Volume 17 Number 13 1989 Nucleic Acids Research

Nucleotide sequence, genomic organization and evolution of a major repetitive DNA family in tilapia (*Oreochromis mossambicus/hornorum*)

Jonathan M.Wright

Department of Biology, Dalhousie University, Halifax, Nova Scotia B3H 4J1, Canada

Received April 7, 1989; Revised and Accepted May 31, 1989 EMBL accession no. X15026

ABSTRACT

A highly repetitive DNA sequence from tilapia (*Oreochromis mossambicus/hornorum*) has been cloned and sequenced. It is a tandemly arrayed sequence of 237 bp and constitutes 7% of the fish genome. The copy number of the repeat is approximately 3×10^5 per haploid genome. DNA sequence analysis of 7 cloned repeats revealed a high degree of conservation of the monomeric unit. Within the monomeric unit, a 9 bp AT rich motif is regularly spaced approximately 30 bp apart and may represent the progenitor of the amplified sequence. One cloned repeat, Ti-14, contained a 30 bp deletion at a position flanked by a 7 bp direct repeat. The Ti-14 sequence appears to have been amplified independently of the major 237 bp tandem array. A higher-order repeat unit, defined by longer-range periodicities revealed by restriction endonuclease digestion, is further imposed on the tandem array.

INTRODUCTION

Eucaryotic genomes contain highly reiterated DNA elements of related sequence (1). These repetitive sequences can vary in copy number from $10-10^6$ per haploid genome and constitute less than 1% to greater than 66% of the nuclear DNA (2). They occur in two prevalent patterns: As short (average 300 bp) and long (average 6000 bp) sequences interspersed with single-copy or repetitive sequences (3–5). The structural organisation of dispersed repetitive DNA suggests their duplication and dispersion proceeds by either DNA-mediated transposition or by retrotransposition of viral and non-viral RNA intermediates (6). Alternatively, repetitive sequences may be clustered in the vicinity of centromeres, telomeres, or less frequently, in interstitial regions of chromosomes (2,7). These localized repetitive DNA families may consist of repeating dinucleotides, such as $(CA)_n$ or $(CT)_n$, or satellite DNAs which are characterized by tandem repetition of a monomeric unit. The monomeric unit can vary in length from a simple sequence of a few nucleotides to several thousand nucleotides. Although satellite DNA was originally isolated from bulk DNA by isopycnic centrifugation (2), more recently the term encompasses any tandemly repeated sequence (8).

Tandemly arrayed repetitive sequences have attracted considerable attention from evolutionary biologists primarily because different but closely related species often show satellite DNA profiles which are characteristic for that species only (2). Attempts have been made to assign a functional role to satellite DNA in both cellular and evolutionary processes. However, these functional hypotheses have not been supported by experimental data (see 7). Others (9–11) have proposed that these repeated sequences have no function at all, and without any effect on phenotype, satellite sequences can arise, spread throughout the genome and maintain themselves in a 'functional vaccum'. Despite extensive study

of satellite DNAs from mammals (12–15), amphibians (16) and invertebrates (17,18), their role, if any, in genome structure and evolution, or genetic regulation, still remains obscure.

With the exception of one report (19), no information is available on the sequence, genomic organisation and evolution of satellite DNA in teleost fishes, a large evolutionary class of animals. In order to advance our understanding of genome organisation and taxonomy of teleost fishes, a systematic survey of cloned repetitive sequences from representatives of the tilapias has been initiated in this laboratory. In this report I describe the cloning, sequence and genomic organization of a satellite DNA family from *Oreochromis mossambicus/hornorum*, an important species in tropical aquaculture.

MATERIALS AND METHODS

Preparation of genomic DNA

Live specimens of tilapia (*Oreochromis mossambicus/hornorum*) were obtained from a breeding stock in its fourth generation at Dalhousie University from Dr. R. Doyle. Blood was obtained from anaesthetized fish by cardiac puncture. Approximately 1 mL of blood was subjected to centrifugation at 50×g for 10 min to sediment cells. The cells were washed with saline (0.85% NaCl) and recovered by centrifugation. Following resuspension in 5 mL of 100 mM Tris-HCl, pH 8.0, 40 mM EDTA, the cells were lysed by addition of an equal volume of 100 mM Tris-HCl, pH 8.0, 40 mM EDTA and 0.2% SDS. An equal volume of saline-saturated phenol was mixed thoroughly, but gently, with the lysed cell suspension and the emulsion subjected to centrifugation at 50×g for 5 min. The aqueous phase was removed and the phenol extraction repeated. The aqueous phase was extracted with an equal volume of chloroform:isoamyl-alcohol (24:1 v/v) and then 0.1 volume of 4M ammonium acetate was added. An equal volume of isopropanol was added and the precipitated DNA was wound onto a glass rod. The DNA was dried *in vacuo* and redissolved in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA.

