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High-resolution mapping of mammalian genes by *in situ* hybridization to free chromatin

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ABSTRACT **Fluorescence *in situ* hybridization to metaphase chromosomes or chromatin fibers in interphase nuclei is a powerful technique in mapping genes and DNA segments to specific chromosome region. We have been able to release the chromatin fibers from cells arrested at G_1 and G_2 phases using different drugs and a simple alkaline lysis procedure. We have also demonstrated specific hybridization of fluorescence-labeled probes to single-copy genomic DNA sequences on the free chromatins. Fluorescence *in situ* hybridization signals have been detected for sequences separated as close as 21 kilobase pairs and as far as 350 kilobase pairs, with excellent correspondence between the observed and expected distances. The resolution of this technique should approach 10 kilobase pairs and its coverage should span millions of base pairs. Therefore, free chromatin mapping can be generally used to study the structure and organization of mammalian genomes.**

Determination of the physical locations of genes and DNA segments on individual chromosomes is an important aspect of genome research. Correct orientation and ordering of these markers are also crucial in identification of disease genes on the basis of chromosome location. Besides crude mapping methods, such as the use of interspecific somatic cell hybrids containing various subsets or portions of human chromosomes, isotope-labeled probe hybridization to genomic DNA in metaphase chromosomes presents a direct approach to localization of genes to specific chromosomal regions with high precision. In combination with fluorescence-labeling (1–5), the resolving power of *in situ* hybridization has been greatly improved; it is also possible to assign relative positions of genes and DNA segments as close as 1 megabase (Mb) apart.

More recently, the introduction of fluorescence *in situ* hybridization (FISH) with less-condensed chromatin of interphase nuclei or pronuclei (6, 7) further increases the resolution, to around 50 kilobase pairs (kb). A major limitation of FISH mapping with interphase nuclei, however, is that the chromatin fibers are organized three-dimensionally, so that gene order can only be inferred by estimating the maximal distance between two probes. Interphase FISH mapping becomes less accurate as the distances between probes increase and the interpretation is complex for multiple fluorescence conjugate data. On the other hand, although methods such as pulsed-field gel electrophoresis and cloning with yeast artificial chromosomes often permit accurate short-range ordering of specific genomic regions, these techniques will have limited applications for the entire genome until sufficient evenly spaced probes are available.

In routine examination of human metaphase chromosome preparations by light microscopy, different chromatin structures can sometimes be identified. In addition to the typical condensed mitotic chromosomes and spherical interphase nuclei containing uncondensed chromatin fibers, several other more elongated structures may be detected (8) (Fig. 1). Some of these structures have a spindle shape with tapered ends and some have long dispersed rope-like ends well separated from one another. It has been shown that these structures are chromatin fibers released from nuclei and that they can be readily stained with DNA-specific dyes such as Feulgen and 4',6-diamidino-2-phenylindole (DAPI) (8–10). The term "free chromatin" has thus been used to describe these released chromatin fibers.

In this report, we demonstrate that the free chromatins released from interphase nuclei may be used for gene mapping. We show that single-copy DNA sequences in these structures can be specifically detected by FISH without the use of sophisticated imaging. The resolution of this technique is estimated to be around 10 kb and it should be broadly useful for physical mapping and ordering of genes in mammalian or other complex genomes.

MATERIALS AND METHODS

Cosmids and Somatic Cells. The cosmid clones cM58-3.6, CF14, cNH24, cJ21, and cW10-20 were derived from the 7q31 region as described (11). Four of these cosmids were mapped upstream of the cystic fibrosis transmembrane conductance regulator gene (*CFTR*), whereas cosmid cW10-20 was found to contain exons 4, 5, 6a, 6b, and 7 of the gene. The human hamster somatic hybrid cell line 4AF/102 (12) contained a single human chromosome 7 as its only human material; the other cell hybrid, ATCC no. GM10323, was specific for human chromosome 21.

