was purified from the culture supernatant of hybridomas maintained in serum-free media (Nutridoma SP, Boehringer Mannheim, FRG) by precipitation with ammonium sulphate.

Assays

Immunoblotting and dot-blotting assays were done as specified by Djabali et al. (1991). The integrity of the vimentin material after coincubation with antibodies or synthetic peptides was assessed by SDS-PAGE (Laemmli, 1970). Assembly mixtures were applied to carbon-coated 300 mesh EM grids and the samples were stained with 1.5% uranyl acetate for 3 min at room temperature. After air-drying, the structures formed were visualised in a Philips 301 electron microscope operated at 80 kV.

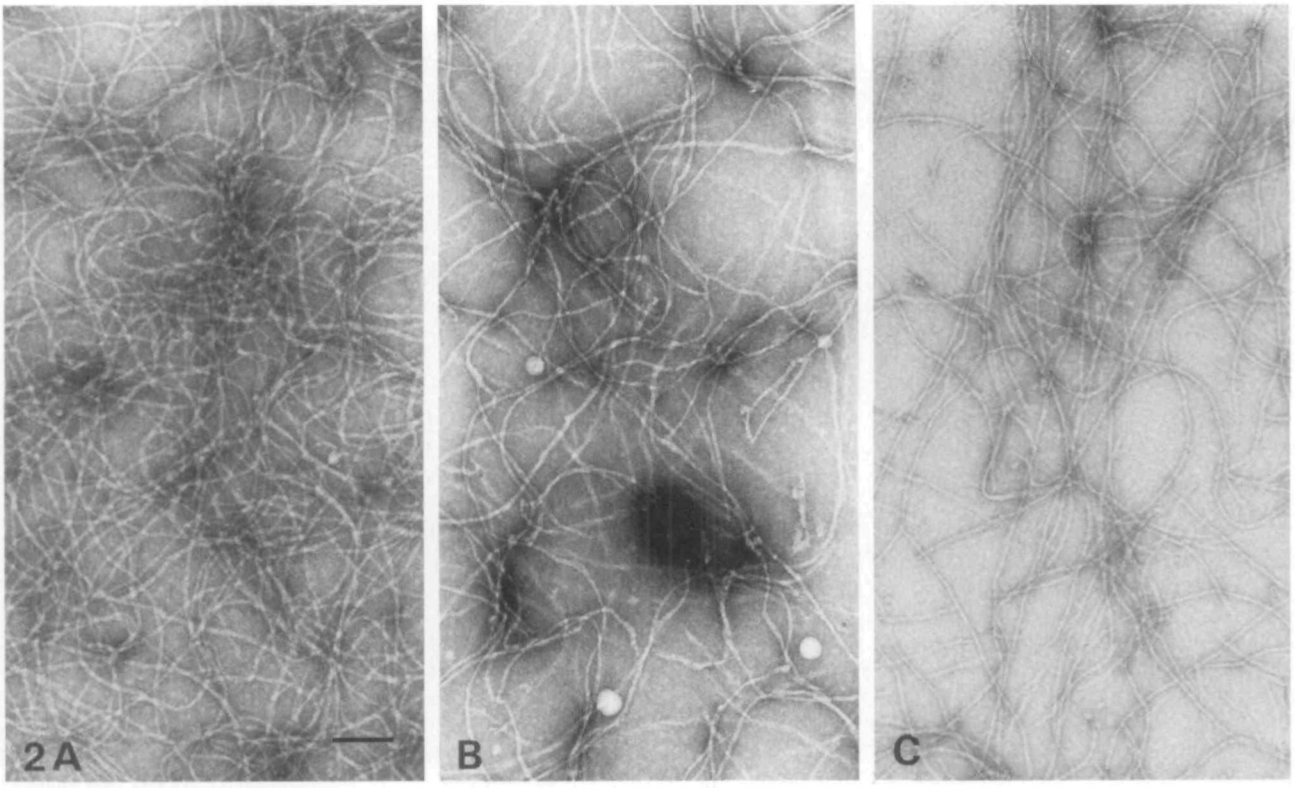


Fig. 2. Lack of a effect on vimentin assembly by control peptides. Vimentin protofilaments were assessed for their ability to polymerise after a preincubation with a 100-fold molar excess of peptides V I (A), PI (B) and LAT I (C) (for sequence assignments see Materials and methods). Bar, 100 nm.

