All intermediate filament proteins possess three distinct domains: heads, rod and tail, and subdomains within the rod called helices 1A, 1B, 2A, and 2B. Subunit packing within a filament is a consequence of interactions among these domains. Several such interactions are known, but probably many more contribute to stabilizing filament structure. We examined a number of such potential interactions using the yeast two-hybrid system. Domains or subdomains of murine vimentin, a Type III intermediate filament protein, were fused with either the DNA-binding or trans-activating domain of GAL4, a transcription factor. Interaction between the vimentin domains/subdomains functionally reconstituted GAL4, thereby activating transcription of a GAL1-LacZ reporter gene. The oligomeric state at which the interactions took place, i.e. whether the domains/subdomains were dimeric or tetrameric as they interacted, was also determined. These studies revealed a number of interesting interactions, among which was a strong homotypic binding of helix 2B to form tetramers. They also demonstrated a lack of interaction among others expected to do so based on current structural models. From these results we deduced which of the candidates for interactions, suggested by current models, were true protein-protein interactions and which represented nearest-neighbors only. Thus, the $A_{11}$ and $A_{22}$ modes of molecular alignment identified by Steinert et al. (Steinert, P. M., Marekov, L. N., Fraser, R. D. B., and Parry, D. A. D. (1993) J. Mol. Biol. 230, 436-452) are probably true interactions, whereas the $A_{12}$ and $A_{2N}$ modes may describe adjacent but non-interacting molecules.

A family of about 40 related proteins make up the intermediate filaments (IFs), a major class of cytoskeletal elements in most eukaryotic cells. There are several types of IF proteins, based on protein and gene structure: Types I and II are the keratins, which form IFs in epithelial cells; vimentin and desmin are Type III IF proteins, as are peripherin and glial fibrillary acidic protein, two proteins found in the nervous system. Vimentin is expressed in cells of mesenchymal origin, and desmin is muscle-specific. The neurofilament triplet proteins, NF-L, NF-M, NF-H, and $\alpha$-internexin, are found only in neurons and are Type IV proteins. Type V IF proteins are the nuclear lamins and Type VI consists of a single protein, nestin. Several new IF proteins of limited tissue distribution, filensin, phakinin, and tanabin, also have been identified recently (reviewed by Fuchs and Weber (1994) and Klymkowsky (1995)). The unifying structural principle of this family of proteins is the presence of a tripartite motif, a central, $\sim 310$-residue-long, $\alpha$-helical rod domain and flanking non-$\alpha$-helical head and tail domains.

The assembly of 10-nm wide filaments from these highly asymmetric molecules is a complex process. Because the protein molecules are extended, and are packed in a roughly parallel (or anti-parallel) but staggered fashion within the filament, a large number of potential interactions between adjacent molecules is possible. Only a few of these interactions have been identified or characterized. Another complicating factor is that, although several intermediate assembly steps, e.g. the formation of dimers and tetramers, have been identified experimentally (reviewed by Parry and Steinert (1992) and Stewart (1993)), recent detailed structural studies (Steinert, 1991b) suggest that a continuum of oligomers exists beyond the dimer stage. This effectively increases the number of possible protein-protein interactions that may occur within an assembled IF.

The most well known interaction during IF assembly is the lateral association of the $\alpha$-helical rod domains via hydrophobic interactions to form dimeric coiled-coils. This association is thought to lead to formation of the filament backbone, and is made possible by the interaction of hydrophobic amino acids located at the first and fourth positions of a 7-residue repeat present in the primary sequence of the rod domain. Additionally, the rod domain contributes to filament structure on a higher level of organization. The external surface of the dimeric coiled-coil exhibits alternate zones of positive and negative charges (Parry et al., 1977; McLachlan and Stewart, 1982), much like those found in other $\alpha$-helical proteins such as the myosin rod (Atkinson and Stewart, 1992) and tropomyosin (Hitchcock-DeGregori and Varnell, 1990). Recent experimental evidence, obtained by a deletion mutagenesis approach, strongly suggests that electrostatic interactions involving these charged zones are a major driving force for the formation of tetramers and/or higher order structures (Meng et al., 1994).

