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Cytoskeleton Association and Virion Incorporation of the Human Immunodeficiency Virus Type 1 Vif Protein

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The human immunodeficiency virus type 1 (HIV-1) Vif protein has an important role in the regulation of virus infectivity. This function of Vif is cell type specific, and virions produced in the absence of Vif in restrictive cells have greatly reduced infectivity. We show here that the intracellular localization of Vif is dependent on the presence of the intermediate filament vimentin. Fractionation of acutely infected T cells or transiently transfected HeLa cells demonstrates the existence of a soluble and a cytoskeletal form and to a lesser extent the presence of a detergent-extractable form of Vif. Confocal microscopy suggests that in HeLa cells, Vif is predominantly present in the cytoplasm and closely colocalizes with the intermediate filament vimentin. Treatment of cells with drugs affecting the structure of vimentin filaments affect the localization of Vif accordingly, indicating a close association of Vif with this cytoskeletal component. The association of Vif with vimentin can cause the collapse of the intermediate filament network into a perinuclear aggregate. In contrast, analysis of Vif in vimentin-negative cells reveals significant staining of the nucleus and the nuclear membrane in addition to diffuse cytoplasmic staining. In addition to the association of Vif with intermediate filaments, analyses of virion preparations demonstrate that Vif is incorporated into virus particles. In sucrose density gradients, Vif cosediments with capsid proteins even after detergent treatment of virus preparations, suggesting that Vif is associated with the inner core of HIV particles. We propose a model in which Vif has a crucial function as a virion component either by regulating virus maturation or following virus entry into a host cell possibly involving an interaction with the cellular cytoskeletal network.

The human immunodeficiency virus type 1 (HIV-1) *vif* gene is an accessory gene encoded by all lentiviruses except equine infectious anemia virus (33). Its gene product is a 23-kDa basic protein which is produced late in the infection cycle in a Rev-dependent manner (14, 38). Deletions in the *vif* gene have been associated with a reduction or the loss of viral infectivity (9, 25, 47), a phenomenon which is largely host cell dependent (2, 3, 8, 11, 35, 49) and can vary in its extent by several orders of magnitude (9, 25, 47). In permissive cells, such as HeLa, COS, C8166, Jurkat, U937, or SupT1 (8, 11, 35) cells, production of infectious particles does not require a functional *vif* gene product, suggesting that these cells express a cellular factor which is functionally homologous to Vif or, alternatively, that these cells lack an inhibitory factor that is normally neutralized by Vif. In contrast, *vif*-deficient viruses produced from nonpermissive cells, such as H9 cells, CEM cells, peripheral blood mononuclear cells, or macrophages (3, 6, 8, 11, 12, 43, 49), are noninfectious regardless of the permissiveness of the target cells (3, 11, 49). Although the existence of cellular factors that in place of Vif can regulate infectivity of HIV-1 particles has been widely speculated, no information as to the identity of such cellular determinants is available. The recent identification of an HIV-2 isolate, $HIV2_{KR}$, whose *vif*-defective variants exhibit cell type restrictions that are distinct from those observed for *vif*-deficient HIV-1, however, could point to quantitative or qualitative differences in the responsiveness to a cellular factor(s) (34). Nevertheless, the fact that *vif* genes from HIV-1, HIV-2, and simian immunodeficiency virus can functionally complement each other (34, 40) suggests a common mechanistic basis for Vif function. The defect in viruses synthesized by restrictive cells in the absence of Vif cannot be complemented by the presence of Vif in recipient cells (3, 49). It is possible that Vif is required at the time of virus production in the producer cell, possibly playing a role in virion assembly, for example, by posttranslationally modifying one or several of the virion components. Such a potential function of Vif is supported by the observation of morphological aberrancies in *vif*-defective virions produced in restrictive cells which were not observed with wild-type virions or with *vif*-defective virions produced in permissive cells (3, 23). On the other hand, it is conceivable that Vif is incorporated into progeny virions, where it could perform a crucial function during the early phase of infection of a new host cell. This is consistent with the observation that *vif*-defective HIV-1 particles produced in nonpermissive cells can bind to and penetrate host cells but are impaired in viral DNA synthesis (3, 6, 43, 49), suggesting that Vif regulates a postpenetration step in the early phase of the virus life cycle.

The biochemical function of Vif is still unclear. On the basis of a similarity of Vif with a family of cysteine proteases, Guy and coworkers had originally proposed that Vif might be a protease targeting the carboxy-terminal end of the cytoplasmic domain of the Env glycoprotein (21). Even though Vif has been implicated in regulating incorporation of Env into virions (3, 35), a proteolytic activity of Vif has so far not been demonstrated, and the processing of the C-terminal end of gp41 as suggested by Guy and coworkers (21) could not be confirmed by others (11, 49). Nevertheless, it was suggested that Vif is required at the time of particle production in the host cell for regulating virus assembly or maturation (2, 3, 11, 35, 49). Vif has been shown to associate with the cytoplasmic face of cellular membranes (17), a phenomenon that involves basic residues located in the C-terminal portion of Vif (18). Vif muta-

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tions that disrupt membrane association also affect Vif function (18), suggesting a correlation between membrane binding and Vif activity.

To gain further insights into the function of Vif, we investigated its subcellular localization by using cell fractionation assays as well as confocal microscopy, and we assessed the potential incorporation of Vif into progeny virions. Our results demonstrate that in transfected HeLa cells as well as in acutely infected A3.01 cells, a significant portion of Vif is present in a soluble form in the cytoplasm of cells. A smaller portion of Vif protein was recovered by detergent extraction of the residual cell fraction. Interestingly, we found that almost half of the total intracellular Vif protein is associated with the cytoskeletal fraction and resists extraction with detergents or high-salt buffers. Cytoskeletal association of Vif was confirmed by confocal microscopy, which revealed a close colocalization of Vif with the intermediate filaments (IFs) vimentin and keratin. The association of Vif with the cytoskeleton is specific and can result in the complete collapse of these IF networks accompanied by the formation of perinuclear aggregates containing Vif, vimentin, and keratin.

Sucrose density gradient analyses of virion preparations from cells overexpressing Vif demonstrate that Vif is incorporated into virus particles. Detergent treatment of virus preparations prior to sucrose gradient analysis released the envelope associated MA protein (p17) but not Vif from the virions. It is therefore unlikely that Vif is nonspecifically attached to the viral surface; rather, it appears to be incorporated into virus particles in association with the inner core, possibly through an interaction with the capsid proteins.

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MATERIALS AND METHODS

Plasmids. The construction of pNL-A1 has been described elsewhere (47). This plasmid encodes all viral proteins except for *gag* and *pol* products. The *vif* gene in pNL-A1 is derived from a cDNA clone (47) and is in its sequence closely related to the HXB2 *vif* gene (not shown). A *vif*-deficient variant, pNL-A1ΔVif, was constructed by deleting a *Nde*I-*Pfl*MI fragment from pNL-A1. The resulting mutant has the capacity to express the first 28 amino acids of Vif followed by 10 nonspecific residues. A similar mutation was introduced into the *vif* gene of pNL4-3 (1), resulting in pNL4-3ΔVif. PNL-A1/vif$_{flag}$ encodes a full-length Vif protein carrying the eight-amino-acid FLAG epitope (DYKDDDDK) at its C terminus. This plasmid was constructed by PCR-based mutagenesis using the primers GTCAGG GAGTCT CCATAG AATGGA GGAAAA AGAG (5′) and TTGCAG AATTCT AGATCA CTTGTC GTCATC GTCTTT ATAATC GT GTCC ATTCAT TGTGTGG (3′) for amplification on pNL-A1 template DNA. The resulting PCR fragment was digested with *Pfl*MI and *Eco*RI and cloned into the corresponding sites of pNL-A1. PNL-A1/vif$_{flag}$ does not encode a functional *vpr* gene because of the insertion of the epitope tag into the overlapping Vif open reading frame. For exogenous expression of the human vimentin gene tagged at its carboxy terminus with the octapeptide FLAG epitope, a 694-bp *Eco*RI-*Cla*I fragment from pCMV-VIM (42) encoding the 5′ half of vimentin was cloned into the *Eco*RI-*Cla*I sites of pHIV-T4$_{flag}$ (46), creating pHIV-vim1. The 3′ half of the vimentin gene was PCR amplified by using the primers GGAACAGCAT GTC CAAATCG ATGTGG (5′) and AACTATCGAT TTCAAGGTCA TCGTG ATG (3′), the latter creating a *Cla*I site in place of the vimentin termination codon. The *Cla*I-digested PCR product was then cloned into pHIV-vim1. The resulting plasmid, pHIV-vim$_{flag}$, encodes the full-length vimentin protein followed by the eight-amino-acid FLAG epitope. As a result of the creation of a *Cla*I site, a nonspecific isoleucine residue is inserted between the C terminus of vimentin and the FLAG epitope. Expression of vimentin from pHIV-vim$_{flag}$ is dependent on the presence of the HIV-1 Tat transcriptional activator.

Antisera. Serum from an asymptomatic HIV-1-seropositive patient was used to detect HIV-1-specific proteins. The serum does not recognize Vif or Nef and only poorly reacts with gp120 in immunoblot assays. Polyclonal monospecific antisera to Vif, Vpu, and Nef were produced in rabbits against *Escherichia coli*-derived fusion proteins (31, 46) and were used for all immunoprecipitation and immunocytochemical analyses. For immunoblotting, a HIV-1$_{HXB2}$ Vif antiserum, obtained from D. Gabuzda (17) through the NIH AIDS Research and Reference Reagent Program, was used for detection of Vif. A monoclonal antibody to vimentin (V9) was purchased from Boehringer Mannheim Corp., Indianapolis, Ind. Tubulin and keratin specific antisera were obtained from Sigma Chemical Company, St. Louis, Mo., and rhodamine-phalloidin for the staining of actin filaments (R-415) was obtained from Molecular Probes, Inc., Eugene, Ore. The monoclonal antibody M2, recognizing the FLAG epitope, is a product of Eastman Kodak, New Haven, Conn. Fluorescein isothiocyanate (FITC)-, Texas red-, or Cy5-conjugated secondary antibodies were obtained from Jackson ImmunoResearch Laboratories, West Grove, Pa.

Buffers. 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) buffer consists of 50 mM Tris-hydrochloride (pH 8.0), 5 mM EDTA, 100 mM NaCl, and 0.5% (vol/vol) CHAPS (Calbiochem). CHAPS-deoxycholate (DOC) buffer is CHAPS-buffer containing 0.2% (wt/vol) DOC. Sample buffer consists of 4% sodium dodecyl sulfate (SDS), 125 mM Tris-HCl (pH 6.8), 10% 2-mercaptoethanol, 10% glycerol, and 0.002% bromophenol blue.

Cells and transfection. HeLa cells, COS cells, BHK cells, or SW13 cells were propagated in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). H9 cells or A3.01 cells were cultivated in complete RPMI 1640 medium supplemented with 10% FBS (RPMI 1640-FBS). Stocks of peripheral blood mononuclear cells were prepared from gradient-isolated lymphocytes of a healthy HIV-seronegative individual by using lymphocyte separation medium (Organon Teknica Corp., Durham, N.C.) and were stored in liquid nitrogen. Cells were either stimulated for 2 days with phytohemagglutinin (2 mg/ml) or left untreated.