Preparation of single-stranded and replicative forms of recombinant bacteriophage M13

M13 bacteriophage mp18 and recombinant phage DNA was purified according to Messing (20). DNA from the replicative form was prepared by the alkaline lysis procedure essentially as described by Maniatis *et al* (21).

Endonuclease digestion, ligation and transformation

All restriction endonuclease digestion and ligation reactions were conducted according to the conditions recommended by the suppliers (Bethesda Research Laboratories, Gaithersburg, MD; Amersham Corporation, Oakville, Ontario; Boehringer-Mannheim, Ontario). Competent *Escherichia coli*, was prepared and transformed by standard procedures (22).

Southern blot transfer

DNA fragments were size fractionated by electrophoresis on 1.3% agarose horizontal slab gels in the presence of 90 mM Tris-borate, 90 mM boric acid, 2 mM EDTA and 0.5 μg/μl ethidium bromide. The gels were destained and photographed using a UV transilluminator. Prior to blotting, the DNA was denatured by incubation of the gel in 0.5 M NaOH, 1.5 NaCl, for 30 min, followed by incubation in 1.5 M Tris-HCl (pH 7.4), 1.5 M NaCl. Transfer to Hybond-N filter (Amersham Corporation) was performed according to Southern (23) using a reservoir of 20×SSPE (3.6 M NaCl, 0.2M Sodium phosphate, 0.02M EDTA, pH 7.7). After blotting for 48 hours, the filters were washed in 2×SSPE and baked for 2 hr at 80°C *in vacuo*.

Radiolabelling of DNA

Genomic DNA and purified RF M13 DNA was radiolabelled with ^{32}P-dATP and ^{32}P-dATP by nick-translation (24). Hybridization probes were prepared from single-stranded M13 DNA clones by primer extension using the universal primer (20). Routinely, the specific activity of the radiolabelled DNA was $1-5\times10^8$ cpm/μg.

Hybridization

Filters were incubated for 1−2 hr at 42°C in a solution of 50% formamide, 5×SSPE, 1×Denhart's (0.1% B.S.A., 0.1% ficol, 0.1% polyvinylpyrrolidone), 0.1% SDS, 0.1 mM EDTA and 100 μg/mL yeast tRNA. Approximately $1-2\times10^5$ cpm/mL of radiolabelled probe, denatured by boiling for 5 min, was added to the hybridization mixture. Hybridization proceeded at 42°C for 24−48 hr. The filters were washed twice in 2×SSPE, 0.1% SDS at room temperature for 15 min, at 65° in 1×SSPE, 0.1% SDS for 15 min. and then at 65°C in 0.1×SSPE, 0.1% SDS for 15 mins. The filters were exposed to X-ray film for 2−24 hr.

Nucleotide sequence analysis

DNA sequencing was performed according to the chain terminating dideoxy method (25) using protocols for template preparation and gradient gel electrophoresis of Bankier and Barrell (26).

RESULTS AND DISCUSSION

Cloning and nucleotide sequence of the Hae III repeat

Digestion of tilapia DNA with restriction endonuclease *Hae* III, followed by electrophoresis on 1.3% low melt agarose, revealed a prominent restriction fragment of approximately 240 bp. The fragment was purified, ligated with *Hinc* II digested M13 mp18 and used to transform *Escherichia coli*. Fifty-five recombinant phage were recovered and lysates of individual phage were dotted onto nylon filter. Following hybridization to total tilapia DNA, 48 clones gave strong hybridization signals indicating the presence of a repetitive sequence. Seven of the recombinant phage were selected for further analysis.

