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The organization and composition of the ribosomal RNA gene non-transcribed spacer of *D. busckii* is unique among the drosophilids

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Summary

Several ribosomal RNA (rRNA) genes from *D. busckii* were cloned and characterized. The prominent repeat classes have lengths of 12·8 and 13·6 kb and lack 28S introns. rRNA genes were cloned containing 28S insertions which exhibit heterogeneity in size and sequence. The non-transcribed spacer (NTS) contains two regions composed of different repeated sequences that exhibit pronounced instability in HB 101. NTS region II, centrally located within the NTS, contains predominately 11 or 16 *Hin*cII generated 160 bp repeats. NTS region III, next to the 18S gene, contains repeats that are variable in number, and are either heterogeneous in length or are dispersed within unique sequences. The organization and composition of the rRNA gene NTS of *D. busckii* is different in comparison to the NTSs of other drosophilids. In addition, the pronounced instability of two different NTS regions is unique in comparison to all other cloned rRNA genes.

1. Introduction

Drosophila busckii is the exclusive member of the subgenus *Dorsilopha*, and is only distantly related to *D. melanogaster*, *D. hydei*, and *D. virilis* (Throckmorton, 1975). The X chromosomal nucleolus organizer region of *D. busckii* is unlike that of most other drosophilids by virtue of the substantially reduced size of the heterochromatic blocks which bound this region. The reduction in size of these chromosomal regions is thought to be a consequence of the virtual lack of satellite sequences within the *D. busckii* genome (Calvet & Krider, unpublished data). Ribosomal RNA genes have been characterized from members of the *Sophophoran* (i.e. *D. melanogaster*) and the *Drosophila* (i.e. *D. hydei* and *D. virilis*) radiations within the genus *Drosophila*. In the interest of extending the observations on ribosomal RNA gene structure within the genus *Drosophila*, and to take advantage of unique attributes of the nucleolus organizer region of *D. busckii*, we have characterized the ribosomal RNA genes from *D. busckii*, a member of the *Hirtodrosophila* radiation.

The genes coding for the 18S and 28S ribosomal RNAs have been cloned from numerous species representing a diverse array of phylogenetic classes. The ribosomal DNA (rDNA) sequences coding for the rRNAs are largely invariant in contrast to the sequences comprising the non-transcribed spacer (NTS). The NTSs from *Tetrahymena* (Niles *et al.* 1981), sea urchin (Simmen *et al.* 1985), *Drosophila* (Beckingham, 1982), *Xenopus* (Bosely *et al.* 1979), mouse (Arnheim, 1979), rat (Yang-Yen *et al.* 1985; Yavachev *et al.* 1986), and human (Volpe *et al.* 1985) rRNA genes contain from one to three regions of tandemly reiterated sequences. The repeated NTS sequences have been the focus of recent investigations directed toward their organization and function.

One set of NTS reiterated sequences in *Xenopus* and *Drosophila melanogaster* contains an incomplete copy of the RNA polymerase I promotor (Moss, 1982; Coen & Dover, 1982). These repeated elements enhance rDNA transcription in *Xenopus* oocyte microinjection (Sollner-Webb *et al.* 1983; Reeder *et al.* 1983) and in *D. melanogaster in vitro* transcription studies (Kohorn & Rae, 1982). It is postulated that the NTS promotor element reiterations in *Xenopus* function *in vivo* to enhance rDNA transcription (Moss, 1983) and in addition, are the basis for nucleolar dominance in *Xenopus* hybrids (Reeder & Roan, 1984). The mouse NTS contains an array of repeated sequences adjoining the transcription termination site that have been implicated in the termination process (Grummt *et al.* 1985).

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In this report we show that the NTS of *D. busckii* contains at least two different arrays of repeated elements and present evidence that is consistent with the existence of a third array. The organization of the set of repeated elements proximal to the 18S gene in *D. busckii* is different in comparison to analogous elements found in other drosophilids studied to date. The significance of this organization in relation to rRNA gene expression is discussed. In addition, two of the tandemly arrayed sequence clusters are shown to exhibit pronounced instability when propagated in HB 101 resulting in the incremental loss of NTS sequences.

