GENOME ANALYSIS

Volume 1

Genetic and Physical Mapping

KAY E. DAVIES &
SHIRLEY M. TILGHMAN
editors

GENETIC and PHYSICAL MAPPING

Edited by

Kay E. Davies
University of Oxford

Shirley M. Tilghman
Princeton University

Volume 1 / GENOME ANALYSIS

Cold Spring Harbor Laboratory Press 1990

Genome Analysis Volume 1
Genetic and Physical Mapping

Printed in the United States of America
ISBN 0-87969-358-4
ISSN 1050-8430

Cover and book design by Leon Bolognesi & Associates, Inc.

Cartoon on p. 30 by John Chase is reprinted from *Science,* vol. 243, Feb. 10, 1989, p. 786.

All Cold Spring Harbor Laboratory Press publications may be ordered directly from Cold Spring Harbor Laboratory Press, 10 Skyline Drive, Plainview, New York 11803. Phone: 1-800-843-4388. In New York (516) 367-8423. FAX: (516) 367-8432.

Preface

It was more than a century ago when Gregor Mendel discovered that inherited traits are controlled by the cellular units which we now call genes. During the last 20 years, since the development of recombinant DNA technology, there have been rapid advances in our understanding of the regulation and function of these genes. The sequencing of individual genes and their flanking regions has led to the recognition of important sequence motifs for the control of transcription. These studies are being carried out in several different organisms as not all of the questions can be addressed in humans, where relatively few mutations are available.

In addition to the intense study of particular genes, considerable efforts have gone into the genetic mapping of several species. A genetic map of the human genome is now being refined, and new highly polymorphic markers will enable the mapping of rare traits as well as the unraveling of polygenetic disorders such as diabetes. Physical maps of whole human chromosomes are now almost complete, and overlapping contig maps of the human genome in cosmids and YACS will become available in the next few years. This will be a valuable resource for the understanding of all human diseases including cancer.

The genetic and physical mapping efforts in humans are also being carried out in other organisms such as the mouse. The implications of this are obvious because of the need to study other organisms to assay function. *Drosophila* and the nematode *Caenorhabditis elegans* have been extensively mapped for this reason.

The amount of information that has accumulated in recent years on genome structure and function is staggering. For example, cloning in yeast was very much in its infancy at the time of the first Cold Spring Harbor Laboratory meeting on *Homo sapiens* (1986) and is now a necessity in most human genetics laboratories. The polymerase chain reaction, developed by workers at Perkin-Elmer Cetus, not only enables novel analyses to be performed, but also permits the generation of data many times faster than three years ago. Genetic diagnosis used to take two weeks to perform and can now be completed in a few hours.

This series of volumes is devoted to reviews of mapping genomes

and aims to highlight some of the most significant and interesting advances in the area of gene mapping, arrangement, and function. We hope that this will enable even those working on the periphery of the field to grasp the exciting advances that are being made.

We are grateful to all of the authors for their hard work and enthusiasm in writing such excellent contributions. We are also greatly indebted to the staff of Cold Spring Harbor Laboratory Press, especially Nancy Ford and her colleagues Dorothy Brown and Mary Cozza, who have worked so hard to ensure rapid publication of this first volume in the series. We hope that the scientific community will find these reviews as much a pleasure to read as we did.

Kay E. Davies
Shirley Tilghman
August, 1990

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A Fluorescence In Situ Hybridization Approach for Gene Mapping and the Study of Nuclear Organization

Jeanne Bentley Lawrence

Department of Cell Biology
University of Massachusetts Medical School
Worcester, Massachusetts 01655

In situ hybridization procedures that directly couple molecular and cytological information have very recently had a surge of success in powerful new applications. Pivotal to these advances has been technological improvements that allow the precise visualization of single sequences within individual metaphase and interphase cells. This has important implications for human gene mapping and clinical cytogenetics. These techniques provide not only an important tool for determining linear arrangements of genes on individual chromosomes, but also a powerful approach for analysis of their three-dimensional organization and expression within the genome. Hence, the ability to detect individual genes, viral genomes, or primary nuclear transcripts sensitively has significance for cell and developmental biology as well.

