MULTIPLE GENE SITES FOR 5S AND 18 + 28S

RNA ON CHROMOSOMES OF *GLYPTOTENDIPES*

BARBIPES (STAEGER)

WU-NAN WEN, PEDRO E. LEÓN, and DONALD R. HAGUE

From the Biology Department, University of Oregon, Eugene, Oregon 97403

ABSTRACT

Ribosomal RNAs (28 + 18S and 5S) and 4S RNA extracted from the chironomid *Glyptotendipes barbipes* were iodinated in vitro with ^{125}I and hybridized to the salivary gland chromosomes of *G. barbipes* and *Drosophila melanogaster*. Iodinated 18 + 28 S RNA labeled three puffed sites with associated nucleoli on chromosomes IR, IIL, and IIIL of *G. barbipes* and the nucleolar organizer of *Drosophila*. Labeled 5S RNA hybridized to three sites on chromosome IIIR, two sites on chromosome IIR and one site in a Balbiani ring on chromosome IV of *Glyptotendipes*. Most of the label produced by this RNA was localized seven bands away from the centromere on the right arm of chromosome III, and we consider this to be the main site complementary to 5S RNA in the chironomid. This same RNA preparation specifically labeled the 56 EF region of chromosome IIR of *Drosophila* which has been shown previously to be the only site labeled when hybridized with homologous 5S RNA. Hybridization of *G. barbipes* chromosomes with iodinated 4S RNA produced no clearly localized labeled sites over the exposure periods studied.

INTRODUCTION

Multiple Gene Sites for 5S and 18 + 28S RNA on Chromosomes of Glyptotendipes barbipes (Staeger)

The location of gene sequences in eukaryotic chromosomes can be visualized directly by *in situ* hybridization of the denatured chromosomal DNA with radioactive nucleic acids (6, 15, 24, 34). Ideally, the RNA or DNA that is hybridized to the chromosome should be clearly characterized and of high specific activity, and its complement in the genome repeated at each locus. Also, the energy of the β-rays emitted by the isotope must be adequately low to produce high resolution autoradiographs.

Polytene chromosomes of dipterans offer an excellent substrate for *in situ* hybridization since their size and banded appearance allow precise localization of the label. DNA in these chromosomes is laterally repeated and it is tandemly repeated as well at loci such as those coding for 18 + 28S, 5S, and 4S RNA (40, 42, 43, 46). Dipteran salivary gland chromosomes have been successfully hybridized with 4S RNA (44), 5S RNA (35, 49), 18 + 28S RNA (17, 26, 35, 36), highly repeated satellite DNAs (12, 14, 21, 41), and putative messenger RNAs (26, 37).

Highly radioactive RNA can be produced by reacting RNA in vitro with ^{125}I (7, 39). This reaction introduces ^{125}I into the cytosine residues

of RNA without altering the thermal stability of the RNA-DNA hybrids on membrane filters (18). Recently, iodinated 5S RNA has been successfully utilized in cytological hybridization with *Drosophila melanogaster* salivary gland chromosomes and diploid plant and animal chromosomes with good autoradiographic resolution (39, 50).

We have isolated 4S, 5S, and 18 + 28S RNA from the chironomid *Glyptotendipes barbipes* (Staeger) and iodinated each RNA species in vitro with ^{125}I. The iodinated products were hybridized to salivary gland chromosomes of fourth instar larvae of the same animal. Chironomid flies are particularly interesting since their excellent cytology and large size allow manipulation of individual puffs in salivary gland chromosomes, making it possible to investigate the mechanisms of transcription and the nature of the chromomere (8, 9, 10). We felt that such chromosomes would permit precise localization of label in relation to bands and interbands. We were interested in determining how many 5S loci occur in the presence of several nucleolus organizers that had been identified by cytological observation (4, 5, 47). Finally, we wished to attempt localization of 4S gene sequences on the chromosomes of *G. barbipes* by this hybridization technique. Some of the sites complementary to 4S have been identified in *D. melanogaster*, and these appear to be widely spread over the genome (44, 45). It was of interest to determine if the 4S sites were spread out similarly or were more tightly clustered in this chironomid dipteran.

MATERIALS AND METHODS

Animals

Larvae of *G. barbipes* were collected from sewage lagoons in Creswell, Lane County, Oregon during the summer of 1973. Salivary gland chromosomes from *D. melanogaster* (Oregon R X Canton S) were used for hybridization controls.