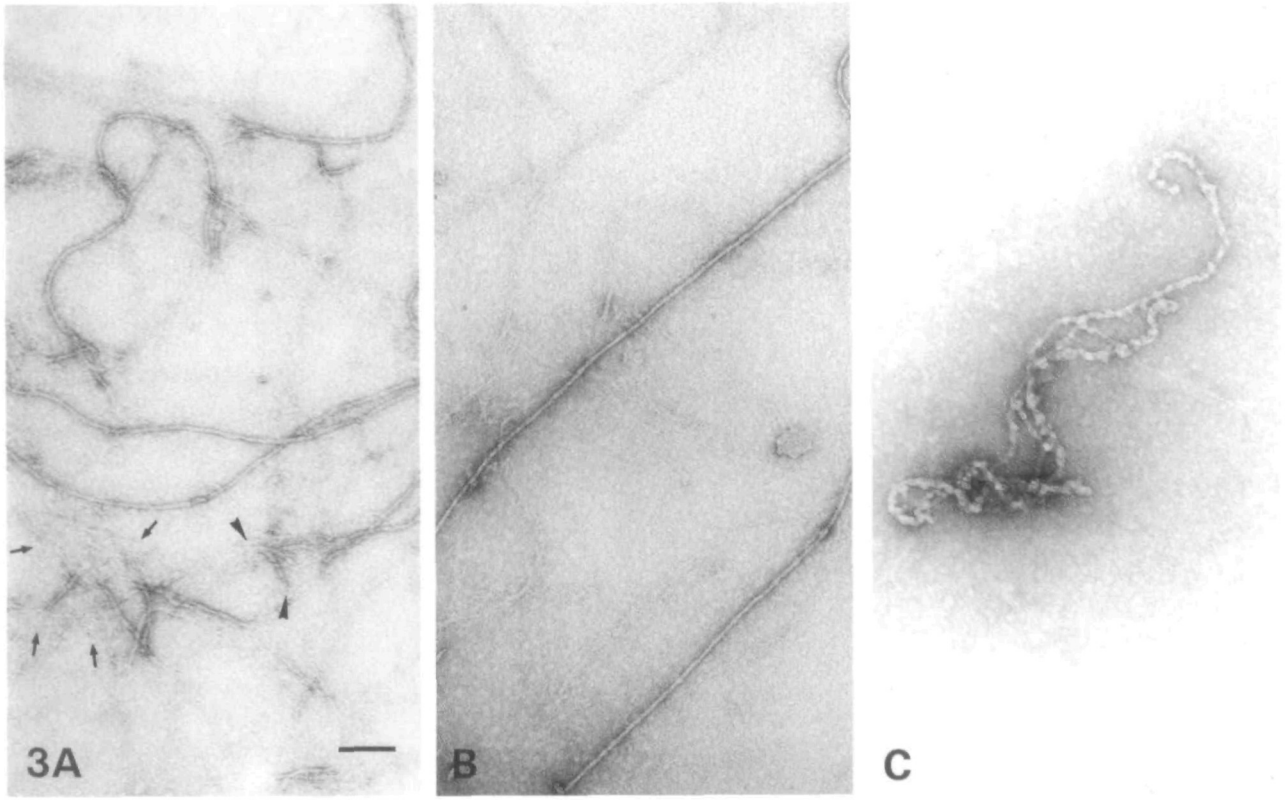


Fig. 3. Time-course of the disassembly of preformed vimentin IFs by C-2. Vimentin filaments were assembled by adjusting the salt of a protofilament solution (750 μg/ml) to 150 mM KCl and incubating the samples for 60 min at 37°C. After this, a 50-fold excess of C-2 peptide (in isotonic salt) was added (final vimentin concentration 150 μg/ml) and further incubations ensued for 10 (A), 60 (B) and 120 min (C) at 37°C. Note the gradual fragmentation of the vimentin polymers (arrowheads), the transient 'straight rod' morphologies of the remaining IFs (B) and the appearence of twisted aggregated filaments at longer times of incubation (C). Unravelled filaments are indicated by thin arrows. Bar, 100 nm.