Figure 1 shows the nucleotide sequence of the seven cloned *Hae* III repeats. Six of the clones were approximately 237 bp in length and exhibited less than 2% sequence variation relative to a derived consensus sequence. One cloned repeat sequence, Ti-14, contained a 30 bp deletion at a position flanked by a 7 bp direct repeat. The presence of the 7 bp direct repeat suggests that the 30 bp deletion occurred by intrastrand base pairing and subsequent excision. Evidence presented later indicates that the deletion did not arise during cloning and propagation of the sequence in *E. coli*. In addition to the 30 bp deletion, Ti-14 displayed greater sequence variation to the consensus sequence (92% homology) than the other cloned *Hae* III repeats.

An imperfect 9 bp AT rich motif with the consensus sequence (CTGAAAAAC) is spaced approximately every 30 bp throughout the *Hae* III repeat (Figure 1). Two of the 9 bp sequences at positions 168−185 and 190−208 appear to have been duplicated in tandem. The presence of this regularly spaced 9 bp AT rich motif within the repeat suggests an underlying oligonucleotide sequence is the progenitor of the monomer unit. This monomer unit may have arisen by tandem duplication of a 30 bp progenitor with subsequent divergence in the duplicated sequence. Similarly, Horz and Altenburger (13) deduced a 30 bp progenitor sequence with an A rich nonanucleotide stretch in a mouse satellite DNA.

Genomic organisation of the Hae III repeat

Highly repeated sequences occur in two prevalent patterns: either dispersed throughout

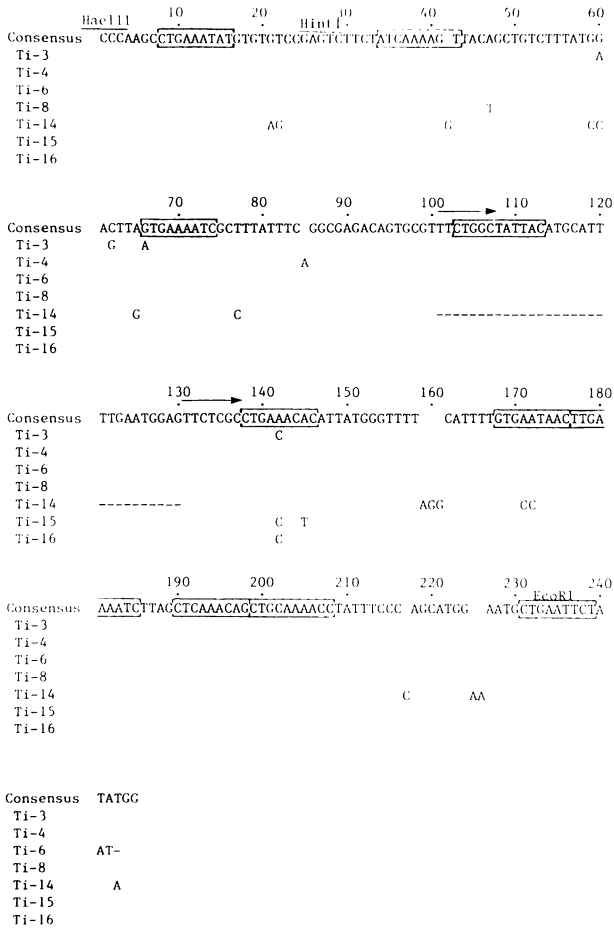

Figure 1. Nucleotide sequence of seven cloned *Hae* III repeats. A consensus sequence is shown above where an unique consensus base was assigned if the base was present in more than 50% of the repeat sequences. Only base changes to the consensus sequence are shown in individual repeats. The position of base gaps in repeat or consensus sequences are based entirely on the maximum homology and are otherwise arbitrary. A dash (—) signifies a deletion. Arrows indicate the position of a direct repeat. Important restriction endonuclease recognition sequences are shown. Boxed sequences outline the regularly spaced 9 bp AT rich motif.

the genome or clustered in tandem arrays. In order to determine the organisation of the *Hae* III repeat, tilapia genomic DNA was subjected to increasingly limited digestion with *Hae* III. Southern blot and hybridization to radiolabelled Ti-16 revealed a ladder of bands in the lane containing DNA digested to completion (Figure 2a). The 'steps' in the ladder correspond to multimers of the 237 bp monomer which arise presumably due to mutation in the *Hae* III recognition sequence in some subsets of the repeat. With decreasing digestion of genomic DNA (lanes left to right), an increasing number of higher molecular weight fragments hybridized to Ti-16, with the major bands corresponding to multimers of 237