2. Materials and Methods

(i) *Construction of recombinant plasmids and isolation of rDNA clones*

DNA was isolated from *D. busckii* female wild-type flies reared on 0·6% agar, 5·0% corn syrup, 5·0% dry brewer's yeast, 7·5% cornmeal, 0·7% linseed meal, and 0·1% Tegosept. The DNA was digested to completion with *Pst* I, layered on a 10–40% sucrose gradient, and spun for 20 h at 25000 rpm at 4 °C. Fractions were collected, and those containing rDNA fragments were identified by Southern analysis using an rDNA clone from *D. melanogaster* (pDmra51, David *et al.* 1978) as a probe. *Pst* I digested pBR322 and *D. busckii* DNA were ligated with T4 DNA ligase and used to transform *E. coli* HB 101 (Hanahan, 1983). The rec A-phenotype of the HB 101 host was confirmed by assay of its UV sensitivity. Colonies harbouring rDNA recombinant plasmids were identified by colony hybridization using ^{32}p-labelled 28S B-2 and 28S B-1/A sequence fragments isolated from the *D. melanogaster* rDNA clone pDmra56 (Dawid *et al.* 1978). The transformed bacteria were plated at a density that allowed the independent isolation of colonies. Positive colonies were removed from the master plates and used to inoculate 10·0 ml LB cultures. These cultures are designated as primary cultures. Secondary cultures were generated from primary cultures by using colonies isolated by streak plating for the inoculum of 10·0 ml LB cultures.

(ii) *Restriction maps, R-loop formation, and electron microscopy*

Restriction mapping, agarose gel electrophoresis, DNA transfer to nitrocellulose, and hybridizations were performed by standard techniques. The 18S and 28S rRNAs used for the R-loop analysis were isolated from *D. busckii* larvae (Tartoff & Perry, 1970). R-loops between the 18S and 28S rRNAs and rDNA clones were formed essentially as described by Barnett & Rae (1979). The preparations were visualized and photographed with a Philips 300 electron microscope.

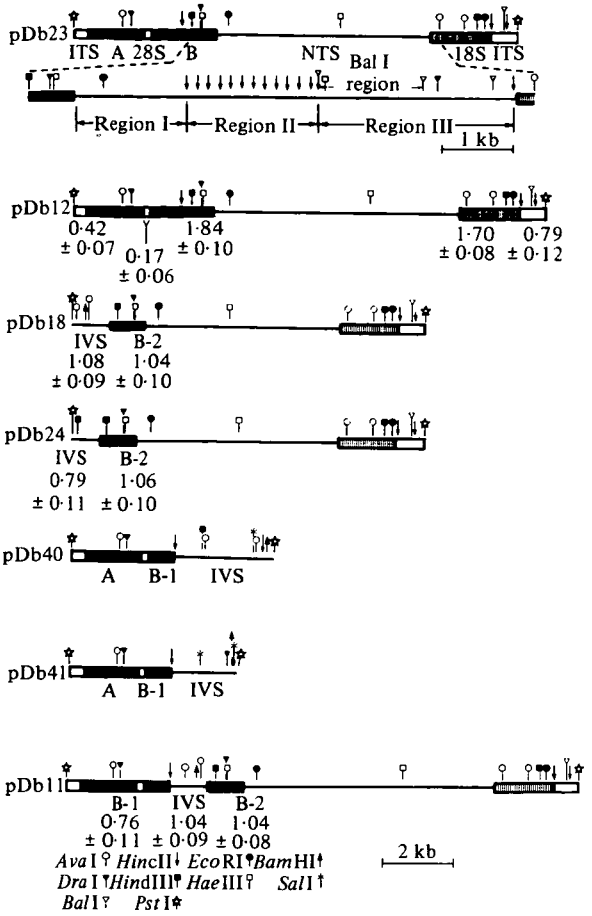

Fig. 1. Maps and lengths of the indicated segments of cloned ribosomal RNA gene fragments representing complete and partial coding units. The 28S A and B coding regions are represented by solid bars, the 18S coding region by vertical hatching, the internal transcribed spacer (ITS) and the 28S gap by open bars, and the non-transcribed spacer (NTS) and intervening sequences (IVS) by lines. The lengths of the molecules given in kilobases were taken from measurements of R-loop molecules formed between *D. busckii* 18S and 28S rRNAs and the indicated clones. Each measurement, shown with its standard deviation, is the average of at least 20 determinations. Not all *Dra* I, *Hae*III, and *Hin*dIII sites have been mapped in any of the described clones. Not all *Hin*dIII sites have been mapped in clones pDb23, pDb12, pDb18, pDb24, and pDb11. Not all *Ava* I sites have been mapped in the IVS of clone pDb40.

