Identification of a Novel Nuclear Domain

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Abstract. For most known nuclear domains (ND), specific functions have been identified. In this report we used murine mAbs and human autoantibodies to investigate precisely circumscribed structures 0.2–0.3 μm in diameter which appear as "nuclear dots" distributed throughout the nucleoplasm. Nuclear dots are metabolically stable and resistant to nuclease digestion and salt extraction. The localization of nuclear dots is separate from kinetochores, centromeres, sites of mRNA processing and tRNA synthesis, nuclear bodies, and chromosomes. The nuclear dots, therefore, represent a novel ND. Nuclear dots break down as cells enter metaphase and reassemble at telophase. In interphase cells, nuclear dots are frequently "paired," and some are visible as "doublets" when stained with one particular antiserum. The number of dot doublets increased when quiescent cells were stimulated with serum although the total number of dots did not change substantially. One of the antigens was identified as a protein with a molecular mass of ~55 kD showing three charge isomers in the pI range of 7.4 to 7.7. Autoantibodies affinity purified from this nuclear dot protein (NDP-55) show nuclear dots exclusively. Nuclear dot-negative rat liver parenchymal cells became positive after chemical hepatectomy, suggesting involvement of the NDP-55 in the proliferative state of cells.

MORPHOLOGICAL studies have shown the nucleus to be composed of structural domains involved in various aspects of the major functions of the nucleus, transcription and replication (Bouteille et al., 1974; Hancock and Boulikas, 1982; Ringertz et al., 1986; Newport and Forbes, 1987; Fu and Maniatis, 1990; Spector, 1990). Different nuclear domains (ND)[1] have been identified from a structural and functional perspective. The nuclear membrane and the nucleolus, for example, represent nuclear landmarks at a gross morphological level. The structure and function of other domains are less well understood, partly because functional compartmentalization, with the exception of the nucleolus, is not as apparent within the nucleus as it is in the cytoplasm. However, the interphase nucleus appears to be organized into domains occupied by individual chromosomes (Agard and Sedat, 1983; Mannuelides, 1985) and RNP complexes with associated RNA-processing activities (Lerner et al., 1981; Reuter et al., 1984; Nyman et al., 1986; Ringertz et al., 1986; Gall and Callan, 1989; Piñol-Roma et al., 1989). Less defined border regions of perichromatin and perinucleolar chromatin have been described as well as fibrillar nucleoplasmic structures of uncertain function called nuclear bodies (Bouteille et al., 1974; LeGoascogne and Baulieu, 1977; Vagner-Capodano et al., 1982; Chaly et al., 1983*a*,*b*). Recent studies have described nuclear bodies as a heterogeneous group of nuclear inclusions 0.2–1.2 μm in diameter, which appear as fibrous granules encapsulated by a proteinaceous shell present only in activated cells (Padykula et al., 1981; Fitzgerald and Padykula, 1983; Brasch et al., 1989; Chaly et al., 1989).

Some of the known details of nuclear substructure and morphology were obtained using immunological methods. For instance, immunological probes were used to determine that the nuclear envelope was composed of lamins that undergo cell cycle-dependent phosphorylation (Gerace and Blobel, 1980), and that the nucleolus has fibrillogranular and "fibrillar center" components containing fibrillarin and RNA polymerase I, respectively (Scheer and Rose, 1984; Bouvier et al., 1985; Reimer et al., 1988). Antibody staining of mammalian cells was used to identify centers of mRNA processing (RNP antigen) and tRNA synthesis (La antigen), which showed a coarsely speckled nuclear immunofluorescence pattern (Sharp et al., 1976; Billings et al., 1982; Rinke and Steitz, 1982; Petersson et al., 1984; Stefano, 1984; Habets et al., 1985; Nyman et al., 1986; Carmo-Fonseca et al., 1989; Fu and Maniatis, 1990). The nucleolar (rRNA), RNP (mRNA), and La (tRNA) domains relate to the transcriptional duties of RNA polymerase I, II, and III, respectively.

Similarly, sites of DNA replication were detected using antibodies to nucleotides incorporated into newly synthesized chromatin (Nakayasu and Berezney, 1989; Mills et al., 1989) or with antibodies to proteins involved in the replication process such as proliferating cell nuclear antigen (Bravo and MacDonald-Bravo, 1987). Using these methods, several hundred discrete granular sites of DNA replication were identified as cells traverse the S phase.

1. *Abbreviations used in this paper*: KM, Kern matrix; ND, nuclear domain; NDP, nuclear dot protein.

The coarsely speckled nuclear RNP immunofluorescence patterns differ recognizably from the staining pattern of centromeric antigens which detect individual centromeres, or kinetochores in interphase nuclei. These sites are often paired, and match the number of chromosomes (Brenner et al., 1981; Brinkley et al., 1984). The kinetochore domains recognized by antibodies to three kinetochore polypeptides (Earnshaw and Rothfield, 1985) are present in both interphase and mitotic cells (Earnshaw et al., 1987; Kingwell and Rattner, 1987; Palmer et al., 1987), although the kinetochore is functional only during mitosis. Telomeres, present at the natural ends of eukaryotic chromosomes, contain a reiterated GC-rich terminal nucleotide sequence and may have terminal transferase activity (Gottschling and Zakian, 1986; Cech, 1988; Morin, 1989). The telomeric domain has been shown on each human chromosome through in vivo hybridization with a complimentary biotinylated oligonucleotide probe (Moyzis et al., 1988). Cytologically, telomeres are positioned at the nuclear envelope in interphase cells and are present in prophase cells in pairs corresponding to telomeric sequences on sister chromatids (Blackburn and Szostak, 1984).

In this study, the immunocytochemical and immunochemical reactivities of human autoantibodies showing a discrete punctate nuclear dot pattern were investigated. We compared the distribution patterns between nuclear dot antigens and several of the known nuclear components described above to determine if protein immunofluorescence patterns overlap, and thus belong to the same functional domain. Our studies were facilitated by using two mAbs showing the nuclear dot pattern and by using other specific autoantibodies and mAbs to label known NDs as reference points throughout the nucleus.

Materials and Methods

Antibodies

Over 1,700 different human autoimmune sera were screened by immunofluorescence microscopy for autoantibodies that recognized a nuclear dot pattern. Several sera were selected for further studies, specifically, serum 455 (lupoid carcinoma), and serum 1157 (primary biliary cirrhosis). Additional autoantibodies are present in these sera showing centriolar (serum 455) and nuclear rim, centriolar, and mitochondrial (serum 1157) staining patterns. Autoantibodies present in serum 455 have been partially characterized (Freundlich et al., 1988). Two mAbs were used that recognized nuclear dot patterns similar to the autoantibodies. One mAb 1150, an IgG, was derived from a human lymphocyte antigen; the other mAb 138, an IgM, was generated from a β-galactosidase fusion protein selected by screening a human cDNA expression library with autoantibodies. Antibodies from various sources were used to determine any colocalization of known ND and nuclear dots, including autoantibodies recognizing CENP-A, B, and C polypeptides, an mAb recognizing nucleolar fibrillarin, an mAb and autoantibodies recognizing RNP components, and an mAb recognizing the La antigen. Antisera were titered by sequential dilution to a useful range of 1:500 to 1:2,000 before use.

