Repeated Nucleotide Sequence Arrays in Balbiani Ring 1 of
Chironomus tentans Contain Internally Nonrepeating and
Subrepeating Elements*

(Received for publication, November 22, 1982)

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Balbiani rings in Chironomus are large puffs on
salivary gland polytene chromosomes that contain
functionally related, but nonidentical genes that code
for tissue-specific secretory polypeptides. In situ hy-
bridization was used to select a recombinant plasmid
(pCtBR1-1) that contained an insert of Chironomus
tentans genomic DNA that originated from Balbiani
ring 1. Mapping with restriction endonucleases dem-
onstrated that the insert was 385 bp (base pair) and
it contained duplicate clusters of certain cleavage sites
about 250 bp apart. This repeat was shown to be part
of tandem sequence arrays in the genome by hybridi-
zation of radioactive pCtBR1-1 to nitrocellulose blots
containing limit and partial restriction endonuclease
digests of nuclear DNA. Subsequent sequence analysis
of the cloned DNA confirmed the presence of a com-
plete copy of a 246-bp repeat comprised of a 114-bp
internally nonrepeating segment and a 132-bp segment
containing four 33-bp subrepeats. The subrepeats ap-
parently evolved from a simple 9-bp sequence encoding
a consensus tripeptide (Lys-Pro-Ser) in which the first
two codons (AAA-CCA) were highly conserved at the
nucleotide level. Comparisons between intragenic and
interspecific (BRb in Chironomus thummi) copies of
corresponding sequences revealed that, during the ev-
olution of these tandemly repeated protein-coding se-
quences, internally nonrepeated segments were highly
conserved and most likely became interspersed by var-
iable segments containing subrepeats that arose from
reduplication and divergence of 9-bp repeats.

Two types of sequence repetitions have been described
that occur within protein-coding genes. In one group, a short
nucleotide sequence is tandemly duplicated within the coding
portion of a gene and is reflected by a repeating amino acid
sequence of functional significance (1, 2). An alternative
repetition pattern is found among members of multigene
families (3, 4) where short intragenic sequence homologies
exist between domains of certain functionally related but
nonidentical genes (5–9). Nucleotide and amino acid sequence
data reveal that an underlying evolutionary feature of these
genes is that they appear to have evolved by reduplication,
divergence, and even translocation of a small ancestral se-
quence that ultimately yields discrete genes that may possess
overlapping phenotypic functions.

A group of functionally related, but nonidentical genes code
for discrete polypeptides that make up saliva which is secreted
from the salivary glands of Chironomus larvae (10, 11). Cy-
tologically, two of these genes can be observed as large chro-
mosomal puffs, known as BR1 and BR2 in Chironomus
tentans, on salivary gland polytene chromosomes. BR1 and
BR2 transcripts appear similar in that they are 75 S RNA
molecules (12, 13) with internal sequence repetition (14–16).
However, in situ hybridization of BR1 versus BR2 RNAs
detect little, if any, sequence homology (14), and their rates
and patterns of reassociation to genomic DNA are distinctly
different (16). Both transcripts emerge from the nucleus as
37-kb cytoplasmic RNAs (17) that can direct in vitro trans-
lation of incomplete secretory polypeptides (18). Secretory polypep-
tides are unusually large (19, 20), yet they have a simple
amino acid composition (21) and tryptic peptide pattern (18),
indicative of short, repeated amino acid sequences. These
repeated sequences presumably permit overlapping alignment
of individual polypeptides during polymerization of a long,
highly elastic, salivary fiber that is used by larvae to spin a
protective cylindrical tube (11).

Because BR genes afford an opportunity to examine the
evolution of tandemly repeated protein coding sequences at
the intragenic, interspecific, and interspecific levels, it is desir-
able to obtain nucleotide sequence data for their basic repeating
units. It is becoming increasingly apparent that the organi-
ization of repeated nucleotide sequences in this system will
have to be derived from a series of small cloned DNA frag-
ments since larger fragments are remarkably unstable (22–
24). Nucleotide sequence data is currently available for BR1
(24, 25) and BR2 (26) in C. tentans as well as their homologous
genes, BRb (27) and BRc (23, 28) in Chironomus thummi,
and BR2 in Chironomus pallidivittatus (29). In each instance,
the existence of tandem arrays within a BR gene has been
indicated by hybridizing cloned DNA to genomic Southern
blots (30). The emerging pattern of BR gene structure is one
of tandem arrays which contain an alternating pattern of
internally repeating and nonrepeating nucleotide sequences
(23, 25–29).