Additionally, less well recognized interactions involving the head and tail domains also contribute to IF assembly (Birkenberger and Ip, 1990; Eckelt et al., 1992; Herrmann et al., 1992; Makarova et al., 1994; Rogers et al., 1995). It is generally agreed that the head domain of IF proteins is essential for filament assembly, and it has been demonstrated in numerous studies that experimental removal of this domain results in assembly incompetence. Involvement of the tail domain is a matter of some controversy. While it seems clear that removal of the tail does not appreciably hinder in vitro assembly of
certain IF proteins, in other cases it has been reported to result in structural aberrations in vitro, in abortive assembly in cells (Kauffman et al., 1985; Quinlan et al., 1989; Makarova et al., 1994; Bader et al., 1991), or in unusual localization of the IF protein to the nucleus (Rogers et al., 1995).

The higher the level of filament organization, the less is known about how oligomeric subunits are arranged within the filament, and what forces drive their formation. For example, although there is strong evidence indicating that two α-helices form a coiled-coil dimer by interactions between hydrophobic residues, what causes the monomers to be aligned in-register and in parallel is not known. Also not yet identified are the forces (and sequences) that drive the formation of tetramers, and filament elongation. At which level of organization does a subunit stagger occur to generate the characteristic 22-nm repeat seen in all IFs? How are IF assembly and disassembly regulated in vivo? While it is widely believed that these interactions are functions of individual IF protein domains, testing this hypothesis has been primarily carried out by a limited number of approaches, in vitro mutagenesis to look for loss of function in transfected cells or transgenic animals, or in vitro approaches such as reassembly and chemical cross-linking studies. Given that significant differences have been observed between the assembly behavior of some IF proteins in vivo and in vitro (Raats et al., 1991; Eckelt et al., 1992; McCormick et al., 1993), it seems desirable that these interactions be studied in an alternative context. In this paper, we describe our recent efforts in identifying such interactions that are involved in the assembly and maintenance of IF structure, using the two-hybrid system (Fields and Song, 1989). Our results have allowed us to examine more closely certain interactions that have been suggested to occur by current structural models.

MATERIALS AND METHODS

Yeast Strain and Media—Saccharomyces cerevisiae strain PCY2 (MATa Δgalα galα8 URA3: GAL1-Lαc2 : lys2-801 hsl1::his3 Δ1.000001 trpl-Δ63 leu2-3,112-101 his4) (Sikorski and Hieter, 1989) was used for all assays. Yeast cultures were grown at 30°C in either YEPD medium (1% yeast extract, 2% peptone, 0.004% adenine sulfate, and 2% glucose) or glucose minimal medium (MinGlu; Yocum et al., 1984)) except when they were used for the β-galactosidase assay. In the latter case, yeast was cultured in galactose minimal medium (MinGal, in which the glucose was replaced by 2% (w/v) galactose (Yocum et al., 1984) and 2% (w/v) glycerol and 2% (v/v) ethanol were used as carbon sources.

Yeast Transformation and β-Galactosidase Assay—Transformation of yeast was performed according to the procedure of Hill et al. (1991) except that a 50-μl aliquot of competent yeast cells was transformed with 0.5 μg of plasmid DNA without carrier DNA. For β-galactosidase assays, yeast colonies were picked and dispensed into 2 ml of MinGlu medium supplemented with the appropriate amino acids and grown at 30°C with shaking for 48–72 h. The cells were then pelleted by low speed centrifugation and resuspended in 2 ml of MinGal medium to induce expression of protein under the control of the GAL1 promoter. For β-galactosidase activity was measured fluorometrically, using 4-methylumbelliferone β-β-galactopyranoside (4-MUG, Fluka) as substrate. Yeast culture samples (100 μl) from MinGal medium were pipetted into a 1.5-ml Eppendorf tube and pelleted in a microcentrifuge at 37°C for 30 min. The reactions were terminated by addition of 400 μl of stop solution (0.1 M glycine, pH 10.3). The amount of 4-MUG released from 4-MUG was determined using a Gilford Fluoro IV spectrophotometer (Gilford, Oberlin, OH), with excitation at 360 nm and emission at 450 nm. Results reported were obtained from at least three independent determinations. Variations were less than 20%.

