The EMBO Journal vol.4 no.3 pp.799–804, 1985

Long tandem arrays of complex repeat units in *Chironomus* telomeres

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Communicated by J.-E. Edström

A cloned 340-bp DNA fragment excised by *Eco*RI from the *Chironomus pallividittatus* genome has been localized to the telomeres by *in situ* hybridization as well as to connectives between telomeres. No hybridization was observed in other regions of the chromosomes. Another cloned *Eco*RI fragment, 525 bp long has also been studied. This represents a partial duplication of the 340-bp sequence. Genomic blot hybridization experiments show that the 340-bp sequence is a representative monomeric unit of tandemly repeated arrays which account for 1.2% of the *Chironomus* genome, on average 300 kb per telomere. The repeat unit contains two types of subrepeats each present twice per repeat unit. Northern blot hybridization experiments show that the telomere-associated sequences are transcribed into a discrete RNA species ~20 kb in size. The evolution of this telomere-associated DNA is discussed.

Key words: DNA evolution/microcloning/tandem repeats/telomeres/transcription

Introduction

Chromosome telomeres are constructed such that complete replication of the end of the linear chromosomal DNA is possible. Telomeres interact with the nuclear envelope both in germ cells (Dancis and Holmquist, 1979, for references) and somatic cells (Agard and Sedat, 1983). This interaction may be of importance for chromosome pairing and for DNA replication. Telomeres are involved in end-to-end association of chromosomes but they also have properties that prevent permanent fusion of chromosome ends to one another (McClintock, 1941). Determination of primary DNA structure in telomeres should be the first step in approaching function. Indeed much structural information is already available in lower eukaryotes. Simple tandemly repeated sequences which can be formulated as $5'C_{(1-8)}A/T_{(1-4)}3'$ have been found at telomeres (Blackburn, 1984, for references). Szostak and Blackburn (1982) have shown that a terminal restriction fragment of *Tetrahymena* rDNA can satisfy some requirements of telomeres in yeast. More recently a mechanism has been suggested that allows addition of simple tandemly repeated sequences during replication (Bernards *et al.*, 1983; Van der Ploeg *et al.*, 1984; Shampay *et al.*, 1984; Walmsley *et al.*, 1984). Interestingly, in the *Xenopus* oocyte, rDNA of *Tetrahymena* is able to replicate and after several rounds of replication the rDNA was longer (Berg *et al.*, 1982). This suggests that a similar mechanism is present in higher eukaryotes.

For higher eukaryotes, in contrast to lower eukaryotes, study of telomeres at the nucleotide level is just at the beginning. Some telomeric DNA fragments have been isolated from *Drosophila* (Rubin, 1978; Young *et al.*, 1983; Renkawitz-Pohl and Bialojan, 1984; Steinemann, 1984) and rye (Bedbrook *et al.*, 1980). The *Drosophila* telomere has a complex structure containing both unique and repeated sequences and the rye telomere contains several families of tandemly repeated sequences.

We have isolated DNA fragments from *Chironomus* chromosomes which hybridize specifically to telomeres and are members of a class of 340-bp repeat units representing >1% of the genome. The units have a complex structure in which components can be distinguished with different modes of evolution. The DNA fragments also hybridize to a high mol. wt. RNA.

Results

Chromosomal localization

Two *Eco*RI fragments designated as the 340-bp sequence and the 525-bp sequence were isolated from a microcloned DNA library of chromosome IV by probing with ^{32}P-labelled RNAs which have been fractionated between 18S ribosomal and 75S Balbiani ring RNAs on Sepharose 2B-CL column chromatography. The pCp306 and pCp309 are subclones from the original lambda clones into the *Eco*RI site of pUC8 and have the 340- and 525-bp sequences as the inserts, respectively.

Upon *in situ* hybridization, biotinylated probes prepared from pCp306 or pCp309 plasmid DNA hybridize to all the telomeres of the four pairs of chromosomes (somatic pairing) except the left end of chromosome IV (Figure 1a). This end contains the kinetochore (Beermann, 1955) and thus may not be considered a genuine telomere. Sometimes connectives between two chromosome ends are observed. The biotinylated probe shows hybridization to the connectives as well (Figure 1b). These observations indicate that the 340-bp sequence locates close to the extreme end of chromosomal DNA.

