Volume 10 Number 23 1982 Nucleic Acids Research

Spacer size heterogeneity in ribosomal DNA of *Chironomus thummi* is due to a 120 bp repeat homologous to a predominantly centromeric repeated sequence

N.Israelewski and E.R.Schmidt

Lehrstuhl für Genetik, Ruhr-Universität Bochum, D-4630 Bochum, FRG

Received 2 September 1982; Revised and Accepted 3 November 1982

ABSTRACT

The rDNA of Ch. tepperi is homogeneous in structure with a repeat size of 8.4 kb. This size seems to be typical for the basic repeat unit in Chironomus species. Ch. th. piger rDNA cistrons are slightly increased in length (9.0 kb). In the non-transcribed spacer (NTS) an appr. 0.18 kb segment is additionally present in about 50 % of the repeats. Ch. th. thummi DNA contains largely heterogeneous rDNA repeats, mainly between 10 and 16 kb. The heterogeneity is due to varying numbers of 120 bp elements present in the NTS. The different spacer size classes are not randomly distributed. The short repetitive 120 bp elements (Cla I elements) hybridize in situ with the nucleolus and with centromere regions. The Cla I elements are regularly present in the thummi NTS, but are absent in the piger NTS. Only very few piger rDNA cistrons may contain Cla I elements.

INTRODUCTION

In our present investigation we have analyzed the rDNA structure of the subspecies Chironomus thummi thummi and Ch. th. piger. In Ch. th. thummi and in Ch. melanotus, another Chironomid of the thummi subgroup (1), the rDNA was shown to be extremely heterogeneous in length. It was proposed that a relationship between centromeric heterochromatin and rDNA heterogeneity exists (2). Ch. th. thummi has considerably more centromeric heterochromatin than Ch. th. piger. This difference coincides with a significant difference in genome size visible as small duplications predominantly in the centromere regions (3). The duplication events in Ch. th. thummi also indicate that Ch. th. piger is the phylogenetically older species (4). The difference in heterochromatin content was also confirmed by application of the C-banding method (5) corresponding to a six-fold increase of the amount of repetitive sequences in the Ch. th. thummi DNA relative to Ch. th. piger (6). Most of this repetitive DNA is AT rich ($8o^{o}$(th)DNA) and hybridizes in situ to those bands of thummi polytene chromosomes which are known to be the sites of duplication events (7). The $8o^{o}$(th)DNA fraction (~ 10 % of the total DNA)

© IRL Press Limited, Oxford, England.

0305-1048/82/1023-7689 $2.00/0

contains appr. 30 % highly repetitive DNA sequences, characterized by a Cla I restriction site and a basic repeat size of appr. 120 bp (8).

The question concerning the relationship between heterochromatin content and structure of rDNA (2), and the correlation of increasing amount of repetitive sequences and the evolutionary growth of genome size (7, 8) prompted us to analyse the Ch. thummi rDNA in detail. The results were compared with those of Ch. tepperi (for cytology see: 9). Previous unpublished results indicated that the rDNA of Ch. tepperi can be included as a standard in the investigation, although Ch. thummi and Ch. tepperi are only distantly related. The results supplement earlier cytogenetic and biochemical studies on the nucleolar organizer regions in polytene chromosomes of European and Australian Chironomus species (10 - 15).

MATERIALS AND METHODS

Animals

Larvae of laboratory stocks of Ch. tepperi, Ch. th. thummi and Ch. th. piger were reared as described (16). Prior to DNA isolation, last instar larvae were selected and kept at 8^{o} C in tap water for 2 - 3 d.

DNA Isolation

The method employed is essentially that described by Schmidt et al.(7).

RNA Isolation

RNA was isolated (17) from ovaries of Ephestia kuehniella. Ovaries were collected and washed in ice-cold buffer (0.15 M NaCl, 0.01 M EDTA, 0.05 M Hepes, pH 7.5). The ovaries were recovered by centrifugation (500 g, 0^{o} C), weighed and resuspended in 10 ml buffer containing 0.5 % (v/v) Nonidet P40. Cells were ruptured in a glass-teflon Potter Elvehjem homogenizer by several strokes and the nuclei were removed (900 g, 0^{o} C, 5 min). To the 10 ml supernatant fraction 0.01 g SDS and 14 g solid CsCl were added and dissolved. 0.2 ul Diethylpyrocarbonate were added per mg of ovaries. After centrifugation (5000 g, 15^{o} C, 45 min) the RNA containing CsCl solution was filtered through GF/C filters (Whatman) and precipitated with ethanol. The RNA was dissolved in 0.15 M Na-acetate, 0.5 % SDS, pH 6.0 (CH_3COOH) and size selected on a 5 - 25 % (w/v) sucrose gradient.