Cell Culture

Stock cultures of HEp2 (human epidermoid carcinoma, larynx), WI38 (human diploid, lung), 3T3 (Balb/c mouse, fibroblast), Indian muntjac (fibroblast, skin), and explanted chicken embryo fibroblast cells were maintained in MEM supplemented with 10% (vol/vol) FCS. MCF-7 (human adenocarcinoma, breast) and MDA-MD231 (human adenocarcinoma, breast) cells were maintained in MEM supplemented with non-essential amino acids, sodium pyruvate, and 10% (vol/vol) FCS. All cells were grown in a humidified atmosphere of 5% CO_2 and 95% air. In certain experiments, 0.25–25 μg/ml cycloheximide, 15 μg/ml puromycin, or 2–25 μg/ml α-amanitin were added to growing cultures of WI38 or HEp2 cells for varying lengths of time to inhibit protein or RNA synthesis, respectively, as described by Jacob (1973).

WI38 cells were seeded at low density (1×10^4 cells/cm^2) onto sterile 12-mm glass coverslips placed in 24-well plates (Falcon Labware, Oxnard, CA). After 2 d the cells were rinsed three times with serum-free medium and were then deprived of serum for 8 d. We replenished the serum of the quiescent cells according to a staggered time series. Under these conditions DNA synthesis began 15–18 h after the addition of serum (Phillips and Cristofalo, 1981).

Cryofixation of Tissue

Sections of liver from male Wistar rats were excised, immersed immediately in OCT compound (no. 4583; Tissue-TEK, Miles Laboratories, Inc., Elkhardt, IL), and frozen in liquid nitrogen for cryosectioning. In certain experiments male rats weighing ~300 g received a single intragastric dose of CCl_4 at 0.25 ml/100 g body wt 48 h before killing to induce liver regeneration (Smuckler et al., 1976; Tournier et al., 1988). The CCl_4 was mixed with an equal volume of corn oil before gavage. Control animals received an equivalent volume of corn oil. Water was supplied ad lib.

Immunofluorescence Microscopy

Nuclear antigen localization was determined in cultured cells grown on glass coverslips or tissue sections deposited onto glass coverslips after cells were fixed for 5 min on ice with freshly prepared 1% (wt/vol) paraformaldehyde in PBS, pH 7.4. After three washes with PBS, cells were permeabilized for 20 min on ice with 0.2% (vol/vol) Triton X-100 (Sigma Chemical Co., St. Louis, MO) in PBS. Permeabilized cells were allowed to react with titered autoimmune sera or mAb diluted in PBS for 1 h at room temperature. Avidin-fluorescein or avidin-Texas Red were complexed with primary antibodies through biotinylated secondary antibodies (Vector Laboratories, Burlingame, CA). Goat anti-human IgG conjugated with fluorescein was used in double label immunofluorescence experiments. Cells were stained with 0.5 μg/ml bis-benzimide (Hoechst 33258; American Hoechst, San Diego, CA) in PBS to visualize DNA before mounting using Fluoromount G (Fisher Scientific, Springfield, NJ). Fluorescence images were recorded using a Nikon Optiphot with a Plan 100× objective. Blue excitation for fluorescein, green excitation for Texas Red, and violet excitation for bis-benzimide were used. P3200 black-and-white print film or EES P800/1600 color reversal film (Eastman Kodak Co., Rochester, NY) were used for photography.

In Situ Cell Fractionation

HEp2 cells grown on glass coverslips were extracted under varying conditions or digested with enzymes to determine biochemical properties of the nuclear antigens and to develop a strategy for the enrichment of nuclear proteins for immunoblotting. In situ cell fractionation was used because immunofluorescence was the only assay for nuclear dots. The method of Staufenbiel and Deppert (1984) was modified resulting in a "nuclear matrix" preparation devoid of DNA, RNA, and soluble nuclear proteins. Briefly, cells were washed three times at 4°C for 15 min with Kern matrix (KM) buffer (10 mM MES, pH 6.2, 10 mM NaCl, 1.5 mM $MgCl_2$, 0.5 μM PMSF, 10% (vol/vol) glycerol) to affix the cells to glass coverslips. No other fixation was used. All reactions were subsequent to this pretreatment. The immunofluorescence assay was used after each step to assess the effects of treatments on nuclear dots, and other nuclear components such as RNP, histones, kinetochores, and nucleolar antigens. After pretreatment, cells were washed twice (3 and 27 min) with KM buffer containing 1% (vol/vol) NP-40 at 4°C, with gentle shaking. Washes with KM buffer as before to remove detergent preceded digestions with nucleases. Nuclease digestions (30 min at 25°C) were carried out in 24-well plates (Falcon Labware) (2 ml vol) using RNase A (50 μg/ml), DNase I (0.2 μg/ml), and micrococcal nuclease (1.0 μg/ml) (Gibco Bethesda Research Laboratories, Gaithersburg, MD). After nuclease digestions, cells were washed three times with KM buffer containing 2.0 M NaCl at 4°C for 15 min per wash with gentle shaking, followed by three washes with KM buffer as before.

Protein Analysis

Preparations of residual nuclear proteins enriched for the nuclear dot antigen were collected by washing flasks with 5 ml of PBS buffer containing 0.1% (wt/vol) SDS and 0.5 μM PMSF, then dialyzed exhaustively against

