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Forum domain in *Drosophila melanogaster cut* locus possesses looped domains inside

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ABSTRACT

We have studied the relationship between chromosomal forum domains and looped domains in the *cut* locus of *Drosophila melanogaster*. Forum domains were earlier detected by separation in pulsed-field gels of 50–150 kb chromosomal DNA fragments obtained after spontaneous non-random degradation of chromosomes. We have localized the boundary region where cleavage sites are scattered between two forum domains in the regulatory region of the *cut* locus. We have sequenced a 13 kb region spanning few kilobases from distal domain, the boundary region and part of the proximal forum domain where several scaffold associated regions (SARs) were observed. We conclude that forum domains and looped domains are physically different types of domains and belong to different levels of organization in eukaryotic chromosomes. The boundary region between the neighboring forum domains in the *cut* locus possesses the *Doc* element insertion and a micro-satellite stretch and thus might remind a small island of heterochromatin and correspond to so-called intercalary heterochromatin that is known to be located in the 7B1-2 band where the major part of the *cut* locus is reside.

INTRODUCTION

In eukaryotic chromosomes DNA is involved in several levels of organization. The first level represents the 10 nm fiber containing 145 bp DNA turns around nucleosomes with 30–50 bp of linker DNA (1). The second level is the 30 nm fiber in which the nucleosomal string is packed in a solenoid-like structure (2,3). At present our knowledge about higher levels is far from certain, although several lines of evidence support the idea that the next level(s) must compact DNA at least 100-fold (4).

Increasing evidence supports the looped domains concept, according to which the higher order structures include topologically independent loops that are attached to a nuclear scaffold formed by proteins. The DNA loops originally were observed by electron microscopy (5) and then analyzed biochemically (6–9). In recent years this concept was also explored in order to explain how genes could be isolated from the influence (activation or repression) of foreign enhancers or silencers, that are not bona fide regulatory elements for the particular gene (10,11). Structural and functional studies support the idea that the chromosomal loops correspond to a series of discrete domains possessing independent units of gene activity and delimited by boundaries.

Three types of elements are known to be involved in the formation of chromosomal domains as independent units of gene expression. Structural analysis of DNA binding to nuclear scaffold revealed scaffold associated region (SAR) sequences. They are AT-rich stretches of several hundred bases in length, often containing topoisomerase II cleavage sites and have been found near genes (12). Functional analysis of SAR sequences led to the conclusion that they possess a limited functional capacity to act as insulators (13–15) and could rather serve to anchor domains mechanically to the nuclear scaffold, forming the structural loops. The next type of elements identified in functionally independent domains are sequences denoted as locus control regions (LCRs). LCRs are regulatory sequences that provide tissue-specific expression of genes in active domains. These elements contain binding sites for regulatory proteins and are associated with DNase hypersensitive sites (16,17). The third type of known elements in autonomous functional domains are insulators. These elements are defined as sequences that prevent activation or repression from regulatory sequences of neighboring chromosomal domains. The best characterized insulators were detected in molecular studies of short nuclease-resistant sequences around the hsp70 gene (scs/scs′ elements) or in genetic studies of regulatory sequences of the *gypsy* mobile element (18–20). It was demonstrated that a boundary element from the chicken genome serves as an insulator in the *Drosophila* genome. This means that *Drosophila* regulatory protein(s) can bind to heterologous insulators *in vivo*, suggesting a dramatic conservation of insulating mechanisms in evolution (21). Recently, a protein component of an scs′ element was identified and localized to hundreds of interbands and many puff boundaries on polytene chromosomes (20).

Although our knowledge about higher order chromosomal structures has clearly grown during the last few years, our understanding of the hierarchy of chromosome domains and of boundary elements which might specify different levels of chromosomal architecture and determine both the local and distant regulation of gene expression is at a rudimentary stage. Thus, it is of importance to use varied approaches to identify and

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analyze the higher levels in chromosome organization. Several years ago we started to study domains that could be released very early in the course of spontaneous degradation of eukaryotic chromosomes in cells included in low-melt agarose blocks (22). We assumed that all known approaches take more time and thus probably do not preserve sensitive chromosomal structures, although they could detect more persistent structures such as looped domains. We showed that DNA fragments of 50–150 kb are released by this method (forum domains). The data indicated the non-random degradation of chromosomal DNA and a method for mapping forum domains by end-labeled probes was elaborated. Fingerprinting patterns of forum domains and total DNA suggested that forum domains might reflect the existence of a periodic distribution of some higher order chromosomal structures in which domains are protected from degradation (23).

Here we report the data on the relationship between forum domains and looped domains delimited by SARs in the sequenced portion of the *cut* locus of *Drosophila melanogaster*. The boundary region where cleavage sites are scattered between two forum domains was mapped. It has some properties of a small heterochromatin island. Several SARs were found inside the 10 kb sequenced portion of the proximal forum domain, suggesting that forum domains and looped domains are physically different types of domains and belong to different levels of organization in eukaryotic chromosomes.

