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SARs on an 835 kb DNA fragment from the *Drosophila* genome

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We have investigated the loop organization of a 835 kilobases DNA fragment from the *Drosophila* genome. This analysis has focused on the perodicity of the distribution of anchoring sequences (SARs) and its relationship to the distribution of A,T-rich regions, transcription units, repeated elements, putative replication origins and topoisomerase II cleavege sites. Altogether, the data support the idea of an active participation of SARs to the structural organization and functioning of this eukaryotic genome.

Numerous reports support the idea that regulation of gene expression in eukaryotes depends partly on structural organization of DNA within the nucleus [1]. Since several years [1–4], we have been contributing to the progress in this idea by studying the structural and functional organization of *Drosophila* genome. More precisely, we are investigating the relationship between the various aspects of the genome functioning and the DNA loop organization. Our analysis has been concentrated at a supragenic level and should be regarded as a link between investigations of specific genetic systems, a few tens of kilobases long, and investigations of the whole genome. This analysis concerns an 835 kb DNA continuum (D835) which, for numerous reasons, is representative of the whole *Drosophila* genome [1–4].

We have established the loop organization of D835, characterized the Scaffold Attached Regions[1] and looked into the topological relationship between distribution of SARs and parameters characterizing the genome activity. For this purpose, specific statistical programs have been set up. Particular attention has been devoted to the relationship between SARs and replication origins (ORIs). The presented paper recalls previous results [1–4], presents data to be detailed elsewhere (Brun, C. *et al.* 1994 and Jullien, N. *et al.*, 1994: manuscripts submitted to *Nucleic Acids Res.*) and preliminary data of recent work (R. Miassod *et al.*, unpublished). All these data are discussed with respect to the role of SARs.

MATERIALS AND METHODS

Chromosome walking, phage and plasmid DNA preparation, Southern analyses. All procedures as described in [5].

SAR mapping in Drosophila and test of binding to the yeast scaffold. Scaffolds from *Drosophila* were prepared by the LIS procedure and SARs were mapped as described in [6]. SAR limits were refined by the *in vitro* rebinding assay described in [7]. Purification of yeast nuclei, preparation of yeast scaffolds and binding assays were performed as reported in [7].

[1]Abbreviations: SAR, scaffold attached region; ARS, autonomously replicating sequence; ORI, replication origin; LIS, lithium diiodosalicylate.

ARS identification. DNA fragments to be tested were cloned into the YIp5 cloning vector [8] and the yeast strain (*S. cerevisiae*) YNN27 was transformed according to [9]. The location of the YIp5 derivatives within the transformed yeast cells was verified by estimating plasmid loss during growth under non selective conditions.

Topoisomerase II cleavage sites. The procedure to identify cleavage sites of DNA by topoisomerase II was derived from [10].

Statistical analyses. Home-built programs that were used have been described in [1] and (Brun, C. *et al.*, 1994: submitted to *Nucleic Acids Res.*).

RESULTS

Mapping SARs on D835

D835 was cloned from the X chromosome, using conventional chromosome walking on genomic libraries, and building physical maps for seven restriction enzymes usually applied (*Eco*RI, *Bam*HI, *Hin*dIII, *Sal*I, *Xho*I, *Xba*I and *Sst*I) that recognize palindromes made of both A,T and C,G. Maps were also established for restriction enzymes recognizing particular palindromic motifs (Brun, C. *et al.*, 1994: submitted to *Nucleic Acids Res.*). These were *Dra*I, *Sst*I, *Pac*I and *Swa*I, that recognize motifs made exclusively of A and T (A,T-cutters), and *Nar*I, *Bss*HII, *Apa*I, *Eag*I, *Sma*I, *Not*I and *Sfi*I, that recognize motifs made exclusively of C and G (C,G-cutters). Maps referring to the A,T-cutters are shown in Fig. 1.

Scaffolds were prepared by the LIS procedure. SARs were localized by probing Southern transfers of total, free and scaffold-bound DNA with DNA from a whole set of seventy representative recombinant phages. SARs limits were established to the nearest *Eco*RI, *Bam*HI and *Hin*dIII site. In some instances, limits were refined by using the *in vitro* rebinding assay. On the whole, eighty six restriction fragments harboring SARs were found to be scattered over D835.