Two hours prior to transfection, the medium was replaced with 5 ml of fresh DMEM-FBS. Calcium phosphate-precipitated (20) plasmid DNA was added to the cells. After 4 h, the medium was removed, and the cells were subjected to a glycerol shock for 2.5 min as described elsewhere (19). The cultures were then washed once with phosphate-buffered saline (PBS) and maintained in 5 ml of DMEM-FBS. For immunocytochemistry, cells were scraped and reseeded onto coverslips as described below.

Immunocytochemistry. For the analysis of monolayer cells such as HeLa or SW13, cells were scraped off the flasks after transfection and reseeded into 12-well plates containing 0.13-mm-thick coverslips. Cells were grown for 15 to 24 h at 37°C in DMEM containing 10% FBS. In some experiments, demecolcine (0.1 μg/ml) was added 3 h following transfection and replating of cells. For the analysis of suspension cultures, cells were washed once with PBS and pelleted onto coverslips in a cytocentrifuge (Cytospin2; Shandon). Cells were then fixed at −20°C in precooled methanol (−20°C) for 10 min and subsequently washed twice in PBS. Coverslips were stored in PBS at 4°C until use. For antibody staining, coverslips were incubated in a humid chamber at 37°C for 1 h with primary antibodies at appropriate dilutions in 1% bovine serum albumin (BSA) in PBS. Coverslips were washed once in PBS (5 min, room temperature) and incubated with FITC-, Texas red-, or Cy5-conjugated secondary antibodies (diluted in 1% BSA in PBS) for 30 min at 37°C in a humid chamber. Coverslips were then washed twice with PBS and mounted onto microscope slides with glycerol gelatin (Sigma) containing 0.1 M *N*-propyl gallate (Sigma) to prevent bleaching. For nuclear counterstaining, coverslips were incubated for 2 min at room temperature with a solution of ethidium bromide (0.1 μg/ml) in PBS immediately before the final washes. Actin staining with rhodamine-phalloidin was performed as follows. A 5-μl aliquot of rhodamine-phalloidin (200 U/ml in methanol) was dried in a Speed Vac and suspended in 200 μl of PBS. Coverslips were incubated with the phalloidin solution for 30 min at room temperature and then subjected to two quick washes in PBS. Coverslips were mounted onto microscope slides as described above. Mounted coverslips were stored at 4°C in the dark until analyzed by confocal microscopy.

Confocal microscopy. For confocal microscopy, a Zeiss LSM410 inverted laser scanning microscope equipped with a krypton-argon mixed-gas laser was used. Generally, images were acquired with a Plan-Apochromat 63×/1.4 oil immersion objective (Zeiss). Additional magnification (up to fivefold) was achieved using the LSM zoom feature. For two- or three-color analysis, objects were excited by using 488/568-nm laser lines. For two-color analyses, green and red emissions were simultaneously recorded through appropriate filters (515- to 540-nm band pass filter for FITC and 590-nm long-pass filter for Texas red, rhodamine, or ethidium bromide) and stored in separate (red and green) image channels. At the same time, bright-field images (Nomarski optics) were collected and stored in a third (blue) channel. Three-color analysis of Vif, vimentin, and keratin was done as follows. Transfected HeLa cells were simultaneously incubated with primary antibodies (rabbit-anti-Vif; mouse antivimentin, and guinea pig anti-keratin) and secondary antibodies (FITC-conjugated donkey anti-guinea pig, Cy5-conjugated donkey anti-mouse, and Texas red-conjugated donkey anti-rabbit). Confocal images were collected in two steps. First, FITC and Cy5 emissions were collected by using the 488-nm excitation line and filtered through 510- to 540- and 670- to 810-nm band pass filters, respectively. Only minor bleeding of Texas red emissions into the Cy5 channel was observed under those conditions. In a subsequent step, the same image field was rescanned by using the 568-nm excitation line and collecting emissions through a 590- to 610-nm band pass filter. Image quality was enhanced during data acquisition by using the LSM line average feature (8× or 16×). Postaquisition digital image enhancement was performed with the LSM software.

Metabolic labeling and immunoprecipitation. Transfected HeLa cells or infected A3.01 cells were washed once with PBS (10 mM phosphate buffer [pH 7.4], 100 mM NaCl) and starved for 10 min in labeling medium (methionine- and

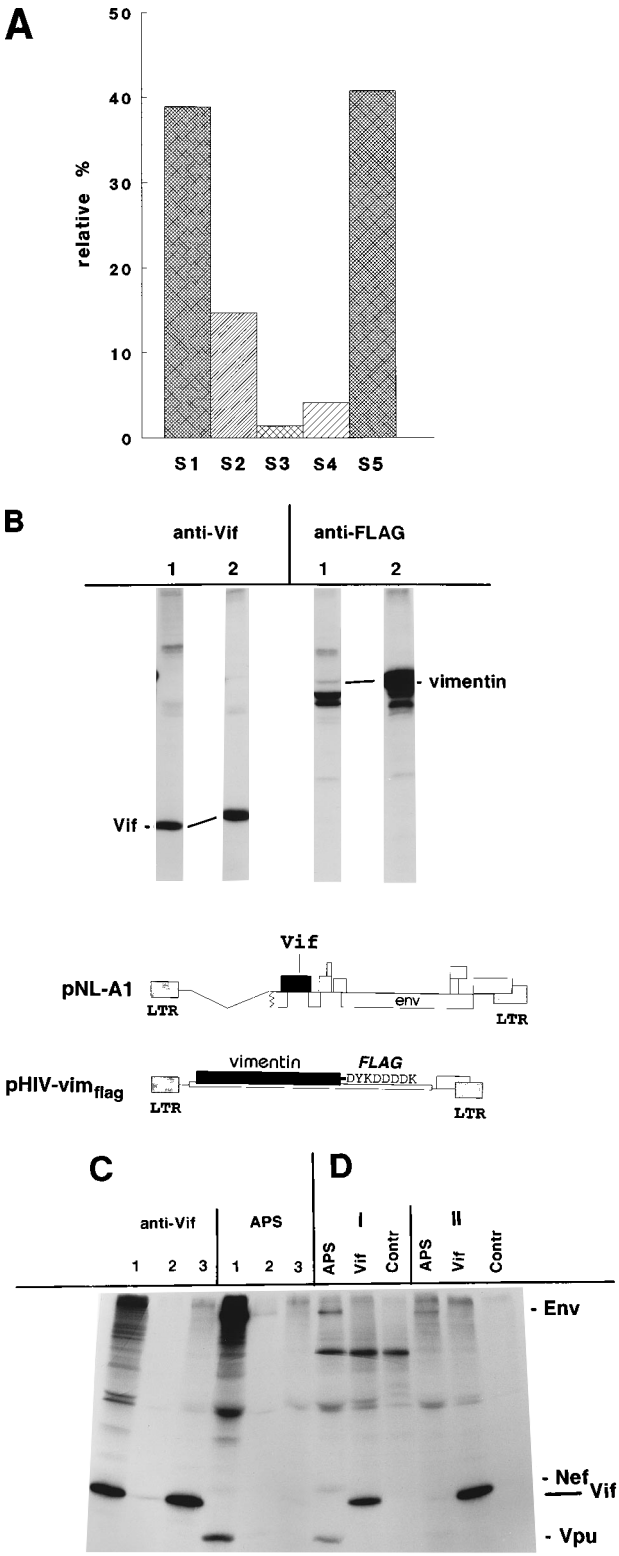


FIG. 1. Extraction of Vif from HeLa cells. (A) Detergent extraction. HeLa cells were transfected with pNL-A1 and metabolically labeled for 2 h with TRAN^{35}S-Label. Cells were fractionated by freezing and thawing in PBS (S1), extracting with CHAPS buffer (S2), Chaps-DOC buffer (S3), or Chaps-DOC–0.1% SDS (S4), or heating in sample buffer (S5). Details of the extraction protocol and compositions of the extraction buffers are described in the text. Extracts were immunoprecipitated with a Vif-specific antiserum and separated on an SDS–12.5% polyacrylamide gel (not shown). The amounts of Vif precipitated from individual fractions were quantitated with a phosphoimager and are presented as the percentages of the total Vif in all fractions combined. (B) High-salt extraction. HeLa cells were cotransfected with pNL-A1 and pHIV-vim$_{flag}$ and metabolically labeled as for panel A. Cells were extracted with 0.6 M KCl–0.1% Triton X-100 in PBS (lanes 1). Residual pellets were extracted by boiling in sample buffer (lanes 2). Individual fractions were immunoprecipitated with a Vif-specific antiserum (anti-Vif) or monoclonal antiserum M2, recognizing the tagged vimentin protein (anti-FLAG). Precipitates were analyzed on SDS–12.5% polyacrylamide gels and then subjected to fluorography. The structure of pNL-A1 and pHIV-vim$_{flag}$ is schematically outlined at the bottom. LTR, long terminal repeat. (C and D) Low-salt extraction of Vif in phosphate-free buffers. HeLa cells were transfected with pNL-A1 and labeled for 2 h as described for panel A. In panel C, cells were extracted with Chaps-DOC buffer (lanes 1), 1% Nonidet P-40 (lanes 2), or sample buffer (lanes 3) as described in the text. All fractions were heated in sample buffer prior to immunoprecipitation with a Vif-specific antiserum (anti-Vif) or an HIV-reactive patient serum (APS). In panel D, cells were extracted with 1% Triton X-100 (panel I). The pellet fraction was extracted by boiling in sample buffer (panel II). Aliquots of each fraction were immunoprecipitated with an HIV-reactive patient serum (APS), a Vif-specific antiserum (Vif), or a nonspecific control serum (Contr). Immunoprecipitates were analyzed on an SDS–12.5% SDS-polyacrylamide gel and subjected to fluorography. The position of Env, Nef, Vif, and Vpu proteins in the gel are indicated on the right.

cysteine-free RPMI 1640 medium (Specialty Media, Inc., Lavalette, N.J.). For cell fractionation studies, cells were labeled for 2 h with TRAN^{35}S-Label (2 mCi/ml; ICN Biomedical, Inc., Costa Mesa, Calif.). Cells were then washed once with PBS to remove unincorporated isotope and lysed, and proteins were extracted. Cell lysates were precleared on GammaBind Plus-Sepharose beads (Pharmacia LKB Biotechnology, Piscataway, N.J.) prior to immunoprecipitation. For identification of Vif in virions, cells were labeled for 2 h as described above in labeling medium (150 μl). After 2 h, 1 ml of RPMI(9:1) (90% labeling medium, 10% complete RPMI 1640) was added, and incubation was continued for 5 h, at which time another 1 ml of RPMI(9:1) was added. Cells were then incubated overnight. The following day, virus-containing supernatants were precleared by centrifugation in a benchtop centrifuge (1,000 rpm, 5 min) and filtered through a 0.45-μm-pore-size filter to remove residual cells and debris. Virions were pelleted by ultracentrifugation in a Beckman SW55 rotor (40 min, 35,000 rpm), suspended in 500 μl of RPMI(9:1), and subjected to 10 to 60% sucrose step gradient centrifugation in a Beckman SW55 rotor (1:15 h, 35,000 rpm). Fourteen fractions (340 μl each) were collected beginning at the top of the gradient and inactivated by heating in 100 μl of sample buffer (5 min, 94°C). Samples were diluted in PBS, and aliquots of each fraction were subjected to immunoprecipitation. A mock gradient was run in parallel to determine the sucrose density in each fraction.