Sequence analysis of clones

Figure 2 shows the nucleotide sequences of the 340-bp and the 525-bp sequences. The A-rich strands are shown from 5′ to 3′ ends. The 340-bp sequence contains two different direct sub-

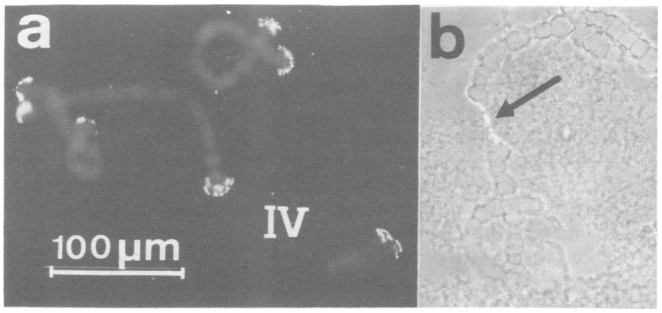

Fig. 1. *In situ* hybridization of polytene chromosomes with biotinylated pCp306 plasmid DNA. Squashes of salivary gland cells were denatured and incubated with biotinylated pCp306 plasmid DNA. The whole chromosome set shows hybridization over all chromosome ends except the kinetochoric end of chromosome IV **(a)**. A connective (arrow) between two chromosome ends is also shown **(b)**. Epifluorescence is used in **(a)** and combined phase contrast illumination and epifluorescence in **(b)**.

