Sensitive, High-Resolution Chromatin and Chromosome Mapping In Situ: Presence and Orientation of Two Closely Integrated Copies of EBV in a Lymphoma Line

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Summary

Here we describe development and application of highly sensitive fluorescence methodology for localization of single-copy sequences in interphase nuclei and metaphase chromosomes by nonisotopic in situ hybridization. Application of this methodology to the investigation of Epstein-Barr virus integration in the Namalwa lymphoma cell line has revealed two EBV genomes closely integrated at the known site on chromosome 1. Detecting sequences as small as 5 kb, we further demonstrate resolution within interphase nuclei of two fragments of the viral genome spaced only 130 kb apart. Results indicate that the viral genomes are in opposite orientations and separated by roughly 340 kb of cellular DNA. This work demonstrates the feasibility and resolving power of interphase chromatin mapping to assess the proximity of closely spaced DNA sequences. Implications for virology, gene mapping, and investigation of nuclear organization are discussed.

Introduction

Development of improved in situ hybridization techniques has made this method an increasingly important molecular tool for detecting specific nucleic acid sequences directly within cells or genomes. Since this methodology was initially described, it has been widely applied to the localization of DNA sequences in polytene chromosomes or highly reiterated sequences in metaphase chromosomes (Gall and Pardue, 1969; Evans et al., 1974). While applications of in situ hybridization have been generally restricted to highly represented sequences, in recent years it has been possible to localize single-copy sequences on metaphase chromosomes using [125]I- or ^{3}H-labeled probes (Gerhard et al., 1981; Harper et al., 1981). Due to the scatter of radioactive disintegrations, the resolution of this approach is limited to only relatively large chromosomal segments. Furthermore, localization of the sequence is not determined directly within a single metaphase spread; because the signal generated is very low relative to the background, identification of the site of localization requires statistical analysis of autoradiographic grain distribution in as many as 50–100 metaphase figures. Because of these limitations, it has not been possible with existing techniques to localize single-copy sequences within the interphase nucleus.

Our laboratory has been involved in the further development and application of in situ hybridization using isotopically and nonisotopically labeled probes (Singer and Ward, 1982; Lawrence and Singer, 1985; Singer et al., 1986) with the general goal of understanding how localization and organization of specific nucleic acid sequences within the cell relate to cell function. We have provided evidence for the nonrandom organization of mRNAs within the cytoplasm of somatic cells: mRNAs for different cytoskeletal proteins are concentrated in specific cellular areas (Lawrence and Singer, 1986). To further this investigation as well as extend it to an analysis of nuclear organization of specific sequences, a method to detect single-copy sequences with high resolution is necessary. Fluorescence detection of nonisotopic probes provides the highest resolution possible at the light microscope level. Since nick translated probes containing a biotinated nucleoside analog were first described by Langer et al. (1981), the higher resolution afforded by a variety of nonisotopic techniques has been applied in situ to analysis of reiterated sequences (Wu and Davidson, 1981; Bauman et al., 1981; Langer-Safer et al., 1982; Manuelidis et al., 1982; Manuelidis and Ward, 1984; Fostel et al., 1984; Albertson, 1984; Pinkel et al., 1986; Hopman et al., 1986) or large (25–50 kb) unique sequences using pooled DNA clones or cosmid vectors as probes (Landegent et al., 1985, 1986; Albertson, 1985). In the work presented here, we describe highly sensitive methodology for localization of individual DNA sequences using fluorescence detection of biotinated probes hybridized in situ. Because of the unusually low background and high hybridization efficiency achieved, this methodology allows direct, high-resolution localization of single sequences not only on chromosomes but, more importantly, within the interphase nucleus.

We have used this approach to investigate the integration of the Epstein-Barr virus genome into a known site on a single homolog of human chromosome 1 of the Namalwa cell line (Henderson et al., 1983). An apparently intact viral genome is present in this Burkitt's lymphoma cell line that produces neither episomes nor viruses (Matsuo et al., 1984). The nuclear organization of the integrated viral genome has been studied by autoradiographic in situ hybridization (Henderson et al., 1983) as well as Southern blot analysis and sequencing (Matsuo et al., 1984) in an effort to understand how the details of viral integration in this line relate to cell transformation and viral gene expression. We have used progressively smaller fragments of the EBV genome to test the sensitivity and resolving power of our technique while conducting a molecular analysis in situ of viral integration in this cell line. Our results reveal the presence of two integrated copies of the EBV genome and further allow us to extrapolate both the orientation of the integrated genomes as well as the approximate distance between them. Finally, analysis of the nuclear localization of the EBV genome integrated into chromosome 1 provides preliminary support for the ordered arrangement of DNA sequences within the interphase nucleus.
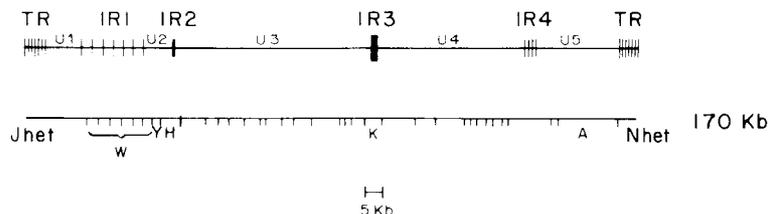
Figure 1. Physical Map of the EBV Genome Indicating the BamHI Restriction Fragments

This map was modified from Henderson et al. (1983). The nomenclature is in accord with that of Skare and Strominger (1980) from whom we derived our clones. Only those fragments that we used as hybridization probes are indicated on the map.

Results

Preparations of metaphase chromosomes and interphase nuclei of the Namalwa cell line were prepared and hybridized as described in Experimental Procedures. The specific methodological protocol described was derived from results of quantitative, analytical experiments for optimization of in situ hybridization to chromosomal DNA. Following an approach similar to that previously employed for characterization of in situ hybridization to mRNA (Lawrence and Singer, 1985; Singer et al., 1987), a series of experiments was conducted utilizing ^{32}P-labeled human genomic DNA hybridized to samples of human nuclei and chromosomes. Hybridization was quantitated by scintillation counting, and the parameters evaluated included probe concentration, hybridization time, probe size, hybridization temperature, chromatin denaturation conditions, chromosome preparation and "hardening," RNAase conditions, and use of nonspecific competitors and acetic anhydride for reduction of backgrounds. While results of all these analyses cannot be detailed here, they were incorporated with aspects of previously existing methodology (Gerhard et al., 1981; Harper and Saunders, 1981) to derive the technique utilized. Presentation of salient technical modifications is restricted to Experimental Procedures except in a few instances where results of quantitative methodological experiments are described below.