We report here the nucleotide sequence of a 348-bp genomic
fragment of BR1 in C. tentans. The cloned fragment contained

The abbreviations used are: BR, Balbiani rings; bp, base pair; kb,
kilobase or kilobase pairs. SR refers to the 132-bp segment of the
246-bp repeat which contains four 33-bp subrepeats; INR refers to
the 114-bp segment of the 246-bp repeat which is internally nonre-
peating.

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§ Recipient of a Dean’s Medical Student Research Stipend.

* This work was supported by the National Institutes of Health
Research Grant GM 26362 from the Institute of General Medical
Sciences and a PROPHET computer terminal from the Biotechnol-
ogy Resources Program, Division of Research Resources. The costs
of publication of this article were defrayed in part by the payment of
page charges. This article must therefore be hereby marked "adver-
tisement" in accordance with 18 U.S.C. Section 1734 solely to indicate
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a complete copy of a 246-bp repeated sequence that is apparently organized as tandem arrays in the genome. The 246-bp repeat was divided into a conserved internally nonrepeating segment and a somewhat more divergent segment that contained subrepeats which may have evolved from simple 9-bp repeats. DArma for BR1 support a recent model (26) for the evolution of 37-kb BR2 genes from a 110 to 120-bp primordial sequence.

EXPERIMENTAL PROCEDURES

Construction, Selection, and Purification of pCtBR1-1—Details have been published regarding the construction of a partial genomic library from which pCtBR1-1 was selected (22). Briefly, BR1 and BR2 sequences were found to reside on 32 to 40-kg fragments after DNA with EcoRI. Such fragments were enriched by gradient sedimentation, randomly sheared, tail, with oligo(dC), and annealed to oligo(dT)-tailed PstI-cleaved pBR322. Recombinants were screened by colony hybridization with radioactive salivary gland 75 S RNA that simultaneously detected BR1 + BR2 sequences. Plasmid DNAs used in this study were purified from detergent lysates of host bacterial cells by two rounds of bound particle precipitation in CsCl gradients containing ethidium bromide (31). Recombinant DNA experiments were conducted under containment conditions prescribed by National Institutes of Health "Guidelines for Research Involving Recombinant DNA Molecules."

Radiolabeling of Nucleic Acids—Purified (17) salivary gland 75 S RNA was partially hydrolyzed and labeled with [γ-32P]ATP using polynucleotide kinase (32). Purified plasmid DNAs used for hybridization probes were labeled by the nick translation reaction (33, 34) using either [3H]dUTTP or [α-32P]dCTP. Phage or plasmid DNAs used as molecular weight markers on gels were labeled by nick translation without the addition of exogenous DNase. DNA fragments cleaved by restriction endonucleases were end-labeled with [γ-32P]ATP in an exchange reaction (35). Radioisotopes were purchased from New England Nuclear.

In Situ Hybridization—Squashed preparations (36) of salivary gland polytene chromosomes were used for in situ hybridization of H-labeled plasmid DNAs. Hybridizations were done in 4 ¥ SET (1 ¥ SET is 0.15 M NaCl, 30 mM Tris- HCl, pH 8.0, and 2 mM EDTA), 30% formamide at 37 °C. Posthybridization treatment included several rinses in excess hybridization media at 37 °C and two rinses in 1 ¥ SET at 65 °C. In some instances, a stringent rinse was performed in 0.1 ¥ SET at 65 °C. Hybridized chromosome preparations were subjected to autoradiography and stained with Giemsa prior to being photographed (37).

Restriction Endonuclease Mapping and Gel Blotting—Restriction endonucleases were purchased from New England Biolabs, Beverly, MA, or Bethesda Research Laboratories. Endonuclease cleavage sites within the insert were initially identified by comparing the cleavage patterns of pCtBR1-1 and pBR322 by electrophoresis in 1% agarose gels (38). Subsequent mapping was done by comparing autoradiograms made from polycrylamide gels (39) containing appropriate single and double enzymatic digests of randomly labeled (nick-translated) versus end-labeled (kinased) samples of the 385-bp insert (excised by PstI) or an internal 250-bp MboI fragment (see Fig. 2A). These data were confirmed by partial digests of end-labeled fragments, according to published procedures (40). Limit or partial endonuclease digests of C. tentans nuclear DNA were fractionated on agarose gels and transferred to nitrocellulose blots (30). 32P-labeled pCtBR1-1 was hybridized to blots in 4 ¥ SET, 30% formamide, 10 ¥ Denhardt's solution (41), 1% sodium dodecyl sulfate, and 0.1% sodium pyrophosphate at 37 °C. Posthybridization washes were the same as described for in situ hybridization. All gels contained internal radioactive molecular weight markers made by cleaving λ DNA with HindIII (42) or PstI with HindIII, HaeIII, or HpaII (43).