RNA or DNA Iodination

18S or 28S RNA or AT rich 80^{o}(th)DNA (7) were in vitro iodinated (18, 19) with the modifications as described (7). Specific activity was 5 - 10 x 10^{6} cpm/ug.

Nick-Translation

The cloned Ch. tentans rDNA repeat pCte 111 (2)(kindly provided by A. Degelmann and C. P. Hollenberg) was nick-translated (20) using ^{32}P-dATP.

DNA Restriction and Gel Electrophoresis

Enzyme incubations and agarose gel electrophoresis were performed as described (8, 21).

Filter Hybridization

Restriction fragments were transferred to nitrocellulose filters (22). The DNA was hybridized with ^{125}I-rRNA in 50% formamide, 5 x SSC, 0.1% SDS at 37^{o} C for 12 - 16 h. For hybridization with ^{32}P nick-translated pCte 111 DNA the filters were pre-incubated 6h at 60^{o} C in Denhardt's solution (23) with the addition of 0.1 % SDS. Hybridization was carried out in Denhardt's solution for 16 h at 60^{o} C. The filters were washed in 2 x SSC at 50^{o} C for one hour and for an additional hour at 20^{o} C, autoradiographed using Kodak XAR Xray film and Dupon Cronex high plus intensifying screens. Exposure time varied from 1 - 10 d.

In Situ Hybridization

Hybridization of 80^{o}(th)DNA (7) or Cla I element (8) to salivary gland chromosomes was performed as described (7) following the method of Singh et al. (24) and pretreatment of the slides (25). DNA was labelled radioactively by nick-translation (20) or iodination (7).

RESULTS

For comparison with the following results, the restriction map of a cloned rDNA cistron of Ch. th. thummi is shown in figure 1.

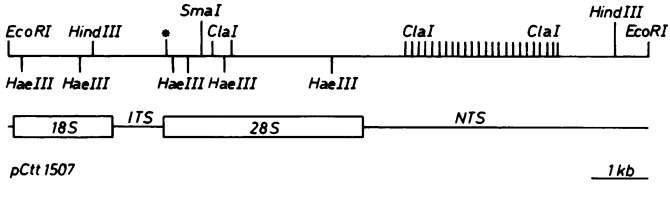

Fig. 1 Restriction map of a cloned Ch. th. thummi rDNA cistron (pCtt 15o7) as elaborated by Schmidt et al. (in press). The asterix indicates a Cla I site that is present in most of the genomic rDNA cistrons, but is absent in clone pCtt 15o7. The NTS contains a repetitive DNA sequence characterized by a Cla I restriction site and a repeat length of ca. 12o bp. Approximately 22 Cla I elements are present in this particular clone. These Cla I elements hybridize with a previously characterized 12o bp sequence which is a member of a predominantly centromeric repeat sequence (8).