Sensitive Detection of EBV Sequences on Chromosomes and in Nuclei: Evidence for Two Integrated Genomes

In initial experiments, samples were hybridized with the BamHI W fragment of EBV, a sequence approximately 3 kb long which is reiterated 6–10 times in the EBV genome (Figure 1). After hybridization, the biotinated W probe was detected using fluorescein-conjugated avidin while in the same sample chromosomes and nuclei were visualized with the DNA fluorochrome, DAPI. Hybridization to Namalwa cells resulted in bright fluorescein spots in each metaphase figure and nucleus. It was immediately apparent by the labeling in identical positions of each sister chromatid on the short arm of chromosome 1 that the spots over metaphase figures represented bona fide signal (Figures 2A and 2B), which is consistent with the previous localization of the EBV genome to 1p35 (Henderson et al., 1983). Hybridization efficiency was high and background spots over chromosomes or glass were few. For instance, of 30 randomly selected metaphase figures, all exhibited labeling on the short arm of chromosome 1, and in all cases both sister chromatids were labeled. Of 65 fluorescent

spots observed, only 5 were randomly dispersed over chromosomes, hence over 92% represented genuine hybridization.

The conclusion that fluorescent spots observed in almost all interphase nuclei represented detection of EBV DNA was supported not only by the high signal-to-noise ratio on our samples, but also by the observation that the number and placement of the spots in nuclei was clearly nonrandom. Most nuclei had two spots that were closely spaced within 0.2–3.3 μm of each other (Figures 3A–3C), whereas nuclear diameters ranged from approximately 10–24 μm. Larger nuclei sometimes had two pairs of spots, as illustrated in Figures 3A and 3B. Since total DAPI fluorescence is directly proportional to DNA content (Coleman et al., 1981), these larger nuclei appear to have a duplicated DNA content relative to diploid G1 cells, and therefore, would be expected to show twice as much signal. The frequency of nuclei showing two pairs of spots (up to 12%) is consistent with the expected proportion of G2 cells in a dividing culture. Further evidence that spots in interphase nuclei represented bona fide hybridization was that they were not observed in negative controls, such as hybridization with pBR322, hybridization of the EBV probe to nuclei of other cell types or to nondenatured Namalwa nuclei, or DNAase-treated Namalwa nuclei. These results indicate that this in situ hybridization methodology can provide sensitive, reproducible detection of approximately 30 kb of EBV DNA in metaphase chromosomes and, more importantly, in interphase nuclei.

We next tested a probe for the BamHI A fragment of EBV, which is a single sequence of 12 kb. Initial experiments revealed very dim fluorescent signals in the correct position on both sister chromatids of chromosome 1. A variety of detectors were tested to increase the intensity of fluorescence, such as rhodamine-conjugated avidin, Texas red-conjugated streptavidin or indirect immunofluorescence using antibodies to biotin. It was found that the fluorescein avidin gave the best combination of low background and good signal. In an attempt to increase signal further, hybridization parameters including probe size, concentration, and time, were varied. We analyzed these parameters quantitatively using ^{32}P-labeled human genomic DNA hybridized to samples of human chromosomes and nuclei (Figure 4). This analysis revealed that hybridization was over within a few hours, presumably due to the rapid reannealment of chromosomal DNA. Although standard chromosomal hybridization techniques call for low probe concentrations in order to avoid high backgrounds (Gerhard et al., 1981; Harper et al., 1981; Harper and Marselle, 1985), our quantitative studies (Fig-
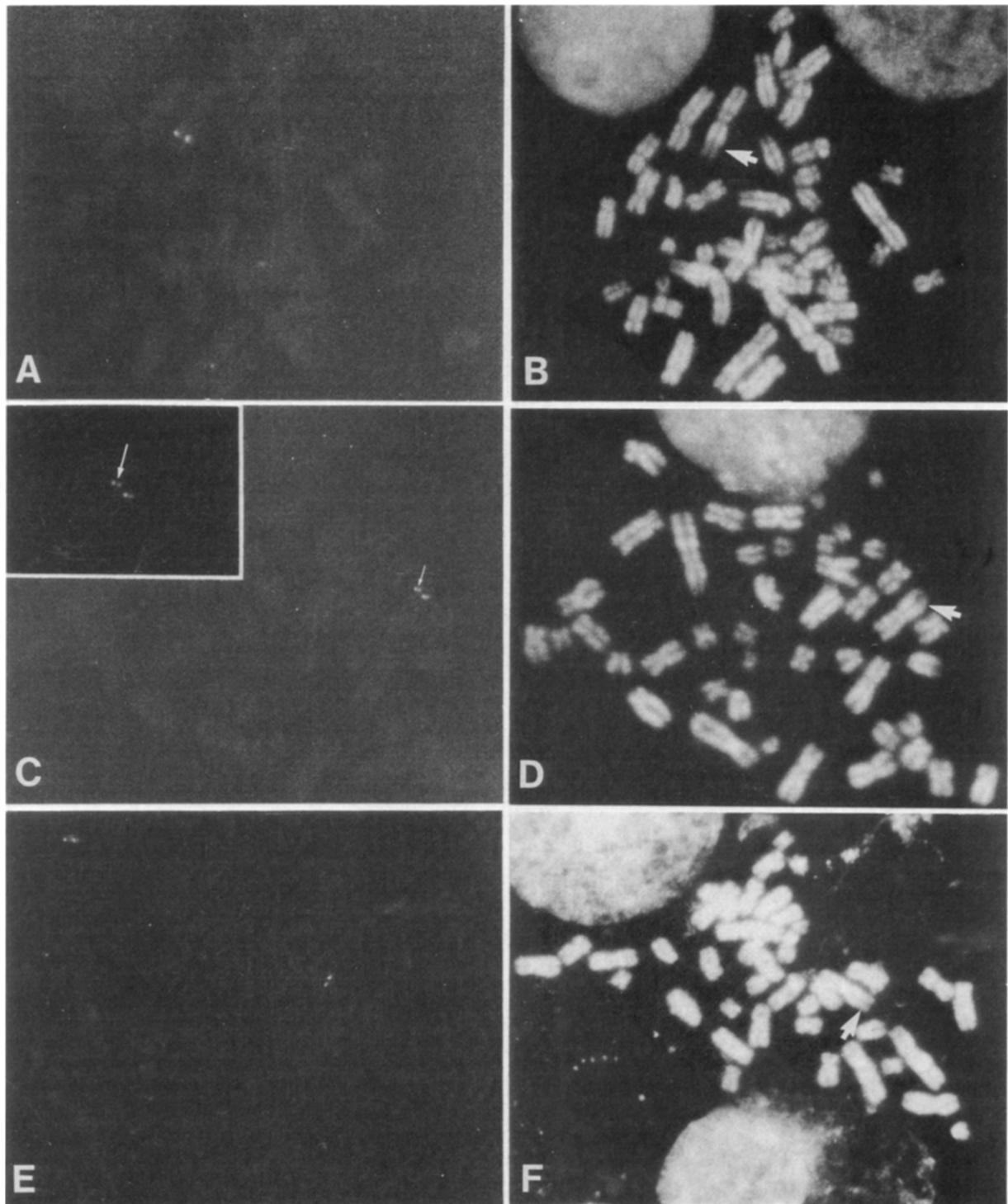
Figure 2. Fluorescence Localization of Single-Copy Sequences on Chromosomes