Immunoprecipitations were conducted as described previously (37), using antibodies preadsorbed to GammaBind Plus-Sepharose beads. Immunoprecipitated proteins were solubilized by boiling in sample buffer and separated by SDS-polyacrylamide gel electrophoresis (PAGE). Gels were soaked for 20 min in 1 M sodium salicylic acid and dried. Radioactive bands were visualized by fluorography. Quantitation was performed with a Fuji BAS 2000 Bio-Image Analyzer.

Immunoblotting. Individual fractions (340 μl each) from sucrose gradients were mixed with 200 μl of sample buffer and boiled for 5 min. Equal aliquots from each fraction (120 μl for Vif blots; 50 μl for blots prepared with AIDS patient serum) were separated on SDS–12.5% polyacrylamide gels and transferred to nitrocellulose membranes (BA83; Schleicher & Schuell, Keene, N.H.). All subsequent steps were performed at room temperature. Membranes were blocked with 5% dry milk in TN buffer (10 mM Tris-hydrochloride [pH 7.4], 150 mM NaCl) for 30 min. Blots were briefly rinsed with TN-T buffer (TN buffer containing 0.3% [vol/vol] Tween 20) and incubated with the first antibody in 3% BSA in TN-T buffer for 3 h. Filters were washed twice with TN-TN buffer (TN-T buffer containing 0.05% [vol/vol] Nonidet P-40) and then twice with TN-T buffer. Filters were then incubated with [^{125}I]protein G (0.1 μCi/ml; (Amersham) for 90 min in 3% BSA in TN-T. Finally, filters were washed twice with TN-TN–10 mM EDTA and then twice with TN buffer. Filters were air dried prior to exposure to Kodak Bio-Max MR film.

RESULTS

Cytoskeleton association of Vif. It was recently reported that Vif associates with cellular membranes (17, 32). To confirm this report and to analyze the intracellular distribution of Vif in more detail, we performed a series of cell fractionation studies including high-salt and detergent extractions. Because of the reported membrane association of Vif, we started out by using increasing detergent strengths to solubilize cellular and viral proteins. For this purpose, HeLa cells were transfected with

pNL-A1 plasmid DNA, which encodes all HIV-1 proteins except *gag* and *pol* products (47). Transfected HeLa cells were metabolically labeled, suspended in PBS, and lysed by three cycles of freezing and thawing (3 min each at −70 and 37°C). Insoluble material was pelleted at 15,000 × *g* for 3 min, and the supernatant containing soluble cytoplasmic proteins (fraction S1) was collected. The pellet was sequentially extracted with CHAPS buffer (fraction S2), CHAPS-DOC (fraction S3), or CHAPS-DOC–0.1% SDS (fraction S4). Each extraction step included a 5-min incubation at room temp followed by 3 min of centrifugation at 15,000 × *g*. Detergent-resistant material was solubilized by boiling in sample buffer (fraction S5). Individual fractions were immunoprecipitated with a Vif-specific antiserum, separated by SDS-PAGE, and subjected to fluorography (not shown). The relative amounts of Vif in the individual fractions were calculated by quantitating the Vif bands on a phosphoimager. As shown in Fig. 1A, Vif was found to be primarily distributed between two fractions, S1 and S5. Only a comparatively small portion of the protein (approximately 15%) could be extracted with detergents (fractions S2 to S4). Similar results were obtained after extraction of Vif from acutely infected T cells (data not shown).

The resistance to detergent extraction could be an indication for cytoskeletal association of Vif. To further investigate this possibility, we compared the intracellular distributions of Vif and vimentin, a cytoskeletal element that is expressed in HeLa cells and which forms stable filamentous structures that are resistant to extraction with high-salt buffers (16). To assess the sensitivity of Vif to high-salt extraction, HeLa cells were cotransfected with pNL-A1 and pHIV-vim$_{flag}$. pHIV-vim$_{flag}$ is a plasmid which encodes human vimentin (42) under the control of the HIV-1 long terminal repeat promoter and carries a FLAG epitope tag at its C terminus (Fig. 1B, bottom). The addition of the FLAG epitope tag permits a discrimination between endogenous vimentin present in HeLa cells and vimentin expressed from pHIV-vim$_{flag}$. Cells were labeled as for Fig. 1A, and the cytoskeletal fraction was prepared by extracting cells with 0.6 M KCl–0.1% Triton X-100 in PBS. Extraction under such conditions removes most cellular proteins but leaves the cytoskeleton intact (16). The cytoskeletal fraction was solubilized by boiling in sample buffer, and the high-salt extract and the cytoskeletal fraction were then subjected to immunoprecipitation with a Vif-specific antiserum or the epitope tag-specific monoclonal antibody M2 to detect vimentin. The result of this experiment shows that Vif was distributed in roughly equal amounts between the high-salt extract (Fig. 1B, anti-Vif, lane 1) and the cytoskeletal fraction (Fig. 1B, anti-Vif, lane 2). In contrast, vimentin was detected almost exclusively in the cytoskeletal fraction (Fig. 1B, anti-FLAG, lane 2). This result is consistent with the results from the detergent extraction (Fig. 1A) and is a further indication that a significant portion of Vif could be associated with cytoskeletal elements.

It is possible that repeated freeze-thaw cycles or the extraction of proteins in high-salt or phosphate buffers cause nonspecific aggregation of Vif, resulting in nonspecific partitioning of Vif to the cytoskeletal fraction in the experiments shown in Fig. 1A and B. To control for such a possible artifact, we performed a series of extractions under low ionic strength. In addition, phosphate buffer was replaced by Tris buffer and cells were not subjected to any freeze-thaw cycles (Fig. 1C and D). Two different protocols were used. In Fig. 1C, HeLa cells transfected with pNL-A1 plasmid DNA were metabolically labeled as described above, extracted with 50 mM Tris-hydrochloride (pH 8.0)–0.5% CHAPS–0.2% DOC (Fig. 1C, lanes 1), and then extracted with 1% Nonidet P-40–50 mM Tris-hydrochloride (pH 8.0) (Fig. 1C, lanes 2). Sample buffer was added to both fractions. Insoluble material (Fig. 1C, lanes 3) was suspended in sample buffer, and all fractions were heated for 5 min at 95°C. All fractions were adjusted to 1 ml with PBS prior to immunoprecipitation with either an HIV-reactive patient serum which was supplemented with a Nef-reactive antiserum (46) or with a Vif-specific antiserum. In Fig. 1D, cells were extracted with 1% Triton X-100–50 mM Tris-hydrochloride (pH 8.0) (panel I), and the insoluble fraction was solubilized by boiling in sample buffer (panel II). Note that samples in panel I of Fig. 1D were not heat denatured prior to immunoprecipitation in this experiment. Equal aliquots of each sample were immunoprecipitated with an HIV-reactive patient serum, a Vif-specific antiserum, or a nonspecific control serum. Independent of the extraction procedure, a significant amount of Vif (50 to 60%) consistently copurified with the cytoskeletal fraction. In contrast, the integral membrane proteins Vpu and Env as well as the membrane-associated Nef protein were almost quantitatively found in the detergent fractions. We therefore conclude that the partitioning of Vif with the cytoskeletal fraction is not due to nonspecific aggregation of Vif but is an inherent feature of this viral protein and most likely reflects the association of Vif with cytoskeletal components. It should be noted that previous studies investigating the subcellular distribution of Vif reported a significant amount of Vif associated with cellular membranes (17, 18, 32). However, these studies did not analyze cytoskeletal fractions of cells and thus ignored the portion of Vif that partitions with that fraction. Our results are therefore not in conflict with but rather extend those earlier findings by demonstrating that, aside from soluble cytoplasmic and membrane-bound forms, a significant portion of Vif is associated with the cytoskeleton.

Intracellular localization of Vif. One previous study reported diffuse cytoplasmic and light nuclear staining for Vif (17). However, this study did not use optical sectioning techniques, nor did it specifically investigate the potential association of Vif with cytoskeletal structures. Thus, to further investigate the intracellular localization of Vif and to verify the suspected cytoskeletal association, we performed a series of immunocytochemical analyses involving confocal microscopy. As a model system, HeLa cells transiently expressing Vif were used since these cells express not only microtubule (Fig. 2A) and microfilament (Fig. 2B) networks but also the IFs vimentin (Fig. 2C) and keratin (Fig. 2D). For analysis of Vif, HeLa cells were transfected with pNL-A1 plasmid DNA, fixed, and stained with a Vif-specific antiserum as described in Materials and Methods. Stained cells were then analyzed by confocal microscopy. Representative results of such studies are shown in Fig. 2E and F. As shown in Fig. 2E, Vif was generally localized to the cytoplasm and exhibited, in part, a filamentous staining pattern. However, Vif was also detectable in the nuclei of cells (Fig. 2E) in amounts that varied from preparation to preparation (not shown). Interestingly, in about 5 to 10% of Vif-expressing cells, Vif formed a distinct perinuclear aggregate (Fig. 2F). Optical sectioning (not shown) of such cells revealed that the Vif aggregates formed spherical structures with a diameter of approximately 5 μm. Both the filamentous staining pattern and the formation of perinuclear aggregates were specific for Vif and not observed when cells were transfected with a Vif-deficient isogenic variant of pNL-A1, pNL-A1ΔVif (not shown). Identical staining patterns were observed when a different Vif-specific antiserum (17) was used (not shown), minimizing the possibility of nonspecific cross-reactivity of the Vif antiserum. The fact that only 20 to 30% of transfected HeLa cells stained positive for Vif also attests to the specificity of the staining reaction and is in good agreement