A SAR subpopulation is localized in A,T-rich regions

The total number of cleavage sites for A,T-cutters or for C,G-cutters recognizing palindromic octanucleotides largely exceeds that expected from the base composition (Brun, C. *et al.*, 1994: submitted to *Nucleic Acids Res.*). The same applies to some of the A,T-cutters and C,G-cutters recognizing palindromic hexanucleotides. This suggests that A and T "clusterize", and that C and G also "clusterize", within a few nucleotides. A further Factorial Correspondence Analysis, at a one kb level, joined to a statistical comparison between distributions of cleavage sites for two A,T-cutters, or two C,G-cutters, or one A,T-cutter and one C,G-cutter, demonstrates that the distribution of A,T-palindromic motifs are associated, as well as that of C,G-palindromic motifs with the exclusion of the first motifs. This indicates that the clustering applies to palindromic hexa- and octa-nucleotides and extends to as much as 1 kb. Indeed, this clustering may concern DNA fragments several tens of kilobases long (Brun, C. *et al.*, 1994: submitted to *Nucleic Acids Res.*). This conclusion also applies to all other A,T-palindromes and C,G-palindromes of a sequenced 330 kb *Drosophila* pseudo-continuum and, more generally, to random A,T-motifs and to random C,G-motifs (Jullien, N. *et al.*, 1994: submitted to *Nucleic Acids Res.*). Thus, we have demonstrated that the *Drosophila* genome is made of alternating A,T-clusters and C,G-clusters. The distribution of SARs was compared to that of cleavage sites for cutters that mark the A,T-clusters (they are *Dra*I and *Ssp*I), and to that of cleavage sites for cutters that mark the C,G-clusters (they are *Nar*I and *Bss*HII). These analyses have demonstrated that a SAR subpopulation is preferentially localized in the A,T-clusters (Brun, C. *et al.*, 1994: submitted to *Nucleic Acids Res.*). However, the A,T-richness does not fully correspond to the presence of SARs: some SARs are found in non A,T-rich regions, or even in C,G-rich regions, at least as evidenced by restriction mapping with specific cutters.

The binding of SARs from D835 is conserved from *Drosophila* to yeast

Samples of the eighty six DNA fragments bearing SARs and of free DNA fragments were tested for their ability to bind to yeast scaffolds, using the *in vitro* rebinding assay. A very strong interspecies conservation of binding was observed, i.e. fragments bound both to the *Drosophila* and yeast scaffolds, or to none of them [3].

