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Detection of DNA domains in *Drosophila*, human, and plant chromosomes possessing mainly 50- to 150-kilobase stretches of DNA

(chromosomal structures/DNA domains/jumping library/pulsed-field gel electrophoresis/sensitive regions)

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ABSTRACT **We have used pulsed-field gel electrophoresis of undigested DNA prepared by cell lysis in agarose with proteinase K detergent treatment and found a resolvable DNA fraction, denoted forum DNA (f-DNA). By changing the pulsed-field gel pulse length from 25 to 4500 sec, to obtain optimal separation in different ranges, we have found f-DNA to occupy a rather broad zone from 2 megabases to 10 kilobases (kb), but mainly at a range between 50 and 150 kb. f-DNA seems to appear as a result of nonrandom spontaneous degradation during cell treatment. The terminal regions of f-DNA segments have been cloned by using a jumping library. The molecular analysis of unique DNA sequence from an anonymous *Drosophila* DNA segment led to the conclusion that f-DNA appears as a result of nonrandom chromosomal DNA cleavage within sensitive regions that occupy a few kilobases. This conclusion was confirmed by detection of rather discrete hybridization bands on pulsed-field gel Southern blots in a region of good separation of undigested f-DNA after hybridization with different unique and repetitive probes. We propose that f-DNA segments may correspond to some regular higher-order structures in the eukaryotic chromosomes.**

From general reasoning and some experimental evidence, it is believed that linear chromosomal DNA must be organized into some kind of superstructures. The string of nucleosomes could form several structural levels in the chromosome, reflecting different features in its organization and function (1). Analysis of these structures in higher eukaryotes is hampered by both the complexity of the chromosomal architecture and the lack of appropriate assay techniques for fine measurements of the basic elements involved.

The study by DNase I treatment of structural changes in chromosomal organization occurring when a gene is activated led to detection of a preferential sensitivity to digestion that extends throughout the transcription unit (1). This sensitivity is specific to the chromosomal regions in which the gene is active. The second phenomenon detected by DNase I digestion is very local alterations in chromosomal structure, which makes it extremely sensitive to DNase I digestion (2). The local changes in structure at 5′ and 3′ regions of genes could be determined by stable sequestration of transcription factors onto promoters that may play a critical role in transcription activation (3).

The study of DNA packaging in interphase and metaphase chromosomes of higher eukaryotes has revealed chromosomal loops to be formed by attachment of specific DNA sites on the nuclear scaffold (4–6). It has been proposed that nontranscribed scaffold binding regions located at the base of looped domains may play roles in chromosome segregation, transcription activation, and replication (4–8).

Recent studies have revealed compositionally (measured as G+C percent) homogeneous vertebrate chromosomal DNA domains spanning several hundred kilobases—so-called isochores (9).

In this paper, we report chromosomal DNA domains detected by pulsed-field gel (PFG) electrophoresis.

MATERIALS AND METHODS

Isolation of DNA. DNA-agarose plugs were prepared as described by Smith *et al.* (10). Cells were pelleted by centrifugation at 2000 rpm, washed with a phosphate-buffered saline (PBS) solution (125 mM NaCl/25 mM sodium phosphate buffer, pH 7–7.2), resuspended to a concentration of 2–3 × 10^7 cells per ml, gently mixed at 43°C with an equal volume of a 1% agarose L (LKB) in PBS solution, and distributed on a mold containing 100-μl wells. The mold was placed on ice for 2–5 min, covered with parafilm, and incubated for 5–20 min at room temperature. The agarose plugs were then placed in Petri dishes containing 0.5 M EDTA (pH 9.5), 1% sodium laurylsarcosine, and 1–2 mg of proteinase K solution per ml for 40–48 hr at 50°C, and stored at 4°C in the same solution. Agarose cell plugs from human sperm were also treated with 2% 2-mercaptoethanol at 50°C during incubation. *Arabidopsis*, tomato, and maize protoplasts were prepared as described (11).

For preparation of total *Drosophila* DNA in solution, anesthetized Oregon-RC flies were homogenized in liquid nitrogen. *Drosophila* forum DNA (f-DNA) preparations for cloning procedures, end labeling, and Southern analysis were isolated by electroelution and dialyzed for 24 hr against 1 liter of 0.01 × TE at 4°C without stirring; DNA was concentrated with solid sucrose (4°C) and redialyzed.