DNA Sequencing—The pCtBR1-1 insert and appropriate fragments were subcloned into the replicative form of bacteriophage M13mp9. The complete insert was subcloned as a PstI fragment. The leftward, central, and rightward RsaI fragments (and adjacent vector sequences, refer to Fig. 2A) were separated by polycrylamide gel electrophoresis and repurified (44), and synthetic EcoRI linkers were added by blunt end ligation (45). The central RsaI fragment was then cleaved with EcoRI to trim linker oligomers and cloned into the EcoRI site of M13mp9 DNA. The leftward and rightward fragments were cleaved with EcoRI + PstI and cloned into phage DNA cleaved by the same enzymes. Recombinant phage containing complementary strands of inserted restriction fragments were sorted by DNA hybridization of phase blots (46). Transcribed versus nontranscribed strands were identified by their ability to form RNase-resistant hybrids with 32P-labeled 75 S RNA. DNA sequencing was performed by using recombinant phage as templates for DNA synthesis in the presence of a primer (47) and dideoxyribonucleoside triphosphate chain terminators (48). Short repeated nucleotide sequence homologies were detected by using Align and Makedotgraph procedures which are based upon a previously published procedure (49) and available on the PROPHET computer network.

RESULTS AND DISCUSSION

Balbiani Ring Origin of pCtBR1-1—pCtBR1-1 is comprised of a fragment of C. tentans nuclear DNA inserted into the PstI recognition site of pBR322 via oligo(dG):oligo(dC) homopolymeric tails. The recombinant was originally selected as a potential BR clone by colony hybridization using radioactive 75 S RNA that contained both BR1 and BR2 sequences. To determine which BR the insert originated from, purified pCtBR1-1 DNA was radiolabeled in vitro with [3H]dTTTP by nick translation and hybridized in situ to squashed preparations of salivary gland polytene chromosomes. Photomicrographs showed that autoradiographic exposure of silver grains occurred over chromosome IV, especially in the region of BR1 (Fig. 1A). Peripheral grains were often seen over BR2, similar
to a previous observation (24), but control experiments indicated that these could be greatly reduced by a stringent posthybridization rinse (Fig. 1B). In parallel experiments it was shown that 3H-labeled pBR322 did not hybridize to polytene chromosomes. We, therefore, conclude that the genomic insert contained within pCtBR1-1 originates from BR1. Hybridization to BR2 was subsequently explained when a partial sequence homology was identified between this recombinant and a cloned BR2 sequence (26).

Endonuclease Cleavage Map and Genomic Organization of the pCtBR1-1 Insert—Restriction endonuclease cleavage mapping of the recombinant (Fig. 2A) indicated that the size of the insert was 385 bp. The central portion of the insert contained four, approximately evenly spaced Sau3A (MboII) sites flanked by two identical clusters of AluI, MboII, HinfI, and RsaI recognition sites about 250 bp apart. These results first suggested that the pCtBR1-1 insert contained a repeated nucleotide sequence.

To determine the organization of the pCtBR1-1 insert within the C. tentans genome, Southern blots (30) containing restriction endonuclease-cleaved fragments of nuclear DNA were probed with 32P-labeled pCtBR1-1. Autoradiograms of blots made from MboII digests revealed a single band of less than 4 kb. Neither enzyme cleaved the pCtBR1-1 insert, yet showed a ladder of bands with a 250-bp interval ranging from 250 bp to at least 2.5 kb (Fig. 2B). Identical results were obtained with HinfI and RsaI (data not shown). These results indicated that the 250-bp MboII fragment of the pCtBR1-1 insert might be organized within BR1 as tandem arrays consisting of at least 10 copies.

