Functional Histone Antibody Fragments Traverse the Nuclear Envelope

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ABSTRACT Factors important in the translocation process of proteins across the nuclear membrane were studied by microinjecting either fluoresceinated nonimmune IgG and $F(ab)_2$ or the corresponding molecules, prepared from antisera to histones, into the nucleus and cytoplasm of human fibroblasts. Intact IgG from both preparations remained at the site of injection regardless of whether it was injected into the nucleus or the cytoplasm. In contrast, nonimmune $F(ab)_2$ distributed uniformly throughout the cell. The $F(ab)_2$ derived from affinity-pure antihistone moves into the nucleus after cytoplasmic injection and remains in the nucleus after nuclear microinjection. The migration of the antihistone $F(ab)_2$ into the nucleus results in inhibition of uridine incorporation in the nuclei of the microinjected cells. We conclude that non-nuclear proteins, devoid of specific signal sequences, traverse the nuclear membrane and accumulate in the nucleus provided their radius of gyration is <55Å and the nucleus contains binding sites for these molecules. These findings support the model of "quasibifunctional binding sites" as a driving force for nuclear accumulation of proteins. The results also indicate that active $F(ab)_2$ fragments, microinjected into somatic cells, can bind to their antigenic sites suggesting that microinjection of active antibody fragments can be used to study the location and function of nuclear components in living cells.

Compartmentalization of components into their proper location is an integral part of cell structure and function. The nuclear membrane divides the cell into two major compartments: the nucleus and cytoplasm. The movement of proteins across the nuclear membrane and accumulation into either the nucleus or cytoplasm seems to depend on several factors such as (*a*) the presence of "signal sequences" in the protein (2, 8–10), (*b*) the presence of nuclear or cytoplasmic binding sites for the protein (3, 12), and (*c*) the size and shape of the molecule (20). Generally, it is difficult to determine which of the above factors is the major driving force in the nuclear accumulation of nuclear proteins. We have "uncoupled" the contribution of putative signal sequences from that of nuclear binding sites by studying the intracellular migration of antihistone IgG and active fragments derived from these. Obviously, the IgG molecules do not contain signal sequences specific for nuclear accumulation. On the other hand, the nucleus contains many binding sites for antihistone antibodies (4). Furthermore, the size of the molecule can be changed by proteolytic digestion without affecting the binding site (24). Nonimmune IgG can serve as appropriate controls since they are identical to antihistone antibodies in all respects, except in their ability to bind to the nuclear chromatin.

Direct microinjection of fluoresceinated molecules into living cells with a glass microcapillary avoids possible artifacts due to cell fusion or fixation and allows immediate observation of the cellular distribution of the microinjected molecules inside living cells. Antibodies introduced into cells by capillary microinjection or by erythrocyte ghost fusion seem to retain their antigen-binding activity. For example, microinjection of the appropriate antibodies neutralized the effect of diphtheria toxin (27), prevented SV40 replication (1), inhibited serum-induced DNA synthesis (18), and caused collapse of the intermediate filament network (16). The microinjection process and introduction of antibodies into the cell does not affect the cell adversely as judged from cell viability (18, 23) and uridine incorporation (Einck and Bustin, unpublished observations).

In the present manuscript, we investigate the in vivo cellular distribution of fluoresceinated IgG and active IgG fragments specific for histones, which have been injected into either the nucleus or cytoplasm of living cells. The studies are aimed at understanding the processes regulating the migration of proteins across the nuclear membrane. In addition, we explore the possibility that antibodies can be used to localize and study the function of defined chromosomal components in

the living cell.

MATERIALS AND METHODS

Preparations of Antigens and Antibodies: The preparation of histones and antisera and the purification of the IgG fraction and affinity-pure IgG were previously described. The specificity of the antibodies was ascertained by immunofluorescence, microcomplement fixation, solid phase radioimmunoassay, enzyme-linked immunoassay, and Western blotting (4). In all cases, the antibodies reacted specifically with the appropriate immunogen. Proteins were fluoresceinated as described previously, except that the labeling was done in carbonate rather than borate buffer (5).

$F(ab)_2$ fragments from the antibodies were prepared by cleavage with pepsin (24). Pepsin (Worthington Biomedical Corp., Freehold, NJ) was added to a solution containing fluorochrome-labeled IgG at 5 mg/ml in 0.2 M acetate buffer, pH 4.0, to bring the substrate-to-enzyme ratio to 100. Digestion pro-

