SuUR Protein Binds to the Boundary Regions Separating Forum Domains in *Drosophila melanogaster*<sup>*</sup>

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Nickolai A. Tchurikov, Olga V. Kretova, Boris K. Chernov, Yulia B. Golova, Igor F. Zhimulev, and Ivan A. Zykov

From the Engelhardt Institute of Molecular Biology Russian Academy of Sciences, Department of Genome Organization, Vavilov Street 32, Moscow 119991, Russia and the Institute of Cytology and Genetics, Siberian Division of Russian Academy of Sciences, Department of Molecular Cytogenetics, Academician Lavrentiev Avenue 10, Novosibirsk, 630090, Russia

Forum domains are 50–150 kb DNA fragments that are released during spontaneous fragmentation of chromosomes. They are separated by islands of putative heterochromatin boundary regions. The SuUR protein, which is involved in the control of chromosome organization, is localized exclusively in heterochromatin and often colocalizes on chromosomes with Polycomb group proteins. To test whether the SuUR protein is associated with boundary regions, we used gel retardation assays and found that the SuUR protein binds specifically to boundary regions and that boundary regions are under-replicated. These results suggest that the regular distribution of boundary regions in chromosomes may represent the dispersion of sites designed for chromosomal silencing.

Eukaryotic chromosomes are organized in two structural components, euchromatin and heterochromatin, corresponding to active (decondensed) or inactive (condensed) chromosomal states, respectively. Small regions of heterochromatin, known as intercalary heterochromatin (I-HC),<sup>1</sup> are scattered throughout the euchromatic regions, but the function of these regions is unknown.

These discrete functional chromosomal domains are formed by the coordinated action of different regulatory elements, and the key elements remain unknown. The finding that genes from euchromatic regions can become epigenetically inactivated after translocation into heterochromatic regions provided the first functional definition for heterochromatin (for review, see Ref. 1). Recently, the original cytogenetic definition of heterochromatin has been supplemented by molecular and biochemical data, providing a deeper understanding of chromosomal structure and function.

Both DNA sequence elements and modifications to bound proteins play a critical role in determining chromosome structure. Recently, the definition of heterochromatic regions has been augmented by the recognition that such regions are marked by the methylation of lysine 9 of histone H3 (2). Repressive chromosomal states can be created and maintained during development for the transcriptional silencing of homeotic genes (for review, see Ref. 3). PcG proteins are required for this type of repressive state (4). These proteins form at least two types of complexes with a number of different proteins. One type possesses histone deacetylase activity and blocks ATP-dependent remodeling of local chromosomal structure (5, 6). Recently, a connection between PcG-mediated gene silencing and the methylation of lysine 27 of histone H3 has been established (7, 8). Another group of proteins, trxG, is required to perpetuate the transcriptionally active state (for review, see Ref. 9). Evidence demonstrates that both PcG and trxG proteins not only control homeotic genes but also play a role in the regulation of other genes important for development (10, 11).

Both PcG and trxG proteins form complexes at specific DNA sequences, PRE (Polycomb response element) and TRE (trithorax response element), which are important for the silencing or activation of neighboring genes (12–14), although only one DNA-binding PcG protein (Pleiohomeotic, also called PHO) and two DNA-binding trxG proteins (GAGA-factor and Zeste) have been described (9, 15). These complexes often form at the response elements indirectly and are recruited by other proteins; it is possible that other PcG or trxG proteins and novel repressing or activating complexes exist (9, 16).

The study of higher order chromosomal structures is hampered by both the complexity of chromosomal architecture and the lack of experimental approaches for the identification of structural components. Ten years ago we began a study of the chromosomal fragments that are released from chromosomes shortly after spontaneous degradation. It has been shown that the early fragmentation is non-random, and chromosomal domains (forum domains) consisting of 50–150 kb DNA stretches were observed in all eukaryotes examined (17). The boundary regions (BRs) between neighboring domains have been studied in *Drosophila* (18), and their properties led to speculation that the biochemically detected BRs could correspond to the cytologically detected I-HC regions.

Recently, the SuUR protein (suppressor of under-replication) has been described (19). SuUR binds exclusively at I-HC regions and often co-localizes with PcG binding sites (19, 20). Changes in SuUR expression dramatically alter the structure of *Drosophila* chromosomes, and increases in protein level lead to the formation of specific structures only in the regions of I-HC (19). These observations led us to ask whether SuUR could bind at BRs. Here we describe results that suggest that SuUR binds to BRs in a specific way and that BRs are under-replicated. We also show that SuUR shares homology with the bromodomain of the Brahma protein, a dominant suppressor of Polycomb mutations (21, 22). The data indicate that BRs cor-

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<sup>1</sup> The abbreviations used are: I-HC, intercalary heterochromatin; SuUR, suppressor of under-replication; PcG, Polycomb group proteins; trxG, trithorax group proteins; PHO, Pleiohomeotic protein.
respond to small islands of I- HC that are regularly dispersed along euchromatic chromosomes. We speculate that this chromosomal organization could be related to the mechanism of chromosomal silencing.

