J Mol Evol (1997) 44:321–326

JOURNAL OF MOLECULAR EVOLUTION

Complex Evolution of Tandem-Repetitive DNA in the *Chironomus thummi* Species Group

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Received: 22 May 1996 / Accepted: 8 October 1996

Abstract. The subspecies *Chironomus thummi thummi* and *C. t. piger* display dramatic differences in the copy number and chromosomal localization of a tandemly repeated DNA family (Cla elements). In order to analyze the evolutionary dynamics of this repeat family, we studied the organization of Cla elements in the related outgroup species *C. luridus.* We find three different patterns of Cla element organization in *C. luridus*, showing that Cla elements may be either strictly tandem-repetitive or be an integral part of two higher-order tandem repeats (i.e., *Hin*f[lur] elements, *Sal*[lur] elements). All three types of Cla-related repeats are localized in the centromeres of *C. luridus* chromosomes. This suggests that the dispersed chromosomal localization of Cla elements in *C. t. thummi* may be the result of an amplification and transposition during evolution of this subspecies.

Key words: Chironomus — Satellite DNA — Tandem-repetitive DNA — Molecular Evolution

Introduction

Closely related species may differ enormously in their genome size (= c-value), in spite of being nearly indistinguishable in morphology and behavior. This seemingly paradoxical situation has been termed the ''c-value paradox'' (for reviews see Gall 1981; Cavalier-Smith 1985). Recently, detailed molecular analyses of genome structures carried out in a multitude of diverse organisms have helped to better explain the broad range of genome sizes found among closely related organisms. It has been recognized that a major portion of the eukaryotic genome is made up of noncoding repetitive DNA sequences. Related species may display significant differences in both the amount and the genomic localization of repetitive DNA. This phenomenon has been reported for animals and plants of widely diverse taxa (Hennig et al. 1970; Schmidt 1981, 1984; Flavell 1986; John 1988; Rubinsztein et al. 1995).

It is still an open question as to whether repetitive DNAs have a general, selectable function (for a detailed discussion of this issue, see John 1988), but it is obvious that even gross changes in the number and genomic organization of repetitive sequences may have little effect on the biology of a species. The possibility that repetitive DNA could play a pivotal role in the speciation process itself has been considered (Rose and Doolittle 1983). However, a role for repetitive DNA in the process of speciation is difficult to prove. Evidence comes from the fact that changes in the composition of the repetitive DNA fraction are often observed in closely related taxa which are in the process of speciation. Detailed phylogenetic analyses of a repetitive DNA family have to be performed before any conclusions may be drawn regarding an evolutionary function for this type of sequence.

In the present study we compare the tandem-repetitive DNA component in genomes of three chironomid species/subspecies. The two subspecies *Chironomus thummi thummi* and *C. t. piger* are quite closely related. Crosses between them produce fertile hybrids (Keyl and Strenzke 1956). In spite of the close relatedness, *C. t. thummi* has

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about 30% more DNA per genome than *C. t. piger* (Keyl 1965). The major part of this ''additional'' DNA consists of several families of tandem-repetitive, minisatellite-like sequences (Schmidt 1981, 1984; Hankeln 1990; Hankeln et al. 1994). One of these repetitive DNA families, the so-called Cla elements, were previously analyzed in detail. They are present in about 70,000 copies in the genome of *C. t. thummi*, while *C. t. piger* contains only approximately 10,000 copies (Schaefer and Schmidt 1981). Moreover, clusters of Cla elements are widely dispersed all over the *C. t. thummi* chromosomes but seem to be limited to the centromeric regions in *C. t. piger* (Schmidt 1984; Hankeln et al. 1994). This situation requires that either (1) an amplification and dispersal of Cla elements have occurred in the *C. t. thummi* subspecies only or that (2) dispersed high-copy-number Cla clusters were already present in the common phylogenetic ancestor of both subspecies and were subsequently eliminated from the chromosomal arm positions in *C. t. piger*. These alternatives imply different genomic mechanisms involved in the rearrangement of Cla elements over evolution. We would like to know which mechanisms of change do apply in this case. A possible way to trace the path of Cla element evolution is to investigate the organization of this sequence family in a third species, which is more distantly related to the *C. thummi* subspecies pair. This outgroup analysis should yield some information on the origin and organization of Cla sequences in a hypothetical ancestral genome, thereby indirectly giving evidence of the mechanisms involved in the evolution of this DNA family. From genomic Southern analyses we know that amongst nine *Chironomus* species tested only *C. t. thummi*, *C. t. piger*, and *C. luridus* produce clear signals upon Cla element hybridization (Hankeln 1990). Another species, *C. halophilus*, only gives a very weak signal on the blot, and none in in situ hybridizations. *C. luridus* and *C. halophilus* are both closely related to the *C. t. thummi* subspecies pair (Keyl 1962). In this study, we therefore investigate the molecular organization of Cla-element-related sequences in the genome of *C. luridus.*