This chapter discusses:

- ❑ the current state of the art and the potential of high-sensitivity fluorescence in situ hybridization methodology
- ❑ recent contributions to this field from our laboratory
- ❑ applications for human genome mapping and for investigating higher-level genome organization
- ❑ a new approach, termed interphase chromatin mapping, that allows the rapid precise localization of closely spaced DNA sequences along the chromatin fiber
- ❑ potential applications for analysis of cytogenetic aberrations

Genome Analysis Volume 1: Genetic and Physical Mapping
 0-87969-358-4/90 $1.00 + 00

INTRODUCTION

Many laboratories have contributed to the development and application of cytological hybridization, with advances being made in stages over a period of 20 years. This technology has progressed from a laborious and time-consuming approach to detect abundant nucleic acid sequences with low resolution to an approach that allows fast, highly precise, and sensitive localization of as little as one molecule per cell. Although it is not possible to review all of the work in this field, this section attempts to summarize briefly the major contributions leading to the current state of the art, before describing work from our laboratory.

The concept of applying molecular hybridization directly to cytological material was initially pioneered by Gall and Pardue (1969) and John et al. (1969). The early phase of the development of this field relied on autoradiographic detection of radioactive probes to localize abundant sequences, such as localization of DNA sequences in amplified polytene chromosomes and highly reiterated sequences on metaphase chromosomes (Evans et al. 1974). During the following decade, applications were generally restricted to highly represented sequences. In 1981, two reports (Gerhard et al. 1981; Harper et al. 1981) showed that it was possible to localize single sequences on metaphase chromosomes by autoradiography of ^{125}I- or ^{3}H-labeled probes. This approach has since been used to map many genes and is the most frequently used in-situ-mapping method to date. However, autoradiography has several significant limitations. Because of the scatter of radioactive disintegrations, the resolution is limited to relatively large chromosomal segments. Furthermore, localization of the sequence is not determined directly within a single cell but requires statistical analysis of grain distribution in as many as 50–100 metaphase figures. In addition, the technique is extremely time-consuming, since weeks are generally required for sufficient autoradiographic exposures. Although autoradiography offers the advantage that cDNA sequences of less than 1 kb can be used routinely for statistical localization, this approach is not used for genomic clones containing repetitive sequences, presumably because of prohibitively high backgrounds.

To overcome the limitations of autoradiography, several laboratories had the foresight to pursue development of innovative nonisotopic detection techniques. One of the earliest nonisotopic in situ detections used an antibody to RNA-DNA hybrids (Rudkin and Stollar 1977). In 1980, a method for direct labeling of fluorochromes to DNA probes was described (Bauman et al. 1980, 1981). Several indirect nonisotopic techniques were developed using chemically modified DNA probes, which are detected after hybridization by affinity reagents. The development of the avidin:biotin system was originated and developed initially using methacrylate spheres or ferritin detectors for electron microscopic

localization of tRNAs or rRNA (Manning et al. 1975; Broker et al. 1978). Subsequently, the incorporation of biotinylated dUTP into a DNA probe (Langer et al. 1981) and detection by a two-step antibiotin antibody reaction after hybridization to amplified sequences in polytene chromosomes were described (Langer-Safer et al. 1982). Probes labeled with AAF (*N*-acetoxy-*N*-acetyl-2-aminofluorene), for which antibodies were readily available, were subsequently reported and successfully applied for detection of abundant sequences (Tchen et al. 1984). In efforts to produce more sensitive or convenient technology, several groups continued to develop alternative labeling techniques, such as mercuration of probes (Dale et al. 1975; Hopman et al. 1986), sulfonation (Verdlov et al. 1974), and direct attachment of horseradish peroxidase (HRP) (Renz and Kurz 1984). Most recently, a very effective system has been described that uses digoxygenin-labeled nucleotides detected by antibodies carrying fluorescent or enzymatic tags (Boehringer Mannheim).