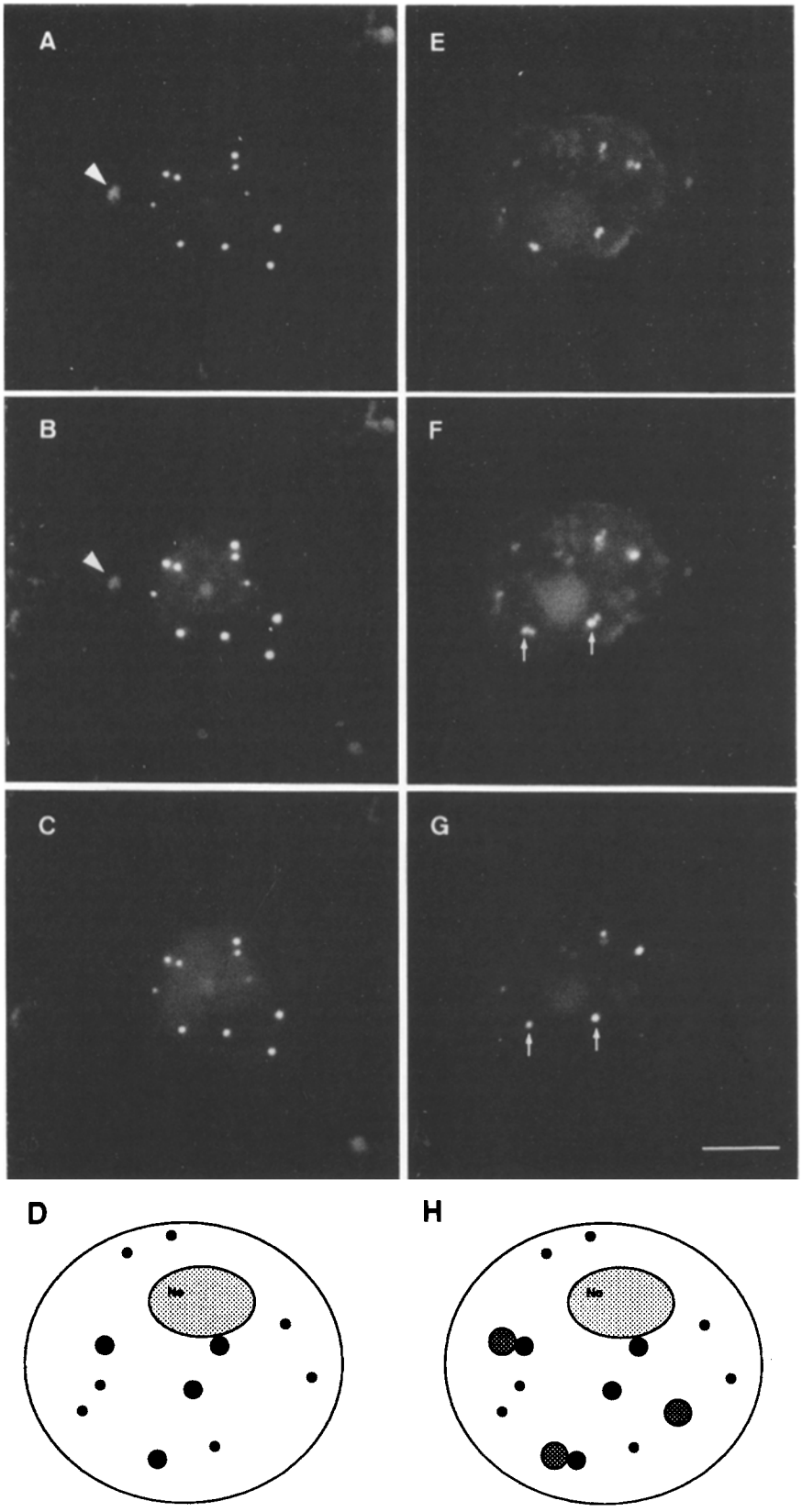

Figure 1. The relationship between autoantibodies in serum 455, serum 1157, and mAb 1150 is shown by single and double exposures of HEp2 cells after double-immunofluorescence labeling with FITC or Texas Red conjugated antibodies. In *A* and *C*, the staining patterns of FITC-labeled autoantibodies in serum 455 and Texas Red–labeled mAb 1150 are shown as single exposures, respectively. In *B*, a double exposure of these two staining patterns shows that the nuclear dots overlap precisely. Additional autoantibodies in serum 455 stain the centrioles which are marked with arrowheads. In *E* and *G*, the staining patterns of FITC-labeled autoantibodies in serum 1157 and Texas Red–labeled mAb 1150 are shown as single exposures, respectively. Autoantibodies in serum 1157 stain several doublet dots (2 contiguous dots). In *F*, a double exposure of these two staining patterns shows that the mAb 1150 recognizes only one of the two nuclear dots which constitute the doublet (arrows in *F* and *G*). In *D* and *H*, a diagram of the nucleus shows the approximate number and spatial distribution of these NDs. In *D* the schematic staining pattern of serum 455 or mAb 1150 is shown. These structures vary in size and frequently appear as pairs. In *H*, the schematic staining pattern of serum 1157 is shown. Bar, 5 μm.

0.1 M ammonium carbonate containing 0.01% (wt/vol) SDS and 0.5 μM PMSF, and lyophilized. Proteins separated on 10% SDS-polyacrylamide gels (Laemmli, 1970) were transferred to 0.22-μm nitrocellulose membrane (Towbin et al., 1979). Biotinylated molecular weight markers (Bio-Rad Diagnostics, Richmond, CA) were coelectrophoresed for size determinations. Nitrocellulose sheets were cut into parallel strips before probing with autoimmune sera or mAbs. Horseradish peroxidase (Vectastain ABC kit; Vector Labs) was used to visualize the immune complex.

Table I. Frequency of Nuclear Dots in Proliferating Human Cells in Vitro

Cells	Serum	*n*	Dots ± SD	Range
HEp2	455	30	12.2 ± 3.2	7–21
	1157	30	14.5 ± 3.5	6–28
	79	30	57.0 ± 8.4	37–85
WI38	455	40	10.1 ± 4.2	4–24
	1157	40	12.9 ± 3.0	0–32
Fibroblast	455	50	26.4 ± 4.1	6–40
Keratinocyte	455	50	8.5 ± 2.9	4–17
Melanocyte	455	50	14.9 ± 4.4	6–23
MCF-7 (−E2)	455	100	14.6 ± 4.4	4–40
MCF-7 (+E2)	455	100	15.7 ± 4.2	6–35
MDA-MB-231 (−E2)	455	100	12.8 ± 4.5	4–40
MDA-MB-231 (+E2)	455	100	12.4 ± 4.0	7–33

Serum 455 stains single nuclear dots; serum 1157 stains the same dots but in addition a variable number of dot doublets; serum 79 stains the kinetochore of each chromosome. The hormone-sensitive MCF-7 and hormone-insensitive MDA-MB-231 cell lines were stained with serum 455 after 48 h of growth in the presence and absence of 17-hydroxy-β-estradiol (E2).

Two-dimensional electrophoresis of residual nuclear proteins was performed according to the method of O'Farrell (1975). Both equilibrium and nonequilibrium buffer systems were used to determine isoelectric points. Proteins separated in ampholines ranging from pH 8 to pH 2 followed by 10% SDS-PAGE, were then transferred to nitrocellulose for immunoblotting. pH gradients were determined by measurements of gel slices and by the position of proteins with known isoelectric points (Dagenais et al., 1984). In other experiments, the apparent migration of the antigen was determined under reducing and nonreducing sample buffer conditions using diagonal electrophoresis (Maul et al., 1984).

Affinity purification of antibodies against a 55-kD antigen was performed according to the method of Smith and Fisher (1984). As a control, immunoglobulin was extracted from an area of the same nitrocellulose sheet that showed no specific band.

Results

We have observed that autoantibodies in selected human autoimmune sera show a discrete punctate nuclear dot immunofluorescence pattern in proliferating cells. Autoantibodies in serum 455 (Fig. 1 *A*) recognize 7–21 single dots per nucleus in HEp2 cells with an average value of 12.2 ± 3.2. These autoantibodies label the same structure as the mAb 1150 as shown by double labeling (Fig. 1, *A–C*). Autoantibodies in serum 1157 (Fig. 1 *E*) recognize 6–28 dots per nucleus in HEp2 cells with an average value of 14.5 ± 3.5. Of these dots, 0–6 are present per cell as doublets (2 contiguous dots). Only one of these doublet dots is recognized by the mAbs (Fig. 1, *E–G*), indicating a defined subpopulation of nuclear dots without the major nuclear dot antigen. The doublet dots are often unequal in size. A schematic of these staining patterns is shown in Fig. 1, *D* and *H*, respectively. Nuclear dots were found in human, monkey, mouse, rat, Indian muntjac, frog, and chicken cells. Table I shows the number of nuclear dots recognized in several human cell lines differing in morphology, ploidy, origin (tumor-derived or primary explant) and hormone sensitivity. When compared with kinetochores (serum 79), a much lower number of nuclear dots was counted in cells stained with antibodies recognizing the nuclear dot pattern. The wide numerical distribution of dots per cell in exponentially growing cells may be due to cell cycle variation.