3. Results

(i) D. busckii *rRNA genes in genomic and cloned DNA*

Digestion of genomic DNA with *Pst* I yields two prominent rDNA size classes of 12·8 and 13·6 kb along with several minor repeat classes. The prominent minor repeat classes are 10–12, 5·8, and 4·8 kb in length (data not shown). The 13·6, 12·8, 10·2, 5·8 and 4·8 kb *Pst* I-generated rDNA fragments were cloned and are represented by clones pDb12, pDb23, pDb18, pDb40, and pDb41, respectively. The locations and lengths of the 18S and 28S coding regions were

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Table 1. *Summary of NTS composition of characterized clones before* (a), *and after* (b) *sequence loss of selected clones*

Clone number	Size of NTS region I	Number of *Hin*cII repeats	Size of NTS region III
(A)			
7, 9, 21	All clones	16, 16, 16	All clones
23, 24	1·7 kb	11, 9	2·7 kb
10, 11, 12	All clones	16, 17, 16	All clones
13, 16, 25	1·7 kb	11, 16, 16	2·75 kb
26, 27, 28	—	16, 16, 11	
17	1·7 kb	12	3·65 kb
18	1·1 kb	9	2·95 kb
19	1·7 kb	12	4·37 kb
22	1·7 kb	9	2·95 kb
29	1·7 kb	11	2.50 kb
(B)			
12-1	All clones	0	2·75 kb
12-2	1·7 kb	1	2·75 kb
12-4	—	5	2·75 kb
12-7	—	0-16	2·50 kb
19-1	—	12	1·50 kb
19-2	—	12	1·60 kb
19-3	—	12	1·80 kb
19-4	—	12	1·85 kb
19-5	—	0	2·05 kb
19-6	—	12	2·10 kb
19-7	—	12	2·30 kb
19-8	—	0	2·40 kb
19-9	—	12	2·50 kb
19-10	—	12	2·80 kb
19-11	—	12	3·25 kb

defined by electron microscopic analysis of hybrid molecules formed between the rDNA clones indicated in Fig. 1 and *D. busckii* 18S and 28S rRNA under R-loop conditions (data not shown). A summary of the R-loop analysis and restriction mapping data is presented in Fig. 1. Clones pDb12 and pDb23 contain complete rDNA coding repeats, with the size difference between them being due to a larger NTS in clone pDb12. The frequent 28S insertions contain at least one *Pst*I site, causing the cloning of most of the insertion harbouring rRNA genes as incomplete repeat units. pDb18 and pDb24 are insertion bearing clones that contain the 28S B-2, NTS, and 18S segments, and part of the internal transcribed spacer. pDb40 and pDb41 are also insertion-harbouring clones that contain part of the internal transcribed spacer and the 28S A and 28S B-1 coding segments. The 28S insertions in the clones described above exhibit unique restriction site patterns and are therefore composed of different sequences. An additional type of rDNA clone, pDb11, was isolated. pDb11 is a complete coding unit with an insertion in the 28S gene. The insertion in pDb11 exhibits the same restriction site pattern as the insertion in pDb18 and thus is likely a truncated version of that insertion (Fig. 1).

(ii) *Definition of the NTS regions*

The NTS is divided into three regions, regions I, II and III, which are described in the following sections (Fig. 1). Heterogeneity in the size of the NTS was found in rDNA clone isolates originating from a single transformation. NTS size heterogeneity as a consequence of sequence instability was found in all rDNA clone isolates examined. In Fig. 1 and Table 1, those clones designated by numbers indicate the putative state of the clone prior to sequence loss. Secondary clones are designated by hyphenated numbers, and are derived from the primary culture of the corresponding numbered clone after sequence loss (Table 1).

(iii) *NTS region I*

NTS region I contains the sequences from the 3′ end of the 28S gene to the first *Hin*cII site of NTS region II (Fig. 1). Digestion by *Hae*III and *Hin*cII produces a fragment containing NTS region I and part of the 28S gene. In clone pDb18 this fragment is 1·1 kb while all other clones contain a 1·7 kb fragment. To assess size variability of NTS region I and determine the relative proportion of rRNA genes represented by clones pDb12 and pDb18, a *Hae*III/*Hin*cII digest of fly DNA was examined and compared to *Hae*III/

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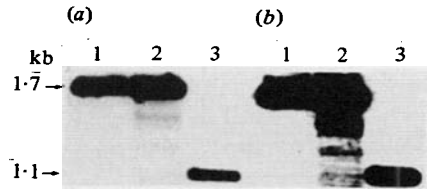

Fig. 2. Assessment of the heterogeneity of NTS region I. (*a*) Clones pDb12 and pDb18, lanes 1 and 3, respectively, and female adult fly DNA, lane 2, were digested with *Hinc*II and *Hae*III. The fragments were separated on an agarose gel, transferred to nitrocellulose, and hybridized with a ^{32}P labelled *Hae*III/*Hinc*II-generated fragment containing part of the 28S gene and NTS region I from pDb12. (*b*) A longer exposure of fig. 2(*a*).