Fluorescent or enzymatic reporter molecules were most frequently used to detect probes labeled by various means. Commonly used enzymes have been HRP and alkaline phosphatase. The enzymatic detection methods require extra steps to convert the substrate to a visible product; however, they have an advantage over fluorescence in that the enzyme reaction can be prolonged in order to amplify signals and the signals do not fade. Fluorescent tags are advantageous in that they provide the highest resolution possible with the light microscope and are easily adapted for multicolor labeling and for quantitation and processing by a variety of instrumentations. Fluorescein isothiocyanate (FITC) and rhodamine isothiocyanate (TRITC) are the most frequently used, but other fluorochromes with different spectral properties are becoming more widely available. Efforts to improve the detection from fluorescent tags have been directed at amplification of the detector, such as by antibody-stacking techniques (Pinkel et al. 1986), and through the use of sensitive digital cameras and computerized image enhancement.

During the past decade, different nonisotopic labeling techniques using fluorescent or enzymatic reporters have been variously applied for detecting highly repeated DNA sequences or abundant mRNAs. For example, nonisotopic hybridization to satellite DNA was reported as early as 1982 (Manuelidis et al.), amplified sequences were localized in polytene chromosomes (Wu and Davidson 1981; Langer-Safer et al. 1982; Kress et al. 1985), and clustered genes for rRNAs were detected on metaphase chromosomes (van Prooijen-Knegt et al. 1982; Albertson 1985). Although nonisotopic detection became more widely applied for highly represented sequences, the detection of single-copy genes during this period was done almost exclusively by autoradiography. There were a few reports of some success in the nonisotopic detection of large (25–50 kb) unique sequences using specialized procedures to amplify signals from either AAF-labeled probes using interference reflection mi-

croscopy (Landegent et al. 1985) or biotin-labeled probes for mapping in *Caenorhabditis elegans* using image processing (Albertson 1985). These procedures were not widely adopted over radioactive techniques, largely because of limitations of sensitivity and reproducibility in a growing number of nonisotopic methods. Recently, the overall performance of the technology in terms of a combination of hybridization efficiency, signal-to-noise ratios, sensitivity, and resolution was sufficiently enhanced to allow fluorescence in situ hybridization to emerge as an invaluable tool with far-reaching applications in genetic, cell biological, virological, and clinical investigations.

The difficulty of applying these techniques for single-sequence detection had been generally considered a consequence of limitations in detection sensitivity. However, the most recent advances in the success of this technology use innovative detection techniques described several years earlier and derive primarily from improvements in hybridization conditions and other parameters of the total process. Our laboratory's contribution has not been in the development of detection techniques but in analytical investigation of the most successful conditions for high-efficiency cytological hybridization with nonisotopic probes.

QUANTITATIVE ANALYSIS OF HYBRIDIZATION PARAMETERS

Conceptually and technically, the process of hybridization and detection of specific nucleic acids within cytological material is complicated relative to the straightforward situation in which isolated nucleic acids stripped of protein are bound to a uniform substance, such as a nitrocellulose filter. For analytical purposes, the steps of in situ hybridization can be divided into three main components, each of which is essential for success:

1. *Preservation.* Target sequences must be well-preserved throughout hybridization but remain in an accessible state within the biological material. Although this is especially critical for RNA, it is an important consideration for detection of nuclear or chromosomal DNA as well.
2. *Hybridization.* The probe must hybridize the target molecules with high efficiency without substantial nonspecific adherence to biological material, which is inherently reactive.
3. *Detection.* The reporter must bind to the probe in sufficient quantities to give a detectable signal, while sources of nonspecific background from the detector or the cellular material itself are minimized.

Failure of any one parameter in any of these components results in a lower signal-to-noise ratio and a loss of sensitivity for a given detection system. The potential of nonisotopic probe technology could not be fully realized until important parameters were identified, optimized, and put together in the appropriate combination to push the practical capabilities of this technology closer to its theoretical potential. To this end, we emphasized quantitative characterization of hybridization and detection parameters to provide higher hybridization efficiency. The goal for single-copy genes was to obtain hybridization efficiencies high enough to provide sister-chromatid labeling and nonstatistical localization. Microscopy was generally too laborious and subject to internal variation to allow a very thorough analysis of numerous hybridization parameters and their various combinations. An analytical approach was implemented that allowed rapid quantitation of numerous parameters and allowed the success of the preservation, hybridization, and detection components to be evaluated separately. This was initially done for detecting cytoplasmic mRNAs and later extended to nuclear DNA (Lawrence and Singer 1985; Singer et al. 1986, 1987; Lawrence et al. 1988). A number of nonobvious parameters were sorted out and unexpectedly found to be important.