Materials and Methods

C. t. thummi and *C. t. piger* were from permanent laboratory cultures at our institute. *C. luridus* animals were collected as egg masses from wildlife habitats by Prof. H.-G. Keyl (Bochum) and were reared in the lab to the fourth larval instar. Species were diagnosed cytologically by inspection of salivary gland polytene chromosome banding pattern (Keyl and Keyl 1959).

Chromosomal DNA was prepared from fourth-instar larvae as described by Schmidt et al. (1980). Genomic libraries of all species analyzed were constructed in lambda vector EMBL3 (Frischauf et al. 1983) after partial *Sau*3A digestion of genomic DNA and size fractionation of 15–20-kb fragments by gel electrophoresis. Recombinant phage DNA was in vitro packaged by using the Gigapack Gold extracts (Stratagene). The library was screened according to the method of Benton and Davis (1977) with a ^{32}P-labeled (Feinberg and Vogelstein 1983) heterologous Cla element probe from *C. t. thummi*. Relaxed hybridization and washing conditions were applied (2 × SSC; 55°C). Phage DNA was prepared by an unpublished method of W. Marzluff, involving polyethylene glycol precipitation of phage particles and subsequent sodium dodecylsulfate lysis.

Restriction fragments from recombinant clones hybridizing to the Cla element probe were subcloned into pUC18 (Yanisch-Perron et al. 1985) by standard techniques. Sequencing was performed on double-stranded templates (Chen and Seeburg 1985) with some modifications as described by Toneguzzo et al. (1988), using the T7 DNA polymerase sequencing kit from Pharmacia (Freiburg, FRG). Sequence compilation and editing was carried out by the DNASTAR software (London, UK). DNA sequences have been deposited in the EMBL database under the accession numbers X95597 and X95598.

In situ hybridization on salivary gland polytene chromosomes was performed using biotin-labeled probes with subsequent immunodetection (Langer-Safer et al. 1982). Details of the protocol are published in Hankeln and Schmidt (1987) and Schmidt et al. (1988).

Results

Characterization of Cla-Element-Containing Clones from C. luridus

Preliminary Southern and dot-blot hybridizations suggested that *C. luridus* contains sequences with similarity to *C. thummi* Cla elements. Therefore we constructed a *C. luridus Sau*3A genomic DNA library in lambda vector EMBL 3; 30,000 recombinant phages (approximately one genome equivalent) of the genomic library were screened with a cloned *C. thummi* dimeric Cla element probe under relaxed stringency hybridization/washing conditions (2 × SSC/55°C). We obtained three independent, relatively weakly hybridizing clones, of which two (Clalu 1 and 2) were arbitrarily chosen for a more detailed molecular characterization.