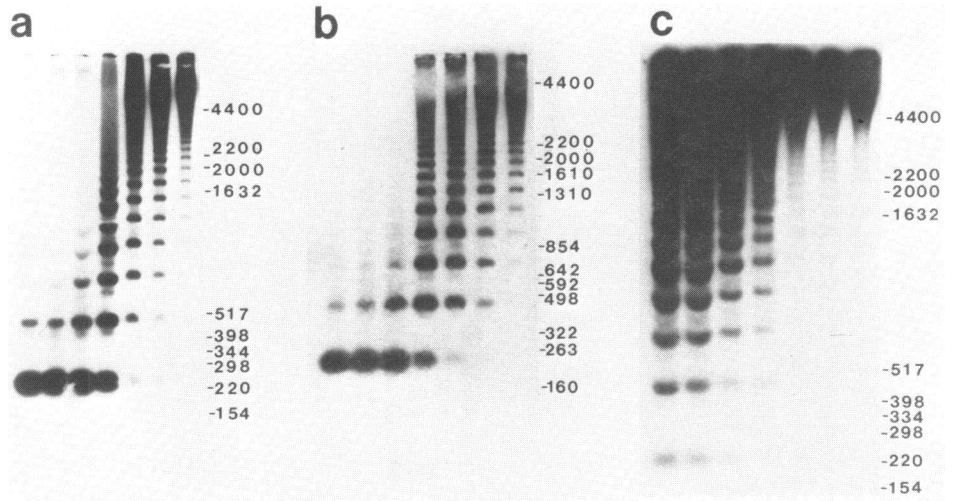

Figure 2. Southern analysis of partial digests of 10 μg/lane total tilapia DNA obtained with *Hae* III (panel a), *Eco* R1 (panel b) and *Hinf* I (panel c), using Ti-16 as a hybridization probe. Units of enzyme/μg DNA (lanes left to right) 5, 2.5, 1, 0.5, 0.25, 0.1 and 0.05. Molecular weight markers are shown to the left of each panel.

bp. As many as 20 bands can be readily resolved in the autoradiogram demonstrating that the repeat is arrayed in tandem, with some subsets containing more than twenty monomeric units.

A similar pattern of hybridizing fragments was generated by partial digestion of tilapia DNA with restriction endonuclease *Eco* RI (Figure 2b) which has a recognition sequence at position 233 in the *Hae* III repeat (Figure 1). The partial digestion pattern generated by *Hae* III and *Eco* RI demonstrates that the repeat is tandemly arrayed in the same orientation and therefore the repeated sequences exhibits an organisation consistent with satellite DNAs from other species (17).

A minor ladder of bands consisting of multiples of a 214 bp fragment is evident in the partial *Hae* III and *Eco* RI digests of tilapia DNA. This minor ladder corresponds to steps equivalent in size to the Ti-14 sequence and suggests deletion of 30 bp in the 237 bp monomer followed by subsequent amplification of the Ti-14 sequence. Because the Ti-14 sequence is found as a discrete ladder of 214 bp oligomers, and not as a combination of oligomers of 214 bp and 237 bp fragments, this is indirect evidence that the 214 bp repeat is organized in a tandem array of at least 5 monomers in length, but unlinked to the major subsets of the 237 bp tandem array. The segregation of these two different types of homologous sequences in different regions argues for the evolution of each from a common ancestral sequence by a mechanism of sequence divergence followed by an amplification mechanism that keeps more closely related sequences in closer proximity. More definitive proof of this unlinked organisation for the minor and major subsets of the satellite DNA should be gained from sequence analysis of cloned longer arrays.

Methylation of the Hae III repeat

Digestion of tilapia DNA with *Hinf* I produced a markedly different pattern of restriction fragments that hybridized to Ti-16 than did *Hae* III and *Eco* RI limited digests. Instead

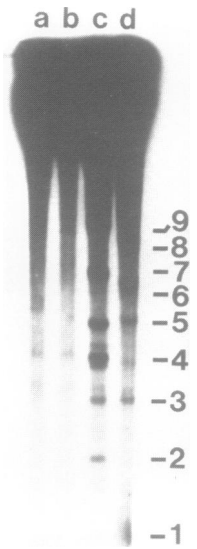

Figure 3. Southern analysis of total genomic DNA hybridized to Ti-16. Restriction endonucleases used are: *Bam* HI (a); *Cla* I (b); *Hind* III (c) and *Pst* I (d). Numbers to the left of the autoradiogram indicate multimers of the 237 bp repeat.