Some of the key parameters identified for single-sequence detection by this quantitative approach are illustrated below, and other technical details can be found in previously published reports (see Lawrence et al. 1988, 1989, 1990a) and in the brief protocol provided at the end of the chapter.

Reducing avidin background for detection of biotinylated probes

We initially used ^{125}I-labeled avidin to assess a means of reducing the widely reported nonspecific adherence of this protein to cytological material, as summarized in Table 1 (Singer et al. 1987). The main conclusion of this work was that the presence of phosphate-buffered saline (PBS), which had been generally used previously (Langer-Safer et al. 1982; Singer and Ward 1982), promoted high nonspecific sticking of avidin. This was to some extent surprising because avidin diluted in PBS was routinely used with success to detect biotinylated probes on filters. The nonspecific sticking of avidin can be reduced by approximately 90% using sodium citrate, preferably of higher salt concentration (4× SSC). Neither acetic anhydride, nor detergents, nor changes in pH reduced the avidin background. The various chemically modified forms of avidin, developed commercially to reduce its background, are unnecessary for this purpose and in some cases give less-intense signals than fluorescein-avidin.

Table 1 ^{125}I-labeled avidin bound to cells with various treatments (average of duplicate samples)

Treatment	Counts per minute
No cells (glass coverslip; 1× SSC)	139
With cells (1× SSC)	1,348
Acetic-anhydride-treated cells (1× SSC)	1,365
Triton-treated cells (1× SSC)	1,905
Cells in PBS	16,406
Cells in PBS with 100 μg/ml cold avidin	1,288
Cells in PBS with 1 mg/ml cold avidin	928
Cells in 4× SSC (pH 5.2)	1,174
Cells in 4× SSC (pH 6.2)	844
Cells in 4× SSC (pH 7.2)	1,235
Cells in 4× SSC (pH 8.2)	747

Data from Singer et al. (1987).

Hybridization kinetics

Further analysis demonstrated that the fluorescein-avidin detection, when applied under appropriate conditions, was extremely sensitive but that the hybridization component was failing (Fig. 1A).

Figure 1 (*A*) An early attempt at fluorescence detection of hybridization to single-copy genes, illustrating a common result of hybridization with low probe concentrations (no specific signal) and large probe fragment size (high spotty background). This experiment used probes labeled with both ^{32}P and biotin and showed that the detection component was working with sensitivity, but that the hybridization component was failing. The hybridization conditions led to high background and no specific signal. (*B*) Total human genomic DNA or *Escherichia coli* DNA was nick-translated with ^{32}P dCTP and hybridized to samples of HeLa cell chromosome preparations, and the results were quantitated by scintillation. *Effect of time:* (*Open squares*) Human DNA probe; (*closed squares*) *E. coli* control probe to assess background. Hybridization was carried out 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 carried out at 37°C for 16 hr, and each point represents the average of duplicate samples. (Reprinted, with permission, from Lawrence et al. 1988.) (*C*) Fluorescence detection of sequences within interphase nuclei. The EBV *Bam*HI W probe was hybridized to Namalwa cell nuclei. Note that two viral genomes are visible in the smaller G_1 nucleus (*right*), whereas four signals are observed in the larger tetraploid or G_2 nucleus. (Reprinted, with permission, from Lawrence et al. 1988.) (*D*) Two probes (*Bam*HI W and A), separated by 130 kb within the EBV genome, were hybridized simultaneously to Namalwa cell nuclei. The presence of four tightly clustered spots of two different intensities (due to two different-size target sequences) is observed in many G_1 nuclei. (Reprinted, with permission, from Lawrence et al. 1988.) (*E*) Diagram illustrating the interpretation of the pattern shown in *D* and described in the text.