Ribonucleic Acid Preparation

Approximately 20 g (fresh weight) of *G. barbipes* larvae were washed with distilled water, frozen in liquid nitrogen, and lyophilized to dryness. RNA was extracted at 4°C following the procedure of Monier (31, 32) with several modifications. Larvae were homogenized in 80 ml of Tris buffer [0.01 M Tris-Cl, pH 7.5; 0.01 M Mg $(C_2H_3O_2)_2$; 0.1 M NaCl and 0.001 M β-mercaptoethanol]. The extraction volume was increased to 120 ml with buffer, and protein was precipitated by addition of an equal volume of redistilled, buffered phenol. Potassium acetate (pH 5.2) was added to a final concentration of 3%, and nucleic acids were precipitated

with three volumes of absolute ethanol. After standing for 12 h at $-20°C$, the nucleic acids were pelleted, dried, and applied to a DEAE-cellulose column in the Tris buffer at 0.3 M NaCl. Unbound A_{260} absorbing material was removed by washing with the loading buffer, and RNA (approximately 500 A_{260} units) was eluted with Tris buffer at a NaCl concentration of 1 M. RNA was precipitated as before, redissolved in 0.01 M $NaC_2H_3O_2$ buffer (pH 5.0) containing 0.75 M NaCl and 1% (vol/vol) methanol, and 120 A_{260} units were applied to a column (1.0 × 120 cm) of Sephadex G-100 (Pharmacia Fine Chemicals, Piscataway, N. J.). The RNA fractions obtained from the column were precipitated and redissolved in deionized water.

Analytical Gel Electrophoresis

Acrylamide gel electrophoresis methods of Loening (28) were used to analyze the fractions from the Sephadex G-100 column. Gels of 0.5 cm diameter and 7.5 cm length were prepared. For analysis of fraction I, 2.4% acrylamide was used, while 9% gels were used for analysis of fractions II and III. All tubes were prerun at 5 mA per tube before loading samples to allow sodium dodecyl sulfate (SDS) to enter the gels. After adding samples of approximately 50 μg, the current was reduced to 2 mA per tube for 1.5 h and then increased to 4 mA for 1 h. Gels were stained in acridine orange or pyronine Y (29). When ^{125}I-RNA was analyzed, gels were stained and destained as required, and then frozen, sliced, and counted in a Gamma spectrometer (Picker Nuclear, White Plains, N. Y.).

Iodination of RNA

RNA was iodinated with carrier-free ^{125}I (New England Nuclear, Boston, Mass.) following the procedure described by Prensky et al. (39). Immediately after iodination of the 4S and 5S RNA fractions the entire reaction mixture containing 5 μg of RNA was applied to a small DEAE-cellulose column (0.7 × 10 cm) equilibrated with a solution of 0.01 M $NaC_2H_3O_2$ (pH 5.0), 0.01 M $Mg(C_2H_3O_2)_2$, and 0.3 M NaCl. The resin was washed with 25 ml of this salt solution and RNA was eluted by increasing the NaCl concentration to 1 M. Unstably bound iodine was removed from the RNA by bringing the pH of the solution to 9.5 with 1.0 M $NH_4C_2H_3O_2$ and 0.5 M NH_4OH and incubating for 20 min at 60°C (7, 21). After this incubation, $Mg(C_2H_3O_2)_2$ was added to a final concentration of 0.01 M and RNA was precipitated in a siliconized tube with 3 vol of ethanol. After standing overnight at $-20°C$, RNA was pelleted by spinning at 8,000 g for 45 min in a swinging bucket rotor. The dried pellet was dissolved in 0.5 ml of 0.01 M NaCl and applied to a Sephadex G-25 column (1.0 × 27 cm). Labeled RNA emerging in the void volume was precipitated as above and dissolved in the hybridization mixture.

Large ribosomal RNA was treated in the same way except that free iodine was removed on a G-50 Sephadex

column. In each case the specific activity of the final product was estimated by counting 5-μl samples in a liquid scintillation spectrometer at an efficiency of 65%. Specific activities of the iodinated RNA fractions used in the hybridization experiments were estimated as 1.59 $\times$ 10^7 dpm/μg, 2.68 $\times$ 10^6 dpm/μg and 7.77 $\times$ 10^6 dpm/μg for 4S RNA, 5S RNA, and 18 + 28S RNA, respectively.

In Situ Hybridization

The hybridization reaction was carried out according to the procedure of Gall and Pardue (16) as modified by Wimber and Steffensen (49). Salivary glands were squashed in 45% acetic acid, fixed in ethanol:acetic acid (3:1 vol/vol), treated with pancreatic RNase, and then denatured with formamide. 20 μl of the iodinated RNA dissolved in 2X SSC (pH 7.2) and 50% formamide was applied to each slide. Except where otherwise indicated, 7.0 A_{260} units of unlabeled yeast 4S RNA, purchased from Sigma Chemical Co. (St. Louis, Mo.) and further purified on benzoylated DEAE-cellulose, were included in the reaction mixture to reduce background label (33). Hybridization was allowed to proceed for 4 h at 40°C, and then the slides were extensively washed with 2X SSC, treated with pancreatic RNase and dipped in NTB-2 liquid emulsion diluted 1:1 with distilled water. Emulsions were exposed for the periods indicated in the figure legends, and the preparations were developed and stained with Giemsa dye.