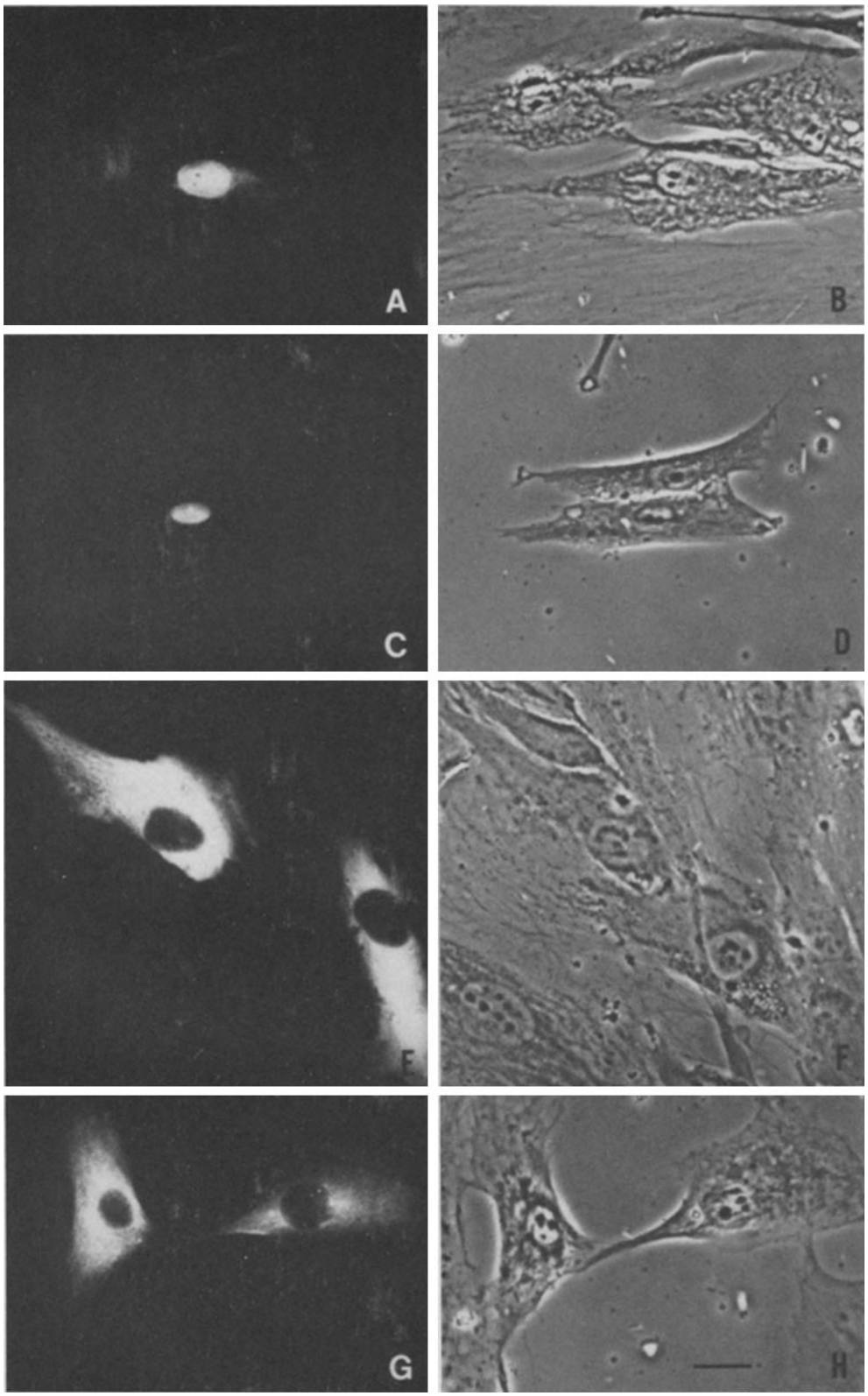

FIGURE 1 Fluoresceinated IgG molecules remain at the site of injection 3 h after injection. The IgG were injected into either the nucleus (*A–D*) or cytoplasm (*E–H*) and kept in media 3 h prior to fixing and processing for fluorescence. Bar, 20 μm. × 450.

ceeded at 37°C for 8 h. The digest was dialyzed against PBS and passed over a 2-ml column containing protein A-Sepharose. The column was equilibrated and eluted in PBS. The unadsorbed fraction contained the $F(ab)_2$ portion of the IgG. The purity of the proteins was monitored by PAGE in buffers containing SDS. The activity of the IgG and $F(ab)_2$ was monitored by an enzyme-linked immune assay (6, 11). Nonimmune IgG, $F(ab)_2$, and Fc were obtained from either Miles Laboratories Inc. (Elkhart, IN) or Cappel Laboratories Inc., (Cochranville, PA).

Cell Culture: Human fibroblasts (KD), obtained from Dr. R. S. Day (National Cancer Institute), were maintained in Dulbecco's modified Eagle medium (Gibco Laboratories, Grand Island, NY) with 10% fetal bovine serum. Cells for microinjection were passed several times without allowing them to come to confluence and then passed to microslide chambers (Titertek, Miles Laboratories Inc.) 24–72 h before injection.