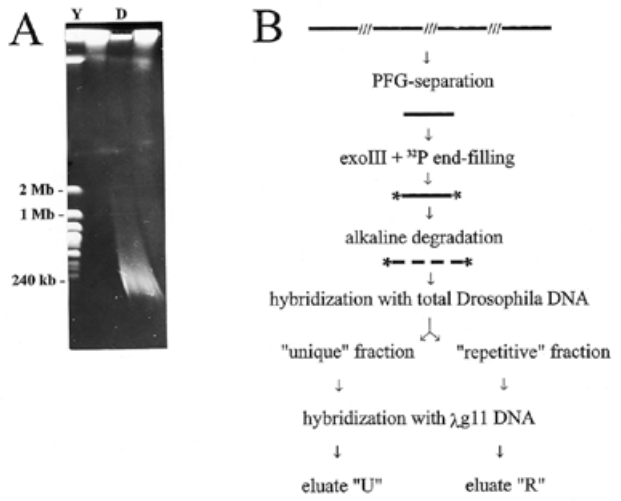

Figure 1. PFG separation and preparation of end-labeled forum domain probes. (**A**) PFG separation of forum DNA sample. Total uncleaved *Drosophila* DNA sample (D) was prepared as described in Materials and Methods and run for 72 h at 400 s pulses in Pulsophor system (Pharmacia). *Saccharomyces cerevisiae* chromosomes (Y) were used as size marker. (**B**) Scheme for preparation of end-labeled forum DNA probes (see Materials and Methods).

MATERIALS AND METHODS

Preparation of forum domains samples

Anaesthetized flies (200 mg) were homogenized in Dounce homogenizer at 0°C in insect cell-culture medium, filtered through nylon, pelleted, washed with a PBS solution (125 mM NaCl; 25 mM Na-phosphate buffer, pH 7.2), resuspended in 4 ml of PBS, gently mixed with an equal volume of 1% agarose-L (LKB) in PBS solution and distributed on a mold containing 100 µl wells. The mold was covered with Parafilm and placed on ice for 3 min. The agarose plugs were then placed in Petri dishes containing 0.5 M EDTA (pH 9.5), 1% sodium lauroylsarcosine and 2 mg/ml proteinase K, incubated at 50°C for 48 h and stored in the same solution at 4°C. Usually one plug corresponded to 15 flies. The DNA–agarose plugs were used in pulsed field gel (PFG) electrophoresis to check the DNA quality. Forum DNA domains for end-labeling were isolated as follows. Five to seven plugs were washed in Eppendorf tubes with 1.5 ml 1× TE (10 mM Tris–HCl, pH 7.4) supplemented with 1 mM PMSF at room temperature four times for 1 h each wash. Then the plugs were washed three times with 1× TE buffer alone for 2 h each. Washed plugs were placed in a dialysis bag containing 0.7 ml 0.5× TBE buffer, 0.5 mg/ml EtBr and electrophoresed for 2 h in mini-gel apparatus at 10 V/cm. The next steps were performed without any stirring at 4°C. Agarose plugs were removed and the dialysis bag was dialyzed for 24 h against 0.01× TE. DNA was then concentrated on solid agarose and redialyzed. Aliquots were used for PFG electrophoresis and run on mini-gel to check the quality and amount of DNA.

The end-labeled probes were prepared as follows (Fig. 1B). About 10 µg of eluted DNA was treated by *Escherichia coli* exonuclease III (Promega) in the condition that allows the removal of ~50 bp. The amount of enzyme and time of incubation were selected in preliminary experiments with mixed up λ *Hin*dIII–*Eco*RI DNA fragments and alkaline agarose gel electrophoresis. After exonuclease III treatment the sample was incubated at 65°C for 10 min, dialyzed in a small bag overnight and concentrated on solid sucrose. The filling in reaction with the Klenow fragment of *E.coli* DNA polymerase I (Promega) was performed in the presence of 500 µCi of [α-^{32}P]dATP (Russia, specific activity >6000 Ci/mmol) in solution containing three unlabeled dNTPs, 0.1 M HEPES, pH 6.9; 10 mM $MgCl_2$; 10 mM DTT; 0.07 M KCl and 10 U of enzyme for 15 min at 14°C. The DNA sample was then precipitated, incubated in 0.1 N NaOH at 100°C for 20 min, cooled, neutralized, and purified through the G-50 Sephadex column.

Fractionation of the probe was performed by hybridization with 0.5 mg of total *D.melanogaster* DNA immobilized on nitrocellulose membrane in 2 ml of the solution containing 4× SSC, 0.1% SDS, 10× Denhardt's solution and 20 µg/ml tRNA at 65°C for 48 h. The fraction depleted in repeats ('unique' probe) was collected. The pieces of nitrocellulose filter were then washed in 4× SSC, 0.1% SDS for 1 h at 65°C and the 'repeated fraction' was eluted from the filter in 0.1% SDS solution at 100°C. Similarly, both fractions were purified by overnight hybridization with 5 µg of λ clone g11 DNA.