RESULTS

Cytology

In the salivary glands of G. barbipes there are four polytene chromosomes, three large metacentrics, and one telocentric, each with a very conspicuous knob of centromeric heterochromatin (4, 5, 47). The chromosomes of the salivary glands do not associate into a chromocenter although the centromeres and telomeres of two, three, or all of the chromosomes are often ectopically paired (4, 5, 47). The position of the nucleoli, Balbiani rings, and centromeres, as well as the arm ratio and other salient features of these chromosomes conform closely to those of the European species of G. barbipes described by Walter (47). The nucleolus organizers are located on chromosome I (D-2), chromosome II (C-2), and chromosome III (A-2). Large regions of centromeric heterochromatin are found on chromosome I (C-3), chromosome II (C-3), chromosome III (C-1), and chromosome IV (D-2). Chromosome IV has two Balbiani rings at A-2 and B-1, of which the latter (3) or both (4) have been previously denoted as nucleolus organizer regions.

In the population we have studied there are two chromosomal arms, IR and IIL, that show polymorphism for rearrangements, probably inversions. The position of the rearrangements on chromosomes I and II corresponds with the location of those described by Basrur (4) for this same species from Ontario, Canada. Basrur also described a complex rearrangement of the included type in chromosome IIIR involving a band located beside the centromere and several bands in the tip of the same arm. The population we studied exhibits the condition described by Basrur (p. 603 of reference 4, Fig. 7 a) for the inversion homozygote of chromosome IIIR.

Chromatography

Chromatography of the RNA from G. barbipes on Sephadex G-100 resolved three distinct fractions (Fig. 1), an 18 + 28S (peak 1), a 5S (peak 2), and a 4S fraction (peak 3) according to Monier and Feunteun (32). The mobility of RNA from each peak was assayed on acrylamide gels in comparison with appropriate standards. RNA from G-100 peak 1 and reticulocyte 18 + 28S RNA showed the same mobility on 2.4% gels and separated into two distinct bands. RNA from peaks 2 and 3 showed mobilities on 9% gels indistinguishable from D. melanogaster (Fig. 2). It is evident, however, that the 5S preparation contained some material having the same mobility as 4S RNA (Fig. 2 B).

Localization of Genes for 18 + 28S RNA

Iodinated 28 + 18S ribosomal RNA (rRNA) hybridized exclusively to three of the puffs in the polytene chromosomes of G. barbipes (Fig. 3). Chromosome IR showed label over two consecutive bands in a puffed region at D-2 which is associated with a nucleolus. Unusually good cyto-
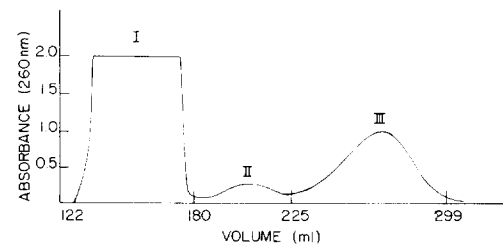