For rapid mass screening for β-galactosidase positive yeast colonies, plates were replica-plated onto nitrocellulose membrane, which were then stained with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside as substrate, as described by Ausubel et al. (1987).

Yeast Vector and Plasmid Construction—Yeast shuttle vector plasmids containing the GAL4 DNA-binding domain (pPC62) and the GAL4 transactivation domain (pPC68) (Chevray and Nathans, 1992) were gifts from Drs. P. Chevray and D. Nathans (J ohn Hopkins University, Baltimore, MD). The plasmid pMA431, containing the full-length GAL4 DNA, which was used as a positive control, was a gift from Dr. J un Ma (Children’s Hospital Research Foundation, Cincinnati, OH). The full-length murine vimentin cDNA (BS-Vim, in pBluescript) was a gift from Dr. Robert Evans (University of Colorado Medical School, Denver, CO) and the human keratin 8 and keratin 18 cDNA were gifts from Dr. Bishar Omary (Stanford University, Palo Alto, CA).

All plasmids constructions (Fig. 1 and Table I) were performed in Escherichia coli strain XL-1 Blue (Stratagene, La Jolla, CA) and bacteria were grown in LB medium. The wild type full-length vimentin cDNA was inserted between the unique SalI and NotI cloning sites of both yeast fusion vectors, pPC62 and pPC68. A sense oligonucleotide, V-P-5 (5′-GCCATGTCGACCAGGT-3′) and the T7 promoter primer (Life Technologies, Inc., Grand Island, NY) in the antisense orientation, were used to amplify the vimentin cDNA and, at the same time, create a unique SalI site at the 5′ terminus. After restriction with SalI and NotI and ligation, the plasmids, p62-wtVim and p86-wtVim, were obtained.

A SalI/PstI fragment encoding the vimentin head domain (amino acids 1–106) was introduced into pPC62 to yield p62-VimH. From this resulting plasmid, a SalI/NotI fragment was excised and subcloned into pPC62 to yield p86-VimT. All other truncations of vimentin were constructed by polymerase chain reaction-based cloning strategies. For construction of p62-Vim1B, primers P-V-6 (5′-GATAAGTAGCAGTCAGGAGGAGGCGG-3′) and P-V-7 (5′-GGCCAGACTAGGGCGATGCTCTCTCTCTCTCTCTCT-3′) were used to amplify the appropriate fragment from BS-Vim. Similarly, a 735-base pair fragment for Vim1B was obtained by amplification using primers P-V-5 and P-V-7. Vim2B, a 363-base pair fragment, was amplified from BS-Vim using a sense primer P-V-8 (5′-GCTTCTGTCGAGCGTAACTGCGAATTTGTC-3′) and the antisense primer, P-V-12 (5′-CCCGCACTGAACTCTGCTCTCTCTCT-3′). The resulting fragment was subcloned between the SalI and SpeI sites of pPC62 and pPC68 to yield p62-Vim2B and p86-Vim2B, respectively. Finally, VimT, a 165-base pair fragment that encodes the vimentin tail domain, was similarly constructed, using primer P-V-9 (5′-CCCTTGCGACTGTCCTGCTCTCTCTCTCACC-3′) (which added a SalI site at the 5′ terminus) and the T7 promoter primer. After restriction with SalI and NotI and ligation into pPC62 and pPC68, p62-VimT and p86-VimT were obtained.

The keratin K8 encoding plasmids, p62-K8 and p86-K8, were obtained by polymerase chain reaction amplification using PUC-K8 as template. The 5′ primer, K8-P-10 (5′-GATAAGTAGCAGTCAGGCTCC-3′) was used to insert sequence encoding a SalI restriction site 5′ to the K8 start site. The 3′ primer, the M13 reverse primer (Promega, Madison, WI), adds two restriction sites, XbaI and EcoRI, from the PUC18 vector polynkynucleotide, to the 3′ end of the keratin cDNA. The Sall/XbaI fragment was then subcloned into pPC62 to...
obtain p62-K8, and the SalI/EcoRI fragment from the same polymerase chain reaction product was subcloned into pPC62 to obtain p62-K8. Similarly, using BS-K18 as template, primer K18-P-11 (5'-AGTCGACGATGAGCTTCACCACTCGC-3') yielded a 9.9-kb XbaI fragment from the same polymerase chain reaction fragment containing a 9-bp K-18 coding region, and a 3'-XbaI site was obtained. The K-18 fragment was introduced into pPC62 to yield p62-K18. From this, a SalI/NcoI fragment was subcloned into pPC60 to yield p68-K18.