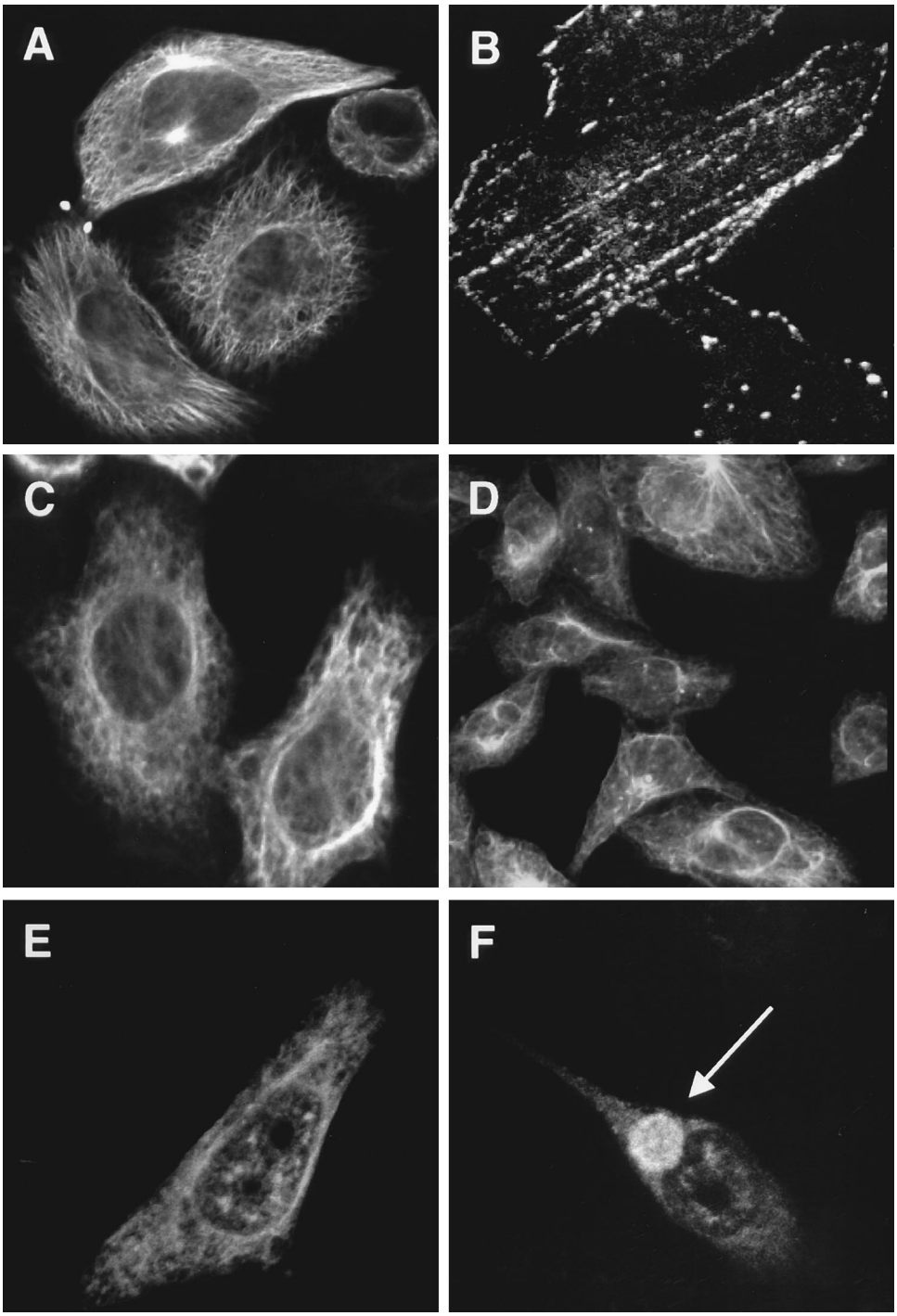


FIG. 2. Indirect immunofluorescence analysis of cytoskeletal components in HeLa cells and analysis of the subcellular localization of Vif. (A to D) HeLa were stained with antibodies to tubulin (A), vimentin (C), or keratin (D). Actin filaments were stained with rhodamine-phalloidin (B). (E to F) HeLa cells were transfected with pNL-A1 and stained 24 h after transfection with a Vif-specific antiserum. Confocal images were acquired with a Zeiss LSM410 laser scanning microscope. Vif was found primarily in the cytoplasm; however, some punctate nuclear staining of Vif is apparent in panels E and F. The image shown in panel E represents the typical Vif pattern observed in HeLa cells and closely resembles that of vimentin (C). In some cells, Vif was concentrated at a perinuclear site (panel F, arrow).

with the efficiency of calcium phosphate transfections generally observed in our hands.

Colocalization of Vif and vimentin. The IF vimentin generally forms stable filamentous structures extending from the nuclear membrane to the plasma membrane (for a review, see reference 10). However, aggregation similar to that observed for Vif in Fig. 2F was observed previously for IFs following microinjection of anti-IF antibodies (26, 28). Similarly, expression of a truncated form of desmoplakin lacking a domain critical for the attachment of IFs to desmosomes at the cell surface caused a structural reorganization of the IF network which was paralleled by a coalignment of vimentin with trun-

FIG. 3. Indirect double-label immunofluorescence of HeLa cells transfected with pNL-A1. Cells were stained 24 h after transfection with antibodies to Vif and vimentin (A to C) or Vif and tubulin (D to F). (A) Two-color overlay of the Vif (B) and vimentin (C) patterns. (D) Overlay of Vif (E) and tubulin (F) patterns. Vif appears red; vimentin or tubulin appear green. Areas of colocalization are yellow. Arrows point to perinuclear aggregation sites of Vif and vimentin. Microtubule networks were not affected by Vif.

cated desmoplakin molecules (44). These observations led us to investigate whether the observed aggregation of Vif in our experiments is accompanied by a simultaneous reorganization of vimentin or other cytoskeletal components. For this purpose, transfected HeLa cells were doubly stained for Vif and vimentin (Fig. 3A to C) or Vif and tubulin (Fig. 3D to F), and confocal images were simultaneously acquired for both fluorochromes. We found that in all cells in which perinuclear aggregation of Vif had occurred, not only had vimentin collapsed into a perinuclear aggregate, but the Vif and vimentin aggregates (Fig. 3B and C, respectively) colocalized (Fig. 3A). No such aggregates were observed for tubulin (Fig. 3D to F) or actin (data not shown), suggesting that the observed effects are specific for vimentin and possibly caused by an interaction of Vif with this IF. Aggregation of vimentin was not observed when cells were transfected with *vif*-defective isogenic variants of pNL-A1 (data not shown), indicating that the effects observed are specific for Vif and not caused by transient expression of other HIV-1 proteins encoded by pNL-A1 such as Vpr, Vpu, Env, or Nef. It is interesting that while vimentin filaments almost quantitatively collapsed (Fig. 3C), aggregation of Vif was only partial (Fig. 3B and E) and residual, diffuse cytoplasmic staining was apparent. This is most likely due to the presence of soluble and/or membrane-associated Vif in these cells.

IF networks are known to respond to pharmacological agents such as colchicine (reviewed in reference 27) by collapsing into spirals around the nucleus. We used this feature to further verify the association of Vif with IFs. Treatment of HeLa cells with the colchicine derivative demecolcine resulted in the appearance of thick cage-like bundles of vimentin which were concentrated around the nucleus (Fig. 4A). Staining of the same cell for Vif revealed a very similar staining pattern (Fig. 4B) which closely coaligned with the vimentin pattern (evident by the yellow color in Fig. 4C). It should be noted that the staining patterns observed for Vif and vimentin after demecolcine treatment vary and are not strictly limited to the structure shown in Fig. 4A. However, Vif invariably colocalized with vimentin, independent of the structure it assumed.

To investigate whether Vif has a general affinity for IF networks or aligns exclusively with vimentin, the possible coalignment of Vif with keratin, another type of IF, was analyzed in HeLa cells. For this analysis, cells were not subjected to drug treatment. A filamentous staining pattern similar to that observed for vimentin in the previous experiments was observed for keratin (not shown). In about 5 to 10% of the Vif-positive cells, keratin and Vif coaggregated into structures similar to those seen for Vif and vimentin (Fig. 4E). Three-color confocal microscopy confirmed that the perinuclear aggregates contained Vif and vimentin as well as keratin (apparent from the white color in Fig. 4F). These results therefore suggest that Vif has a general affinity for IFs and, under certain conditions, is able to cause aggregation of both vimentin and keratin fila-

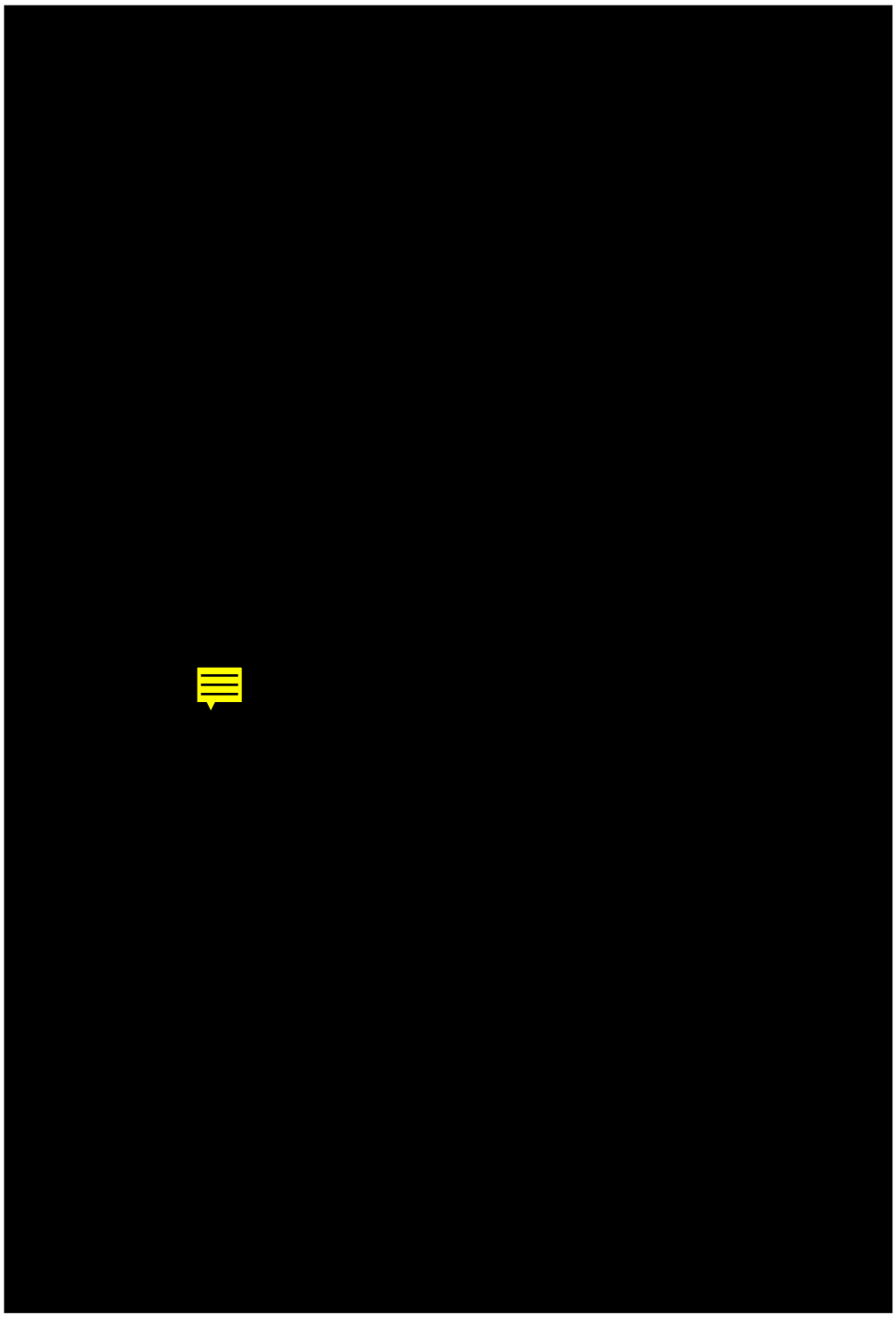


FIG. 4. Indirect double or triple immunofluorescence of HeLa cells transfected with pNL-A1. (A to D) Cells were treated with demecolcine (0.1 μg/ml) for 15 h following transfection. Cells were stained for vimentin (A) and Vif (B), and confocal images were simultaneously acquired as for Fig. 3. (C) Two-color overlay of panels A and B. Vif appears red; vimentin appears green. (D) Nomarski bright-field image. Demecolcine treatment of HeLa cells resulted in a structural reorganization of vimentin IFs. The pattern for Vif changed accordingly, suggesting a close association of Vif and vimentin. (E) Transfected HeLa cells were stained for Vif (red) and keratin (green). In cells exhibiting perinuclear aggregates of Vif, keratin filaments had collapsed as well (arrow). (F) Three-color analysis of Vif (blue), vimentin (red), and keratin (green) demonstrates that perinuclear aggregates contain all three proteins (apparent by the white color; arrows).

ments. Association of Vif with keratin filaments could also explain the filamentous staining observed for Vif at the edges of the cell shown in Fig. 4B which does not appear to colocalize with vimentin filaments (red staining in Fig. 4C).