of a prominent band of 237 bp and two minor bands of 474 bp and 711 bp hybridizing to Ti-16 in the complete digestion of tilapia DNA, as was seen with *Hae* III and *Eco* RI digestion, *Hinf* I digestion generated a ladder of numerous bands with increasingly stronger hybridization of Ti-16 to higher molecular weight DNA fragments (Figure 2c). The major fragments correspond in size to multimers of the 237 bp monomeric unit. Few monomers of the repeat were released by *Hinf* 1 digestion. A ladder of minor *Hinf* I fragments hybridized to Ti-16 corresponding in size to multimers of a 214 bp monomeric unit, which presumably are related to the Ti-14 sequence. Partial digestion with *Hinf* I did not generate a loss of the lower molecular weight bands with a concomitant increase in hybridization to higher molecular weight restriction fragments as was seen with *Hae* III and *Eco* RI digests of tilapia DNA. With increasing partial digestion by *Hinf* I, the hybridization signal at each multimer of the repeat diminished equally with the appearance of increased hybridization to restriction fragments greater than 20 Kbp.

One interpretation for this result is that the *Hinf* I recognition sequence has mutated in most of the *Hae* III repeats. This is unlikely as all seven cloned repeats contained a *Hinf* I recognition sequence that was susceptible to digestion with this restriction endonuclease after passage through *E. coli*. A more plausible explanation is that internal monomeric units of the tandem array are methylated at the adenine residue of the *Hinf* I recognition sequence (27). *Hinf* I is reported to cleave the hemimethylated sequence GANTmC (28). To obtain the digestion pattern shown in figure 2c, the *Hinf* I recognition sequence in the two flanking monomeric units of subsets of the tandem array must be

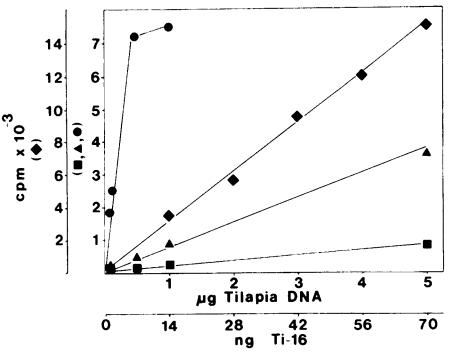

Figure 4. Estimation of copy number of the *Hae* III repeat. Graded amounts of tilapia genomic DNA digested with *Eco* R1 and graded amounts of Ti-16 DNA digested with *Bam* HI and *Hind* III were hybridized to the Ti-16 insert following Southern blotting. The regions of the filter containing radioactivity were detected by autoradiography and then excised. The amount of radioactivity that hybridized to Ti-16 inserts, and multimers of the repeat was plotted as a function of the quantity of DNA immobilized. Ti-16 (◆); monomer (●), dimer (▲) and trimer (■) of repeat in tilapia DNA.

unmethylated and therefore susceptible to digestion with *Hinf* I. Thus, cleavage with *Hinf* I of a trimer with an internal monomer methylated at the *Hinf* I recognition sequence would release a dimeric unit of the tandem array; a tetramer with two internal monomers, methylated at the *Hinf* I recognition sequence would release a trimeric unit of the tandem array; etc. Furthermore, these discrete internally methylated tandem arrays of dimers, trimers, tetramers etc., must be interspersed with non-repeat DNA, otherwise partial digestion with *Hinf* I should have produced loss of the shorter tandem arrays with the appearance of longer tandem arrays. This did not occur (Figure 2, compare panels a and c).

The methylation of the *Hinf* I recognition sequence is unusual, as the quantity of 6-methyladenine in animal DNA is normally very small, with most methylation being found as 5-methylcytosine (29). It is not possible to readily test this hypothesis as no isoschizomer is available that cleaves the methylated *Hinf* I recognition sequence. The data suggests, however, that methylation, a common modification of repetitive DNA (30), may occur as 6-methyladenine in the *Hinf* I recognition sequence of tilapia satellite DNA.