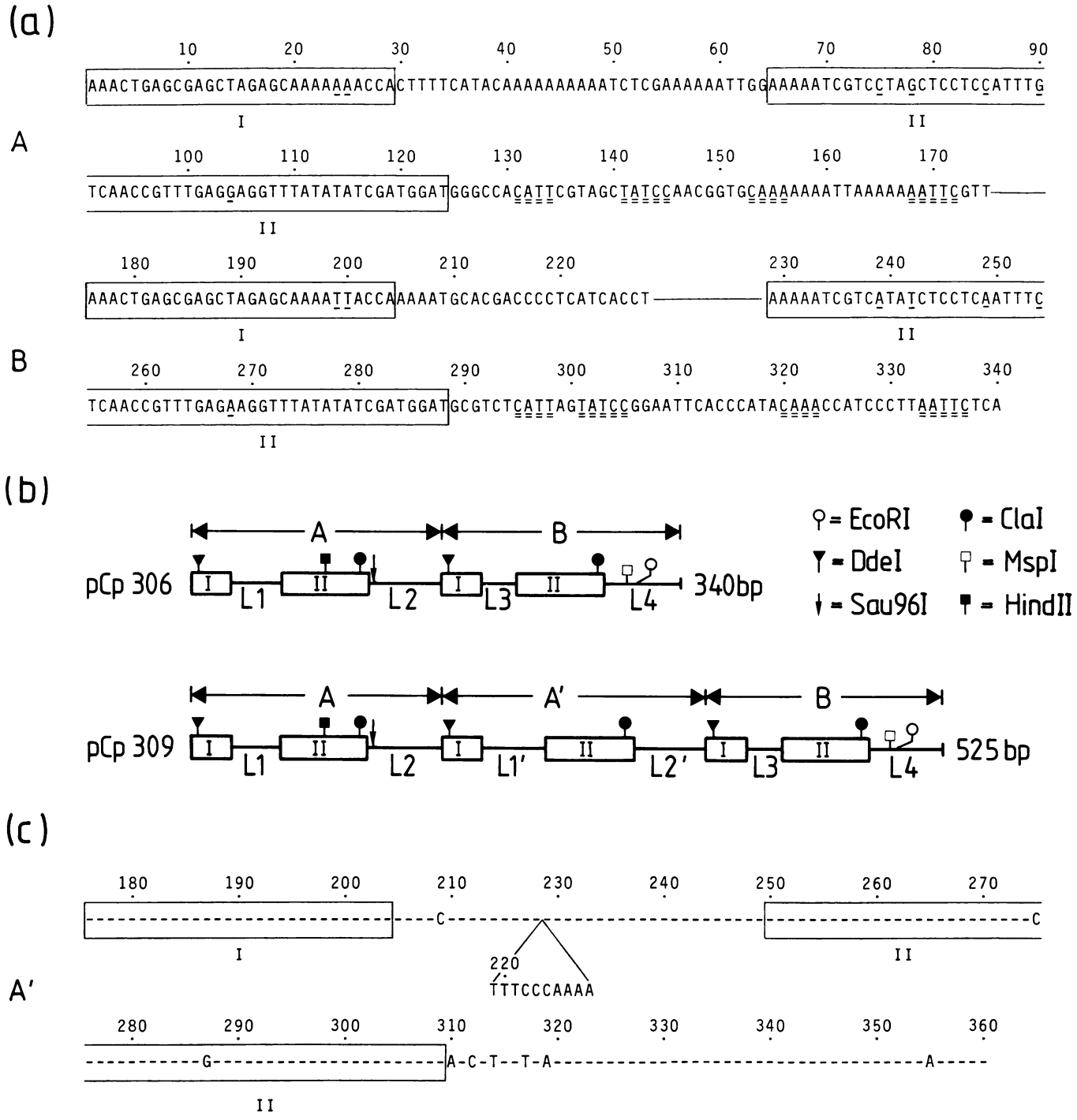

Fig. 2. Nucleotide sequences of the pCp306 (340 bp) and pCp309 (525 bp) inserts. The upper part **(a)** shows the sequence of the 340-bp (pCp306) insert. The middle part **(b)** shows a diagram of the pCp306 and pCp309 inserts and the lower part **(c)** shows the part of the pCp309 which is a duplication of part of pCp306. As shown by the diagram, the pCp306 insert consists of two parts, A and B. In the pCp309 insert, a duplication of A results in A′ and the order is A, A′ and B. Each A or B part contains the subrepeats I and II shown in boxes, also in the upper and lower parts of the illustration. In **(a)**, base substitutions between subrepeats are underlined. Sequences between subrepeats are designated L-regions, L1, L2, L3 and L4 for pCp306. In the duplicated A′, the corresponding L-regions are L1′ and L2′. Homologies between the L-regions L2 and L4 in the pCp306 insert are shown by double underlining. A and B regions of the pCp309 insert are identical to A and B regions in the pCp306 insert, respectively. Therefore in **(c)** only the A′ part is shown. Dash (-) indicates identity with A whereas base substitutions are indicated as well as a 10-bp large insertion in L1′. Restriction sites used for analysis of genomic organization are indicated in **(b)**. The *Eco*RI site (position 308) for the pCp306 insert in **(a)** is the cloning site. For convenience for sequence comparison and since the 340-bp sequence is tandemly arranged, the nucleotide sequences after the *Eco*RI site (308 – 340) were moved from their original location 5′ to the subrepeat I in the A half to their present location at the 3′ end of the whole repeat unit.

repeats (I,II), each in two copies. The longer subrepeat (II) is 60 bp with five mismatches between the two copies and the shorter one (I) is 29 bp with two mismatches. The two subrepeats are arranged in an alternating order and the distance between the longer subrepeats is 164 bp and between the shorter subrepeats 175 bp. For designation of the sequences between the subrepeats we propose the term L-region (Linking-region). There is no significant homology between L1 and L3, whereas for L2 and

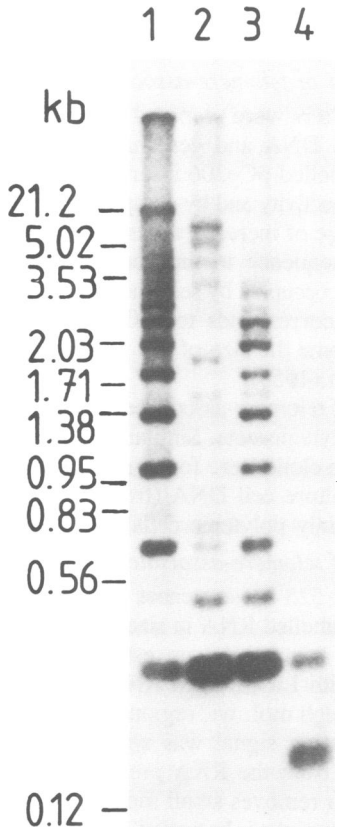

Fig. 3. Southern blot of genomic DNA digested with restriction enzymes probed with labelled pCp306 insert. 1 μg of genomic DNA was digested with restriction enzymes, separated on 1.0% agarose gel and transferred to nitrocellulose filter. The filter was hybridized to labelled pCp306 insert. **Lane 1** is digestion with *Msp*I; **2**, *Hind*II; **3**, *Sau*96I; **4**, *Cla*I, respectively. (^{32}P-labelled λ DNA fragments digested with *Eco*RI and *Hind*III were used as size marker.)

