THE DEVELOPMENT OF A 120 BASEPAIR REPETITIVE DNA SEQUENCE IN *CHIRONOMUS THUMMI* IS CORRELATED TO THE DUPLICATION OF DEFINED CHROMOSOMAL SEGMENTS

Erwin R. SCHMIDT
Lehrstuhl für Genetik, Ruhr-Universität, Postfach 10 21 48, 4630 Bochum, FRG

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1. Introduction

In the dipteran species *Chironomus thummi*, two subspecies exist which differ in their genome size by ~30%. If chromosomes of *Ch. th. thummi*, the subspecies with the higher DNA content/genome, are compared with chromosomes of *Ch. th. piger*, one can find that numerous chromosomal bands of *Ch. th. thummi* are thicker than homologous bands of *Ch. th. piger*. Cytophotochemical measurements of the DNA content of single chromosomal bands have shown, that the thicker bands contain always 2^n times (n = 1,2,3,4) as much DNA as the homologous bands in *Ch. th. piger* chromosomes [1]. This has been interpreted as an evolutionary DNA increase by DNA duplication series.

The analysis of the DNA of the two subspecies has revealed another difference between *Ch. th. thummi* and *Ch. th. piger*: an early melting AT-rich DNA subfraction is present in the *Ch. th. thummi* DNA which is not detectable in *Ch. th. piger* DNA. This DNA fraction (~10% of the total DNA) called '80°C-*th*-DNA' according to its melting point contains ~30% highly repetitive DNA sequences. In situ hybridizations with polytene chromosomes of *Ch. th. piger* × *thummi* F_1-hybrids have shown that the 80°C-*th*-DNA is located in these chromosomal bands which are known to have the increased DNA content. Furthermore, in *Ch. th. thummi* these sequences are also present in chromosomal sites, where cytologically no duplication can be recognized. In *Ch. th. piger*, however, these sequences are present in a very low concentration and hybridize only to the centromeres of all 4 chromosomes [2].

The highly repetitive portion of the 80°C-*th*-DNA resembles a satellite sequence. It reassociates very fast and hybridizes in situ to the centromeric heterochromatin in various chironomid species. In addition, there are some relationships with 2 AT-rich satellite DNAs found in another chironomid, *Glyptotendipes barbipes*. The repetitive 80°C-*th*-DNA as well as the *G. barbipes* satellite sequences hybridizes in situ to the same chromosomal segments [3] and, hence, might be partially an identical sequence. These data indicated that repetitive DNA sequences are involved in the evolutionary process of DNA duplication. The analysis of the DNA of *Ch. th. thummi* and *Ch. th. piger* with a variety of restriction endonuclease has confirmed this presumption.

2. Materials and methods

Larvae of *Ch. th. thummi* and of *Ch. th. piger* were obtained from permanent breedings kept in our laboratory.

The DNA was prepared from crude nuclear preparations obtained from homogenized larvae as in [2]. The digestion of the DNA with various restriction endonucleases was carried out as recommended by the manufacturer. *Eco*RII digestions were carried out as in [4]. Completeness of digestion was controlled by adding either λ,φ X 174 RF, or plasmid pBR 322 DNA to the digestion mixture. The restriction endonucleases *Hae*III and *Msp*I were purchased from New England Biolabs (Beverly MA), *Bst*EII, *Hin*dIII and *Pst*I from BRL (Neu Isenburg), *Eco*RI, *Hpa*II and *Cla*I from Boehringer (Mannheim) and *Eco*RII was a generous gift of Dr R. Eichenlaub (Ruhr-Universität Bochum). Electrophoresis of the DNA restriction

fragments was carried out on 0.8–1.6% agarose vertical slab gels (20 × 15 × 0.2 cm) using Tris (36 mM), NaH_2PO_4 (30 mM) and Na_2EDTA (1 mM), or Tris-borate (90 mM) (pH 8.2), Na_2EDTA (1.25 mM) as running buffer. For M_r determinations, the gels were calibrated with λ × *Hin*dIII and φ × 174 × *Hae*III DNA fragments. The gels were stained with ethidium bromide (5 μg/ml) and photographed with UV-transillumination using a Polaroid MP 4 Land camera, equipped with an orange filter. For quantitative evaluation of the gels, the negatives of the photographs were scanned with a Joyce-Loebl microdensitometer and the integration areas under single peaks were obtained using a Videoplan-digitizer (Kontron, Eching). In situ hybridizations were carried out according to [5] and dot filter hybridization as in [6].