FIGURE 1 Chromatography of G. barbipes RNA on Sephadex G-100. Center portions of peaks were collected for analysis by gel electrophoresis and iodination.
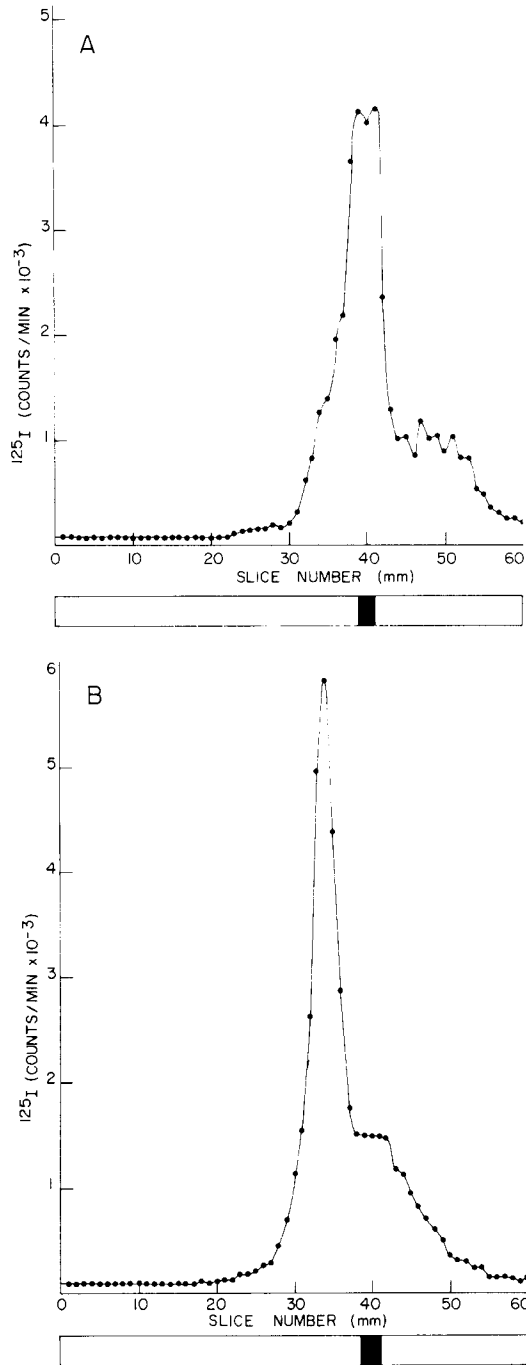
FIGURE 2 Coelectrophoresis of iodinated *Glyptoten-dipes* 5S RNA with: (A) unlabeled *Glyptotendipes* 5S RNA and (B) *Drosophila* 4S RNA. The gels, which are represented under each radioactivity profile, were stained with acridine orange and the position of the band was determined before they were sliced.

logical preservation of hybrids exposed for 67 h afforded clear resolution of the label over two bands of this chromosome (Fig. 4). Chromosome IIL was heavily labeled at C-2, but because of the extreme state of puffing we were unable to determine the disposition of the silver grains in relation to bands and interbands.

We have also observed interchromosomal connections bridging the two rRNA sites on chromosomes IR and IIL in certain of our preparations (Fig. 5). Label over these connections demonstrates the presence here of DNA complementary to 18 + 28S rRNA.

The third 18 + 28S RNA hybridization site is located over a band on chromosome IIIL (A-2) as shown in Fig. 3. This site is commonly less densely labeled than those on chromosomes IR and IIL.

Even after long exposures (30 days) the iodinated 18 + 28S chironomid RNA labeled only the three sites in chromosomes I, II, and III. In one control preparation we challenged salivary gland chromosome preparations from *G. barbipes* with iodinated 18 + 28S RNA prepared from lily callus tissue (R. White and W. Wen, unpublished observation). This RNA preparation also specifically labeled the same three sites on the chironomid chromosomes. When polytene chromosomes of *D. melanogaster* were hybridized with *G. barbipes* 18 + 28S RNA only the nucleolar organizer region became labeled.

Localization of 5S RNA Gene Sequences

Iodinated 5S RNA clearly labeled several sites in the polytene chromosomes of *G. barbipes* after 67 h exposure (Fig. 6). Three of these sites correspond to those labeled by the 18 + 28S RNA, and label on these three puffs is completely abolished when unlabeled 18 + 28S RNA is included in the 5S hybridization mixture. The most strongly labeled site after 5S hybridization is located on the seventh band from the centromere on chromosome IIIR (C-3). This locus is still evident after 700-fold competition with cold 18 + 28S RNA.

After longer exposures, five sites, two on chromosome IIL (E-2 and F-3), two on chromosome IIIR (C-3 and D-3), and one on chromosome IV (Balbiani ring, B-1) were labeled in addition to the main 5S band on chromosome IIIR (Figs. 7, 8, 9). None of these sites is labeled by our 4S or 18 + 28S RNA preparation after equivalent exposure periods (Fig. 10). We conclude that these labeled

FIGURE 3   Autoradiograph of *Glyptotendipes* salivary gland chromosomes hybridized with iodinated 18 + 28S RNA. Three sites are labeled: chromosome IR at D-2 (arrow no. 1), chromosome IIL at C-2 (arrow no. 2) and chromosome IIIL at A-2 (arrow no. 3). Exposure time 107 h. Scale: 20 µm. × 1,250.
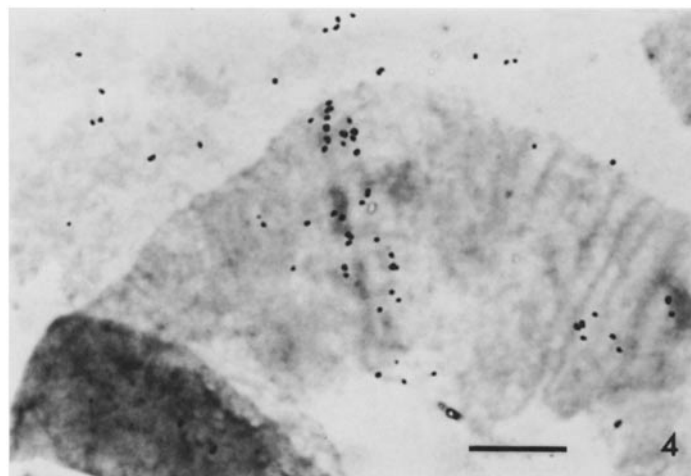