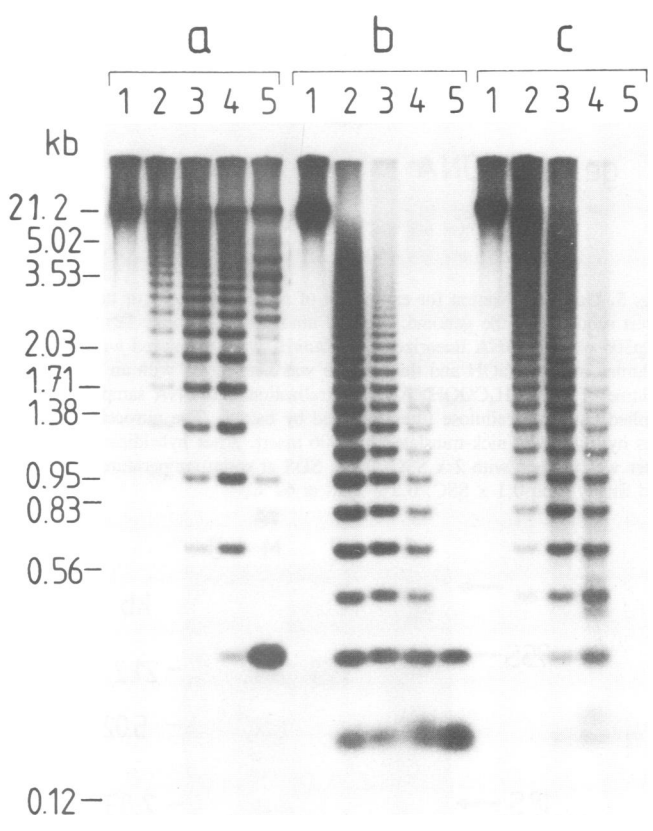

Fig. 4. Partial digestion of genomic DNA with *Eco*RI, *Cla*I and *Dde*I. 5 μg of genomic DNA were incubated with 5–10 units of restriction enzyme in 250 μl reaction mixture. After various times of incubation 50 μl aliquots were withdrawn, DNA was precipitated with ethanol, separated on 1.2% agarose gel, transferred to nitrocellulose filter and hybridized to ^{32}P-labelled pCp306 insert. Digestion with *Eco*RI **(a)** *Cla*I **(b)** and *Dde*I **(c)** is shown. Incubation time is 0 min **(1)**, 5 min **(2)**, 10 min **(3)**, 20 min **(4)** and 90 min **(5)**. (^{32}P-labelled λ DNA fragments digested with *Eco*RI and *Hind*III were used as size marker.)

L4 some homologies can be traced (double underlining in Figure 2a). Computer analysis did not reveal any further significant direct repeats within the 340-bp unit. With one exception (position 252–269, Figure 2a), there was little evidence for inverted repeats. There is no open reading frame for the pCp306 insert.

The 525-bp sequence contains a region which is a duplication of about half of the 340-bp sequence (its A-half, see Figure 2). The subrepeats of the duplicated part, A′ are more similar to the A subrepeats (two mismatches) than to the subrepeats in the B-half (seven mismatches). The corresponding L-regions in A and A′ are largely homologous with a major alteration of an insertion of 10 bases in the first L-region of A′ (L1′ in Figure 2b).

Genomic organization

To confirm the cloning results and to study the arrangement of the cloned sequences in the genome we digested genomic DNA from *Chironomus* with restriction enzymes cutting once (*Msp*I, *Hind*II, *Sau*96I) or twice (*Cla*I) in the pCp306 insert (Figure 2b). Digested DNA was separated on agarose ge's and transferred to nitrocellulose filters followed by hybri lization to nick-translated pCp306 insert DNA. Figure 3 shows an autoradiogram. In all cases, a band corresponding to 340 bp is apparent. Upon digestion with *Cla*I, a band corresponding to ~170 bp can be seen as well as a 340-bp band (lane 4 in Figure 3). These two bands account for most of the hybridization. *Msp*I, *Hind*II and *Sau*96I give bands at multiples of 340 bp and faint bands around 500 bp (lanes 1, 2 and 3) (~1.5-mers). In addition to weak bands of 340-bp multimers, *Hind*II digestion gives bands between 340 bp multimers (lane 2). Upon digestion with *Hind*II, *Msp*I and *Sau*96I, a 170-bp band was not detectable. These results together with the fact that the corresponding L-regions are largely dissimilar (Figure 2a) suggest that the 340-bp sequence is a representative monomeric unit of highly repetitive sequences. Another suggestion from the results of Figure 3 is that these sequences are organized in tandemly repeated arrays.