3. Results

Digestion of total nuclear *Ch. th. thummi* DNA with *Cla*I produces DNA fragments which form a strikingly prominent ladder of bands after electrophoresis (fig.1). The DNA length of the fastest moving band has been determined to be 120 ± 4 basepairs according to its electrophoretic mobility relative to *Hae*III fragments of φ × 174 RF DNA. The other prominent bands are roughly multiples of the basic repeat length. In partial digests, one can detect bands up to the band representing the dimer. However, the trimer and the following multimeric bands are always found split up into doublet bands which differ in length by ~20 basepairs (trimer) to 40 basepairs (hexamer) (fig.1c). After prolonged digestion with increased amounts of *Cla*I enzyme, most of the higher multimeric fragments are converted to monomer, dimer and trimer fragments (fig.2a). These fragments are then resistant to further digestion. The relative amounts obtained upon complete digestion are 3.5% monomer, 1.0% dimer and 0.4% trimer fragments as measured relative to the total amount of digested DNA. This means that ~5% of the *Ch. th. thummi* DNA is organized as highly repetitive DNA with a repeat length of 120 basepairs, recognized by the *Cla*I restriction enzyme. Beside the fragments fitting into the ladder of 120 basepair, there are two faint bands visible after *Cla*I digestion; one represents a 1.5-mer (~180 basepairs) and the other represents a 0.75-mer (~90 basepairs) (fig.1c). This has been found also in repetitive sequences of other organisms [7–10].

With all other restriction endonucleases (see section 2), no restriction pattern similar to the *Cla*I cleavage patterns was obtained.

*Cla*I digestion of *Ch. th. piger* DNA: The subspecies with the lower DNA content, does not display a prominent ladder of bands corresponding to the 120 basepairs ladder obtained with *Ch. th. thummi* DNA (fig.1b). However, if *Ch. th. piger* DNA is digested completely with *Cla*I, very faint bands are present at the monomer and dimer position (fig.1b, white arrow). This shows that the 120 basepair sequence is also present in *Ch. th. piger* DNA, although in a much lower copy number. We also performed dot

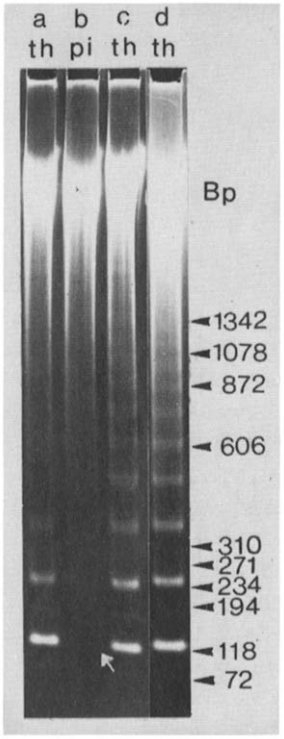

Fig.1. Cleavage pattern of *Ch. th. thummi* DNA (a,c,d) and *Ch. th. piger* DNA (b) with *Cla*I restriction endonuclease. (a,b) Complete digestion: 25 μg DNA were incubated with 10 units *Cla*I for 16 h at 37°C and then an additional 10 units *Cla*I were added and the incubation continued for 8 h. (c,d) Partial digestion of *Ch. th. thummi* DNA: (c) 10 units *Cla*I/20 μg DNA were incubated for 19 h at 37°C and with an additional 5 units incubated for 5 h; (d) 10 units *Cla*I/20 μg DNA incubated for 19 h at 37°C. In separate experiments λDNA was included in the digestion mixture. The λDNA is also not cleaved completely under the conditions used for the DNA in (c,d). The positions of the M_r standard DNA are indicated in the right hand-margin 1.4% agarose gel. White arrow: 120 basepair band of *Ch. th. piger* DNA.

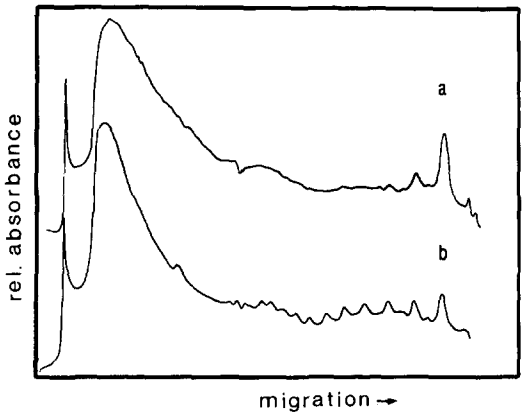

Fig.2. Densitometrical tracings of *ClaI* digested *Ch. th. thummi* DNA separated on a 1.3% agarose gel: (a) complete digestion, 20 μg DNA were incubated with 10 units *ClaI* for 16 h, then an additional 10 units *ClaI* enzyme were added and the incubation continued for 12 h, and finally 5 units added and incubated for 2 h. Further incubation or addition of more enzyme did not alter the cleavage pattern; (b) partial digestion, 20 μg DNA were incubated with 10 units *ClaI* enzyme for 12 h at 37°C, followed by addition of 10 units *ClaI* enzyme and incubation for 6 h.

