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Dynamic Changes in the Higher-Level Chromatin Organization of Specific Sequences Revealed by In Situ Hybridization to Nuclear Halos

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Abstract. A novel approach to study the higher level packaging of specific DNA sequences has been developed by coupling high-resolution fluorescence hybridization with biochemical fractionation to remove histones and distend DNA loops to form morphologically reproducible nuclear "halos." Results demonstrate consistent differences in the organization of specific sequences, and further suggest a relationship to functional activity. Pulse-incorporated bromodeoxyuridine representing nascent replicating DNA localized with the base of the chromatin loops in discrete clustered patterns characteristic of intact cells, whereas at increasing chase times, the replicated DNA was consistently found further out on the extended region of the halo. Fluorescence hybridization to unique loci for four transcriptionally inactive sequences produced long strings of signal extending out onto the DNA halo or "loop," whereas four transcriptionally active sequences

remained tightly condensed as single spots within the residual nucleus. In contrast, in non-extracted cells, all sequences studied typically remained condensed as single spots of fluorescence signal. Interestingly, two transcriptionally active, tandemly repeated gene clusters exhibited strikingly different packaging by this assay. Analysis of specific genes in single cells during the cell cycle revealed changes in packaging between S-phase and non S-phase cells, and further suggested a dramatic difference in the structural associations in mitotic and interphase chromatin. These results are consistent with and suggestive of a loop domain organization of chromatin packaging involving both stable and transient structural associations, and provide precedent for an approach whereby different biochemical fractionation methods may be used to unravel various aspects of the complex higher-level organization of the genome.

THOUGH the length of naked DNA from a single human cell is approximately one meter, it is packaged into a nucleus with a typical diameter of 10 μm . Despite its enormous condensation and tight complexing with protein into a highly viscous nucleus, genomic DNA, only a few percent of which is expressed within any given cell, remains appropriately and perhaps selectively accessible to transcription factors and the transcriptional machinery (for review see Van Holde, 1988). It is well known that DNA is complexed with histones to form the 10-nm and 30-nm chromatin fibers, condensing DNA length \sim 50-fold (Weisbrod, 1982). Beyond this, much less is known concerning the higher-level packaging and further condensation of the 30-nm fiber. However, there is strong evidence that one step involves packaging of chromatin into loops typically estimated to be between 50–200 kb (for review see Nelson et al., 1986; Gasser and Laemmli, 1987; Jackson et al., 1990; Zlatanova and van Holde, 1992). The other levels of packaging that exist or the nature of the associations that maintain higher-level chromatin structure remain largely unknown.

Clearly, the way the genome is packaged in interphase nuclei and metaphase chromosomes is of fundamental importance for understanding the mechanics of nuclear structure and basic cell function. However, DNA packaging may also play a significant role in developmental regulation of gene expression. For example, changes in DNase I sensitivity that can extend tens of kilobases beyond the dimensions of the involved gene itself accompany changes in specific gene expression and are believed to reflect alterations in chromatin organization (Weintraub and Groudine, 1976; Weisbrod, 1982; for review see Gasser and Laemmli, 1987). Even at the crude level of chromatin packaging represented by the metaphase chromosome, there is evidence for differential higher-level packaging of functional subsets of DNA: chromosome bands reflect a clustering of gene rich, early replicating, and Alu-rich DNA, which is concentrated in large chromatin structures or domains termed light G-bands (Ganner and Evans, 1971; Holmquist et al., 1982; Manuelidis and Ward, 1984; Korenberg and Rykowski, 1988; Kerem et al., 1984; Gazit et al., 1982; for review see Bickmore and Sumner, 1989). The structural basis for various changes in chromatin structure or even the level of packaging at which they occur remains almost entirely unknown. For example, neither the structural basis for long-range DNase

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sensitivity surrounding active genes nor the chromatin packaging that creates cytogenetic bands is understood, but both likely involve changes in chromatin folding above the level of the nucleosome. One level which may be involved in these or other changes in chromatin packaging may be the formation and higher-level packaging of loop domains.

The work presented here describes the development and application of a novel approach for directly visualizing the packaging of specific DNA segments within single cells, by coupling fluorescence detection of individual genes with procedures reported to distend DNA loops by extraction of histones and other soluble proteins. Evidence for the existence of chromatin loops resulted from extraction of interphase nuclei with detergent and 2 M NaCl followed by ethidium bromide staining to reveal a residual "nucleoid" or "matrix" surrounded by a halo of positively supercoiled DNA (Cook and Brazell, 1976; McCready et al., 1980; Vogelstein et al., 1980). Paulson and Laemmli (1977) directly demonstrated individual DNA loops emanating from the metaphase chromosomes "scaffold" by electron microscopy. These studies provided elegant empirical visual evidence for the packaging of DNA into loops but could not demonstrate whether specific sequences occupy reproducible positions with respect to loop domains or determine whether these domains change with cell cycle and mitosis. In this report, we interpret the distended DNA to reflect the loops of DNA believed to exist *in vivo* and will refer to these putative *in vivo* structures as "loop domains." The rationale for the work described here is to augment the analytical power of the cytological approach by directly visualizing the distribution of specific DNA sequences within halo preparations of nuclei and chromosomes.

To date the investigation of specific sequences and their relationship to putative loop domains has relied upon indirect approaches involving extraction of isolated nuclei, DNA digestion, and subsequent fractionation by electrophoresis to characterize the DNA which binds the residual nuclear matrix (for example: Robinson et al., 1983; Ciejek et al., 1983; Cockerill and Garrard, 1986; Gasser and Laemmli, 1986; Dijkwel and Hamlin, 1988). These sequences, thought to localize to the base of chromatin loops (for review see Gasser and Laemmli, 1987; Cockerill and Garrard, 1986; Zlatanova and van Holde, 1992) are frequently termed matrix attachment regions (MARs)¹ or scaffold attachment regions (SARs), depending on the nuclear extraction method used. While the composition and nature of the matrix or the scaffold, which constitutes only 5% of nuclear protein (Fey et al., 1986), remain largely unknown and somewhat controversial, substantial evidence suggests that this insoluble non-chromatin nuclear material has a role in organizing DNA into loop domains (for review see Nelson et al., 1986; Fey et al., 1991; Jackson, 1991; Stuurman et al., 1992). A host of studies using a variety of fractionation techniques indicate that replicating DNA (for example: Berezney and Coffey, 1975; Vogelstein et al., 1980; McCready et al., 1980; Nakayasu and Berezney, 1989; Vaughn et al., 1990; for review see Berezney, 1991; Cook, 1991) and transcriptionally active sequences (for example: Robinson et al., 1983; Ciejek et al., 1983; for review see

Zlatanova and van Holde, 1992) are preferentially associated with the matrix, although the latter is not universally accepted (see Discussion).

Coupling *in situ* hybridization with methods to visualize DNA halos provides a molecular cytological assay for specific gene packaging that potentially allows the physical relation of individual sequences to proposed loop domains and to the residual matrix or scaffold to be examined directly and simultaneously. Moreover, DNA packaging can be investigated in single cells and in different phases of the cell cycle, avoiding the limitations of averaging heterogeneous cell populations. Our results using this assay clearly demonstrate sequence specific DNA packaging and are consistent with the concept of DNA loop organization that has implications for not only the folding and condensation of the genome, but for its replication and transcription as well. Moreover, results indicate that dynamic changes in chromatin packaging occur in relation to both cell cycle and gene expression, revealing a marked difference in the loop/matrix organization of a given sequence between interphase and mitotic chromatin.

Materials and Methods

In developing the methods for this study, several approaches for both the production and preservation of looped DNA on nuclear halos were tested. Two different compounds are widely used to remove soluble proteins, including histones, from nuclei to reveal loops of DNA: NaCl (Vogelstein et al., 1980; McCready et al., 1980), or isotonic lithium diiodosalicylate (LIS; Mirkovitch et al., 1984). Similarities and differences in the DNA loops produced by these two procedures remains controversial (Jackson et al., 1990). Our original experiment using 2 M NaCl extraction resulted in a more uniform distribution of DNA around the residual nucleus and produced more consistent results than LIS, although for the two genes studied using both methods, essentially identical results were obtained. We therefore focused on using 2 M NaCl extraction to provide for an empirical survey of representative sequences during different phases of the cell cycle.

Several protocols were compared to identify a fixation procedure that maximized preservation of nuclear halo morphology through subsequent denaturation and hybridization. Those tested included various methods for aldehyde or alcohol fixation. However, the approach that most effectively preserved the halos intact during subsequent steps was baking at 70°C for 2 h.

Cell Culture

Namalwa cells (CRL 1432, Amer. Type Culture Collection, Rockville, MD), a single X-chromosome lymphoma cell line containing integrated Epstein-Barr virus DNA (Henderson et al., 1983; Lawrence et al., 1988), were grown as suspension cultures in RPMI medium with 10% FCS (GIBCO BRL, Gaithersburg, MD) at 37°C. Human diploid fibroblast cells (Detroit 551; CCL 110, Amer. Type Culture Collection) were grown on glass coverslips in DME containing 10% FCS (GIBCO BRL) at 37°C. For DNA replication studies, unsynchronized cells were cultured in the presence of 25 µg/ml of bromodeoxyuridine (BrdU; Boehringer Mannheim Co., Indianapolis, IN) for 15 min, and then harvested or washed 2× with copious amounts of DME, and then cultured in normal media for the specified chase time before the halo preparation. Generally 40–50% of cells (those in S-phase) were labeled.

Enrichment for Mitotic Cells

To obtain a cell population enriched for mitotic cells, human HeLa S3 cells (CCL 2.2, Amer. Type Culture Collection) were grown in T-25 culture flasks (Becton Dickinson and Co., Franklin Lakes, NJ) to 70% confluence in DME containing 10% FCS (GIBCO BRL) at 37°C. Dead or detached cells were washed-off in media, fresh media added, and the flask then sharply struck several times to dislodge cells undergoing mitosis. The detached cells were recovered by centrifugation at 500 g for 5 min and resuspended in PBS.