We have confirmed this notion by partial digestion of genomic DNA. Figure 4 shows autoradiograms from partial digestions with *Eco*RI, *Cla*I and *Dde*I. Recognition sequences for *Cla*I and *Dde*I appear once in each longer and shorter subrepeat, respectively (Figure 2). Upon digestion with *Eco*RI, ladders with bands that are multiples of 340 bp can be seen and as digestion time increases, the monomer band becomes more intense. After complete digestion, in addition to the 340-bp band, which is the most intense band in lane 5 of Figure 4a, bands corresponding to multiples of 340 bp can be seen. This is most likely due to heterogeneity of the 340-bp sequences with occasional base substitutions. Extra bands (outside of ladder positions) might represent junctions between 340 bp sequences and flanking sequences. *Cla*I and *Dde*I give ladders with multiples of ~170 bp instead of 340 bp. After complete digestion, *Dde*I gives almost

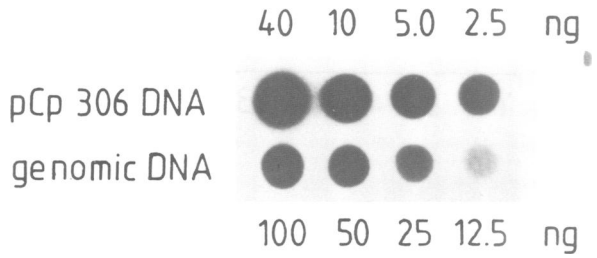

Fig. 5. Dot hybridization for estimation of relative amounts of the pCp306 insert sequence in the genome. Defined amounts of genomic DNA and pCp306 plasmid DNA linearized with *Bam*HI were denatured with 0.2 volumes of 2 N NaOH and the mixture was neutralized with an equal volume of 2 M NH_4COOH. After neutralization, the DNA samples were applied onto nitrocellulose filter followed by baking. The nitrocellulose filter was hybridized to nick-translated pCp306 insert. After hybridization the filter was washed with 2 x SSC, 0.2% SDS at room temperature five times and finally with 0.1 x SSC, 0.2% SDS at 62°C.

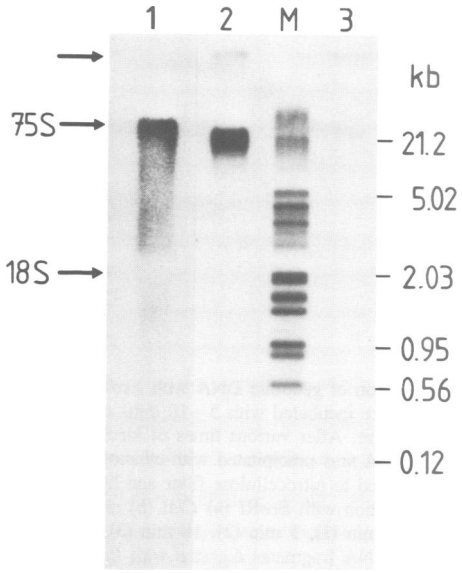

Fig. 6. Northern blot hybridization of salivary gland cellular RNA. RNA was prepared from salivary glands, treated with LiCl to remove DNA and was separated on 1.0% agarose gel containing 2.2 M formaldehyde followed by transfer to nitrocellulose filter. The filter was hybridized to nick-translated pCp306 insert **(lane 2)**. **Lane 3:** prior to gel electrophoresis an RNA sample was incubated with 50 μg/ml heat-treated RNase A at 30°C for 10 min. **Lane 1:** nitrocellulose filter was rehybridized to nick-translated pCp90 DNA (Galler *et al.*, 1984) to show the position of 75S (37 kb, Case and Daneholt, 1978) Balbiani ring mRNA. The position of 18S rRNA was determined by staining the nitrocellulose filter with methylene blue after autoradiography. The arrow above 75S indicates the origin. ^{32}P-Labelled λ DNA fragments, digested with *Eco*RI and *Hind*III were treated in the same way as the RNA preparation and used as size markers **(lane M)**.

only the 170-bp band; *Cla*I gives a 170-bp band as well as a 340-bp band, which suggests that at least one of the *Cla*I sites within the 340-bp sequence is frequently degenerated. These results suggest that most of the 340-bp sequences are organized in tandemly repeated arrays.