Serum-deprived WI38 cells, arrested in G_0-phase and stained with serum 455 had the same large range of nuclear dots between individual cells. After refeeding (i.e., after the reinitiation of the cell cycle; Phillips and Cristofalo, 1981), no significant change in the number of dots at hours 0, 6, 18, or 24 after serum replenishment was observed. However, changes in the immunofluorescence pattern of cells stained with serum 1157 were noted. In serum deprived cells, only 0–5 (1.2 ± 1.2) double dots per cell were counted (Fig. 2 *A*), whereas after 18 h 0–9 double dots (3.2 + 1.8) were counted per nucleus (Fig. 2 *B*). The change in the appearance of cells stained with serum 1157 from mostly single dots in the 0-h control to single and double nuclear dot staining pattern 18 h after serum stimulation is significant ($p < 0.01$) and seems to correspond with reinitiation of the cell cycle.

By double-labeling cells with nuclear dot antibodies and antibodies to known NDs that have appearances somewhat similar to nuclear dots, we investigated the relative position

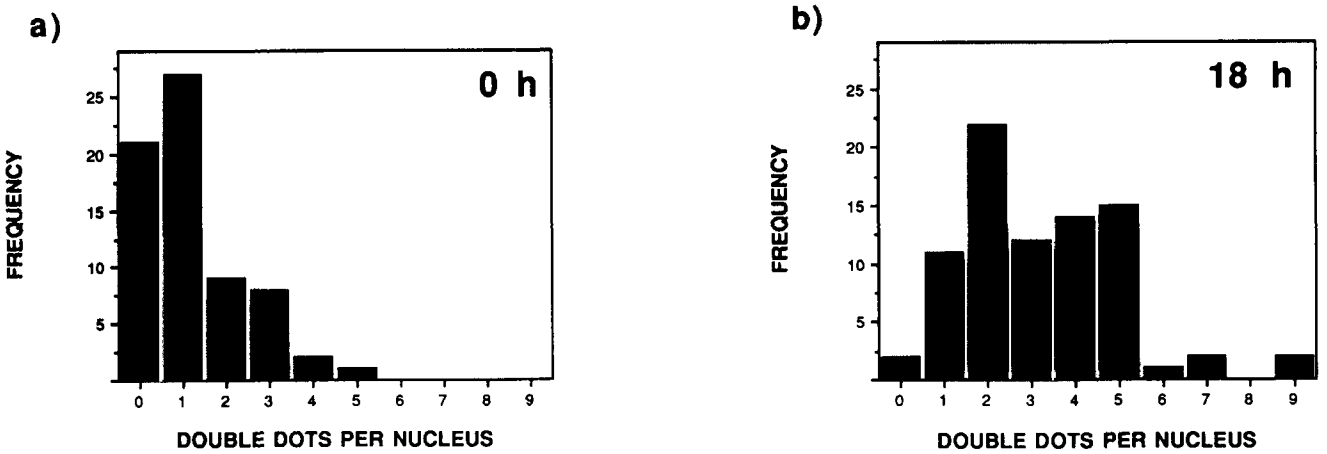

Figure 2. Histogram showing an apparent increase in the number of double dots present in populations of WI38 cells. Nuclear dots in WI38 cell cultures starved of serum for 8 d, and WI38 cell cultures refed serum, were counted in 100 cells. The ordinate shows the number of cells observed. The abscissa shows the number of double dots visible within the nucleus. In *A*, a histogram showing the distribution of dot doublets counted for the 0-h control is shown. In *B*, a histogram showing the distribution of dot doublets counted for cells 18 h after serum replenishment is shown.