In an attempt to estimate the maximum number of tandem sequence arrays in BR1, blots containing nuclear DNA cleaved with TaqI or HaeIII were hybridized with 32P-labeled pCtBR1-1. Neither enzyme cleaved the pCtBR1-1 insert, yet each enzyme cleaved most of the C. tentans DNA to fragments of less than 4 kb. TaqI digests mainly showed hybridization with 19- and 22-kb bands, while HaeIII digests had a prominent 30-kb band (Figs. 3, C and D). These same bands hybridized to 32P-labeled 75 S RNA (data not shown) which is highly enriched in BR1 + BR2 sequences, although we could not tell if they contained any nontranscribed flanking sequences. Nonetheless, these results raised the possibility that a substantial portion of a 37-kb BR1 gene may be comprised of tandem arrays of the 250-bp repeated sequence contained within the recombinant. We subsequently found that the faint 28-kb bands obtained in both digests could be eliminated by the same stringent rinse used after in situ hybridization. Thus, we conclude that these bands also result from a sequence homology with BR2.

Nucleotide and Amino Acid Sequence—The DNA sequencing strategy employed in this study involved subcloning the entire 385-bp insert and appropriate subfragments (Fig. 2A) into the replicative form (double-stranded DNA) of bacteriophage M13mp9. This procedure allowed us to unambiguously identify recombinants containing transcribed versus nontranscribed strands by filter hybridization with 32P-labeled 75 S RNA (Fig. 2B). All segments were sequenced at least twice and 95% of the sequence was confirmed by independently sequencing complementary strands of the original insert. Due to the presence of a long homopolymeric sequence at the 3'-end of the insert (Fig. 2A), it was not possible to obtain confirmation of the 10-bp sequence between the nearest RsaI and AluI sites on the nontranscribed strand (Fig. 2B).

The nucleotide and encoded amino acid sequence of the nontranscribed strand of the pCtBR1-1 insert is presented in Fig. 4. The insert contained 348 bp of C. tentans DNA and homopolymeric stretches of (dG)7 and (dC)7 located at the transcriptional 5'- and 3'-ends, respectively. Direct evidence was found for the repeated sequence previously suggested by endonuclease cleavage mapping (Fig. 2A) and genomic blotting experiments (Fig. 3). The repeat length was 246 bp, beginning at nucleotide position 10 and extending through to position 255. The sequence beginning at position 256 corresponds to the start of an incomplete (95/246 bp) copy of what...
Repeated Nucleotide Sequences in Balbiani Ring 1

Fig. 4. The nucleotide sequence and derived amino acid sequence of the pCtBR1-1 insert. The recognition sequences for various restriction endonucleases are underlined in the DNA sequence. Portions of the sequence enclosed in boxes represent subrepeats (see Fig. 2).

would have been the next tandem array. The 246-bp repeat had 42% C + G content, in agreement with the base composition of BR1 RNA (50). The amino acid sequence was derived from the only open translational reading frame found in the transcribed strand. The next two reading frames were interrupted by two and 24 termination codons, respectively. About 64% of the encoded polypeptide consisted of only Lys, Pro, Ser, and Arg residues (Table I). These same four amino acids have been identified as the most abundant residues in hydrolysates of the putative BR1 polypeptide.2 Ser codons frequently occurred within sequences that were compatible with reported phosphorylation sites in other proteins (51). We have found that greater than 80% of $^{32}$P incorporated into the putative BR1 product in vivo can be recovered as phosphoserine.3

The 246-bp Repeat Contains an Internally Nonrepeating Segment and a Segment with Subrepeats—While this manuscript was in preparation, models of BR gene structure were published which predicted that segments of highly repeated sequences alternated with nonrepeated sequence segments (23, 25-29). The 246-bp repeat within the pCtBR1-1 insert could similarly be divided into two regions consisting of distinctive nucleotide sequence patterns. These regions were designated as INR and a segment containing SR based upon a computer-assisted search for internally repeated nucleotide sequences. Examination of the complete nucleotide sequence indicated that the INR and SR segments alternate with one another and that two SR/INR junctions occurred within the pCtBR1-1 insert (Figs. 2C and 4).

The INR1 segment (Fig. 2C) contained 114 bp extending from nucleotide position 10 through 123, inclusive (Fig. 4). No obvious patterns of internal sequence repetition were found within INR1, and the clustered AluI, MboII, HinfI, and Rsal recognition sites were located within this segment (compare Figs. 2, A and C). A second, partial INR segment (INR2) begins at nucleotide position 256 and extends 93 bp toward the 3'-end of the insert. These INR segments exhibited remarkable sequence homology with only a single base difference occurring between them. The nucleotide at position 54 within INR1 was Ado, while the corresponding location within INR2 (position 282) had Thd. This variation invoked a silent mutation in the wobble position of an Ala codon; thus, the amino acid sequence is perfectly conserved throughout the homologous regions of INR1 and INR2.