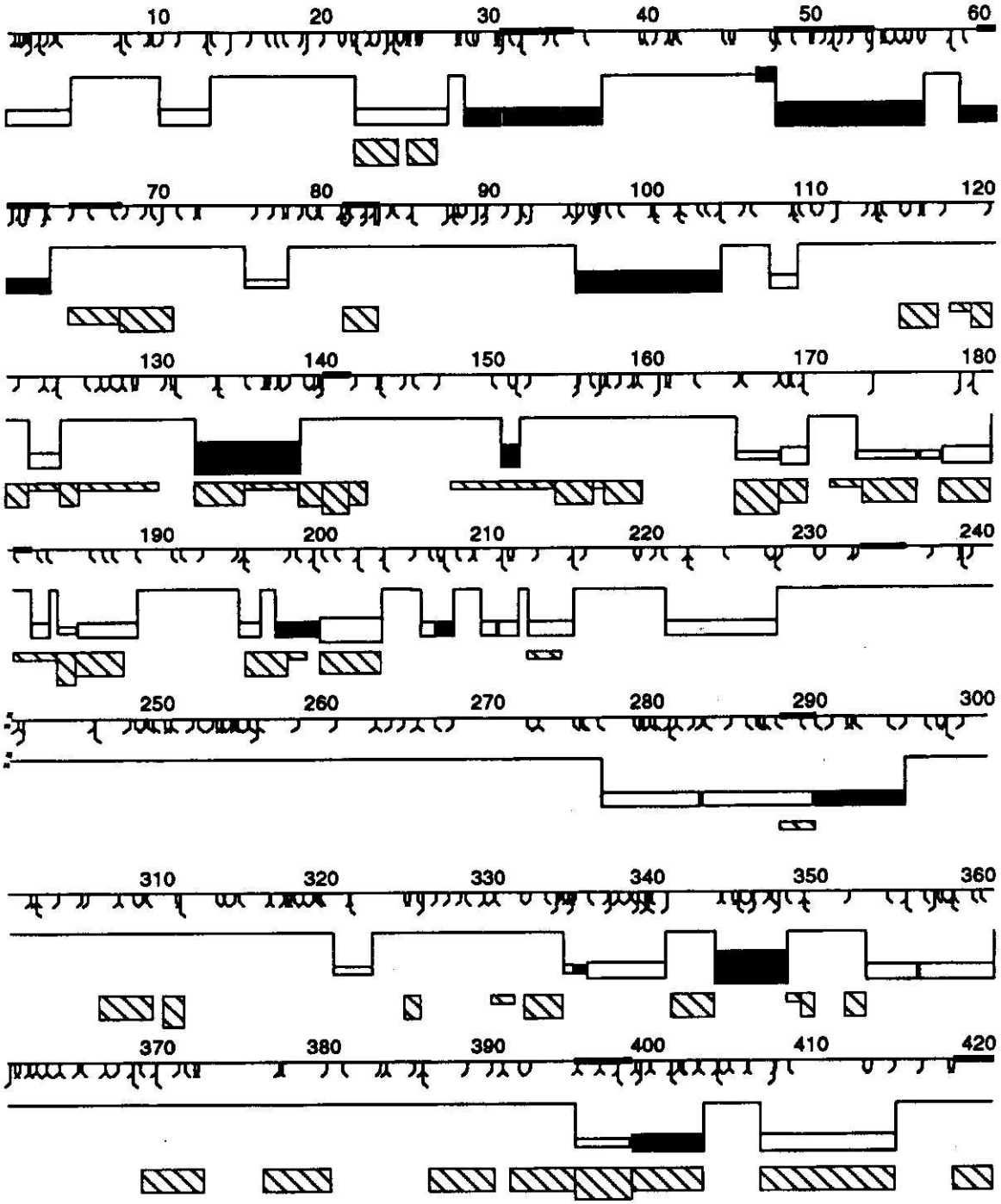

Fig. 1. Structural organization of an 835 kb long DNA fragment from the Drosophila X chromosome.
The structural organization of D835 is schematized as fourteen successive rows organized as follows. **Physical map**: The scale is given in kb, along a horizontal line, running from 0 to 835. Only cleavage sites for the A,T-cutters are shown; ᵀᵀᵀᵀ are, respectively, for *Dra*I, *Ssp*I, *Pac*I and *Swa*I. **SARs**: The DNA loop organization is schematized. Upper horizontal lines are free DNA regions. Lower horizontal rectangles, either empty or filled, are restriction fragments, delineated to the nearest *Eco*RI, *Hin*dIII or *Bam*HI site, showing a SAR activity. The height of the rectangle is proportional to the strength of binding to the scaffold.