Microinjection: Cells were microinjected by the Graessman technique

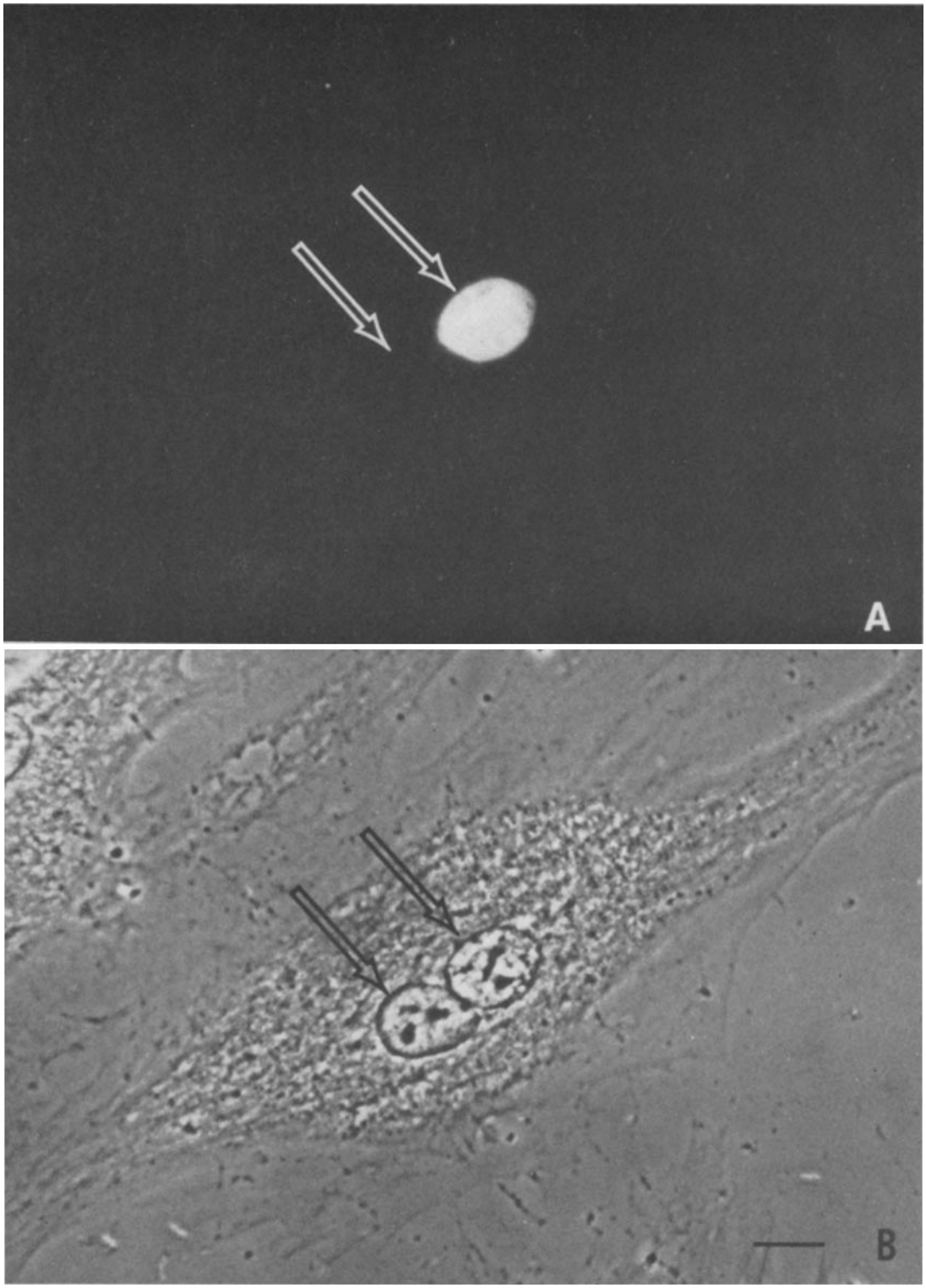

FIGURE 2 Intact IgG does not equilibrate between contiguous nuclei (arrows). One of the nuclei present in a binucleated cell has been injected with fluoresceinated IgG and photographed 3 h after injection. (*A*) Fluorescence micrograph; (*B*) phase-contrast micrograph. Bar, 20 μm. × 600.