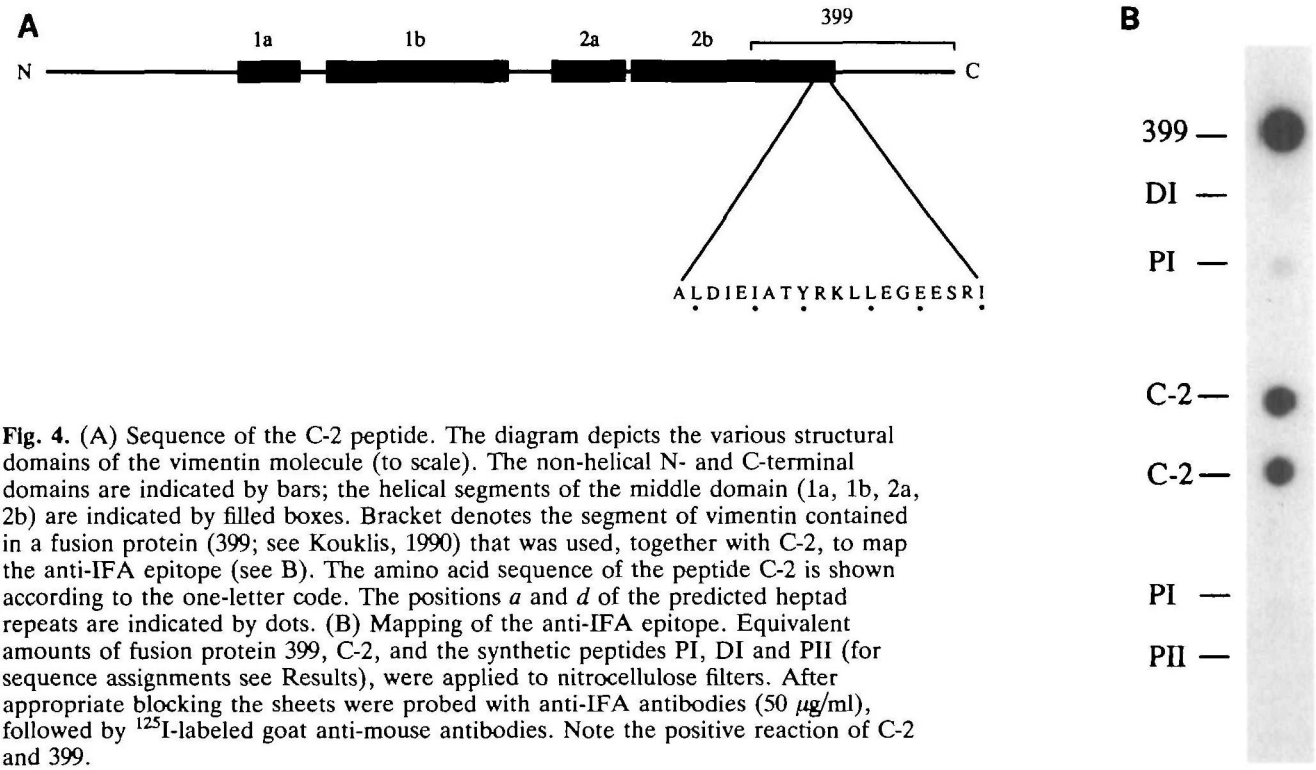


Fig. 4. (A) Sequence of the C-2 peptide. The diagram depicts the various structural domains of the vimentin molecule (to scale). The non-helical N- and C-terminal domains are indicated by bars; the helical segments of the middle domain (1a, 1b, 2a, 2b) are indicated by filled boxes. Bracket denotes the segment of vimentin contained in a fusion protein (399; see Kouklis, 1990) that was used, together with C-2, to map the anti-IFA epitope (see B). The amino acid sequence of the peptide C-2 is shown according to the one-letter code. The positions *a* and *d* of the predicted heptad repeats are indicated by dots. (B) Mapping of the anti-IFA epitope. Equivalent amounts of fusion protein 399, C-2, and the synthetic peptides PI, DI and PII (for sequence assignments see Results), were applied to nitrocellulose filters. After appropriate blocking the sheets were probed with anti-IFA antibodies (50 μg/ml), followed by ^{125}I-labeled goat anti-mouse antibodies. Note the positive reaction of C-2 and 399.

Microinjection

Injection of 3T3 cells was performed exactly as specified by Kouklis et al. (1991).

Results

In vitro effects of the C-2 peptide

To examine the role of the coil 2b sequence motif in vimentin filament assembly, we preincubated a standard amount of vimentin protofilaments (EM profile shown in Fig. 1A) with increasing amounts of C-2 for 60 min at room temperature. After this preincubation, in vitro polymerisation of vimentin was induced by increasing the ionic strength to 150 mM KCl and further incubating the reaction mixtures for another 60 min at 37°C.

Fig. 1B demonstrates that, in control samples, increasing the ionic strength of the vimentin protofilament solution results in numerous, unbranched 10 nm filaments. Addition of small amounts of C-2 (e.g. a 10-fold molar excess) does not visibly affect the ability of vimentin protofilaments to polymerise into normal-looking IFs (data not shown). However, when the same experiment is repeated with a 50 to 70-fold excess of peptide, two major effects are detected on vimentin polymerisation. First, much fewer filaments are seen throughout the fields (Fig. 1C-E). Second, the few filaments that do form under these conditions are shorter than the normal IFs (Fig. 1C-E, thin dark arrowheads) and appear either unravelled (Fig. 1C, light arrowheads), or thicker (Fig. 1F, thick arrowheads) than usual. Varying the time of preincubation of vimentin protofilaments with C-2 shows that the peptide effects are rather rapid (<10 min; data not shown).

To examine the specificity of the C-2 effects, we coincubated vimentin protofilaments with several other synthetic peptide preparations and assessed the assembly products. Fig. 2A demonstrates that a peptide modelled after the tail domain of vimentin, which is adjacent to C-2 (V I, residues 432-446; Quax et al., 1983), has no effect on IF assembly, even at a 100-fold molar excess. Other peptides, like P I for example, patterned according to the tail domain of rat peripherin (residues 405-434; Leonard et al., 1988) and LAT I (modelled after the chicken lamin A tail domain, residues 562-583; Peter et al., 1989) also show no significant effect on the length and general appearance of the IFs (Fig. 2B, C). (The 'rough' surface of the

