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# Characterization of highly and moderately repetitive 500 bp *Eco* RI fragments from *Xenopus laevis* DNA

Sabine Hummel, Wolfgang Meyerhof, Elke Korge and Walter Knöchel

Institut für Molekularbiologie und Biochemie, Freie Universität Berlin, Arnimallee 22, D-1000 Berlin 33, FRG

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ABSTRACT

Three different types of repetitive Eco RI fragments, which comigrate within a visible band of approximately 500 bp at gel electrophoresis of Xenopus laevis DNA Eco RI digests have been cloned and sequenced. These sequences are designated as Repetitive Eco RI Monomers: REM 1, REM 2 and REM 3. The sequences contain direct repeats, inverted repeats and palindromic elements. Genomic organization of the most abundant sequence (REM 1; 0.4 % of total DNA) is that of an interspersed sequence. REM 2 (0.08 %) is partly organized as an interspersed element and partly found in tandem arrangement, whereas REM 3 (0.02 %) represents the tandemly repeated monomeric unit of a satellite DNA. In situ hybridization has shown that REM 1 and REM 2 sequences are found on most chromosomes, REM 1 being preferentially located on specific chromosomal loci. REM 3 is located near the centromere region of only one chromosome pair (presumably number 1). Hybridization of Northern blots from RNAs of different developmental stages revealed that REM 1, REM 2 and REM 3 sequences are transcribed and that transcription is under developmental control.

## INTRODUCTION

Similar to nearly all higher eucaryotic organisms the genome of the South African clawed toad, Xenopus laevis, contains a high proportion of repetitive sequences which have been estimated to account for approximately 20-30 % of total DNA in this species (1). During the last years substantial amounts of nucleotide sequencing data on different repetitive DNA families of Xenopus have accumulated. According to their genomic organization the sequenced family members can arbitrarily be subdivided into two groups, i.e., those of dispersed or interspersed and those of tandem arrangement. To the first group belong the sequences of X 132 (2), C 19 (3), 1723 element (4), pJCC 31 (5) (homologous to repeat 9 (6)),

to the latter class belong the sequences of major oocyte 5 S DNA (7, 8), trace oocyte and somatic 5 S DNA (9), satellite 1 (10) (homologous to OAX (11), RHM 2 and RHM 5 (12)), SB 18 (13) and U 2 DNA (14). However, it has to be mentioned that some interspersed sequences contain smaller sequence elements in itself, which are organized in tandem like arrangement (for examples: X 132 (2) and 1723 element (4)), or members of a tandem repeat family are found as orphons (15), thereby making a commitment to any of the two groups arbitrarily. With the exception of SB 18 (13) at least parts of all these repetitive sequences are transcribed, even if in some cases a functional role of transcripts could not yet be elucidated and the transcription is in some cases confined to certain developmental stages (for references: see 2-9, 16, 17). The compilation of sequenced repetitive DNA elements can be continued with the ribosomal repeat units, the repetitious tRNA coding genes and it seems reasonable to add repetitious polymerase II transcribed gene clusters like that of the histone genes.

By the present investigation we have characterized the sequences, which give rise to the formation of a visible band by gel electrophoresis of Eco RI digests of Xenopus DNA and therefore have to represent repetitious DNA fragments. We have analyzed their nucleotide sequences, genomic organization, chromosomal location and transcriptional behaviour.

## MATERIALS AND METHODS

### Isolation and gel electrophoresis of DNA and RNA

Genomic DNA was isolated from liver nuclei of an adult Xenopus laevis female according to the proteinase K/phenol extraction procedure (18). Eco RI digests were run on 1 % agarose gels in 0.03 M Tris (pH 7.6), 0.03 M $NaH_2PO_4$, 1 mM EDTA or on 6 % polyacrylamide gels in 0.05 M Tris/borate (pH 8.3), 1 mM EDTA. RNA was extracted from 10 embryos, each, of different developmental stages by using the procedure of Probst et al. (19) and subsequently purified by CsCl gradient centrifugation. Glyoxylated RNA was run on 1.25 % agarose gels in 0.01 M Na-phosphate (pH 7.0) (20).