Cell Culture and Drug Treatment. Lymphocytes isolated from healthy donors or human cord blood were cultured in α-minimal essential medium (α-MEM) supplemented with 10% fetal calf serum and phytohemaglutinin, in a CO_2 incubator at 37°C for 48–52 hr. In an attempt to increase the proportion of free chromatin fibers in subsequent slide preparation, the cultures were treated with various concentrations of *N*-[4-(9-acridinylamino)-3-methoxyphenyl]methanesulfonamide (*m*-AMSA, gift of the Drug Synthesis Branch, National Cancer Institute, Bethesda, MD) for 2 hr as indicated. For use with the alkaline-release procedure (see below), the lymphocyte cultures were treated with thymidine (0.3 mg/ml) for 20 hr. The synchronized cultures were then washed three times with serum-free medium to release the thymidine block and incubated for ≈10 hr in α-MEM to enrich for free chromatins from the G_1 phase. To obtain fibroblastoid cells at the G_1 phase, the cultures were allowed to grow to confluency for 2–4 days after routine subculture.

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Abbreviations: FISH, fluorescence *in situ* hybridization; DAPI, 4',6-diamidino-2-phenylindole; *m*-AMSA, *N*-[4-(9-acridinylamino)-3-methoxyphenyl]methanesulfonamide; FITC, fluorescein isothiocyanate.

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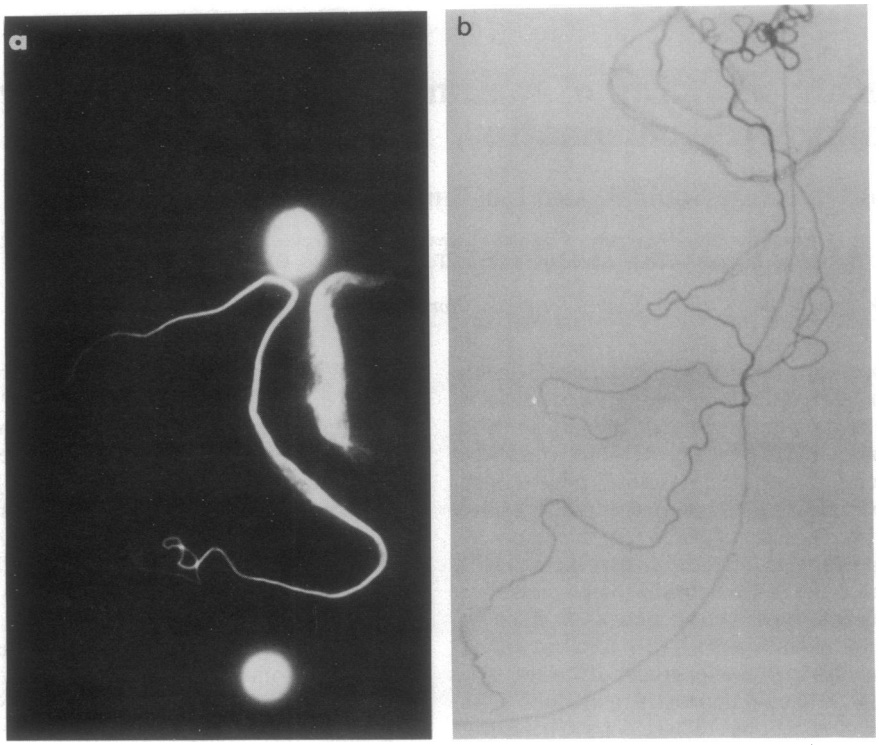

FIG. 1. Detection of free chromatin structures by DNA binding dyes. (×900.) (*a*) Two interphase nuclei and two spindle-like free chromatins after staining with DAPI. (*b*) Elongated free chromatins stained with Giemsa. The procedures were the same as those in Table 1.

Microscope Slide Preparation. The cells were collected and subjected to the standard hypotonic treatment, with KCl (0.4%) for 10 min at 37°C, and rinsed twice with 3:1 methanol:acetic acid (8–10). The cell suspension was dropped on ice-cold slides and air-dried for subsequent cytogenetic and hybridization analyses. The proportion of free chromatin in each preparation was examined after staining the slides with 3% Giemsa solution (pH 6.8; Fisher Diagnostics) for 10 min.

Alkaline Release of Free Chromatin. The harvested cells were resuspended in a borate buffer (1 mM sodium borate adjusted to pH ≈10 with NaOH and 0.5–2% KCl) at room temperature for 2–10 min. Since different types of cells responded differently to this alkaline-releasing procedure, it was necessary to adjust the time of incubation and KCl concentration accordingly. The free chromatin preparations were then fixed with 3:1 methanol:acetic acid and the slides were prepared in the usual manner.