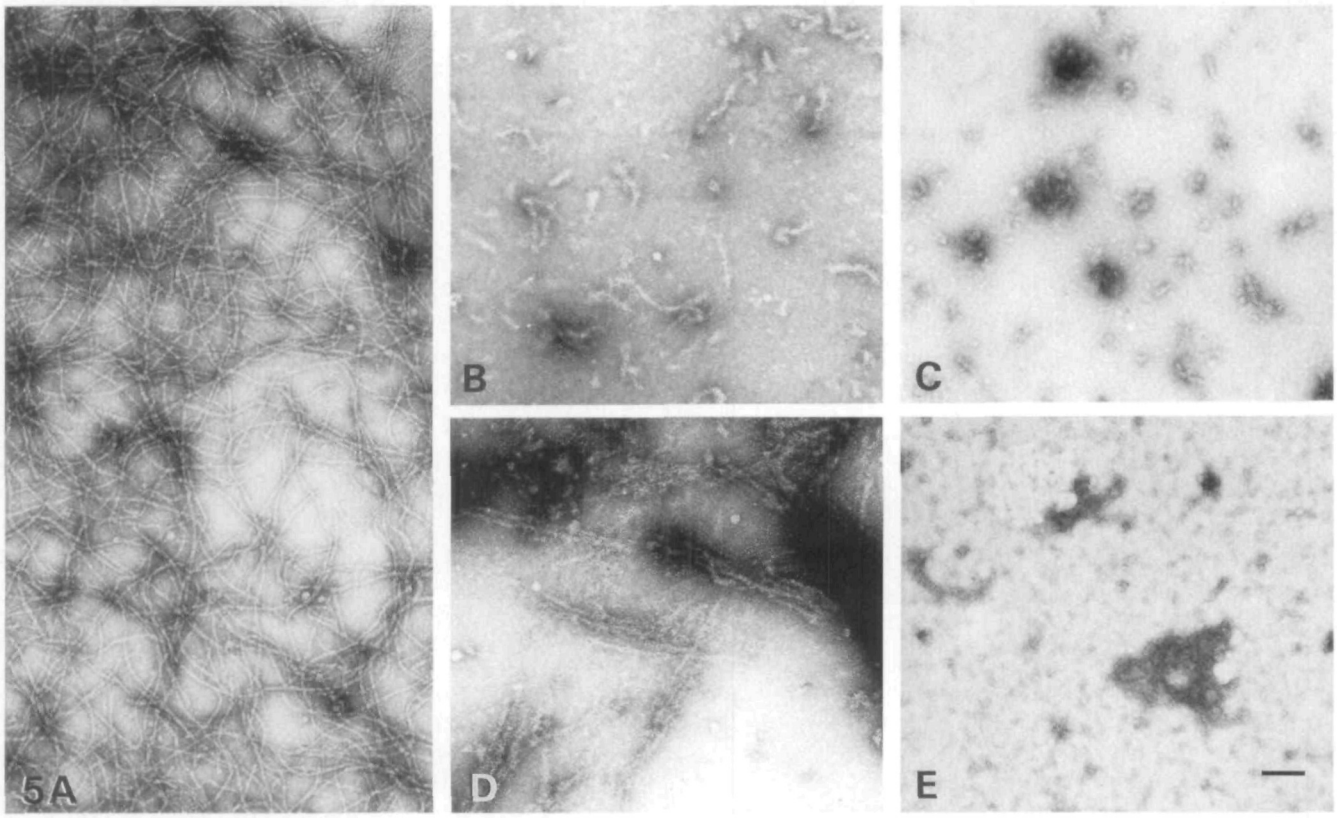


Fig. 5. Dose-dependence of the effects of anti-IFA antibodies on vimentin assembly. (A, B, C) Experiments with vimentin protofilaments. Vimentin protofilaments were preincubated with 20 μg/ml (A), 200 μg/ml (B) and 700 μg/ml (C) of purified anti-IFA IgG for 60 min at room temperature. After this, polymerisation was induced (as above) and samples were visualised by electron microscopy. (D,E) Dose-dependence of the effects of anti-IFA antibodies on preformed vimentin filaments. Vimentin filaments were assembled as in Fig. 1 and then incubated with 500 μg/ml (D) and 2 mg/ml (E) of anti-IFA for 120 min at 37°C. Note the gradual fragmentation of the vimentin IFs into short filament pieces and aggregated filaments. Bar, 100 nm.