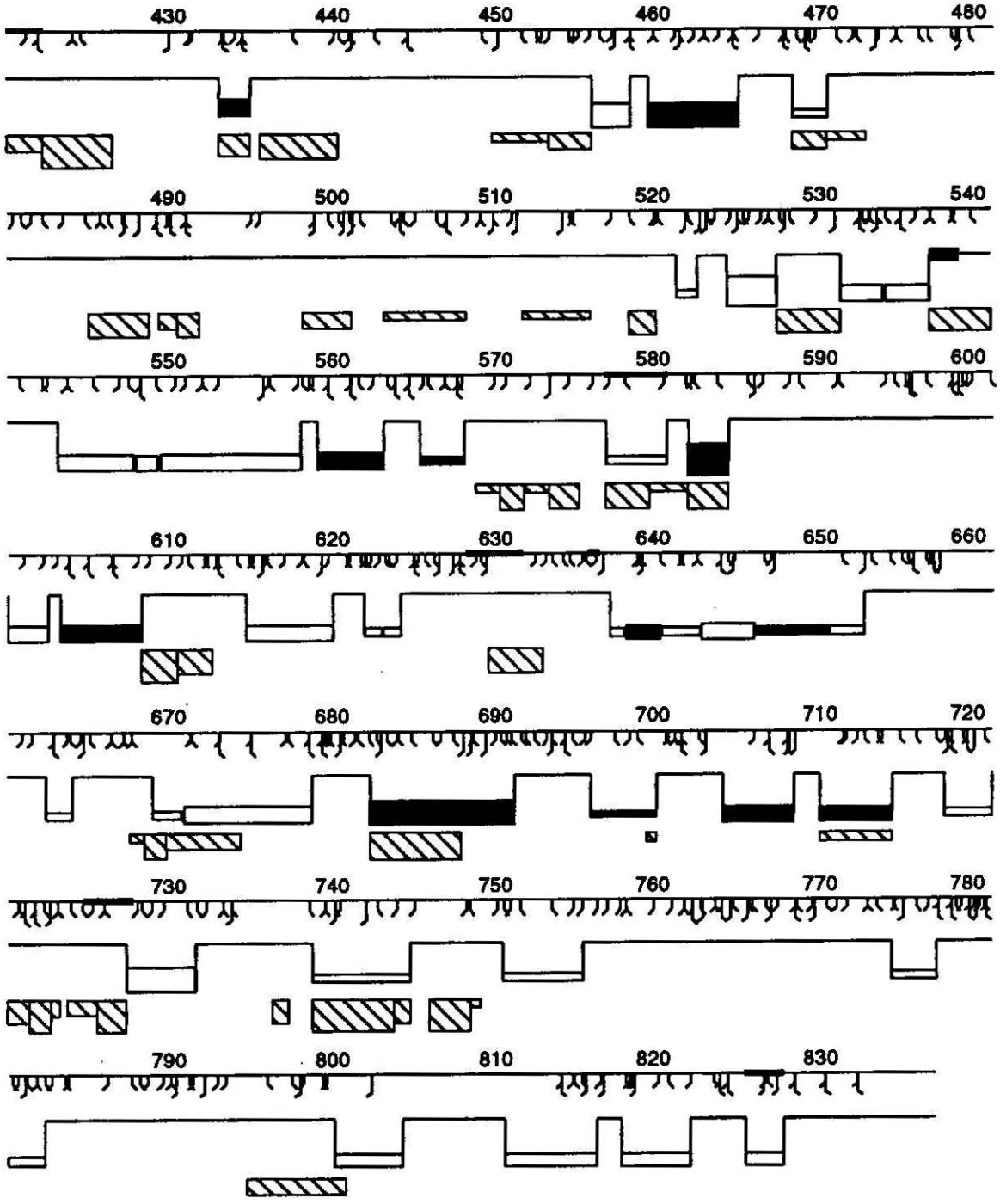

Fig. 1. Continued

ARSs: When the restriction fragment bearing a SAR also displays an ARS activity, it is filled in. The two filled rectangles in the upper part of the loop organization are for restriction fragments, still delineated to the nearest *Eco*RI, *Hin*dIII or *Bam*HI site, that have an ARS activity, but no SAR activity. **Transcribed sequences**: Parts of D835 that are expressed in 0–18 h old embryos are shown as striped horizontal rectangles, under the schematized scaffolding. They are delineated to the nearest *Eco*RI, *Hin*dIII, *Bam*HI, *Sal*I, *Xho*I, *Xba*I or *Sst*I site. The height of the rectangle is proportional to the intensity of transcription. **Repeated sequences**: Middle repeated sequences are shown as thick bars, along the horizontal line for the scale. They are delineated to the nearest *Eco*RI, *Hin*dIII or *Bam*HI site.

SARs are periodically disposed on the DNA molecule

A special statistic program has been devised to detect a possible periodicity of SAR localization [1]. This program consists in subdividing D835 into units of equal length, then generating a numerical distribution characteristic of the SAR distribution, and comparing it to the theoretical periods distribution while varying either the period value, or the size of the periodical object, or the starting points for comparison between the real and theoretical distributions. Application of this program has shown that there is a potential contact point between D835 and the scaffold every 12–13 kb, or multiples thereof, and a systematic contact every 57–64 kb (Table 1).