*Hinc*II digested clones pDb12 and pDb18 (Fig. 2*a*). In the genomic digest a single prominent fragment of 1·7 kb co-migrated with the fragment originating from region I of clone pDb12. In addition, several minor fragments of less than 1·7 kb were evident after a longer exposure, including one that co-migrates with the *Hae*III/*Hinc*II 1·1 kb fragment from pDb18 (Fig. 2*b*).

(iv) *NTS region II*

Region II of the NTS, centrally located within the NTS (Fig. 1), contains sequences exhibiting pronounced instability when maintained in HB 101. Changes in the composition of NTS region II were detected by Southern analysis after digestion of the clones by *Hae*III. Digestion with *Hae*III yields a fragment (termed the *Hae*III-1 fragment) containing part of the 28S B-2 coding unit, NTS region I, and NTS region II (Fig. 1). Since no evidence of clonal sequence loss was detected in the 28S gene or in NTS region I, a change in *Hae*III-1 fragment size must be due to sequence instability in NTS region II. Digestion of rDNA clones from primary cultures with *Hae*III results in an array of *Hae*III-1 fragments differing in size by increments of 160 bp. All rDNA clones from primary cultures exhibit sequence instability in NTS region II, and contain either 10, 12, 13, 17 or 18 *Hae*III-1 fragments (Fig. 3*a*). Heterogeneity in the size of the largest *Hae*III-1 fragment is evident in comparisons between clones. In most clones this fragment is either 4·46 or 3·66 kb, whereas the rest of the clones contain 4·62, 3·82, or 3·34 kb fragments. Instability of NTS region II in clones with 10 *Hae*III-1 fragments (lane 5) is not as pronounced in comparison to clones containing more than 10 fragments (lanes 1–4). NTS region II instability is evident in clone pDb24 after a longer exposure (lane 6). Since heterogeneity in the size of NTS region II is found in comparisons between clones, and because the region exhibits instability resulting in an array of fragments differing in size by increments of 160 bp, a tandemly repeated array of 160 bp elements is inferred to exist in NTS region II. The degree of sequence conservation between the 160 bp elements is unknown. However, an area of sufficient homology exists to render NTS region II unstable.

In order to determine the number of 160 bp elements within NTS region II, secondary isolates from the primary culture of clone pDb12 were prepared. Clones pDb12-1 and pDb12-2 contain *Hae*III-1 fragments of 1·90 and 2·06 kb, respectively, indicating that clone pDb12-2 contains one additional 160 bp repeat compared to clone pDb12-1 (Fig. 3*b*). Digestion