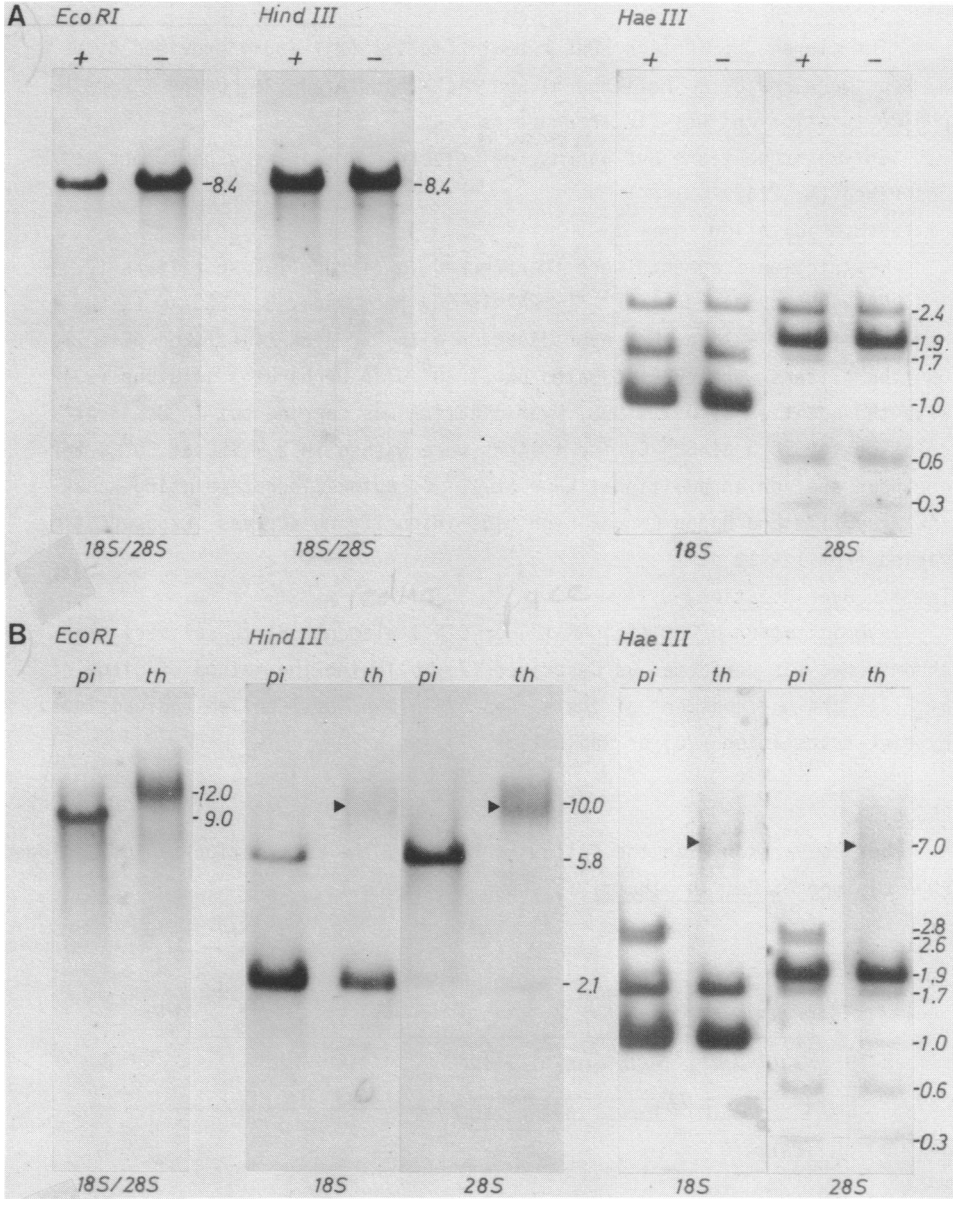

Fig. 2 Restriction pattern of DNA of Ch. tepperi, Ch. th. piger and Ch. th. thummi after digestion with Eco RI, Hind III and Hae III. Restriction fragments were separated on 1 % agarose gels in Tris-phosphate buffer. The hybridization probe used (18s/28s; 18s, 28s) is indicated at the bottom of the lanes. (a) Ch. tepperi, +stock and -stock. The 8.4 kb Eco RI and Hind III fragments represent the cistron length. The Hae III restriction

pattern does not reveal differences between the two tepperi stocks. The 1.7 kb Hae III fragment contains the ITS sequence, the 2.4 kb fragment the complete NTS region. (b) Ch. th. piger (pi) and Ch. th. thummi (th). The Eco RI fragment represents the cistron length of 9.0 kb in piger. In thummi, the Eco RI fragment is heterogeneous in size leading to a "smear" around 12 kb. After Hind III and Hae III digestion, the length heterogeneity is confined to only one fragment containing the NTS region (arrow). The corresponding piger fragments (5.8 kb - Hind III and 2.6 - 2.8 - Hae III) do not display similar extended length heterogeneity.

In Ch. th. thummi and Ch. th. piger the rRNA genes are clustered at a single site on chromosome IV. In Ch. tepperi + stock, two nucleoli are present on chromosomes I and IV, whereas the - stock lacks the rDNA cistrons in chr. IV.

The rDNA cistrons of the + and - stock of tepperi seem to be identical with regard to the restriction patterns (Fig. 2 a). The Eco RI digestion generates one band at 8.4 kb. Hind III produces a fragment of the same size, while Hae III cuts the rDNA into 6 fragments. There is no obvious length heterogeneity in any of the fragments. Except for the 2.4 kb fragment, the lengths of the Hae III fragments and their specifity of hybridization for 18S and 28S rRNA correspond to those fragments of cloned Ch. th. thummi rDNA which are located in the transcribed portion of the cistron (comp. Fig. 1). Thus the 2.4 kb Hae III fragment of tepperi includes the complete NTS region and, correspondingly, hybridizes weakly with both 18S and 28S rRNA.