1. *Abbreviations used in this paper:* LIS, isotonic lithium diiodosalicylate; MARs, matrix attachment regions; SARs, scaffold attachment regions.

Nuclear Halo Preparation

Our standard procedure was as follows: 1.5×10^6 Namalwa cells or mitotic HeLa cells were pelleted at 650 g for 5 min and washed two times in PBS. Cells were then resuspended to a final concentration of 1.5×10^6 cells/ml in isotonic CSK buffer (10 mM Pipes [pH 7.8]; 100 mM NaCl; 0.3 M Sucrose; 3 mM MgCl₂) (Fey et al., 1986) with 0.5% Triton X-100 and incubated on ice for 15 min. 1.5×10^5 nuclei were then centrifuged onto coverslips for 5 min at 500 g using a Cytospin 2 cytological centrifuge (Shandon Inc., Pittsburgh, PA). The coverslips were then rinsed twice in PBS for 2 min, followed by extraction in 2 M NaCl buffer (2 M NaCl; 10 mM Pipes [pH 6.8]; 10 mM EDTA; 0.1% Digitonin [Sigma Chem. Co., St. Louis, MO]; 0.05 mM Spermine; 0.125 mM Spermidine) (modified from: Vogelstein et al., 1980; Robinson et al., 1983; Lewis and Laemmli, 1982) for 4 min. After this extraction of soluble proteins, nuclear halos were prepared for baking by sequential 1 min rinses in 10 \times , 5 \times , 2 \times PBS, followed by 1 \times PBS for 2 min and sequential 1 min rinses in 10, 30, 70, and 95% ethanol. Coverslips were air-dried and nuclear halos fixed by baking at 70°C for 2 h (Lawrence et al., 1988). Fibroblast nuclear halos were prepared as above except that the cytospin step was omitted. Alternatively, nuclear halos were prepared by isolation of nuclei in 0.1% Digitonin buffer (0.05 mM Spermine, 0.125 mM Spermidine, 0.5 mM EDTA, 1.0% Thioglycol, 20 mM KCl, 5 mM Hepes [pH 7.4], and 0.1% Digitonin) followed by extraction in LIS buffer (5 mM Hepes/ KOH [pH 7.4], 0.25 mM Spermine, 2 mM KCl, 0.1% Digitonin, 25 mM 3,5-diiodosalicylic acid, 2 mM EDTA). The overall protocol was as above except that nuclear halos were dehydrated in the graded series of ethanol directly after extraction in LIS buffer. To prepare extracted cells without preserving nuclear morphology, "cytopun" cells were placed directly into the 2 M NaCl buffer, and then processed according to the remaining halo procedure.

DNA Probes and Antibodies

For fluorescence in situ DNA hybridization, the following probes were used (also see Table I). 24A2 (provided by Louis Kunkel, Children's Hospital Medical Center, Boston, MA) and 15ADMD (provided by C. Thomas Caskey, Baylor College of Medicine, Houston, TX) correspond to two regions of the dystrophin gene \sim 650 kb apart. LAO 1 and BER 226 are clones from the ribosomal protein RPS4X gene locus, and are from David Page, Whitehead Institute (Cambridge, MA). Plasmid pTP18 was obtained from Alan Weiner, Yale University (New Haven, CT). Cosmid C3 was provided by Frank Ruddle, Yale University. Phage clone p8-1A and plasmid pH5SB were provided by Leslie Leinwand and Randall Little, respectively, from Albert Einstein University, New York, NY. Phage clone HHG 6, containing the closely linked histone H3 and H4 genes, was from Janet Stein, University of Massachusetts Medical Center (Worcester, MA). The alphoid centromere repeats p3.6 and L1.84 were obtained from Huntington Willard, Stanford University (Stanford, CA) and Jan Baumann, University of Amsterdam (Amsterdam, The Netherlands), respectively. A mouse monoclonal antibody reactive to the SC-35 spliceosome assembly factor was obtained from Tom Maniatis (Harvard University, Cambridge, MA). Antiserum to lamin B protein was provided by Ted Fey, University of Massachusetts Medical Center. Antiserum to the gp120 nuclear pore protein was provided by Gunter Blobel (Rockefeller University, New York, NY). A monoclonal antibody against the nuclear mitotic apparatus protein, NuMA, was obtained from Matritech, Inc. (Cambridge, MA).

Hybridization and Detection

Hybridization and detection of nick translated probes was performed according to previously established protocols (Lawrence et al., 1988; Johnson et al., 1991). DNA probes were nick translated using biotin-11-dUTP or digoxigenin-16-dUTP (Boehringer Mannheim) and electrophoresed on agarose gels to verify that fragment sizes of 200–500 base pairs were obtained (Lawrence et al., 1989). Briefly, halos were placed in PBS for 10 min then denatured in 70% Formamide, 2 \times SSC at 70°C for 2 min, dehydrated in cold 70% and 100% EtOH for 5 min each, and allowed to air dry. Alternatively, to insure removal of RNA, halos were denatured in 0.2N NaOH in 70% EtOH for 5 min at room temperature (Carter et al., 1991), and then dehydrated as described above. For each sample, 50 ng of labeled probe, mixed with competitor nucleic acids (10 μ g of sonicated salmon sperm DNA, 10 μ g of *E. coli* tRNA, and 10 μ g of COT-1 DNA [GIBCO BRL]) was resuspended in 10 μ l deionized formamide and denatured at 70°C for 2 min. Hybridization was allowed to proceed 3 h to overnight in a buffer consisting of 50% formamide, 20% dextran sulfate, 10% BSA, and 2 \times SSC (1 \times SSC is 150 mM NaCl, 30 mM sodium citrate). For

hybridization to Epstein-Barr virus RNA, the halo preparations were not denatured. After hybridization, biotin or digoxigenin-labeled samples were incubated in solutions (1:500 in 4 \times SSC/1% BSA) of either FITC, Texas red, or rhodamine-conjugated avidin (2.5 mg/ml; Boehringer Mannheim), DTAF, rhodamine or Cy3-conjugated streptavidin (1.0 mg/ml; Jackson ImmunoResearch Laboratories, Inc. West Grove, PA), or FITC or rhodamine-conjugated sheep anti-digoxigenin antibodies (200 μ g/ml; Boehringer Mannheim) for 45 min at 37°C. Samples were then rinsed at room temperature for 10 min each in 4 \times SSC; 4 \times SSC/0.1% Triton X-100; then 4 \times SSC (Lawrence et al., 1988; Johnson et al., 1991).

Immunofluorescence

Incorporated BrdU was detected with a FITC-conjugated anti-BrdU antibody (Boehringer Mannheim) at a dilution of 3:500 in PBS/1% BSA for 45 min at 37°C, and then washed three times for 10 min in PBS. For antibody detection of the lamin B, gp120 nuclear pore protein, and SC-35 spliceosome assembly factor, nuclear halos were incubated with the appropriate antibody for 45 min in PBS/1% BSA at 37°C and detected with a fluorochrome-conjugated secondary antibody (Organon Teknika/Cappell Division, Durham, NC) at a 1:100 dilution in nPBS/1% BSA for 30 min at 37°C.

Simultaneous Detection of DNA and Protein in Nuclear Halo Preparations

For simultaneous detection of a DNA probe corresponding to the U2 snRNA gene and of spliceosome assembly factor SC-35, the in situ DNA hybridization and fluorochrome detection of probe DNA was performed as described above, and the DNA signal "fixed" by incubation in 4% paraformaldehyde for 5 min. The sample was then incubated with anti-SC-35 for 90 min, washed and detected as above. For simultaneous detection of the U2 DNA probe and the nuclear mitotic apparatus protein NuMA, the sample was first incubated with anti-NuMA in 4 \times SSC/1% BSA at 37°C for 90 min, detected with FITC donkey anti-mouse IgG (Jackson ImmunoResearch Inc.) at a dilution of 1:500 in 4 \times SSC/1% BSA for 45 min, and the fluorescent signal fixed by paraformaldehyde treatment as above. The halo preparation was then denatured in formamide and DNA in situ hybridization performed and detected as described above.

Microscopy

The DNA in nuclear halos was visualized by staining with propidium iodide (50 μ g/ml) or DAPI (4,6-Diamidino-2-phenylindole, 1 μ g/ml) in PBS for 1 min. Samples were examined at 630 \times or 1,000 \times using a Zeiss Axioskop microscope equipped for epifluorescence photomicroscopy. Color photographs were taken using Ektachrome ASA 400 film (Kodak, Rochester, NY). Black and white photographs were taken with T-Max ASA 400 film (Kodak). Alternatively digital images were obtained using a Photometrics CCD camera (Photometrics Ltd., Tucson, AZ) in conjunction with multiple-band pass filters that allow for precise alignment of color images (Johnson and Lawrence, 1991).

Scoring the Position of Individual Sequences within Nuclear Halos

For the data presented in Figs. 6–8 the amount of "looping" or extension of a given sequence was determined by its position within the nuclear halo in a large number of cells. Because the pattern of individual sequences was variable they were assigned to one of 7 categories: (1) a single spot "inside" the residual nucleus; (2) a pair of closely spaced spots inside; (3) a "string" of spots inside; (4) a string "crossing" the edge of the residual nucleus; (5) a string completely "out" on the looped portion of the halo; (6) a pair of spots crossing; or (7) a pair of spots out. In the presentation of the data herein, categories 1 and 2 were pooled and called "non-extended," and categories 3–7 were pooled and called "extended." Pairs of spots on the looped portion of the halo (categories 6 and 7) were rarely seen, thus these two categories generally accounted for less than 10 percent of the extended category. Single spots on the extended portion of the halo were even less often seen and were not scored. In most experiments S-phase cells were labeled by BrdU incorporation into replicating DNA just before harvesting. The BrdU was then visualized simultaneously with the gene probe, allowing all signals to be further categorized as deriving from an S- or non-S-phase cell. Typically, experiments were only considered scoreable if >85% of halos had gene signal. Scoring was done in a "blind" fashion in which the

person scoring did not know the identity of probe and most experiments were scored independently by two different investigators and the data pooled.