Recombinant DNA clones containing different size fragments of the EBV genome were labeled with biotin and hybridized to preparations of metaphase chromosomes. No image processing or intensification devices were utilized. All final magnifications are 1700x, except inset of Figure C is 2600x.

(A, C, E) Fluorescein-avidin detection of hybridized probe.

(B, D, F) DAPI fluorescence staining for total DNA. Arrows indicate the position of label on chromosome 1.

(A, B) Probe for BamHI W (21-30kb).

(C, D) Probe for BamHI A (12 kb); inset shows greater enlargement of the label on chromosome 1 indicating the paired signals on each of the two sister chromatids.

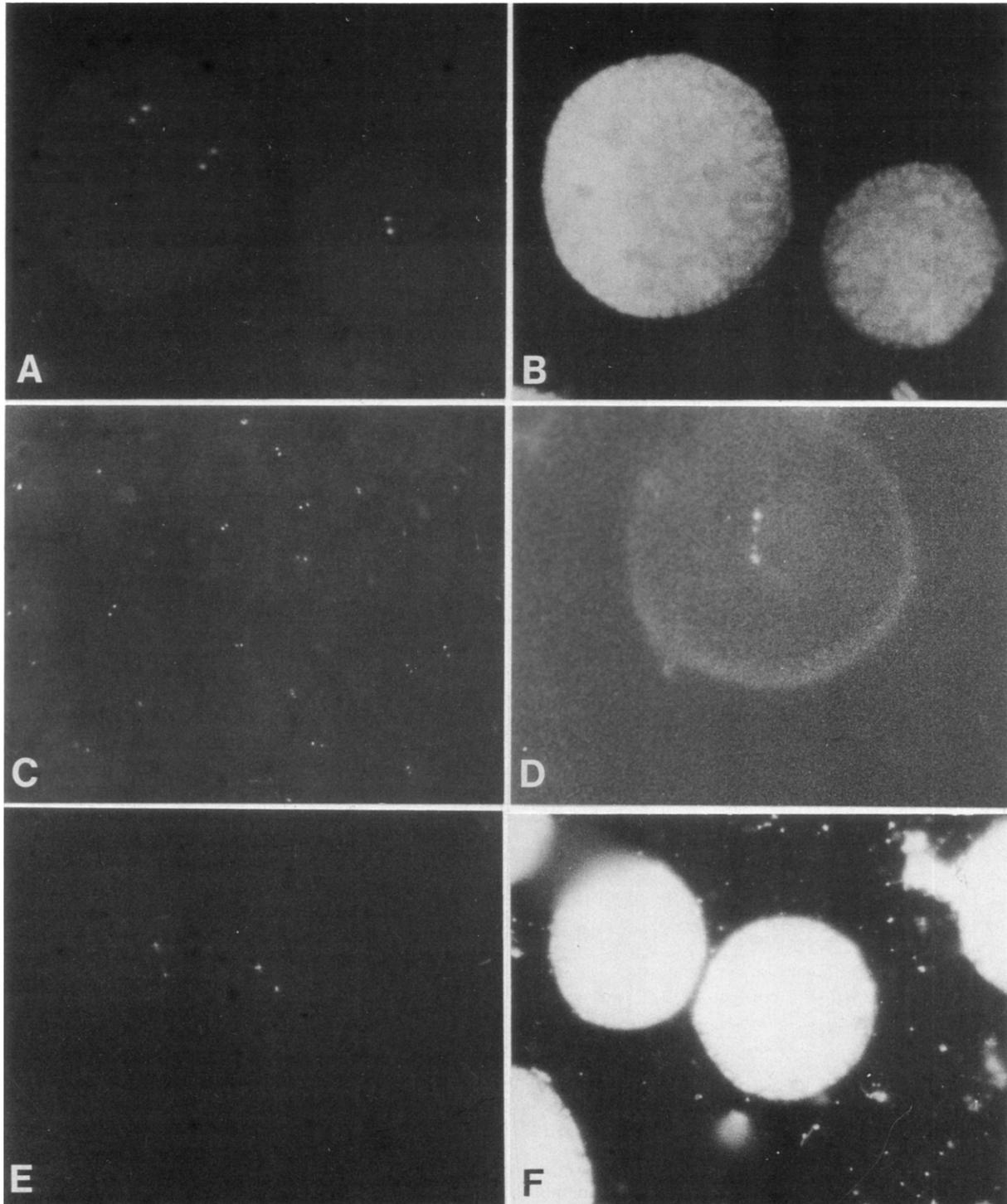(E, F) Probe for BamHI Y/H (7 kb).