The digestion of Ch. th. piger DNA with Eco RI, Hind III and Hae III (pi - lanes in Fig. 2 b) revealed some differences between tepperi and piger. Eco RI generates one fragment of cistron length (9.0 kb). Hind III produces two fragments, one (5.8 kb) hybridizing mainly with 28S and the other one (2.1 kb) with 18S rRNA. The sum of the two fragments is only 7.9 kb, so that a third Hind III fragment must be postulated which is not transcribed and which contains NTS sequences. The Hae III restriction pattern of piger rDNA is very similar to the one in tepperi, except that the fragment carrying the NTS region is slightly longer in piger and exists as two variants, i.e. one 2.6 and one 2.8 kb.

In Ch. th. thummi the positions of the restriction sites for Eco RI, Hind III and Hae III (th - lanes in Fig. 2 b) are in principle the same as in Ch. th. piger. However, the rDNA cistrons are significantly longer and very heterogeneous in length: Eco RI produces a "smear" ranging from appr. 10 to 16 kb. Hind III generates length-heterogeneous fragments (8 - 14 kb) hybridizing mainly with 28S rRNA. By comparison with figure 1 these fragments contain the NTS region.The 2.1 kb fragment is the same as the one in piger and it contains 18S sequences. The Hae III pattern of thummi rDNA reveals that the heterogeneous

fragments (5 to 10 kb) contain mainly the NTS region because they hybridize only weakly with 18S or 28S rRNA and the remaining five Hae III fragments do not differ in length nor in specifity of hybridization between thummi and piger.

Southern hybridization of the digested DNA of thummi and piger with cloned rDNA of Ch. tentans (containing also the NTS sequence) gives much stronger signals (Fig. 3). In overexposed autoradiographs (Eco RI and Hind III lanes) a minor heterogeneous population of rDNA fragments is also seen in the piger DNA. However, most of these fragments are smaller than one cistron length (Eco RI digest) and only few are longer. Variants of cistrons with smaller sizes are also visible in thummi DNA.In gels more suitable to separate large fragments (Hae III digest) the two NTS containing fragments of piger, 2.6 and 2.8 kb,

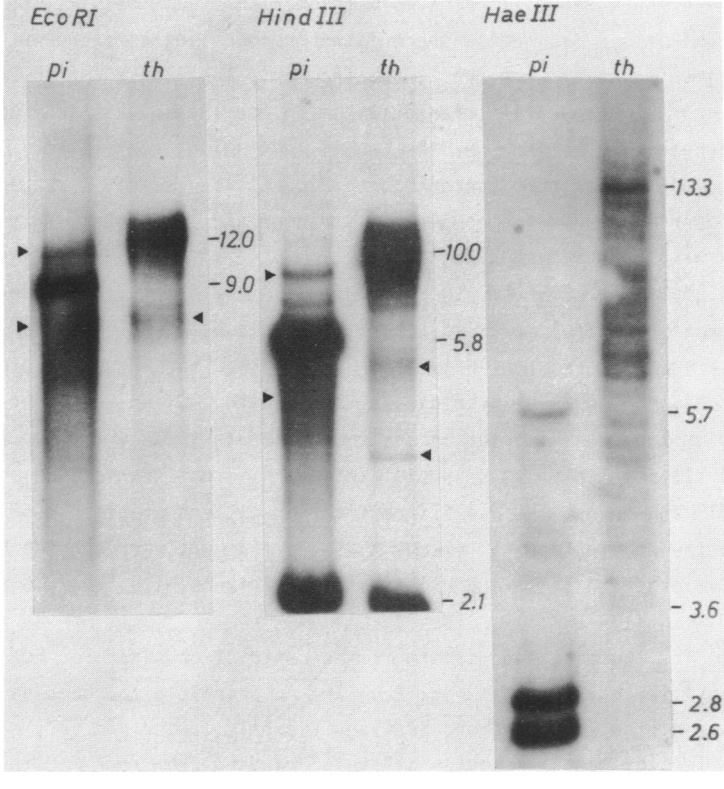

Fig. 3 Southern hybridization of restricted piger and thummi DNA with cloned tentans rDNA (pCte 111). Autoradiographs are overexposed in order to show minor fractions of rDNA (arrow). Hae III restricted DNA (right panel, o.8 % agarose gels) was used in order to show the fragments containing the NTS region of piger and thummi at higher resolution.