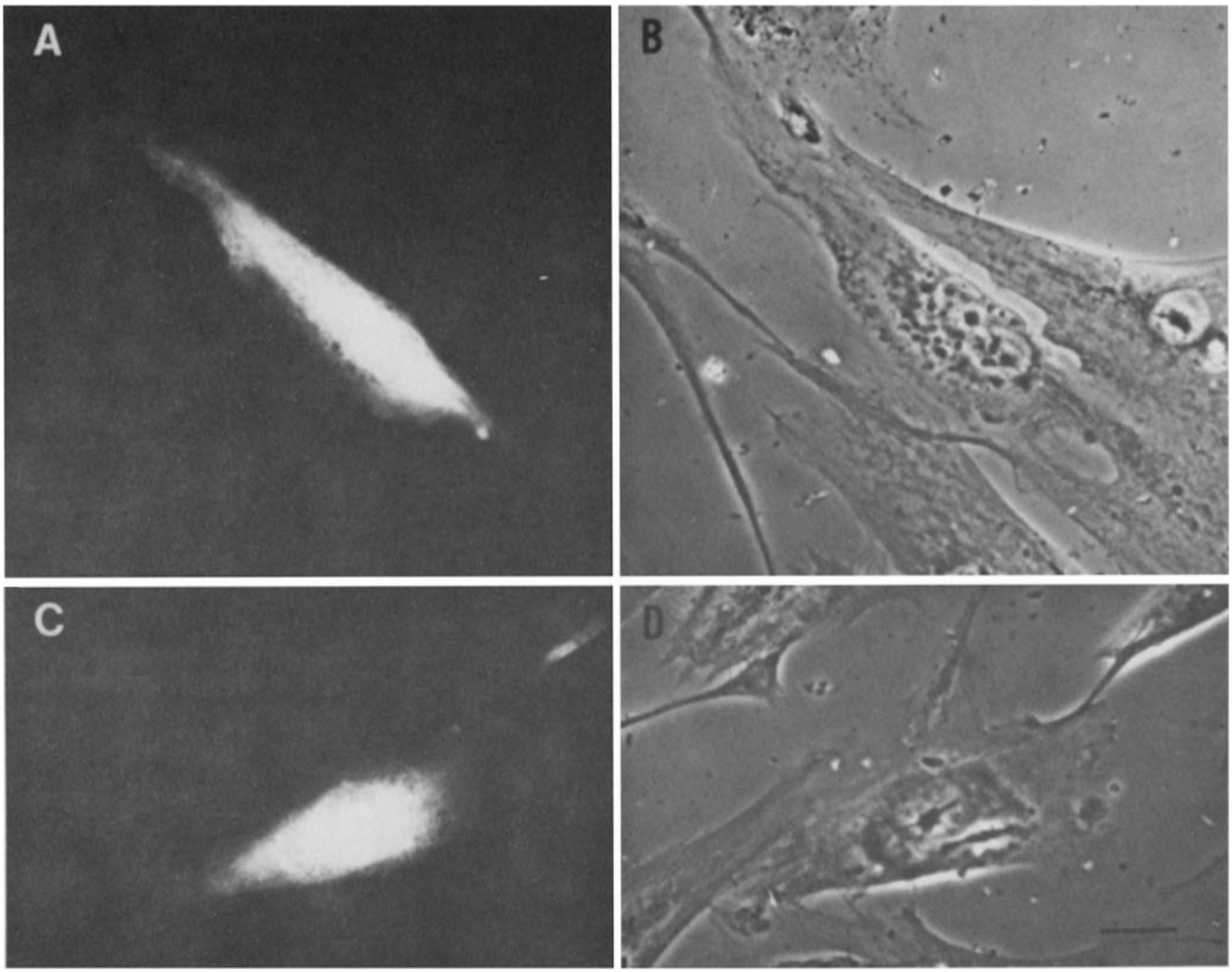

FIGURE 3 Antibody fragments can traverse the nuclear envelope. The F(ab)$_2$ fragment derived from nonimmune IgG, when injected into either the nucleus (*A* and *B*) or cytoplasm (*C* and *D*), disperses evenly throughout the cell. The equilibrium state, shown here, is reached in 30–60 min. Bar, 20 μm. × 550.

(13). Antibody was concentrated to 1–10 mg/ml in dilute phosphate buffer (0.065 M potassium phosphate buffer, pH 7.2) by Amicon pressure filtration (Amicon Corp., Danvers, MA) or in a hollow fiber. The sample was placed in a small glass capillary needle and injected into either the nucleus or cytoplasm of the cultured cells. The average volume injected into the cells was 5×10^{-11} ml.

Microscopy: Microinjection and subsequent antibody movement could be monitored by observing the cells with a Leitz inverted phase microscope equipped with ultraviolet epifluorescence, a silicon intensified target–video camera (DAGE-M.T.I. model 65 MKII) and a video monitor. The image on the monitor was photographed with a Nikon single-lens reflex camera and 55-mm macro lens using Kodak 2415 film and POTA developing. Alternatively, the cells were washed in saline, fixed in 10% formalin, at 4°C for 30 min, and photographed with a Zeiss photomicroscope III. The labeled injected molecules were photographed under epifluorescent illumination and cellular morphology was observed under phase optics.

Labeling and Autoradiography: 1 h before injection, the uridine pool was depleted by making the media 20 mM in glucosamine. Following injection, the cells were incubated in 200 μCi/ml [^{3}H]uridine in preconditioned media for 60 min. The cells were washed in Puck's saline, fixed with 10% formalin in saline for 30 min at 4°C in the dark, processed for autoradiography using Kodak NTB-3 emulsion, exposed at room temperature for 7–10 d, developed in Dektol (Kodak) 1:4, fixed, and observed in a Zeiss photomicroscope III.

RESULTS

Intact IgG Does Not Traverse the Nuclear Membrane

Initial experiments were aimed to study whether the nuclear envelope is a barrier to antibody migration. Fluoresceinated control IgG were injected into either the nucleus or the cytoplasm of KD fibroblasts. The fluorescent IgG molecules remain confined at the site of injection regardless of whether it was the nucleus or the cytoplasm. This confinement is not temporary since the IgG microinjected into either the nucleus or the cytoplasm remains compartmentalized even 3 h after injection (Fig. 1). A binucleated cell in which one of the two contiguous nuclei was microinjected is shown in Fig. 2. The IgG is contained by the nuclear membrane of the injected nucleus and does not equilibrate between the two nuclei. We conclude that the intact IgG molecule does not traverse the nuclear membrane.