**EXPERIMENTAL PROCEDURES**

**Isolation of DNA and Pulsed Field Gel (PFGE) Electrophoresis—**Preparation of forum domains was performed as described earlier (17, 18). Anesthetized flies were homogenized in a phosphate-buffered saline solution containing 125 mM NaCl and 25 mM sodium phosphate buffer, pH 7.2 at 0 °C. Cells were collected by centrifugation and washed in the same solution. Cells were resuspended in phosphate-buffered saline at a concentration of 2-3 × 10^6 cells per ml, gently mixed at 43 °C with an equal volume of 1% agarose (LRB) in the phosphate-buffered saline solution, and distributed on a mold containing 100-μl wells. The mold was incubated for 5-10 min on ice. The agarose plugs were then incubated at 50 °C for 48 h in the solution containing 0.5 mM EDTA (pH 9.5), 1% sodium lauryl sarcosine, and 2 mg/ml proteinase K and stored in the same solution at 4 °C. Portions of the agarose-DNA plugs containing 1–5 μg of DNA were used for pulsed field gel electrophoresis (LKB Pulsaphor system) at pulse times of 100 s as described (17).

**Cloning Procedures—**The cloning of the BR from the 70C region using the supF selectable marker and the modified jumping library technique was described earlier (17). The BR from the 84D region was cloned using end-labeled forum DNA probes (18). About 5 μg of DNA, eluted after pulsed field gel electrophoresis (see the scheme in Fig. 1B), was treated with E. coli exonuclease III (Promega) under a condition that removes ~100–300 bases. After the exonuclease III treatment, the sample was incubated at 65 °C for 10 min, dialyzed, and concentrated on solid sucrose. A fill-in reaction with the Klenow fragment of E. coli DNA polymerase I (Promega) was performed in the presence of 500 μCi of [α-32P]dATP (REMB; specific activity ~6000 Ci/mmol) in a solution containing three unlabeled dNTPs. After alkaline fragmentation, the fraction of the DNA probe depleted in repeats (18) was used for hybridization with the restriction fragments of clones comprising the 84D region. The BR-containing fragments were sequenced and subcloned using PCR with the specific primers containing the following artificial restriction sites: the A subfragment of the BR DNA domains, denoted forum domains. Two approaches were used for the mapping and cloning of BRs, a modified jumping library technique and mapping with end-labeled probes (17, 18). The scheme in Fig. 1B illustrates the procedures used for isolation of two BRs, one from the 84D region and another from the 70C region. To study the BRs, spanning regions ~700 bp in length where DNA cleavage takes place during excision of the domains, we isolated clones corresponding to undamaged regions between individual forum domains in *Drosophila melanogaster*.

To study the binding of SuUR we used the BRs separating different forum domains in chromosome 3R, one from region 70C and another from region 84D (18). The cloned regions were localized by in situ hybridization on polytene chromosomes, and the result was confirmed by the FLY BLAST search using the FlyBase/GadFly genome annotation database. The clones corresponding to the undamaged regions were subcloned into 200–300-bp fragments using PCR. SuUR protein binding to the BRs was measured using gel retardation assays.

Nuclear protein extracts were isolated from flies of three genetic backgrounds, namely Oregon RC wild-type stock, the transgenic hs-SuUR stock producing high levels of SuUR protein under the control of the hs70 promoter, and a mutant homozygous SuUR– stock. It was shown earlier that the protein is expressed in wild-type lines at a low level (20, 23). Fig. 2A shows that the protein extract, isolated after heat shock treatment of the transgenic stock, demonstrates strong binding and retardation of 32P-labeled DNA corresponding to the 210-bp fragment from the distal part of the 84D-BR (subfragment A; see scheme in Fig. 2A). Quantitative analysis revealed that up to 30% of the DNA is present in two retarded bands. Binding with the extract from the Oregon RC stock was not detected (Fig. 2B), suggesting low levels of SuUR protein in normal stocks. Apparently, the upper retarded band corresponds to homodimers or heterodimers of SuUR. Two super-shifted bands were observed after the addition of SuUR antibodies (Fig. 2B) (20). They most likely correspond to the doublet of the retarded bands, and each supershifted band has a SuUR subunit. In experiments with GAGA and PHO antibodies, no change in the patterns of the retardation was detected (not shown). GAGA is a DNA-binding trxG protein (15), whereas PHO is the only known PcG protein directly binding to DNA (24). This indicates that neither the BRs nor SuUR bind to these regulatory proteins under the conditions used.