Nuclear scaffold isolation

Drosophila embryos (2–3 g; 0–18 h) were collected, dechorionated in a 5% sodium hypochlorite solution for 5 min and purified by floatation in 15% sucrose (w/v) solution. Unless otherwise indicated, all subsequent steps were performed at 0–4°C using modified protocol described by Mirkovicht *et al*. (8). Embryos were homogenized with a Dounce homogenizer in 15 ml of a cold of isolation buffer containing 3.8 mM Tris–HCl, pH 7.8; 0.05 mM spermine; 0.125 mM spermidine; 0.5 mM EDTA; 1% thiodyglycol (v/v), 20 mM KCl and supplemented with 1 mM PMSF, 5 µg/ml aprotinin (Sigma), 0.1% digitonin (Fluka) and filtered through

nylon. The nuclei were pelleted (15 min; 2500 *g*), resuspended, and repeatedly washed in the same buffer. The final pellet was suspended in isolation buffer (to 50–100 OD_{260} units of nuclei) and incubated at 37°C for 20 min. Then 4 vol of 37°C buffer containing 5 mM HEPES/NaOH, pH 7.4; 2 mM EDTA/KOH, pH 7.4; 2 mM KCl; 0.25 mM spermine; 20 mM 3,5-diiodosalicylic acid, lithium salt (Sigma) and supplement (see above) were added and incubation was performed for 5 min at room temperature. Then 1 vol of SM buffer containing 20 mM Tris–HCl, pH 7.8; 0.05 mM spermine; 0.125 mM spermidine; 20 mM KCl; 70 mM NaCl; 10 mM $MgCl_2$ and supplement was added and sample was centrifuged at 4°C (15 min; 1000 *g*). The solution was discarded and pellet was repeatedly (three times) washed with SM buffer at 0°C. The final pellet was washed with SM buffer without supplement and resuspended in 3–5 ml of this buffer. Up to 1000 U/ml each of *Sau*3A, *Hae*III and *Hin*fI enzymes were added and incubated at 37°C on a shaker. The nuclear scaffold was pelleted at 4°C (15 min; 3000 *g*), washed with buffer containing 20 mM Tris–HCl, pH 7.8; 0.05 mM spermine; 0.125 mM spermidine; 20 mM KCl; 70 mM NaCl and 0.1 mM EDTA, pelleted, and finally suspended in 2 ml of solution containing 10 mM Tris–HCl, pH 7.8; 0.025 mM spermine; 0.063 mM spermidine; 10 mM KCl; 35 mM NaCl and stored at –20°C after the addition of 1 vol of glycerol.

Assay of DNA binding to nuclear scaffold

We have used the *in vitro* binding procedure described by Cockerill and Garrard (24), with some modifications. Unlabeled competitors, sonicated *D.melanogaster* and pUC12 DNAs were used. Their concentrations were selected in preliminary experiments with plasmid containing histone gene repeat digested with *Eco*RI, *Xho*I and *Bam*HI (8). End-labeled DNA fragments (200×10^3 c.p.m.; 0.1 µg) and 10 µl of nuclear scaffold preparation (see above) were incubated in 300 µl of incubation buffer containing 10 mM Tris–HCl, pH 7.8; 0.025 mM spermine; 0.063 mM spermidine; 10 mM KCl; 35 mM NaCl, 100 µg/ml BSA, 1–4 µg of *Drosophila* and 10–20 µg of pUC12 DNA competitors. After incubation on a shaker at 23°C for 2 h the probe was centrifuged at 4°C in an Eppendorf centrifuge (15 min; 12 000 r.p.m.). The pellet was washed with 300 µl of the same buffer and then suspended in 50 µl of the incubation buffer. Scaffold bound DNA was solubilized in 1% SDS, then NaCl and EDTA were added to 200 and 10 mM, respectively, and overnight digestion with proteinase K (0.5 mg/ml) was performed at 50°C. After addition of 10 µg of tRNA carrier, the sample was phenol-extracted and ethanol precipitated. DNA fragments were electrophoretically separated on a 1% vertical agarose gel in standard Tris–acetate buffer (or in 5% native polyacrylamide gel in TBE buffer), washed for 10 min in 7% TCA, twice in ethanol, 10 min each, dried and autoradiographed.

Standard procedures

Standard procedures of restriction–hybridization analysis, preparation of nick-translated probes, synthesis of random hexamers-primed cDNA probes and PFG electrophoresis were performed as described earlier (22). Nucleotide sequences were determined by the Sanger dideoxy chain termination method.

Sequence analysis

Nucleic acid and protein sequences were analyzed with the Genebee programs (25). Sequence similarity comparisons were also done with Gapped BLAST programs (26).

GenBank accession numbers

The accession numbers for the sequences reported in this paper are U89926 and U90540.