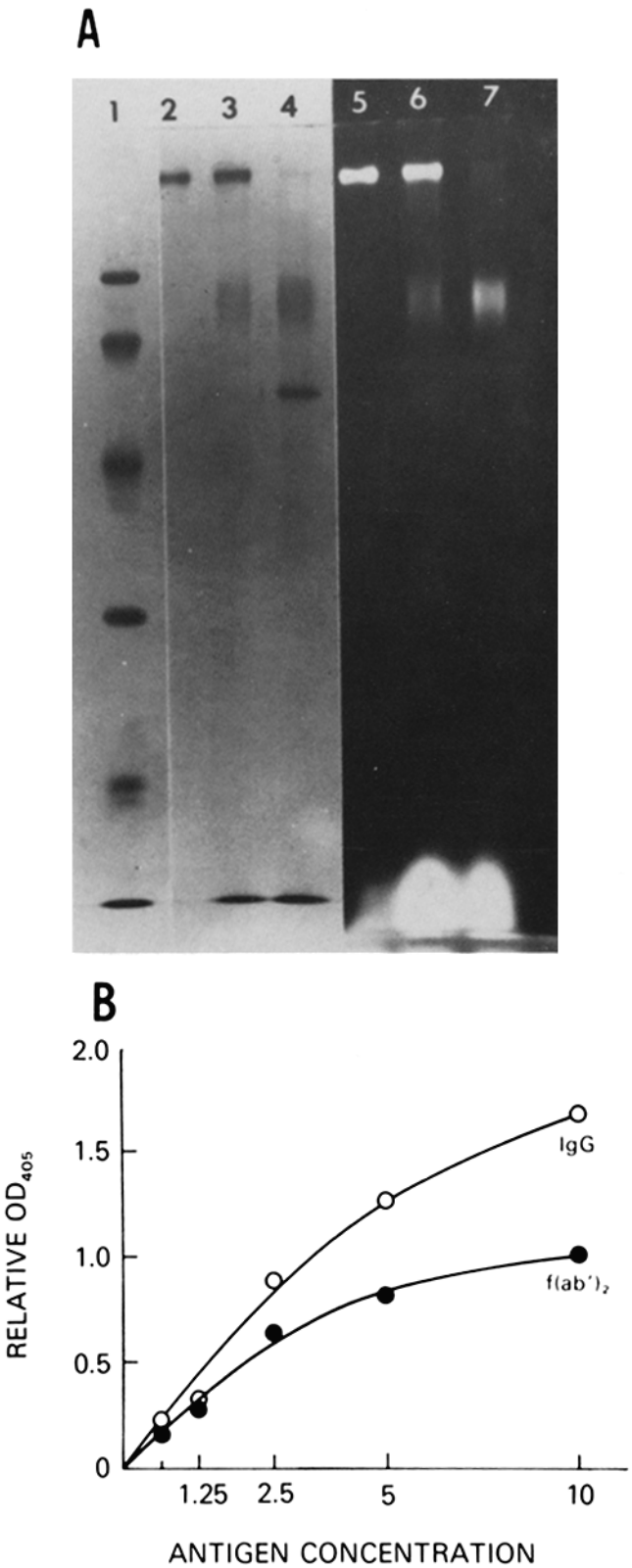

FIGURE 4 Purification of active $F(ab)_2$ fragments from antiH2A and antiH2B IgG. (*A*) lanes *1–4*, Coomassie Blue staining; lanes *5–7*, fluorescence photograph. Lane *1*, molecular weight standards: lactalbumin, 14,400; trypsin inhibitor, 20,100; carbonic anhydrase, 30,000; ovalbumin, 43,000; BSA, 67,000; and phosphorylase *b*, 94,000. Lanes *2* and *5*, affinity-purified IgG before digestion. Lanes *3* and *6*, affinity-purified IgG after mild pepsin digestion. Lanes *4* and *7*, $F(ab)_2$ after protein A purification (note unfluoresceinated carrier protein). (*B*) Enzyme-linked solid phase immunoassay showing that the pepsin-cleaved fragments retain antigen binding. Control antiserum was 0 for all antigen concentrations. Antibody dilutions were 1:500 from original serum volume. Antigen concentration is given in μg/ml.

Nonimmune $F(ab)_2$ Fragments Traverse the Nuclear Membrane

The movement of molecules across the nuclear membrane is governed either by specific signals within the molecule or by its size. Bonner has suggested that components with a radius of up to 45Å enter the nucleus rapidly while substances with a radius >45Å enter the nucleus very slowly (3). The IgG molecule is rod shaped with a radius of gyration of ~55Å (25). The $F(ab)_2$ molecule has a radius of ~35Å. To test whether the more compact $F(ab)_2$ molecule could traverse the nuclear membrane, we injected fluoresceinated $F(ab)_2$ molecules into either the nucleus or cytoplasm of KD cells. The photomicrographs presented in Fig. 3 indicate that $F(ab)_2$ molecules traverse the nuclear membrane and distribute throughout the cell within 30–60 min regardless of whether injection was made into the nucleus (Fig. 3, *A* and *B*) or cytoplasm (Fig. 3, *C* and *D*). We have tested for the possibility that the Fc protein in the intact IgG prevents its movement across the nuclear membrane by injecting fluoresceinated Fc fragments. The Fc fragments injected into the cytoplasm also diffused through the nuclear membrane. We conclude, therefore, that in the case of IgG the radius of the molecule may affect its migration across the nuclear membrane.

Functional Antihistone Antibody Fragments Concentrate in the Nuclei

The results presented in the previous section were obtained with IgG and IgG fragments derived from nonimmune sera. We next investigated whether IgG molecules with known specificity for nuclear proteins behave in a similar way. IgG fractions obtained from either antiH2B, antiH3, or antiH2A sera were conjugated with fluorescein and injected into the cytoplasm of KD cells. In all cases the fluorescence remained in the cytoplasm. Since <10% of the IgG fraction contains IgG molecules reacting with histones (M. Bustin, unpublished observation), the experiments were repeated with affinity-purified antiH2B and antiH2A. The results indicated that the affinity-purified IgG molecules remained in the cytoplasm and did not traverse the nuclear membrane.

$F(ab)_2$ fragments were generated by pepsin digestion of fluorescein-labeled, affinity-purified antiH2A and antiH3. The $F(ab)_2$ portion labeled with the fluorochrome can be separated from nondigested material by passage through columns of protein A-Sepharose (Fig. 4 *A*). The $F(ab)_2$ fragments retain their immunological activity as determined by a solid phase immunoassay (Fig. 5 *B*).