PFG Electrophoresis. Portions of the original agarose-DNA plugs (5–100 μl) containing 1–10 μg of DNA were used for electrophoresis without any restriction enzyme digestion. The gels were run on an LKB Pulsaphor system.

Cloning Procedures. The cloning of forum DNA (f-DNA) termini was performed as follows. About 10 μg of high molecular weight f-DNA prepared as described was treated with the Klenow fragment of *Escherichia coli* DNA polymerase I in the presence of all four dNTPs to maximize the number of molecules with perfectly blunt ends (see scheme of Fig. 2), dialyzed, and concentrated on solid sucrose (4°C). The DNA was ligated with 1:100 molar excess of dephosphorylated *Not* I adaptors. To remove free adaptors, the sample was electrophoresed in a 1% agarose minigel, and the f-DNA band was eluted. The ligation with 400:1 molar excess of *supF*-containing *Not* I ends was performed in 300 μl of solution containing 0.1 M NaCl, 50 mM Tris·HCl (pH 7.4), 8 mM $MgCl_2$, 9 mM 2-mercaptoethanol, 7 μM ATP, and 10

Abbreviations: Mb, megabase(s); PFG, pulsed-field gel(s); f-DNA, forum DNA.

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units of T4 DNA ligase at 4°C for 16 hr. After heating at 65°C for 10 min, the reaction mixture was diluted to 500 μl and digested with *Eco*RI enzyme. The DNA was used for cloning in λL47.1 arms as described (12). For plating on 5-bromo-4-chloro-3-indolyl β-D-galactoside/isopropyl β-D-thiogalactoside agar, the 3103 *E. coli lac7* amber *recA* host was used. About 3–5% of plaques were blue (*supF*).

Hybridization Probes. The end-labeled f-DNA probe was prepared as follows. About 5–10 μg of f-DNA was eluted as described and treated with exonuclease III. The amount of exonuclease III and incubation time at 37°C were preselected in the experiments with λ DNA, allowing removal of not more than 4.3 kilobases (kb) of sequences. Then the sample was heated for 10 min at 65°C, dialyzed, and concentrated. The fill-in reaction was performed in 200 μl of solution containing [α-^{32}P]dATP- and [α-^{32}P]dCTP-labeled (specific activity, >3000 μCi/mmol; 400 μCi each; 1 Ci = 37 GBq) and unlabeled dGTP and TTP, 0.1 M Hepes (pH 6.9), 10 mM $MgCl_2$, 10 mM dithiothreitol, 0.07 M KCl, and 10 units of Klenow fragment of *E. coli* DNA polymerase I for 15 min at 14°C. Then DNA was precipitated and incubated in 0.1 M NaOH solution at 100°C for 20 min, chilled, neutralized, and purified through a G-50 Sephadex column.

Nick-translation of DNA probes and hybridization procedures in stringent conditions were performed as described (13).

RESULTS

Detection of the DNA Fraction Migrating from Undigested DNA-Agarose Plugs. Total cellular high molecular weight DNA samples were prepared in agarose from *Drosophila* cells, human cells, and plant protoplasts as described. The samples were loaded directly onto a PFG to detect DNA that may be spontaneously excised from chromosomes. We proposed that damage of chromosomes occurring just before cell lysis may provide some information on the chromosomal structures. We have found a resolvable DNA fraction that comprises from 3–5% to ≈50% of total DNA. The amount depends on both the length of incubation of agarose plugs before EDTA/lauroylsarcosine/proteinase K treatment and the source of cells and can be easily reproduced.