Preparation of Cell Extract and Immunoblotting—Protein extracts of S. cerevisiae for immunoblotting were prepared essentially as described by Bram and Kornberg (1985) with the following modifications. Culture cells (50 ml) were chilled on ice for 10 min, pelleted at 4°C by centrifugation at 3000 rpm, washed once with cold water, and resuspended in a 1 ml of cold buffer A (25 mM Tris-HCl, pH 7.5, 50 mM KCl, 5 mM MgCl2, 0.1 mM EDTA, 0.5 mM dithiotreitol, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 2 mM pepstatin A, and 0.6 mM leupeptin). 0.5 ml of cell suspension was transferred to a 1.5-ml Eppendorf tube, placed into liquid nitrogen for 3 min, and allowed to thaw for 20 min on ice. Following addition of a three-fourth volume of 0.45-mm acid-washed glass beads, the cells were lysed with eight 30-s pulses of a vortex mixer interspersed with equal periods of cooling on ice. The cell lysate was separated from the glass beads, the beads rinsed with 0.2 ml of buffer A, and the recovered volumes combined. The cell lysate was then mixed with one-third volume of 3 × Laemmli electrophoresis sample buffer, placed in a boiling water bath for 2 min, centrifuged, and the supernatant saved for electrophoresis and Western blot analyses. Protein content in cell lysates was determined by the method of Bradford (1976).

Immunodetection on Western blots was performed essentially according to the method of Towbin et al. (1979). A rabbit antiserum, Vim11091, raised against full-length murine vimentin expressed in E. coli, was used as the primary antibody at a dilution of 1:200. Alkaline phosphatase-conjugated affinity-purified, goat-anti-rabbit IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was used as the secondary antibody at a dilution of 1:1200.

RESULTS

Strategy and Controls—The two-hybrid system exploits the fact that the yeast transcription activator, GAL4, consists of two separate and essential domains, the DNA binding (DB) domain and the transcription activating (TA) domain, that must be in proximity to one another to function (Ma and Ptashne, 1987). It was designed originally as a method for screening expression libraries based on interaction between a protein and its ligands (Fields and Song, 1989). In this mode of use, the cDNA of the protein of interest is subcloned into a vector such that it is expressed as a fusion protein with either the DB or TA domain, while the library to be screened is subcloned into a second vector such that it is expressed as fusion proteins with the other GAL4 domain. Upon co-transformation of yeast with both plasmids, interaction between the fusion protein of interest with fusion proteins represented in the library then leads to its screening and subsequent identification of binding partners (for example, see Chevray and Nathans (1992)). In our study, we simplified the two-hybrid system for examining interactions between given pairs of IF protein domains, by substituting the expression library with a second IF protein domain. Interaction between the IF protein domains would then reconstitute activity of the GAL4 protein and transcription of a GAL1-lacZ reporter gene would ensue.

We used two vectors, pPC62 (the DB vector) and pPC86 (the TA vector) (Chevray and Nathans, 1992). Complementary DNAs encoding full-length IF proteins or individual domains were subcloned into these vectors, and the fusion plasmids were used pairwise to transform yeast strain PCY2, which carried a genomic copy of the GAL1 promoter driving expression of lacZ. Initially, interactions were detected qualitatively by staining nitrocellulose replicates of yeast plates with 5-bromo-4-chloro-3-indolyl β-D-galactosidase. Quantitation was carried out by expanding positive yeast colonies and assaying for β-galactosidase activity in cell homogenates derived from such colonies, using the fluorescent substrate, 4-MUG. In transformations involving pairs of IF protein domains, the experiments were always repeated by interchanging inserts and vectors, to control for potential steric effects that could result from differences in spatial juxtaposition of the IF protein domain relative to the GAL4 domain when they were expressed as a fusion protein. In most cases, swapping of vectors and inserts in this manner resulted in differences in β-galactosidase activity of only 10–20%, but more importantly, it did not alter the strength of the interaction relative to others that were studied.