Localization of Vif in cells lacking IFs. IFs are prominent components of the cytoskeleton and nuclear envelope of most eukaryotic cell types (for a review, see reference 45). In HeLa cells, which express vimentin and keratin cytoplasmic IFs, Vif is predominantly found in the cytoplasm and, for a large part, in close association with IF networks. To study the influence of

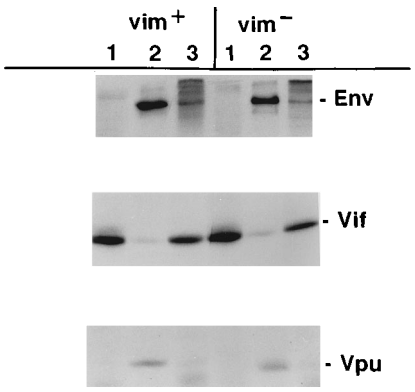


FIG. 5. Extraction of Vif from cells lacking cytoplasmic IFs. SW13 cells either expressing (vim$^+$) or lacking (vim$^-$) cytoplasmic IFs were transfected with pNL-A1. Cells were metabolically labeled as described for Fig. 1. Cells were sequentially fractionated by freezing and thawing in PBS (lanes 1), extraction with CHAPS-DOC (lanes 2), and boiling in sample buffer (lanes 3). Extracts were immunoprecipitated with antibodies specific for Env, Vif, or Vpu, separated on an SDS–12.5% polyacrylamide gel, and subjected to fluorography. Samples were run in different lanes of the same gel. Only the regions containing the Env, Vif, or Vpu proteins are shown.

cytoplasmic IFs on the subcellular distribution of Vif, we made use of a human adrenal tumor cell line, SW13 (vim$^-$), which lacks detectable levels of vimentin and keratin (36). This cell line is derived from the vimentin-positive cell line SW13 (vim$^+$) (36), which we used in parallel as a control. If the resistance of Vif to detergent extraction observed in Fig. 1 is due to the stable association of Vif with cytoplasmic IFs, then the lack of such elements in SW13 (vim$^-$) cells should increase the solubility of Vif. At the same time, the observed filamentous staining pattern observed for Vif in Fig. 2E should change and no perinuclear aggregates of Vif such as the ones shown in Fig. 2F should be detectable.

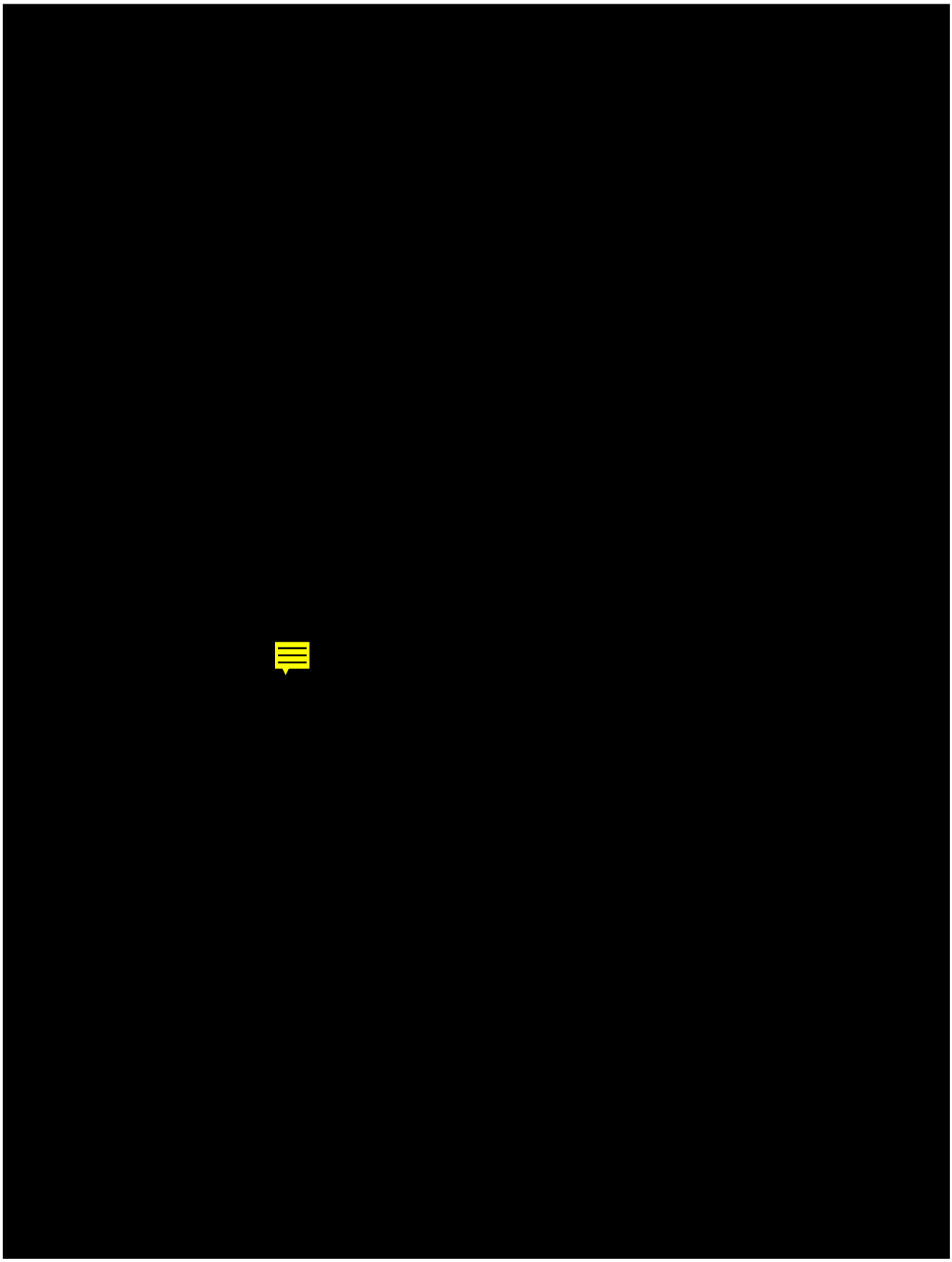
To test this hypothesis, vimentin-deficient SW13 (vim$^-$) cells and, as a control, vimentin-positive SW13 (vim$^+$) cells were transfected with pNL-A1 plasmid DNA. Cells were metabolically labeled, fractionated by freezing and thawing in PBS, and extracted with CHAPS-DOC buffer. Residual material was solubilized by boiling in sample buffer. Extracts were immunoprecipitated with a Vif-specific antiserum and analyzed by fluorography following gel electrophoresis. Surprisingly, even in the absence of cytoplasmic IFs, approximately 50% of Vif was resistant to extraction (Fig. 5, lanes 3). Most of the remaining Vif protein was present in the soluble fraction (Fig. 5, lanes 1), and only a minor portion of Vif was detergent extractable (Fig. 5, lanes 2). The membrane-associated HIV-1 proteins Vpu and gp160 (Env) were almost quantitatively found in the detergent fraction, attesting to the efficiency of the detergent extraction (Fig. 5, lanes 2). The finding that the solubility of Vif is not increased in the absence of cytoplasmic IFs could be an indication that Vif does not directly interact with IFs but links up with IF-associated factors which might be present in SW13 (vim$^-$) cells in conjunction with other cytoskeletal elements. Alternatively, it is possible that in the absence of cytoplasmic IFs, Vif has an increased affinity for nuclear IFs.

To further investigate the localization of Vif in SW13 cells, transfected SW13 cells were analyzed by confocal microscopy (Fig. 6). Cells were doubly stained for Vif and vimentin, and two-color confocal images were simultaneously acquired. As in HeLa cells, Vif was predominantly found in the cytoplasm of vimentin-positive cells in structures that closely colocalized with vimentin (Fig. 6A to F). The structure of the vimentin network in Vif-expressing cells was significantly altered (red arrows in Fig. 6D) and resulted in a perinuclear concentration of the IF, again attesting to the effect of Vif on IF organization. Vif-negative cells exhibited normal filamentous patterns of vimentin (green arrows in Fig. 6D). In contrast, in vimentin-negative cells (Fig. 6G to M), a significant portion of Vif was localized to the nucleus and the nuclear membrane (arrows in Fig. 6I and M). The organization of the tubulin network in SW13 (vim$^-$) cells (Fig. 6L) was comparable to that found in SW13 (vim$^+$) cells (not shown). Diffuse cytoplasmic Vif-staining observed in Fig. 6I and M likely represents soluble Vif present in these cells as identified in Fig. 5 (lanes 1). The results from this experiment suggest that in the absence of cytoplasmic IF structures, Vif has a tendency to relocate to the nucleus. The resistance of Vif to detergent extraction in SW13 (vim$^-$) cells observed in Fig. 5 is possibly due to an association of Vif with nuclear lamins, which are members of the IF family and are integral components of the nuclear envelope (for a review, see reference 15). Thus, the intracellular distribution of Vif is affected by the presence or absence of cytoplasmic IFs.

Association of Vif with virus particles. The results from the experiments presented above demonstrate that Vif has an affinity for and coaligns with intermediate filaments. However, the question of how such an interaction could be involved in the regulation of viral infectivity has not been addressed. It could be argued that association of Vif with IFs helps in the targeting of Vif to a critical intracellular site; however, the infectivity of virus particles produced from vimentin-negative SW13 cells is comparable to that derived from vimentin-positive SW13 or HeLa cells (not shown), suggesting that the presence of vimentin in the donor cells has no impact on the infectivity of progeny virions. On the other hand, studies by Liu and coworkers demonstrate that 60 to 100 molecules of Vif are present in virions (29). This finding raises the possibility that virus infectivity is dependent on the presence or absence of Vif in virions, and it could be argued that the affinity of Vif for IFs as well as its ability to translocate to the nucleus could facilitate the translocation of incoming virus particles from the cell surface to the nucleus.