are clearly separated and they hybridize with appr. equal intensity (the size difference netween the two fragments has been determined in additional blots to be appr. 0.18 kb). The origin of the faintly hybridizing piger fragments at higher molecular weights is unknown. In the Hae III digestion of thummi DNA it is demonstrated that the'smear' can be resolved into a large number of bands, ranging in size between appr. 3 to 13 kb or longer. The length difference from one band to the next is appr. 0.1 - 0.15 kb. However, as seen in the region 3 to 6 kb some bands, i.e. NTS size classes, are underrepresented.

On Cla I digestion and Southern hybridization with 18S or 28S rRNA the heterogeneity in the thummi rDNA is no longer demonstrable (Fig. 4 a). Due to the Cla I sites in the thummi NTS fragments of 3.o and 4.5 kb are produced which are present only in very small amounts in the piger rDNA. Nearly all of the piger rDNA cistrons lack Cla I sites in the NTS leading

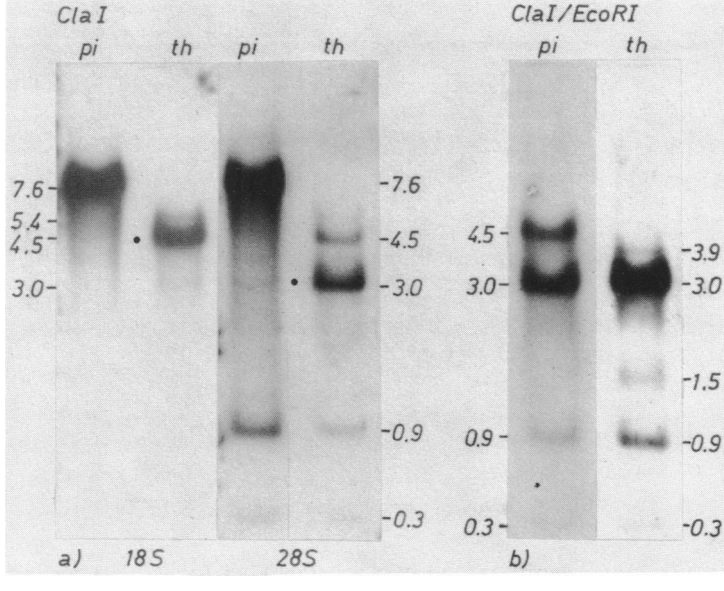

Fig. 4 (a) Cla I digestion pattern of DNA of Ch. th. piger (pi) and Ch. th. thummi (th) after hybridization with 18s or 28s rRNA, as indicated at the bottom of the lanes. The heterogeneity of thummi rDNA is no longer visible. Cla I sites in the NTS of thummi lead to the 4.5 and 3.o kb fragments (•), whereas the main fraction of piger rDNA has no Cla I site in the NTS. (b) Cla I - Eco RI double digest of piger and thummi rDNA probed with pCte 111 tentans rDNA. The 1.5 kb Cla I - Eco RI fragment in thummi adjoins the Cla I elements in the NTS. The faint band at 3.9 kb is due to variants of thummi rDNA cistrons lacking a Cla I restriction site (comp. Fig. 1).

to the 7.6 kb fragment. The 28S specific o.3 and o.9 kb fragments are the same in piger and thummi. In addition, a faintly hybridizing 5.4 kb Cla I fragment in thummi is observed. This represents a rare modification (absence) of a Cla I site at the left of 28S (comp. Fig. 1). The sum of the thummi Cla I fragments give only 8.7 kb, thus from 1 to 7 kb are missing when compared to the 1o to 16 kb cistron length (Eco RI digest, Fig. 2 b). This is due to digestion of a part of the NTS into 12o bp Cla I elements (comp. Fig. 1).