The 132-bp SR segment was characterized by four direct repeats (designated SR1, SR2, SR3, and SR4) of a 33-bp sequence (Figs. 2C and 4). Although these direct repeats had considerable sequence homology, more sequence heterogeneity exists between them than between INR segments. SR2 and SR4 had complete homology in their DNA sequence and contained the apparent consenus nucleotide and amino acid sequence for subrepeats within pCtBR1-1. SR3 was 97% (30/31) homologous with the consensus sequence. This implied that it represented the distal three codons at the 3'-end of the insert. The INR segments exhibited remarkable sequence homology with only a single base difference occurring between them. The nucleotide at position 54 within INR1 was Ado, while the corresponding location within INR2 (position 282) had Thd. This variation invoked a silent mutation in the wobble position of an Ala codon; thus, the amino acid sequence is perfectly conserved throughout the homologous regions of INR1 and INR2.

Distribution of Amino Acids and Codons between INR and SR Segments—Certain amino acids and codons were not randomly distributed between INR and SR segments of the pCtBR1-1 insert. The INR1 segment was characterized by containing 70% (7/10) of the Arg residues found within the complete (INR1 + SR) 246-bp repeat (Table I). Less frequently occurring amino acids (Cys, Asn, Met, Phe, and Thr), that individually accounted for less than 7% of the residues,
Table I

| Amino acid | Per cent composition | Possible codons | Frequency of occurrence |
|------------|----------------------|----------------|------------------------|
|            | INR1 | SR | Total | INR1 | SR |
| Lys        | 4.9  | 14.6 | 19.5  | AAA | 3  | 8 |
|            | 1    | 4   | 5     | AAG | 1  | 4 |
| Pro        | 12   | 14.6 | 15.9  | CCA | 1  | 7 |
|            | 0.5  | 0   | 0.5   | CCU | 0  | 5 |
| Ser        | 6.1  | 9.8  | 15.9  | UCA | 1  | 0 |
|            | 0    | 0   | 0     | UCG | 0  | 0 |
|            | 0.5  | 0   | 0.5   | UCC | 1  | 0 |
|            | 0.5  | 0   | 0.5   | AGU | 2  | 3 |
| Arg        | 8.5  | 3.7  | 12.2  | AGA | 7  | 3 |
|            | 0.5  | 0   | 0.5   | AGG | 0  | 0 |
|            | 0    | 0   | 0     | CGA | 0  | 0 |
|            | 0.5  | 0   | 0.5   | CGU | 0  | 0 |
|            | 0    | 0   | 0     | AGC | 1  | 1 |
| Gly        | 3.6  | 4.9  | 8.5   | GGA | 0  | 4 |
|            | 0    | 0   | 0     | GGU | 3  | 0 |
|            | 0    | 0   | 0     | GGG | 0  | 0 |
|            | 0    | 0   | 0     | GGC | 0  | 0 |
| Glu        | 2.4  | 6.1  | 8.5   | GAA | 2  | 1 |
|            | 0    | 0   | 0     | GAG | 0  | 4 |
|            | 0.5  | 0   | 0.5   | GCU | 0  | 0 |
|            | 0.5  | 0   | 0.5   | GCC | 1  | 0 |
| Ala        | 7.3  | 0    | 7.3   | GGG | 0  | 0 |
| Cys        | 4.9  | 0    | 4.9   | UGU | 2  | 0 |
| Asn        | 3.7  | 0    | 3.7   | UGC | 2  | 0 |
| Met        | 1.2  | 0    | 1.2   | UUG | 0  | 0 |
| Phe        | 1.2  | 0    | 1.2   | UUC | 1  | 0 |
| Thr        | 1.2  | 0    | 1.2   | ACA | 0  | 0 |
|            | 0    | 0   | 0     | ACU | 1  | 0 |
|            | 0    | 0   | 0     | AGC | 0  | 0 |
|            | 0    | 0   | 0     | ACC | 0  | 0 |

were also sequestered in INR1. The 4 Cys residues were preceded by either Arg (nucleotide positions 10 and 88) or Lys (positions 46 and 82). Three of these dipeptides were spaced 10 residues apart from each other and the fourth dipeptide immediately followed the third. Although Gly residues were nearly evenly distributed between INR and SR segments, GGU codons were restricted to INR1 while GGA codons were restricted to the SR segment (Table I).