Strong SARs and weak SARs

Twelve restriction fragments harboring SARs hybridize exclusively with the scaffold-bound DNA fraction, whereas all other restriction fragments bearing SARs hybridize both to the scaffold-bound DNA and to the free DNA fractions. The former SARs are named strong SARs, and the latter ones weak SARs [1]. Strong SARs organize D835 into twelve consecutive loops ranging from 15 to 115 kb in size. Weak SARs subdivide these large loops into smaller ones. Two explanations may account for this observation. One is that strong and weak SARs are of the same nature, but they have distinct affinity for the scaffold: the DNA-scaffold interaction for strong SARs is strong so that it resists the scaffold preparation procedure, whereas the DNA-scaffold interaction for weak SARs is weaker, so that it is partly lost. Another explanation is that strong SARs are interacting with the scaffold in every nucleus, whereas weak SARs are interacting with the scaffold only in a nuclei subpopulation, so that the twelve basic loops are realized in every cell, but their subdivision into smaller loops is specific for a given cell type.

SARs are unique sequences, but they share cross-homologies

Next point whether SARs are members of repeated families, or unique sequences, has been examined. Southern transfers bearing DNA from the seventy recombinant phage set were digested with combinations of seven conventional restriction enzymes, then hybridized with a total genomic DNA probe. Restriction fragments visualized at short exposure times were those harboring repeated sequences.

These fragments, in turn, were used to probe Southern transfers of genomic DNA. This has allowed identification of the repeated sequences displaying multiple genomic localization. As detailed elsewhere [4], twenty two repeated sequences has thus been evidenced (Fig. 1). Twelve of them have a polydisperse chromosomal localization. In their vast majority, SARs are distinct from the repeated sequences (Fig. 1 and Table 1). However, although the limits of SARs and those of repeats are distinct, cross-hybridization between SARs is observed at a low stringency. More precisely, the complex cross-homology network preferentially concerns the SAR subpopulation which is periodically disposed on the DNA molecule.

Middle repeated sequences are positioned next to SARs

When the numerical distribution generated for repeats is slided along that of SARs, a high coincidence between the two distributions is observed, as soon as the sliding value exceeds 3 kb (Table 1). The coincidence is maximum for a 5 kb sliding, then, with a further increase of sliding, non significant values for the statistical parameters are again observed. This demonstrates that repeats are positioned a few kb apart from SARs.

Strong SARs are generally localized in non coding regions, whereas weak SARs may be present in expressed parts of the DNA molecule

The point whether SARs are coding sequences, or not, has been examined. Coding sequences were localized on D835 by hybridizing a cDNA copy of total *Drosophila* mRNAs to Southern transfers bearing DNA from the recombinant phage set, digested with combinations of restriction enzymes ([2] and Fig. 1). The lack of restriction sites has hampered precision in the limits of SARs and of expressed regions in D835. Statistical comparisons between the distribution of expressed regions and the distribution of SARs (both strong and weak) show no significant statistical correlations (Table 1). However, if one considers strong and weak SARs separately, it appears that the major part of strong SARs is localized outside transcrip-

Table 1
Statistical comparison between the distribution of SARs and that of sequences of biological interest on D835

Distribution of:	Compared to the distribution of SARs		
	X^{2a}	PInf[b]	PSup[c]
Markers of A,T-clusters			
*Dra*I cleavage sites	NS	NS	NS
*Ssp*I cleavage sites	+	+	–
*Swa*I cleavage sites	+++	+++	–
Markers of C,G-clusters			
*Bss*HII cleavage sites	NS	NS	NS
*Nar*I cleavage sites	+	–	+
Theoretical distributions[d]			
with a 12–13 kb period value	+++	+++	–
with a 24–27 kb period value	+++	+++	–
with a 37–40 kb period value	+++	+++	–
with a 57–64 kb period value	+++	+++	–
Expressed regions	NS	NS	NS
Repeated regions[e]			
without sliding the distribution	+++	–	+++
sliding the distribution by 1 kb	NS	NS	NS
sliding the distribution by 2 kb	NS	NS	NS
sliding the distribution by 3 kb	++	++	–
sliding the distribution by 4 kb	+++	+++	–
sliding the distribution by 5 kb	+++	+++	–
Cross-homologous families[f]			
1 cross-homology	+++	+++	–
2 cross-homologies	+++	+++	–
3 cross-homologies	+++	+++	–
4 cross-homologies	+++	+++	–
5 cross-homologies or more	+++	+++	–
ARSs	+++	+++	–
Strong topoisomerase II cleavage sites[g]			
without sliding the distribution	NS	NS	NS
sliding the distribution by + 1 kb	NS	NS	NS
sliding the distribution by + 2 kb	NS	NS	NS
sliding the distribution by + 3 kb	++	++	–
sliding the distribution by + 4 kb	+++	+++	–