Cloning of 500 bp fragments and nucleotide sequencing

500 bp Eco RI fragments eluted from an polyacrylamide gel were ligated into the Eco RI cleavage site of pBR322. Sequences identified by colony screening (21) were released from pBR322 and were recloned in the Eco RI site of M13mp8 (22). Sequencing of REM 1 and REM 2 was done with 5' end labelled Bgl II fragments (REM 1) or with separated strands (REM 2) according to the chemical procedure of Maxam and Gilbert (23). All three types of fragments were also sequenced from M13 clones containing the sequences in opposite directions according to the enzymatic procedure of Sanger et al. (24).

DNA and RNA blotting

DNA and RNA were transferred to nitrocellulose paper according to the procedures described by Southern (25) and by Thomas (26), respectively. DNA blots were hybridized with $^{32}P$ nick translated REM fragments in aqueous solution at 65 $^{o}C$ and RNA blots were hybridized with the same probes in 50 % formamide at 37 $^{o}C$. DNA blots were finally washed in 0.1 x SSC at 50 $^{o}C$ and RNA blots were finally washed in 0.2 x SSC at room temperature. Exposures to Kodak XAR-5 films were done at -70 $^{o}C$ for varying times.

In situ hybridization

Mitotic chromosomes were prepared from dissected brain tissue of Xenopus laevis tadpoles (stages 39-41; (27)). Animals were pretreated with 0.1 % colchicine in 0.5 % NaCl for 2 h. After 30 min in 0.075 M KCl the cells were fixed in ethanol/acetic acid (3/1). Chromosomes were squashed in 45 % acetic acid and dehydrated. In situ hybridizations were performed according to the procedures described by Pardue and Gall (28) and Hennig (29). Chromosome preparations were treated with 50 % acetic acid for 5 min at room temperature, denatured by boiling in 0.1 x SSC for 1 min and immediately dehydrated in ethanol. $^{3}H$ nick translated REM fragments were purified on Sephadex G 75 columns and had specific radioactivities of 2-3 x $10^{7}$ dpm/µg. Chromosome preparations were hybridized with 5 x $10^{5}$ dpm/10 µl of labelled DNA for 18 h at 64 $^{o}C$ in 3 x SSC. The uncovered slides were extensively wash-

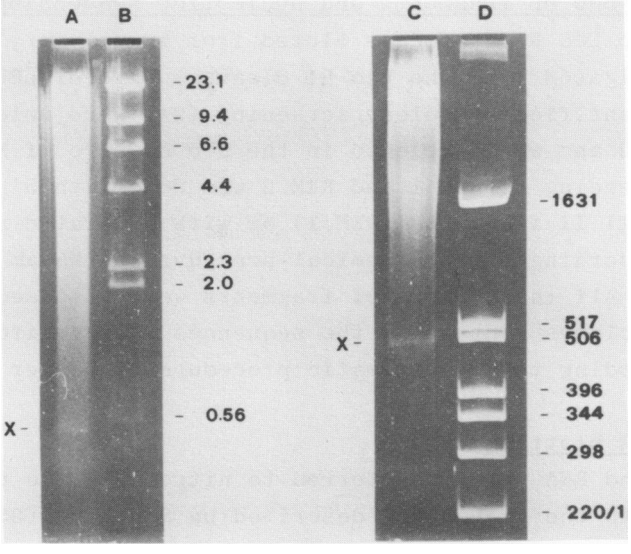


Figure 1: Left: Agarose gel electrophoresis of Xenopus DNA digested with Eco RI (A) and λ DNA digested with Hind III (B). Right: Polyacrylamide gel electrophoresis of Xenopus DNA digested with Eco RI (C) and pBR322 DNA digested with Hinf I (D). X designates position of 500 bp band in Xenopus DNA digests.

ed in 2 x SSC at 60 °C for 2 h, followed by 2-4 h wash at room temperature. Autoradiography was carried out with Ilford K 5 nuclear research emulsion for 10-16 days. Preparations were developed with Kodak D 19 and chromosomes stained in 5 % Giemsa staining solution.