RESULTS

Precise localization of the boundary region between two forum domains in the *cut* locus

Earlier we localized the boundary region between two forum domains inside a 5.6 kb *Eco*RI fragment in the clone g11 clone (22). To localize precisely this region we have used the end-labeled forum DNA probes from *D.melanogaster*. Figure 1A shows a typical separation pattern of forum DNA in a PFG. The forum DNA preparations were slightly treated with exonuclease III followed by filling in 3′ recessed termini with Klenow fragment of DNA polymerase I in the presence of a ^{32}P-labeled precursor (Fig. 1B and Materials and Methods). The DNA probe was hybridized with an excess of total *D.melanogaster* DNA immobilized on a nitrocellulose filter. In this way, the 'unique' (depleted for repeat sequences) and 'repeated' sequences were isolated. In order to reduce the background in Southern blot analysis we performed pre-hybridization of 'unique' and 'repeated' probes with an excess of clone g11 DNA immobilized on a nitrocellulose filter. Finally, the purified [^{32}P]DNA probes capable of binding to clone g11 sequences were selected and used for Southern analysis of identical blots, containing the subcloned fragments produced by *Eco*RI and *Hin*dIII digestion of clone g11 DNA (Fig. 2A). It was found that the 'unique' fraction of the end-labeled forum DNA probe binds extensively to a 3.3 kb *Eco*RI–*Hin*dIII fragment from the left border of the clone g11 stretch, while the 'repetitive' probe reveals only slight binding to the same fragment. No other signals were detected by the end-labeled forum DNA probe. On the other hand, the fractions from total nick-translated *D.melanogaster* DNA revealed another pattern of hybridization: 'unique' probe binds to all fragments at the same level and 'repetitive' fraction binds very extensively to the 3.3 kb *Eco*RI–*Hin*dIII fragment. Figure 2A also shows the signal after hybridization of a [^{32}P]cDNA preparation. No signal was detected after hybridization of a cDNA repetitive fraction (not shown). The actively transcribed sequences were detected in the 3.1 kb *Hin*dIII fragment (Fig. 2A).

We conclude from the data above that the boundary region between neighboring forum domains is located inside 3.3 kb *Eco*RI–*Hin*dIII fragment. The fragment also possesses a repetitive sequence, but the signal from the end-labeled forum DNA probe clearly comes from the adjacent unique sequence. We have sequenced the entire 13 425 bp stretch of the clone g11 (GenBank accession number U89926). It was found that the repetitive sequence in the 3.3 kb *Eco*RI–*Hin*dIII fragment corresponds to a 2282 bp stretch of the *Doc* element (see the scheme in Fig. 2C). In an attempt to narrow the region that separates the two forum domains in the *cut* locus and to reduce the signal from the repetitive element, we have hybridized the 'unique' fraction of end-labeled forum DNA preparation from *Drosophila simulans* (the closest relative of *D.melanogaster*) to Southern blots containing *Hin*fI fragments of the 3.3 kb *Eco*RI–*Hin*dIII stretch

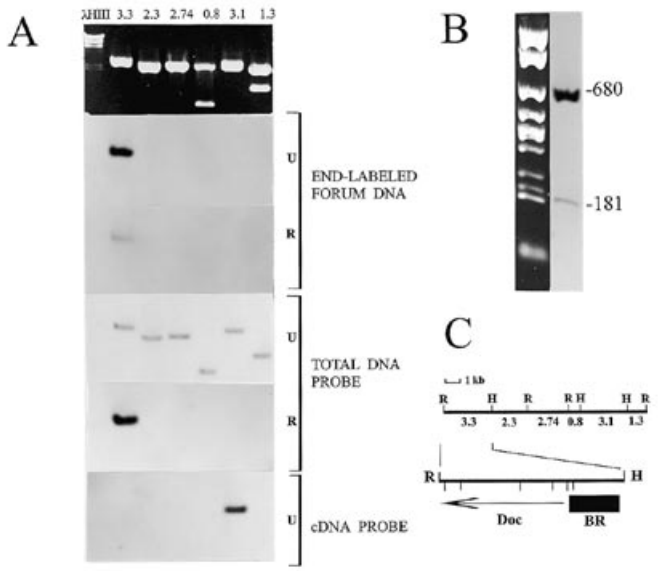

Figure 2. Mapping of BR between adjacent forum domains in the *cut* locus inside clone g11. (**A**) Hybridization of probes with identical blots containing *Eco*RI–*Hin*dIII, *Eco*RI or *Hin*dIII fragments from clone g11 subcloned in pUC12 vector. Numberings on lanes refer to the fragment lengths in kb. λ-*Hin*dIII (λHIII) was used as a size marker. Excised 3.3, 2.3, 2.74 and 3.1 kb fragments after short run are separated very close to pUC vector. 'Unique', U, and 'repeated', R, probes of end-labeled *D.melanogaster* DNA forum DNA sample were used. Total nick-translated *D.melanogaster* DNA was used as a control (U and R fractions were prepared by hybridization as described for forum DNA; see Materials and Methods). [^{32}P]cDNA sample was prepared on embryonic mRNA with random 6 bp primers and fractionated by hybridization as indicated above. (**B**) Hybridization of *Eco*RI–*Hin*dIII–*Hin*fI fragments of plasmid clone containing 3.3 kb *Eco*RI–*Hin*dIII stretch with end-labeled forum DNA preparation from *D.simulans*. The probe was prepared as shown in Figure 1B and described in Materials and Methods. Numbering indicates fragments in bp. (**C**) Physical map of clone g11. 3.3, 2.3, 0.8, 1.3 kb *Eco*RI–*Hin*dIII; 2.74 kb *Eco*RI and 3.1 kb *Hin*dIII fragments were subcloned. *Hin*fI-map of 3.3 kb region is shown lower. BR segment is boxed. *Doc* element is shown by a horizontal arrow.

subcloned in pUC12 vector. Both genomic Southern analysis with *cut*-probes and *in situ* hybridization on polytene chromosomes indicate that there is no *Doc* element in this position in the *D.simulans* line (N.A.Tchurikov, unpublished). End-labeling of *D.simulans* forum DNA preparation was performed after very slight treatment with exonuclease III. Figure 2B shows that the signal comes mostly from two adjacent *Hin*fI fragments of 680 and 181 bp in length. Thus we conclude that the boundary region (BR) between the two forum domains inside the *cut* locus spans ~700 bp as shown in the scheme (Fig. 2C).