[a] NS, or +, ++ and +++ means non significant, or non randomness at a $P > 0.95$, $P > 0.999$ confidence value, respectively. [b] NS, or –, or +, ++ and +++ means non significant, or exclusion, or association at a $P > 0.95$, $P > 0.99$ and $P > 0.999$ confidence value, respectively. [c] NS, or –, or +, ++ and +++ means non significant, or association, or exclusion at a $P > 0.95$, $P > 0.99$ and $P > 0.999$ confidence value, respectively. [d] The theoretical distributions were computer generated [1]. [e] Each repeat was slided to the nearest SAR, by 1 kb increment [4]. [f] These are cross-homologies between SARs; thus, the statistical comparison concerns only the SAR-ARS subclass [4]. [g] Each cleavage site was slided towards the nearest SAR, by 1 kb increment.

tion units, whereas weak SARs are localized partly outside, and partly within transcription units.

SARs and topoisomerase II cleavage sites

Topoisomerase II plays a structural role in scaffolds. It is a major protein constituent of scaffolds from metaphase chromosomes [11], and it is also present in scaffolds from interphase chromosomes [12]. Moreover, the purified topoisomerase II preferentially binds and aggregates SAR-containing fragments *in vitro* [13]. Topoisomerase II also displays an enzymic activity necessary to the disentanglement of daughter chromatids at mitosis, as well as to the control of DNA torsional stress [14]. It was therefore of interest to see whether, in D835, this structural role could also be accompanied by enzymic activity. We have therefore examined the relationship between the distribution of SARs and that of topoisomerase II cleavage sites, on D835. Preliminary results (R. Miassod *et al.*, unpublished) have shown that the enzyme preferentially cleaves DNA in the vicinity of SARs (Table 1).

SARs and sequences able to replicate autonomously (ARSs) co-map

Previous analyses at the level of the whole nucleus showed that the newly replicated DNA was enriched in the scaffold-bound DNA fraction [15]. It was thus of interest to look at ORIs on D835. Because of limitation in sensitivity of the two-dimensional chromatography techniques used to localize ORI in large-sized genomes [16], we have carried out this investigation at the level of ARSs, i.e. of sequences replicating autonomously in a cell system, when incorporated into a plasmid unable to replicate by itself. For convenience, ARS activity has been tested in the yeast system. This was fully justified by the above mentioned observation that the binding of SARs to the scaffold is conserved from *Drosophila* to yeast.

The twelve restriction fragments bearing strong SARs, fifty nine of the seventy four fragments bearing weak SARs and a representative sample of free DNA, i.e. thirty eight fragments, either adjacent to SARs or centrally positioned within loops, were tested for ARS activity. A total of twenty seven ARSs have been identified (Fig. 1). A statistical analysis has shown that the distribution of an ARS subpopulation is associated to the distribution of cleavage sites for cutters that mark the A,T-clusters in contrast to that of cutters that mark the C,G-clusters. However, another ARS subpopulation is not localized in the A,T-rich clusters.

Two sets of data demonstrate that SAR and ARS activities are linked. First, although there are less ARSs than SARs (twenty seven and eighty six, respectively), all ARSs but two are present in SAR-containing fragments (Table 1). The two ARSs making exception are located in restriction fragments immediately adjacent to SAR-containing fragments. Thus ARSs and SARs co-map. Second, there is a direct relationship between the strength of binding of SAR subclasses and the ARS activity: the stronger the binding for a given subclass, the higher the percentage of ARS identified within the SAR subpopulation [3]. The possibility that the co-mapping may just reflect the size of the restriction fragments used to look for the activity, or reflect a fortuitous co-recruitment of both activities in A,T-rich regions, has been completely eliminated ([3] and Brun, C. *et al.*, 1994: submitted to *Nucleic Acids Res.*).