The bands around 500 bp for *Msp*I, *Hind*II, *Sau*96I and *Eco*RI after complete digestion are probably not due to occasional extra restriction sites half-way between the normal sites in the 340-bp sequence in combination with occasional losses of the normal sites. If this were the case it would create bands also at half monomer size and this was not observed, in contrast to the results after complete *Cla*I digestion. The ~1.5-mer band is probably caused by occasional units representing partial duplications of monomers like that represented by the pCp309 insert.

Genomic content of telomere-associated DNA

Nitrocellulose filters were prepared with increasing amounts of pCp306 plasmid DNA and genomic DNA. Such filters were hybridized to labelled pCp306 insert (Figure 5). Each spot was counted for radioactivity and by comparing the radioactivity within the linear range of increase we estimated the relative amount of the 340-bp sequence in the genome. About 1.2% of the genomic DNA is occupied by sequences hybridizing to the 340-bp sequence. This corresponds to 6500 copies of this sequence, assuming a genome the size of 0.2 pg (Daneholt and Edström, 1967; Beermann, 1955).

The content of telomere-associated sequences is unlikely to be related to the polytenic state. Similar amounts of DNA hybridizing to the 340-bp clone were found in the sibling species *C. tentans* in tissue culture cell DNA (from diploid cells) and larval DNA (from mainly polytenic cells) (data not shown).

Transcription of telomere-associated DNA

The 340-bp and 525-bp sequences were originally isolated by screening with labelled RNA in size between 18S and 75S. The identity of the transcript was established in Northern blot hybridization with LiCl-treated RNA. One distinct signal was detected in the high mol. wt. region (~20 kb) (Figure 6). Most of the hybridization signal was sensitive to ribonuclease. To eliminate DNA from the RNA preparation we used LiCl precipitation, which removes small mol. wt. RNA as well as most of the DNA. There was, however, no hybridization in the low mol. wt. region even when an RNA preparation without LiCl treatment was used. Thus at least a part of the telomere-associated sequences is transcribed into high mol. wt. RNA.

Discussion

We have isolated two related DNA sequences both of which are members of a class of highly repeated sequences that represent >1% of the *Chironomus* genome. As far as could be determined by *in situ* hybridization, all of the sequences are localized at the chromosome ends. We also observed hybridization over connectives between chromosome ends, suggesting that the hybridizing sequences are close to or extending to the ends. Nevertheless it is not known whether there are any even more distal sequences of different character. Therefore, according to the suggestions of Blackburn and Szostak (1984), these sequences should, at least for the time being, be classified as telomere-associated sequences.

Amount and arrangement of the telomere-associated sequences

The telomere-associated sequences constituted as much as 1.2% of the genome. Although we cannot rigorously exclude the presence of the 340-bp sequences in interstitial locations, sequences as large as 340 bp are likely to be detectable according to our experience of *in situ* hybridization with biotinylated probes in *Chironomus* polytene chromosomes. Thus the length occupied by the 340-bp sequences per telomere corresponds to an average of ~300 kb. Although tandem repeats have been observed in association with telomere-associated as well as telomeric sequences (Blackburn and Szostak, 1984) it is only in rye that long tandem arrays have been observed similar to the *Chironomus* arrangement (Bedbrook *et al.*, 1980). The tandem repeats observed in *Drosophila* consist of a few copies (Rubin, 1978; Young *et al.*, 1983; Steinemann, 1984). Telomere-associated sequences in rye are localized in heterochromatic regions (Bedbrook *et al.*, 1980). In *Chironomus*, however, 300 kb (occupied by the

telomere-associated sequence) correspond to 3 – 5 average size chromosome bands (Daneholt and Edström, 1967), whereas the heterochromatin occupies only thin or submicroscopic disks at the chromosome ends, likely to represent considerably less than 300 kb (Beermann and Bahr, 1954; Beermann, 1955). Thus most of the telomere-associated sequences in *Chironomus* extend over euchromatic regions.