We initially attempted to confirm the presence of virion-associated Vif by immunoblotting concentrated virion preparations from HIV-infected A3.01 cells with a Vif-specific antiserum. Similar to the results of Borman et al. (3), only small amounts of Vif were detectable under such conditions (data not shown). To enhance the sensitivity of our assay, epitope-tagged Vif protein was expressed in *trans* from plasmid pNL-A1/vif$_{flag}$, which overexpresses Vif about 15-fold relative to the full-length molecular clone pNL4-3. To prevent interference with the incorporation of Vif$_{flag}$ into virus particles, expression of wild-type Vif from pNL4-3 was suppressed by introducing a deletion into the *vif* gene. The resulting mutant, pNL4-3ΔVif, is defective for Vif but expresses normal levels of the other viral proteins, including Vpr, as judged by immunofluorescence analysis (not shown).

Virion-association of Vif$_{flag}$ was first analyzed by immunoblotting. HeLa cells were cotransfected with pNL4-3ΔVif and pNLA-1/Vif$_{flag}$ plasmid DNAs, and virus-containing culture supernatants were collected over a 48-h time period following transfection. Virus particles were concentrated by ultracentrifugation and analyzed on 10 to 60% sucrose gradients. Individual fractions were collected beginning at the top of the gradient, separated on protein gels, and blotted against an HIV-reactive patient serum or a Vif-specific antiserum (Fig. 7). As shown in Fig. 7A, viral proteins were concentrated in fractions 8 to 10, corresponding to densities of 1.14 to 1.18, which is indicative for the presence of virus particles. Smaller amounts



of viral proteins were detectable in adjacent fractions. Interestingly, the Vif-specific antiserum identified the 23-kDa Vif protein, which was largely concentrated in fractions 8 and 9 and, to a lesser extent, in fraction 10. The fact that Vif migrates at a similar density as HIV-1 particles is indicative of its virion association.

To rule out a fortuitous comigration of non-virion-associated Vif with HIV particles in sucrose gradients, virus particles were treated with mild detergent (0.05% Triton X-100 in PBS) prior to sucrose gradient centrifugation. Detergent treatment is expected to disrupt viral envelopes as well as contaminating cellular membrane vesicles, resulting in the release of proteins associated with those structures. This experiment should also address the question of whether Vif is nonspecifically incorporated into virions as a result of its affinity for cellular membranes (17). Immunoblotting of individual gradient fractions with an HIV-reactive antiserum (Fig. 7B, APS) confirmed the complete dissociation of the viral envelopes, since the membrane-associated matrix protein (p17) was found exclusively at the top of the gradient (fractions 1 to 3). The main capsid component (p24) was found broadly distributed throughout the gradient, indicating that a fraction of the HIV core particles had been disrupted by the detergent treatment, resulting in structures with lighter densities than wild-type virus particles. Nevertheless, it appears that a portion of the virions resisted the detergent treatment and migrated with a density characteristic of virus particles. Interestingly, the migration of the $p55^{gag}$ precursor protein appeared to be largely unaffected by the detergent treatment (Fig. 7B, APS) and was associated with particles similar in density to non-detergent-treated particles (compare Fig. 7A and B, APS). Analysis of the same gradient fractions from the detergent-treated virus with a Vif-specific antiserum revealed a distribution of Vif between fractions 5 and 10, corresponding to densities of 1.09 to 1.18. The broadening of the Vif-containing fractions parallels the broadening of the fractions containing $p55^{gag}$ and $p24^{gag}$. However, Vif could not be detected at the top of the gradient, eliminating the possibility of a nonspecific attachment of Vif to viral or cellular membranes and suggesting a close association of Vif with the viral core structure. The absence of Vif from the top of the gradient could also be an indication that only a fraction of virus particles contain Vif, and it is conceivable that resistance of virions to detergent treatment is correlated with the presence of Vif in viral particles. No Vif-specific signal was detected when pNL4-3ΔVif was expressed alone (Fig. 7C), attesting to the specificity of the signal seen with the Vif-specific antiserum in Fig. 7A and B. In addition, no extracellular Vif was detectable when Vif was expressed in the absence of HIV particles (not shown).

To independently demonstrate the presence of Vif in virus preparations and to rule out cross-reactivity of the Vif-specific antibody with the $p24^{gag}$ capsid protein which in Fig. 7 migrates at a mobility similar to that of Vif, we performed a side-by-side comparison of sucrose gradient fractions of metabolically labeled virions following immunoprecipitation with an antiserum to the epitope-tagged Vif protein or a Vif-negative HIV-reactive patient serum. HeLa cells were cotransfected with pNL4-3ΔVif and pNL-A1/vif_{flag} plasmid DNAs, and cells were metabolically labeled with TRAN^{35}S-Label for 20 h as described in Materials and Methods. Concentrated virus preparations were subjected to sucrose gradient centrifugation, and individual fractions were collected as described for Fig. 7 and heat inactivated. Aliquots of each fraction were immunoprecipitated with an HIV-reactive patient serum (30%; Fig. 8, lanes a) or an antibody, M2, to the FLAG epitope (70%; Fig. 8, lanes b) and analyzed by SDS-PAGE. Only fractions 7 through 12, containing virus particles, are shown. The remaining fractions did not contain significant amounts of viral proteins (not shown). Heat denaturation of viral proteins caused a reduction of the affinity of the AIDS patient serum for $p24^{gag}$, while the affinity of monoclonal antibody M2 for the epitope-tagged Vif protein was unaltered (not shown). This feature allowed us to analyze Vif and $p24^{gag}$ in adjacent lanes without interference due to overexposure of the dominant $p24^{gag}$. In agreement with the results from Fig. 7, Vif was present in all fractions containing virion proteins. Vif was clearly distinguishable from $p24^{gag}$ by its faster mobility in the gel.

DISCUSSION

One of the key findings of our study is the association of Vif with IFs. It is apparent from our immunocytochemical analyses (Fig. 3F, 4, and 5E and F) that the presence of Vif affects the structure of IFs and can lead to a complete collapse of the IF network, resulting in the formation of perinuclear aggregates containing Vif and IFs. While the mechanistic basis for this phenomenon is still unclear, available evidence suggests that it involves an interaction between Vif and vimentin. First, Vif was never observed to form perinuclear aggregates in cells lacking cytoplasmic IFs. Similarly, the collapse of vimentin IFs was observed only in cells expressing Vif, suggesting the requirement for a close physical interaction between these two factors. Also, while collapse of vimentin IF networks has been previously observed following microinjection of HIV-1 protease into human skin fibroblasts (24), there is no evidence that would support such a mechanism for Vif. The effect of HIV-1 protease on vimentin is the result of a proteolytic cleavage of vimentin at a site in the C-terminal tail domain and is therefore irreversible (39). In contrast, we have preliminary evidence suggesting that the observed effect of Vif on IFs is reversible (46) and thus more consistent with a mechanism that does not involve permanent modification of IF components. It is interesting that Vif was found to have a more pronounced effect on the IF structure when de novo-synthesized vimentin was analyzed. While newly synthesized vimentin was found to be efficiently incorporated into existing IF networks in the absence of Vif, the presence of Vif resulted in the formation of aberrant vimentin structures, including perinuclear aggregates, in almost all cells (46). This finding suggests that Vif has a more profound effect on the growth of IF networks than on preexisting IF structures and is consistent with a mechanism involving protein-protein interactions between Vif and vimentin. Attempts to demonstrate a direct interaction between Vif and vimentin by means of coimmunoprecipitation have so far been hampered by the fact that vimentin filaments are very stable and little if any soluble vimentin is present in cells (41). How-

FIG. 6. Cytoskeletal elements affect the subcellular distribution of Vif. Vimentin-positive (A to F) and vimentin-negative (G to M) SW13 cells were transfected with pNL-A1. Indirect double-label immunofluorescence with antibodies to Vif and vimentin or tubulin was done 24 h following transfection. Confocal images of two representative fields are shown for each cell type. (A, D, G, K) Two-color overlays of the vimentin (B, E, and H) or tubulin (L) and Vif (C, F, I, and M) patterns. In vimentin-positive cells, Vif colocalized with vimentin. The vimentin patterns in Vif-positive cells (red arrows in panel D) are distinct from the vimentin network in Vif-negative cells (green arrows in panel D). In SW13 (vim^-) cells (G to M), Vif was found predominantly in the nucleus or associated with the nuclear membrane (arrows in panels I and M).

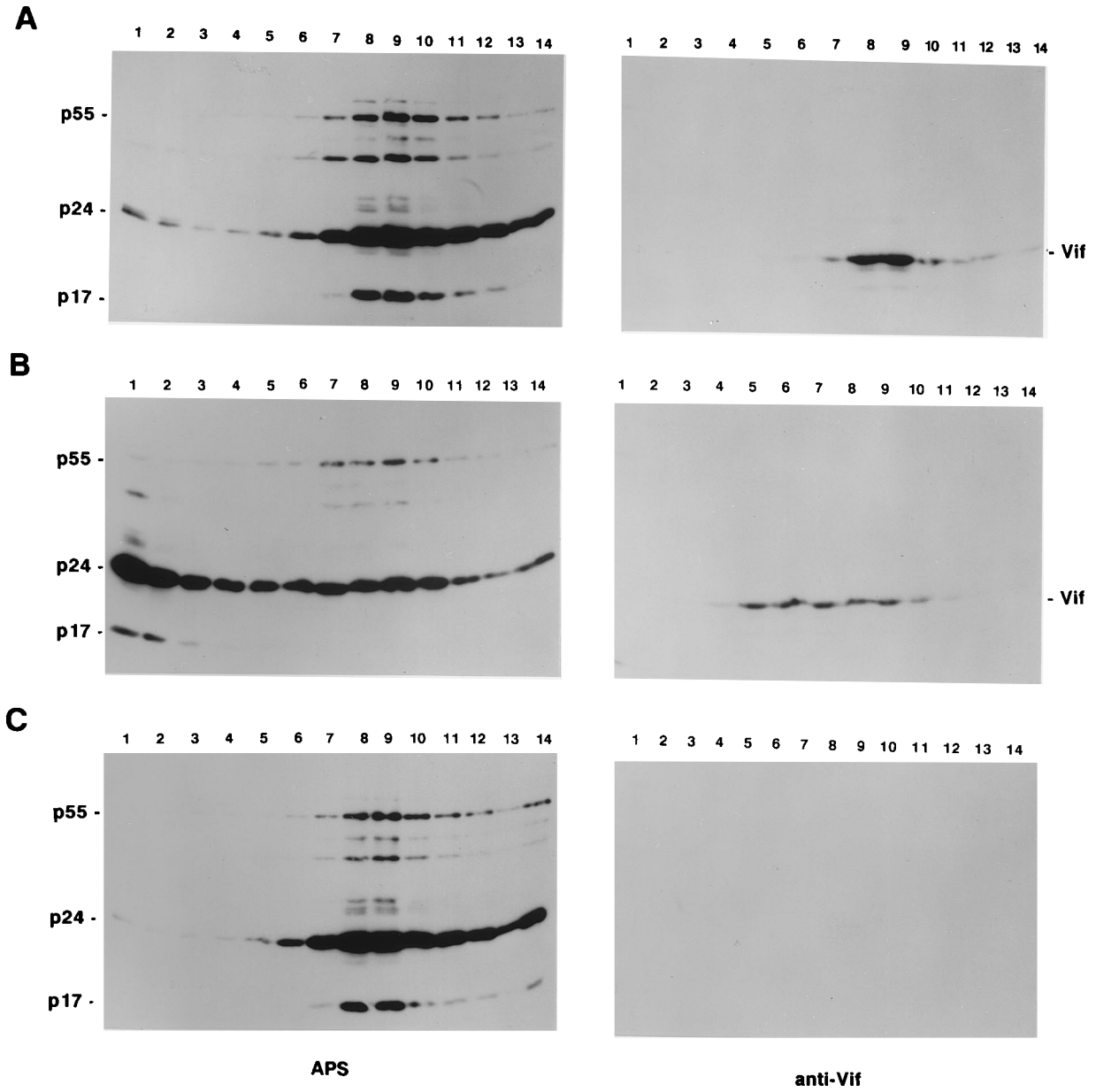


FIG. 7. Immunoblot analysis of Vif in virus particles. Concentrated virus preparations were produced in transiently transfected HeLa cells as described in the text and subjected to sucrose density gradient centrifugation. Aliquots of individual fractions were separated on SDS–12.5% polyacrylamide gels, transferred to nitrocellulose filters, and incubated with an HIV-reactive patient serum (APS) or a Vif-specific polyclonal antiserum (anti-Vif [17]). (A and B) Particles derived from cells expressing Vif; (C) particles derived from cells lacking Vif. In panel B, virus preparations were treated with 0.05% Triton X-100 prior to sucrose gradient centrifugation. The positions of the main capsid components are indicated on the left. Vif is identified on the right.