SARs, ARSs and an ORI

As already stated, the direct mapping of ORI by the two-dimensional chromatography technique is not possible in the *Drosophila* genome. However, in the particular case of the rDNA region which is repeated several hundred times, this is feasible. Therefore, as a further step towards the analysis of the relationship between SARs, ARSs and ORI, we have performed on rDNA an analysis similar to that on D835, and, in addition, we have run these chromatographic analyses [17]. Here again, SAR and ARS activities co-map, in the internal transcribed spacer and in the intergenic spacer + external transcribed spacer (Fig. 2). Moreover, one ORI has been identified in the intergenic spacer + external transcribed spacer, i.e. this ORI co-maps with the SAR and ARS. Therefore, at least in this study system, SAR, ARS and an ORI do coincide.

DISCUSSION

The data that we have obtained on D835, together with those on rDNA, and those reported by others (review in [1]) strongly sup-

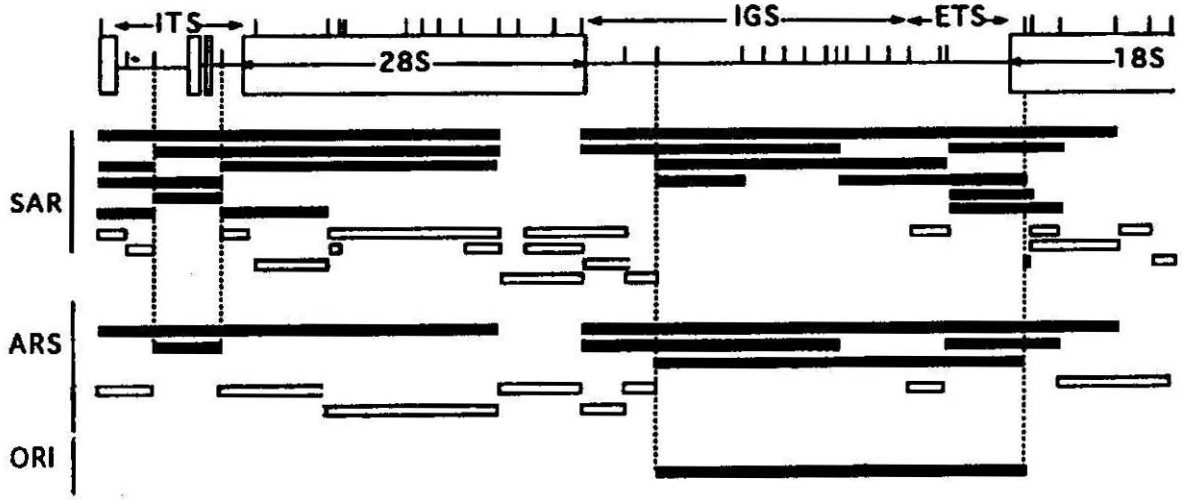

Fig. 2. Structural organization of the Drosophila rDNA.
The rDNA unit is schematized as empty rectangles for the conserved transcribed parts (18S, 28S and the two small rRNAs) and lines for the non conserved parts of the unit, i.e. the internal transcribed spacer (ITS), the intergenic spacer (IGS) and the external transcribed spacer (ETS). Small vertical bars correspond to convenient cleavage sites by restriction enzymes. Empty rectangles are restriction fragments having neither SAR nor ARS activity. Filled in rectangles are restriction fragments displaying a SAR, or an ARS, or an ORI activity. Broken vertical lines delineate one region showing both SAR and ARS activities, and another region showing simultaneously SAR, ARS and ORI activities.

port the idea that SARs play active roles in the nucleus.