Fig. 1 shows the ethidium bromide (EtdBr) staining pattern of gels. Changing the PFG pulse length from 25 to 400 sec considerably affects the migration pattern of DNA. One or two regions of lower resolution at a switching interval of 25 or 100 sec appear as 1- and 2-megabase (Mb) bands (Fig. 1 *A* and *B*). These bands originate from smeared DNA because, after separation at pulse times of 400 sec, which allows efficient separation up to 2 Mb, no discrete bands in the region of 1 or 2 Mb could be seen (Fig. 1*D*). Thus, the migrating DNA fraction has a length of 10 kb to ≈2 Mb, but it mainly separates in the region of 50–150 kb. The latter region, after more efficient separation at a pulse time of 100 sec and a short duration run, reveals a small amount of DNA that migrates as a discrete band (Fig. 1*B*). This migrating DNA was denoted f-DNA (named after the forum in ancient Rome, by analogy with a city "structural element"). The pattern of f-DNA is indicative of nonrandom degradation of chromosomal DNA. We have studied whether added naked DNA could be subjected to degradation in agarose plugs during the procedure used for preparation of f-DNA. No degradation of λ DNA fragments was observed in these experiments (Fig. 1*C*).

Study of the Terminal Regions of f-DNA Segments. The observation that rather large DNA fragments spontaneously excise from chromosomal DNA immediately raises the question of whether f-DNA segments have specific borders. To look for molecular evidence for specific or casual damage of chromosomes that may produce f-DNA stretches, we cloned the terminal regions from *Drosophila* f-DNA. We have used modified jumping library techniques to isolate a number of clones, each containing a pair of fragments corresponding to two different f-DNA segments. One of the clones has been studied in more detail. It contains two *Eco*RI/*Not* I fragments, one of which is 1.3 kb long and possesses unique DNA