FISH. FISH was performed according to published procedures (1). Briefly, the slides were aged for 20–30 days prior to denaturation by 70% formamide in 2× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate) at 70°C for 3 min and followed by dehydration in ethanol. Total human DNA or cosmid probes were prepared according to standard procedures. Probes were labeled with biotinylated dUTP (the BRL BioNick labeling system). Approximately 20 ng of the biotinylated probe was added to each slide together with 10 μg of salmon sperm DNA in 12 μl of hybridization buffer (50% formamide/1× SSC/10% dextran sulfate).

For total human DNA probe, hybridization was performed for 16–20 hr at 37°C. Post-hybridization washing consisted of three 5-min immersions in 50% formamide and 2× SSC, followed by 2× SSC and 0.1× SSC at 42°C. The slides were then immersed in a solution containing 3% bovine serum albumin (BSA) and 4× SSC for 30 min prior to incubating in 5 μg of fluorescein isothiocyanate (FITC) per ml conjugated to avidine (Vector Laboratories) in 1% BSA/0.1% Tween 20/4× SSC. Unbound fluorophores were removed by three 3-min rinses in the same solution without FITC and the slides were counterstained with 40 μg of propidium iodide per ml in phosphate-buffered saline (PBS) or with 0.2 μg of DAPI per ml. After a final washing in PBS for 5 min, the slides were mounted in 90% glycerol with 20 mM Tris·HCl (pH 8) and 2.3% 1,4-diazabicyclooctane (DAPCO antifade).

The procedure for cosmid probes was similar to that described above, except for the slide baking (60–65°C, 1–3 hr) and inclusion of RNase treatment (60 min at 37°C with 100 μg of RNase A per ml in 2× SSC), repetitive sequence suppression (15–30 min prehybridization at 37°C with 2 μg of sonicated total human DNA per slide), and signal amplification (with biotinylated goat anti-avidine antibody and FITC-avidine; Vector Laboratories).

Microscopy and Distance Analysis. The photographs were taken with a Nikon Microphot-FXA epifluorescence microscope equipped with dual-band FITC/Texas red filters (Omega Optical, Brattleboro, VT). Kodak color Ektachrome P800/1600 "push level 2" E-6P professional film was used with typical exposure times of 30–90 sec. The distance measurements were obtained from projected images of photographic slides.

RESULTS

As expected, the proportion of extended chromatin structures in a conventional metaphase preparation from peripheral blood culture was generally low. Typical free chromatins were operationally defined as bundles of fibrous structures at least five times longer than the mean diameter of the nuclei. Based on the analysis of >100 individuals, the average frequency of such structures among the interphase nuclei and mitotic figures was around 0.3% (data not shown) and there appeared to be significant variation in the proportion of these structures among samples from different individuals. The proportion of free chromatin structures, however, could be increased with various reagents and culture conditions; for example, the anti-neoplastic drug pingyanymycin was effec-

tive in inducing free chromatin from lymphocyte preparations (10).

In our preliminary study in which *m*-AMSA was used, as much as 2.1% of the structures were found to be typical free chromatins (Table 1). Fifteen percent of these structures reached 200–300 μm in length. There appeared to be an optimal concentration of *m*-AMSA and the effective concentration varied among different individuals (data not shown).

Since nuclear lamins could be disrupted by high pH treatment (13), the effect of pH of the cell resuspension buffer was investigated. The result showed that the proportion of free chromatins could be greatly increased by the high pH buffer, particularly for fibroblastoid cell lines, which were generally less responsive to *m*-AMSA (details to be published elsewhere). Different cell lines responded differently to the high pH protocol but many showed close to complete release of free chromatins. Therefore, the alkaline-releasing method was used in most of the subsequent studies.

To investigate whether these highly extended chromatin fibers could be used for gene mapping, we examined the hybridization pattern of human DNA in human/rodent somatic cell hybrid lines using FISH with a biotin-labeled total human DNA probe. In the control experiment (Fig. 2 *a* and *c*), a bright hybridization signal could be readily identified for the single, metaphase chromosome 7 in a background of hamster chromosomes in the cell line 4AF/102 (12), demonstrating the specificity of the probe. The hybridization pattern with free chromatin structures released from cell cultures at the G_1 phase was then examined. As shown in Fig. 2*d*, a long, thin, and somewhat discontinuous hybridization signal could be detected among each of the fibrous free chromatin bundles.