FIGURE 4   Autoradiograph of chromosome IR showing two consecutive bands labeled with iodinated 18 + 28S RNA. Exposure time 67 h. Scale: 10 µm. × 3,750.
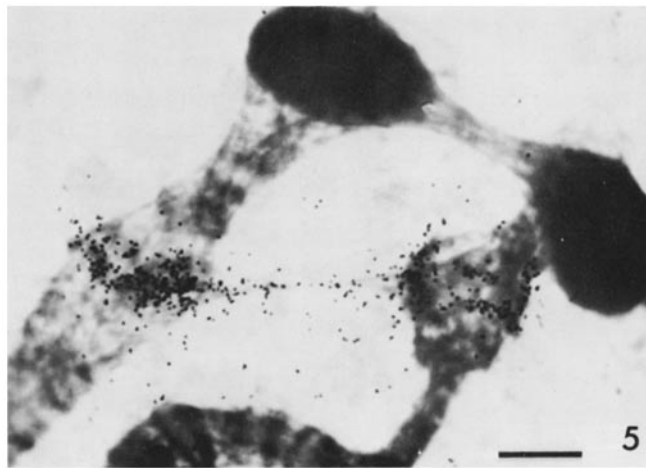
FIGURE 5 Autoradiograph of a chromatin bridge hybridized with iodinated 18 + 28S RNA between the nucleolus organizers on chromosomes IR (left) and IIL (right). The centromeres of these two chromosomes are ectopically paired. Exposure time 17 days. Scale: 10 μm. × 3,200.
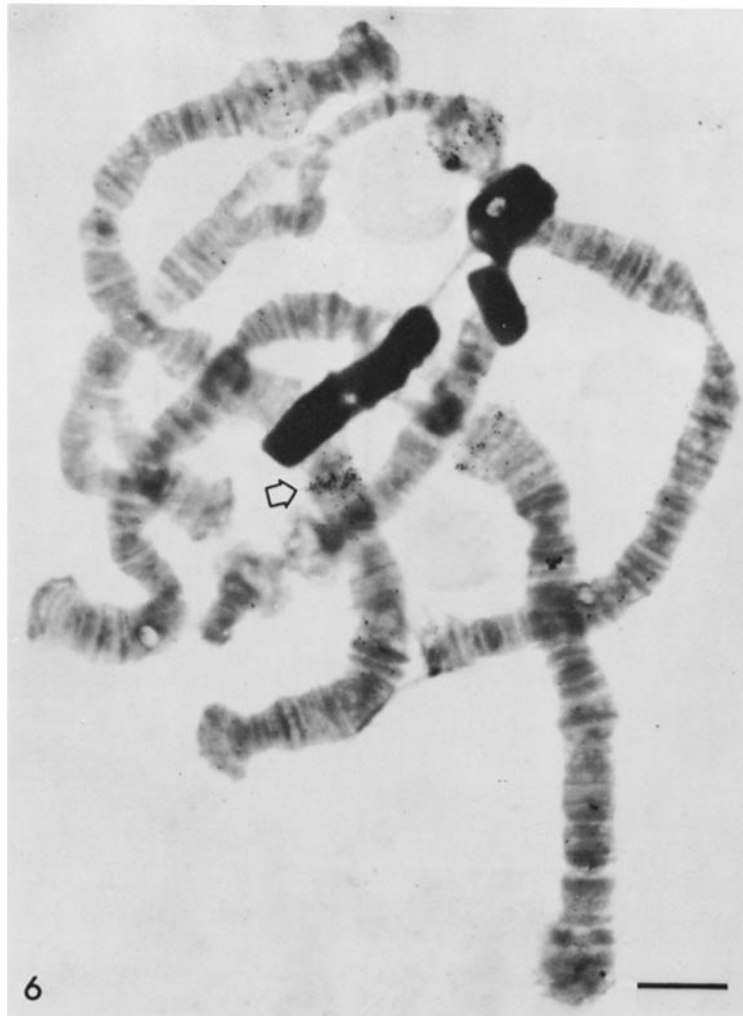