The position of the Cla I elements in thummi rDNA was mapped by double digestion probed with the tentans rDNA clone (Fig. 4 b). The 4.5 kb

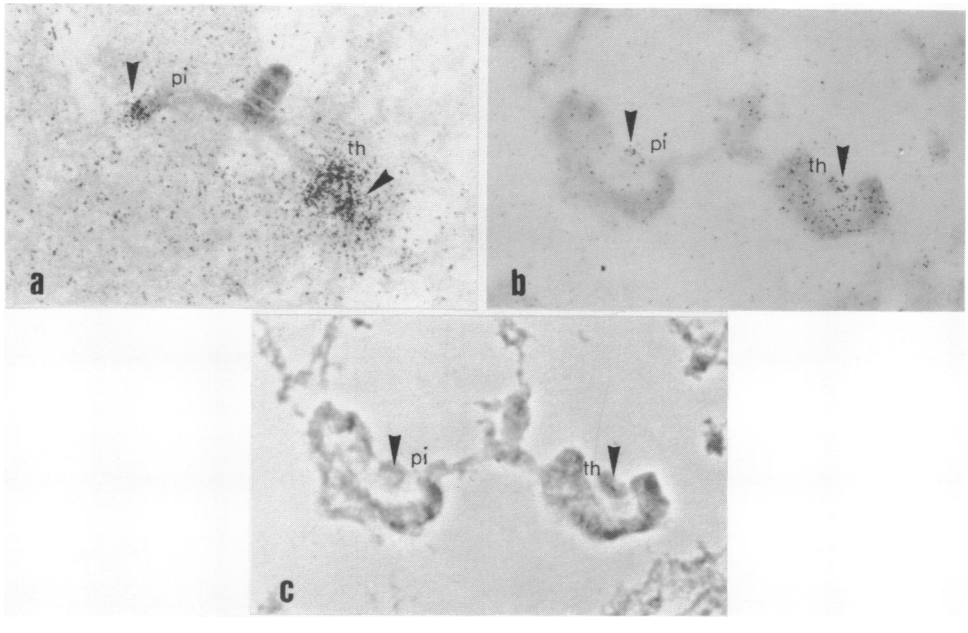

Fig. 5 (a) In situ hybridization of ^{125}I-labelled 8o^{o}(th)DNA to the nucleoli of salivary gland chromosomes of the hybrid of Ch. th. piger x Ch. th. thummi. The centromere regions (arrow) and the nucleolar organizer regions (pi, th) in the unpaired section of the hybrid chromosome IV are indicated (we are grateful to Prof. Dr. H.-G. Keyl for providing the photograph and determining the nucleolar organizer regions). (b) In situ hybridization of the 12o bp Cla I element (^{3}H-labelled) with the hybrid polytene chromosome IV. (c) The same as in (b) but phase contrast. The different intensities of labelling in figure 5 b when compared with figure 5 a is due to the different isotopes used. In the case of hybridization with Cla I elements the exposure was not prolonged in order to demonstrate also the label at the centromeres in thummi and piger.

Cla I fragment is digested by Eco RI into a 3 kb and 1.5 kb fragment. While the 3 kb Cla I - Eco RI fragment comigrates with the 3 kb Cla I - Cla I fragment, the 1.5 kb Cla I - Eco RI fragment hybridizes weakly, probably due to a limited sequence homology between thummi and the tentans NTS sequences. In comparison, the piger rDNA lacks the 1.5 kb fragment. Thus, the distance of the Cla I elements in genomic rDNA is 1.5 kb measured from the Eco RI site within the NTS. It is the same position as in cloned rDNA (comp. Fig. 1).

The in situ hybridization of the $8o^{o}$(th)DNA (enriched for repetitive DNA) (7) to salivary gland chromosomes of piger x thummi hybrids shows a quantitative difference in hybridization between the thummi and piger nucleolus (Fig. 5 a). Using Cla I elements as the hybridization probe, a significant label is seen only over the thummi nucleolus (Fig. 5 b). In addition, the centromeres of thummi and piger chromosome IV are labelled.

DISCUSSION

The repeated units of rDNA of the Australian Ch. tepperi are homogeneous in size with a repeat length of 8.4 kb. This repeat length corresponds precisely to the size reported for other distantly related Chironomus species (2) and, therefore, seems to represent the basic repeat length of rDNA in Chironomus.

The rDNA repeats of Ch. th. piger are slightly longer (9.0 kb). Minor heterogeneous rDNA fractions have sizes smaller than one repeated unit. This is not simply due to degradation of the DNA. These repeats might result from deletions in some of the rDNA repeats (26, 27) or might also represent orphons (28). The slight increase in size of piger rDNA repeats when compared with the basic repeat is due to additional DNA within the NTS region. Furthermore, the findings show that two NTS size classes are present in Ch. th. piger which differ by about 0.18 kb.