ever, the fact that binding of IF-associated factors to intermediate filaments (44) or microinjection of IF-specific antibodies (26, 28) causes disruption of the IF structure and its collapse into perinuclear aggregates similar to those observed for Vif makes it attractive to speculate about a physical interaction between Vif and vimentin. It is unlikely that the Vif-mediated changes in the structure of IF are due to the overexpression of Vif, since aggregation of vimentin was observed in cells expressing low or high levels of Vif (data not shown). It is possible that the Vif-induced aggregation of IFs is a cell cycle-dependent phenomenon. Since IFs undergo cell cycle-dependent reorganization (5), it is conceivable that the presence of Vif causes aggregation of IFs as they undergo reorganization during cell division. This would also explain why only a fraction of Vif-expressing cells exhibits aggregation of IFs at any given time.

It is unclear whether the partitioning of Vif between soluble, membrane, and cytoskeleton-associated forms reflects biochemical modifications of this protein or simply represents a steady-state equilibrium. The immunocytochemical techniques used in this study do not generally allow one to differentiate between individual forms of Vif. However, detergent extraction of cells, while readily eliminating positive staining for membrane-associated marker proteins such as CD4, did not affect the filamentous Vif staining (46) and is therefore clearly attributable to its association with IFs. It is interesting that the

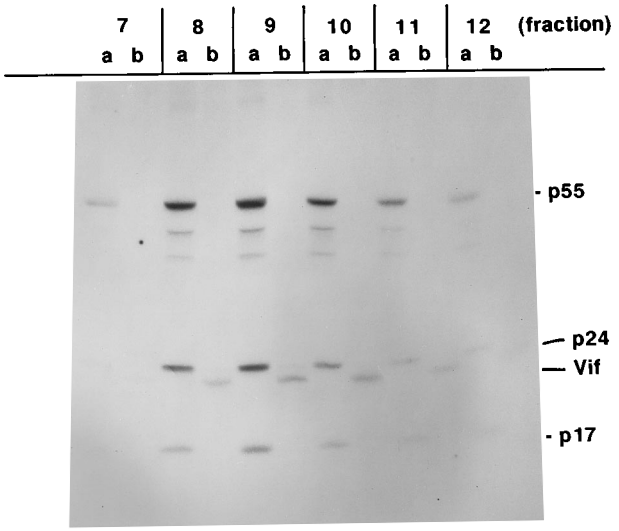


FIG. 8. Immunoprecipitation of virion-associated Vif. Transiently transfected HeLa cells were metabolically labeled with TRAN^{35}S-Label as described in the text and subjected to sucrose gradient centrifugation. Individual fractions were heat denatured and immunoprecipitated with an HIV-reactive patient serum (lanes a) or the FLAG-specific monoclonal antibody M2, recognizing the tagged Vif protein (lanes b). Only fractions 7 to 12 of the gradient are shown. The positions of the major capsid components and of Vif in the gel are indicated on the right.

absence of the cytoplasmic IFs vimentin and keratin in SW13 (vim$^-$) cells does not significantly increase the solubility of Vif but rather appears to result in an increased partitioning of Vif to the nucleus or the nuclear envelope. It is possible that this phenomenon is caused by an association of Vif with nuclear lamins, which belong to the family of intermediate filaments and constitute an integral part of the nuclear envelope. Association of Vif with the nuclear envelope was not generally apparent in cells expressing cytoplasmic IFs even though punctate nuclear staining of Vif was common in most cells analyzed.

The available data do not currently allow us to fully appreciate the importance of Vif-vimentin interactions and in particular the significance of Vif-induced reorganization of IF networks and their importance for virus replication. However, it is worth noting that similar effects have been observed with other viral proteins as well. For example, the adenovirus E1B 19-kDa protein, which plays a role in oncogenic transformation of infected cells, specifically associates with and disrupts the organization of IFs and the nuclear lamina (50). It was speculated that the resulting morphological changes are necessary to prevent abnormal cytopathic effects (50). Similarly, the human papillomavirus (HPV) E1-E4 protein causes a collapse of the cytokeratin network following infection of human keratinocytes (7). The effect of E1-E4 is specific for cytokeratin and is assumed to be caused by a close physical interaction of E1-E4 with cytokeratin IFs (7). In this case, it was argued that destruction of the cytokeratin matrix benefits HPV by destroying a physical barrier that could interfere with virus release from the surface of the skin (7). The effects of the adenovirus 19-kDa and the HPV E1-E4 proteins on intermediate filaments are quite distinct. However, in both cases they serve a specific purpose and are beneficial for the virus. It is likely that the Vif-induced alterations in the IF structure of infected cells are similarly beneficial for HIV replication.

The presence or absence of vimentin in virus-producing SW13 cells did not significantly influence the effect of Vif on virus infectivity, suggesting that the interaction of Vif with vimentin in virus-producing cells is irrelevant and unrelated to the biological phenotype of Vif-deficient HIV particles. However, it is conceivable that the affinity of Vif for vimentin plays a role during infection of a target cell. Primary T cells, macrophages, and various T-cell lines analyzed in our study all showed expression of vimentin (data not shown). Since Vif-defective virions are able to penetrate target cells (30, 49) but are blocked at some early step in the replication cycle, it is possible that Vif regulates a postpenetration-preintegration step of the virus life cycle. One of the prerequisites for such a potential function of Vif is its incorporation into progeny virions.

Borman and coworkers recently reported the presence of extremely small quantities of Vif in cell-free preparations of wild-type HIV particles (3). However, they suggested that Vif incorporation into virus particles might be due to nonspecific encapsidation. In contrast, Liu and coworkers demonstrate incorporation of significant amounts of Vif into virions as well as association of Vif with viral core structures (29). Our own attempts to identify Vif in virions from HIV-infected T-cell cultures were similarly hindered by the relatively low expression of this viral protein in HIV-infected cells. To overcome this problem, we overexpressed epitope-tagged Vif in *trans* in HeLa cells and analyzed Vif incorporation into virions by using immunoblot and immunoprecipitation analyses. The overexpression of Vif and the increased sensitivity of our assay enabled us to clearly demonstrate incorporation of Vif into HIV particles. Consistent with the results of Liu et al. (29), we found that virion-associated Vif is resistant to mild detergent treatment under conditions in which the p17 matrix component is quantitatively released from virus particles, which suggests that Vif is stably associated with the viral core. Given these findings, it is tempting to speculate that regulation of viral infectivity involves incorporation of Vif into virions, where Vif could control a postbudding step during virus maturation or catalyze an early process following viral entry into a target cell. The question of whether Vif is specifically incorporated into virions through an association with other virion components remains to be investigated. However, preliminary results suggest that Vif is capable of binding to capsid proteins (46), and we are currently investigating whether such an association regulates virion incorporation of Vif.

The design of our experiments, which involved overexpression of Vif in virus-producing cells, does not allow precise calculations of the number of Vif molecules packaged into viral particles. However, we estimate that the amounts of Vif present in virus particles are comparable to those of reverse transcriptase or integrase, which is in agreement with calculations by Liu et al. (29). Since the biochemical function of Vif is obscure, it is impossible to predict how much Vif would have to be incorporated into virions to be functionally relevant or how much Vif protein would be required to explain the biological phenotypes associated with its function. It is possible that by analogy to the viral reverse transcriptase or integrase components, only few molecules of Vif are actually required to perform an essential function during virus replication. In any case, a potential function of Vif from within viral particles is not in conflict with previous observations which demonstrate that particles produced in restrictive cell types in the absence of Vif exhibit aberrant morphologies (3, 23). In fact, it could be argued that the absence of Vif from virions is responsible for the observed alterations in virion morphology. A function of Vif as a virion component is also not in conflict with earlier observations that Vif present in target cells cannot support infection by *vif*-deficient virions produced in restrictive cells (3,

49). It is possible that the observed association of Vif with the viral core occurs only during virus assembly and cannot be supplemented in *trans* at a later stage.

From the results presented in this study as well as published data, we can only speculate as to how the affinity of Vif for IFs and its presence in virions could increase virus infectivity. It can be assumed that the principal deficiency of Vif-defective virions is their inability to perform one or several critical steps of the virus replication cycle that follow penetration of a target cell but precede integration of the virus into the host genome. One of the steps that could thus be affected is the transport of incoming virus particles to the nucleus. Since intermediate filaments directly connect nuclear and plasma membranes (for a review, see reference 10), they could serve as a guide to direct virus particles toward the nucleus. Vif, as part of the inner core of HIV and because of its affinity for vimentin, could consequently serve as an anchor molecule and allow the two-dimensional movement of virus particles along IFs from the plasma membrane to the nuclear membrane. Such a guided movement of virus particles would be significantly more efficient than undirected diffusion. Nuclear import could then be accomplished with the assistance of Vpr or $p17^{gag}$, both of which are virion components and have been implicated in nuclear import of preintegration complexes (4, 13, 22, 48). Since the absence of Vif from virions would prevent directional movement of incoming virions and result in undirected diffusion, translocation of virions to the nucleus would be significantly less efficient. This would only occasionally result in a successful infection and could explain the reported 100- to 1,000-fold reduced infectivity of Vif-defective HIV particles (9, 47).