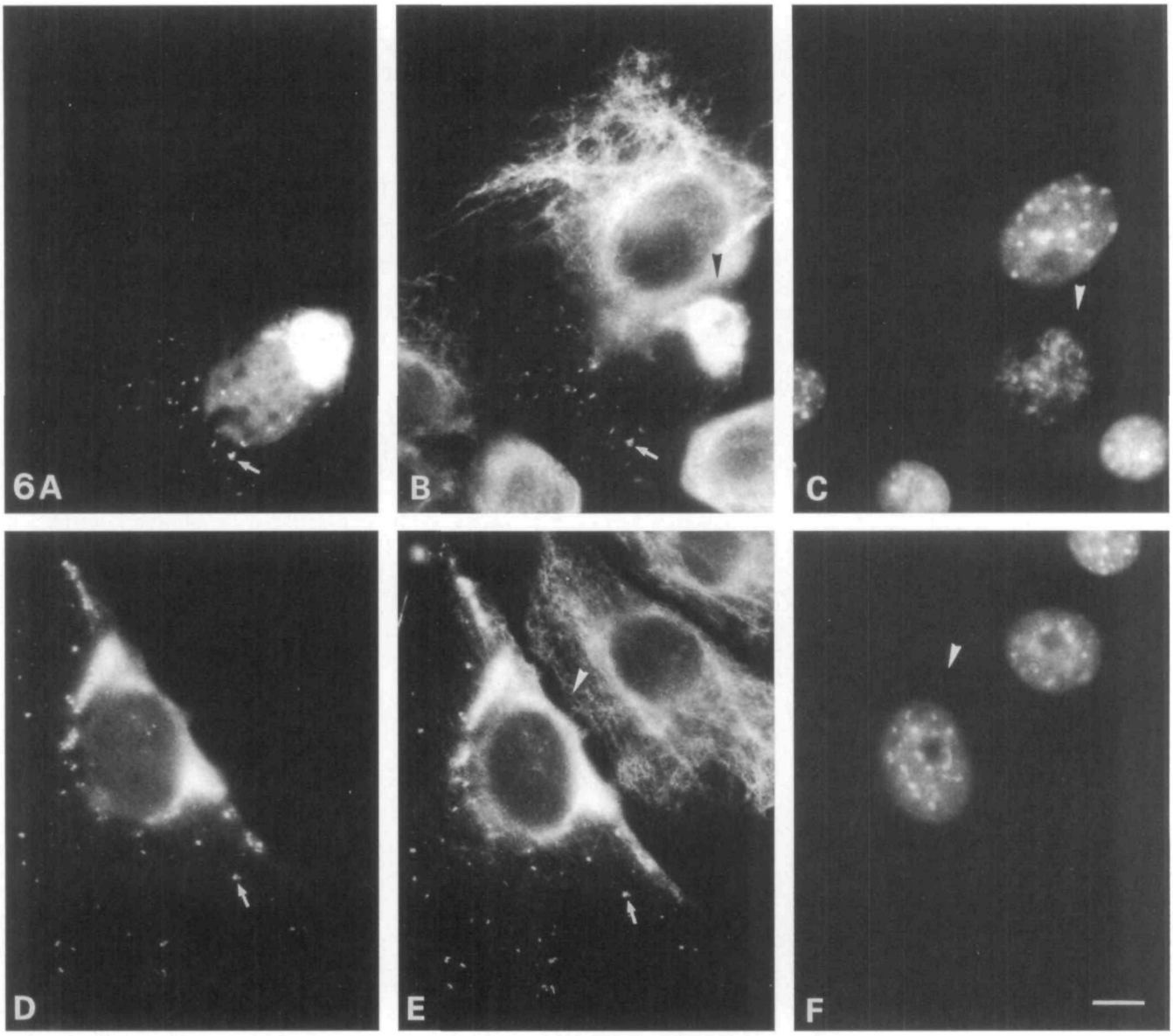


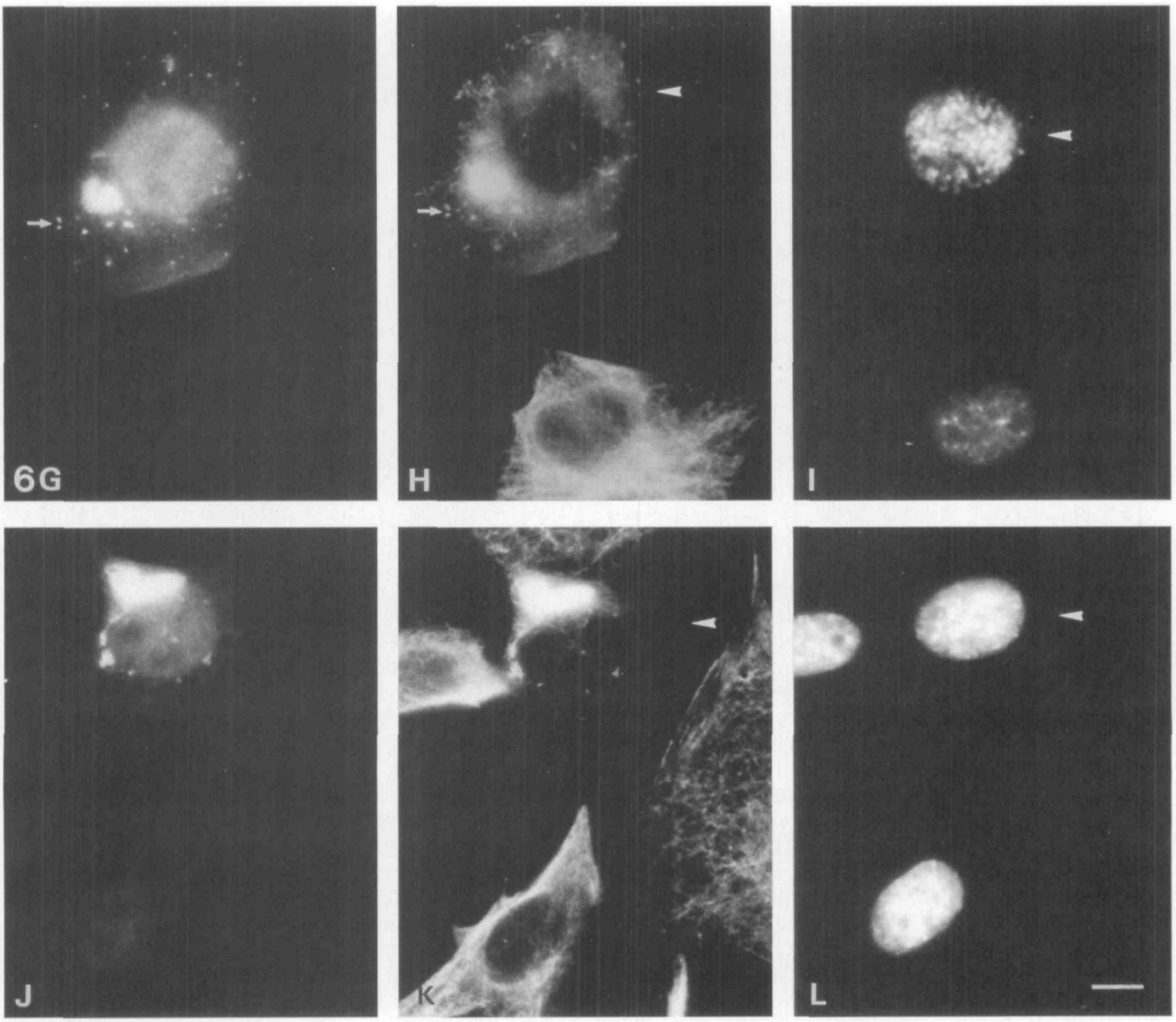
Fig. 6. Microinjection of anti-IFA into the cytoplasm of 3T3 cells. Anti-IFA (8 mg/ml) was injected into the cytoplasm of 3T3 cells. The cells were fixed at 4.5 h (A-C), 8 h (D-F), 21 h (G-I), and 42 h (J-L) and examined by indirect immunofluorescence. Vimentin IFs were localised using a goat anti-vimentin polyclonal serum and rhodamine-labeled rabbit anti-goat antibodies; the injected cells were identified in the same coverslips by FITC-labeled rabbit anti-mouse antibodies; DNA was stained by DAPI. (A, D, G, J) The localization of the injected antibody; (B, E, H, K) the distribution of vimentin; (C, F, I, L) the DNA staining of the same cells at each time point. Arrowheads indicate the microinjected cells; arrows indicate amorphous aggregates of vimentin, dispersed in the cytoplasm. Bar, 6 nm.