In another experiment, biotin-labeled total human DNA probe was incubated with free chromatin preparations from a somatic cell hybrid line containing chromosome 21 as its only human chromosomal material as well as from 4AF/102. Again, elongated thread-like hybridization signals were detected in the chromosome 21-only cell line, as in 4AF/102 (data not shown).

The above studies therefore demonstrate that free chromatin structures are greatly extended chromosomes and that they can be readily detected by FISH. The discontinuous hybridization patterns observed are probably due to the uneven distribution of repetitive DNA along human chromosomes (14).

To show that free chromatin could be used for gene mapping, we next examined the hybridization pattern with a set of five cosmids in a region containing the cystic fibrosis transmembrane conductance regulator gene (7q31) for which a detailed physical map was known (Fig. 3) (11). To avoid possible confusion of hybridization signals from the sister chromatids blocked at the G_2 phase by *m*-AMSA, we also switched to the use of cell culture at the G_1 phase. Since a single human chromosome 7 was present in 4AF/104, there should be only one set of hybridization signals in the free chromatin prepared from the G_1 phase culture of this cell line.

Table 1. Dose-responsive induction of free chromatin structures from human lymphocytes (of a single donor) with *m*-AMSA

m-AMSA, μg/ml	Structures examined, %	Free chromatin, %	Mitotic figures, %	Interphase nuclei, %
0	10,129	0.24	4.2	95.6
0.5	10,299	0.28	1.5	98.2
1.0	9,285	0.69	1.1	98.2
5.0	9,494	0.72	0.4	98.3
10	9,830	2.1	0.4	97.5
20	9,846	1.6	0.4	98.0
40	9,814	1.6	0.3	98.2

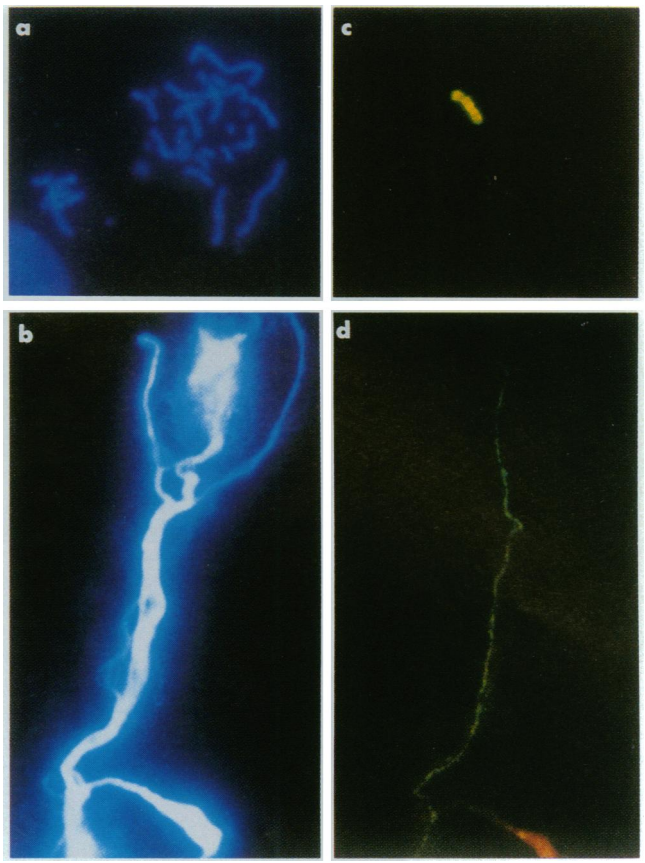

FIG. 2. Visualization of free chromatin by the FISH technique. (×700.) (*a*) The chromosome 7-specific somatic hybrid cell line 4AF/102 metaphase spread stained with DAPI to show the hamster and human chromosomes. (*b*) DAPI staining of free chromatin from the same cell line to show the total hamster and human DNA content. (*c*) The same metaphase preparation as in *a* with FISH detection showing hybridization of human chromosome 7. (*d*) Human chromosome 7 visualized as a long fiber in the free chromatin after FISH and FITC detection.

Accordingly, after testing in conventional FISH mapping with metaphase chromosomes (Fig. 4*a*) and interface nuclei (Fig. 4*b*), these probes were used in hybridization with the free chromatin preparations. As shown in Fig. 4 *c–f*, discrete fluorescent signals were readily detectable with cosmid probes in different combinations—two, three, or four spots could be identified with the corresponding number of hybridization regions (Fig. 3)—demonstrating the feasibility of this mapping technique with single-copy DNA sequences. The efficiency of hybridization with free chromatins had not been fully evaluated, but it appeared to be roughly the same as mapping with interphase nuclei (≈90%).