FIGURE 6 Autoradiograph of *Glyptotendipes* chromosomes hybridized with iodinated 5S RNA after 67 h exposure. Most of the label is localized over a band seven bands away from the centromere at C-2 on chromosome IIIR (arrow). The nucleolus organizers are lightly labeled. Scale: 20 μm. × 1,125.
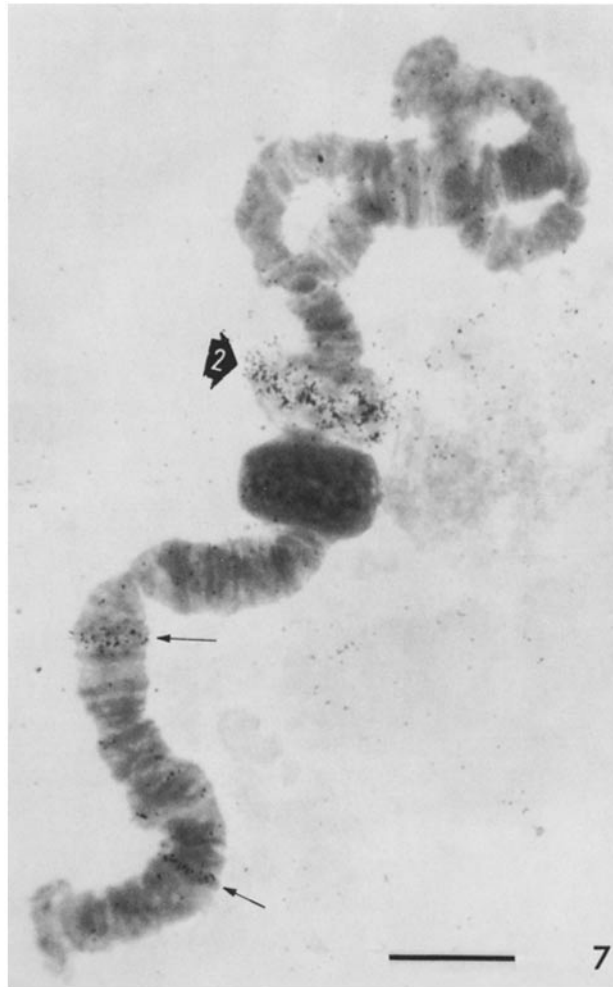
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FIGURE 7 Autoradiograph of chromosome number two of *Glyptotendipes* after 17 days exposure to the
hybrid formed with iodinated 5S RNA. The nucleolus organizer (arrow no. 2) is heavily labeled; in addition
two other sites (thin arrows) on E-2 and F-3 are also distinctly labeled. These other sites are interpreted to
represent additional loci for the small ribosomal gene. Scale: 20 μm. × 2,000.

loci also represent genes coding for 5S RNA
although the appreciably smaller number of silver
grains over these sites (relative to the main 5S site)
suggests that the DNA sequences here are less
repeated. Alternatively, these labeled loci could
represent DNA sequences homologous to con-
taminating RNA present in our 5S RNA prepara-
tion and unresolvable by our fractionation proce-
dures.

Hybridization of *G. barbipes* 5S RNA to *D.
malanogaster* chromosomes produces labeled loci
limited to the 5S site (region 56 E–F of the right

arm of chromosome II) and the nucleolar orga-
nizer region (Fig. 11).

4S RNA Hybridization Studies

Hybridization of *G. barbipes* chromosomes with
homologous 4S RNA causes no clearly labeled
regions to appear. After 17 days of exposure, label
that appears to be above background levels is
spread over large regions of the chromosomes
(Fig. 10). These results are consistent with the
notion that the 4S genes are neither highly re-
peated nor clustered at a few sites in the chromo-

somes of this organism. The possibility remains that our conditions of annealing 4S RNA did not allow sufficient hybridization to obtain detectable label.

DISCUSSION

Our results indicate that there are three nucleolus organizing sites in the chironomid *G. barbipes* complementary to 18 + 28S rRNA, one main site complementary to 5S RNA, and five subsidiary sites where, possibly, 5S RNA sequences are located.

A number of conclusions drawn from our results depend on the specificity of the iodinated reagents. Several criteria of specificity were used. Gel electrophoresis confirmed our interpretation of the identity of the three peaks obtained from G-100 chromatography. We observed that the mobility of the 5S RNA was not noticeably affected by incorporation of ^{125}I into the molecules, in accordance with Getz et al. (18). Electrophoresis did indicate that the iodinated 5S RNA was contaminated with molecules having the same mobility as

4S RNA. Results of hybridization of *D. melanogaster* chromosomes with 5S RNA demonstrated that the 5S material also contained fragments of large rRNA in agreement with the results of Wimber and Steffensen (45, 49). However, competition experiments permitted us to interpret our results despite the presence of these contaminants.