filaments in samples coincubated with control peptides is always observed when vimentin assembly is performed in the presence of high concentrations of other proteins.)

To investigate the influence of C-2 on the stability of preformed IFs, we incubated preassembled vimentin filaments with a 50-fold molar excess of C-2 for various periods of time and examined the state of the IFs by negative staining and electron microscopy. Fig. 3A shows that after 10 min of coincubation the filaments are already fewer and appear fragmented. After 60 min normal filaments are very scarce and most of the surviving IFs posseses a peculiar 'straight rod' morphology (Fig. 3B). After 120 min extensive fragmentation is detected, resulting exclusively in short twisted structures that are decorated with globular material (Fig. 3C). Repetition of these experiments using control peptides (previously reported by Djabali et al., 1991 and Kouklis et al., 1991) shows no such effects on the stability of preformed vimentin filaments.

In vitro effects of anti-IFA

To investigate further the role of the coil 2b sequence in filament assembly and to confirm the previously described observations on the C-2 peptide, we performed assembly studies in the presence of the anti-IFA



antibody. Anti-IFA is a monoclonal antibody previously characterised for its ability to recognise most forms of cytoplasmic IF proteins (Pruss et al., 1981) and nuclear lamins (Osborn and Weber, 1986). The epitope of this antibody has been mapped near the C-terminal end of the helical domain in previous studies with partially cleaved IF proteins (Geisler et al., 1983) or mutant proteins expressed in *Escherichia coli* (Hatzfeld and Weber, 1991; Magin et al., 1987). To test this assignment directly, we examined the ability of anti-IFA to react with the synthetic peptide C-2 that comprises 20 residues of the conserved coil 2b region, or with a fusion protein (399) that contains the 100 C-terminal residues of mouse vimentin (for construction, see Kouklis, 1990). As seen in Fig. 4B, consistent with the previous data, the monoclonal antibody reacts with C-2 and 399, but not with control peptides. Thus, anti-IFA provides a highly specific reagent that recognises an epitope within the consensus coil 2b sequence.

In one series of experiments, vimentin protofilaments were mixed with various amounts of anti-IFA and, after inducing polymerisation, the assembled structures were visualised by negative staining and electron microscopy. Fig. 5A shows that up to 20 μg/ml of anti-IFA (molar ratio between vimentin and antibody ~1:1) there is no visible effect on vimentin assembly. However, upon further increase in the antibody concentration (10-fold excess), the filaments become progressively shorter (Fig. 5B), until no filamentous material could be detected (Fig. 5C). No filament cross-linking or bundling has been detected in the course of these experiments. Irrelevant antibodies (IgGs) did not give the same result, although some slight aggregation of filaments was consistently observed (data not shown).

To complete this analysis, we incubated preformed vimentin filaments with various amounts of anti-IFA for a fixed period of 2 h. Data depicted in Fig. 5D and E document that the antibody effectively fragments

preassembled IFs into short filament pieces that have a tendency to aggregate laterally. However, this effect apparently requires antibody concentrations higher than the ones needed to prevent assembly of soluble protofilaments (consult legend to Fig. 5).

In vivo effects of anti-IFA

Microinjection of anti-IFA into the cytoplasm of interphasic 3T3 cells causes the following changes in the architecture of vimentin IFs. First, 4.5 h to 42 h post-injection, the cells are found to contain a mass of collapsed IFs located near the nucleus. Second, already from the early time points, numerous amorphous vimentin aggregates are observed in the cytoplasm of the same specimens (Fig. 6). The collapsing effect of anti-IFA is consistent with previous observations in 3T3 cells (Klymkowsky, 1981). However, although a 'spotty' appearence of cytokeratin material has been noticed before in epithelial cells microinjected with anti-keratin antibodies (Lane and Klymkowsky, 1982), such a phenomenon has not been previously observed in 3T3 cells that contain vimentin filaments.