Figure 3. Fluorescence Detection and Localization of Sequences within Interphase Nuclei

(A, C, D, E) Fluorescein-avidin detection of hybridized probes, as indicated below.

(B, F) DAPI fluorescence staining of total DNA.

(A, B) Detection using the BamHI W probe. Note that the smaller (G1) cell has one pair of closely spaced spots whereas the larger (G2 or tetraploid) cell has two pairs of spots. (Total magnification, 1600×).

(C) Randomly selected field showing many nuclei hybridized with the W probe. (700×).

(D) Nucleus hybridized with W and A probes simultaneously, showing the presence of four tightly clustered spots of two different fluorescent intensities. (2450×).

(E, F) Detection of BamHI K probe hybridized within interphase nuclei. Each spot represents hybridization to 5 kb of target DNA. (1600×).

PROBE CONCENTRATION (ng)

HYBRIDIZATION TIME (hrs)
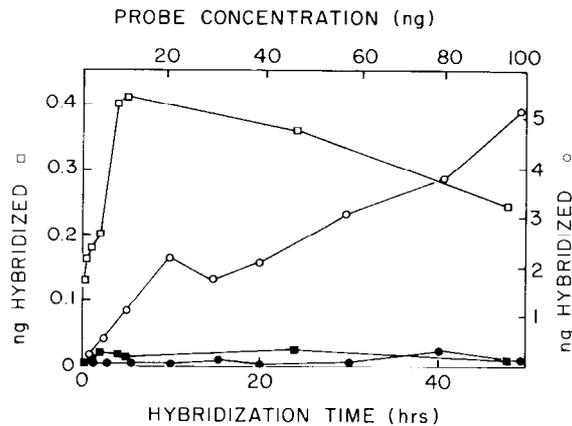
Figure 4. Quantitative Analysis of In Situ Hybridization Parameters

Total human genomic DNA or E. coli control DNA was nick translated with [^{32}P]dCTP and hybridized to samples of human HeLa cell chromosome preparations made at uniform densities on glass coverslips. Results were quantitated in a scintillation counter.

Effect of Time: open squares, human DNA probe; closed squares, E. coli control probe to assess background. Hybridization was for the times indicated at 37°C at a constant concentration of 4 ng/10 μl/sample. Each point represents the average of triplicate samples.

Effect of Concentration: the probe concentration is expressed as the nanograms of probe applied per sample in 10 μl of volume. Open circles, human DNA probe; closed circles, E. coli control probe to assess background. Hybridization was at 37°C for 16 hr and each point represents the average of duplicate samples.

ure 4) indicated that high probe concentrations could provide significantly increased signal, presumably by allowing the probe to compete more efficiently with the rapid reannealing of DNA in chromatin. In contrast, the background increased only slightly relative to the increase in signal. When probe concentration of the EBV A fragment clone was increased from 0.2 μg/ml to 5.0 μg/ml, the intensity of fluorescent signal increased markedly, such that the signal with A probe was easily visualized on chromosomes as well as in nuclei (Figures 2C and 2D). Background was not significantly increased. As observed with probe to the W fragment, most nuclei had two closely spaced fluorescent spots. Of 20 randomly selected metaphase spreads, 18 were labeled on the short arm of chromosome 1 and, in 17 of these, both sister chromatids were labeled. In these experiments, even highly condensed, morphologically poor chromosome spreads generally exhibited hybridization.

We next proceeded to still smaller probes of the EBV genome, using the Y/H fragment (7 kb) and the K fragment (5 kb) of viral DNA successively. Each of these probes generated visible signal that was localized at the appropriate site on the short arm of chromosome 1 (Figures 2E and 2F). Greater than 70% of metaphase spreads were labeled with either the Y/H or the K probe, and generally both sister chromatids showed signal. As with the W and A fragments, hybridization with either K or Y/H most frequently produced a pair of closely spaced fluorescent spots within interphase nuclei (Figure 3E). It should be noted that the K fragment signal was visible despite the

fact that this fragment contains some repetitive sequences in the IR-3 region that have homology to cellular DNA (Heller et al., 1982) and may cause a slightly higher fluorescence through the nucleus. Hence, these results demonstrate that this methodology is sensitive enough to detect as little as 5 kb of a single sequence.

Our results and previous results using autoradiographic in situ hybridization (Henderson et al., 1983) indicate that a single homolog of chromosome 1 is the only chromosome to exhibit label in Namalwa cells. Therefore, the consistent observation that, regardless of the EBV genome fragment used, closely spaced fluorescent signals are observed in most interphase nuclei strongly suggests that the two spots represent two copies of the EBV genome incorporated at this site on chromosome 1. The spots clearly had a nonrandom distribution with respect to each other in that the distance separating them exhibited a relatively narrow range of 0.2 μm to slightly over 3 μm. This result is consistent with two copies of EBV integrated close together on the same strand of DNA. The observation that larger nuclei, many of which may be either in G2 of the cell cycle or tetraploid, most frequently have two "pairs" of spots (Figures 3A and 3B) is further consistent with this interpretation. However, the most convincing evidence for two EBV genomes integrated at this site is that close examination of metaphase chromosomes, in which the DNA is more condensed and the two signals may coalesce, shows that the signal on each sister chromatid consists of a doublet (Figure 2C inset). High magnification (1000×) revealed that in many or most metaphase figures the signal on each chromatid of chromosome 1 could be resolved as two very closely spaced fluorescent spots (up to 0.4 μm apart). Hence the G2 content of the metaphase cell also produces two pairs of spots, with the spots in each pair approximately an order of magnitude closer than in the interphase nucleus, due to the increased condensation of DNA into chromosomes.

Analysis of the Orientation and Proximity of the Two EBV Genomes

The presence of two EBV genomes would not be resolvable by autoradiographic in situ hybridization used in previous studies (Henderson et al., 1983). Although it had been estimated from earlier kinetic hybridization studies that there were two copies of the EBV genome in Namalwa cells (Pritchett et al., 1976), later restriction enzyme analysis of the cloned cellular DNA flanking the EBV genome indicated that there was only one set of viral-cell junction sequences (Matsuo et al., 1984). Our results indicating the presence of two viral genomes, coupled with evidence for only one set of viral-cell junction sequences, suggests that a duplication of viral and adjacent cellular sequences occurred during or after integration and has been stably maintained in this cell line.