Mapping of SARs inside the clone g11 insert

In order to study the relationship between forum and looped domains we carried out mapping experiments for a rough estimate of the number of looped domains in the clone g11. We used the approach suggested by Cockerill and Garrard (24) for *in vitro* binding of labeled DNA restriction fragments with nuclear scaffold preparations in the presence of unlabeled *D.melanogaster* and pUC12 DNA competitors (Materials and Methods). After incubation, probes were centrifuged. Pellets containing nuclear scaffold-associated labeled fragments were purified and electrophoretically separated. The conditions for binding and the concentrations of competitors were selected in preliminary experiments with *Drosophila* histone gene restriction fragments as positive controls for SAR mapping (8,24).

Figure 3A shows the results of mapping. The maximum sizes of the detected SARs were determined inside the corresponding restriction fragments. SARs were found on the 2.3 kb *Hin*dIII–*Eco*RI fragment, in a rather short 0.55 kb region on the 2.74 kb *Eco*RI fragment and on 0.8 and 1.3 kb *Hin*dIII–*Eco*RI fragments. No binding of nuclear scaffold preparations was detected with the 3.3 kb *Eco*RI–*Hin*dIII and 3.1 kb *Hin*dIII fragments. It is known that large DNA fragments even containing SARs tend to bind weakly (27). To be sure that we had not missed some attachment sites in the largest 3.3 kb *Eco*RI–*Hin*dIII fragment, we used *Hin*fI-labeled subfragments of the isolated fragment for *in vitro* binding experiments and again no binding was detected. The scheme in Figure 3A shows that the 1.97 and 0.16 kb *Eco*RI–*Hin*dIII fragments from the central 2.74 kb *Eco*RI fragment do not show SAR binding. Thus, there are at least four SARs in the clone g11 sequence that border internal regions between.

SARs that bind *in vitro* with nuclear scaffold proteins do not share extensive sequence homology but possess AT-rich stretches, ATATTT hexamers and topoisomerase II sites (24,28). Sequence analysis revealed 17 ATATTT sequences and 10 topoisomerase II sites inside the detected SARs (Fig. 3A). All four SARs detected revealed high-affinity binding in the presence of the well-known SAR sequence from *Drosophila* histone cluster (not shown) and thus meet a criterion suggested for screening functional SAR elements (8,28).

The 1.97 and 3.1 kb DNA stretches between SARs should correspond to the loops (loop1 and loop2, respectively; Fig. 3A). Figure 2A shows that the 3.1 kb *Hin*dIII fragment is actively transcribed in embryos. No signal was detected with the same probe from the 2.74 *Eco*RI fragment possessing 1.97 kb stretch. Two cDNA clones were isolated with 1.97 and 3.1 DNA probes from imago and embryonic cDNA libraries, respectively (data to be published separately). The data indicate that the two loops detected possess different transcription patterns. Figure 4 summarizes the data on physical mapping of BR and SARs inside clone g11 sequence and shows the position of the region in the whole *cut* locus spanning ~240 kb in *D.melanogaster* X chromosome (29). Distal and proximal forum domains extend beyond the 240 kb cloned segment and are >105 and 135 kb long, respectively (22).

BR sequence from another genomic region

Earlier we described the boundary region between the neighboring forum domains from an anonymous genomic region (22). Now we have determined both 1.3 kb fragment sequence that was cloned using the jumping library technique from the end of an individual forum domain and compared this sequence with the corresponding 1.8 kb fragment sequence from the undamaged genomic region (GenBank accession number U90540). The cleavage site for this individual cloned forum DNA terminus in the corresponding BR region (where fragmentation sites between two forum domains are scattered) was determined. It was speculated whether topoisomerase II is responsible for the endogenous cleavage of chromosomes that produces PFG-detectable forum domains (32). The cleavage site sequence GGCTGGG↓CTGCCAA does not correspond to the consensus sequence of *Drosophila* topoisomerase II—GTN A/T A C/T ↓ATTNATNN A/G (30). Moreover, in the ~700 bp BR where the cleavage sites in forum preparation were mapped