In clone Clalu 2, a 1.1-kb *Sal*I fragment was shown by Southern hybridization to contain the Cla-element-related sequences. The fragment was subcloned and sequenced (Fig. 1A). The *Sal*I site at the 5′ end of the sequence proved to be of genomic origin, while the 3′ *Sal*I site was vector derived. The genomic DNA thus terminates with a 3′ *Sau*3A site. The 1.1-kb *Sal*I fragment from Clalu 2 contains two copies of a tandemly repeated DNA sequence (Fig. 1A, boxed). The repeat in the sequenced fragment is 434 bp long. Repeat A putatively represents the complete copy. The second repeat (B), however, is shortened by 46 bp due to the fact that an internal *Sau*3A site of this element was cleaved during cloning and construction of the DNA library. The two repeats are identical in sequence over the 388 bp, which can be compared. Due to the presence of a characteristic *Hin*fI restriction site, we termed this new tandem repeat family ''*Hin*f(lur) elements.'' Within both *Hin*f(lur) copies, we find a 133-bp stretch revealing 78% sequence similarity to the Cla element consensus sequence from *C. thummi* (Schmidt 1984; Fig. 1B). The Cla portion of the *Hin*f(lur) element is equivalent to 1.16 Cla elements.

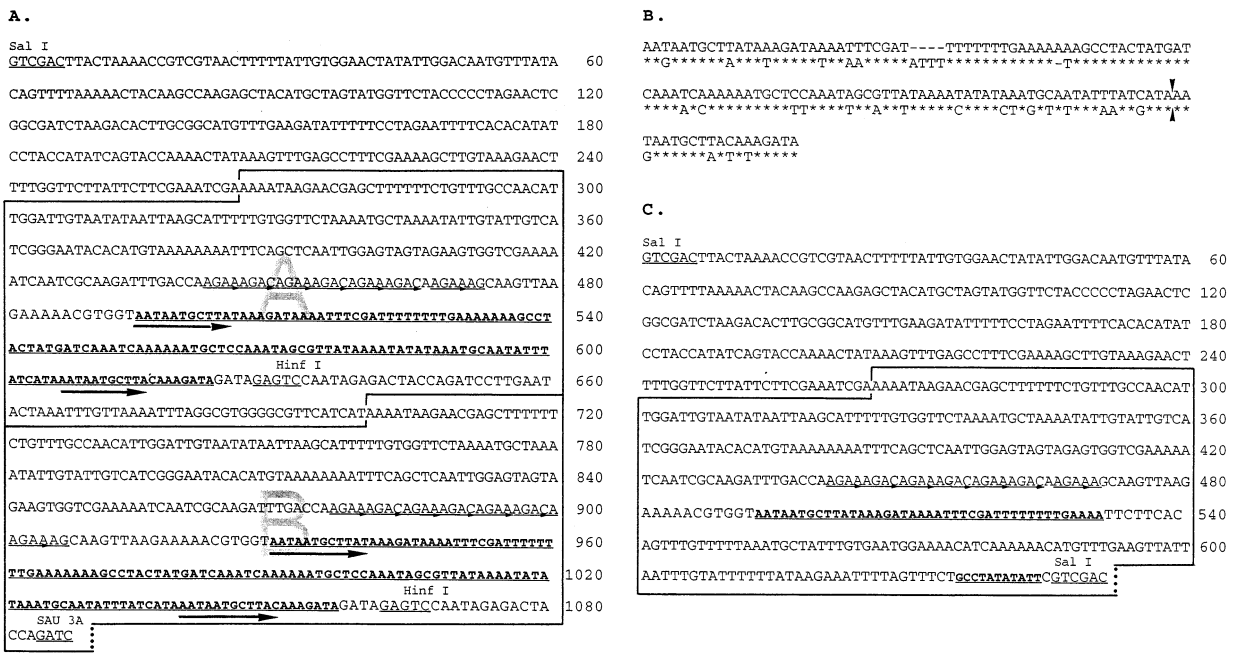

Fig. 1. A Nucleotide sequence of the 1.1-kb *Sal*I fragment from clone Clalu 2, containing Cla-element-related sequences. The sequence starts with the genomic *Sal*I site (at the right end in Fig. 3B) and ends with the genomic *Sau* 3A site used for cloning into the lambda vector. *Hinf*(lur) tandem repeats are boxed. The second repeat *B* is incomplete due to the cloning strategy. The characteristic *Hin*fI sites are indicated (Hf). The Cla element portion of the *Hinf*(lur) repeat is *underlined and printed in bold characters.* A simple sequence repetition is marked by *short arrows.* The *long arrows* indicate the portion of the Cla-element-related sequence which is present twice in the complex *Hinf*(lur) repeat. **B** Comparison of the 133-bp Cla element portion of the *Hinf*(lur) element (*upper line*) to the Cla element consensus sequence from *C.t. thummi* (*lower line;* Schmidt 1984). Identical nucleotides are marked by *asterisks;* insertions/deletions, by *dashes. Arrow-heads* indicate the normal end of one Cla repeat unit and the start of the second, incomplete Cla repeat. **C** Nucleotide sequence of the 651-bp *Sal*(lur) repeat from clone Clalu 1. The sequence starts and ends with a *Sal*I site. The *Hinf*(lur)-element-related part of the sequence is *boxed.* A truncated Cla element sequence of 52 bp (*underlined and printed bold*) is interrupted by a 101-bp non-Cla sequence. This insertion is flanked by 4-bp direct repeats (*arrows*). A stretch of simple sequence repetitions is *underlined by multiple arrows.*