The sequences are distributed between seven telomeres. It is possible that sequences within a certain telomere show greater similarity than sequences from different telomeres, as was shown for a similar telomere associated repeat unit from *C. thummi* (Carmona *et al.*, in preparation). It is also possible that within a certain telomere there are sequence differences between repeat units. Degeneracy shown in the genomic blots may be attributed to one or both of these causes.

It is of particular interest that telomere-associated sequences are not measurably present at the kinetochoric end of chromosome IV. This means that these sequences are probably not an obligatory component of a chromosome end, i.e., replication of the chromosomal DNA ends is not necessarily dependent on these sequences. On the other hand the sequences might well be involved in recognition of other chromosome ends. In fact end-to-end association occurs between telomeric chromosome ends but not between the kinetochoric end and the telomeric ends (Bauer, 1936).

Transcription product

Northern blot hybridization experiments showed that at least parts of the 340-bp sequence are transcribed. Telomere-associated sequences in *Drosophila* hybridize to a series of poly(A)$^+$ transcripts, considerably smaller than the present product (Renkawitz-Pohl and Bialojan, 1984).

In our material no evidence has been obtained for chromosomal location of the transcripts. Although a distinct *in situ* hybridization with pCp306 as probe was obtained over the telomeres without prior chromosome denaturation, this was also the case when the squashes had been treated with ribonuclease (data not shown). In the related species *C. thummi* a clone has been isolated which also represents telomere-associated sequences and hybridizes to RNA of a similar size. Such RNA is located in a telomeric Balbiani ring that can be induced by heat shock (Carmona *et al.*, in preparation). At present there is no information as to the function of the telomeric transcript except that the 340-bp sequence does not have any open reading frame. For this purpose its structure as well as cellular localization should be established. Considering the average amount of telomere-associated sequences, probably ~300 kb per telomere, and the size of the transcript, ~20 kb, it is of particular interest to learn how transcription units are arranged in relation to telomere associated sequences.

Evolution of the telomere-associated sequences

The 340-bp sequence is a representative monomeric unit which is organized in long tandem arrays in the telomeres. It is likely that the 340-bp unit evolved from a 170-bp ancestral sequence, not only in view of the subrepeat arrangement, but also because of the traces of homology between L2 and L4. Furthermore, inspection of the 525-bp sequence indicates that duplication of an ~170-bp unit (the A-half) has occurred in this case.

The various regions in the putative ancestral 170-bp unit have evolved very differently after duplication. The members of the L1-L3 pair diverged quickly from each other. Members of the L2-L4 pair have diverged less rapidly since some homology remains, and the size difference is moderate. The two copies of each subrepeat have, finally, diverged very little from each other.

These different rates are well demonstrated, at an earlier stage of divergence, in the comparison of the A′ with the A part in the 525-bp sequence. Both subrepeats in A′ remain almost identical with those in A. The L2-L2′ pair shows a few differences, whereas the L1-L1′ pair has diverged more, mainly due to a 10-nucleotide insertion. Thus, the regional differences in divergence between A and B parts of the 340-bp unit are expressed also at the early stage of divergence represented by A′.

The similarity between the two copies of each subrepeat does not necessarily indicate slow evolution. On the contrary, some lines of evidence suggest that telomere-associated DNA evolves quite rapidly. In yeast such DNA shows an unusual degree of polymorphism among different strains (Chan and Tye, 1983). In a related species, *Chironomus thummi*, there is a 170-bp repeat unit of telomere-associated sequences which does not show any significant homology to the 340-bp sequence in *C. pallidivittatus* although it has similar properties (Carmona *et al.*, in preparation). Consequently, the apparent conservation of the subrepeat parts should be understood also in the light of the rapid evolution of the telomere-associated sequences.

Materials and methods

Cloning of telomere-associated sequences

The telomere-specific clones described here were obtained from an experiment with the aim of cloning sequences from Balbiani ring (BR) 3. Chromosome squashes were prepared as previously described (Scalenghe *et al.*, 1981) and the BR3 region was isolated from one chromosome IV by microdissection. Microcloning was performed as previously described (Scalenghe *et al.*, 1981) using λ641 as vector and a library from this chromosomal region was obtained. Two clones, containing the 340-bp sequence and the 525-bp sequence, respectively, hybridized to a labelled RNA fraction between 18S rRNA and 75S Balbiani ring mRNA upon Sepharose 2B-CL column chromatography (Rydlander *et al.*, 1980). The inserts of these clones, subcloned into pUC8 hybridized *in situ* to the telomeres. To control this unexpected result we cloned DNA after isolating telomeres. Screening of plaques from such experiments yielded a much higher percentage of positive clones. Such clones also contained 340-bp inserts. Therefore we think that a minute contamination from the telomere of chromosome IV, which is relatively close to BR3, was responsible for the original positive clones.