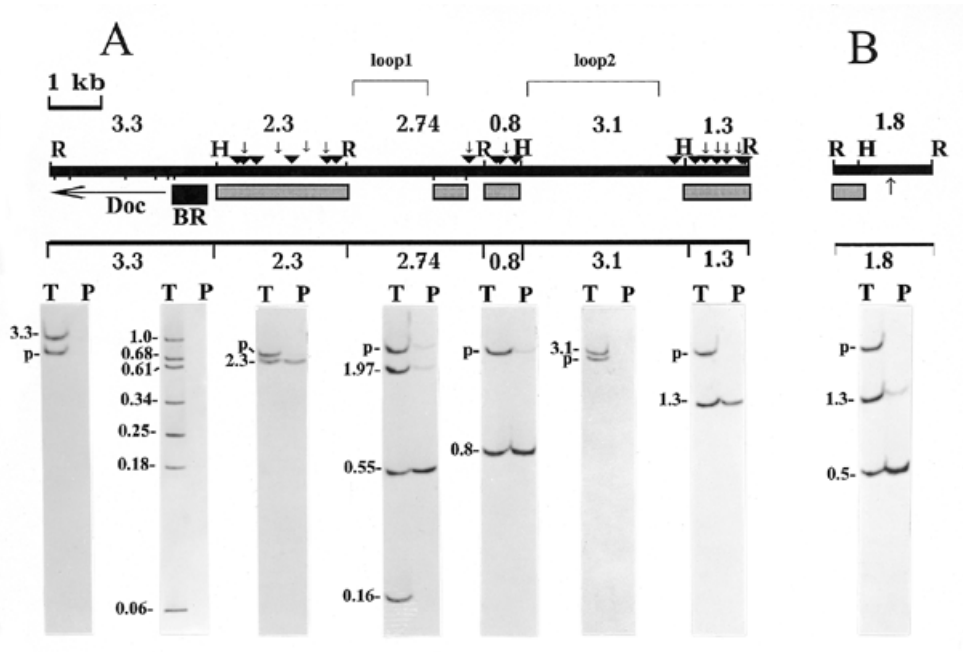

Figure 3. Mapping of SARs inside clone g11 from the *cut* locus (**A**) and inside the 1.8 kb fragment from an anonymous genomic region (**B**). The top line presents the restriction fragments of clone g11. *Eco*RI (R) and *Hin*dIII (H) sites are indicated. Results of *in vitro* binding to scaffold preparation for each fragment are shown below. Subcloned fragments were usually digested by *Eco*RI and *Hin*dIII (3.1 kb fragment was excised by *Hin*dIII alone). *Hin*dIII sites inside 2.74 kb *Eco*RI fragment are shown on the map under the solid line. T, total ^{32}P-labeled DNA fragments; P, pellet fraction isolated during scaffold binding experiments as described in Materials and Methods; lengths of restriction fragments are shown in kb; p, pUC12 plasmid. Shaded bars present the SARs. Brackets above the map show loops. The solid bar corresponds to the BR between forum domains. *Doc* element is indicated by a horizontal arrow. Vertical arrows above the map locate positions of *Drosophila* topoisomerase II sites that fit 13/15 bp match within the detected SARs. Solid arrowheads indicate positions of ATATTT hexamers. (B) Mapping of the SAR inside the 1.8 kb *Eco*RI fragment from λ1.3 where junction of two forum domains was described earlier (22). Indications are the same as in (A). The vertical arrow under the map indicates the position of cut site determined by sequencing of both cloned DNA fragment from forum DNA terminus using jumping library technique and undamaged 1.8 kb fragment.

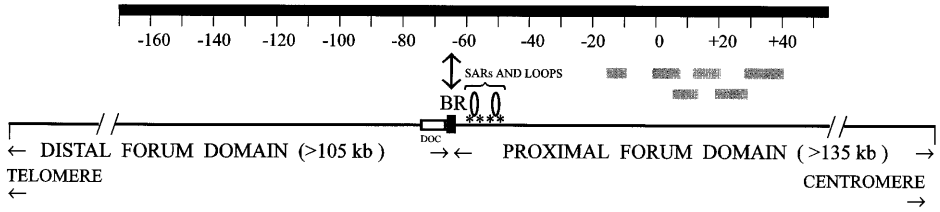

Figure 4. Forum domains in the *cut* locus and position of BR. Solid line represents the cloned segment possessing the entire *cut* locus from *Drosophila* X chromosome in coordinates as described earlier (29). The scale refers to DNA length in kb. The relative positions of *Doc* element, BR and detected SARs and loops are shown (not to scale) below. The telomeric and centromeric forum domains extend beyond the cloned region (22). Bars indicate localization of known transcripts in the locus (29,34,35).

earlier (22), there are no topoisomerase II sites. The same is true for the BR from the *cut* locus cloned in clone g11. It may indicate that some other endogenous nuclease(s) produces cleavage of chromosomal DNA in the course of preparation of forum DNA.

We have studied whether there is an SAR sequence on the 1.8 kb *Eco*RI fragment. Figure 3B shows that an SAR is located at ~250 bp distance from the BR. Thus, although SARs and BRs can exist close to one another, they are physically different elements and possess different properties.