Thus, the Cla elements, which are the dominant tandem-repetitive DNA family in *C. thummi,* are an integral part of a larger tandem repeat in the genome of *C. luridus.*

In lambda clone Clalu 1, a 0.65-kb *Sal* fragment was found which cross-hybridizes strongly with the *Hinf*(lur)-element-containing 1.1-kb fragment from Clalu 2, but only very weakly with the *C. thummi* Cla element probe (results not shown). The 0.65-kb fragment was therefore subcloned and sequenced, too (Fig. 1C). Both flanking *Sal*I sites are of genomic origin. The sequence comparison between Clalu1 and Clalu 2 shows that the first 532 bp at the 5′ end of the 0.65-kb Clalu 1 fragment is identical to the corresponding 5′ part of the 1.1-kb fragment from Clalu 2. The 0.65-kb Clalu 1 fragment thus contains the 5′ flanking DNA plus the first half of a *Hinf*(lur) element. Within this *Hinf*(lur) element-homologous region, however, the region containing the Cla element is scrambled: Only 51 bp of Cla element sequence is left, and this Cla-element-related part is interrupted by a 101-bp non-Cla-element sequence. This interruption is flanked by 4-bp direct repeats, reminiscent of target-site duplications often being generated during integration of mobile DNA sequences. This possible target-site duplication, however, cannot simply be explained by a duplication of 4 bp of Cla sequence, and its origin therefore remains unclear.

Gel electrophoresis of *Sal*I-cut Clalu 1 DNA generates a banding pattern in which the 0.65-kb band stains overproportionately strong. This shows that multiple copies of this fragment are present in Clalu 1. Furthermore, after Southern hybridization of *Sal*I-digested Clalu 1 DNA with the 0.65-kb *Sal*I repeat, multiples of the 0.65-kb fragment appear as hybridizing bands (data not shown). This is a clear indication that the 0.65-kb fragment is organized in a tandemly repeated way and that this fragment represents an independent repeating unit. We call it the ''*Sal*(lur) element.''

At least part of the *Sal*(lur) element sequence seems to be present as a repetitive DNA in different *Chironomus* species, e.g., *C. tentans, C. pallidivittatus, C. t. thummi, C. t. piger, C. pseudothummi,* and *C. plumosus* (genomic Southern blot results not shown).