The 344-bp fragment from the BR, corresponding to the 70C region (subfragment A on a scheme, Fig. 2C), demonstrates a similar binding pattern in which only extracts from heat shock-treated transgenic stock lead to a shift of the BR probe, and, again, two retarded bands are observed. The subfragments B and C, corresponding to the 84D-BR (see scheme in Fig. 2, A and B), do not appear to bind SuUR under the same conditions (not shown). The same is true for the subfragment B from 70C-BR. These data point to the specificity of the binding of SuUR within particular regions of the BRs. In our experiments we used a protocol in which the addition of 32P-labeled DNA was performed after pre- incubation of the reaction mixture with an excess of pUC-12 plasmid DNA for 5–15 min. The

**Computer Analysis—**BLAST search of the *Drosophila* genome was performed through the Berkeley Drosophila Genome Project (Fly BLAST; www.fruitfly.org/blast/blast_form.html). Protein BLAST and BLAST 2 sequence searches on the NCBI server were performed for a study of homologous protein sequences using the default parameters (www.ncbi.nlm.nih.gov/blast). Accession numbers of SuUR and Brahma protein sequences are NM_080096 and P25439, respectively.
specific character of the binding was also confirmed by self-competition experiments in which the pre-incubation was performed with an excess of the same unlabelled DNA fragment (Fig. 2B).

Fig. 3 shows the results of competition experiments with excess DNA from another BR. 32P-labeled DNA of the A subfragment from 84D-BR was added to a mixture containing nuclear extract from the transgenic stock and an excess of 70C-BR (70C-BR, A subfragment). The latter was as effective a competitor as the cold 84D-BR itself (84D-BR, A fragment). Thus, 70C-BR DNA competes with the formation of the retarded complexes obtained with the 84D-BR, indicating that both BRs bind the same protein. As expected, a nonspecific competitor, the B subfragment of 84D-BR, does not prevent the binding of the SuUR protein with the labeled DNA. Some other nonspecific DNAs were also found to be incapable of preventing binding of SuUR to the BR (not shown). Nuclear extract isolated from the heat shock-treated Oregon RC stock does not reveal any binding (Fig. 3), indicating that the protein binding to the sequences is not some unknown protein induced by heat shock. It suggests that the production of SuUR in the transgenic stock occurs after heat shock treatment and that SuUR interacts with the BRs studied. The data strongly support the conclusion that BRs bind SuUR in a specific manner. There was no consensus sequence detected between the two BRs tested. This may indicate that SuUR binding is not sequence-specific but structure-specific, although it could not be excluded that some degenerate sequences are involved in the specific binding.

Analysis of BRs Under-replication—We next asked if these BRs are under-replicated and thus correspond to the I-HC regions that were detected cytologically in bands 70C and 84D (25, 26). We compared the amount of DNA from brains (non-polytenic organ) and salivary glands (polytenic organ) hybridizing with 32P-labeled RNA probes that were synthesized on the BR fragments. We used a set of five identical Southern blots obtained after fractionation of equal volumes of the same preparation of total DNAs from brains and glands as described earlier (19). Subsequent hybridization experiments were per-
formed with five blots each, using different probes. Fig. 4A shows that both of the BRs studied are indeed under-replicated. Quantitation of the autoradiographic signals was used to determine the relative levels of under-replication. The relative levels of under-replication in bands 70C and 84D are 5- and 2-fold, respectively (Fig. 4A). Differences of the relative level of polyteny of 2-fold or more are significant (23). Bands 70C and 84D were previously described as the regions in which visible reorganization of chromatin took place upon overexpression of the *SuUR* gene in the salivary glands and where the *SuUR* protein was localized by immunofluorescence (20, 27). Our molecular data on *SuUR* binding to 70C-BR and 84D-BR are clearly consistent with these observations.

**SuUR Protein Has Several Domains That Are Homologous to the Brahma trxG Protein**—It was demonstrated earlier (19) that the N terminus of *SuUR* possesses significant similarity with the N-terminal portions of the ATPase/helicase domains of the SNF2 family of transcription activators (*e.g.* SNF2, STH1, Brahma, and MOT1). We present additional data showing homology between the *SuUR* and trxG proteins. We also detected two additional regions of *SuUR*, which share 42% identity with the entire bromodomain of the Brahma protein (22) and its downstream region (Fig. 5). This is surprising, because *SuUR* is associated with the I-HC regions, where chromatin is condensed and transcription should be repressed. One possible explanation is that PcG- and trxG-containing complexes share some protein(s) and that this domain of *SuUR* interacts with other proteins during the remodeling of local chromosomal configuration regardless of transcription activation. Another possibility is that the brahma and *SuUR* genes have a common origin but different functions. In any case, these observations provide additional arguments in favor of the idea that functional division into PcG repressors and trxG activators may be oversimplified (9, 28).