Sequence homologies to BR and loops detected in the clone g11

BR sequences from the *cut* locus and the anonymous chromosomal region do not reveal extensive sequence homology. A short region of homology from the left borders of both BRs is shown in Figure 5A. The BR sequence from the *cut* locus also reveals 45 bp homology region with sequences, located at different distances around genes in different genomes. Some of them are shown in Figure 5B. This region contains a micro-satellite-like motif possessing dipyrimidine/dipurine tandem repeat of the form $(TCAG)_{11}$. It is known that micro-satellite stretches may reside near genes, inside introns and in heterochromatin regions. Their function is unknown yet.

Figure 5B presents homology regions found in 1.97 and 3.1 kb loops (loop1 and loop2, respectively). Loop1 also shows similarity with the *rough* gene from *D.melanogaster* that includes mostly intron stretch and exon segment (not shown, the data to be published separately). The *rough* gene controls cellular interactions in development (31). It is known that the *cut* locus is involved in

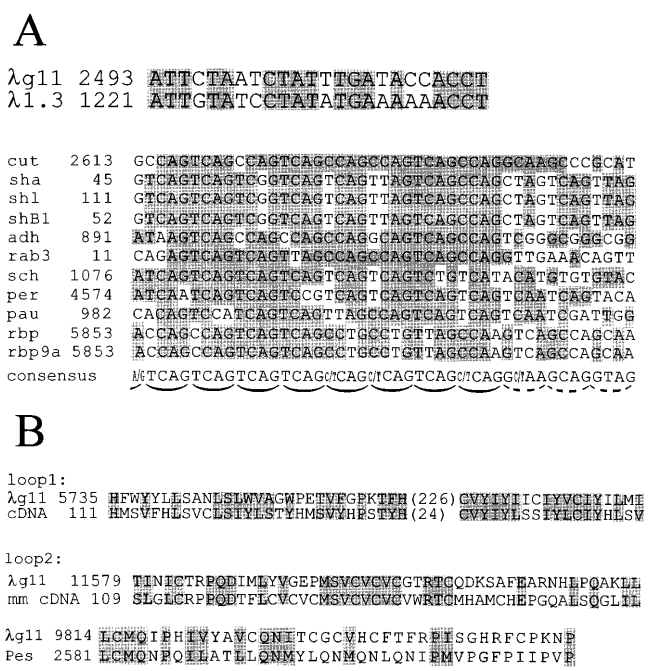

Figure 5. Sequence homologies of the detected BR and loops. (**A**) Homologies of BR. Short region of homology between the BR from the *cut* locus (clone g11) and from anonymous region (λ1.3) is shown. Multiple alignment represents regions reminding micro-satellite stretches of BR from the *cut* locus and non-coding sequences around different genes. Sha, shl and shB1 correspond to regions possessing the members of *Drosophila* shaker gene family (AC# X07131, X07134 and X06742, respectively); adh, segment containing the *Drosophila* Adh gene (AC# L36303); rab3, *Drosophila* GTP-binding protein encoding region (AC# M64621); sch, Schistosoma protease gene region (AC# J03946); per, *D.virilis* region containing *per* gene (AC# X13877); pau, the region from *D.virilis* containing gene possessing PAU domain (AC# U14723); rbp and rbp9a, regions of *Drosophila* genome possessing members of RNA-binding protein genes (AC# S55886 and L04930, respectively). (**B**) Homology regions of the loop1 and loop2 detected at the distal portion of telomeric forum domain in the *cut* locus. Numberings for ORFs from clone g11 stretch and for Pes protein are indicated in bp numbers of the corresponding DNA sequences. cDNA, murine cDNA clone (AC# Q62098); mm cDNA, *Mus musculus* cDNA clone (AC# AA107143); Pes, *C.elegans* fork head transcription factor controlling early embryogenesis (AC# Z28375). Numbers in brackets refer to the residues that separate homologous segments. The positions of identical or chemically similar amino acids are shaded.

local interactions among cells in early development (32). Loop2 shows homology with the *Caenorhabditis elegans pes-1* gene that specifies the fork-head transcription factor important for early embryogenesis (33). The data support our finding of two transcribed loops at the terminus of the telomeric forum domain in the locus.

DISCUSSION

Forum domains were identified in experiments based on non-random degradation of chromosomal DNA by endogenous nucleases in different eukaryotic organisms. For forum domain isolation we included live cells in physiological conditions in low-melt agarose plugs and then put the plugs into strong 0.5 M EDTA–1% laurylsarcosine–2 mg/ml protease K containing mixture (Materials and Methods). Chromosomal DNA damage presumably takes place just before cell lysis or shortly after it. Thus, we believe that our approach for detection and analysis of sensitive chromosomal structures is the most adequate available one. Alternative procedures for isolation of DNA domains usually include isolation of nuclei or extraction of proteins from chromosomes. These need more time and thus probably do not preserve sensitive chromosomal structures, although they could detect more persistent structures, such as looped domains.