Curiously, shortly after introduction of anti-IFA into the cytoplasm, one notices a slight accumulation of the injected antibody into the nucleus (see Fig. 6A). This accumulation becomes very obvious at later time points (Fig. 6G and J) and cannot be explained by entrapment of the antibody in the daughter nuclei of divided cells (since it is seen in the majority of the injected cells at early time points). Considering that the nuclear lamins possess a coil 2b motif that is very similar to that of vimentin (Fisher et al., 1986; McKeon et al., 1986; Höger et al., 1988), we thought that anti-IFA might be transported into the nucleus, driven by its affinity for nuclear lamins (despite the exclusion limits imposed at the nuclear pores). To examine this possibility, we injected anti-IFA directly into the nucleus and examined its distribution.

Inspection of the cells 1 h, 4 h and 24 h after injecting the anti-IFA into the nucleus does not reveal any changes in the lamin distribution and localisation (Fig. 7B, E and H), whereas the injected antibody is found throughout the nucleoplasm rather than concentrated around the nuclear lamina (Fig 7A, D). After 4 h, anti-IFA starts 'leaking' out of the nucleus (Fig. 7D, G). At later time points, most of the antibody is transported to the cytoplasm where it produces the effects on vimentin filaments already described. These results suggest that anti-IFA can diffuse in and out of the nucleus, bypassing the barrier of the nuclear pores. Since this does not appear to be mediated by binding to the lamin proteins (which are either masked by associated chromatin, or not affected by anti-IFA in vivo), it remains unclear how such a process is mediated.

Discussion

Using a peptide analogue of the coil 2b consensus motif and an antibody specifically reacting with the same site we have reinvestigated the role of one of the two conserved regions of IF proteins. Both of these agents disassemble preassembled vimentin filaments and block polymerisation of vimentin protofilaments. Such effects can be explained in two ways. According to one scenario, protofilaments with their coil 2b sites blocked by anti-IFA, or neutralized by competition from C-2, may possess a lesser propensity to self-associate, or may assemble very slowly. Alternatively, the filaments assembled from such protofilaments may be unstable, easily fragmenting under in vitro conditions.

Consistent with the former explanation is the remarkable scarcity of long, normal-looking IFs after incubation of vimentin protofilaments with the two interfering agents. Because the effects of C-2 and anti-IFA require more than stoichiometric amounts of the inhibitors, the simplest interpretation would be that protofilaments with their coil 2b site blocked by anti-IFA antibodies, or with their coil 2b-'acceptor' sites neutralized by C-2, become unavailable for assembly. This could effectively reduce the pool of polymerisation-competent protofilaments and result in a much lesser degree of filament formation. The fragmentation of preassembled filaments can also be explained by the same 'titration' mechanism, which would prevent incorporation of new protofilaments, while the dissociation of protofilaments continues at the same rate. If this interpretation were correct, one would conclude that in vitro assembled IFs are dynamic structures, i.e. that polymerised protofilaments are in equilibrium with non-polymerised subunits.

As indicated in Results, the most frequent profile of vimentin polymers assembled in the presence of C-2 or anti-IFA is that of short filaments. At face value, this would suggest a direct involvement of the coil 2b region in filament elongation. However, since the mechanisms that lead to assembly of oligomers higher than the tetramer are at present not well understood, one does not know whether a decrease in the length of the IFs is a direct effect, or a consequence, of protofilament misalignment. Hatzfeld and Weber (1992) obtained several forms of IF aggregates after co-incubation of urea-solubilised cytokeratins and desmin with a synthetic peptide similar to C-2. Although (at equivalent inhibitor concentrations) the corresponding filament profiles are slightly different from the ones we have generated using vimentin protofilaments, it is clear that both the elongation and the lateral arrangements of different IF proteins are affected by C-2. Thus, it would seem reasonable to suspect that this region is involved in multiple molecular associations.

Current structural models of IF assembly (Stewart et al., 1989) are topologically compatible with idea that the coil 2b region in one dimer may align and interact with the coil 2b region of another dimer (in an anti-parallel fashion). In addition, recent binding studies reveal that the N-terminal head region of vimentin strongly interacts, in a salt-resistant fashion, with chymotryptic peptides containing the coil 2b and the coil 1a region (Traub et al., 1992). This is in line with previous data suggesting a staggered antiparallel arrangement of the coiled-coil dimers (Potschka et al.,