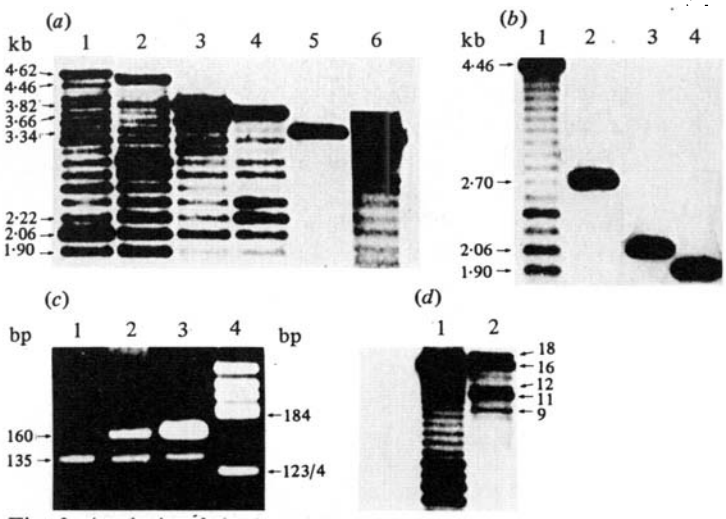

Fig. 3. Analysis of the instability, organization, composition, and heterogeneity of NTS region II. (*a*) Clones pDb30, pDb12, pDb17, pDb28, and pDb24 (lanes 1–5, respectively) were digested with *Hae*III, the fragments separated on an agarose gel, transferred to nitrocellulose, and hybridized with a ^{32}P labelled *Hae*III/*Hinc*II-generated fragment containing part of the 28S gene and NTS region I from pDb12. Lane 6 is a longer exposure of lane 5. (*b*) Clones pDb12-7, pDb12-4, pDb12-2, and pDb12-1 (lanes 1–4, respectively) were treated as described in fig. 3(*a*). (*c*) Clones pDb12-1, pDb12-2, and pDb12-4 (lanes 1–3, respectively) were digested with *Hinc*II, the fragments separated on a 4% NuSieve (FMC) agarose gel, and stained with ethidium bromide. Lane 4 contains a *Hae*III digest of pBR322 to serve as fragment size markers. (*d*) Clone pDb12-7 (lane 1) and DNA from adult female flies (lane 2) were treated as described in fig. 3(*a*). The numbers at the side of lane 2 indicate the number of *Hinc*II repeats within the respective fragments.

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of clones pDb12-1 and pDb12-2 with *Hin*cII results in identical restriction fragment profiles, with the exception of a 160 bp fragment from clone pDb12-2 which is absent in the digest of clone pDb12-1 (Fig. 3*c*). Approximately the same amount of DNA from clones pDb12-1 (lane 1) and pDb12-2 (lane 2) were loaded on the gel. The intensity of the 135 bp fragments in lanes 1 and 2 are comparable, whereas no 160 bp fragment is evident in lane 1. These data indicate that clone pDb12-1 arose from a deletion event within NTS region II, and contains no *Hin*cII excisable 160 bp repeats. The 160 bp repeats within NTS region II that are generated by digestion with *Hin*cII are termed 160 bp *Hin*cII repeats. If the deletion event occurred through inter-repeat homologous recombination, one 160 bp element refractory to *Hin*cII excision would remain in pDb12-1, assuming the presence of a single *Hin*cII site per repeat. Clone pDb12-2 would therefore contain two 160 bp repeats in tandem array, with one repeat being excisable with *Hin*cII. Alternatively, if intra-repeat recombination occurred, clone pDb12-1 could contain no 160 bp repeats. Since the secondary isolate pDb12-4 contains a 2·70 kb *Hae*III-1 fragment, it would be expected to contain five 160 bp *Hin*cII repeats (Fig. 3*b*). *Hin*cII digested clone pDb12-4 was loaded on the gel in the same amount as clone pDb12-2 (Fig. 3*c*). The intensity of the 160 bp fragment from pDb12-4 in lane 3 is several fold that of the 160 bp fragment in lane 2 from clone pDb12-2. From these data the largest *Hae*III-1 fragments from the primary cultures exhibiting 10, 12, 13, 17, and 18 *Hae*III-1 fragments are inferred to contain 9, 11, 12, 16, and 17 160 bp *Hin*cII repeats, respectively.

The configuration of NTS region II in *Hae*III digested genomic rRNA genes was examined and compared to *Hae*III digested clone pDb12-7 in order to determine which fragment within each array shown in Fig. 3A represents its original state (Fig. 3*d*). This analysis also allows assessment of the relative proportion of rRNA genes represented by the clones shown in Fig. 3(*a*). DNA prepared from the culture of pDb12-7 contains *Hae*III-1 fragments with 9, 11, 12 and 16 160 bp *Hin*cII repeats, which match the largest *Hae*III-1 fragments from the clones in Fig. 3*a*. As is shown in Fig. 2, NTS region I is highly conserved. Therefore, *Hae*III digestion of genomic rRNA genes will result in fragments of equal size to and in register with specific *Hae*III-1 fragments from clone pDb12-7. These fragments will be reflective of the original state of NTS region II within rDNA clones. Genomic fragments containing the same number of *Hin*cII repeats as the largest *Hae*III-1 fragments within cloned rRNA genes are evident. The prominent genomic fragments correspond to pDb12-7 fragments containing 11 or 16 160 bp *Hin*cII repeats (Fig. 3*d*). Of several fragments that are less frequent, those containing 9, 12, and 18 160 bp *Hin*cII repeats are most prominent. The largest *Hae*III-1 fragments from all rDNA clones analyzed contain either 9, 11, 12, 16, or 17 160 bp *Hin*cII repeats. These fragments, with the exception of those containing 17 *Hin*cII repeats, correspond in size with the prominent or the most frequent minor *Hae*III genomic fragments. These findings support the conclusion that the largest *Hae*III-1 fragments represent the original configuration of NTS region II prior to transformation of HB 101. The array of *Hae*III-1 fragments within primary cultures would therefore probably have arisen from deletion events.