A similar quantitative approach was applied for optimizing hybridization to chromosomal and nuclear DNAs. To generate sufficient signal for rapid quantitation of many samples, we used total human DNA labeled with ^{32}P as a probe for hybridization to standard preparations of chromosomes. Numerous technical parameters were tested, and two are presented here to illustrate the approach.

The efficiency of hybridization to single-copy sequences was generally low as analyzed by autoradiographic techniques, and standard protocols called for long hybridization times (1–4 days) and low concentrations of probe (<1 ng per sample) (Gerhard et al. 1981; Harper et al. 1981; Harper and Marselle 1985). In Figure 1B, the kinetics of the in situ hybridization process were evaluated on the basis of triplicate samples in replicate experiments. The striking feature of these results was that hybridization rose very sharply, with the reaction being one-third maximal in just 10 minutes and essentially complete within 4 hours. In some experiments, a slight decrease in signal occurred with prolonged incubations, probably because of dissolution of the sample. Since hybridization of low-copy sequences should continue to occur over a longer period of time, these results first indicated that the rapid reannealment of chromosomal DNA may limit hybridization efficiency and

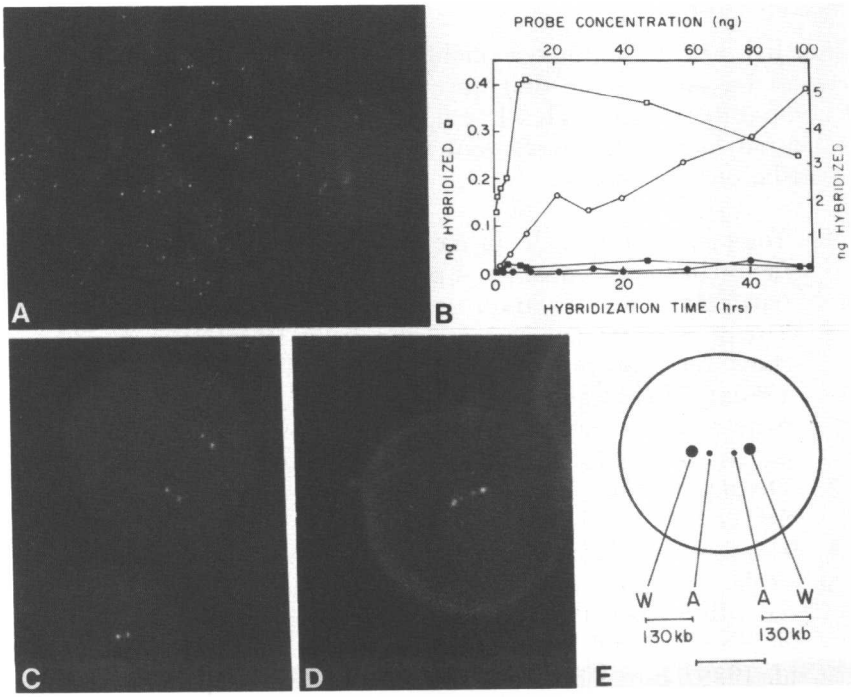

Figure 1 (*See facing page for legend.*)

should be competed with extremely high probe concentrations, rather than prolonged hybridization times and low probe concentrations.

These conclusions led us to evaluate the effect of probe concentration on signal and noise, since high probe concentrations are generally considered prohibitive because of increased background. Repeated experiments yielded similar results, as illustrated in Figure 1B. Note that hybridization was still rising rapidly at 200 ng per coverslip (10 μg/ml or over 100 times more DNA than was used routinely in hybridizations), whereas background increased only very gradually. Hence, at high probe concentrations, the extent of hybridization is much greater, although the signal-to-noise ratio can be as good as or better than that at low concentrations, since hybridization rises more sharply than background. These results suggested that better hybridization efficiencies could be obtained by changing in situ hybridization strategies to relatively short hybridization times and high probe concentrations, which would ordinarily be expected to be well above saturation. However, in other experiments, it was determined that high concentrations of nonisotopic probes, such as biotinylated probes, could only be used successfully if another key parameter, the probe fragment size, was carefully controlled.