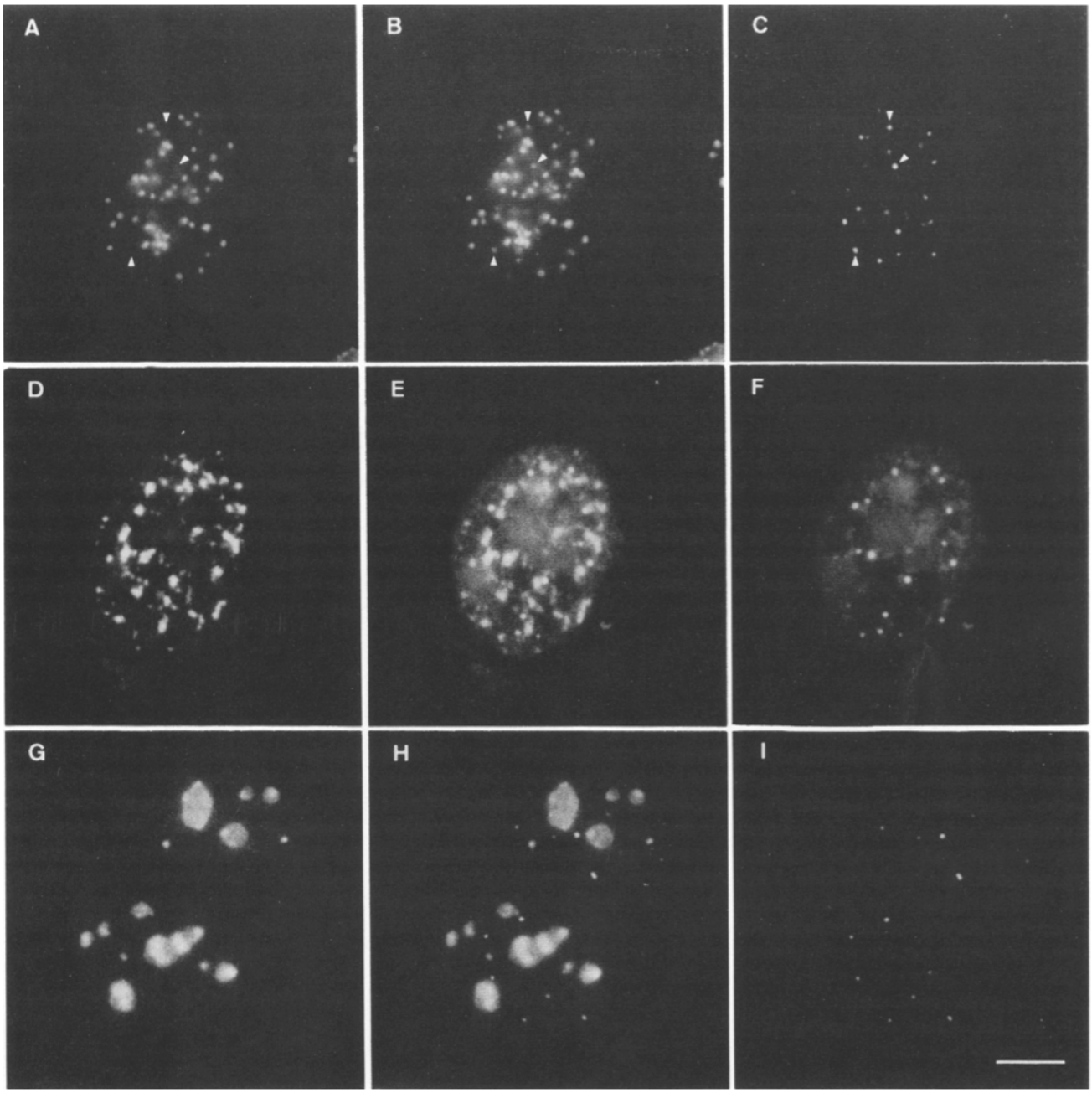

Figure 3. Single and double exposures of interphase HEp2 cells after double label immunofluorescence staining with antibodies that recognize nuclear dots and antibodies that recognize known NDs. In *A* and *C*, staining patterns of FITC labeled autoantibodies against kinetochores and Texas Red labeled mAb 1150 are shown as single exposures, respectively. In *B*, a double exposure of these two staining patterns indicates that the staining of nuclear dots (*arrowheads*) does not superimpose with kinetochore staining. In *D* and *F*, staining patterns of Texas Red–labeled mAb 90–20 (anti-RNP) and FITC-labeled autoantibodies in serum 455 are shown as single exposures, respectively. In *E*, a double exposure of these two staining patterns indicates that the immunofluorescence patterns of nuclear dots and the RNP domain do not superimpose, but often appear to juxtapose. In *G* and *I*, staining patterns of Texas Red–labeled mAb 364–5 (anti-nucleolar) and FITC labeled autoantibodies in serum 455 are shown as single exposures, respectively. In *H*, a double exposure of these two staining patterns indicates that the immunofluorescence pattern of nuclear dots and the nucleolar domain are distinct. Bar, 5 μm.

of these structures to each other. Nuclear dots and kinetochores do not coincide (Fig. 3, *A–C*). This was particularly apparent in color photographs and in areas of low kinetochore staining (*arrowheads*). The dissimilarity was even more apparent on Indian muntjac cells because of the low chromosome number and nature of kinetochores in this species (Brinkley et al., 1984). In Indian muntjac cells the nuclear dot antigen appeared throughout the nucleoplasm, whereas kinetochores were clustered in one specific area in the nucleoplasm (not shown). Since the mitotic distribution and disappearance of the nuclear dot pattern was reminiscent of those recorded for RNP, we double-labeled cells with antibodies recognizing the domains of RNA polymerase I, II, and III products. Double staining of interphase HEp2 cells

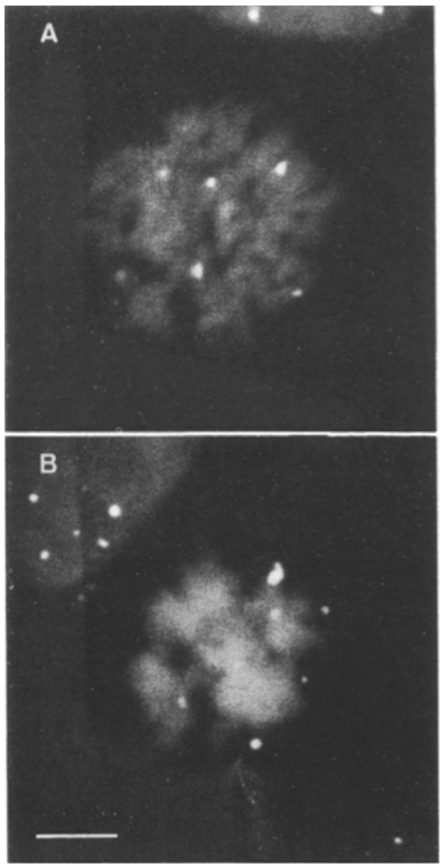

Figure 4. Localization of nuclear dot structures in prophase HEp2 cells by double exposure of antibodies conjugated to FITC and bis-benzimide stained chromatin. Antigens recognized by FITC labeled autoantibodies in serum 455, are localized in (*A*) early prophase and (*B*) late prophase by a double exposure with bis-benzimide fluorescence. Bar, 5 μm.

with RNP antibodies (mAb 90–20) and the nuclear dot autoantibodies in serum 455 showed that nuclear dots do not coincide with, although often are positioned directly adjacent to, the strongly labeled RNP domain (Fig. 3, *D–F*). When cells were stained with mAb 364–5 reacting with fibrillarin and serum 455, we observed that the nuclear dot antigen and the nucleolar domain, especially the smaller sized nucleoli, do not coincide (Fig. 3, *G–I*). Also, the pattern of cells stained by an mAb against the La antigen and serum 455 did not coincide, suggesting that the nuclear dot antigen does not correlate with tRNA or 5S RNA synthesis (not shown).

Because the nuclear dot structures frequently appeared as pairs or doublets (see Fig. 1 *A* and *E*), we assumed a chromosomal location either as specialized kinetochores, centromeres, or telomeres. However, the immunofluorescence patterns of mitotic cells stained with nuclear dot antibodies and kinetochore antibodies showed an obvious difference in localization. Antigens recognized by serum 455 antibodies were present as discrete nuclear dots dispersed throughout the nucleoplasm in early prophase (double exposure with bis-benzimide-stained chromatin; Fig. 4 *A*), but by late prophase (Fig. 4 *B*) appeared close to the periphery of the chromosomes. We also noted on prophase cells that the nuclear dot antigens are often seen adjacent to areas without bis-benzimide staining. The number of nuclear dot structures during prophase was four to eight per cell. No staining was observed on metaphase or anaphase cells, but by late telophase, the structures reappeared in the nucleus.

To test whether protein or RNA synthesis inhibitors would affect the number of nuclear dots, cultures of HEp2 cells and WI38 cells were exposed from 0 to 24 h to varying concentrations of cycloheximide, puromycin, or α-amanitin. After exposure to 15 μg/ml puromycin, no effects were noted. However, ~30% of cells treated with cycloheximide (concentrations as low as 0.25 μg/ml) showed an altered immunofluorescence pattern, which appeared as a pattern of more and much finer dots (not shown). No effects were noted when WI38 cells were treated with 2–25 μg/ml concentrations of α-amanitin for lengths of time varying from 0 to 24 h. The antigenic nuclear structures seemed, therefore, to be extremely stable and not to turn over recognizably.

Nuclear bodies recognized only ultrastructurally have a fine fibrillar component (Bouteille et al., 1974; Chaly et al.,

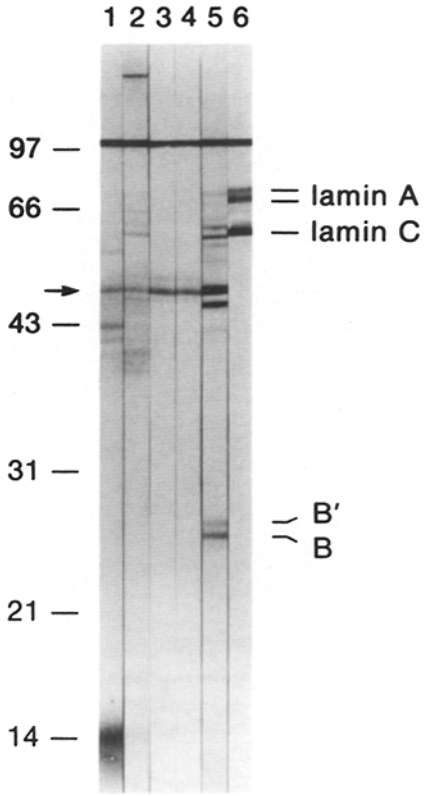

Figure 5. Immunoblotting of residual nuclear proteins with nuclear dot positive human autoantibodies and mAbs. After separation in 10% SDS polyacrylamide gels residual nuclear proteins were transferred to 0.22 μm nitrocellulose. Parallel strips were individually stained with (*1*) serum 455, (*2*) serum 1157, (*3*) mAb 1150, (*4*) mAb 138, (*5*) serum 516, and (*6*) serum 1753. Lanes *5* and *6* show the position of known proteins (B,B', lamins) used as an internal mol wt standard. The position of biotinylated molecular weight standards are indicated at the left. An arrow marks the position of a 55-kD polypeptide common to the nuclear dot positive human antisera and murine mAbs. Control strips (not shown) were negative. The black ink mark near the top of the nitrocellulose is for realignment of strips after staining.