(v) *NTS region III*

NTS region III is defined as the region between the 3′ end of NTS region II and the 18S rRNA gene (Fig. 1). This region also exhibits pronounced instability when maintained in HB 101. Changes in the size of NTS region III were detected by Southern analysis after digestion with *Hin*cII. Digestion of rDNA clones with *Hin*cII yields a fragment (termed the *Hin*cII-1 fragment) that contains only NTS region III. *Hin*cII-1 fragment size heterogeneity was found in comparisons between clones. These fragments range in size from 2·50 to 4·37 kb with fragments of 2·70 and 2·75 kb

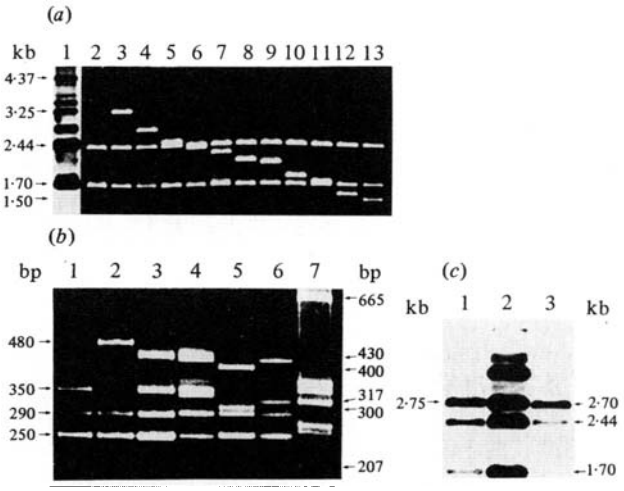

Fig. 4. Analysis of the instability, organization, composition, and heterogeneity of NTS region III. (*a*) Clones pDb19 (lanes 1 and 2) and pDb19-11 through pDb19-1 (lanes 3–13, respectively) were digested with *Hin*cII, the fragments separated on an agarose gel, and stained with ethidium bromide. The *Hin*cII digest of clone pDb19 in lane 1 from another gel was transferred to nitrocellulose and hybridized with ^{32}P labelled pDb12. The 2·44 kb fragment contains the 28S B-2 and NTS region I segments and the 1·7 kb fragment contains the 18S coding segment. (*b*) Clones pDb19-8, pDb19-9, pDb19-10, pDb19-11, pDb7, and pDb12 were digested with *Bal* I, the fragments separated on a 4% NuSieve agarose gel, and stained with ethidium bromide. Lane 7 contains a Sau 3A1 digest of pBR322 to serve as fragment size markers. (*c*) Clones pDb12 and pDb7 (lanes 1 and 3, respectively) and female adult fly DNA were digested with *Hin*cII, the fragments separated on an agarose gel, transferred to nitrocellulose, and hybridized with ^{32}P labelled pDb12. The 2·44 and 1·7 kb fragments are the same as those described in Fig. 4(*a*).

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being most common. *Hinc*II digests of clones pDb17, pDb18, pDb19, and pDb22 from primary cultures produce an array of *Hinc*II-1 fragments. These fragments do not differ in size by multiples of a single incremental unit as was found in NTS region II (e.g. pDb19, Fig. 4*a*, lanes 1 and 2). Size heterogeneity in the *Hinc*II-1 fragment was also found when comparing commonly derived secondary isolates (e.g. clone pDb12-7 *vs.* pDb12-1, Table 1).

To investigate the organization of NTS region III, secondary isolates were prepared from the primary culture of clone pDb19. Within 20 secondary isolates eleven different *Hinc*II-1 fragments were found, ranging in size from 1·5 to 3·25 kb (Fig. 4(*a*) and Table 1). The smallest size difference between contiguous *Hinc*II-1 fragments in the array shown in Fig. 4*a* is approximately 50 bp and the largest difference 500 bp. The largest *Hinc*II-1 fragment found in the pDb19 primary culture is 4·37 kb (Fig. 4*a*, lanes 1 and 2).