$F(ab)_2$ fragments derived from affinity-pure antihistone move into the nucleus following cytoplasm injection. The gradual translocation of the fluorescence from the cytoplasm to the nucleus is clearly seen in Fig. 6. The fluorescence, which originally was exclusively cytoplasmic (Fig. 5 *A*, 0 min), is almost fully nuclear after 30 min. The relative intensity of the nuclear fluorescence as compared with the cytoplasmic fluorescence further increases 60 min after injection. While the rate at which the antibody fragment traverses the nuclear

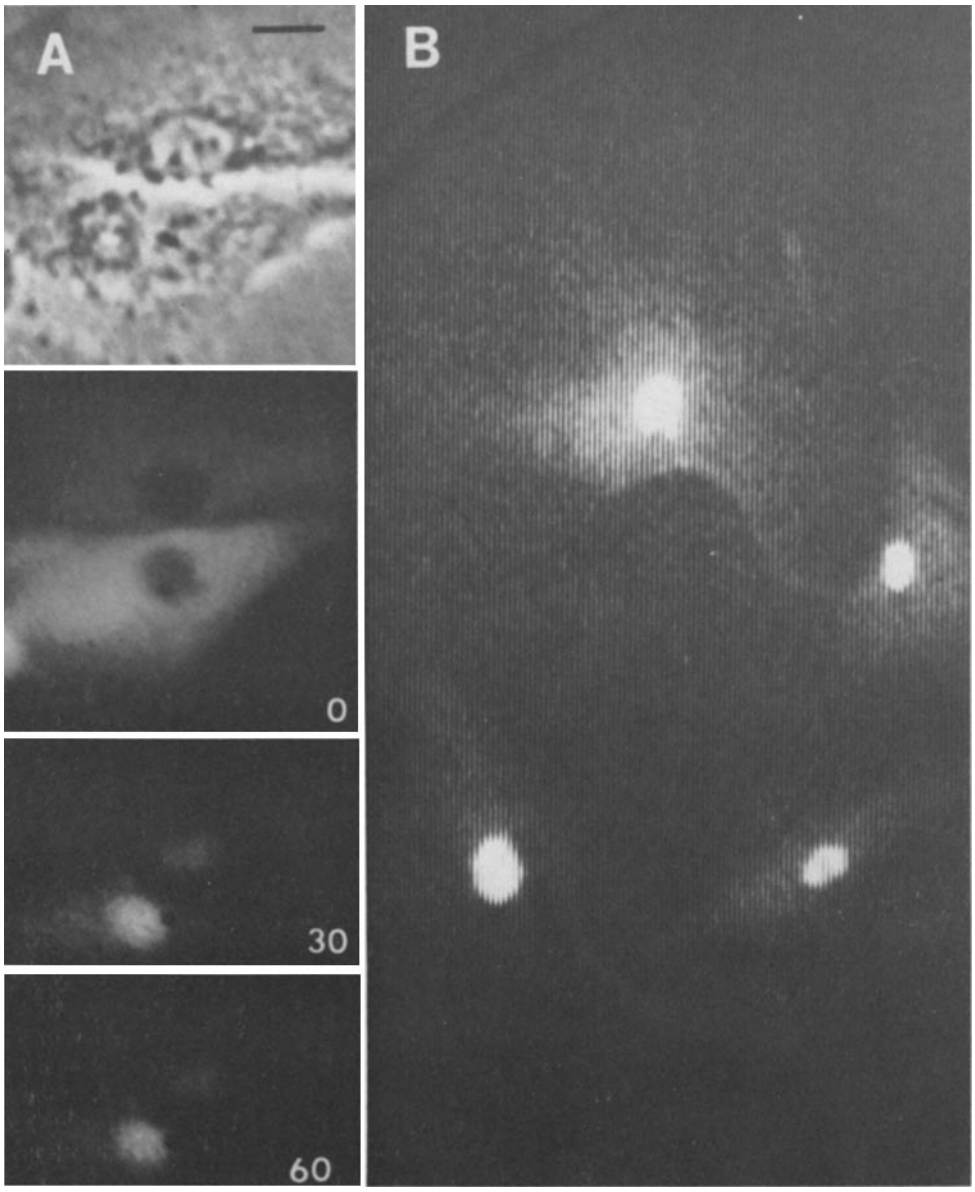

FIGURE 5 $F(ab)_2$ fragments prepared from affinity-pure antiH2A move into the nucleus following cytoplasmic injection. The antibody movement in the cells was continuously monitored with an SIT video camera (see Materials and Methods). The photomicrographs shown are the images displayed by the video camera on a video screen. (*A*) Gradual translocation of fluorescence from the cytoplasm in the nucleus. The top panel shows two cells under phase optics. The second panel shows the corresponding cells, immediately (0 min) after injection of fluoresceinated $F(ab)_2$ into the cytoplasm. Note that the nucleus is devoid of fluorescence. The third panel shows the cells 30 min after injection, and the bottom panel shows the cells 60 min after injection. (*B*) Several cells 60 min after cytoplasmic injection of fluoresceinated $F(ab)_2$ from antiH2A. Bar, 20 μm. × 500.

membrane varies among cells, eventually all the cells in the field display predominantly nuclear fluorescence (Fig. 5*B*). $F(ab)_2$ fragments injected into the nucleus remain there (data not shown). We conclude that the $F(ab)_2$ fragment can home in on the appropriate antigenic site within the living cells.

During these studies we noted that, compared with preimmune IgG and $F(ab)_2$ fragments, active antibody molecules and $F(ab)_2$ fragments have a markedly diminished half-life inside the nucleus. Approximately 5–15 min after microinjection of fluoresceinated immune molecules, the fluorescent pattern becomes very sharp. The fluorescent image then becomes less distinct and less intense over the next 1–2 h. Almost all the fluorescence is removed from the nucleus as well as the cytoplasm within 6 h after injection. In contrast, nonimmune IgG or $F(ab)_2$ can be detected in the cell even 24 h after injection.