To investigate the possibility that the two viral genomes are separated by cellular DNA, as well as to test further the resolving power of our technology, we conducted experiments to determine if hybridization to regions A and W, sequences at opposite ends of the EBV genome (Figure 1), could be simultaneously and individually visual-

ized within the interphase nucleus. Samples were reacted with a mixture of 5 μg/ml each of A and W probes and hybridization detected with fluorescein-avidin as above. Results suggested that in many nuclei, hybridization to the A fragment of each EBV genome could be visually resolved from hybridization to the corresponding W fragment, separated by 130 kb. This was indicated by the presence of four tightly clustered spots discernible in many interphase nuclei. Generally, two of the spots were dimmer and two were brighter, as evident in Figure 3D. Since in any given experiment, samples hybridized with the A fragment (12 kb) consistently showed dimmer signal than samples hybridized under identical conditions with the W fragment (30 kb), we surmised that the two dimmer fluorescent spots represent A and that the brighter spots represent W. Note that the four uniformly bright spots observed in larger G2 or tetraploid nuclei hybridized with just W probe (Figure 3A) are qualitatively different from the clustered spots of two different intensities observed in smaller (presumably G1) nuclei hybridized simultaneously with A and W. Occasionally, the configuration of these four spots appeared in an extended linear array, as illustrated in Figure 3D. The arrangement of low and high intensity fluorescent signals in this linear array allows us to orient the two integrated EBV genomes as W–A–A–W. The presence of four distinct signals indicates a separation of the two genomes and is consistent with the duplication or insertion of cellular sequences between them. Furthermore, by comparing the W–A distances to the A–A distance, we can estimate the amount of DNA separating the two genomes. For example, in the cell in Figure 2B, the distance between W–A (bright–dim) was in both cases 0.9 μm, whereas the distance between A–A was 1.5 μm. Since it is known that W and A are approximately 130 kb apart in each EBV genome, we estimate that the two genomes are separated by roughly 220 kb of DNA.

If the orientation of the integrated viruses is indeed W–A–A–W, as the above results suggest, then the distance between the two signals generated by hybridization with the BamHI A probe alone should be, on the average, less than that generated with just the BamHI W probe. Separate hybridizations with each probe were conducted and the average distance between paired signals was determined from photographs of randomly chosen cells. Based on 125 determinations of the distance between paired signals with the A probe, the average distance (± 95% confidence limits) was 0.99 (± 0.093) μm. In contrast, the average distance between the two fluorescent spots of each pair was 1.74 (± 0.144) μm for the W probe based upon 150 determinations. The difference between these two means is highly significant statistically with P less than 0.001, confirming the above results that indicate that the EBV genome is integrated as an inverted repeat in a W–A–A–W orientation. These data also provide an independent and more accurate means of assessing the distance between the two genomes based on a large statistical population. From this approach, we calculate that approximately 340 kb of DNA separates the two viral genomes. The two estimates of this distance (220 kb and 340 kb), which were derived by two different approaches, are

relatively close, given the expected difficulty of approximating molecular distances based on in situ hybridization.

Is the EBV Genome Randomly Localized in Interphase Nuclei?

The ability to detect individual DNA sequences within interphase nuclei allows us to examine the nuclear localization of these sequences. During the course of these experiments, it was consistently noted that the EBV genome, integrated into the short arm of chromosome 1, was not detected near the periphery of the nucleus but occupied a more central region. An apparent nonrandom localization was observed despite the fact that the nuclei in these preparations were free to rotate randomly during slide preparation such that any defined localization might be obscured. A quantitative analysis was performed in which the EBV integration site (on chromosome 1) was localized in over 100 interphase nuclei with respect to distance from the center. For each nucleus, this distance was expressed as a fraction of the nuclear radius and plotted in a standardized model, as summarized in Figure 5. As illustrated, signal was almost never observed in the area of the nucleus circumscribed by the outer 20% of the radius, even though this spheroid constitutes 49% of the nuclear volume. If the region on chromosome 1 marked by the EBV genome were randomly distributed in nuclei, it would lie in this outer sphere approximately 49% of the time. However, because we observe the nucleus in two dimensions rather than three, we would not expect to observe all 49% of the signal in the spheroid defined by the outer 20% of the radius. We calculate (see Experimental Procedures) that if sequence distribution were random, we would expect to observe approximately 17.6% of the signal in this outer region of the nucleus. Hence, of 130 determinations, we expect approximately 23 in this region, if distribution were random. However, we observe this only once near the inner margin of this outer spheroid. No signal was observed in the outer 18% of the radius (see Figure 5). These results indicate that the EBV integration site on chromosome 1 is nonrandomly localized within an inner sphere of the nucleus representing only 51% of the nuclear volume.

Discussion

This paper describes the high resolution localization of single-copy nucleic acid sequences in metaphase chromosomes and interphase nuclei, made possible using improved methodology for in situ hybridization and fluorescence detection of biotinated probes applied to the visualization of Epstein-Barr virus integration in a lymphoma cell line. The sensitivity and low background of the method we describe allowed us to unambiguously and reproducibly detect 5 kb of the EBV genome in a single interphase nucleus. The high resolution of the fluorescence detection method revealed the presence of two integrated EBV genomes on each sister chromatid of chromosome 1. This resolving power, when applied to less condensed chromatin within interphase nuclei, made it possible to visualize simultaneously two separate frag-
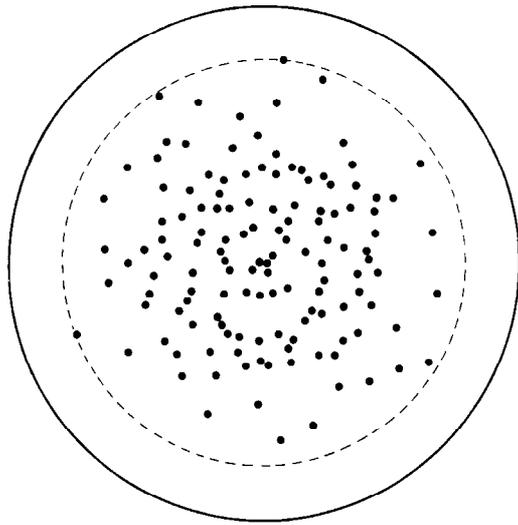
Figure 5. Distribution of the Integrated EBV Genome in Interphase Nuclei