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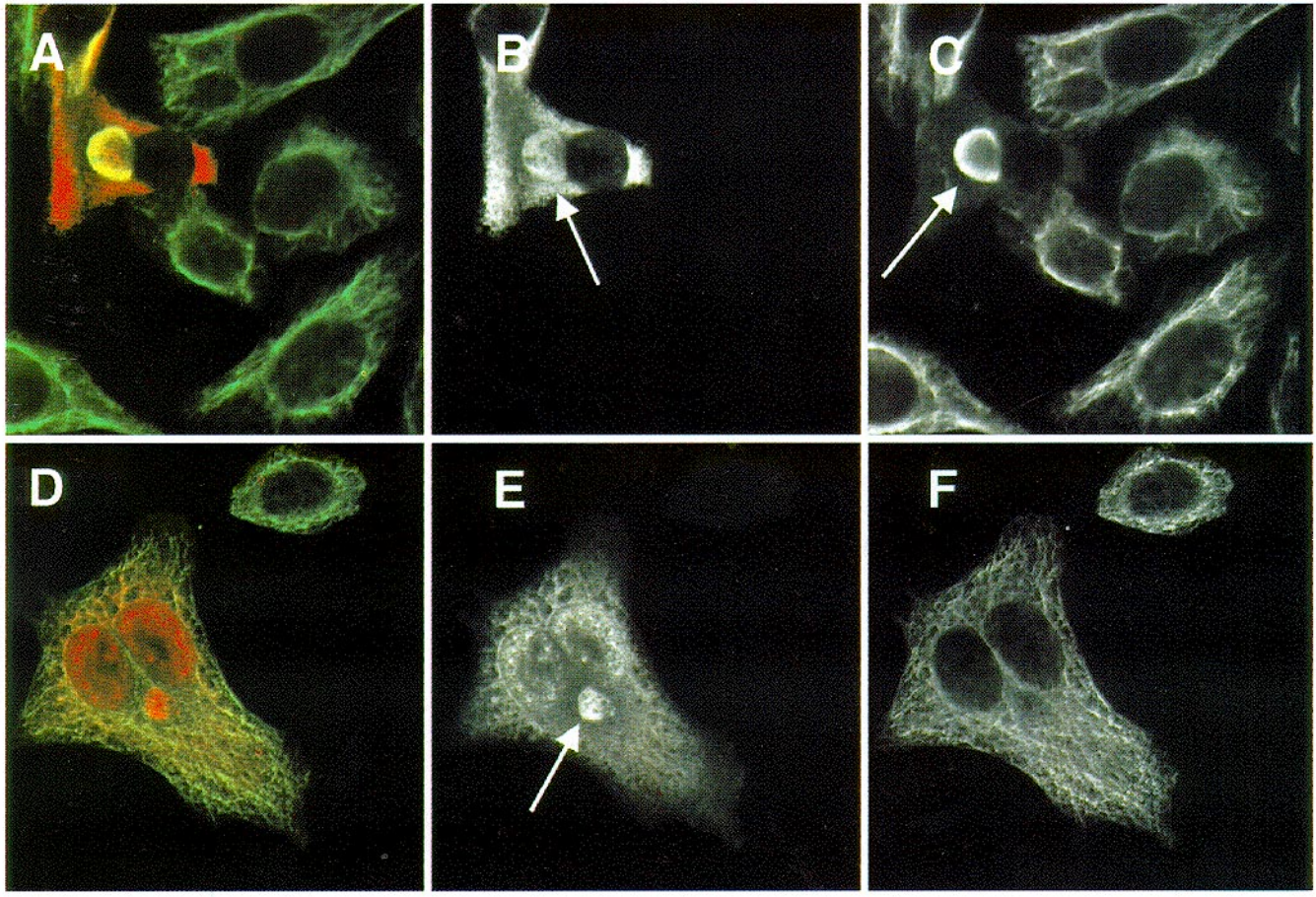
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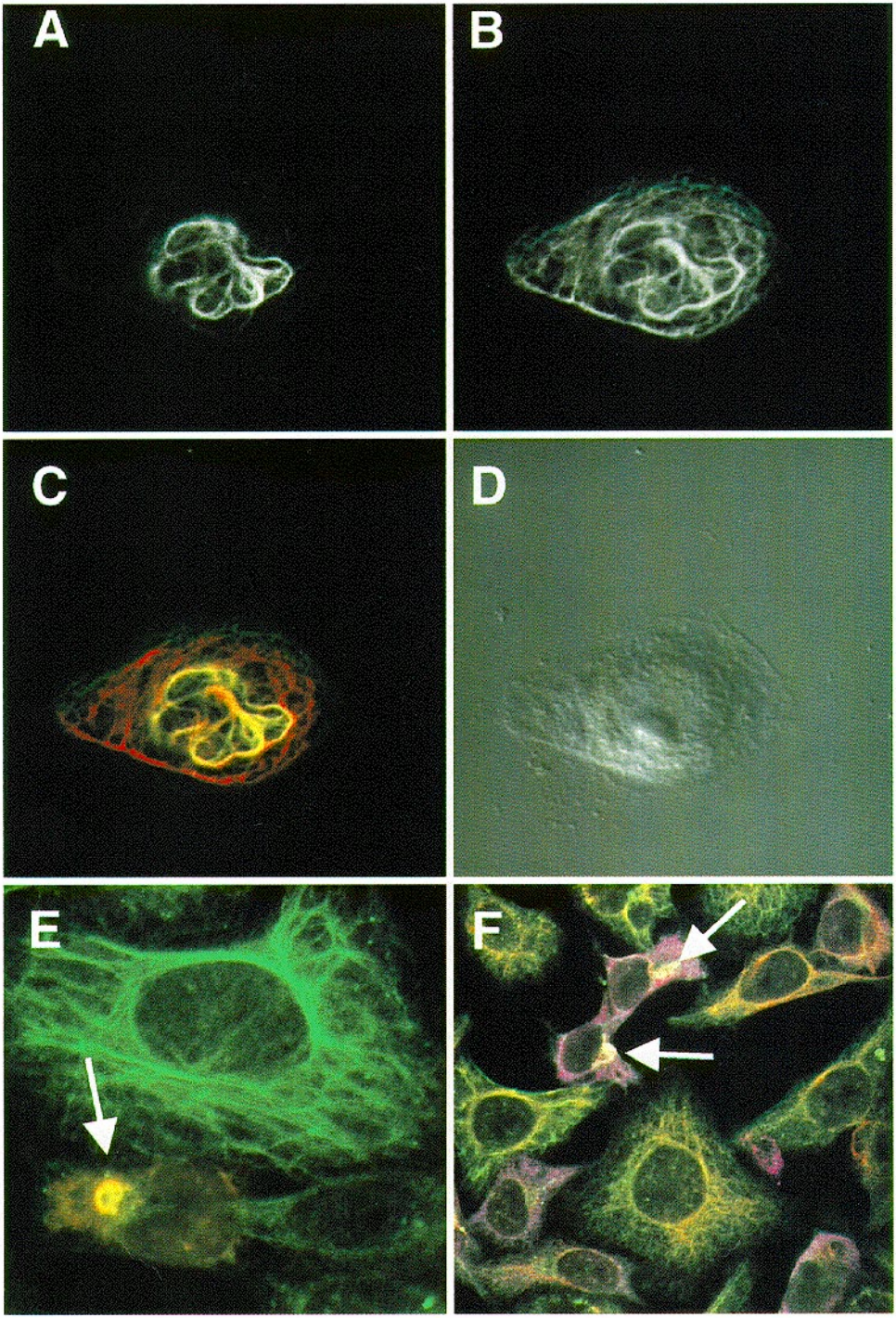
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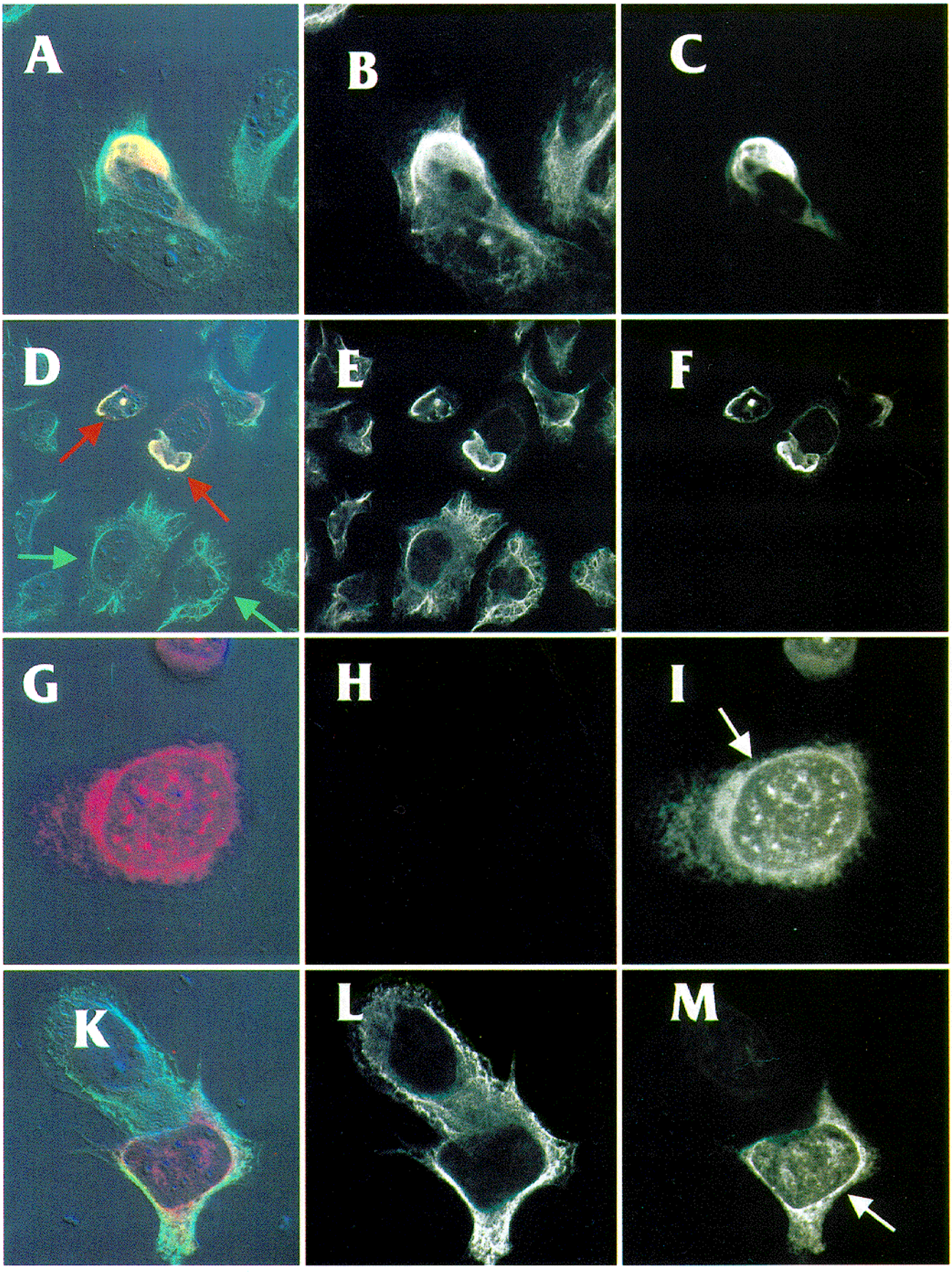
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