Functional Test for $F(ab)_2$ Translocation

Microinjection of antibodies to histones into the nuclei of the oocytes in *Pleurodeles waltlii* brings about a retraction in the transcription loops in the lampbrush chromosomes present in these nuclei (22). We have found that in somatic cells, microinjection of antihistone IgG into the nuclei of cells inhibits transcription (28). Thus, antibodies can be used for studying the in vivo function of chromosomal antigens. We reasoned that if IgG cannot traverse the nuclear membrane but active $F(ab)_2$ can, then cytoplasmic injection of IgG should not inhibit transcription while cytoplasmic injection of $F(ab)_2$ should. To test this, the uridine pool in KD human fibroblasts was depleted by making the media in which the cell grew 20 mM in glucosamine. After microinjection of antibodies or $F(ab)_2$ fragment [^{3}H]uridine was added to the media and the cells were processed for autoradiography. The microinjected cells were visualized by fluorescence. Representative pictures of cells injected with various IgG preparations are shown in Fig. 6. Microinjection of fluoresceinated control IgG into the nucleus did not affect nuclear uridine incorporation in the microinjected cell (Fig. 6, *A* and *B*). In contrast, nuclear microinjection of antiH3 IgG (Fig. 6, *C* and *D*) caused a significant reduction of nuclear uridine incorporation as evidenced by the significant reduction in autoradiographic grains

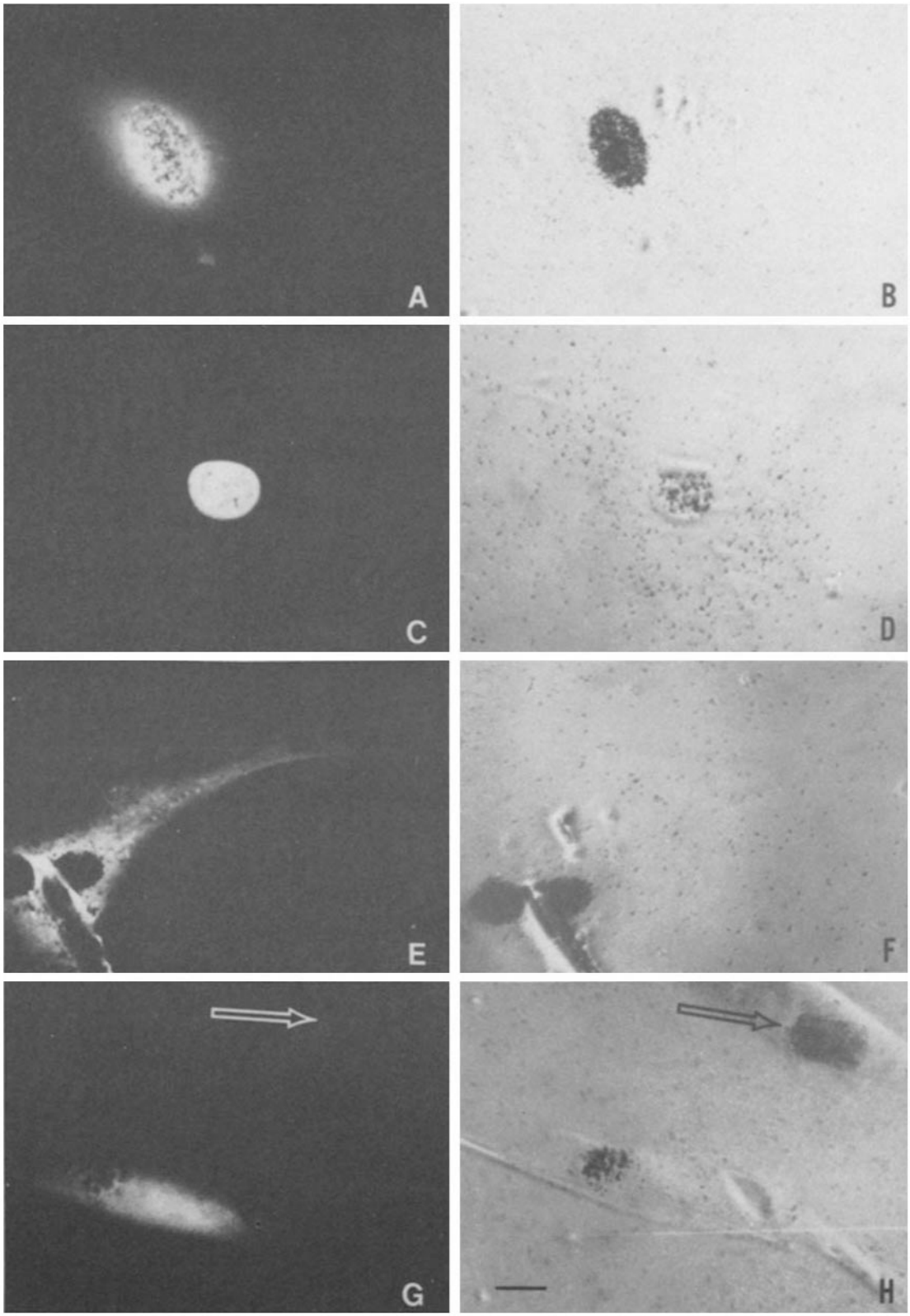

FIGURE 6 Functional test for $F(ab)_2$ migration across the nuclear membrane. $F(ab)_2$ and IgG were injected into cells in which the uridine pool was depleted. After microinjection, the cells were incubated with [^{3}H]uridine, and the relative efficiency of transcription was assessed by autoradiography. (*A* and *B*) Nonimmune IgG injected into the nucleus; (*C* and *D*) antiH3 IgG injected into the nucleus; (*E* and *F*) antiH3 IgG injected into the cytoplasm; (*G* and *H*) $F(ab)_2$ derived from antiH3 injected into the cytoplasm. Arrows in *G* and *H* point to a noninjected cell. Note the difference in nuclear [^{3}H]uridine incorporation between the injected and noninjected cell. Bar, 20 μm. × 400.