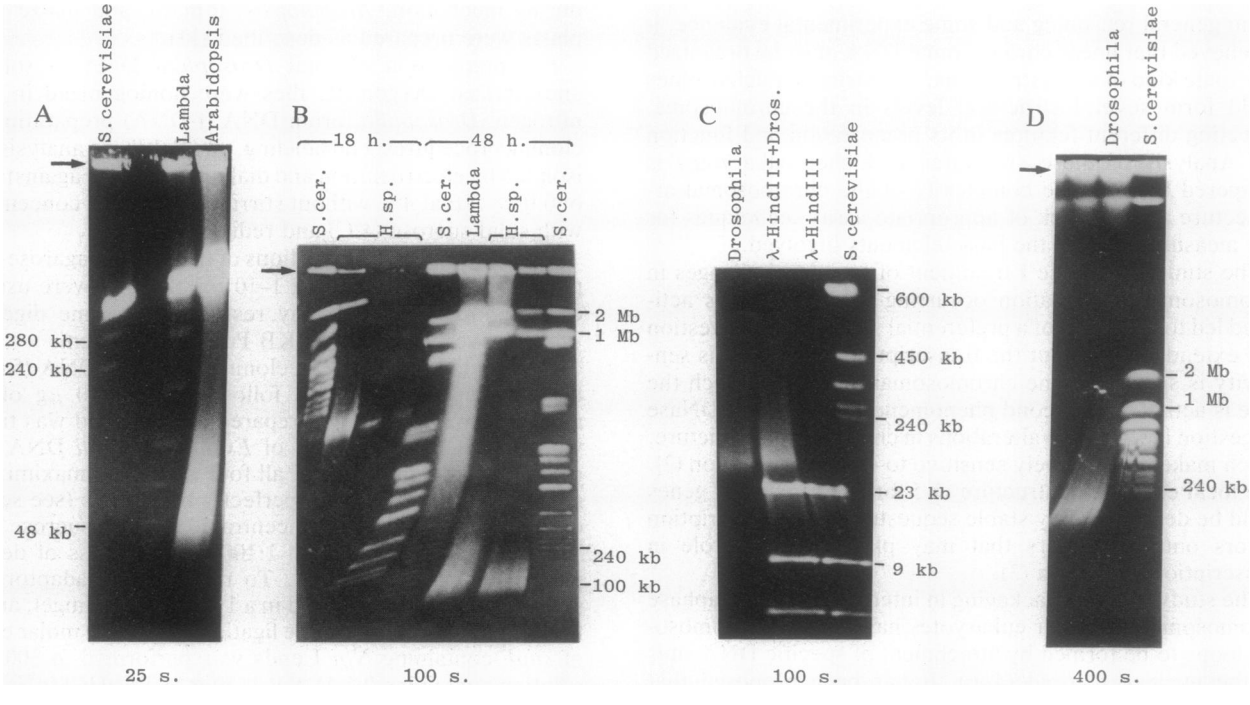

FIG. 1. Electrophoretic separation of DNA from DNA-agarose plugs containing total undigested DNA. Total uncleaved DNA samples were isolated from human sperm (H.sp.), *Drosophila*, and *Arabidopsis* after 5–20 min of incubation of cells in agarose as described. *Saccharomyces cerevisiae* (S. cer.) chromosomes and a λ ladder were used as size markers. Arrows indicate the slots. EtdBr-stained PFGs with different pulse times are shown. Gels were run from 18 to 72 hr (*A*, 50 hr; *B*, 18 or 48 hr; *C*, 56 hr; *D*, 72 hr). (*C*) Separation of naked DNA fragments into agarose blocks (with or without *Drosophila* cells), which were used for the DNA isolation procedure.

sequences. This fragment was used as a probe for cloning the corresponding undamaged genomic DNA sequence (λ1.3), which was mapped by standard molecular techniques. As shown in Fig. 2*B*, a 1.3-kb fragment containing an artificial *Not* I site corresponds to a 1.8-kb *Eco*RI fragment lacking *Not* I sites.

To test the characteristics of degradation in this particular region, we prepared a probe containing end-labeled f-DNA. DNA termini were treated by exonuclease III followed by filling in 3′ recessed termini with Klenow fragment of DNA polymerase I in the presence of ^{32}P-labeled precursors. Fig. 2*C* shows the result of hybridization with the probe after fragmentation to 200–400 base pairs. There are *Eco*RI fragments of the λ1.3 clone that hybridize preferentially with the probe. Short repetitive elements and immobilization of DNA fragments on the Southern filter do not interfere with the experiment because the total *Drosophila* DNA probe exhibits a rather uniform pattern. We suppose that during the end-labeling procedure some f-DNA fragments are sheared. But this apparently happens to an extent that allows labeling predominantly at the ends of the original DNA molecules. Interestingly, we observed in λ1.3 two regions of efficient hybridization with f-DNA ends. This suggests that in this genomic region there are two f-DNA termini separated by several kilobases of DNA, which apparently are attacked upon spontaneous degradation of chromosomes. The cloned terminus from one f-DNA segment also corresponds to the sensitive region between these two neighboring segments (Fig. 2*B*).

These results encouraged us to determine the characteristics of the DNA damage inside the sensitive region by genomic Southern analysis of f-DNA digested by *Eco*RI endonuclease. Fig. 2*D* shows the results of genomic Southern analysis of the f-DNA preparation, DNA remaining in original DNA-agarose plugs after elution of the f-DNA fraction (forum$^-$ DNA), and total DNA samples. Surprisingly, we found practically no difference between the hybridization patterns of f-DNA and total DNA samples, to say nothing of some smeared DNA on f-DNA and forum$^-$ DNA lanes. One possible explanation is that in this experiment we analyzed whole f-DNA stretches, which, as a result of partial digestion, may contain mostly undamaged 1.3-kb sequence in internal regions. In contrast, in the previous experiment (Fig. 2*B*) we studied the ends of f-DNA segments. We believe the results of both experiments may indicate that cut sites are scattered inside the sensitive region and that there is no precise site similar to hypersensitive DNase I sites that could be detected as discrete additional bands on genomic Southern autoradiographs (14). This conclusion is supported by observation of