A series of quantitative controls were undertaken, using MUG fluorescence measurements, to ensure that positive β-galactosidase activity truly reflected interactions between IF domains (Table I). These included co-transformation with one or both vectors lacking insert, and transformation with only one vector. Without exception, such transformations resulted in extremely low levels of β-galactosidase activity, the highest of which amounted to 0.13% of that reached by co-transformation with both vectors encoding full-length vimentin (see below).

Full-length IF Proteins Expressed in Yeast Cells Interact Strongly—In contrast to control transformations, when the full-length vimentin-DB and vimentin-TA fusion proteins were expressed in PCY2 cells, they interacted robustly, resulting in β-galactosidase activity that was readily detected either qualitatively, by 5-bromo-4-chloro-3-indolyl β-D-galactosidase staining of nitrocellulose replicates (not shown), or quantitatively, by MUG fluorescence (Table III, row 1). In fact, the interaction was so strong that, in the latter assay, the reconstituted β-galactosidase activity was 46% of that obtained by transformation with a full-length GAL4 DNA (data not shown). To ascertain that the transformed yeast cells were indeed expressing vimentin as fusion proteins with either the GAL4 TA or DB domain, a cell extract was made from a positive yeast clone and subjected to immunoblotting, using a polyclonal antiserum directed against recombinant murine vimentin. A prominent but diffused immunoreactive band of molecular mass ~75–80 kDa was detected in the blot (Fig. 2), consistent with the sizes expected for the TA- and DB-vimentin fusion proteins.

An additional measure of confidence that the IF protein interactions detected by using the two-hybrid were specific was provided by transformation experiments using keratins K8 and K18 (Table II). Keratin IFs are obligate heteropolymers, i.e. they must contain one Type 1 and one Type 2 keratin. Thus, one would expect interaction to occur only when yeast cells...
were co-transformed with plasmids encoding one Type 1 and one Type 2 keratins, but not in a co-transformation with both plasmids encoding either Type 1 or Type 2 keratins. This was indeed the case. Strong interaction was detected when co-transformation was carried out with one plasmid encoding K8, a Type 2 keratin, and the other encoding K18, a Type 1 keratin (Table II, rows 1 and 2), but when both plasmids encoded either K8 or K18 (Table II, rows 3 and 4), β-galactosidase activities fell to about 3% of the K8/K18 co-transformation. When one keratin was omitted from the transformation (Table II, rows 5 and 6), β-galactosidase activity fell to baseline levels. Interestingly, K18, but not K8, interacted quite strongly with full-length vimentin (Table II, rows 7–10). A similar interaction between an acidic keratin, K14, and vimentin has also been reported (Steinert et al., 1993c). However, as suggested by these investigators, this interaction probably does not proceed beyond the dimer stage and is likely to be non-productive for filament formation.

**Fig. 2. Yeast cells transformed with p62wtVim and p86wtVim express vimentin protein.** Immunoblot analysis of a cytoplasmic homogenate of yeast transformed with the two vectors encoding full-length vimentin as fusion proteins with the TA and DB domains of GAL4. Left lane, molecular mass markers at 205, 94, 68, and 45 kDa. Center lane, PCY2 cell homogenate probed with a polyclonal anti-vimentin antibodies, revealing a reactive product at about 80 kDa, consistent with the size of GAL4-vimentin fusion polypeptides. Right lane, purified recombinant vimentin.