**DISCUSSION**

The main result of this study demonstrates that *SuUR* is a DNA-binding protein that interacts in a specific way with BRs, sequences that separate forum domains. Our experimental data demonstrate that the two BRs studied bind the same protein, *SuUR*. In the *SuUR*-binding region within BRs, the fragmentation of chromosomes during the isolation of forum domains occurs. The I-HC regions in thick 70C and 84D bands were established by a number of criteria such as ectopic pair-
SuUR Protein Binds to Boundaries of Forum Domains

The positions of identical or chemically similar amino acids are shaded. The bromodomain in the Brahma protein is indicated by a bar.

Fig. 4. Analysis of DNA under-replication in two BRs and their positions in the genome annotation data base of Drosophila. A, fragments from the 84D-BR and 70C-BR regions were tested. Total DNA preparations from brains (b) and salivary glands (g) were isolated from the third instar Oregon RC larvae and used for Southern hybridizations as described previously (20). The control probes corresponding to the under-replicated R270 fragment (20) and the normally replicated regions of the 84D-BR and 70C-BR were isolated from the third instar Oregon RC larvae and used for Southern hybridization. The control probes corresponding to the normally replicated regions of the 84D-BR and 70C-BR were isolated from the third instar Oregon RC larvae and used for Southern hybridization. The control probes corresponding to the normally replicated regions of the 84D-BR and 70C-BR were isolated from the third instar Oregon RC larvae and used for Southern hybridization. The control probes corresponding to the normally replicated regions of the 84D-BR and 70C-BR were isolated from the third instar Oregon RC larvae and used for Southern hybridization.

Fig. 5. Region of homology between SuUR and Brahma proteins. The positions of identical or chemically similar amino acids are shaded. The bromodomain in the Brahma protein is indicated by a bar.
and weak points. Different I-HC regions are involved in these contacts, suggesting their apparent role in the spatial organization of chromosomes. The cytological data on the distribution of I-HC regions were obtained mostly during the study of chromosomes in salivary glands. The pattern of I-HC regions is probably changed during development and has constant and variable sites. This might explain why, on Southern blots, fractionated forum domains reveal discrete bands corresponding only to housekeeping histone or ribosomal genes (17).

Up to 50% of the genomic DNA is excised into 50–150-kb forum domains during non-random fragmentation of chromosomes from different species (17). In diploid cells, the loss of SuUR function leads to the disappearance of late replication (27). These facts, taken together with the data on SuUR binding within BRs, indicate that BRs (I-HC regions) are weak chromosomal sites and that SuUR binds within these weak chromosomal points in both polytene and diploid cells. It is probable that the borders of forum domains are involved in some structures that are inaccessible to earlier replication and that SuUR, directly binding with DNA in these regions, plays a role in the formation of such structures. The indirect evidence in favor of the unusual structural capacities of these regions is the fact that I-HC regions are involved in ectopic pairing. Our working hypothesis is that BRs are permanently breakable in all cell types. SuUR directly binds to these DNA target sites in a tissue-specific way during the formation of active I-HC islands involved in the formation of a repressive state of large chromosomal segments in the course of differentiation.

SuUR possesses ATPase/helicase domains found in the SNF2 family of trxG proteins in which it catalyzes ATP-dependent alterations in nucleosomal organization (28). Thus, SuUR could be considered a putative chromatin-remodeling factor, but the nature of its effect is not clear. SuUR also has a bromodomain, which is characteristic of a number of trxG proteins. Recently, it was demonstrated that the specificity of bromodomain function is determined, in part, by the complex with which it interacts. In catalytic subunits of SAGA and SNF complexes, the bromodomain is dispensable for acetylation but is required to anchor these complexes to acetylated promoter nucleosomes (29). We propose that SuUR recognizes specific DNA motifs (sequences or secondary structures) in chromosomes and becomes a hallmark in the formation of the silenced state of chromatin in these regions. It may locally associate with other proteins to produce this state. Our data from the gel retardation experiments support a view that other proteins are involved in the formation of a repressive state in the I-HC regions. SuUR may also initiate ectopic contacts of chromosomes after enhancement of SuUR expression, which are discussed above, support this idea. The detailed study of protein complexes containing SuUR may answer this and other questions regarding chromosome structure and the regulation of gene expression.

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