Results

Preparation and Morphological Characterization of Nuclear Halos

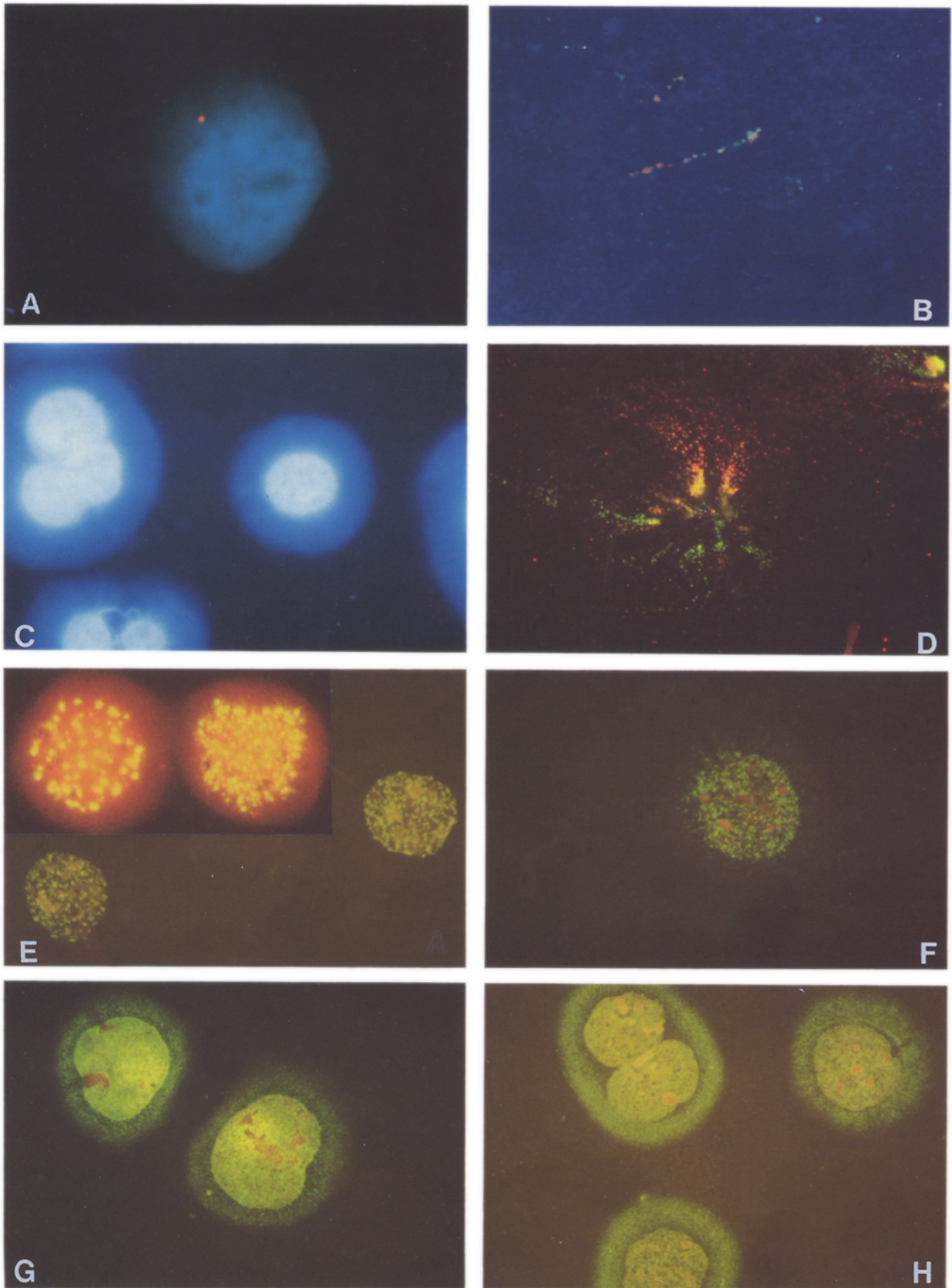
This work required the development of an experimental approach coupling high-sensitivity fluorescence hybridization with nuclear extraction methods to examine aspects of chromatin packaging. This involved: (a) producing nuclear halos by biochemical fractionation to remove histones and distend DNA while maintaining loop structure and association with the residual nucleus; (b) preserving the delicate loop preparations so that they remain essentially intact through in situ hybridization procedures; (c) high-resolution detection of specific single-copy sequences within halo preparations. Many procedures can be used to decondense DNA for in situ hybridization, however many of these result in highly variable and apparently random threads of DNA smeared erratically across the slide, with little if any retention of native morphology (Fig. 1 *B*). These procedures may have use for ultra-high resolution (below 50 kb) gene mapping (Wiegant et al., 1992; Heng et al., 1992; Lawrence et al., 1992), but contrast sharply with those implemented here, the objective of which is to extend DNA while preserving loop domains with as much nuclear structure as possible. Testing of a number of variables revealed several to be critical to success and significantly impact the results obtained. These include methods for extracting proteins, for cell fixation, and for denaturation and dehydration. The bulk of this work used a 2 M NaCl extraction procedure modified from Vogelstein et al. (1980) that results in reproducible structures in which a regular, uniform halo of DNA circumscribed the residual nucleus (Fig. 1 *C*; Fig. 2). A limited amount of analysis was also done with procedures using lithium diiodosalicylate (Mirkovitch et al., 1984) that, for two sequences studied, yielded results essentially identical to those obtained by NaCl extraction. Despite the fact that the 2 M NaCl extraction removes lipids and the vast majority of nuclear protein (for review see Fey et al., 1991), the resulting structures

retained several aspects of native nuclear morphology. Immunofluorescence staining for nuclear pores (Fig. 3, *A-D*) and lamins (not shown) indicated that these structures essentially remained intact with similar distributions before and after 2 M NaCl treatment. This was also seen for the nuclear mitotic apparatus protein NuMA (data not shown). As illustrated in Fig. 3, *E-H*, the spliceosome assembly factor SC-35, which colocalizes with snRNP/poly A RNA-rich transcript domains (Fu and Maniatis, 1990; Carter et al., 1991, 1993) is well preserved in these halo preparations, consistent with a previous report that SC-35 is retained in nuclear matrices, whereas snRNP antigens are more easily extracted (Spector et al., 1991). In agreement with reports that nuclear RNA is retained within the nuclear matrix (see Fey et al., 1986; Xing and Lawrence, 1991; and for review see Fey et al., 1991; Verheijen et al., 1988), fluorescence hybridization to a specific viral transcript showed nuclear "tracks" of Epstein Barr Virus RNA in over 90% of residual nuclei (not shown). As described below, detailed studies of the distribution of replicating DNA and transcriptionally active sequences within the halo structures provided further evidence for the preservation of functionally relevant structural associations.

High-Resolution Visualization of Replicating DNA within Nuclear Halos

Previous work based on either biochemical fractionation of cell populations (Berezney and Coffey, 1975; Pardoll et al., 1980) or autoradiographic analysis of nuclear halos (Vogelstein et al., 1980; McCready et al., 1980) indicated that newly replicating DNA was preferentially associated with the residual nucleus, and therefore may be expected to be at the base of extended DNA loops. Use of a fluorescence assay for replicating DNA in halos made it possible to investigate the loop domain organization of replicating DNA with higher resolution and detail, and at the same time provided a means to verify that our specific protocol did not randomly extend DNA but preserved physiologically relevant associations. Nuclear halos were prepared from cells grown in the presence of the modified nucleoside bromodeoxyuridine (BrdU) for 15 min, followed by a chase period ranging from 0 to 18 h. In S-phase cells which were labeled but not chased

Figure 1. (A) Hybridization and fluorescent in situ hybridization detection of the X-linked dystrophin gene in an intact Namalwa cell nucleus. Cells were prepared by Triton extraction, as diagrammed in Fig. 2, step 1, and the 16-kb probe (24A2, see Table I) detected with rhodamine (red spot) and total DNA stained with DAPI (blue). As reported elsewhere (Lawrence et al., 1990), single probes up to a few hundred kb long or pairs of probes ~50–100 kb apart consistently appear as a single spot in intact cells and nuclei. (B) Hybridization and detection of adjacent 10 kb sequences (one labeled green, the other red) in cellular DNA prepared using a procedure that does not preserve nuclear morphology (see Materials and Methods), resulting in extensive random smearing of cellular DNA (blue). Note that when cellular DNA is drastically distended in this way these tandemly positioned probes are readily detected and resolved as strings of signal. (C) Namalwa cell nuclear halos. Cells were extracted as described in the text and outlined in Fig. 2, steps 1 and 2, and then photographed after staining with DAPI (blue). After fixation, halo preparations like these were used for characterization and hybridization throughout the study. (D) A single Namalwa cell nuclear halo hybridized with satellite DNA probes specific for the chromosome 17 (green) and chromosome 19 (red) centromere repeats. (E–H) Visualization of replicated DNA on nuclear halos. Namalwa cells were treated with BrdU for 15 min, and then washed and grown for various times in normal media (see Materials and Methods). Nuclear halos were then prepared and incorporated BrdU detected by immunofluorescence (green). (E) 0 h (No chase). Halos were not counterstained, hence are not visible due to the complete absence of BrdU on the looped out DNA. (Inset) 0 h time point from a separate experiment in which total DNA was stained with propidium iodide (red). Note that the recently replicated DNA containing BrdU remains completely within the residual nucleus. Also note that the two residual nuclei have different patterns of replication foci, likely representing cells in late and early S-phase (Nakayasu and Berezney, 1989), suggesting that overall nuclear morphology is well preserved. (F) 1 h chase. (G) 6 h chase. (H) 18 h chase. Note progressive appearance of BrdU label on looped portion of halos.