A good correlation was also found between the distance determined from the hybridization signals and their physical map in kb (Fig. 5). For example, the intervals (average distance ± standard deviation) between the four probe-hybridizing regions (cM58-3.6, CF14, cJ21, and cW10-20), measured from midpoint to midpoint in a pair-wise manner, were 0.9 ± 0.3 μm, 2.14 ± 0.48 μm, and 4.0 ± 1.1 μm, which corresponded well with 63, 167, and 301 kb from fine restric-

FIG. 3. Physical map of the region containing the five cosmids used in this study. The map was determined by detailed restriction mapping of cloned DNA (11).

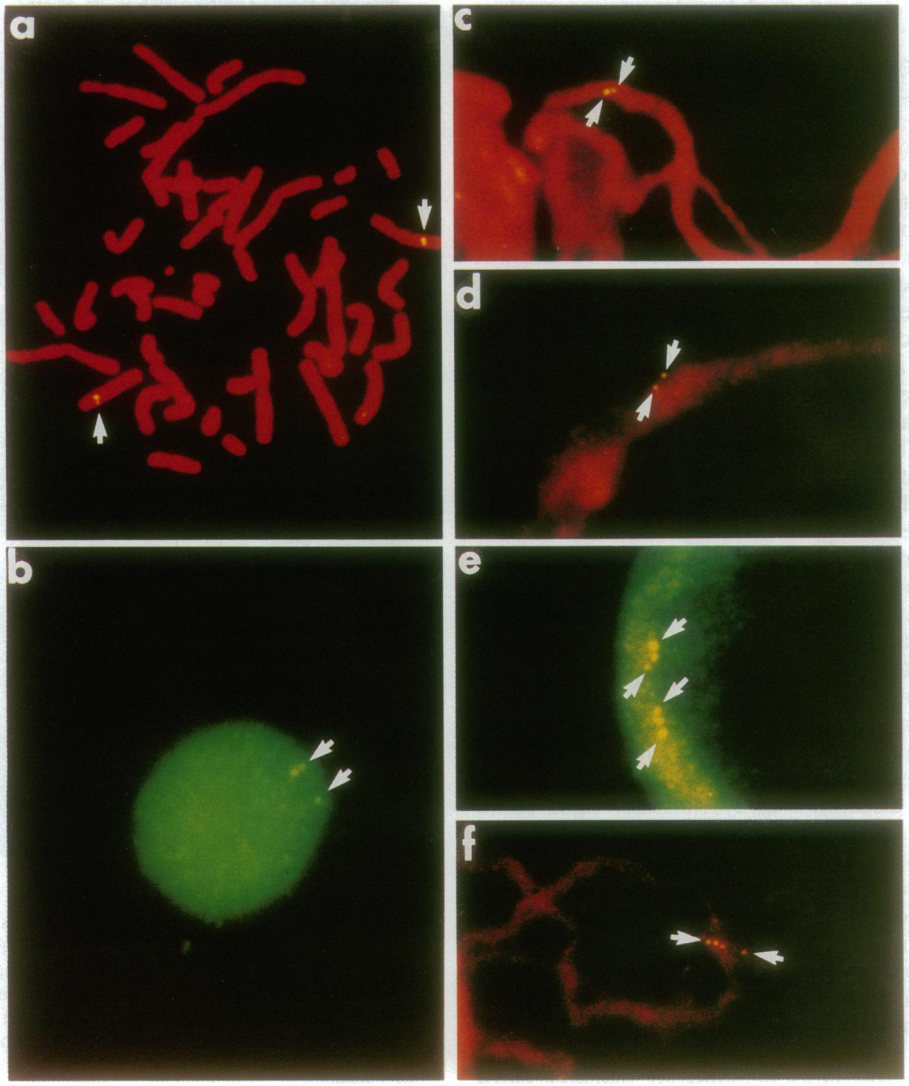

FIG. 4. Detection of single-copy sequences in metaphase chromosome, interphase nuclei, and free chromatin preparations by FISH. (×1300.) (*a*) Metaphase chromosomes from human diploid lymphocyte culture were hybridized with four cosmid probes, cM58-3.6, CF14, cJ21, and cW10-20 together, spanning a total distance of 341 kb (see Fig. 3). (*b*) Hybridization of the same probes as in *a* with G_1 phase nucleus; arrows indicate two sets of signals. (*c*, *d*, and *f*) Results of hybridization with different combinations of the cosmids to free chromatin prepared from G_1 phase nuclei of cultured lymphocytes; only a section of free chromatins showing one set of hybridization signals is shown. (*e*) Results of hybridization to somatic hybrid cell line 4AF/102; the two sets of hybridization signals represent the two sister chromatids of chromosome 7 at the G_2 phase. Probes used are as follows: (*c*) cNH24 and cJ21; (*d*) cM58-3.6 and cJ21; (*e*) cM58-3.6, CF14, and cJ21; (*f*) cM58-3.6, CF14, cJ21, and cW10-20.

tion mapping (Fig. 3) (11), respectively. Further, although the boundaries of each hybridization region could not be precisely determined, the signals were clearly discernible even for the two closest probes (cNH24 and cJ21), which were only 21 kb apart (Fig. 4*c*, the mean space measured between the two signals was 0.3 μm).

DISCUSSION

Using a simple alkaline-releasing buffer and FISH, we have developed a free chromatin mapping technique useful for determining gene orders in mammalian genomes. The utility of this technique compares well with all of the advantages offered by mapping with the interphase nuclei or pronuclei. The added advantage of the free chromatin mapping technique is the fact that the hybridization signals are detected along the extended fibers on the same plain, whereas those obtained with the interphase nuclei are distributed three-dimensionally. Thus, although it may be tricky to determine the order of multiple genes with interphase nuclei, the task can be achieved with relative ease with free chromatins. Using the present protocol, we have been able to determine the order of three or more probes in a single hybridization reaction with a few (typically one to three) properly prepared slides.