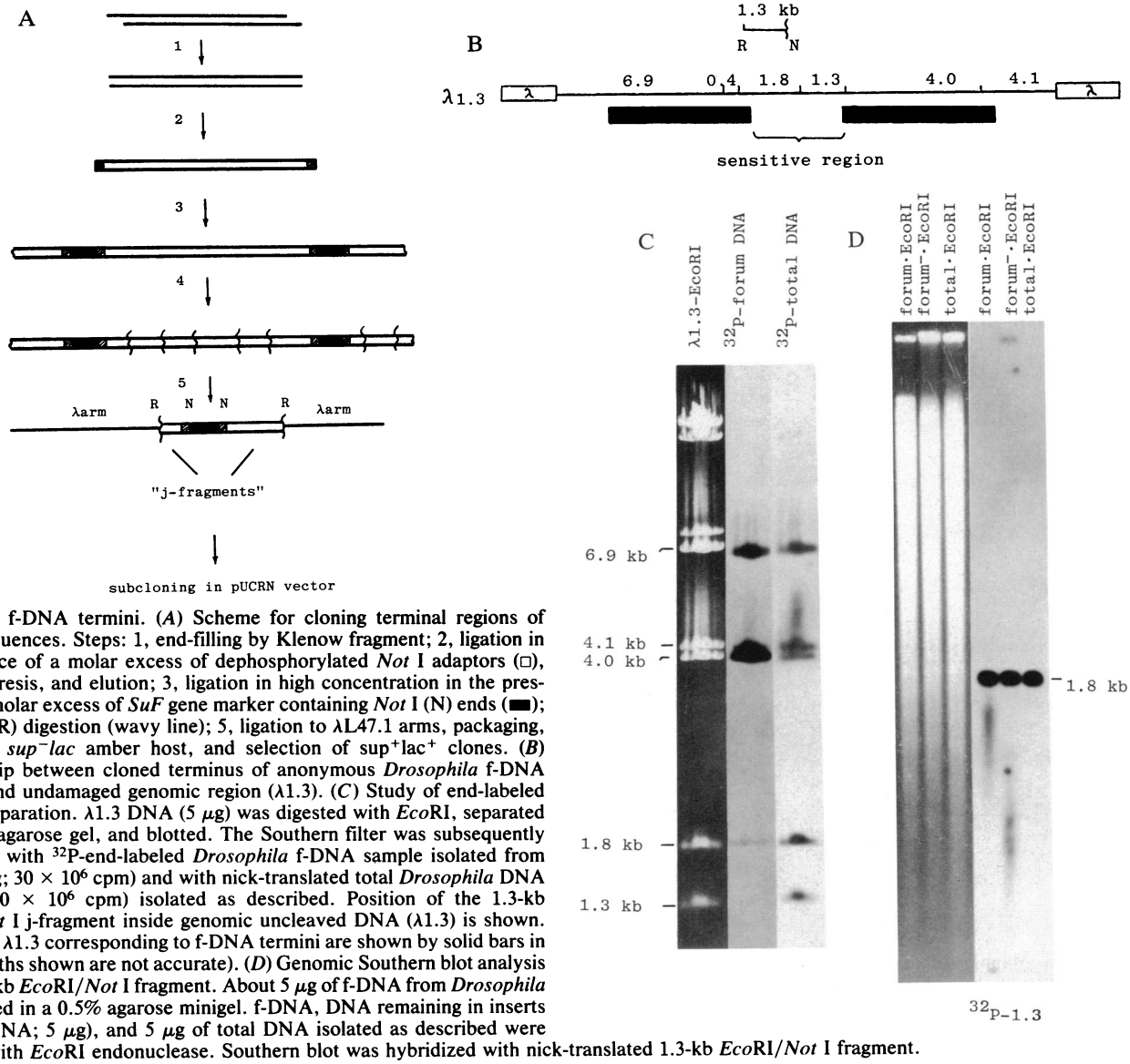

FIG. 2. f-DNA termini. (*A*) Scheme for cloning terminal regions of f-DNA sequences. Steps: 1, end-filling by Klenow fragment; 2, ligation in the presence of a molar excess of dephosphorylated *Not* I adaptors (□), electrophoresis, and elution; 3, ligation in high concentration in the presence of a molar excess of *SuF* gene marker containing *Not* I (N) ends (■); 4, *Eco*RI (R) digestion (wavy line); 5, ligation to λL47.1 arms, packaging, plating on *sup*$^-$*lac* amber host, and selection of sup$^+$lac$^+$ clones. (*B*) Relationship between cloned terminus of anonymous *Drosophila* f-DNA segment and undamaged genomic region (λ1.3). (*C*) Study of end-labeled f-DNA preparation. λ1.3 DNA (5 μg) was digested with *Eco*RI, separated in a 0.8% agarose gel, and blotted. The Southern filter was subsequently hybridized with ^{32}P-end-labeled *Drosophila* f-DNA sample isolated from flies (10 μg; 30 × 10^6 cpm) and with nick-translated total *Drosophila* DNA (0.5 μg; 10 × 10^6 cpm) isolated as described. Position of the 1.3-kb *Eco*RI/*Not* I j-fragment inside genomic uncleaved DNA (λ1.3) is shown. Regions of λ1.3 corresponding to f-DNA termini are shown by solid bars in *B* (the lengths shown are not accurate). (*D*) Genomic Southern blot analysis of the 1.3-kb *Eco*RI/*Not* I fragment. About 5 μg of f-DNA from *Drosophila* was isolated in a 0.5% agarose minigel. f-DNA, DNA remaining in inserts (forum$^-$ DNA; 5 μg), and 5 μg of total DNA isolated as described were digested with *Eco*RI endonuclease. Southern blot was hybridized with nick-translated 1.3-kb *Eco*RI/*Not* I fragment.

the cloned copy of the f-DNA segment terminus, which covers some part of the sensitive region (Fig. 2*B*).

Mapping of f-DNA Segments Inside the *cut* Locus of *Drosophila melanogaster*. To understand further the nature of the sensitive regions, we attempted to map the f-DNA segment ends inside the *Drosophila cut* locus, which was cloned earlier in a region spanning 240 kb (12). The Southern blot containing *Eco*RI digestion fragments of λ clones from this chromosomal walk was hybridized with the end-labeled *Drosophila* f-DNA probe. In five different experiments, only one 5.6-kb fragment in the λg11 clone was found to hybridize to the probe (Fig. 3*A*).

The 5.6-kb *Eco*RI fragment is not transcribed and genetically corresponds to the regulatory region of the *cut* locus (12, 15, 16). The nearest transcribed regions are located 25 kb downstream (15, 16). Therefore, in the *cut* locus, mainly located in 7B1.2 (dense bands in the *Drosophila* X chromosome), there are at least two neighboring f-DNA segments that are separated by a sensitive region residing at the −65 coordinate. The distal and proximal segments extend beyond our walk and are more than 105 and 135 kb long, respectively.