Each spot represents the placement of the EBV genome relative to the center of the nucleus and randomized 360°. Because our analysis is of freely rotated nuclei, the distribution presented provides three-dimensional information (see Results). The data were derived from photographs of 134 randomly selected interphase nuclei after hybridization with the BamHI W probe for the EBV genome. For each nucleus both the radius and the distance from the center of the nucleus to the most distal signal was determined using an automated digital planimeter. The distance to each signal was expressed as a fraction of the corresponding nuclear radius and results plotted above.

ments at opposite ends of the EBV genome, separated by only 130 kb of DNA. Results of these analyses indicate that in these lymphoma cells the two EBV genomes are in reverse orientation (W–A–A–W) and that they are separated by roughly 340 kb of cellular DNA. Finally, our results suggest that the EBV genome, integrated into chromosome 1, is centrally localized within interphase nuclei. This work illustrates an innovative application of in situ hybridization to derive biological information not obtained by previously existing techniques. The significance of both the general methodology as well as the specific virological results obtained is discussed below.

The methodological approach described here has implications for several areas of biomedical research. Most apparent is gene mapping by in situ hybridization. Current autoradiographic in situ hybridization techniques provide rather crude resolution relative to chromosome dimensions, since the scatter of tritium is 1–2 μm in both directions (Rogers, 1979). Therefore, the 0.1 μm resolution of fluorescence provides approximately 20-fold greater precision in the localization of sequences on chromosomes. A striking feature of our results is that the signal appears as small discrete fluorescent spots that can be precisely positioned on the chromosome. This improves upon resolution that has previously been illustrated using nonisotopic in situ hybridization (Landegent et al., 1985; Albertson, 1985; Pinkel et al., 1986). While providing a significantly greater degree of both sensitivity and resolution, the method described here is straightforward and

convenient compared with other methods that involve the use of avidin amplification systems (Pinkel et al., 1986), interference reflection microscopy (Landegent et al., 1985, 1986) or image processing (Albertson, 1985). The high hybridization efficiency obtained, coupled with the ability to resolve labeling of both sister chromatids, provides a rapid means of identifying true hybridization. Therefore, the laborious statistical analysis required by autoradiographic localization techniques is unnecessary. Although our emphasis in this work was not on detailed mapping to metaphase chromosomes, the fluorescein-avidin detection method is compatible with both DAPI fluorescent banding and Giemsa staining and circumvents the need for photographic emulsion, which can interfere with other cytogenetic banding techniques.

Perhaps more important than the sensitive fluorescence detection of genes on chromosomes is the detection of single-copy sequences in interphase nuclei. The ability to detect as little as 5 kb of a specific sequence at interphase makes possible a high resolution localization of two sequences with respect to one another by an approach that we refer to as "interphase chromatin mapping." As demonstrated by the analysis of two EBV sequences 130 kb apart, the less condensed state of interphase chromatin makes it possible to determine the proximity of two closely spaced DNA sequences with much greater resolution than is obtainable on chromosomes. We approximate from our measurements that the condensation of DNA is at least 10-fold less in chromatin than in chromosomes. Coupling the 10- to 20-fold greater resolution of fluorescence with the less condensed state of interphase chromatin allows two closely linked sequences to be discernible with approximately 200-fold greater resolution than by autoradiographic techniques on chromosomes. Such resolving power may prove valuable where the genetic linkage of two sequences needs to be determined, as in providing a linkage map of the human genome. This could facilitate identification of restriction fragment length polymorphisms useful for prenatal diagnosis of a given disease by allowing a rapid, initial screening for the most appropriate sequences to be used in more time-consuming family pedigree analysis. While we have been successful at detecting single copy cellular sequences, it remains to be determined if the limit of sensitivity is the same for viral and cellular sequences. Work in progress to increase the sensitivity of detection to 1–2 kb would make this approach more widely applicable. Use of image processing or avidin-biotin amplification systems (Pinkel et al., 1986) could enhance sensitivity still further beyond the 5 kb currently visible through the microscope. Detection of the biotinated probe using a streptavidin-alkaline phosphatase reaction (Unger et al., 1986; Singer et al., 1986) can provide comparable or improved sensitivity as recently reported by Garson et al. (1987). However, we have found that the resolution and background of this enzymatic technique is substantially worse than that of fluorescence.

We expect that this methodology may contribute significantly to the investigation of nuclear organization in interphase cells. Efforts to understand the relationship between gene organization and gene function may be most

appropriately applied to chromatin in its functional state, at interphase. In recent years, much has been learned about the nucleosome structure of chromatin (reviewed in Hamkalo and Rattner, 1980; Georgiev, 1981; Weisbrod, 1982), but, as yet, little is known about the higher-level organization of genes within the nucleus. Since first proposed (Rabl, 1885), an order to the three-dimensional distribution of chromatin in the interphase nucleus has been suggested by a variety of cytological observations (Comings, 1968, 1980; Agard and Sedat, 1983; Manuelidis, 1984, 1985; Cremer et al., 1982; Schardin et al., 1985; Hochstrasser et al., 1986; Hochstrasser and Sedat, 1987), and it has been suggested that functional chromatin is localized in specific nuclear domains (Hutchinson and Weintraub, 1985). However, the investigation of nuclear organization is at an early stage and has been restricted primarily to repetitive sequences or nuclear landmarks, such as the nucleolus. What we describe here is a tool for localization of specific single-copy genes within nuclei. While a precise positioning of nuclear sequences would only become apparent in studying highly oriented cells, analysis of freely rotated nuclei is advantageous in that it allows visualization of sequence localization within all three axes of rotation, yielding three-dimensional information. Restriction of the EBV genome to an inner sphere of the nucleus representing only 50% of the volume strongly indicates higher-level order within interphase chromatin and illustrates the potential of this approach for further investigations of nuclear organization, which should be facilitated by our recent success at reproducing this methodology in nuclei of intact cells.