In the present study, the high-resolution power of the free chromatin mapping technique has been demonstrated with single-colored FISH, which could distinguish the signals from two DNA sequences separated by 21 kb. An excellent correspondence between the observed and expected distances has been obtained for genomic DNA sequences as far as 350 kb apart. With the use of multicolor fluorescence conjugates, it is reasonable to speculate that free chromatin mapping would readily resolve gene sequences separated by as little as 10 kb. Moreover, our preliminary data (not shown) indicated that the technique could allow simultaneous detection of signals spanning several Mb. Therefore, free chromatin mapping represents an extremely powerful technique for genome analysis and it should complement the currently existing physical and genetic mapping strategies in any large-scale efforts.

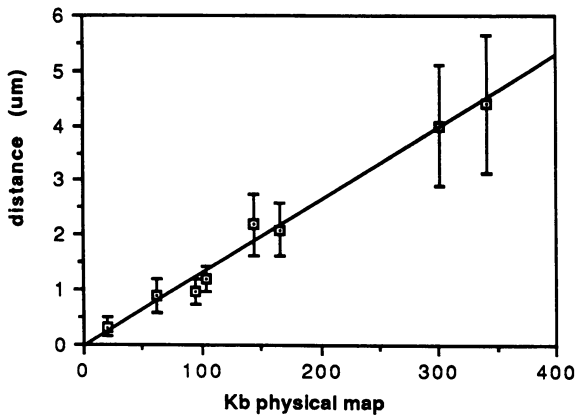

FIG. 5. Correlation between the expected and observed physical distances in free chromatin mapping. Each measurement was performed 10–50 times with hybridization signals derived from slides of the same preparation; the average distance is shown with standard deviation.

It is curious that hybridization with a 40-kb cosmid with the chromatin fibers appears as dots rather than as elongated "strings." The following explanations may be considered: (*i*) the signals detected are amplified through the use of secondary antibody, (*ii*) the concentration of signal may be highest in the middle of the probe hybridizing region and the signal becomes even more rounded due to light scattering, and (*iii*) part of the hybridization region might be suppressed by the competitive (total human DNA) probe and therefore the signal does not cover the entire cosmid. When large DNA fragments (such as those contained in yeast artificial chromosomes) were used as probes in our more recent studies, however, a "string-like" hybridization signal has been observed as anticipated (data not shown).

Our data also show that the physical distance estimate is not as accurate for probes separated far apart as for those located in close proximity. Fortunately, the variability in distance estimation can be easily overcome by inclusion of an internal ruler (e.g., a pair of well-studied probes). In the present example, the mean distances derived from multiple measurements do show the expected difference between 300 and 350 kb.