SARs might participate in the physical packaging of the DNA molecule within the nucleoplasm and the nucleoli. Several results support this view. We have shown that SARs are scattered over the 835 kb long DNA molecule, as well as over the rDNA molecule. We have also demonstrated that there exists a periodicity in SAR positioning, which is an expected result, if one takes into account a regular basic DNA packaging. Lastly, we have demonstrated the alternation of A,T-, and C,G-clusters, both in the *Drosophila* and human genomes, and we have shown that a SAR subpopulation is preferentially localized in the A,T-clusters. This is in complete agreement with a recent data showing an A,T-path corresponding to loop bases, along the axis of metaphase chromosomes [18].

SARs might be implicated in the transcriptional process in three ways. First, they may organize the DNA molecule into independent transcriptional domains. We have distinguished strong SARs and weak SARs and proposed two explanations for this observation. The interpretation according to which strong SARs contact the scaffold in every nucleus, whereas weak SARs are interacting with the scaffold only in some nuclei, implies a dynamic view of the DNA scaffolding. Strong SARs will define basic loops, common to all nuclei, whereas weak SARs will delineate specific smaller loops in some nuclei. This subdividing could change, however, according to specific developmental programs. Consequently, small loops delimited by weak SARs should be considered as closed domains in which the transcription of genetic units is submitted to a specific control. Second, "transcription factories" have been evidenced in agarose-encapsulated nuclei and it was suggested that they were localized on a physical support that could be scaffold [19]. In agreement with this proposal, the anchoring of DNA might facilitate the recruitment of protein factors necessary to the basic transcription machinery, or/and might allow interaction between transcription factors bound to distant sequences on the DNA molecule [20]. Third, it is known that positive supercoiling of the DNA molecule accumulates downstream transcribed units [14]. Efficient cleavage of DNA by topoisomerase II at the level of SARs, which relieves DNA supercoiling generated by the progression of transcription, can be considered as an efficient system advantageous for the cell.

SARs might also be involved in the recombination process. In support of this hypothesis is our observation of a spatial contiguity between SARs and repeats. In the *Drosophila* genome, the major part of repeated sequences are mobile genetic elements that can move from one chromosomal localization to another [21]. Among the twenty two middle repeated sequences that we have identified on D835, none of them corresponding to any presently known repeated *Drosophila* element, we have demonstrated that at least twelve of them have a multiple chromosomal localization. If they are mobile elements, then our observation of a spatial contiguity between SARs and repeats makes sense. It can be speculated that SARs create a micro-environment favorable to the recruitment of protein partners necessary to the recombination process, so that these mobile genetic elements can re-integrate the genome. The observation that the mobile P-elements frequently re-insert at 5′ position to genes, on one hand, and that SARs are also frequently positioned upstream of transcription units, on the other hand, supports this speculation [22].

Lastly, SARs might play an active role in the replication process, possibly, at the level of initiation. In support of this idea we report that ARSs, which are scattered along D835, and at a frequency compatible with the known size of replicons in the *Drosophila* genome [23], co-map with SARs. Moreover, at least in one case, that of the rDNA system, we report a coincidence between SARs, ARSs and ORI. Several lines of evidence, obtained by us and by others [24], show that SARs and ARSs are distinct sequences but that they are very close to each other and that they cooperate. It is again tempting to speculate that SARs create nucleus subregions in which all partner proteins for the unwinding of DNA and the initiation of replication are recruited. There may be also other levels at which SARs interfere with replication. If the recently characterized "replication factories" are localized on the scaffold (or nuclear matrix), as suggested [25], then our report on strong cleavage of DNA by topoisomerase II next to SARs is of interest. It is an obvious advantage for the elongation process to have it occurring at the level of a nuclear substructure, the SARs, where topoisomerase II concentrates and preferentially cleaves the DNA molecule, thus disentangling daughter DNA molecules.

In conclusion, our investigation of SARs has been deliberately placed at a supragenic level. There were two drawbacks to this choice. One concerned limitation in the precision of the data, due to the large size of the examined DNA. The other one was due to the necessity of developing computerized statistical tools to handle the data. However, two obvious advantages have been achieved. Firstly, some conclusions reached, in particular the periodicity of SARs and the co-mapping of SARs with ARSs could not be reached in studies on particular genetic systems. Secondly, because of the representativeness of the DNA sample analyzed, all conclusions reached are probably valid for the whole genome. On the whole, the data strongly support the notion of an active participation of SARs in the structural organization and functioning of the eukaryotic genome.

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