Preparation of genomic DNA

Genomic DNA was prepared from *C. pallidittatus* larvae according to Blin and Stafford (1976). 500 larvae were frozen with liquid nitrogen, ground to powder and suspended in 10 ml of 0.5 M EDTA, 2 mg proteinase K, 0.5% sarcosyl. After incubation during rotation at 50°C for 3 h, the mixture was extracted with phenol and dialyzed against 50 mM Tris-HCl, 10 mM EDTA (pH8.0) overnight. DNA was purified by centrifugation in CsCl gradient containing ethidium bromide, and dialyzed against 10 mM Tris-HCl, 1 mM EDTA (pH 8.0).

Preparation of RNA

RNA was prepared from isolated salivary glands according to a modification of the method described by Rydlander *et al.* (1980). After the first precipitation of RNA by ethanol it was dissolved in 30 mM Tris-HCl (pH 7.6), 1 mM EDTA, 0.5% SDS. To remove DNA, one third volume of 6 M LiCl was added and the mixture was kept overnight at +4°C. After centrifugation, the supernatant was removed, which contained most of the DNA and small mol. wt. RNAs. The precipitate was dissolved with 30 mM Tris-HCl (pH 7.6), 1 mM EDTA, 0.5% SDS and two volumes of ethanol were added to the solution. RNA was recovered by centrifugation and the LiCl precipitation step was repeated. Finally RNA was dissolved in 20 mM Tris-HCl (pH 7.6), 0.5% SDS and 0.05 volumes of 2 M NaCl was added. RNA was recovered by ethanol precipitation.

Transfer of nucleic acids to nitrocellulose filter

1 μg of genomic DNA, digested with various restriction enzymes, separated on 1.0% agarose gel, was transferred to nitrocellulose filter according to Southern (1975).

About 5 μg of RNA was fractionated by electrophoresis on 1.0% agarose gel containing 2.2 M formaldehyde (Lehrach *et al.*, 1977). After electrophoresis, the gel was treated with 50 mM NaOH, 10 mM NaCl at room temperature for 45 min and neutralized with 0.1 M Tris-HCl (pH 8.0). RNA was transferred to nitrocellulose filter by the Southern method.

Dot hybridization was done according to Kafatos *et al.* (1979).

Filter hybridization

Nitrocellulose filters were incubated in 50% formamide, 50 mM phosphate buffer (pH 7.0), 5 x SSC, 5 x Denhardt's solution (Denhardt, 1966) containing 100 μg/ml denatured *Escherichia coli* DNA and DNA probe at 42°C.

After the hybridization reaction, filters were washed with 2 x SSC, 0.2% SDS at room temperature (Thomas, 1980). If stringent washing conditions were necessary, filters were further washed with 0.1 x SSC, 0.2% SDS at 62°C for 60 min.

In situ hybridization

The fixed squashes of salivary gland cells were denatured with 0.07 N NaOH for 3 min prior to the hybridization reaction. Biotinylated probe was prepared according to the method of Langer-Safer *et al.* (1982) with biotinylated UTP (Enzo Biochemicals). Hybridization was done at 58°C in 0.6 M NaCl, 50 mM phosphate buffer (pH 7.2), 1 x Denhardt's solution. Hybridized biotinylated probe was bound to anti-biotin goat IgG (Enzo Biochemicals) and visualized with rhodamine-labelled rabbit anti-goat IgG (Miles).

Determination of nucleotide sequence

Insert DNA fragment of pCp306 and pCp309 plasmid were prepared, labelled with polynucleotide kinase and [γ-^{32}P]ATP (3000 Ci/mmol, Amersham) and two strands were separated. Nucleotide sequence was determined for both strands according to Maxam and Gilbert (1980).

Acknowledgements

We thank Dr. R. Galler for helping us in the sequence analysis by computer and Dr. E. Schmidt (Bochum) for critical reading of our manuscript. We are also indebted to Dr. R. M. Widmer (Zürich) for the gift of DNA from tissue culture cells of *Chironomus tentans*.

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Received on 30 November 1984; revised on 8 January 1985