Hierarchical organisation of the Hae III repeat

Digestion of tilapia genomic DNA with selected restriction endonucleases, whose recognition sequences were absent in the seven cloned repeats, produced a reverse ladder in the autoradiogram with increased hybridization of Ti-16 to larger DNA fragments (Fig. 3). The majority of major fragments that hybridized to Ti-16 were multimers of 237 bp, and this pattern of hybridization to Ti-16 indicates the restriction sites appeared by mutation in some repeats. Digestion of tilapia DNA with *Hind* III released major fragments resolved in the autoradiogram consisting of 2, 3, 4, 5, 7 and 9 monomeric units of the 237 bp repeat, but not abundant fragments consisting of 1, 6 and 8 monomeric units. Whereas, complete digestion with *Pst* I released abundant fragments hybridizing to Ti-16 with sizes corresponding to 3, 5, 6, 8 and 9 monomers of the repeat, but not fragments consisting of 1, 2 and 4 monomeric units. Apparently a hierarchial order is superimposed on the repeat of the monomeric unit where mutations have occurred within monomers giving rise to very specific subsets of the tandem arrays. These multiple periodicities have been

observed in satellite DNA of other organisms (7,12), and for human α-satellite, the organisation and nucleotide sequence of multimeric higher-order repeat units are specific for a particular chromosome (8, 31, 32).

Copy number of the Hae III repeat

Clone Ti-16 was used to estimate the number of copies of the *Hae* III repeat in the tilapia genome. Serial dilutions of known quantities of tilapia DNA digested with *Eco* RI and Ti-16 DNA were size-fractionated by gel electrophoresis and blot-hybridized (23) to the Ti-16 insert. Following autoradiography, the X-ray film was used as a template to excise regions of the filter that contained hybridized DNA. The amount of radioactivity that hybridized to monomer, dimer and trimer subsets of the repeat in the tilapia genomic DNA, and to the Ti-16 insert, was determined by liquid scintillation spectrometry. Figure 4 shows the amount of radioactivity that hybridized to various DNA restriction fragments as a function of quantity of DNA immobilized on the nylon filter. Based on the amount of radioactivity that hybridized to the 237 bp, 474 bp and 711 bp fragments released by digestion of tilapia genomic DNA with *Eco* RI, the monomer, dimer and trimer units of the repeat represent 93%, 6% and 1%, respectively, of satellite sequences in the tilapia genome. Approximately 35 ng of Ti-16 hybridized to the same amount of Ti-16 probe as 0.5 μg of tilapia DNA (7500 cpm above background). Therefore, approximately 7% of the tilapia genome consist of the *Hae* III tandem array. With a 'c' value of 1 − 1.2 pg (33,34), there are approximately 3×10^5 copies of the *Hae* III repeat per haploid genome.

Homology of Hae III repeat to DNA sequences in various organisms

Hybridization of Ti-16 DNA to a 'Zoo'-blot at low stringency (filter washed at 65°C in 1×SSPE) showed that little or no homology exists between this tilapia satellite sequence and DNA sequences present in the genomes of human, mouse, Atlantic salmon (*Salmo sala*), rainbow trout (*Salmo gairdneri*), Atlantic cod (*Gadus morhua*), haddock (*Melanogrammus aeglefinus*), pollock (*Pollachius virens*), goosefish (*Lophius americanus*), goldfish (*Carassius auratus*), Medaka (*Oryzias latipes*), *Artemia salina* and *Physarum polycephalum*. Only after long exposure was a weak hybridization signal detected in DNA samples of Atlantic salmon, rainbow trout, cod, haddock and pollock (data not shown). Due to the high AT content of the tilapia satellite sequence (61%) and the conditions of low stringency used in the hybridization reaction, this result does not provide compelling evidence of significant homology between the satellite DNA of tilapia and DNA sequences in these fishes.

In conclusion, I have cloned and sequenced representatives of a highly repeated DNA family from tilapia. Based on its size, tandem repetition, abundance and sequence conservation among cloned representative members, this repetitive DNA displays all the characteristics of a satellite DNA. A higher-order repeat unit, defined by longer-range periodicities revealed by restriction endonuclease digestion, is superimposed on the tandem array consistent with the hierarchical organisation of satellite DNA in a number of vertebrate and invertebrate genomes (7, 12).

ACKNOWLEDGEMENTS

I thank: Sophie Beiger for technical help in preparation of tilapia genomic DNA; Dr. Roger Doyle for stimulating my interest in tilapias as an experimental system; Dr. C. Helleiner for various fish DNA samples; and Dr. B. Pohadjak for his generous gift of M13mp18 DNA. This work was generously supported by grants from the Natural Sciences and

Engineering Research Council of Canada to J.M. Wright and the International Development Research Centre to Dr. R. Doyle.