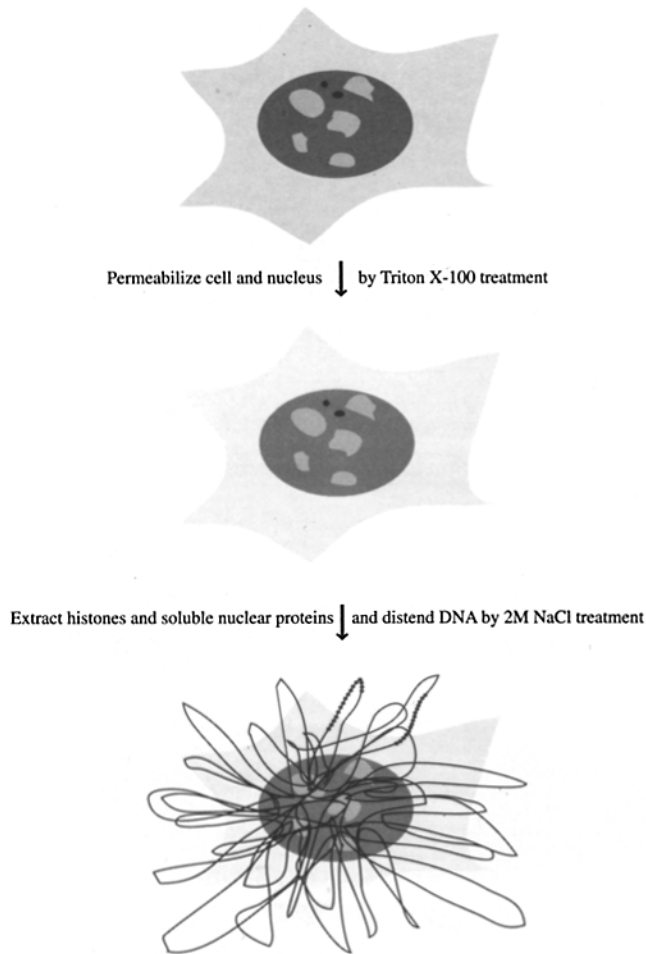


Figure 2. Schematic representation of nuclear halo preparation. Intact cells (*top*) were washed extensively in PBS then permeabilized in an isotonic buffer containing Triton X-100 (*middle*). The cells were then extracted with NaCl to remove histones and other soluble proteins leaving an intact residual nucleus surrounded by loops of DNA (*bottom*). For details see Materials and Methods. Signal from a probe hybridization to a given sequence that “loops out” is represented by the dark spots in the intact nucleus (*middle*) and the string of signal on a DNA loop of the nuclear halo (*bottom*). For single-copy probes which extended on the loop, the length of signal was generally about 1–2 μm for every 10 kb of probe length (10 kb of fully extended β -helical DNA would measure 3.4 μm). Namalwa cell nuclei typically measure 10–15 μm in diameter and the main body of the looped DNA generally extended $\sim 10 \mu\text{m}$ beyond the border of the residual nucleus; however a very light amount of DAPI stained DNA was often seen beyond the bulk of the halo. Thus the size of the loops was estimated to be in the 50–200-kb range as reported by others (see text).

essentially all incorporated BrdU was retained within the residual nucleus (see Fig. 1 *E*), indicating that the halo preparation protocol preserved a differential structural association of this entire functional class of DNA. Furthermore, the same associations that exist within the intact cell were found to be preserved in halos, as shown by the retention of discrete clustered patterns for early and late replicating DNA (Fig. 1 *E*; inset). As expected, these discrete clusters, also seen in human diploid fibroblasts (not shown), were most appar-

ent for the shorter chase times in which the label had not yet moved out onto the extended portion of the loops. After 1, 2, 4, and 6 h chase times, labels progressively moved out onto the extended portion of the halo, with virtually all labeled cells at a given time point showing a similar pattern. Movement of replicated DNA onto the extended portion of the loop was very slight after a one hour chase (Fig. 1 *F*) and maximal after six hours (Fig. 1, *G* and *H*). After the 18-h chase, an occasional cell showed significantly less BrdU signal in the halo than did the majority, indicative of cells that had completed one cell cycle and were in the process of a second round of replication. The precise spatial retention of replicating DNA at the base of the halo structure and its apparent temporal movement onto the extended DNA halo after replication is consistent with a preferential localization of replicating DNA at the base of chromatin loops. This clear change in halo position of labeled DNA at different times after replication demonstrates that the biochemical fractionation used does not extend DNA at random but reproducibly differentiates physiologically relevant subclasses of DNA. As illustrated below, this defined organization with respect to replicating DNA is preserved throughout hybridization (for example, Fig. 5 *H*).

Distribution of Centromeric Repeats

Initial attempts to hybridize specific sequences to DNA halos prepared using conditions that maintain their morphology were undertaken using the centromere-specific satellite sequence probes p3.6 and L1.84 (Waye and Willard, 1986; Devilee et al., 1986; also see Table I for a complete list of probes used). The results showed a majority of the signal as a spray of many strings of fluorescence signal emanating from a discrete origin within the residual nucleus (Fig. 1 *D*) instead of two large but tightly condensed spots within the nucleus as has been shown for centromeres in unfractionated cells (for example, Carter et al., 1991).

Also apparent in these initial experiments was that individual threads of signal did not form a solid line of fluorescence, but consistently had a spotted appearance. To investigate whether this reflected the hybridization of individual probe molecules along the DNA strand, two discretely labeled probes for the same satellite sequence were hybridized simultaneously to halo preparations and detected in two different colors. The results demonstrated that the spotted appearance was not due primarily to the hybridization of individual probe molecules, since individual spots generally contained both labels (Fig. 4, *A–B*). Furthermore, the spacing of the signals was highly variable, suggesting that they do not represent the precise distribution of repetitive sequences along the extended DNA. The spots likely reflect incomplete hybridization efficiency, in part caused by reannealing of the DNA strand to itself and competing with hybridization of the probe. In addition to providing a large target sequence with which to establish hybridization conditions for halo preparations, these results provide preliminary evidence that the satellite sequences are packaged in many loops which are not bound along their length to the residual matrix structure. This is in contrast to the behavior observed for two other large tandemly repeated sequences (see below).

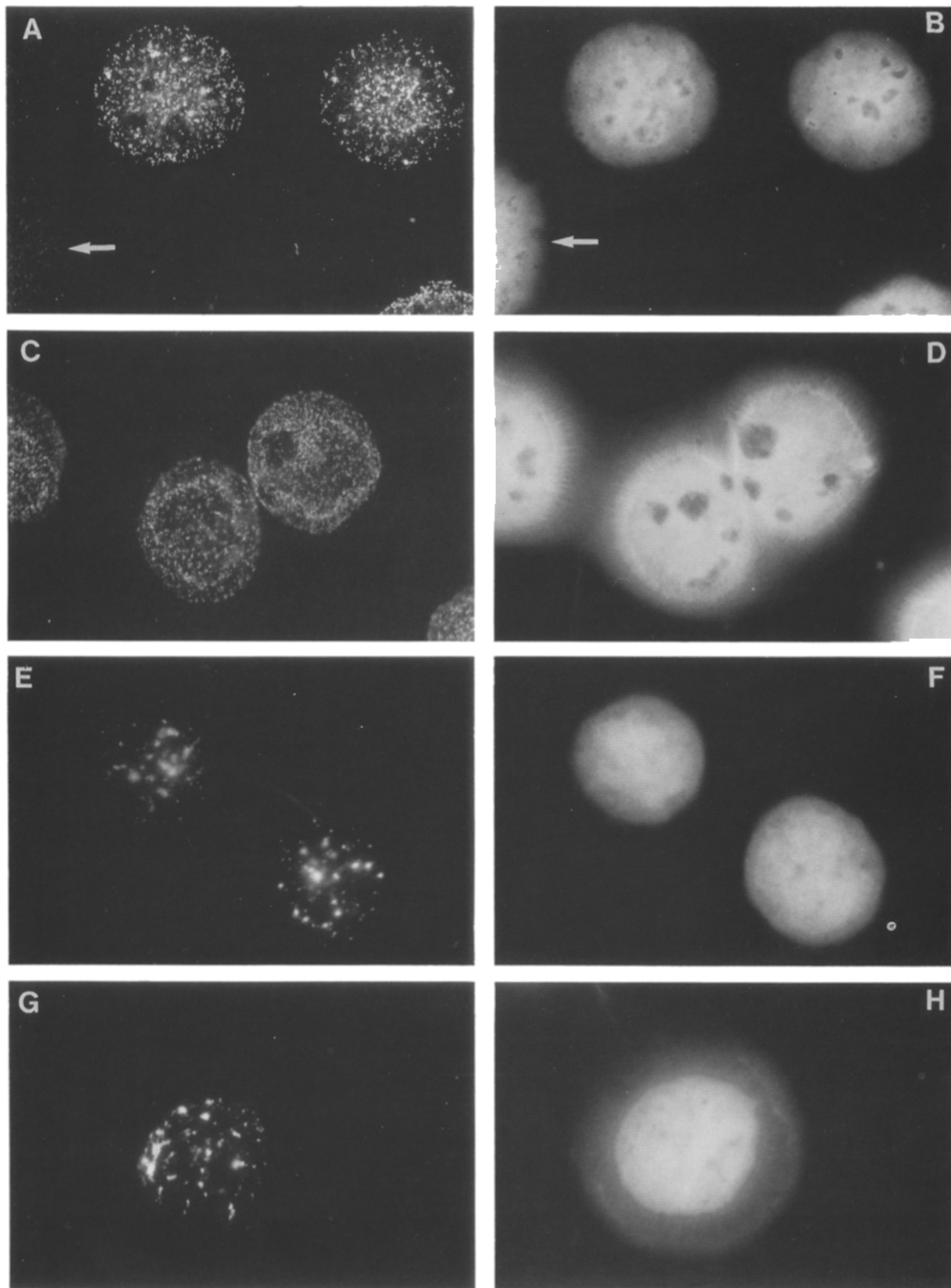


Figure 3. (A and E) Detection of nuclear pore proteins (A) and SC-35 (E) by immunofluorescence in intact Namalwa cell nuclei (prepared as in Fig. 2, step 1). The cell at left in A is believed to be mitotic due to the absence of nuclear pore staining (arrow). (B and F) Total DNA stain (DAPI) for cells in A and E. (C and G) Detection of nuclear pore proteins (C) and SC-35 (G) by immunofluorescence in Namalwa cell nuclear halos (prepared as in Fig. 2, steps 1 and 2). (D and H) Total DNA stain (DAPI) for cells in C and G.