The rDNA of Ch. th. thummi is completely heterogeneous in length (mainly between 10 and 16 kb, very few have sizes up to 40 kb per repeat unit). The results indicate that this heterogeneity has evolved in the NTS region. Generally, NTS sizes are known to differ considerably between eukaryotes in the size range between smaller than 1 kb and appr. 30 kb (27, 29 - 33). In some species the NTS is graduated in size due to NTS subrepeats, in others the NTS is surprisingly homogeneous. In Dipteran species, NTS size variations due to subrepeats were found in Drosophila (30, 34) and in the related species

Calliphora (35) and Sarcophaga (36). The Nematocerans Sciara (29) and Phryne (in prep.) have homogeneous NTS sizes and in Chironomus only some, at least thummi and most probably melanotus, have varying numbers of subrepeats in the NTS. However, subrepeats do not seem to have a distinct interspecies location in the NTS region (compare: Calliphora, Drosophila and Xenopus, 35, 34, 31).

Usually, unequal sister chromatid exchange (SCE) has been attributed as an explanation of how rDNA repeats are driven to homogeneity (37, 38, 39) and the repetitous character of NTS internal sequences indeed suggests that these sequences are the site of frequent recombination (40). However, the fact that distinct NTS size classes are underrepresented in Calliphora, Drosophila (35, 34) and in Ch. th. thummi additionally suggests a selective advantage that restricts the heterogeneity to within preferred lengths.

Also the size of the subrepeats does not seem to vary at random among species (60 - 120 - 240 - 350 bp for Xenopus, Chironomus, Drosophila, Calliphora). Apparently, there must exist a selective pressure for a distinct size frame, suggesting that one function of NTS subrepeats may be in some way correlated with the repeat size. This could be coupled with the correct phasing of nucleosomes vis-a-vis important regulation sequences (41, 42).

Unusually, in Ch. th. thummi the Cla I NTS subrepeats are not restricted to the nucleolus. They are also found at sites were a geometric increase of DNA in polytene chromosome bands can be observed (3, 8). At least the multiple position of Cla I elements is reminiscent of mouse NTS rDNA sequences found also elsewhere throughout the genome (43). The fact that the Cla I elements are predominantly localized at the centromeres of all chromosomes of Ch. th. thummi (8) closely parallels the distribution of repeated sequence tracts separating newt histone gene clusters. These repeats are members of a predominantly centromeric satellite DNA with a repeat size of appr. 225 bp (44). This is about twice the length of the Ch. th. thummi Cla I element.

In Ch. th. thummi the frequency of ocurrence of Cla I elements decreases with the distance from the centromere (8). Also the rDNA clusters are preferentially located in a centromere-near position in Chironomus species (15). For various species, including plants and animals, Lima de Faria (45, 46) demonstrated a preferential position of rDNA sequences in the chromosome arms. Krystal et al. (47) suggested that identical positions of rDNA clusters could affect genetic exchanges among rDNA repeats. In the future we can ask if the frequency of occurrence of Cla I elements in the chromosome arms is coupled with the selective advantage that determines the position of the rDNA in relation to the centromere and telomere. Another question of interest is how

the Cla I elements have entered the thummi NTS. Since Ch. th. piger is the phylogenetically older subspecies (4) in which the Cla I elements seem to be clustered only at the centromeres (8), one can assume that during evolution the Ch. th. thummi Cla I elements have evolved mobility. Therefore, it would be interesting to characterize both Cla I elements of thummi and of piger.

ACKNOWLEDGEMENTS

Thanks are due to Prof. Dr. H.-G. Keyl for critically reading of the manuscript. We are grateful to Dr. A. Degelmann and Prof. Dr. C. P. Hollenberg (Tübingen and Düsseldorf) for providing the cloned Ch. tentans rDNA. We thank Dr. E. A. Godwin for her help and advice in preparing the manuscript. This research was supported by grants of the Deutsche Forschungsgemeinschaft (Ke 41,15 and Schm 523,3).