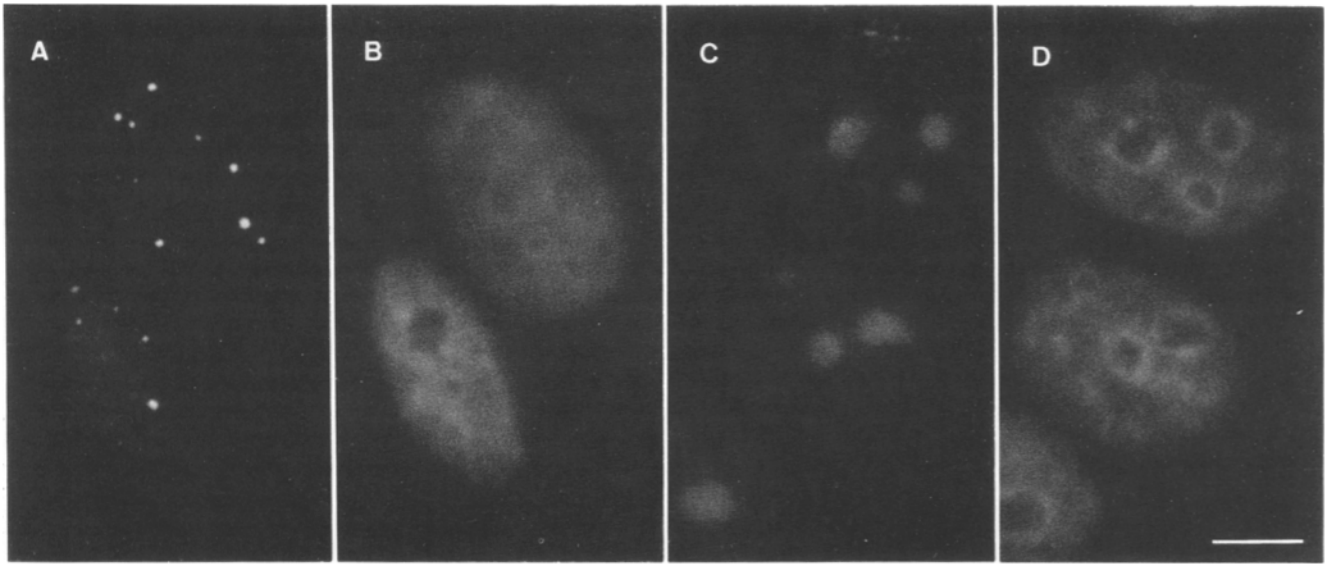

Figure 6. Immunofluorescence staining pattern of serum 1157 autoantibodies affinity purified from the 55-kD band. The method of Smith and Fisher (1984) was used to recover antibodies from the 55-kD band that were used to stain HEp2 cells. In *A*, single nuclear dots are detected using purified antibodies with no other nuclear staining visible. *C* shows staining using extractions from areas of nitrocellulose without bands. *B* and *D* show bis-benzimide staining to indicate nuclear position. Bar, 5 μm.

1983*a*). To test whether the nuclear dots are equivalent to the nuclear bodies, we visualized the nuclear dots by immunocytochemistry at the electron microscope level. Their average diameter ranged from 0.2 to 0.3 μm and no fibrillar component could be seen (not shown). We also tested MCF-7 cells, which are responsive to hormonal stimuli resulting in a dramatic increase in the number of nuclear bodies. No striking increase in the number of nuclear dots was found (Table I) upon hormone treatment. These findings indicate that nuclear dots are not equivalents of nuclear bodies.

The nuclear dot antigen is present in extremely low quantities and is not detectable in immunoblots of whole cells. Therefore, we combined in situ biochemical fractionation with the immunofluorescence assay to determine any sensitivity of the antigens to extractions and enzymatic digestions and to determine a procedure for enrichment of the antigen. Once the effects of individual treatments were assessed by immunofluorescence, a series of sequential extractions and digestions was selected which resulted in a particularly clear image of the nuclear dots (not shown). This series consisted of washes with low salt, low pH buffer, followed by extractions with NP-40, digestions with RNase A, DNase I and micrococcal nuclease, and washes with 2.0 M NaCl. We determined, using the immunofluorescence assay, that antigens recognized by mAb 364-5 (anti-nucleolar), mAb 90-20 (anti-RNP), and mAb 1415 (anti-histone) were removed as a result of these treatments. Nuclear dot antigens and kinetochore antigens remained. Chaotropic concentrations of $MgCl_2$, deoxycholate, SDS extraction, or protease removed the cells from the substrate, thereby abolishing the assay system. Most of the nuclear dot antigen seems extremely hydrophobic and stably attached to a nuclear matrix component.

Larger quantities of residual nuclear proteins were obtained from HEp2 cells sequentially treated as described above. When residual nuclear proteins were separated by 10% SDS-PAGE and stained with Coomassie blue, only the most abundant polypeptide species were apparent, primarily polypeptides with molecular weights similar to lamins, keratins, vimentin, nuclear pore elements, actin, and desmin (not shown), as reported elsewhere (Staufenbiel and Deppert, 1984; Zackroff et al., 1984; Goldman et al., 1986; He et al., 1990). When transferred to nitrocellulose and reacted with antibodies, less abundant polypeptides were detected. Using antikinetochore antisera, we were able to detect the three polypeptides of the centromere (CENP-A, B, and C) (Earnshaw and Rothfield, 1985) apparently intact, indicating that we had achieved a substantial enrichment and that any proteolysis was negligible (data not shown). Fig. 5 shows parallel strips of nitrocellulose after immunoblotting of residual nuclear proteins using nuclear dot positive human autoantibodies (sera 455 and 1157; lanes *1* and *2*, respectively) and the two murine mAbs showing the nuclear dot staining pattern (lanes *3* and *4*, respectively). Lanes 5 and 6 are stained with antisera recognizing several antigens including B,B′, and lamins which serve as an internal mol wt standard. The black ink line at the top of the nitrocellulose is for realignment of strips. The position of a band common to nuclear dot positive antibodies is shown by an arrow. An apparent molecular mass of 55 kD was determined for the protein, which is independent of dithiothreitol in the sample buffer as judged from diagonal electrophoresis (not shown).

Autoantibodies in serum 1157, a serum which also contains nuclear rim and centriolar and mitochondrial antibodies, were affinity purified from the 55-kD band. These affinity-purified antibodies stain only single nuclear dots with no nuclear rim, centriolar, or mitochondrial staining evident (Fig. 6 *A*; bis-benzimide staining, Fig. 6 *B*). Equivalent areas of nitrocellulose, where no specific band was present, were subjected to this extraction and show background levels of immunofluorescence only (Fig. 6 *C*; bis-benzimide staining, Fig. 6 *D*).