Genomic Organization of the Cla-Element Homologous Sequences in C. luridus

The chromosomal localization of clones Clalu 1 and 2 as well as of subcloned *Sal*(lur) elements was analyzed by in situ hybridization to *C. luridus* polytene chromosomes. Both clones show a strong signal at the centromeric end of the acrocentric chromosome IV. Addition-

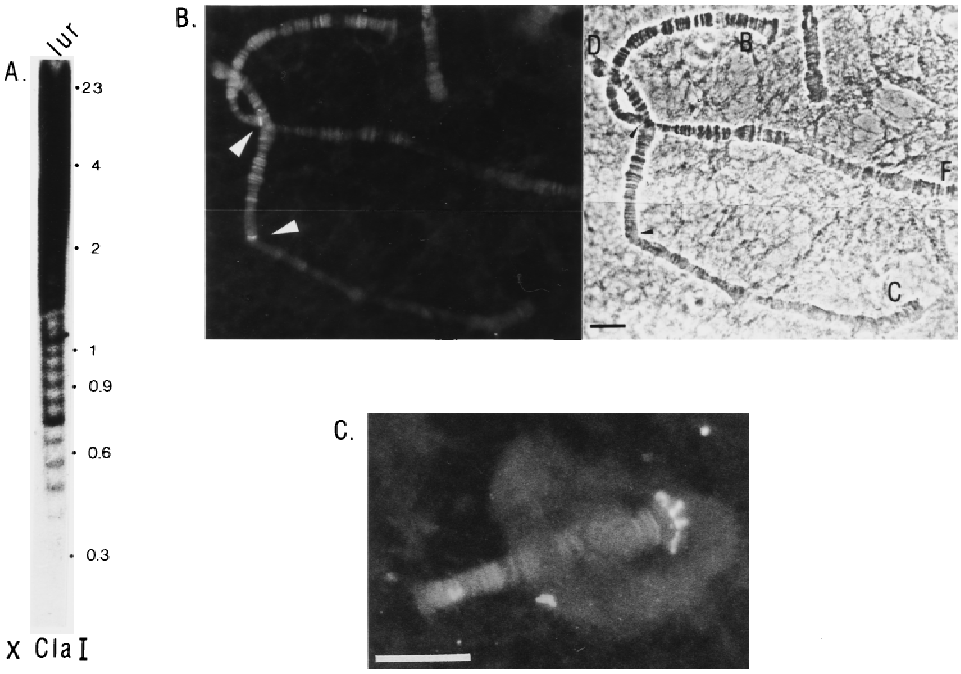

Fig. 2. Genomic organization and chromosomal distribution of Cla-element-related sequences in *C. luridus.* **A** Southern hybridization of *Cla*I-digested *C. luridus* genomic DNA with radioactively labeled heterologous Cla elements from *C. t. thummi* as probe. The ''ladder'' of bands is typical for tandem-repetitive DNA, showing a repeat unit length of 80–90 bp. Molecular weights are given at the right margin (in kb). **B** (Left) In situ hybridization of *C. luridus* polytene chromosomes with biotin-labeled Cla elements from *C. t. thummi.* Only the centromeric regions of chromosomes *B/F* and *C/D* (arm designations according to Keyl 1962) show weak signals (see arrows). (Right) The corresponding phase-contrast micrograph. Bar represents 10 μm. **C** The subcloned *Sal*(lur) element from Clalu 1 shows a strong in situ hybridization signal only at the centromeric heterochromatin of the acrocentric chromosome IV. This chromosomal site probably represents the origin of both *Hin*f(lur) and *Sal*(lur) elements (see Results).

ally, each one hybridizes less strongly to an additional site on one of the large chromosomes (not shown). The subcloned *Sal*(lur) element probe hybridizes merely at the centromere of chromosome IV (Fig. 2C). These results suggests that *Sal*(lur) elements and *Hin*f(lur) elements may both originate from this chromosomal region.