Table I. Description of Hybridization Probes Used

Gene	Chr.	Probe designation	Insert size	Sequence organization and transcriptional activity in cell studied
Dystrophin	X	24A2 15A DMD	15 kb 16 kb	Sequences ~650-kb apart on the 2.5-Mb single-copy dystrophin gene; both inactive*
Ribosomal protein RPS4X	X	LAO 1	14.1 kb	Single copy; active
U2 snRNA gene cluster	17	pTP18	5.8 kb	~120 kb cluster of 5.8 kb repeats; active
Nerve growth factor receptor	17	C3	32 kb	Single-copy gene; inactive‡
α -cardiac myosin heavy chain	14	p8-1A	13 kb	Single-copy gene; inactive§
Histone H3, H4	1	HHG 6	15.4 kb	Two adjacent single-copy genes; active
5S RNA gene cluster	1	pH5SB	2.3 kb	~200 kb cluster of 2.3 kb repeats; active
Centromere repeat (alphoid)	17	p3.6	2.5 kb	~500–1,000 copies; non-coding
Centromere repeat (alphoid)	18	L1.84	0.68 kb	~2,000 copies; non-coding

Ribosomal protein RPS4X, the U2 snRNA genes, histones H3 and H4, and the 5S rRNA genes are constitutively expressed in all cell lines used. Chromosome 17 and 18 alphoid repeats are non-coding.

* Dystrophin gene expression was detected in only one out of 500–1,000 cultured lymphoblast cells by PCR, and was attributed to basal expression (Chelly et al., 1988).

‡ Nerve growth factor receptor was detected by immunofluorescence in only 1 of 15 lymphoma biopsy specimens, and heterogeneously in 3 of 15 specimens (Garin-Chesa et al., 1988).

§ α -Cardiac myosin heavy chain is expressed in atrial and ventricular cardiac muscle (Saez et al., 1987).

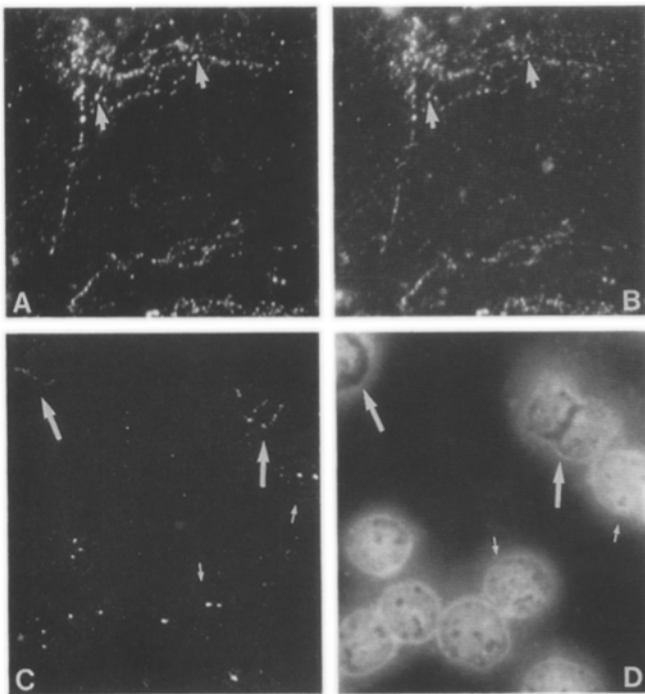


Figure 4. (A–B) Distribution of probe for chromosome 17 satellite sequence in Namalwa cell nuclear halo preparation. Separate samples of probe p3.6 (Table I) were labeled with either biotin- or digoxigenin-conjugated nucleotides, and then hybridized simultaneously to the same sample and detected using different fluorochromes. Note that in most cases the signal from the biotin-labeled probe (A, arrows) localizes to the same individual spots as the digoxigenin-labeled probe (B, arrows), indicating that each spot

Detection and Behavior of Specific Active and Inactive Genes

Having identified successful hybridization conditions for nuclear halos, it was then of primary interest to examine and compare the behavior of different genes. It is important to note that under conditions that remove RNA as used here (see Materials and Methods) hybridization of genomic probes to unfractionated nuclei results in a single pinpoint of fluorescence, as illustrated in Figs. 1 A and 2, due to the high condensation of DNA complexed with histone proteins. This has been well documented in gene mapping studies for virtually hundreds of sequences (as large as a few hundred kb) within interphase nuclei of cytogenetic preparations or paraformaldehyde-fixed intact cells (Lawrence et al., 1988, 1990; Trask et al., 1989, 1991). To investigate the packaging of individual genes after release of DNA loops, the first sequence hybridized was an internal segment of the human dystrophin gene, a 2.5-mb gene that is expressed primarily in muscle and is inactive in lymphocytes (Chelly et al., 1988). It was immediately apparent, that unlike its condensed state in intact nuclei (see Fig. 1 A and Lawrence et al., 1990), in a majority of nuclear halos the DNA detected

represents hybridization of multiple probe molecules. (C) Localization of histone H3/H4 sequence (see Table I) within Namalwa cell nuclear halos. Note that the signal for this transcriptionally active sequence remains tightly condensed within the residual nucleus in halos from interphase cells (small arrows), but is extended in halos from cells believed to be mitotic (large arrows). (D) Total DNA (DAPI) stain for cells in C.

by this probe was released by 2 M NaCl extraction, such that it became extended on the DNA halo and was visualized as a string of small dots after hybridization of the 15-kb probe (Fig. 5, A–C). A second 16-kb dystrophin probe to a region ~650 kb away from the first probe (see Table I) showed a similar behavior. The strings of signal were generally 2–3 microns long, with the longest being 4–5 microns. Thus the length of the extended signal was usually about half that expected for fully extended DNA (5.1 microns for a 15-kb sequence) and well above the expected condensation for either the 10- or 30-nm fibers (see Fig. 2 legend). This result was observed in multiple experiments and is schematically represented in Fig. 2.

Different results were observed for the packaging of the second gene studied, RPS4X, that encodes a ribosomal protein expressed in all cell types tested (Fisher et al., 1990). This transcriptionally active sequence was chosen first for comparison to dystrophin sequences because it is also located on the X chromosome, circumventing the possibility that differential chromosome location within the nucleus could affect the perceived position of sequences with respect to the halo of DNA loops. The 14-kb RPS4X probe signal remained in the residual nucleus as a single discrete spot, similar to that seen in non-fractionated nuclei (Fig. 5 C). Results were quantitated in hundreds of cells in multiple experiments in which the RPS4X probe and the dystrophin probes were analyzed and scored blind by two independent investigators. Data are presented in Fig. 6, which for reasons described below are based on non-S-phase cells. In 86% of nuclear halos the signal from RPS4X was retained as a single spot within the residual nucleus. Similar results were seen in a single experiment done using a probe covering a separate region of the RPS4X gene (data not shown). In contrast, both of the inactive dystrophin sequences produced a string of signal on the extended portion of the loop in >70% of nuclear halos. Analysis using several other probes provided stronger support for the conclusion that individual sequences exhibit characteristic differences in packaging which could be directly visualized by *in situ* hybridization to halo preparations. Using the same approach, packaging of several gene sequences with varying transcriptional activity, size, and chromosomal location, including nerve growth factor receptor, α -cardiac myosin heavy chain, histone (H3 and H4), and U2 snRNA was evaluated. As summarized in Fig. 6 (see also Fig. 5, D and E), sequences demonstrated essentially one of two behaviors: they either remained as a single spot within the residual nucleus, or they produced a string of beaded signal on the extended halo, frequently apparent outside the residual nucleus. Also indicated in Fig. 6, in each case the transcriptionally active sequences most frequently remained as a single spot within the residual nucleus, whereas the transcriptionally inactive sequences typically were found on the extended portion of the halo. For example, the myosin heavy chain gene distributed with high frequency on the halo (after 2 M NaCl or LIS extraction, see legend to Fig. 6), in contrast to the transcriptionally active RPS4X, histone, and the U2 snRNA gene cluster (Fig. 5, D–G, Fig. 6). Despite the large size of the U2 snRNA gene cluster (~120 kb), the probe signal remained consistently as a single condensed spot within the nucleus.

These results provide direct visual evidence for the differential packaging of specific DNA sequences and fur-

ther suggest that this correlates with gene activity, with inactive gene sequences positioned on the extended DNA halo and active sequences remaining as a condensed spot associated with the residual nucleus.