**Table II**

| Plasmids co-transformed | Fluorescencea | β-galactosidase activityb |
|-------------------------|---------------|--------------------------|
|                         | arbitrary units | %                        |
| 1 p62-K8                | + p86-K18     | 6400.0                   | 100.0                     |
| 2 p62-K18               | + p86-K8      | 5300.0                   | 82.7                      |
| 3 p62-K8                | + p86-K18     | 166.3                    | 2.6                       |
| 4 p62-K18               | + p86-K18     | 222.6                    | 3.4                       |
| 5 p62                   | + p86-K8      | 13.1                     | 0.2                       |
| 6 p62                   | + p86-K8      | 10.0                     | 0.1                       |
| 7 p62-K8                | + p86-wtVim   | 377                      | 5.9                       |
| 8 p62-wtVim             | + p86-K8      | 546                      | 8.5                       |
| 9 p62-K8                | + p86-wtVim   | 4640                     | 72.5                      |
| 10 p62-wtVim            | + p86-K18     | 4550                     | 71.1                      |

a 4-MUG fluorescence, assayed as described under "Materials and Methods."

b Expressed as percent of the p62-K8 + p86-K18 co-transformation (row 1).

**Fig. 3. Dimers and tetramers are both formed when IF proteins interact in PCY2 cells.** Immunoblot analysis of two cytoplasmic homogenates of PCY2 cells transformed such that they expressed vimentin helix 1B-GAL4 (lane 2) and helix 2B-GAL4 fusion proteins (lane 3). Lane 1, molecular mass markers at 205, 94, 68, and 45 kDa. Lane 4, Comassie Blue-stained gel of the homogenate used for lane 2; that used for lane 3 was virtually identical. The homogenates were cross-linked with glutaraldehyde before immunoblotting to stabilize dimers and tetramers. The lower bands in lanes 2 and 3 are dimers of the fusion proteins and the upper bands are tetramers of the same species.

**Results from a sample taken from a co-transformation with both plasmids encoding helix 1B is shown in Fig. 3, lane 2. Two immunoreactive bands, with molecular mass of ∼55 and 100 kDa are found. The former has a size appropriate for a DB-Vim1B/TA-Vim1B dimer, while the latter has a size appropriate for a tetramer. A similar sample, from a co-transformation with helix 2B-encoding vectors, is shown in lane 3 of Fig. 3. Again, immunoreactive bands of sizes compatible with DB-Vim2B/TA-Vim2B dimers and tetramers are evident. Thus, we conclude that the β-galactosidase activity we detected in our transformations was indicative of interactions between IF proteins at the level of both the dimer and the tetramer. We do not have direct information as to whether our GAL4 domain-IF fusion proteins further assemble into 10-nm filaments in PCY2 cells; however, because the fusion proteins contain sizable amounts of TA or DB domains (110 and 144 residues, respectively), which are probably sufficiently large to interfere sterically with subunit packing during filament assembly, it is unlikely that filaments as such are formed.

**Interactions between IF Protein Domains**—The two-hybrid system offers an opportunity to examine, in an in vivo setting, whether two given IF protein domains interact. In turn, this should allow one to map the interactions that take place along the IF protein molecules as they coalesce to form oligomers and, ultimately, filaments and filamentous networks. Interactions between domains of the vimentin molecule were examined by co-transforming individual pairs of plasmids encoding the relevant domains (Fig. 1). Transformations and the resulting β-galactosidase activity are presented in Table III. The strongest interaction was detected between subdomains of the rod. For example, homotypic interaction between helix 1B segments, between the amino half of the vimentin molecule (H1B) and also between helix 2B segments, resulted in β-galactosidase activities 18, 44, and 52%, respectively, of that attained by transformation using full-length vimentin molecules. Of interest is that amino halves of vimentin interacted significantly more strongly than helix 1B segments alone (44% versus 18%), even though head domains themselves did not interact to any significant extent (see below).

Several co-transformations that resulted in low levels of β-galactosidase activity are also noteworthy. Among these are ones to screen for interaction between helices 1 and 2B, predicted to overlap in several models of IF structure (Stewart et al., 1989; Geisler et al., 1992; Heins et al., 1993; Steinert et al., 1993a). When co-expressed in PCY2 yeast cells, neither helix 1B alone, nor domain H1B, encompassing the entire amino half of the vimentin polypeptide, interacted significantly with helix 2B, which contained the most highly conserved COOH-termi-
Specific association between and argue against the possibility that they arose from nonspecific in the context of the assembly properties of IF proteins, (Table II) demonstrates that the detected interaction swere
tors (Table II) demonstrates that the detected interactions were
tual end of the rod domain (Table III, rows 5–8). The level of β-galactosidase activity attained in these transformations was less than 1% of that produced by homotypic interaction between full-length vimentin polypeptides. Also of considerable interest is the apparent lack of interaction between vimentin head domains, between tail domains, and between head and tail domains (Table III, rows 9–18). Interaction among these pairs of domains resulted in 2% or less of the activity attained by interaction between full-length vimentin polypeptides.