Isoelectric focusing followed by size separation and immunoblotting with the mAb 1150 shows that the 55-kD polypep-

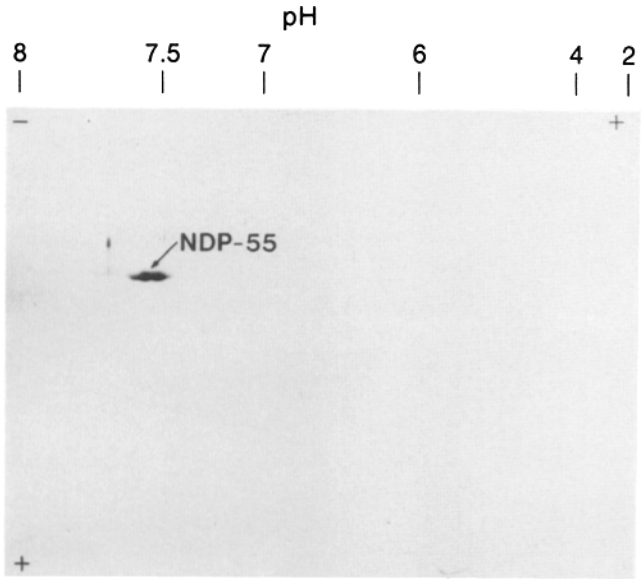

Figure 7. Isoelectric focusing and size separation followed by immunoblotting of NDP-55 polypeptide. After separation in pH 8.0 to pH 2.0 ampholines using equilibrium buffer conditions, electrophoresis in 10% SDS polyacrylamide was performed. Residual nuclear proteins were transferred to nitrocellulose. Immunoblotting with mAb 1150 detected charge isomers of NDP-55, with mol wts of ~55 kD. Three equally separated spots are detected at pI 7.4–7.7.

tide has an apparent pI value of 7.4–7.7 (Fig. 7). Three charge isomers were apparent as equally separated spots. The physical properties of the other nuclear dot–associated proteins including the additional antigen of the dot doublet seen with serum 1157 have not yet been determined and may only be verified after the development of additional mAbs.

All of our initial immunofluorescence studies utilized tissue culture cells. To test for the presence of nuclear dots in differentiated cell types, we stained cryosectioned rat liver tissue. Kinetochore staining was observed indicating nuclear antigens were accessible and recognizable under these conditions (Fig. 8 *A*). Nuclear dot antigens were not detected when control liver was stained (Fig. 8 *B*, *arrowheads* mark centrioles). Nuclear dots in parenchymal cells, however, reacted strongly in liver sections of rats 48 h after CCl_4 was administered to induce liver regeneration (Fig. 8 *C*). These experimental conditions effectively induce the parenchymal cells to go through the cell cycle and divide. Mitotic cells were observed only in sections of liver from rats receiving CCl_4.

Discussion

The multitude of functions taking place in the nucleus have not been reflected in its recognizable structural diversity. The apparent simplicity of the early ultrastructural images of the cross-sectioned nucleus did not afford assignment of functional domains with any detailed resolution. Recently developed techniques show that individual chromosomes occupy precisely circumscribed spaces (Agard and Sedat, 1983; Mannuelides, 1985) indicating a functional partitioning of areas perceived to be homogeneous by ultrastructural analysis. Structural investigations had defined a few large extrachromosomal spaces or domains like the nucleolus and the nuclear envelope besides the dense heterochromatin and the dispersed euchromatin. Histochemically, that is through the regressive staining methods (Granboulan and Granboulan, 1965), a network of densely staining RNP could be visualized ultrastructurally. This extrachromosomal space was later separated into functional domains corresponding to products of RNA polymerase II or RNA polymerase III. In both cases the functional elucidation and structural localization was strongly aided by antibodies to proteinaceous components of these domains (Lerner and Steitz, 1979; Habets et al., 1985; Ringertz et al., 1986; Montzka and Steitz, 1988; Spector, 1990). Other NDs, visualized only indirectly through antibodies, have no known function in the interphase nucleus. For example, the functional kinetochore is recognizable only at the electron microscopic level as a specific structure on chromosomes (Brinkley and Stubblefield, 1970), but antibodies to three major proteins label this otherwise unrecognizable structure in interphase as precise entities (Moroi et al., 1980) and allowed a timing of its duplication towards the end of the cell cycle (Brenner et al., 1981).

We can segregate additional NDs as we find new and specific probes. Through the recognition of a new nuclear dot staining pattern using human autoantibodies of a patient with lupoid carcinoma (Freundlich et al., 1988), we selected other sera with similar patterns. We also generated mAbs showing the same nuclear dot pattern. These antibody probes allowed us to differentiate between other similar nuclear structures, to quantitate the new nuclear structure, to observe changes when quiescent cells are stimulated to proliferate, and to detect and characterize one polypeptide of these domains.

We observed this distinct punctate nuclear dot immunofluorescence pattern in human as well as monkey, mouse, rat, Indian muntjac, frog, and chicken cell lines, indicating that the labeled structure is present at least in all vertebrates. The size varies considerably but an average of 0.3 μm was established from measurements of immunoelectron micrographs. We recognized that an apparent pairing of equal-sized structures existed that implied a chromosomal location. However, the numerical analysis showed a wide distribution not consistent with a permanent chromosomal site, ploidy, species, or cell morphology. The possibility that numerical variation is due to cell cycle changes was excluded leaving the possibility of high turnover. Blocking RNA and protein synthesis eliminated this possibility. In fact, we were struck with the temporal as well as physical stability of the antigen. We saw double dots where one was often smaller than the other. Such images are suggestive of budding or replication, if a chromosomal site was only temporarily expressed during the cell cycle. Quantitative data support the idea of budding in that the number of single dots did not increase during serum stimulation of WI38 cells, but the double dots increased from a very infrequent presence to approximately three. Both chromosomal location or activation of a newly replicated chromatid could accommodate such an interpretation.

Initially, we considered the nuclear dot structures to be individual or specific centromeres or kinetochores with specific antigens showing differential staining patterns similar to the one shown with the mAb against the CENP-B antigen (Earnshaw et al., 1987), but more extreme. However, double labeling showed no correlation with the centromere