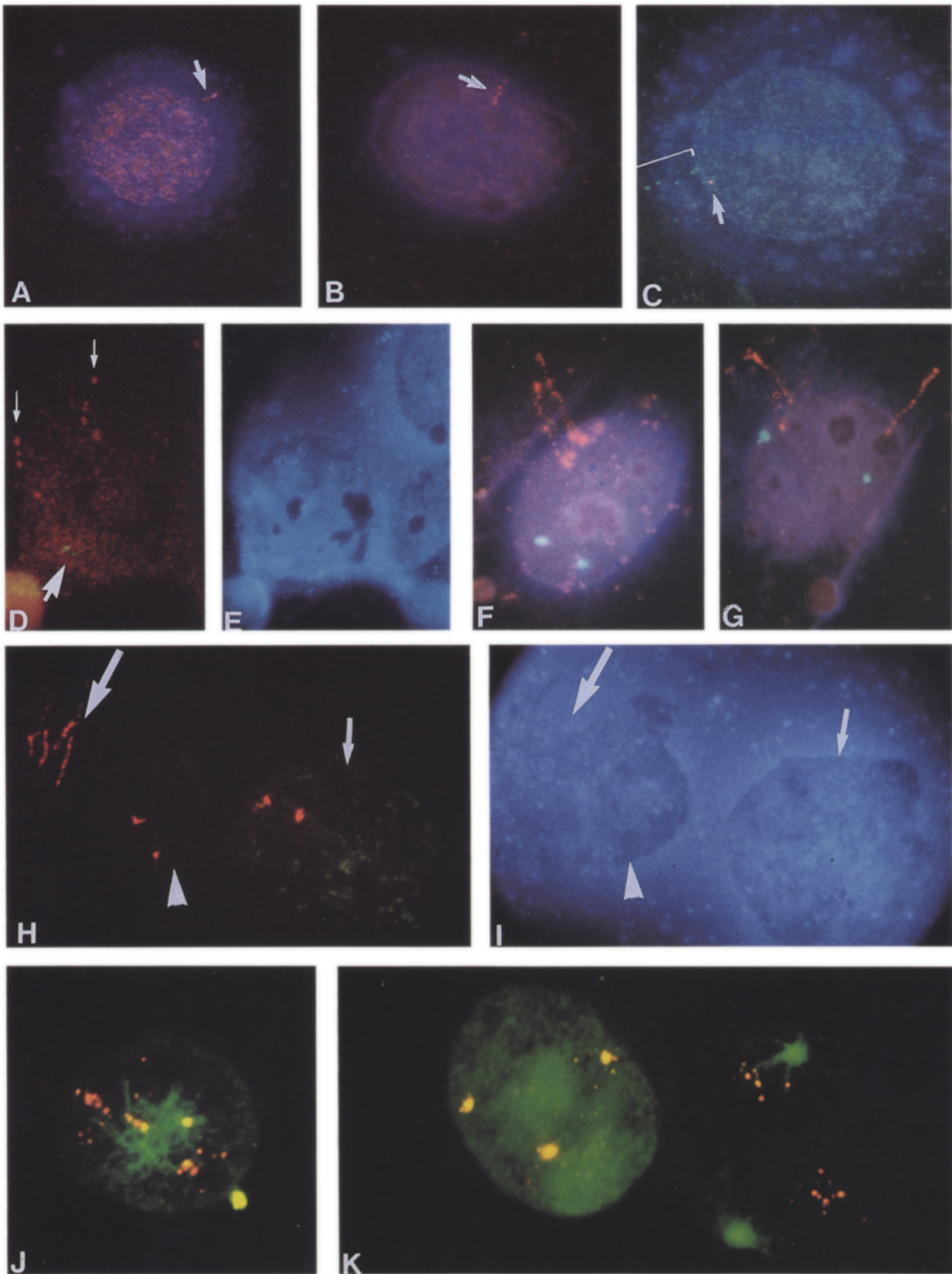
Changes in Specific Gene Packaging during the Cell Cycle: Effects of Replication

The above results demonstrate clear differences in packaging of different sequences which for all eight sequences studied correlates with transcriptional activity. However, for all sequences some degree of packaging variability was seen between cells in a population. This heterogeneity was highest in initial experiments using unsynchronized cells where generally 20–35% of cells exhibited what we refer to as “atypical” chromatin packaging relative to the rest of the population. While some internal variation is likely technical in origin (see below), we reasoned that a significant component may result from potential biological differences in gene packaging during the cell cycle. In particular, changes in the DNA packaging of specific sequences during S-phase may result if there is movement of DNA with respect to loop attachment sites during DNA replication. Hence, an inactive sequence, typically freely extended in this assay, could become more tightly associated with the matrix during that portion of S-phase when it is replicated. Similarly, the ordinarily tight association of active sequences with insoluble nuclear structure may be modified during S-phase as a consequence of movement or rearrangement of associations during replication.

To examine the effects of replication on the positions of these genes relative to the DNA halo, before fractionation and subsequent hybridization, cells were pulsed with BrdU for fifteen minutes to label those in S-phase. Simultaneous analysis of cell cycle stage and halo position of the RPS4X and dystrophin genes, for which the highest level of variability was seen, indicated that the atypical appearance seen in unsynchronized populations could be reduced dramatically by eliminating S-phase cells from the analysis. For example, for both dystrophin gene probes the number of cells producing an atypical single spot within the matrix instead of an extended string of signal out on the loop was <30% in non-S-phase cells, but rose to ~45% during S-phase. Similarly, for RPS4X the percent of atypical signal, in this case extended on the DNA halo, was 14% in non-S-phase cells and 36% during S-phase. This same analysis was repeated with all of the probes described above with an increase in atypical signals being seen during S-phase for all eight sequences (see Fig. 8).

Collectively, these results support the theory that some DNA attachment sites are gained and lost as a result of or during replication. They also explain a significant portion of the heterogeneity observed when non-synchronous cell populations are studied, strengthening the conclusion that the general differences in behavior observed between transcriptionally active and inactive sequences reflect bona fide differences in their *in vivo* associations.

Despite eliminating S-phase cells from the study population, some heterogeneity of results for a given sequence within a cell population was still observed. The belief that this relatively small fraction of atypical cells remaining, ~15–20% or less, is due primarily to technical variation is supported by comparisons between the behavior of identical



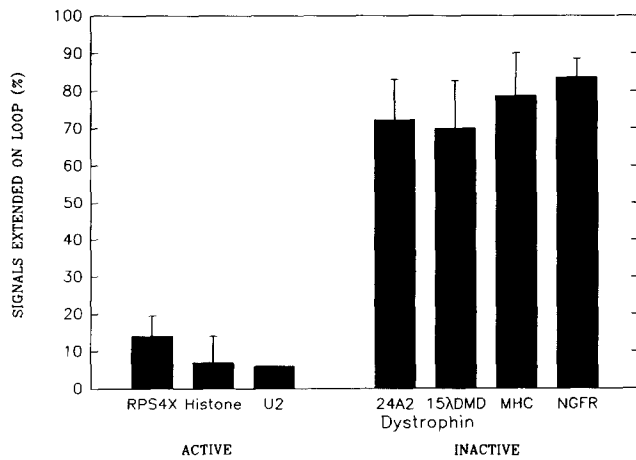


Figure 6. Quantitation of percent of gene signals which were extended on the loop portion of individual halos. Namalwa cell nuclear halos were prepared as shown in Fig. 2, hybridized and individual gene signals on a given halo scored in "blind" fashion as either "extended" or "nonextended." The data are graphed according to the percent of signals in a given experiment that were extended (see Materials and Methods for details of scoring and Table I for probes used), and represent data from non-S-phase cells only. Each bar, with the exception of U2, represents the average (\pm SD) of at least three separate experiments in which $>85\%$ of individual halos had "scoreable" signal. The large U2 RNA gene cluster was consistently preserved as a condensed spot within the residual nucleus in multiple experiments obviating the need for extensive statistical analysis. In single experiments comparing LIS vs NaCl extraction, α -cardiac myosin heavy chain (MHC) and nerve growth factor receptor (NGFR) had 87 and 85% extended signal, respectively, as compared to their respective averages of 79 and 82% with NaCl.

gene sequences from the two homologous chromosomes within the same cell. For example, using either NGFR or histone gene probes there was 85% identity in the packaging observed for the same gene on two homologous chromosomes within the same cell. This is most consistent with each allele on two homologous chromosomes having essentially identical packaging, with the differences in behavior of $\sim 15\%$ resulting from technical variation.

The Unique Behavior of Active Genes in Mitotic Preparations

Although it has long been of interest, little is known concerning the extent to which structural associations that exist in interphase nuclei are maintained during mitosis, and vice-versa. Halos prepared from cycling cell populations were closely examined to attempt to identify mitotic cells and then to examine the packaging of specific sequences within them. Results from two transcriptionally active sequences, histone and U2 snRNA genes, quickly revealed a marked difference in the packaging of interphase and mitotic chromatin for these genes as visualized by this assay. For example, in samples hybridized with either histone or U2 gene probes, which typically are retained as a tight focus of signal in the residual nuclear region of the halo preparation, a small fraction of cells showed unusually long extended strings, comprised of many tiny spots (Fig. 4, C-D; Fig. 5, H-I). Several consistent observations suggested that these cells were mitotic. They lacked visible nucleoli or residual nuclear membrane and they did not incorporate BrdU. Additionally, nuclear pore proteins, known to be absent during mitosis were not detectable by immunofluorescence (see Fig. 3, A and B). On occasion, a black filamentous structure was present, which we believe may represent the chromosome scaffolds shown by others (Paulson and Laemmli, 1977; for review see Gasser and Laemmli, 1987). Analysis of results showed that over 90% of cells identified as mitotic based on the above criteria exhibited extended strings of signals rather than single spots retained on the matrix (Fig. 7).