Detection of Hybridization Bands in the Resolved Area of Undigested f-DNA Segments. The observation of sensitive regions a few kilobases long separating f-DNA segments prompted us to investigate whether such nonprecisely excised large DNA molecules could be detected as discrete bands after separation in PFG. In other words, we decided to analyze large DNA segments by a method that is not sensitive to differences of several kilobases above 50 kb. As shown in Fig. 4, two different unique probes could detect DNA bands in the regions of good separation, which was indicated by a λ ladder separation. In each case, there are several DNA bands on the smeared background. A number of hybridization bands (up to 250 kb) are detected in *Drosophila* f-DNA by the probe containing *Drosophila* histone genes. We have also tested a number of different unique and repetitive DNA probes and concluded that the result varies from a smeared pattern to a clearly banded one, apparently because of some structural peculiarities in different genomic regions. The data presented here strongly support the notion that f-DNA segments appear as a result of specific damage to chromosomes.

DISCUSSION

Origin of f-DNA. Using PFG electrophoresis, we have detected a fraction of DNA excised from eukaryotic chromosomes. In our experiments, cells were incubated in agarose for short times followed by strong treatment with EDTA/detergent/protease. We believe, therefore, that chromosomal DNA damage must take place just before cell lysis or rather rapidly after the beginning of treatment, preventing any enzymatic breakage of DNA. Thus, we think this procedure provides the most adequate available approach for detection of chromosomal structures via analysis of its DNA component. Alternative procedures for molecular analysis, which usually include isolation of nuclei or extraction of histones and other constituents from chromosomes, need more time and thus probably do not preserve sensitive chromosomal structures, although they could detect more persistent structures such as looped domains (17). It is well known that DNA may be protected from nuclease by histones and other proteins. Therefore, the observation of nonrandomly excised DNA stretches from chromosomal DNA led us to propose that f-DNA segments arise from some chromosomal higher-order structures.