## RESULTS

### Identification and cloning of 500 bp Eco RI fragments

Eco RI digests of Xenopus DNA show a visible band after ethidium bromide staining when separated by agarose or polyacrylamide gel electrophoresis. This band migrates at about 500 bp as it is shown in figure 1. DNA fragments within this band were eluted from an acrylamide gel and ligated into the Eco RI cleavage site of pBR322. After transformation of E. coli HB 101 we have randomly selected 60 clones for further investigation. Colony hybridization of these 60 clones with nick translated DNA of the 500 bp band resulted in 36

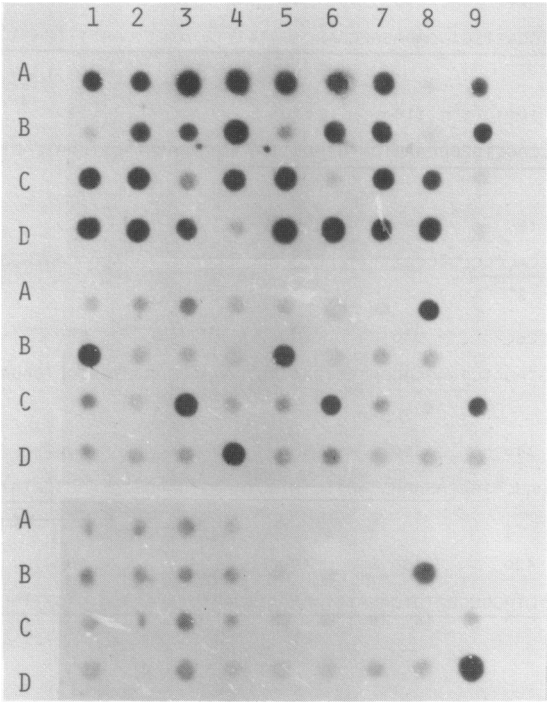


Figure 2: Autoradiograph after colony screening of 36 recombinant clones with nick translated Eco RI inserts of clone A1 (upper part), B1 (middle part) and B3 (lower part).

positive autoradiographic signals. Since the 500 bp band is not only a discrete band but is also overlaid upon a broad smear of Eco RI fragments, it is reasonable to assume that the 24 clones without autoradiographic signals contain those fragments which belong to the 500 bp region of the Eco RI smear. These 24 clones were not further investigated. To check, whether the band consists of only one or of different types of sequences, we have hybridized the $^{32}$P-labelled 500 bp Eco RI fragment of clone A1 (Fig. 2) to the DNA of the remaining 36 bacterial colonies. Out of these 27 showed autoradiographic signals (Fig. 2: upper part). Colony hybridization was continued with the Eco RI insert of clone B1 resulting in the identification of 7 clones (Fig. 2: middle part) and finally we obtained two signals with the probe of clone B8 (Fig. 2: lower part). The fact that all 36 clones could be