Since the free chromatin FISH mapping technique is insensitive to distribution of rare-cutting restriction enzyme recognition sites, it will be particularly useful for studying genomic DNA regions where traditional cloning and long-range restriction mapping have not been straightforward. For example, the technique may be used to define the fine structure and organization of the different types or subfamilies of repetitive elements at the centromeres and telomeres. Similarly, free chromatin FISH mapping may be applied to studies of gene amplification, translocation, and deletion, where genomic DNA rearrangement is often too complex for traditional methods of analysis.

Although the structural basis behind the free chromatin is presently unclear, the utility of the technique should not be affected by this ignorance. Nevertheless, some speculations may be offered to explain the mechanism leading to free chromatin formation. The anti-tumor drug *m*-AMSA used in this study is a potent inhibitor of topoisomerase II, which is thought to be required for chromosome condensation (15, 16). This observation implies that *m*-AMSA directly interferes with chromatin condensation and arrests the chromosomes at a transient stage not normally amenable to routine cytogenetic preparation methods. On the other hand, the high pH buffer may simply destabilize nuclear envelope, releasing chromatin fibers from the cell regardless of the stage of cell cycle. Since the use of cell cultures at the G_1 phase is important for ordering genes or DNA segments, the latter technique is particularly useful when combined with a synchronized cell population. The alkaline buffer is also effective for most of the tested cell types, including lymphoblasts, fibroblasts, and tumor cell lines.

It remains to be determined, however, if the chromatin fibers released from different stages of cell cycle would assume the same structure. Although we have demonstrated that there is a good correlation between the true and measured physical distances for a small region with the same free chromatin preparation and that there is only small variation among chromatin strands when slides from the same preparation are used, there is significant variation between preparations from different procedures (data not shown). In addition, it will be important to investigate if there is any difference between active and inactive chromatins, if the relationship is constant for the entire genome, or if there is variation between different regions of a chromosome or among different cell types. Answers to these questions may provide some insight into chromatin structure itself and into mechanisms of gene regulation during cellular differentiation and development.

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1. Lichter, P., Tang, C.-j. C., Call, K., Hermanson, G., Evans, G. A., Housman, D. & Ward, D. C. (1990) *Science* **247**, 64–69.
2. Fan, Y. S., Davis, L. M. & Show, T. B. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 6223–6227.
3. Lawrence, J. B., Singer, R. H. & McNeil, J. A. (1990) *Science* **249**, 928–932.
4. Trask, B., Pinkel, D. & van den Engh, G. (1989) *Genomics* **5**, 710–717.
5. Ferguson-Smith, M. A. (1991) *Am. J. Hum. Genet.* **48**, 179–182.
6. Trask, B. J. (1991) *Trends Genet.* **7**, 149–154.
7. Brandriff, B., Gordon, L. & Trask, B. (1991) *Genomics* **10**, 75–82.
8. Heng, H. Q. & Chen, W. Y. (1985) *J. Sichuan Univ. Nat. Sci. Ed.* **2**, 105–108.
9. Heng, H. Q. & Chen, W. Y. (1988) *The Nucleus* **29**, 5–8.
10. Heng, H. Q., Chen, W. Y. & Wang, Y. C. (1988) *Mutat. Res.* **199**, 199–205.
11. Rommens, J. M., Iannuzzi, M. C., Kerem, B.-s., Drumm, M. L., Melmer, G., Dean, M., Rozmahel, R., Cole, J. L., Kennedy, D., Hidaka, N., Zsiga, M., Buchwald, M., Riordan, J. R., Tsui, L.-C. & Collins, F. S. (1989) *Science* **245**, 1059–1065.
12. Arfin, S. M., Cirullo, R. E., Arredondo-Vega, F. X. & Smith, M. (1983) *Somatic Cell Genet.* **9**, 517–531.
13. Raymond, Y. (1990) *Cell Biol. Int. Rep.* **14**, 165–171.
14. Moyzis, R. K., Torney, D. C., Meyne, J., Buckingham, J. M., Wu, J. R., Burkes, C., Sirotkin, K. M. & Goad, W. B. (1989) *Genomics* **4**, 273–289.
15. Adachi, Y., Luck, M. & Laemmli, U. K. (1991) *Cell* **64**, 137–148.
16. Drlica, K. & Franco, R. (1988) *Biochemistry* **27**, 2253–2259.