Aspects of these results also relate to the degree of condensation between closely spaced sequences both at interphase and metaphase. Our measurements indicate that the distance between the two EBV genomes is approximately 5- to 10-fold greater in interphase nuclei than on metaphase chromosomes, which is consistent with the relative condensation believed to exist in chromsomes versus chromatin. By comparing the distance between sequences based upon 3.4 A/bp for the linear DNA molecule and the actual measurement of these distances from our results, we estimate condensation between the two EBV genomes, or between the two ends (A and W) of the EBV genome, to be approximately 500- to 1000-fold for chromosomes and 100-fold for chromatin. To our knowledge, the actual condensation between closely spaced sequences has not previously been directly measured. This degree of condensation is almost an order of magnitude less than the 10^4 and 10^3 overall packaging commonly estimated for chromosomes and chromatin, respectively (Lewin, 1985). These estimates are not incompatible, however, when one considers that the overall chromosomal condensation is not achieved by uniform "shrinkage" of the DNA, but is accomplished by extensive looping or folding of the chromatin fiber (see, for example, Hamkalo and Rattner, 1980) such that condensation between closely spaced sequences would not be as great as the overall shortening of the strand from end to end. This is best conveyed diagramatically using a simplified loop model of chromatin structure (see Figure 6). The distance between
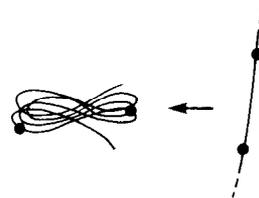

Figure 6. A highly schematic representation of chromatin folding to illustrate how the distance between closely spaced sequences may not be reduced to the same extent as is the overall length of the fiber.

closely spaced sequences will depend upon the way in which the chromatin fiber folds in the particular region between them. This interpretation is visually supported by our data, which consistently show (for example, see Figures 2C and 2E) that resolution of the two EBV genomes at metaphase is possible due primarily because of their separation across the width rather than along the length of each chromatid, presumably reflecting the looping and folding of the chromatin fiber in that region. Finally, interpretations of our results must take into consideration the possibility that the integrated viral genome exhibits less condensation than the rest of the chromosomal DNA and that the hypotonic treatment of nuclei used in our analysis increases the nuclear diameter by a factor of two.

The specific results presented as well as the approach employed have implications for the study of viral gene expression and its relationship to viral integration. The Namalwa cell line is the only EBV-transformed cell line that does not produce episomes and is not inducible to productive infection, although the integrated viral genome appears to be complete (Dambaugh et al., 1986). Information concerning the number and arrangement of EBV genome(s) in this cell line may provide insight into the regulation of EBV gene expression. While it had been suggested that this line provides evidence that one EBV genome is enough to maintain cell transformation (Dambaugh et al., 1986), results presented here raise the possibility that two genomes are required. The finding of one set of viral-cell junction sequences from Southern blot and sequencing analysis (Matsuo et al., 1984) is compatible with a duplication of a portion of the chromosome containing the viral genome and at least several kb of the flanking cellular DNA. That duplication of the EBV genome occurred as part of a larger scale chromosomal rearrangement is suggested by several observations: results reported here demonstrating that there are a few hundred kb of cellular DNA inserted between the two EBV genomes, the presence of a light staining area at this region of the chromosome (our observation as well as Henderson et al., 1983), and sequencing results showing duplication and deletion of some cellular DNA at the viral integration site (Matsuo et al., 1984). Since it is likely that the larger-scale duplication included cellular DNA flanking the EBV genome, it would not be detectable on Southern blots with EBV probes. This illustrates the potential power of in situ hybridization as a complement to filter blot analysis and

as a means of investigating sequence linkages across greater distances than is feasible by chromosome walking techniques. Current investigation of other EBV transformed cell lines using the in situ approach should clarify whether the duplication of viral and, presumably, cellular sequences is characteristic of EBV integration. It is possible that duplication or rearrangement of specific cellular sequences, such as the *Blym* oncogene located near the site of EBV integration (Matsuo et al., 1984), could prove an important factor in cell transformation. The methodological approach described here may facilitate other studies on viral integration and expression and their relationship to pathology. Under certain circumstances it should be possible to screen a heterogeneous population of cells for the presence of as little as one latent virus, as well as to determine if and where the viral genome has integrated into the host genome.

Finally, in our experiments, the signal within interphase nuclei does not represent detection of nuclear transcripts since it remains after RNAase digestion and is only observed when nuclear DNA has been denatured prior to hybridization. However, recent results from our laboratory indicate that modifications of this methodology can be used to visualize nuclear RNAs from actively transcribed genes (unpublished data).

Experimental Procedures

The methodology developed here builds upon previously described in situ hybridization techniques as well as nonisotopic detection techniques (Brahic and Haase, 1978; Langer et al., 1981; Langer-Safer et al., 1982; Harper et al., 1981; Gerhard et al., 1981; Lawrence and Singer, 1985; Singer et al., 1986, 1987). The improvement and success of this methodology does not rest upon any one step. Optimization of technical parameters for preservation of nuclear material, hybridization to double-stranded DNA, and nonisotopic detection, when put together in the appropriate array, collectively result in improved methodology. Among the technical variables (described in detail below) that may differ from previously described protocols and contribute to success are: frozen storage and "hardening" of nuclear preparations prior to processing; RNAase H treatment after hybridization rather than RNAase A treatment prior to denaturation; use of probe concentrations 100–1000× greater than recommended in standard protocols; monitoring of probe fragment size after nick translation to use probe molecules approximately 300–1000 nucleotides in length; staining with fluorescein avidin in 4× SSC rather than PBS; and finally, testing the quality of different lots of key reagents such as methanol, formamide, and fluorescein-avidin.