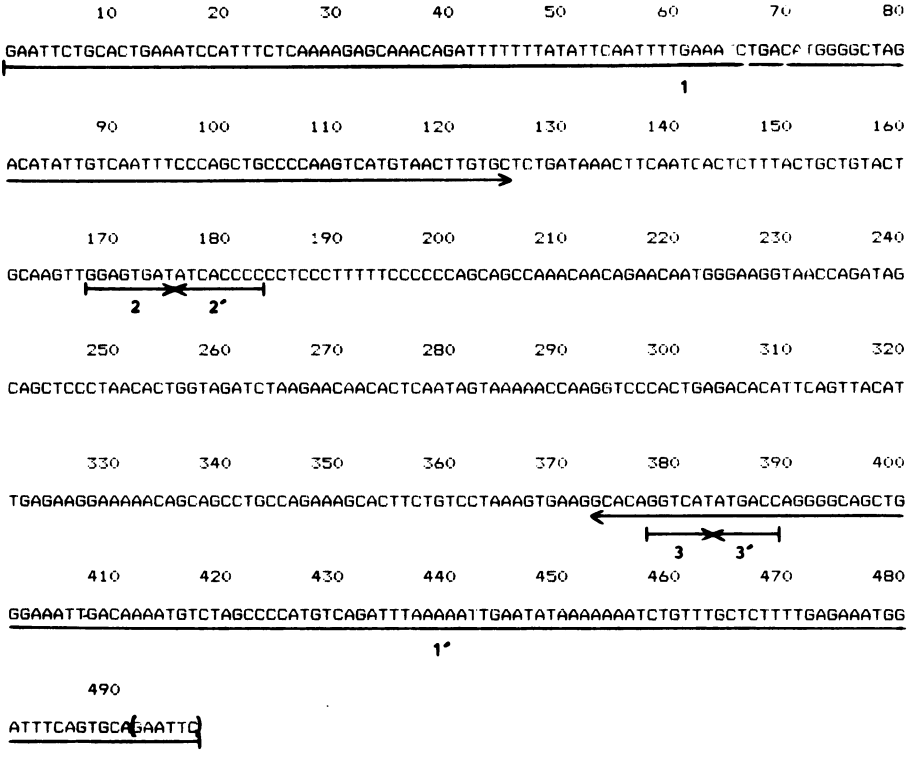


Figure 3: Nucleotide sequence of REM 1 (clone A1). Nucleotides of only one strand are shown from 5' to 3' ends. The underlined regions represent inverted repeats (1/1') and palindromes (2/2', 3/3').

aligned to three different types of sequences, which do not cross-hybridize, does not necessarily mean that we have considered all components of the 500 bp band, but it is rather unlikely that a major component of this band would not be present within as many as 36 clones. Instead, we think to have succeeded in cloning the major components of the 500 bp band which then consequently would consist of mainly three different types of sequences. We have designated these sequences Repetitive Eco RI Monomers: REM 1 (clone A1), REM 2 (clone B1) and REM 3 (clone B8).

Nucleotide sequences

The nucleotide sequences of the three fragments have been investigated by the chemical or after recloning in M13

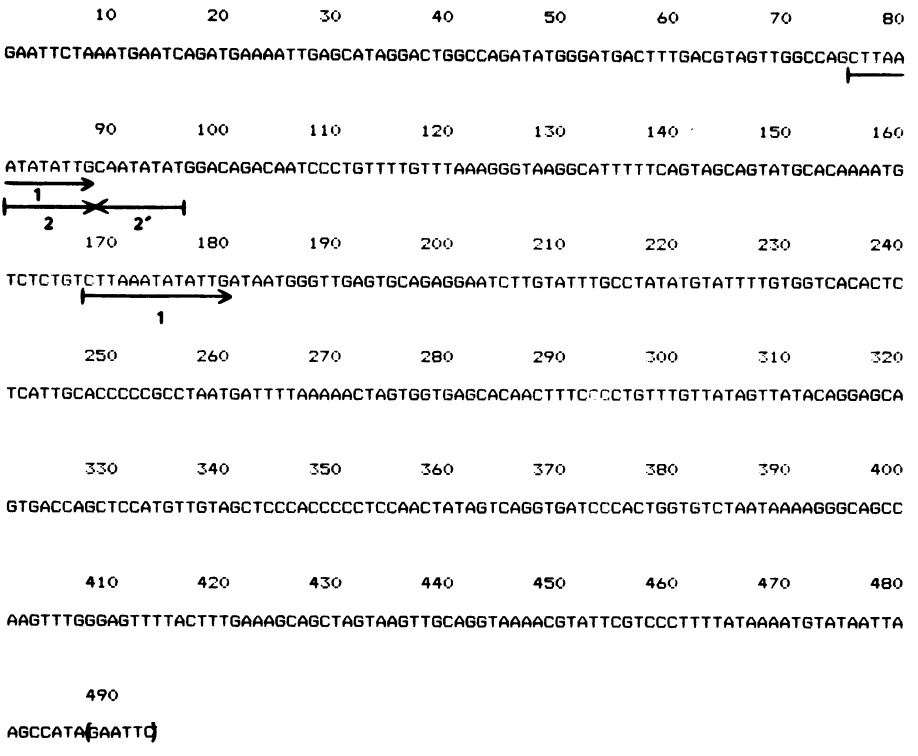


Figure 4: Nucleotide sequence of REM 2 (clone B1). Nucleotides of only one strand are shown from 5' to 3' ends. The underlined regions represent a direct repeat (1) and a palindrome (2/2').