Probe fragment size

For filter hybridization, it is reasonably assumed that two labeled preparations of the same probe that have equivalent specific activities will yield comparable results. This is emphatically not the case for in situ hybridization because the fragment size after labeling (commonly by nick translation) impacts on:

1. The penetration of the probe into the cytological material. This factor has been considered previously by several groups and is particularly important for tissue sections and intact cells.
2. The nonspecific adherence of the probe to cytological material (Lawrence and Singer 1985; Singer et al. 1986; Lawrence et al. 1990a). This is an extremely important parameter for in situ hybridization, especially with nonisotopic probes. Probe sticking has been a very common source of failure in the past.
3. The networking of the probe to amplify signals (Lawrence and Singer 1985).
4. The iterative detection of individual molecules (i.e., if several probe fragments hybridize to a single target sequence, the signal will be distributed differently than the noise). This is most clearly observed for electron microscopy detection (Singer et al. 1989), but it is also a significant factor in generating high signal-to-noise ratios with fluorescence.

Other technical variables

Other parameters found to influence the quality of the results were the frozen storage of slides and their "hardening" by baking before denaturation; minimizing pretreatments prior to denaturation; omitting RNase A digestion prior to hybridization (which is generally unnecessary but can be replaced by RNase H after hybridization); testing of lots of formamide for neutral pH, correct melting point, and effectiveness for hybridization; careful monitoring of time, temperature, and pH during the denaturation step; and autoclaving the dextran sulfate, which chemically modifies it in such a way that it causes less nonspecific sticking of probes at high concentrations. In addition, a variety of other steps that existed in the literature were tested and found to be unnecessary. For example, extensive rinsing of the samples made no difference in the results, and proteinase digestion was generally found to be unnecessary. Acetic anhydride, useful for some cell types with high endogenous backgrounds, is not necessary for chromosomal hybridizations.

HIGH-EFFICIENCY FLUORESCENCE HYBRIDIZATION

We emphasized fluorescence, as did other investigators, because it is the simplest yet highest-resolution detection and because initial attempts indicated that it could be quite sensitive provided hybridization were improved (Fig. 1A). With attention to the variables identified above, we demonstrated that a one-step fluorescein-avidin detection, without amplification or image-processing procedures, could detect single sequences as small as 5 kb by standard fluorescence microscopy (Lawrence et al. 1988). Although first done with biotinylated probes, the same conditions can be used to detect smaller sequences of a few kilobases or less using digoxygenin-labeled probes, for instance, to detect single copies of the human immunodeficiency virus (HIV) genome (Lawrence et al. 1990b). This work showed that it was possible to obtain extremely high hybridization efficiency and low backgrounds, such that bona fide hybridization to fragments of an integrated Epstein-Barr virus (EBV) genome could be confidently identified in greater than 90% of individual metaphase or interphase cells. Identical sister-chromatid labeling, coupled with negligible background (see Fig. 9A), allowed unequivocal localization within a single cell, qualitatively distinct from autoradiographic or enzymatic procedures that were statistical in nature. In addition to applications for chromosome mapping, this work showed it was possible to detect and localize single sequences within interphase nuclei precisely, which had implications for both gene mapping and the study of nuclear organization.

The demonstration that very tightly linked DNA sequences within decondensed interphase nuclei could be resolved by light microscopy

(Lawrence et al. 1988) was an initially surprising outcome of this work and revealed that genes must be farther apart than would be predicted by the total condensation of nuclear DNA, known to be 1:1000 or greater (Lewin 1985). In the Namalwa cell line studied, the EBV genome was integrated on only one chromosome-1 homolog, yet paired fluorescent signals separated by 0.2–3 μm were observed in the vast majority of interphase nuclei. Further analysis provided evidence that the two spots represented two copies of the EBV genome incorporated at a single chromosomal site, which became apparent in the less-condensed interphase nucleus (Fig. 1C). The observation that larger nuclei, either in G_2 phase or tetraploid, frequently had two "pairs" of spots (Fig. 1C) was further consistent with this interpretation. Close examination of metaphase chromosomes, in which the DNA is more condensed and the two signals may coalesce, showed that the signal on each sister chromatid consists of a doublet of two very closely spaced fluorescent spots (up to 0.4 μm apart). Hence, the G_2 content of the metaphase cell also produced two pairs of spots. These results, indicating the presence of two viral genomes, coupled with evidence for only one set of virus-cell junction sequences (Matsuo et al. 1984) suggested that a duplication of viral and adjacent cellular sequences occurred during or after integration.