The size difference between *Hinc*II-1 fragments in the pDb19 series was localized to the area of NTS region III between the last *Hinc*II/*Bal* I site at the junction of NTS regions II and III, and the *Bal* I site before the *Dra* I site (Fig. 1). This region, termed the *Bal* I region, contains multiple unevenly spaced *Bal* I sites. Digestion of clones pDb19-11, pDb19-10, pDb19-9, and pDb19-8 with *Bal* I produced a variable number of fragments ranging in size from approximately 250 to 480 bp (Fig. 4*b*). The intensity of the 250 bp fragment from clones pDb19-10, pDb19-9, and pDb19-8 is greater than the intensity of the 290 bp fragment in each digest, indicating that the 250 bp fragment is present in greater frequency. The size difference between the sum of the *Bal* I fragments from and between clones pDb19-11–pDb19-10, pDb19-10–pDb19-9, and pDb19-9–pDb19-8 (approximately 500, 300 and 130 bp, respectively) is in good agreement with the size difference between the *Hinc*II-1 fragments from these clones (approximately 450, 300 and 120 bp, respectively). As the size of the *Hinc*II-1 fragment decreases, there is a concomitant loss of *Bal* I fragments. These data indicate that the unstable sequences within NTS region III lie within the *Bal* I region, and that these sequences consitute either repeats of heterogeneous length or a dispersed repeated element. On the basis of the unstable nature of sequences within this region, the size heterogeneity found in this region in comparisons between clones, and the presence of multiple *Bal* I sites an array of repeated sequences is inferred to exist in the *Bal* I region of NTS region III. These repeats are termed the *Bal* I repeats. The degree and extent of homology between the repeated sequences within this region is unknown, however sufficient homology exists to render the elements unstable in a rec A^- bacterial host.

The size of the *Hinc*II-1 fragments from clones pDb7 and pDb12 are 2·7 and 2·75 kb, respectively, and correspond to the size of the *Hinc*II-1 fragments present in most of the clones analyzed (Table 1). *Bal* I digests of clones pDb7 and pDb12 were analyzed and compared to *Bal* I digests of selected pDb19 secondary isolates (Fig. 4*b*). The *Bal* I regions of clones pDb7 and pDb12 contain *Bal* I repeats ranging in size from approximately 250 to 430 bp. As was found in some of the pDb19 secondary isolates, the 250 bp *Bal* I fragments from clones pDb7 and pDb12 are present in greater frequency than the other larger *Bal* I repeats. The size difference between the *Hinc*II-1 fragments of clones pDb7 and pDb12 is due to two *Bal* I fragments of pDb12 of slightly greater length (Fig. 4*b*).

To assess size variability and to determine the predominant size class of NTS region III in genomic rDNA repeats, a *Hinc*II digest of genomic DNA was examined and compared to *Hinc*II digests of clones pDb7 and pDb12 (Fig. 4*c*). A single prominent genomic band was found that corresponds in size to the *Hinc*II-1 fragments from clones pDb7 and pDb12. The small size difference (approximately 50 bp) between the *Hinc*II-1 fragments of clones pDb7 and pDb12 did not allow resolution of these fragments or of the corresponding fragments in the genomic digest. Minor *Hinc*II-1 fragments of greater length are apparent in the genomic digest, but the majority of rRNA genes contain a *Hinc*II-1 fragment of 2·7/2·75 kb.

(vi) *The independent nature of the deletion events in NTS region II and III*

All secondary isolates of clone pDb12 (20 in total) except one exhibited sequence loss in NTS region II only. All of these clones retained the *Hae*III site between NTS regions II and III, including pDb12-7 which incurred sequence losses in both regions (Table 1). All secondary isolates of clone pDb19 retained the *Hae*III and *Dra*I sites that bracket the unstable region of NTS region III. Most of these clones exhibited sequence loss only in NTS region III, however, clones pDb19-5 and pDb19-8 incurred sequence losses in both regions II and III (Table 1). These data indicate that the deletion events of NTS regions II and III occur autonomously.

4. Discussion

We have cloned and characterized several rDNA repeating units from *D. busckii*. The repeats share many features common to the rDNAs of related species, but contain numerous differences as well. The organization of the rRNA genes of *D. busckii* is analogous to that of other drosophilids (Beckingham, 1982). The prominent rDNA repeat classes are 12·8 and 13·6 kb in length. Clones were isolated that contain insertions in the 28S coding unit. These insertions exhibit heterogeneity in both size and nucleotide composition, traits shared by the insertions

found in *D. melanogaster*. The majority of rRNA genes in *D. melanogaster* and other drosophilids contain insertions in the 28S gene (Beckingham, 1982). In contrast, the prominent rDNA repeats of *D. bucskii* lack 28S insertions. The most prominent feature distinguishing the rRNA genes of *D. busckii* from other drosophilids lies in the composition and organization of the NTS.