The properties of the sensitive regions described in this paper clearly differ from the properties of DNase hypersensitive sites in at least two ways. The sensitive region reported here spans a few kilobases, so the length of the resulting f-DNA segments should vary in this range. On the contrary, in DNase hypersensitive sites, DNA is cut precisely (14). The second characteristic feature of the sensitive regions is that they reside at a distance of 50–150 kb from each other, while DNase hypersensitive sites are much closer to each other. The nature of the nucleases that produce cleavage of chromosomal structures is unclear. Topoisomerase II could be one of the presumptive candidates.

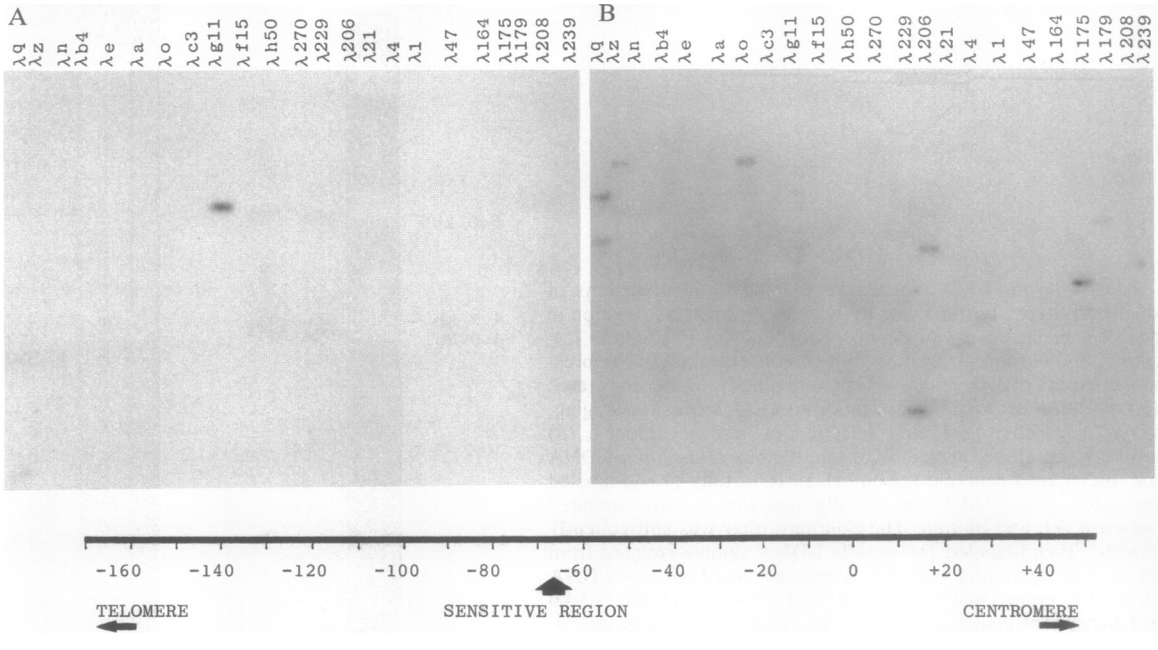

FIG. 3. Mapping of f-DNA segments in the *cut* locus of *D. melanogaster*. Southern blot of *Eco*RI-digested phage clones (0.5 μg per lane) hybridized with the end-labeled f-DNA probe (*A*) and with nick-translated Oregon-R genome DNA (*B*). Repetitive sequences from both probes were removed, at least partly, by hybridization with excess total *Drosophila* DNA immobilized on a nitrocellulose filter. The filter was used earlier to study the distribution of repetitive sequences in the *cut* locus (12). Vertical arrow indicates position (in our coordinates) of a sensitive region (12).