Preparation of Nuclei and Metaphase Figures
The Namalwa cell line (provided by Elliot Kieff) was maintained in suspension in RPMI medium, 10% fetal calf serum (Gibco, NY) and 0.1% Gentamycin (Microbiological Associates). The L8 rat myoblast line was used as a control. Growing cultures were incubated at 37°C with 0.015 μg/ml of colcemid (demicolcine) for 2–4 hr. Cells were then harvested and incubated in 0.075 M KCl at 37°C for 17 min followed by fixation in three changes of fresh 3:1 methanol:acetic acid according to conventional cytogenetic techniques. Cells in suspension were dropped onto clear glass coverslips in a humid environment to promote spreading of chromosomes. Coverslips were then air dried overnight and stored at −80°C with desiccant until further use. Prior to hybridization, chromosome preparations were baked at 60°C for 3 hr to promote further drying and "hardening" of nuclear material.

Probes and Nick Translation
The restriction map of the Epstein-Barr genome is presented in Figure 1 and clones of specific fragments in a pBR322 vector were provided by Jim Skare. Probes used were the W, A, Y/H, and K fragments representing approximately 30, 12, 7, and 5 kb, respectively (Skare and Strominger, 1980). Plasmid DNA was nick translated according to established procedures using biotinated-11-dUTP (Enzo Biochemical, NY). Fragment size was controlled by varying the amount of DNAase (Worthington) in the reaction as determined empirically for each lot of DNAase. Size of probes was determined using alkaline agarose electrophoresis followed by transfer to nitrocellulose filter and visualization using streptavidin and biotinylated alkaline phosphatase (BRL, Gaithersburg, MD). Probe preparations were used if the range of probe fragment sizes after nick translation was between 300 and 1000 nucleotides. Results of quantitative studies using total human genomic DNA as a probe (not shown) demonstrated that larger biotinated probe fragments worked better for hybridization to chromosomes and nuclei than they did for hybridization to whole cells (Lawrence and Singer, 1985; Singer et al., 1986).

Hybridization and Detection
Cytogenetic preparations were incubated for 10 min in 0.1 M triethanolamine and 0.25% acetic anhydride and were then denatured by incubation at 70°C for 2 min in 70% formamide, 2× SSC. Preparations were immediately dehydrated through cold 70%, 95%, and 100% ETOH for 5 min each and then air dried. The hybridization solution was similar to that previously described for detection of mRNA (Lawrence and Singer, 1985) except that higher probe concentrations and larger probe fragment sizes were used. For each sample 2–50 ng of probe (see Results), 5 μg of sonicated salmon sperm DNA (Sigma Chemical, St. Louis, MO), and 20 μg E. coli tRNA (Boehringer Mannheim) were suspended in 5 μl deionized formamide and heated from 70°C–80°C for 10 min. An equal volume of hybridization buffer was then added, so that the final hybridization solution consisted of 2× SSC (0.3 M sodium citrate buffer), 1% BSA and 10% dextran sulfate (Sigma Chemical). Immediately after mixing, the hybridization solution was placed on the samples, covered with parafilm and incubated at 37°C in a humidified chamber overnight. Samples were rinsed for 30 min each in 50% formamide-2× SSC at 37°C, 2× SSC and finally 1× SSC at room temperature. To eliminate the possibility of contaminating cytoplasmic or nuclear RNA, slides were either treated prior to hybridization with 100 μg/ml RNAase A (Sigma Chemical) for 1 hr at 37°C or, preferably, treated after hybridization with RNAase H (BRL, Bethesda, MD). RNAase H was used at 8 U/ml for 1 hr at 37°C in a buffer consisting of 100 mM KCl, 20 mM Tris-HCl, (pH 7.5), 1.5 mM MgCl$_2$, 50 μg/ml BSA, 1 mM DTT, 0.7 mM EDTA, and 13 mM HEPES (Minshull and Hunt, 1986).

Hybridization was detected using avidin conjugated to fluorescein (Enzo Biochemical, NY). Samples were incubated in 2 μg/ml avidin in 4× SSC, 1% BSA for 30 min at room temperature. Previous quantitative studies using ^{125}I-avidin (Singer et al., 1987) showed that the nonspecific sticking of avidin was 10-fold less in 4× SSC than in PBS. Samples were then rinsed at room temperature for 10 min each in 4× SSC, 4× SSC with 0.1% Triton, and then 4× SSC. Samples were stained with the DNA fluorochrome, DAPI (diamino phenylindole) for 5 min at 0.1 μg/ml in PBS and mounted in antibleach mounting medium (Johnson and Nogueira, 1981).

Microscopy
Samples were directly visualized at 1000× magnification on a Zeiss ICM microscope equipped with epifluorescence optics for DAPI (Exciter 365 nm, Reflector 390 nm, Barrier 420 nm) and for fluorescein (Exciter 485 nm, Reflector 510 nm, Barrier 515–560 nm). No image intensification or image processing devices were utilized. Black-and-white pictures were taken with Tri-X (ASA 400 film). Exposure times were generally 2.5 min for fluorescein and a few seconds for DAPI fluorescence. Samples were evaluated within a few weeks after detection since the fluorescent signals faded significantly over time.

Calculations
Estimation of the percentage of signal expected to occur at the peripheral 20% of the nucleus was based upon the assumption that in our preparations we observe the sphere as a relatively flat, two-dimensional object. This assumption is supported by the observations that signal is not only visible, but within the same plane of focus in almost all nuclei (Figure 2C), and that the DAPI fluorescence, which is

directly proportional to DNA content (Coleman et al., 1981), is uniform across the nucleus (Figures 2B and 2F).

We estimate that the probability of observing signal in the region defined by the outer 20% of the nuclear radius is equal to the probability that the sequence actually is in this outer region of the three-dimensional nucleus (49%, based on relative volume) multiplied by the probability that, if it were there, it would appear to be there when viewing the freely rotated nucleus as a two-dimensional object (36%, based on relative surface area). Hence, if sequence distribution within the nucleus were random, we expect 49% × 36% = 17.6% of the observed signal to be in the region defined by the outer 20% of the radius. This estimation assumes that any distortion of sequence distribution that occurs during nuclear flattening is minimal and that, to the extent that such distortion does occur, it would tend to increase still further the amount of signal expected at the periphery.

Acknowledgments

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