The NTS of *D. busckii* rRNA genes contains three distinct regions. Heterogeneity in the size of each NTS region was found in comparisons between clones and in examinations of genomic digests. Heterogeneity in restriction fragment lengths from regions of repeats of multigene families is often indicative of the presence of repeated sequences within that fragment, because differences in the number of repeats within a region give rise to restriction fragment size polymorphisms. In addition, clones containing tandemly arrayed repeats can be unstable when propagated in a bacterial host (Simmen *et al.* 1985; Schmidt *et al.* 1982; Arnheim & Kuehn, 1979; Erickson & Schmickel, 1985). NTS regions II and III both exhibit pronounced instability when maintained in HB 101. Sequences were deleted from NTS region II in increments of 160 bp, and from region III in units ranging from 250 to 480 bp. The instability of NTS regions II and III in conjunction with the nonartifactual size heterogeneity of these regions support the conclusion that NTS regions II and III contain repeated sequences. The size heterogenity of NTS region I observed in genomic digests in conjunction with differences between cloned rDNAs, support the idea that NTS region I also contains a repeated sequence.

The repeats of NTS region II are uniform in size and are organized in a tandem array. In contrast, NTS region III contains repeats that are either heterogeneous in length or are unevenly dispersed within unique sequences. In either case, the organization of NTS region III gives rise to deletion products of heterogeneous length. The repeats within NTS regions II and III contain different restriction sites, and the deletion events of these regions occur independently. Moreover, the *Hinc*II sites within NTS region II are evenly spaced within the array, in contrast to the unevenly spaced *Bal* I sites of region III. The autonomous deletion events in conjunction with the unique restriction sites of each region support the conclusion that at least part of the sequences composing these repeats are different.

The prominent rDNA repeating units of 12·8 and 13·6 kb contain 11 or 16 160 bp *Hinc*II repeats respectively, accounting for the size difference of 0·8 kb between the repeats. Heterogeneity in the number of *Hinc*II repeats present in NTS region II was evident upon examination of genomic digests. However, a stochastic distribution in the number of *Hinc*II repeats within NTS region II is not present, indicating the existence of a homogenization mechanism(s). Among the rRNA genes of drosophilids examined to date, the repeats of NTS region II appear to be unique to the ribosomal RNA genes of *D. busckii* on the basis of size and location within the NTS.

NTS region III is located next to the 18S gene, and contains a region composed of a variable number of *Bal* I fragments that are heterogeneous in size. Of these fragments the 250 bp repeat is most prevalent, and is synonymous with regards to size and location to the repeats proximal to the 18S gene of *D. melanogaster*. The presence of repeated elements in the NTS proximal to the 18S gene of *D. busckii* renders its organization similar to that found in the NTSs in *D. melanogaster*, its sibling species, and the distantly related species *D. virilis* and *D. hydei* (Beckinham, 1982; Coen *et al.* 1982). However, heterogeneity in the length and composition of these repeats is unique to the rRNA genes of *D. busckii*.

The tandemly repeated organizational motif of NTS 18S proximal repeats within the rRNA genes is highly conserved in the drosophilid species within the *Sophophoran* and *Drosophila* radiations as exemplified by *D. melanogaster*, and *D. virilis* and *D. hydei*, respectively. These repeats, which contain partial reiterations of the RNA polymerase I promotor sequence, are thought to function as polymerase I enhancers in *D. melanogaster* (Kohorn & Rae, 1982). The lack of a high degree of sequence conservation within the 18S proximal repeats of *D. busckii* may indicate that these elements lack RNA polymerase I enhancer function. The role of the repeated elements within NTS regions II and III in transcription can be assessed through the use of the rRNA gene NTS deletion clones described in this report.

Instability of tandem sequence arrays within NTSs has been found to occur in *Chironomus* (Schmidt *et al.* 1982), sea urchin (Simmen *et al.* 1985), mouse (Arnheim & Kuehn, 1979), and human (Erickson & Schmickel, 1985) rDNA clones. Repeated elements next to the origin of transcription in the mouse NTS exhibit instability when cloned in lambda gtWes and pBR322 vectors maintained in a rec A^- host. In contrast, the instabilities seen in *Chironomus*, sea urchin, and human NTS tandem repeats (in the vectors pBR322, Charon 4, and Charon 27 and 28, respectively) occur in rec A^+ bacterial hosts. In all of the other cases of NTS instability the loss of sequences occurred in a single tandem array of repeats. Instability of elements in the NTS of *D. busckii* occurs in two distinct regions containing different repeats, making it unique in comparison to other NTS instabilities. No sequence instability has been reported in the NTS tandem arrays present in cloned rRNA genes of *D. melanogaster* or any other drosophilid. Thus, the composition of the *Hinc*II and *Bal* I repeats within the NTS of *D. busckii* differ from the NTS repeats in all other drosophilids by virtue of their unstable behavior in a bacterial host.

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