The striking localization of label over chromosome IIIR (C-2) hybridized with 5S RNA clearly indicates the presence of one main locus complementary to 5S RNA. Additional sites that appear on longer exposure may represent less redundant sequences of this gene. None of these loci is labeled by our 4S or 18 + 28S RNA preparations.

The possibility of 5S RNA sequences at more than one locus is of interest in comparison to work previously published on localization of 5S RNA genes in other organisms. Wimber and Steffensen (49) using tritiated 5S RNA prepared in vivo and Prensky et al. (39) using ^{125}I-5S RNA have found only one locus for 5S RNA in *D. melanogaster*. On the other hand, Attardi and coworkers (1, 3, 19) and Pardue et al. (33) have found multiple
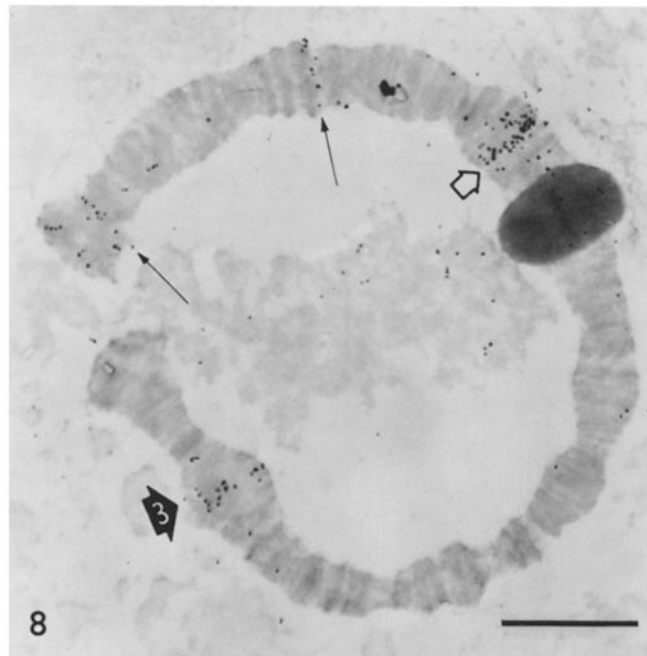

FIGURE 8   Chromosome III hybridized with 5S RNA and exposed to the emulsion for 107 h. The main site which hybridizes to 5S RNA is indicated by an open arrow. Two additional sites on regions C-3 and D-3 are indicated by thin arrows. The nucleolus organizer on IIIL, indicated by arrow no. 3 is also labeled by the 5S RNA preparation. × 1,500.
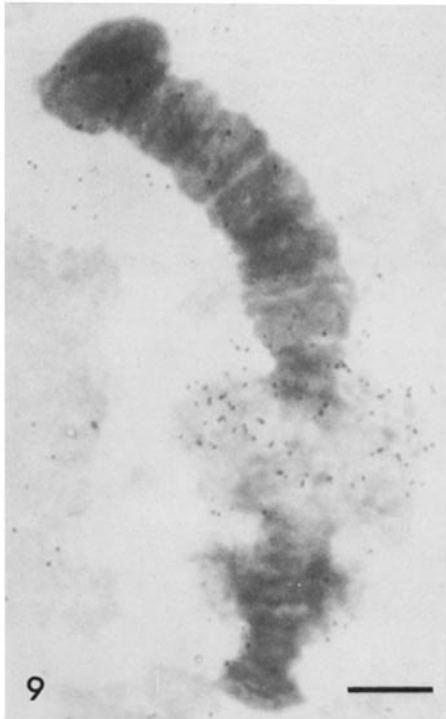
FIGURE 9 Hybridization of iodinated 5S RNA to chromosome IV of *Glyptotendipes*. After 17 days exposure, diffuse label is observed over a Balbiani ring at B-1. Scale: 10 μm. × 2,500.

sites coding for 5S RNA in HeLa cell and *Xenopus laevis* chromosomes, respectively. Experiments are in progress to determine if the several sites sparsely labeled by our 5S RNA are truly homologous to 5S rRNA or represent sites homologous to peculiar contaminants in the 5S RNA preparation.