REFERENCES

1. Britten, R.J. and Kohne, D.E. (1968). Science *161*: 529–540.
2. Skinner, D.M. (1977). Bioscience *27*: 790–796.
3. Singer, M. (1982). Int. Rev. Cytol. *76*: 67–112.
4. Rubin, C.M., Houck, C., Deininger, P.L., Friedman, T. and Schmid, C.W. (1980). Nature (London) *284*: 372–374.
5. Adams, J.W., Kaufman, R.E., Kretschmer, P.J., Harrison, M. and Nienhuis, A.W. (1980) Nucl. Acids Res. *8*: 6113–6127.
6. Weiner, A.M., Deininger, P.L., Efstratiadis, A. (1986). Ann. Rev. Biochem. *55*: 631–661.
7. Miklos, G. (1985). In: *Molecular Evolutionary Genetics* (MacIntyre, J., ed), Plenum Press, New York pp. 241–321.
8. Willard, H.F. and Waye, J.S. (1987). Trends in Genet *3*: 192–198.
9. Doolittle, W.F., and Sapienza, C. (1980). Nature (London) *284*: 601–603.
10. Doolittle, W.F., (1982). In: *Genome Evolution* (Dover, G.A., and Flavell, R.B., eds.) pp. 3–28, Academic Press.
11. Orgel and Crick (1980). Nature (London) *284*: 604–607.
12. Brutlag, D.L., (1980). Ann. Rev. Genet. *14*: 121–144.
13. Horz, W., and Altenburger, W. (1981). Nucl. Acids Res. *9*: 683–696.
14. Arnason, U., Hoglund, M., and Widegren, B. (1984). Chromosoma *89*: 238–242.
15. Novak, U. (1984). Nucl. Acids Res. *12*: 2343–2350.
16. Hummel, S., Meyerhof, W., Korge, E., and Knochel, W. (1984). Nucl. Acids Res. *12*: 4921–4937.
17. Miklos, G.L.G. (1982). In: *Genome Evolution and Phenotypic Variation*. Academic Press, New York, pp. 62–68.
18. Fowler, R.F., and Skinner, D.M., (1985). J. Biol. Chem. *260*: 1296–1303.
19. Datta, U., Dutta, P., and Mandal, R.K., (1988). Gene *62*: 331–336.
20. Messing, J., (1983) In: Methods Enzymol, Academic Press, New York; Vol. 101 pp. 20–78.
21. Maniatis, T., Fritsch, E.F., and Sambrook, J., (1982) In: *Molecular Cloning, A Laboratory Manual*. Cold Spring Harbour Lab. pp. 90–91.
22. Dagert, M., and Ehrlich, S.D., (1979) Gene *6*: 23–28.
23. Southern, E.M., (1975) J. Mol. Biol. *98*: 503–517.
24. Rigby, P.W.J., Dieckmann, M., Rhodes, C. and Berg, D., (1977) J. Mol. Biol. *113*: 236–251.
25. Sanger, F., Nicklen, S., and Coulson, A.R., (1977). Proc. Natl. Acad. Sci. *74*: 5463–5476.
26. Bankier, A.T., and Barrell, B.G. (1983). In: *Nucleic Acids Biochemistry*. Elsevier Scientific Publishers Ireland Ltd. pp. 1–34.
27. Kessler, C., and Holtke, H-J., (1986) Gene *47*: 1–153.
28. Gruenbaum, Y., Cedar, H., and Razin, A., (1981). Nucl. Acids Res. *9*: 2509–2515.
29. Vanyushin, B.F., Tchakeva, S.G., and Belozersky, A.N., (1970) Nature (London) *225*: 948–951.
30. Razin, A., and Riggs, A.D. (1980). Science *210*: 604–610.
31. Mitchell, A.R., Gosden, J.R., and Miller, D.A., (1985). Chromosoma *92*: 369–377.
32. Jorgensen, A.L., Bostock, C.J., and Bak, A.L., (1986) J. Mol. Biol. *187*: 185–196.
33. Hinegardner, R., and Rosen, D.E., (1972). The Amer. Natl. *106*: 621–644.
34. Majumdar, K.C., and McAndrew, B.J., (1986). Genetica *68*: 175–188.

This article, submitted on disc, has been automatically converted into this typeset format by the publisher.