REFERENCES

1. Keyl, H.-G. (1961) Arch. Hydrobiol. 58, 1-6
2. Degelmann, A., Royer, H.-D. and Hollenberg, C.P. (1979) Chromosoma 71, 263-281
3. Keyl, H.-G. (1965) Chromosoma 17, 139-180
4. Keyl, H.-G. (1962) Chromosoma 13, 464-514
5. Hägele, K. (1977) Chromosoma 59, 207-216
6. Schaefer, J. and Schmidt, E.R. (1981) Chromosoma 84, 61-66
7. Schmidt, E.R., Vistorin, G. and Keyl, H.-G. (1980) Chromosoma 76, 35-45
8. Schmidt, E.R. (1981) FEBS Letters 129, 21-24
9. Martin, J. (1974) Chromosoma 45, 91-98
10. Beermann,W. (1960) Chromosoma 11, 263-296
11. Pelling, C. and Beermann, W. (1965) Nat. Cancer Inst. Monogr.23, 393-409
12. Derksen,J., Trendelenburg, M.F., Scheer, U. and Franke, W.W. (1973) Exptl. Cell Res. 80, 476-479
13. Hollenberg, C.P. (1976) Chromosoma 57, 185-197
14. Eigenbrodt, J. (1978) Chromosoma 67, 63-66
15. Lentzios, G. and Stocker, A.J. (1979) Chromosoma 75, 235-258
16. Keyl, H.-G. (1963) Chromosoma 13, 588-599
17. Nabeel, A.A. and Young, B.D. (1976) MSE Appl. Inf. A12/6
18. Commerford, S.L. (1971) Biochemistry 10, 1993-2000
19. Prensky, W. (1976) in Methods in Cell Biology, Prescott, D.M. Ed., Vol. XIII, pp. 121-152, Academic Press, New York.
20. Macgregor, H.C. and Mizuno, S. (1976) Chromosoma 54, 15-25
21. Loening, U.E. (1979) Biochem. J. 102, 251-257
22. Southern, E.M. (1975) J. Molec. Biol. 98, 503-513
23. Denhardt,D.T. (1966) Biochem. Biophys. Res. Commun. 23, 641-646
24. Singh,L., Purdom, I.F. and Jones, K.W. (1977) Chromosoma 60, 377-389
25. Bonner, J.J. and Pardue, M.L. (1976) Chromosoma 58, 87-99
26. Dawid, I.B., Wellauer, P.K. and Long, E.O. (1978) J. Molec. Biol 126, 749-768
27. Files, J.G. and Hirsh, D. (1981) J. Molec. Biol. 149, 223-240
28. Childs,G., Maxson, R., Cohn, R.H. and Kedes, L. (1981) Cell 23, 651-663
29. Renkawitz, R., Gerbi, S.A. and Glätzer, K.H. (1979) Molec. Gen. Genetics 173, 1-13

30. Rae, P.M.M., Barnett, t. and Murtiv, V.L. (1981) Chromosoma 82, 637-655
31. Wellauer, P.K., Dawid, I.B. and Brown, D.D. (1976) J. Molec. Biol. 105, 461-486
32. Wellauer, P.K. and Dawid, I.B. (1979) J. Molec. Biol. 128, 289-303
33. Kominami, R., Urano, Y., Mishima, Y. and Maramatsu, M. (1981) Nucleic Acids Res. 9, 3219-3232
34. Kunz, W., Petersen,G., Renkawitz-Pohl, R., Glätzer, K.H. and Schäfer, M. (1981) Chromosoma 83, 145-158
35. Schäfer, M., Wyman, A.R. and White, R. (1981) J. Molec. Biol. 146, 179-199
36. French, C.K. Fouts, D.L. and Manning, J.E. (1981) Nucleic Acids Res. 9, 2563-2576
37. Smith, G.P. (1973) Cold Spring Harbor Symp. Quant. Biol. 38, 507-513
38. Petes, T.D. (1980) Cell 19, 765-774
39. Szostak, J.W. and Wu, R. (1980) Nature 284, 462-430
40. Wellauer, P.K. and Dawid, I.B. (1978) J. Molec. Biol. 105, 461-486
41. Gottesfeld, J.M. and Bloomer, L.S. (1980) Cell 21, 751-760
42. Samal, B., Worcel, A., Louis C. and Schedl, P. (1981) Cell 23, 401-409
43. Arnheim, N., Seperack, P., Banerji, J., Lang, R.B., Miesfeld, R. and Marcu, K.B. (1980) Cell 22, 179-185
44. Stephenson, E.C. Erba H.P. and Gall, J.G. (1981) Cell 24, 639-647
45. Lima de Faria, A. (1976) Hereditas 83, 1-22
46. Lima de Faria, A. (1980) Hereditas 93, 1-46
47. Krystal, M., D'Eustachio, P., Ruddle, F.H. and Arnheim, N. (1981) Proc. Nat. Acad. Sci. USA 78, 5744-5748