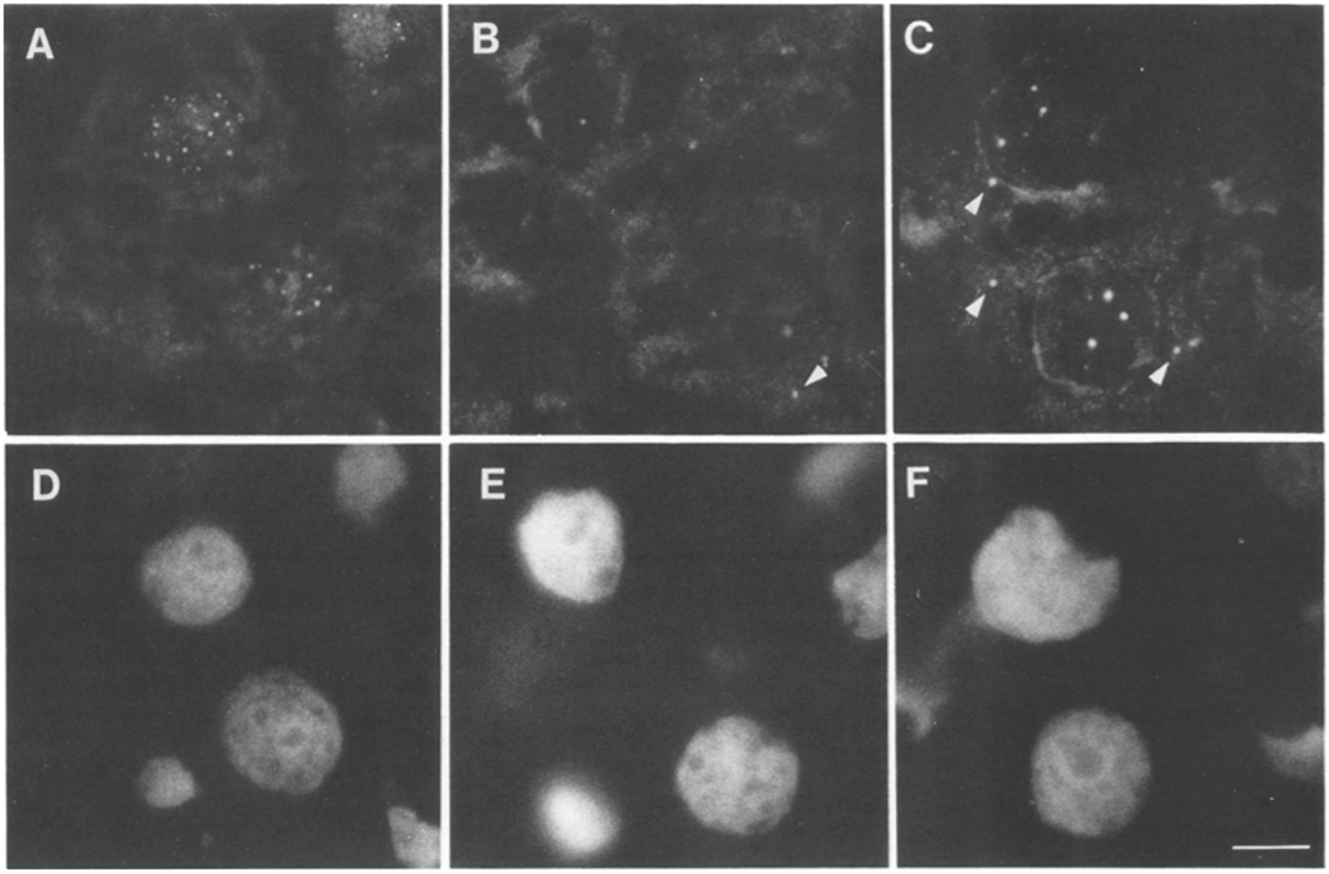

Figure 8. Immunofluorescence staining patterns of rat liver sections stained with autoantibodies. Photographs (*A–F*) of stained rat liver sections from control animals show that although (*A*) kinetochore antigens are accessible and recognizable, nuclear dots are not detected in differentiated tissue (*B*). When rat liver sections were obtained from rats 48 h after receiving CCl_4 to regenerate liver tissue nuclear dots were detected (*C*). *D–F* show corresponding bis-benzimide staining. Arrowheads mark centrioles stained by additional autoantibodies in serum 455. Bar, 5 μm.

effectively eliminating the possibility that these dots represent a subset of kinetochores or centromeres. Double dots as well as paired dots could also have resulted from selective telomere staining. We could not exclude this possibility; however, telomeres are present mostly on the nuclear envelope (Blackburn and Szostak, 1984), and we are therefore not pursuing this line of reasoning any further.

During mitosis the nuclear dots behave like the nucleolus, the mRNA processing sites, or sites of tRNA and 5S RNA synthesis; that is, they disappear or redistribute with the advancing mitotic sequence. However, none of the sera recognizing these nuclear dots precipitated a consistent RNA species.

Nuclear bodies, present in very low numbers in uninduced cells, can be induced by hormonal stimuli in responsive cells resulting in a striking increase in frequency (Padykula et al., 1981; Fitzgerald and Padykula, 1983; Jensen and Brasch, 1985; Brasch et al., 1989; Yu and Ho, 1989) or by induction of T cell proliferation with lectins (Chaly et al., 1983*a*,*b*). Single nuclear bodies as well as complex ones have been described with diameters similar to the nuclear dots we report here. MCF-7 cells provide a model system to study the effects of estrogenic stimulation (Greene et al., 1984; Geier et al., 1985; Heggeler-Bordier et al., 1988). No striking change in the immunofluorescence pattern was noted when unstimulated and 17-β-hydroxyestradiol-stimulated MCF-7 cells were stained with nuclear dot antibodies. No increase of nuclear dots was noted when isolated human lymphocytes were stimulated with phytohemagglutinin (1 μg/ml) for various times from 0 to 48 h. If the structures we describe through binding of specific antibodies recognize the same entity which has become known as nuclear bodies, then we see substantially more or additional ones difficult to visualize by electron microscopy. No specific antibody probes for nuclear bodies exist to allow direct comparison by double label studies. Judged from these ultrastructural findings and induction experiments, nuclear dots and nuclear bodies are not the same.

The interaction of nuclear dots as recognized by the antibodies can be partially deduced from double-label immunofluorescence studies (Fig. 1) and from the immunoblotting data which shows that sera 455 and 1157 and the two mAbs all recognize a 55-kD polypeptide (NDP-55). Since mAb 138 raised against a fusion protein reactive with autoantibodies (Maul, G. G., P. E. Gregory, D. Ziemnicka-Kotula, manuscript submitted for publication) recognize nuclear dots, and affinity purified autoantibodies recovered from Western blots also stain nuclear dots, we conclude that

the 55-kD antigen is part of the nuclear dot structure. We propose that all single nuclear dots contain the NDP-55 polypeptide. When screening the literature concerned with autoantibody localization we found nuclear dot-like images present in sera of a variety of autoimmune diseases (Makinen et al., 1983; Bernstein et al., 1984; Kurki et al., 1984; Cassani et al., 1985; Penner et al., 1986; Szostecki et al., 1987). The prevalence and disease specificity is presently being investigated.

We detected the NDP-55 polypeptide under a variety of one-dimensional and two-dimensional electrophoretic conditions. At least three charge isomers are apparent, which may indicate posttranslational modification. A phosphorylation-dephosphorylation event may provide for solubilization differences like the lamins in mitosis (Gerace and Blobel, 1980). The relatively basic isoelectric point of the NDP-55 polypeptide (pI 7.4-7.7) provides a means of separation from relatively acidic desmin and vimentin with approximately the same molecular mass (54 and 56 kD, pI 5.6 and 5.7, respectively), which are present in this preparation (Dagenais et al., 1984). Since NDP-55 is not polymerized by disulfide bonds as is lamin A and C with similar isoelectric points, NDP-55 can be separated from these residual nuclear proteins by precipitation of lamins under nonreducing conditions.

No correlation with any known nuclear structure has been found and no direct suggestion has emerged for the function of the antigens present in the nuclear dot structure. We observe, however, that all cycling cells (or those recently gone through a cell cycle) contain the nuclear structure, and that noncycling rat liver parenchymal cells do not, except upon induction to replicate. We suggest, therefore, that the antigens may have importance in aspects of the proliferative state.

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