by the enzymatic procedure (see Methods). Direct repeats, inverted repeats and palindromes were searched for by aid of a computer program and results are indicated in the corresponding figures. REM 1 (Fig. 3) contains a long inverted repeat (3 mismatches in 125 nucleotides) and two palindromes (1 mismatch in palindrome 2/2'). Since the inverted repeat includes the 5' and 3' termini of the 491 bp fragment it is reasonable to assume that it further extends within genomic DNA and that only a part of the repeat is present within the REM 1 sequence. The 487 bp sequence of REM 2 exhibits a perfect direct repeat of 13 nucleotides and a palindromic element of 16 nucleotides (Fig. 4). Interestingly, exactly half of this palindrome is represented by part of the direct repeat sequence

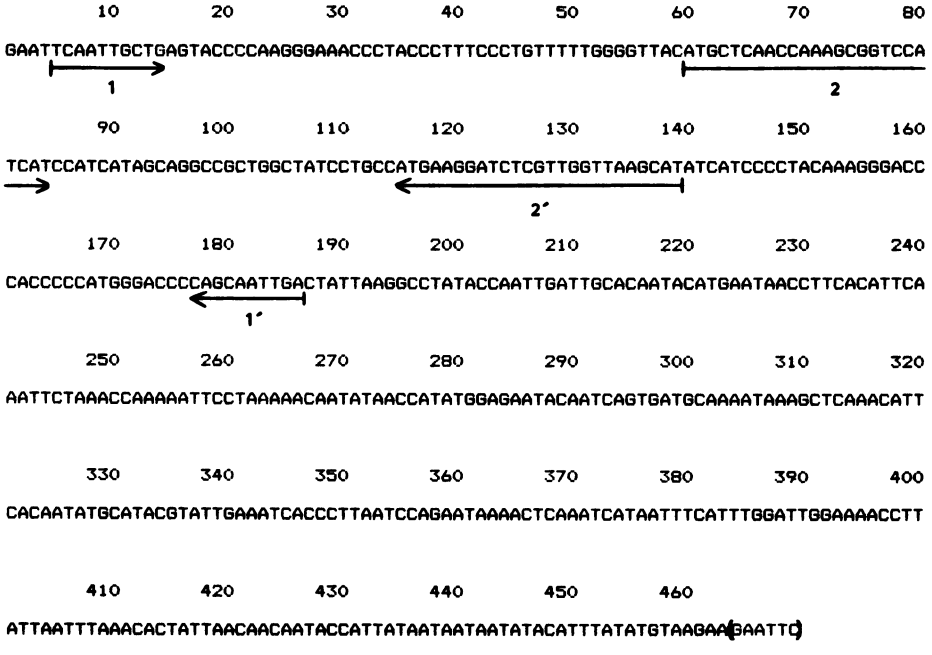


Figure 5: Nucleotide sequence of REM 3 (clone B8). Nucleotides of only one strand are shown from 5' to 3' ends. The underlined regions represent inverted repeats (1/1', 2/2').

(see also discussion). The sequence of REM 3 is slightly shorter than those of REM 1 and REM 2 comprising only 463 nucleotides (Fig. 5). There is a perfect inverted repeat of 10 nucleotides and another inverted repeat which shows 5 mismatches out of 25 nucleotides. Since the shorter repeat frames the longer one, we have also analyzed the nucleotides in between them. Although there are various complementary homonucleotide blocks, it is difficult to judge, whether or not the two repeats were originally derived from a larger one, the nucleotides of which partly diverged during evolution. Search for translational stop codons revealed that they are frequently present within all six reading frames on each of the three sequences (see also discussion).