To confirm the identification of mitotic cells, and to corroborate these results, the pattern of the U2 snRNA gene was investigated in mitotically enriched HeLa cell populations obtained by physical shake-off. Two methods were used to unambiguously identify mitotic cells with the enriched population. First, we used an antibody to NuMA, a nuclear mitotic apparatus protein. In interphase, NuMA is found in a fine punctate pattern overlaying a diffuse protein signal (Fig. 5 K, left). During mitosis, it is reorganized to the polar region of the mitotic apparatus; hence mitotic cells are readily identifiable by the appearance of NuMA decorating the spindle apparatus (Fig. 5, J and K right; Lydersen and Pettijohn, 1980). We observed this pattern for NuMA in

Figure 5. (A-B) Hybridization and detection of probe 24A2 to the transcriptionally inactive dystrophin gene (red; arrow) in Namalwa cell nuclear halos. Two examples of extended or "looped out" signal are shown. Total DNA-blue. (C) Simultaneous detection of X-chromosome probes to the transcriptionally active, non-extended RPS4X gene (red, arrow; probe LAO1) and the inactive, extended dystrophin gene (green, bracket; probe 24A2) in Namalwa cell nuclear halos. See text for discussion of "gaps" between hybridizing signals in the extended dystrophin gene. Total DNA (blue). (D) Namalwa cell nuclear halos hybridized simultaneously with probes to the transcriptionally active histone H3 and H4 gene region (green, large arrow) and the transcriptionally inactive α -cardiac myosin heavy chain gene (red, small arrows). (E) Total DNA stain (blue) of nuclear halos from D. (F and G) Human fibroblast nuclear halos hybridized simultaneously with probes for the U2 snRNA gene cluster (green) and the 5S RNA gene cluster (red; total DNA [blue]). Note the two continuous 5S rDNA loops seen clearly in F. (H) Three Namalwa cell nuclear halos showing simultaneous detection of replicating DNA (green; detection of incorporated BrdU) and the transcriptionally active U2 snRNA gene cluster (red). Three nuclear halos are shown, representing cells in S-phase (right, small arrow; stained positive for BrdU), non-S-phase (middle, arrowhead), and what appears to be mitosis (left, large arrow). Note the extreme distention of the gene signal in the putative mitotic cell. (I) DAPI staining of nuclear halos from H. A structure resembling a residual mitotic scaffold can be seen in upper left corner (large arrow), partially overlaying the residual nucleus from the adjacent halo (arrowhead). (J) Simultaneous detection of the nuclear mitotic apparatus protein NuMA (green) and the transcriptionally active but distended U2 snRNA gene cluster (red) in nuclear halo preparations of mitotic HeLa cells. (K) Differential chromatin packaging of the U2 snRNA gene cluster at interphase versus mitosis. At interphase (left), NuMA (green) is found in a fine punctate pattern overlaying a diffuse protein signal. The U2 gene cluster (yellow) remains essentially tightly condensed in the residual nucleus. In mitosis (right), NuMA is reorganized to the polar regions of the mitotic apparatus and the U2 gene cluster signal (red) is seen to extend.

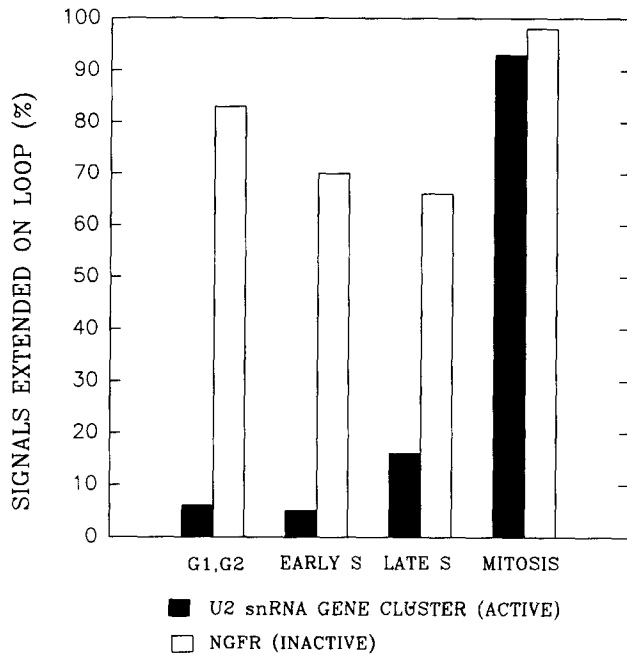


Figure 7. Quantitation of percent of gene signals extended on the loop portion of individual halos throughout the cell cycle. Nuclear halos were prepared from exponentially growing, asynchronous Namalwa cultures that were pulsed for 15 min with BrdU to label cells in S-phase before harvesting, and then hybridized and the gene probe signal and BrdU detected simultaneously (see Fig. 5 H). Thus early and late S-phase cells were identified by the presence and pattern of BrdU-containing replication complexes (see Fig. 1 E). Mitotic cells were identified based on morphology and the absence of nuclear pore antibody staining (see text and Fig. 3 A). Signals were scored and plotted as in Fig. 6.

both intact and nuclear halo cell preparations (data not shown). Second, we observed that the SC-35 spliceosome assembly protein is absent from biochemically fractionated cells during mitosis, consistent with results from Spector et al. (1991) showing a cytoplasmic localization of SC-35 during mitosis. In simultaneous immunofluorescence detection experiments, most cells exhibiting a spindle pattern for NuMA contained no SC-35 (data not shown). When simultaneous *in situ* hybridization and immunofluorescence experiments were performed on the HeLa cell mitotic shake-off halo preparations, ~80% of cells positively identified as mitotic by NuMA staining showed a consistent decondensation and extension of the U2 snRNA gene cluster (Fig. 5, J and K right), as did 85% of cells identified as mitotic based on the absence of SC-35 staining (data not shown). In both series of experiments, the U2 DNA signal in mitotic cells was clearly and consistently more distended than in interphase nuclei, suggesting a profound change in structural organization at mitosis. This differential packaging of DNA during mitosis further underscores the dynamic nature of loop domain/matrix organization during the cell cycle as seen with this assay (Fig. 7). In addition, these results account for another significant fraction of the heterogeneity or atypical class of signals observed within a cycling population.

The 5S RNA Gene Cluster Is Packaged on a Single Loop Which Freely Extends from the Nucleus

After the above experiments, we investigated the organiza-

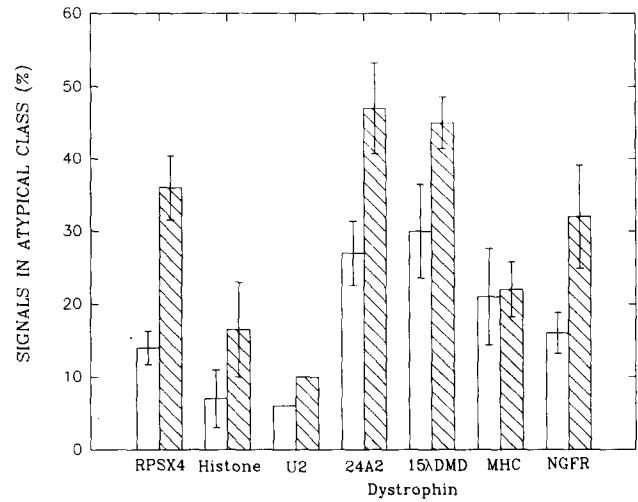


Figure 8. Quantitation of change in atypical class of signal for individual sequences during S-phase. Namalwa cell nuclear halos were hybridized as for Figs. 6 and 7, using a terminal BrdU pulse labeling to identify S-phase cells. The percent of total signal that was judged atypical is shown (i.e., extended signal for RPS4X, histone, and U2, and non-extended signal for 24A2, 15ΔDMD, MHC, and NGFR). Open bars represent non-S-phase cells and hatched bars S-phase cells. Data represent average (\pm SEM) of minimally three separate experiments (with the exception of U2, which represents data from one experiment) in which >85% of individual halos had scoreable signal (see Fig. 6).

tion of the 5S RNA gene complex relative to DNA halos. Interestingly, this cluster of tandemly repeated genes, similar in many respects to the U2 snRNA gene cluster, exhibited a markedly different packaging. In humans, most 5S RNA genes are localized in a cluster of ~100 tandem copies of a 2.3-kb sequence (Little and Braaten, 1989; Sorensen and Frederiksen, 1991) and are transcribed by RNA polymerase III (for review see Geiduschek and Kassavitis, 1990). The U2 gene cluster contains ~20 copies of a 6.2-kb repeat (Van Arsdell and Weiner, 1984; Westin et al., 1984) and is transcribed by RNA polymerase II (for review see Hernandez, 1992). Although the number of genes that are transcribed within either of these human gene clusters has not been determined, both are expressed at extremely abundant levels (Reddy and Busch, 1981). Analysis of halos from human fibroblasts showed that the 5S RNA gene cluster is consistently released on one very large single string from interphase nuclei (Fig. 5, F-G), and in some nuclei this string of signal could be seen to form a loop (Fig. 5 F). Simultaneous two-color hybridization demonstrated that the 5S RNA gene cluster consistently localized on the distended portion of the DNA halos, in the same nuclei in which U2 remains as a condensed spot within the residual nucleus (Fig. 5, F-G). Within a given cell, the allelic sequences on each homologous chromosome behaved in an identical fashion, with most of 5S RNA gene clusters forming a highly extended loop under the same conditions in the same cells in which highly distended U2 gene signals were rarely observed.

The results with the 5S RNA gene cluster are also notably different from the above results obtained with the centromeric satellite sequences, which represent blocks of small repeats comprising on the order of 1-2 Mb of DNA. The 5S RNA gene cluster generally appeared as a single loop or strand emanating from a point of origin within the residual

nucleus (Fig. 5, *F-G*). In contrast, the centromeric sequences produced a burst of signal in a large number of punctate strands (Fig. 1 *D*). These results demonstrate a clear difference in the packaging of tandemly repeated sequences for U2, 5S RNA, and satellite sequences.