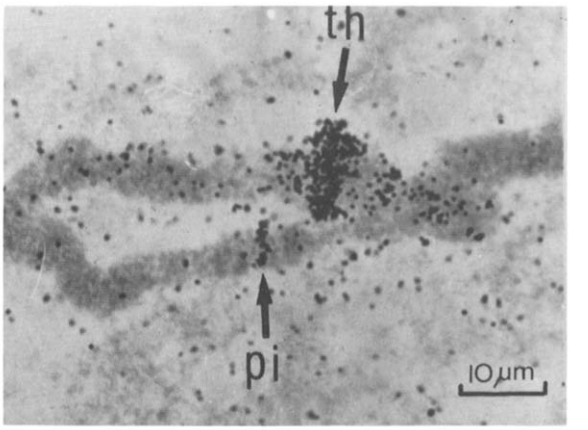

Fig.3. Microautoradiograph of *Ch. th. piger* × *thummi* F_1-hybrid polytene salivary gland chromosomes after in situ hybridization with the isolated 120 basepair monomer DNA fragment labeled radioactivity by nicktranslation as in [11] using all 4 deoxyribonucleotide-triphosphates labeled with tritium. Heavy label is found over the centromeric region of the *Ch. th. thummi* chromosome (→th). Only little label is found over the homologous centromere of the *Ch. th. piger* chromosome (→pi). Note that the homologous chromosomes of *Ch, th. thummi* and *Ch. th. piger* are not somatically paired in this region.

filter hybridization experiments in which the isolated 120 basepair monomer DNA was hybridized to filter bound *Ch. th. thummi* and *Ch. th. piger* DNA. The results (not shown) clearly confirm that the 120 basepair sequence is also present in low concentration in the genome of *Ch. th. piger*.

To localize the 120 basepair sequence within the *Ch. th. thummi* and the *Ch. th. piger* chromosomes, in situ hybridizations were carried out. The isolated 120 basepair sequence was hybridized with the polytene chromosomes of *Ch. th. piger* × *thummi* F_1-hybrids. The result is shown in fig.3. There is a heavy label visible over the centromeric region of the *Ch. th. thummi* chromosome and only a slight label is found over the centromere band of the *Ch. th. piger* chromosome. In the *Ch. th. thummi* chromosome, there is some additional label in the vicinity of the centromere region.

4. Discussion

The comparison of the *ClaI* restriction pattern of the DNA from the two subspecies *Ch. th. thummi* and *Ch. th. piger* revealed a striking difference between the two subspecies: *Ch. th. thummi*, the subspecies with the higher DNA content/genome, contains a tandemly repeated DNA sequence in a highly repetitive, satellite like organization, while *Ch. th. piger*, the subspecies with the lower DNA content, contains the same sequence in a much lower copy number. Thus the development of the highly repetitive 120 basepair sequence is directly correlated to the evolutionary increase in genome size. The higher DNA content of the *Ch. th. thummi* genome is a result of series of duplications of selected chromosomal segments equivalent to bands in polytene chromosomes [1]. Altogether, *Ch. th. thummi* has ~30% more DNA than *Ch. th. piger* according to cytophotometrical measurements [1]. The relative amount of the *ClaI*–120 basepair repetitive DNA fraction, however, is only ~5% of the total *Ch. th. thummi* DNA and this might even be an overestimate due to the unprecise measurement of the bulk DNA from the negatives caused by film saturation in the bulk DNA region. This means that, in addition to the 120 basepair sequence, other DNA sequences must have been multiplied during evolution. We had a low melting point DNA fraction from *Ch. th. thummi*, which represents ~10% of the total DNA. This '80°C-*th*-DNA' is enriched in repetitive DNA sequences (~30% highly repetitive DNA sequences) if compared to total *Ch. th. thummi* DNA (12% highly repetitive

DNA [12]). This DNA fraction hybridizes in situ to the same chromosomal sites as does the 120 basepair sequence and so it seems probable that both DNA fractions have homologous sequences.

The repetition frequency of the 120 basepair sequence can be calculated assuming a haploid genome size of 0.15 pg for *Ch. th. thummi* (Keyl, H.-G., unpublished) and 5% of the total DNA representing the 120 basepair repetitive DNA. The 120 basepair would then be present ~60 000 times in a haploid *Ch. th. thummi* genome. The bulk of this DNA is located in the centromeric regions of the 3 large chromosomes as shown by in situ hybridization. *Ch. th. thummi* and *Ch. th. piger* differ largely in these regions with respect to the DNA content. This is exactly reflected by the results of the in situ hybridization with the isolated 120 basepair monomer DNA. However, as mentioned above the amount of the 120 basepair DNA alone is not sufficient to account for the total difference between the genomes of the two subspecies. Thus one can assume that besides the 120 basepair sequence, other DNA sequences are present in the duplicated regions.

It is not clear whether the 120 basepair sequence has something to do with the initiation of the evolutionary duplication events, but the correlation between the DNA increase and the presence of the 120 basepair sequence is evident. In addition, in F_1-hybrids *thummi* and *piger* chromosomes do not pair somatically in the duplicated chromosomal regions. Whether the 120 basepair sequence is responsible for this phenomenon is not clear.

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