The SR segment contained the majority of Pro (12/13), Lys (12/16), Glu (5/7), and Ser (6/13) residues coded for by the 246-bp repeat (Table I). As a rule, Pro residues were always preceded by a charged amino acid and most frequently found within the tripeptides Lys-Pro-Ser or Arg-Pro-Glu. We noticed that the only Pro residue within INR1 (Table I) was also part of a Lys-Pro-Ser tripeptide located very near SR1 (see Fig. 4, nucleotide positions 106 through 114).

Subrepeats Contain Conserved Tripeptides with a 6-bp Consensus Sequence—The redundant pattern of Lys-Pro-Ser and Arg-Pro-Glu tripeptides implied that the 33-bp subrepeats may have evolved by duplication and divergence of a smaller repeated nucleotide sequence. A computer-assisted alignment revealed a series of tandem 9-bp homologies beginning 18 bp prior to the INR1/SR1 junction and encompassing the adjacent SR segment (nucleotide positions 106 through 255). A 3-bp insertion was required, between the eighth and ninth codon within each subrepeat, that presumably reflects deletion of a codon during the evolution of these repeats (Fig. 5). This essentially converted each subrepeat into a block of four 9-bp repeats. The 9-bp repeats exhibited polarity with regard to conservation in nucleotide selection at each position (Fig. 5). That is, bases were more conserved at the 5'-end of each 9-bp repeat than the 3'-end. In fact, no consensus nucleotide sequence was found for the last three nucleotides of the 9-bp repeat. However, 71% (10/14) of the 3'-terminal trinucleotides aligned at this position were synonymous Ser codons. So while the nucleotide sequence was conserved for only the first 6 bp, Lys-Pro-Ser prevailed as the consensus amino acid sequence.

Intragenic and Interspecific Conservation of Nonrepeating Segments and Subrepeats of BR1—There has been strong intragenic conservation among five independent INR segments from BR1 in C. tentans. A 150-bp BR1 sequence published previously (24) contained a 114-bp overlap (designated here as INR3; Fig. 6) with our INR1 segment. In addition, the recently published (25) nucleotide sequence of a cDNA clone from BR1 contained two nonrepeated segments (designated here as INR4 and INR5; Fig. 6). A minimum of

Consensus Lys Pro Ser

Fig. 5. Derivation of a consensus tripeptide and conserved hexanucleotide sequence by aligning 9-bp repeats within pCtBR1-1. Numbers in parentheses indicate nucleotide positions within the complete sequence (Fig. 4). The sequence reads from left to right, top to bottom. The proposed locations of deleted codons (Δ) are indicated. Each row is designated by its origin in INR or SR segments of the insert (Fig. 2A), then consecutively numbered and lettered as indicated. The consensus nucleotide for each of the first six columns is shown at the bottom along with its frequency of occurrence. Divergent nucleotides are circled. For the last three columns, related codons are shown along with their frequency of occurrence.

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that the ancestral sequence for BR1 in *C. tentans* supported such a model and suggested the BR1 sequence organization found in this study (i.e., 246-bp arrays comprised of INR segments flanked by tandem 9-bp repeats) supported a model and suggested that the ancestral sequence for BR1 in *C. tentans* was also about 105 to 114 bp in length and may be represented by segment INR1. We have noted that INR1 shares about 70% DNA sequence homology with a similar segment in BR2. A detailed intergenic (BR1 versus BR2) comparison of INR segments will be made in conjunction with the description of a variant BR2 sequence (56).

The fundamental functioning unit within secretory polypeptides in *Chironomus* may be reflected by 200 to 300-bp repeats within BR genes. In turn, conserved and variable domains within these units may be represented by alternating INR and SR segments, respectively. Variable domains have tolerated limited divergence in amino composition and length due to variations in short tandemly repeated sequences. However, certain key characteristics were retained, reflected by the occurrence of prevalent di- and tripeptides. Homologous but unequal crossing-over between tandemly repeated sequences can lead to variations in nucleotide sequence and length (52) that can presumably lead to expansion or contraction of gene size (53). To better understand the evolution of BR genes it is desirable to identify major sequence variants that might occur, as well as obtain sequence data from other functionally related genes that code for salivary polypeptides (54, 55).

**Acknowledgments**—Mary Jane Bevill and Virginia Hill provided diligent technical assistance throughout much of this study. Joachim Messing kindly provided us with bacteriophage M13mp9 and detailed protocols regarding its use. We also wish to thank Ronnie Brown for her patience in typing the manuscript and figures containing sequence data.

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Repeated nucleotide sequence arrays in Balbiani ring 1 of Chironomus tentans contain internally nonrepeating and subrepeating elements.

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*J. Biol. Chem.* 1983, 258:7793-7799.

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