**DISCUSSION**

Specificity and Utility of the Approach—The GAL4 two-hybrid system appears to be a powerful tool for studying protein-protein interactions involved in the formation of intermediate filaments. As evident from an extensive battery of controls, the methodology behaves consistently and predictably for IF proteins. Results of transformations using keratin-encoding vectors (Parry et al., 1993) is closely similar to the ones presented here. Domains shaded in each alignment are ones whose interaction was examined by two-hybrid cloning. The high level of interaction between helices 1B (Steinert et al., 1992) and vimentin (Steinert et al., 1993c) is that there are specific domain overlaps within the filament (Fig. 4). For example, the studies of Geisler et al. (1992) identified three lysine cross-linked regions between two desmin dimeric coiled-coils. The locations of two of these regions, EGS1 and EGS2, are compatible with a model of molecular alignment in which the two dimers in a tetramer are paired in an anti-parallel, staggered fashion such that the inner overlapping part of the tetramer is formed by helices 1A, 1B, and 2A (G11, in Fig. 4). This model is consistent with earlier observations, derived from proteolysis studies, that helix 1B could be isolated as a tetramer (Gruen and Woods, 1981a; Woods and Ingles, 1984). In an extensive series of chemical cross-linking studies on keratin pairs (Steinert et al., 1993a, 1993b) and vimentin (Steinert et al., 1993c), Steinert and co-workers identified a number of lysine-lysine and cysteine-cysteine cross-links, which led them to describe four types of domain alignment termed A11, A22, A12, and A21 (Fig. 4). The first three of these describe overlap of the rod domains to varying degrees, whereas the fourth, A21, describes an approximately 10-residue long head-to-tail overlap of helix 2B of one dimer with helix 1A of a second dimer. Interestingly, comparison of the length of the rod (46 nm, Steinert et al., 1993c) versus the well known 21-nm repeat seen in electron micrographs of metal shadowed IF (Milam and Erickson, 1982; Aebi et al., 1983) also led Heins et al. (1993) to propose an end-to-end overlap of the

| Protein Interaction in Intermediate Filament Assembly |  |
|---|---|

**TABLE III**

| Domain interactions | Fluorescence \(^a\) | β-Galactosidase reconstitution \(^b\) |
|---------------------|-----------------|-----------------|
| **Plasmids co-transformed** | **Fluorescence \(^a\)** | **β-Galactosidase reconstitution \(^b\)** |
| 1 | wtVim + wtVim | 13450.0 | 100.0 |
| 2 | Vim1B + Vim1B | 2439.0 | 18.1 |
| 3 | VimH1B + VimH1B | 5965.6 | 44.3 |
| 4 | Vim2B + Vim2B | 6953.0 | 51.6 |
| 5 | Vim1B + Vim2B | 133.1 | 0.9 |
| 6 | Vim1B + Vim1B | 70.0 | 0.5 |
| 7 | VimH1B + Vim2B | 48.4 | 0.4 |
| 8 | Vim2B + VimH1B | 58.7 | 0.4 |
| 9 | VimH + wtVim | 466.7 | 3.4 |
| 10 | VimH + VimH | 141.2 | 1.0 |
| 11 | VimH + Vim1B | 102.0 | 0.7 |
| 12 | VimH + Vim2B | 91.4 | 0.6 |
| 13 | VimH + VimT | 274.5 | 2.0 |
| 14 | VimT + wtVim | 155.6 | 1.1 |
| 15 | VimT + VimH | 109.0 | 0.8 |
| 16 | VimT + Vim1B | 64.5 | 0.4 |
| 17 | VimT + Vim2B | 100.7 | 0.7 |
| 18 | VimT + VimT | 103.2 | 0.7 |

\(^a\) 4-MUG fluorescence, assayed as described under “Materials and Methods.”