Our results demonstrate the presence of three nucleolus organizers in *G. barbipes* associated with chromosomes I, II, and III. Hybridization experiments indicate absence of a nucleolus organizer in chromosome IV contrary to the suggestion made on the basis of cytological observation (4, 5). The organizer sites are located near centromeric or telomeric heterochromatin in accordance with the situation found in many different organisms (51, 52). When chromosomes I and II are united at their centromeres (Fig. 3), the two large ribosomal sites are juxtaposed. The main 5S site and the nucleolus organizers are also closely apposed when the centromeric heterochromatin of chromosome

III fuses with that of chromosome I or II (Fig. 6). It is uncertain whether the localization of the ribosomal DNA sites (18 + 28S and 5S), which gives rise to their apposition when centromeric fusion occurs, is the result of selection acting on chromosomal architecture. Steffensen and Wimber (45) and Holmquist and Steffensen (23) have found occasional attachment of the 5S locus and the nucleolus in *D. melanogaster* and have suggested that such an arrangement may facilitate transport of 5S RNA to the nucleolus. Amaldi and Buongiorno-Nardelli (2) have reported that in Chinese hamster cells 5S RNA hybridizes to the nucleolus periphery. Pardue et al. (33) also have evidence of association between the nucleolus and the 5S locus in chromosomes of *X. laevis* gonial cells. If the apposition of rDNA sites has the functional significance referred to above (45) one might expect simultaneous synthesis of 5S and 18 + 28S RNA. Synthesis starts simultaneously after gastrulation in *X. laevis* (22). However, other evidence indicates nonsimultaneous synthesis of 5S and 18 + 28S RNA (3, 13, 31, 38, 48). Indeed, even if coordinate synthesis of 5S and 18 + 28S RNA does occur, apposition may not be functional for 5S RNA may first enter the cytoplasm and then return to the nucleolus for ribosome assembly (27). It remains unclear, therefore, in *Glyptotendipes* as well as in other organisms that apposition of rRNA transcription sites is significant in the assembling of 5S and 18 + 28S RNA.

The strong puffing activity we find in the nucleolus organizers contrasts sharply with the compact bands of the main 5S site. However, the Balbiani ring on chromosome IV hybridizes with 5S RNA, and it is possible that this locus is active in the fourth instar larvae. Since the state of puffing, per se, is not a rigorous indication of synthetic activity (47), it is impossible to draw definite conclusions from these observations. Incubation of salivary glands with tritiated uridine labels sites of RNA synthesis on the chromosomes, and experiments are in progress to attempt to determine the time of transcription of the putative 5S loci as well as the temporal relationship of this activity to that of the large rRNA genes.

The nucleolus organizer sites in *G. barbipes* differ from those of certain dipterans that possess organizers with stretched out, ramifying chromatin, originating in a band but separate from it (17, 35, 36). In *G. barbipes*, the rDNA associated with

the puffs remains over the chromosome as in other chironomids (37). A remarkable feature of the nucleolus organizers in *G. barbipes* is the localization of the 18 + 28S rDNA in chromosome IR over two consecutive small bands (Fig. 4). The consistency of this observation suggests that this is a common feature of the population we have studied. In contrast, the nucleolar organizer on chromosome III shows label over one band. We would like to point out that the resolution afforded by some of our preparations of the nucleolus

organizer on chromosome I (Fig. 4) supports the notion that transcribed genes are present in bands. It cannot be concluded that the interband in this site is devoid of rDNA sequences, but we can conclude that the band is not wholly devoted to control function.

The occurrence of a clearly labeled inter-chromosomal connection between two of the nucleolar organizers (Fig. 5) demonstrates the existence of rDNA in this chromatin homologous to the two sites connected. A similar observation has

FIGURE 10   Autoradiograph of *Glyptotendipes* chromosomes hybridized with iodinated 4S RNA and competed with "cold" 18 + 28S RNA. 15 days exposure. Label over the chromosomes is above background, but is not clearly localized. Scale: 20 μm. $\times$ 950.

FIGURE 11   Autoradiograph of *D. melanogaster* salivary gland chromosomes hybridized with iodinated 5S RNA from *Glyptotendipes*. Region 56 E-F is labeled (arrow) and also the nucleolus beside it. Exposure time was 107 h. Scale: 10 μm. × 4,300.

been made by Henderson et al. (20) for the human chromosomes carrying the nucleolus organizers. Judging from the amount of label in our preparations, we believe that many strands of DNA have been hybridized with the iodinated 18 + 28S RNA. The occurrence of ectopic pairing among dipteran polytene chromosomes is well documented (reviewed in 25) and, because of the compact nature of the nucleolus organizers in *Glyptotendipes*, we do not believe that this connection is an artifact. How can separate bands in polytene chromosomes become connected by multiple DNA strands? We suggest that a crossover event between the two sites during DNA synthesis leading to polytenization could give rise to multistranded, interchromosomal connection such as we have seen in this chironomid. Such an event would be favored if the two sites are spatially related, and, as most crossing over models require, if the two loci contain complementary nucleotide sequences. However, if the interchromosomal connections of human metaphase chromosomes (20)

are formed in a manner analogous to those we observe in the dipteran, this explanation does not apply directly. Other possible ways to account for these chromosomal associations have been discussed by DuPraw (11).

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