To verify this interpretation and to test further the resolving power of the technology, experiments were conducted to determine whether sequences at opposite ends of a single EBV genome could be simultaneously and individually visualized at interphase. In many nuclei, hybridization to the A fragment of each viral genome could be visually resolved from hybridization to the corresponding W fragment, separated by only 130 kb. Four tightly clustered spots were discernible in many interphase nuclei, two of which were dimmer and two of which were brighter, as shown in Figure 1D. Because the smaller A fragment (12 kb) consistently showed a dimmer signal than the larger W fragment (30 kb), it was surmised that the two dimmer spots represented A and the brighter spots represented W. The four uniformly bright spots observed in larger G_2 or tetraploid nuclei hybridized with just the W probe (Fig. 1C) were qualitatively different from the clustered spots of two different intensities observed in smaller (presumably G_1) nuclei hybridized simultaneously with A and W. Occasionally, the configuration of these four spots appeared in an extended linear array, as shown in Figure 1D, indicating that the orientation of the two integrated EBV genomes was W-A-A-W (Fig. 1E) and that roughly 220 kb of cellular DNA separated them.

These results were corroborated by a different approach. If the W-A-A-W orientation were correct, it would predict that the distance between signals with the W probe alone would be greater than with the A probe alone. Separate hybridizations were conducted, and the average distances between paired signals were determined in 125–150 randomly

selected nuclei. The average distance between A-A was consistently different from that between W-W in the predicted direction (0.99 ± 0.09 vs. 1.74 ± 0.14, respectively). This difference was highly significant statistically ($p < 0.001$), confirming the above results which indicated that the EBV genome integrated as an inverted repeat in a W-A-A-W orientation. These data also provided an independent assessment of the distance between the two genomes as approximately 340 kb. The two estimates of this distance (220 kb and 340 kb) derived by two different approaches were relatively close, given the expected difficulty of approximating molecular distances on the basis of in situ hybridization.

These results led us to propose an approach to gene mapping, termed interphase chromatin mapping (Lawrence et al. 1988), whereby the physical proximity of tightly linked DNA sequences could be directly evaluated by hybridization to interphase nuclei. Studies were then initiated aimed at exploring and further developing the potential of interphase and metaphase fluorescence hybridization for genome analysis, as described below.

APPLICABILITY FOR HUMAN GENE MAPPING

The Human Genome Project is in an early stage of an ongoing worldwide effort to produce a genomic map of markers having an initial resolution of 1–5 cM, upon which a much-higher-resolution map will be based. Figure 2 gives an overview of the range of DNA distance measurements approachable by established mapping techniques and compares these with fluorescent in situ techniques. Genetic recombination analysis is an essential method for localizing disease genes with a limit of resolution of approximately 1 cM (~1 Mb), but it can only be used for sequences containing a restriction-fragment-length polymorphism (Botstein et al. 1980). Sequences less than 1 Mb apart are approachable by the technique of pulsed-field gel electrophoresis (PFGE) (Schwartz and Cantor 1984; Cantor et al. 1988), provided specific restriction enzyme sites are appropriately distributed in the area of interest. Somatic cell hybridization (Ruddle 1973; Ruddle and Creagan 1975) and autoradiographic in situ hybridization (Gerhard et al. 1981; Harper et al. 1981) have proven invaluable for localizing sequences to specific chromosomes or chromosome segments, but these techniques generally provide less resolution, in the range of 10^4 to 10^5 kb. Although both genetic recombination and PFGE are enormously useful approaches, there is a strong need for alternative or complementary physical linkage mapping methods capable of resolving anonymous DNA sequences 1–2 Mb or less apart. It would be particularly valuable if such methodology could be applied across a broad range of genetic distances to help bridge what is