We also used heterologous Cla elements from *C. thummi* for in situ hybridization in *C. luridus* (Fig. 2B). Under moderately stringent hybridization conditions, the *thummi* Cla probe hybridizes selectively at the putative centromeric regions of the large chromosomes *C/D* and *B/F* (chromosome arm designations according to Keyl 1962). The chromosome IV centromere containing the *Hin*f(lur) and *Sal*(lur) repeats did not react with the *thummi* Cla probe under our hybridization conditions. These in situ hybridization results point to the existence of two different Cla-element-related sequence components in *C. luridus:* (1) the *Sal*(lur) and *Hin*f(lur) repeats, located at the centromere of chromosome IV, and (2) Cla sequences more closely related to the Cla family of *C. thummi,* located at the centromeres of chromosomes *C/D* and *B/F.*

By genomic Southern analysis, we could clearly show that in addition to *Hin*f(lur) and *Sal*(lur) elements a third type of Cla-element-related repeat exists in *C. luridus* (Fig. 2A). *Cla*I-digested *C. luridus* DNA hybridized with the heterologous Cla element probe from *C. thummi* produces a ladder of signals characteristic for tandem-repetitive Cla elements. These tandem-repetitive Cla elements in *C. luridus* display a repeat length of 80–90 bp which is different from the repeat length of Cla elements in *C. thummi* (120 bp). Possibly, these tandem-repetitive Cla element clusters are too large or too unstable to be represented in the genomic DNA library from *C. luridus.* Attempts to clone these tandem repeats directly from gel-electrophoresed *C. luridus* genomic DNA were unsuccessful, presumably because of the low copy number of the repeats which could not be seen as distinct bands against the background smear of genomic restriction fragments.

Discussion

Cla elements are a family of tandem-repetitive sequences first found in the genome of the subspecies pair *C. t. thummi* and *C. t. piger* (Schmidt 1981, 1984; Schmidt and Godwin 1983; Hankeln et al. 1994; Kraemer and Schmidt 1994). The present analysis shows that Cla-element-homologous sequences in the related species *C. luridus* are organized at least in three different ways (Fig. 3): (1) Within the *C. luridus* genome we find tandemly repeated, ''naked'' Cla elements, which may be similar to those present in *C. thummi,* albeit showing a shorter

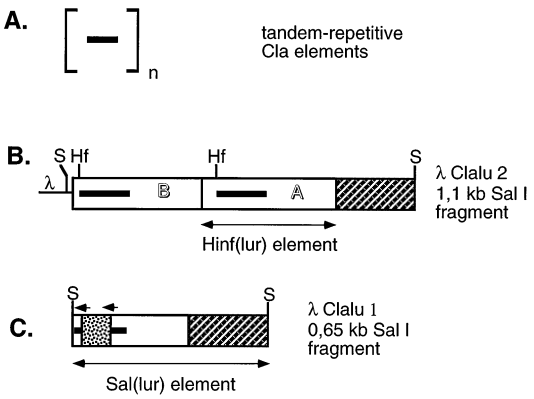

Fig. 3. Cla-element-related sequences in *C. luridus* are organized in three different ways. The evolution of these repeats may be explained by stepwise mutations and reamplifications (see Discussion). We find: **A** ''Naked'' tandem-repetitive Cla elements with a repeat length of 80–90 bp. **B** Complex Cla element sequences (133 bp; *black bars*) which are part of the 434-bp *Hinf*(lur) element in clone Clalu 2. The characteristic *Hin*fI site (*Hf*) and the *Sal*I sites (*S*) used for subcloning are indicated. The left *Sal*I site is derived from the lambda vector. The *Hinf*(lur) element flanking sequence on the right is *shaded.* **C** Remainders of the Cla sequences (*black bars*) are part of the 650-bp *Sal*(lur) supra repeat of clone Clalu 1. A 101-bp non-Cla sequence (*stippled*) has inserted into the truncated Cla element. This insertion is flanked by 4-bp duplications (*small arrows*). The left half of the *Sal*(lur) element (*open box*) corresponds to the *Hinf*(lur) sequence while the right half (*shaded*) corresponds to the *Hinf*(lur) flanking DNA.

repeat length (Fig. 3A). (2) Cla elements in *C. luridus* can be packed together with flanking sequences to give the complex *Hinf*(lur) elements (Fig. 3B), and (3) the Cla element sequences can be further rearranged by insertions and deletions as seen in the *Sal*(lur) tandem repeats (Fig. 3C). These complex repeat structures may have evolved stepwise:

- Within the 434-bp tandem-repetitive *Hinf*(lur) element there is a 133-bp Cla element sequence insertion, corresponding to 116% of a typical *thummi* Cla element. We suggest that the *Hinf*(lur) element has been generated by a transpositional integration of the Cla sequence and subsequent amplification of this sequence together with flanking DNA. Possible target-site duplications which are generally flanking transposed DNA cannot be found at the borders of the Cla sequence. However, we have evidence that Cla elements in *C. t. thummi* may transpose without generating target-site duplications (Hankeln et al. 1994).
- The *Sal*(lur) element may have evolved from the *Hinf*(lur) repeat. It consists of a *Hinf*(lur)element-related part plus a sequence which is known to flank *Hinf*(lur) elements at least in one case (clone Clalu 2). Thus, the ancestor of the *Sal*(lur) element has probably been generated by a co-amplification of one *Hinf*(lur) repeat plus flanking DNA. In another step, the major part of the Cla-element-related sequence has been lost, perhaps during integration of the 101-bp non-Cla sequence.

The example of *Hinf*(lur) and *Sal*(lur) elements shows that complex repetitive sequences are derived from one another during evolution by the process of insertion, deletion, and amplification of DNA together with flanking regions.

These mechanisms of evolution may be typical for tandem-repetitive DNA, since they have also been observed in other organisms. In cucumber, satellite type IV has been formed by insertion of a 180 bp sequence, which is similar to satellite types I–III, into another unrelated sequence (Ganal and Hemleben 1988). Telomeric tandem repeats in *Secale* have been formed by rearrangements and amplification (Flavell 1986), and comparable events have been described in *Xenopus laevis* (Meyerhof et al. 1983, 1987). In the bovine genome, satellites 1.711 (Streeck 1981) and 1.709 (Skowronski et al. 1984) have evolved by integration of DNA into already-existing tandem repeats. Subsequently, the new repeat units containing the insertions were amplified. In humans, subfamilies of satellite I and of alpha satellite have been shown to contain one inserted Alu element per tandem repeat (Frommer et al. 1984; Lund Jorgensen et al. 1986).

Changes within repeats by various mutation mechanisms and subsequent in situ amplification of the new repeat type may lead to the generation of a chromosome-specific organization of repetitive DNA (Lund Jorgensen et al. 1986; Alexandrov et al. 1991; Waye and Willard 1989). The presence of *Hinf*(lur) and *Sal*(lur) repeats in *C. luridus* may be limited to the centromere of chromosome IV. On the other hand, the *C. thummi*-type, ''naked'' Cla sequences in *C. luridus* occur only on chromosomes *C/D* and *B/F*. In addition, they display the *C. luridus*-specific repeat length of 80–90 bp. Similar processes have shaped satellite DNA families in human chromosomes: the 2.4-kb satellite I repeat, which is characterized by the integration of one Alu element per repeat unit, represents a Y chromosome–specific variant (Frommer et al. 1984).

The *thummi*-type Cla elements in *C. luridus* seem to be confined to the putative centromeric regions of two large chromosomes. Concerning the chromosomal distribution, the Cla sequences in *C. luridus* are thus organized like the related repeats in *C. t. piger*. The location of Cla elements in a pericentric position therefore seems to be a phylogenetically ancestral feature. Consequently, the hundreds of euchromatic loci of Cla elements in the subspecies *C. t. thummi* would then be the result of amplification and dispersion events. We would like to conclude that amplification and transposition (rather than elimination) have led to the enormous difference in copy number and genomic distribution of Cla elements in the subspecies *C. t. thummi* and *C. t. piger*. We are currently investigating the question of whether other tandem-repetitive DNA families also show the same behavior during evolution.

Acknowledgments. We would like to thank Prof. H.-G. Keyl (Bochum) for the *C. luridus* larvae, Mrs. Beth lhm for typing the manuscript, and Mrs. Ursula Boell for photographic work. This work was supported by the Deutsche Forschungsgemeinschaft (DFG; Sch 523/3-7).

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