Discussion

The development and application of a novel assay for chromatin packaging has revealed sequence-specific differences that are consistent with a loop/matrix organization of DNA. In addition to providing an empirical survey of the cytological configuration of several specific sequences during the cell cycle in histone depleted nuclei and chromosomes, this work provides the precedent for an approach to investigate higher-order chromatin folding by coupling high-resolution *in situ* hybridization with specific nuclear fractionation techniques. Although we have used primarily a single fractionation protocol (2 M NaCl) throughout these experiments, the same direct microscopic approach should eventually be amenable to studying the molecular cytology of DNA folding using a variety of other techniques to extend chromatin or extract nuclei, each of which may disclose different aspects of chromatin and nuclear architecture.

The distribution of sequences with respect to nuclear halos comprised of putative chromatin loops suggested two clearly different structural arrangements: a given sequence either remained condensed as a single spot within the residual nucleus or it produced a string of tiny spots up to several microns long on the extended portion of the DNA halos. The observed configuration of a sequence in most cases correlated with its functional status, with regard to both replication and transcription. High-resolution visualization of BrdU incorporated DNA in nuclear halo preparations in our assay showed nascent replicating DNA localized at the base, with essentially no signal detectable in the extended portion of the halo. The clustered patterns of replication characteristic of unfractionated cells were retained in nuclear halos, consistent with previous studies showing the native distribution of replicating DNA within the matrix (Nakayasu and Berezney, 1989). The movement of replicated DNA onto the halo progressed over a period of several hours, consistent with a model where DNA replication occurs at a fixed site and replicated DNA has now been released to extend onto the halo. The overall packaging of transcriptionally active and inactive gene sequences was investigated using probes encompassing 10–100 kb of genomic DNA. Quantitative analysis showed that four transcriptionally inactive sequences each produced a string of distended signal, whereas four transcriptionally active sequences remained tightly condensed within the residual nuclear structure after removal of histones and the majority of nuclear protein. Consistent with this, the transcriptionally inactive telomere repeat sequence was shown to distend in decondensed hamster sperm nuclear halos (de Lara et al., 1993). In contrast to the tandemly repeated U2 snRNA gene cluster, the similarly sized 5S RNA tandem gene cluster was not retained in a highly condensed spot, but consistently released on a single highly distended DNA loop. The reason for this very different behavior relative to other transcriptionally active genes is not clear, however, it is interesting to speculate that this difference may re-

late to the fact that the 5S RNA genes are the only sequences studied transcribed by the polymerase III transcriptional machinery. If the difference between 5S and U2 results reflect differences in constitutive internal MARs within the repeat sequences (see below), it is interesting to note that the U2 gene attachment sites are apparently completely lost on the mitotic chromosome.

A major advantage of the experimental approach used here is that sequence packaging in single cells at different stages of the cell cycle can be analyzed, providing evidence for dynamic changes in the behavior of a given DNA segment in S-phase as well as mitotic cells. The higher degree of "atypical behavior" observed for individual gene probes in S-phase as compared to non-S-phase cells supports the results on replicating DNA, indicating that DNA sequences become transiently associated with the matrix or base of the loop during the replication of that specific sequence. Not only are new associations formed during S-phase, but results suggest that the association of transcriptionally active sequences with the matrix may be temporarily abrogated since there is an increase in atypical nuclei in which a transcriptionally active sequence becomes distended on loops. These observations are consistent with the idea that the DNA may be in dynamic flux as it moves relative to replication complexes (Berezney and Coffey, 1975; for review see Cook, 1991; Jackson, 1991; Berezney, 1991) and with studies suggesting that some functionally significant structural rearrangement of DNA may occur during replication, so as to effectuate heritable changes in cell commitment or gene expression (for review see Bodnar et al., 1989a; Villarreal, 1991). Finally, a marked difference in the structural associations of specific genes in mitotic and interphase cells is revealed by this assay, such that sequences which remain tightly condensed within residual interphase nuclei are freely distended from the residual scaffold of mitotic chromosomes. While not conclusive proof, this result is consistent with a model of gene organization comprising multiple attachment sites between each gene within the cluster. In the case of histone, the presence of MARs in the intervening sequences between each of the clustered histone genes has been described in *Drosophila* (Mirkovitch et al., 1984) based on analysis of interphase cells. The fact that typically only one continuous string of signal is observed in extracted mitotic cells indicates that the entire cluster of genes detected by both of the probes used is free to distend as one mitotic chromosome loop, with no attachment sites remaining within the sequence.

Several considerations are important in evaluating the biological implications of the associations observed, including the question of the extent to which they reflect associations within the intact cell. While not specifically demonstrated here, it is plausible that at least two general categories of matrix association may exist *in vivo* (for review see Bodnar, 1988), each of which may eventually be amenable to analysis by this approach. First, there are likely attachments involved in the generic condensation of DNA through the formation of chromatin loops, which may be mediated through permanent sites that would exist in all cell types. Second, as several earlier studies have suggested (for example, Ciejek et al., 1983; Robinson et al., 1983; Phi-Van et al., 1990) and our results support, there may be more transient associations which impact the DNA loop domain organization of a given DNA segment and vary with functional status. Evidence for the

former has been derived from studies in which specific small sequences, typically only several hundred base pairs in length, associate preferentially with the residual scaffold prepared by LIS extraction or bind *in vivo* to a matrix prepared by 2 M NaCl (for example: Mirkovitch et al., 1984; Cockrill and Garrard, 1986). Assuming these associations exist within the intact cell they likely represent constitutive sites for DNA folding. In addition, the potential functional implications of these SARs or MARs as chromatin boundaries which compartmentalize genes relative to regulatory elements has also been suggested by experiments with transgenic mice (discussed in Gasser and Laemmli, 1987) and for DNA viruses, where replication origins and immediate early gene promoter elements localize close to matrix attachment regions (Bodnar et al., 1989; Jankelevitch et al., 1992). Evidence for transient associations is suggested by Jackson and Cook (1993) who report that only transcribed viral minichromosomes are attached to the nuclear skeleton.

In contrast, a potentially different type of association is observed in this study based on visualizing the overall packaging of comparatively long stretches of DNA (in some cases greater than 100 kb) in which the entire sequence appears either associated with the residual nuclear structure or free to distend from it on an extended DNA strand. Because the differential packaging observed is sequence specific and correlates with transcriptional status (for several pol II transcribed genes studied) as well as with replicative status of DNA in general, it is clearly non-random and very likely reflects associations of functional subsets of DNA that exist in some form within the intact cell. However, it cannot be concluded that the packaging observed is a prerequisite rather than a consequence of transcription or replication, since it is possible that the specific DNA sequences are retained in the 2 M NaCl extracted nuclei by virtue of their association with transcription complexes or the DNA polymerase. If so, the polymerases themselves may be associated with an insoluble substructure or "matrix", although the concern has also been raised that the high salt extraction may cause rearrangement of native associations or precipitation of transcription or replication complexes (Mirkovitch et al., 1984; Cook, 1989). However, this is argued against by the results of Jackson and Cook (1993) who find a retention of only transcribed viral "minichromosomes" to the nuclear skeleton after extraction in a physiological buffer that presumably does not precipitate transcription complexes. Also, in the case of replicating DNA where this has been studied extensively, the evidence is strongest that its retention with the insoluble nuclear material is independent of the extraction procedures used, including 2 M NaCl, LIS, or isotonic extraction of nuclei embedded in agarose (for example, Vaughn et al., 1990; for review see Cook, 1991). While we cannot rule out the possibility that in our procedure high salt extraction causes adherence of transcription complexes to the residual nucleus, several observations are less consistent with this. For example in the U2 snRNA gene cluster, only a small portion (<1 kb) of the targeted repeat is transcribed while the remainder (~5 kb) is not (Hernandez, 1992). The resolution of DNA sequences is high enough when the DNA is extended (~2 kb, see Fig. 1 B) that some extension of sequences might be detected from the U2 gene cluster, should it occur. In addition, if a substantial fraction of the 5S RNA genes are indeed transcribed as are the larger ribosomal

RNA genes (Reddy and Busch, 1981), then the free distention of these sequences on an extended loop argues against interpretations based merely on precipitation of large transcription complexes. Finally, it should be noted that in gene mapping methods which apparently extend DNA at random (Wiegant et al., 1992; Lawrence et al., 1992 and above) using 2 M NaCl for extraction, no differential association or "precipitation" of transcriptionally active sequences has been observed, presumably because the specific protocol used results in increased physical disruption of the native structure. Many questions remain concerning the nature of the insoluble nuclear matrix, however our results support and extend the idea that transcriptionally active DNA and replicating DNA are preferentially associated with a highly insoluble non-chromatin nuclear material.

Irrespective of the precise mechanism responsible for the differential packaging of specific sequences, the molecular cytological approach developed provides a means whereby differences in the higher-order folding of individual DNA sequences can be detected and evaluated at a single cell level. This approach provides information on chromatin packaging which is distinct from, but may be complementary to, assays based on differential DNase sensitivity. However the basis for the well-established DNase sensitivity that surrounds active genes in regions of tens to hundreds of kb, or even the basic level of chromatin packaging it reflects, remain essentially unknown. In contrast, it is relatively clear that the differential packaging observed here between specific sequences is clearly most consistent with models of DNA loop organization where these genes would possess different loop/matrix associations. In addition to the specific information obtained using this assay, a major significance of this work may be to provide a novel approach to the study of chromatin organization.

The authors wish to thank Dr. E. G. Fey for helpful discussion, Christine Dunshee for her excellent photographic assistance and Heather-Lyn Haley for her work assisting in the preparation of this manuscript.

This work was supported in part by a National Institutes of Health (NIH) grant R01-GM49254-02, a Human Genome Project grant 2-R01-HG00251-06, and an NIH Research Career Development Award HG00002-03 to J. B. Lawrence, an NIH Postdoctoral Fellowship F32-HG00026-01 to K. F. Carter, and Muscular Dystrophy Association Postdoctoral Fellowships to K. F. Carter and P. T. Moen, Jr.

Received for publication 10 October 1993 and in revised form 26 April 1994.

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