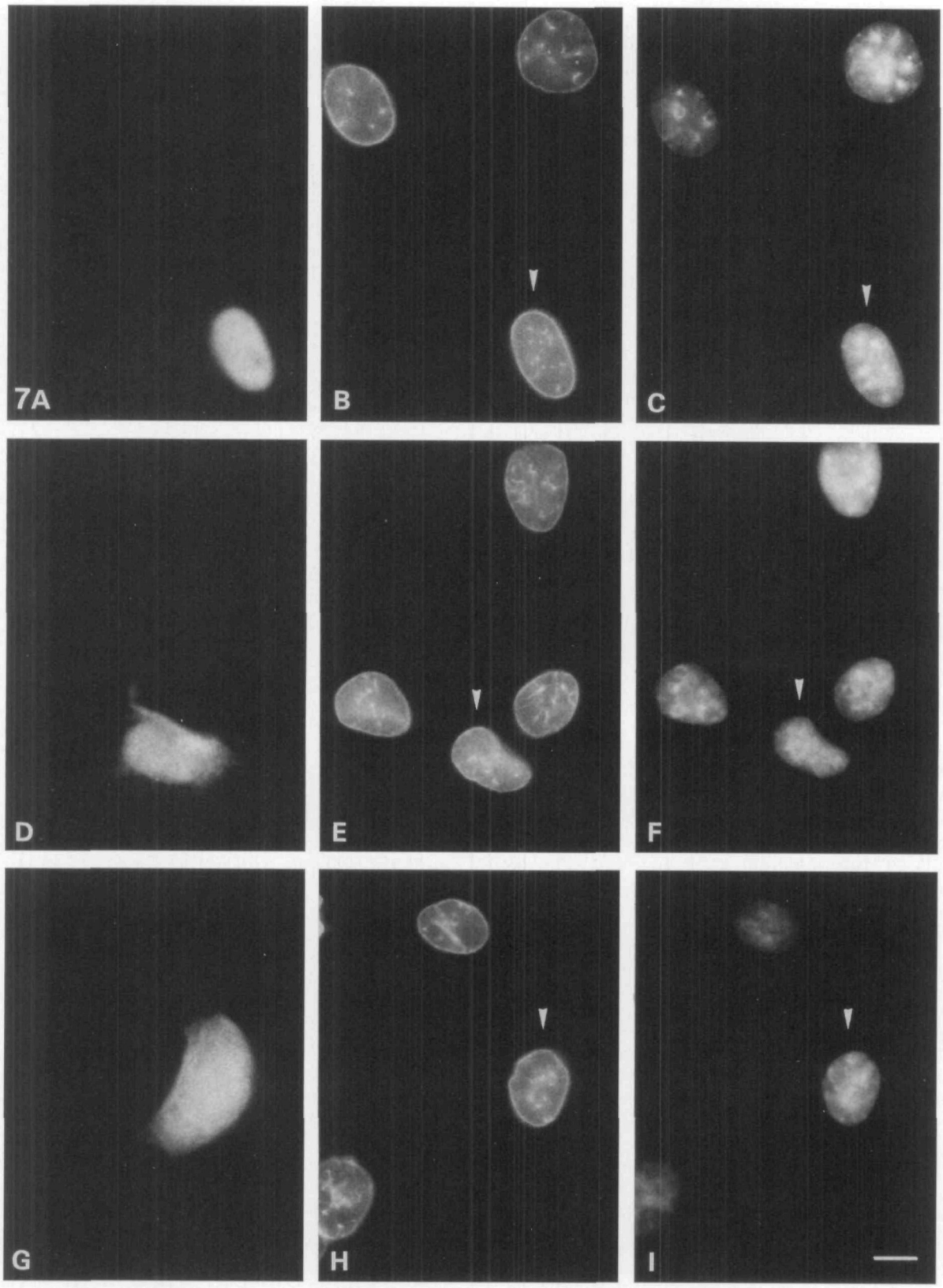


Fig. 7. Microinjection of anti-IFA into the nucleus of 3T3 cells. Anti-IFA (8 mg/ml) was injected into the nucleus of 3T3 cells. The cells were fixed after 1 h (A-C), 4 h (D-F) and 24 h (G-I) and assayed by indirect immunofluoresence microscopy using a rabbit polyclonal serum that recognises all lamins (aLI; Djabali et al., 1991). (A, D, G). The distribution of the injected antibody (localised as in Fig. 6); (B, E, H), the lamina staining; (C, F, I) the DNA staining of the same cells at each time point. Arrowheads indicate the microinjected cells. Note the normal appearance of the nuclear lamina in the injected cells. Bar, 6 nm.

1990), a staggered parallel arrangement of the dimers (Ip, 1988; Hisanaga et al., 1990), or a combination of these two (Steinert, 1991), at the level of the tetramer.

The present in vitro observations match the in vivo data that we have obtained by injecting tissue culture cells with anti-IFA antibodies. Consistent with the fragmentation of preformed filaments by anti-IFA antibodies in vitro, the native vimentin network of 3T3 cells has been found to be disrupted by microinjected anti-IFA. In previous studies we have shown that the masses of vimentin material that appear 24 h after microinjection of anti-IFA have a non-filamentous substructure (see also Kouklis et al., 1991). A somewhat different phenotype (exclusively collapsed IFs) was noticed in earlier studies when anti-IFA was microinjected into 3T3 cells (Klymkowsky, 1981). This effect is probably due to the lower concentrations of the injected antibodies that were used in these previous experiments (3 mg/ml), because from our in vitro data it seems clear that the fragmenting effect of anti-IFA is concentration-dependent.

It has been suggested that the appearance of cytokeratin 'spots' in epithelial cells injected with anti-IFA (which resemble the vimentin aggregates we have described above) may reflect autophagy of IFs induced by the antibody (Klymkowsky et al., 1983; Lane and Klymkowsky, 1982). Although this is a matter of interpretation, we think that the data presented here are more favourable to the idea of specific IF fragmentation, as similar fragments are detected after coincubation of purified components in vitro. It is, nevertheless, possible that autophagocytic mechanisms are indeed activated after IF fragments have been produced as a result of filament disassembly.

In summary, our present results suggest that the coil 2b region may specifically interact with other 'acceptor' sites along the vimentin molecule and that this interaction is important for the normal assembly and stability of IFs.

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This work is dedicated to Elias Brountzos.

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