The relationship between forum domains and looped domains

This study was undertaken to answer the question of the relationship between forum and looped domains in chromosomes. We have performed direct mapping of both types of domains in the junction region of two forum domains in the *cut* locus on the *Drosophila* X chromosome. We have used the approach for the mapping of forum domains using end-labeled probes and mapped a BR ~700 bp in length between the forum domains. In the proximal forum domain in a region spanning 10 kb we have mapped two looped domains delimited by SARs. As this forum domain is >135 kb long (22), one could expect that there is enough room for other loops. Two cDNA clones corresponding to the middle part of this domain were described earlier (34,35). Moreover, six actively transcribed regions were detected in this domain (29). Taken together, these data support our conclusion that several loops are located inside the forum domain. Thus, forum domains and looped domains seem to correspond to different hierarchies of chromosomal domains. We predicted this in our more recent study (22) taking into account the fact that the size of the looped domains in the *Drosophila* genome is shorter (4). For example, in ~500 kb long histone gene cluster each 4.8 kb repeat corresponds to a separate loop (8). Earlier we observed 50 and 120 kb forum domains as hybridization bands on PFG-blots containing fractionated forum domains probed with 4.8 kb histone genes DNA (22). In this study we have detected two loops of ~2 and 3 kb in close proximity which are transcribed at different developmental stages. Eighty-six SARs were mapped on an 800 kb *Drosophila* DNA segment (36). It means that the average size of a loop should be ~10 kb. Thus, the size of looped domains often may be smaller than was suggested in earlier studies. The looped domain concept implies an attractive model of expression of independent units. We speculate that the local regulation is associated with looped domains, while distant regulation could be connected with some another domain type(s). Forum domains possessing several looped domains inside could be one of the presumptive candidates for such distant regulation.

Both types of domain have one feature in common: they are regular periodic domains in eukaryotic chromosomes but they possess different properties. While looped domains are often <50 kb in length, practically no forum domains smaller then 50 kb were detected. Moreover, looped domains easily survive long procedures of nuclei isolation and protein extraction, whereas forum domains are very sensitive to long procedures. Looped domains are delimited by nuclease-resistant protein-protected regions, SARs, that are the anchor points for mechanical formation of loops. SARs bind to nuclear scaffold proteins, are AT-rich and often possess ATATTT hexamers and topoisomerase II sites. The sequences between SARs may correspond to single units of gene regulation. Forum domains are larger, they are delimited by nuclease sensitive regions, BR sequences, where cleavage sites are scattered. BRs do not possess ATATTT motives, topoisomerase II sites and apparently are associated with another endonuclease(s). One can calculate that in the 1.4×10^5 kb *Drosophila* genome

there should be ~1400 forum domains and BRs. Therefore, we conclude that looped domains and forum domains do not present the same level of chromosomal organization detected by different approaches. Taken together, all the available data support the view that forum domains and looped domains correspond to different levels of organization in eukaryotic chromosomes. We consider this study as the first evidence in favour of such supposition.

BR sequences

In the present study we have defined two BR sequences in two sequenced genomic regions. No extensive sequence homology was observed between them. The significance of the 24 bp homologous sequences from the right borders of both BRs is not clear. It is well known that functionally or structurally homologous regulatory sequences are not organized as families of repeated sequences. In contrast, they often occur as very 'degenerate' sequences and exhibit only several more or less 'conserved' positions (e.g. promoter regions, 3′ trailer sequences, SARs, interbands observed in polytene chromosomes, etc.). Whether such conserved positions are characteristic for BRs remains to be determined.

Both of the detected BRs are ~700 bp long. The same result of BR mapping in clone g11 was observed with forum domain preparations from *D.melanogaster* and *D.simulans*. This means that cleavage of chromosomes that releases forum domains takes place at the same regions. In the previous study we demonstrated that the cleavage sites in these regions produced by endogenous nuclease(s) are scattered (22). Thus, the properties of BRs clearly differ from the properties of DNAse hypersensitive sites, where DNA is cleaved precisely by exogenous nuclease (37). The nature and physiological role of the nuclease(s) that excise protected chromosomal domains at the very beginning of chromosome damage, when degradation is non-random, remains to be elucidated.

In the BR from the *cut* locus there is a micro-satellite sequence. Additionally, a *Doc* element and SAR are located in the close neighborhood on both sides of this BR. It is not clear yet whether this arrangement is characteristic for BRs and areas flanking chromosomal domains are preferred target sites for insertion of mobile elements.

It is known that in the 7B region, where the *cut* locus is located in the 7B1-5 bands (38), the intercalary heterochromatin was mapped inside the thick 7B1-2 band by a number of criteria: ectopic pairing, chromosomal breaks and rearrangements, late replication, somatic pairing and presence of repetitive sequences (39). Some properties of the BR from the *cut* locus described here could allow to consider this region as small heterochromatin region inside euchromatin area.

The role of BRs is not clear. Three different types of elements were described on the borders of the functional chromosomal domains: SARs, LCRs and insulators (10). SARs mechanically attach borders of looped domains to the proteinaceous nuclear scaffold. LCRs can span some 15 kb segments of DNA and provide the proper expression of genes at a distance up to 200 kb. Insulators (or silencers) isolate promoters of independent genetic units from the action of any enhancer or silencer from adjacent genetic unit. Clearly, BRs are distinct from SARs. But the possibility that BRs may share some properties of LCRs and/or insulators cannot be excluded. We believe that new experiments to search for proteins that bind with BRs and *in vivo* analysis of BR containing constructs may answer these questions.

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