(compare Fig. 6, *B* and *D*). Similar results were obtained with other histone antibodies. Cytoplasmic microinjection of control $F(ab)_2$ did not affect uridine incorporation (Fig. 6, *E* and *F*). In contrast, cytoplasmic injection of $F(ab)_2$ derived from affinity-pure antiH3 (Fig. 6, *G* and *H*) markedly inhibited uridine incorporation. This is most obvious when the nucleus of the injected cell in Fig. 6, *G* and *H* is compared with that of a noninjected cell in the same panel (marked by arrow) and with those of the injected cells in Fig. 6, *E* and *F*.

DISCUSSION

The purpose of the experiments described was to study factors involved in translocation of proteins across the nuclear membrane and to explore the possibility that antibodies can be used to localize and study the function of nuclear components in living cells. It is not presently clear whether the nuclear membrane has a passive or an active role in nuclear translocation. Goldstein and Ko (12) presented evidence that the outer nuclear membrane has little to do with the selective accumulation and retention of nuclear proteins in the nucleus. On the other hand, evidence has been presented that the migration of proteins across the nuclear membrane is dependent upon "signal sequences", some of which are part of the primary sequence (10) and some of which are not (2, 21). Other factors relevant to protein migration into the nucleus are the size and shape of the molecule (3), the organization of the nuclear membrane during the cell cycle (3, 14), hormone treatment (19), and the presence of nuclear binding sites for the migrating molecules (3, 12).

As mentioned in the introduction, antibody molecules specific for histones are especially useful for studies on nuclear transport. Using the needle microinjection technique (13), we have introduced IgG and $F(ab)_2$ fragments into both the nucleus and the cytoplasm of the cell. The finding that nonimmune IgG do not traverse the nuclear membrane regardless of whether injected into the nucleus or the cytoplasm while $F(ab)_2$ molecules do pinpoints clearly the size of the molecules as an important factor in determining whether a molecule can cross the nuclear membrane. Considering the possibility that the fluoresceination itself may affect the cellular distribution of the injected molecules, we note that while in some cases this may be a factor, it has no influence on the migration of the IgG and IgG fragments since it has been shown (17) that nonimmune, nonfluorescent IgG does not, while Fab and Fc fragments do, migrate from the cytoplasm into the nucleus. Furthermore, antihistone IgG when injected into the cytoplasm also did not penetrate the nuclear envelope, while the $F(ab)_2$ derived from these accumulated in the nucleus did. Electron microscopy studies (25) indicated that the 150,000-dalton IgG molecule is rod shaped and ~110Å long. Thus, the radius of gyration of this molecule is ~55Å and is significantly longer than the $F(ab)_2$ fragment that has a radius of gyration of ~35Å. While other workers have also indicated that the size may be an important factor in nonspecific nuclear transport, it is possible that the fate of foreign proteins is different from that of native cellular proteins. Furthermore, IgG molecules are extracellular proteins possibly possessing signal sequences for excretion out of antibody-producing cells. Since several studies indicated that large nuclear proteins, when injected into the cytoplasm, traverse the nuclear membrane, it seems that within the cell some proteins, such as nucleoplasmin (10), indeed do contain signal sequences for nuclear transport.

The accumulation of antihistone $F(ab)_2$ in the nuclei supports the model of quasibifunctional binding sites (3, 12) as a driving force for nuclear accumulation. Bonner (3) suggested that this model applies even for molecules with a K' of $\sim 10^{-4}$ M. Specific antibodies usually bind to their antigen with a K' $>10^{-6}$ M. Assuming that the average KD nucleus contains 5 pg of DNA, and that the histone-to-DNA weight ratio is 1, it can be calculated that the nucleus contains $\sim 10^7$ molecules of a core histone. The average volume of antihistone introduced into the cell is 10^{-11} ml (13) of a solution containing 1 mg/ml $F(ab)_2$ molecules. This corresponds to $\sim 1 \times 10^6$ $F(ab)_2$ molecules injected. Obviously, there are sufficient binding sites to bind all the active $F(ab)_2$ molecules. In summary, our studies support a model indicating that non-nuclear proteins can migrate across the nuclear membrane and accumulate in the nuclei, provided their radius of gyrations is <55Å and that the nucleus contains binding sites for these molecules.

The movement of the $F(ab)_2$ across the nuclear membrane is significantly slower than the diffusion of microinjected IgG molecules throughout the cytoplasm of living cells. Fluorescence photobleaching experiments (15, 26) yielded a value of 1×10^{-8}–2×10^9 cm^2/s for the diffusion constant of labeled IgG molecules. The process of accumulation of the $F(ab)_2$ in the nucleus takes more than 30 min. It seems, therefore, that the nuclear membrane is a very effective kinetic barrier to the diffusion of large proteins. During these studies we have also noted that the immune IgG molecules are more rapidly removed from the cells than nonimmune IgG. The conformation changes in the IgG upon antigen binding, or the size of the immune complex, may trigger cellular degradative processes such as ubiquination of proteins destined to be proteolyzed (7).

The nuclear membrane serves as a barrier to IgG penetration into the nucleus not only in cells microinjected with glass microcapillary but also in cells injected by the red blood cell ghost fusion technique (17). It seems, therefore, that the results presented here may be applicable to other experimental systems. Our finding that the $F(ab)_2$ specific to a histone, when introduced into the cytoplasm, will migrate to the nucleus and inhibit transcription in a specific way, indicates that this approach will allow the study of the function of chromosomal proteins in the living cell.

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