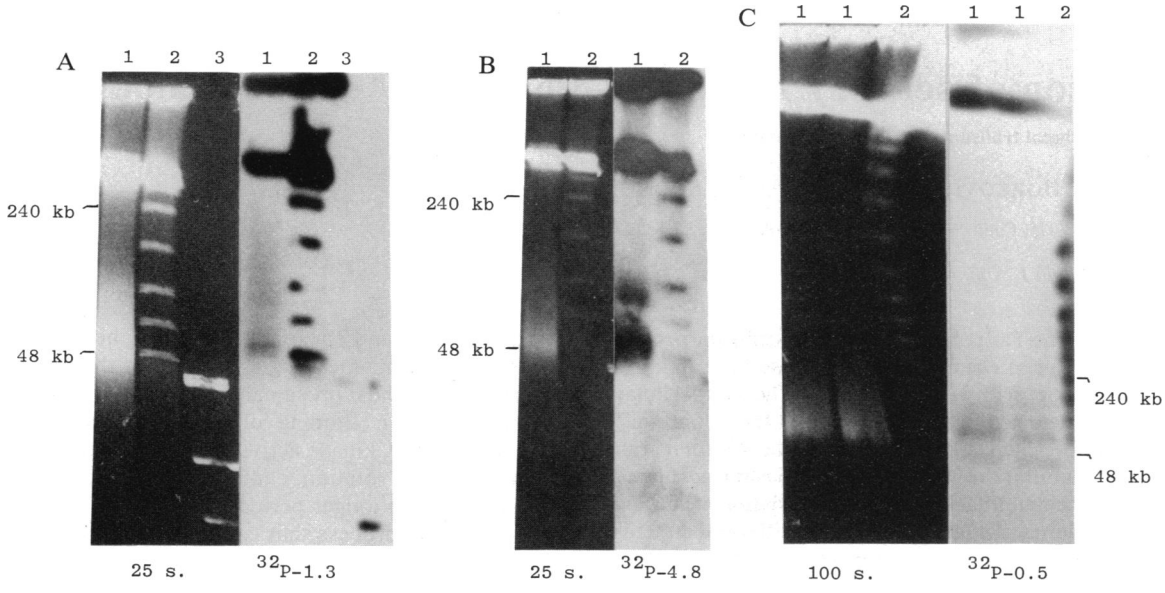

FIG. 4. Detection of specific fragments in f-DNA. *Drosophila* f-DNA was separated with 25-sec (*A* and *B*) or 100-sec (*C*) pulses and blotted. Lanes: 1, *Drosophila*; 2, λ ladder; 3, λ *Hin*dIII. PFG Southern blots were hybridized with ^{32}P-labeled probes. (*A*) Unique *Drosophila* 1.3-kb *Eco*RI/*Not* I j-fragment (see Fig. 2). (*B*) A 4.8-kb *Bam*HI *Drosophila* histone DNA repeat. (*C*) Anonymous unique 0.5-kb *Eco*RI/*Not* I fragment from a *Drosophila* linking library (N.A.T., unpublished data). Nick-translated λ DNA was used to visualize λ ladder size markers. In separate experiments, it was found that ^{32}P λ DNA gives no detectable hybridization in f-DNA lanes.

The Nature of f-DNA. The key experimental finding of this study is detection of nonrandom excised stretches of DNA upon spontaneous endogenous cleavage of chromosomes. These DNA stretches isolated from *Drosophila*, human, and plant cells are 2 Mb to 10 kb, but mainly 50–150 kb, long. f-DNA and total DNA preparations have practically indistinguishable DNA fingerprinting patterns (data not shown). We suppose that these data reflect a specific periodic distribution of some chromosomal structures. This could also reflect the periodic distribution of specific DNA sequences for which endogenous nucleases had an inherent preference. The mapping of a nonrandom cleavage site in the *Drosophila cut* locus, in a region not transcribed and thus not expected to be highly nuclease sensitive, favors higher-order packing of f-DNA domains. At least two types of regular chromosomal structures are described: bands/interbands and looped domains. We suppose that f-DNA segments, which are separated by rather short sensitive regions, may correspond to bands and interbands, respectively.

The relationship of the f-DNA segments to the looped domains seems less attractive to us. The size of the looped domains in *Drosophila* is rather short (18). Moreover, scaffold-attached DNA regions, which are located at the bases of looped domains, easily survive nuclear isolation and protein extraction procedures, whereas f-DNA domains are sensitive to long procedures. It is speculated that the looped domains contain transcription units, whereas f-DNA isolated after a very short incubation is depleted of transcribed sequences (11). However, the final conclusion should be drawn from direct data by mapping of individual f-DNA segments and scaffold attachment sites.

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