\(^b\) Expressed as percent of the p62-wtVim + p86-wtVim co-transformation (row 1).

Fig. 4. Models of IF structure and domain alignments specified by them. This figure is redrawn from Geisler et al. (1992) and Steinert et al. (1993a, 1993b, 1993c) to illustrate the domain alignments specified by current IF models. G11, is the model of Geisler et al. (1992) and A11, A22, A12, and A21 are the alignments of Steinert et al. The model of Heins et al. (1993) is closely similar to the ones presented here. Domains shaded in each alignment are ones whose interaction was examined by two-hybrid cloning. The high level of interaction between helices 1B and between helices 2B to form tetramers, revealed by two-hybrid cloning, is compatible with the hypothesis that the G11, A11, A22, and A12 alignments are the consequence of true domain binding. Conversely, the lack of interaction between helix 1A and helix 2B suggests that the A21 and A21 alignments do not contribute to forces that hold subunits together within a filament.
rod domain of similar dimension.

An important question arises when considering these structural data: which of the domain overlaps represent actual protein-protein interactions that bring the molecules together to form the filament, and which of them represent nearest neighbors that were identified as a result of the proximity of cross-linkable lysine and/or cysteine residues within their sequences? Because we were able to determine whether or not given pairs of IF protein domains interact in a cytoplasmic context, it is possible to examine this question in some detail.

Transformation with both vectors encoding helix 1B-GAL4 fusion proteins produced high levels of β-galactosidase activity (Table III, rows 2 and 3), indicating that helices 1B bind strongly to one another. Accompanying Western blotting experiments showed that helices 1B form tetramers, consistent with the aforementioned observation that helix 1 tetramers could be isolated from proteolyzed wool keratin (Gruen and Woods, 1981; Woods and Ingls, 1984). Thus, we conclude that helix 1B is a site along the molecule at which active interaction occurs to stabilize the structure of an IF. Interestingly, the amino-terminal halves of vimentin, encoded by the VimH1B construct, interacted considerably more strongly than helices 1B alone. As the head domain did not interact with either another head domain or the rod (Table III, rows 9–12), the strength of the homotypic interaction between the amino-terminal halves of vimentin likely stems from the presence of helix 1A, which might have enhanced molecular alignment. An additional transformation using a helix 1 (1A plus 1B) construct should provide a definitive answer.

In light of the fact that a tetramer composed of helix 2 has not been isolated, the finding that helices 2B interacted strongly and produced tetrameric complexes in the two-hybrid system was somewhat surprising. Nonetheless, this interaction is consistent with the A22 alignment of Steigner et al. (1993a, 1993b), and is a logical consequence of the A12 interaction discussed above. This is because the alignment of helices 1B between two rows of dimers as specified by the A22 interaction would necessarily place their helices 2B in close proximity also (see Fig. 4). The larger size of helix 2, and the presence of more proteolytic cleavage sites, may account for the failure to detect intact tetramers composed of helix 2. The fact that both helix 1B and 2B exist as tetramers does not allow one to discriminate between the anti-parallel staggered alignment of dimers, favored by all three models of IF structure, and the parallel unstaggered alignment not favored by any. However, it does suggest against active interaction between dimers arranged in an A12 alignment, because this would have resulted in dimers but not tetramers in an interaction screen.

A second result that suggests a lack of true interactions between dimers in the A12 and ACN types of alignment is the low levels of β-galactosidase activity produced by transformations using the relevant constructs (Table III, rows 7 and 8). VimH1B, which encompassed essentially the amino terminus half of the vimentin polypeptide and includes helix 1A, did not interact with Vim2B, which encompasses the rod’s carboxyl terminus, as would have been predicted by the A12 as well as the ACN modes of alignment (Fig. 4). Although there is little doubt that these regions of adjacent vimentin molecules are situated very close to one another within the filament, as evidenced by the positional data provided by chemical cross-linking (Steinert et al., 1993c), it would seem that the cross-links repre- sent juxtaposing, but not interacting, regions of the molecules.

Finally, equally interesting was the observation that interactions involving the vimentin end domains were rather minimal (Table III, rows 9–18). In the two-hybrid system, the end domains formed neither homodimers nor heterodimers, sug-

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