Genomic organization and estimation of copy numbers

DNA fragments obtained after limited and complete Eco RI digestion of Xenopus DNA were separated by agarose gel elec-

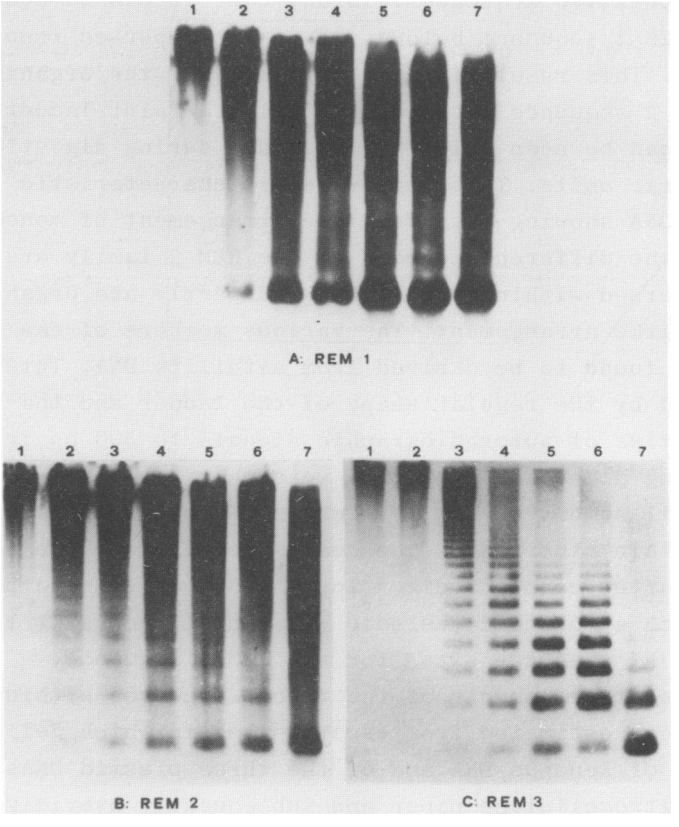


Figure 6: Autoradiographs of DNA blots after hybridization with $^{32}$P-labelled A) REM 1, B) REM 2 and C) REM 3 sequences. Xenopus DNA had been digested with Eco RI (lanes 1-6: 1 U/µg DNA) for: 1.) 0 min, 2.) 1 min, 3.) 2 min, 4.) 3 min, 5.) 10 min, 6.) 30 min, 7.) 60 min (10 U/µg DNA).

trophoresis and subsequently blotted on nitrocellulose paper. The autoradiographs obtained after hybridization to REM 1, REM 2 and REM 3 are shown in figure 6. The REM 1 sequence is located on various fragments of different sizes. After longer incubation times the 500 bp fragment is released but a major portion of this sequence is still present in fragments of higher molecular weight even after completion of DNA digestion by Eco RI. This implies, that in many cases the Eco RI cleavage sites have undergone mutational exchanges or that partial sequence elements of the 500 bp fragment are also present in

related repetitive sequences. In any case it can be concluded, that the REM 1 sequence belongs to an interspersed repetitive DNA family. This result is also obtained for the organization of the REM 2 sequence but, additionally, a faint ladder of multiples can be seen which are degraded during digestion to the monomeric units. This appearance is characteristic for satellite DNA showing a tandem like arrangement of monomers. Therefore the different members of the REM 2 family are partly interspersed within genomic DNA and partly are organized at tandem like arrangement. The various members of the REM 3 family are found to be derived from satellite DNA. This can be followed by the regular shape of the ladder and the shift of intensities of autoradiographic signals to 500 bp fragments during digestion. However, we cannot completely exclude that a small number of family members is interspersed as orphons within other DNA sequences. Moreover, we have noticed that even after complete digestion the dimer seems to be quite stable which also indicates some mutational events at least at the Eco RI cleavage sites for the REM 3 sequence.

The relative amounts of the three sequences within genomic DNA were estimated from experiments, in which defined quantities of Xenopus DNA and of the three plasmid DNAs were fixed to nitrocellulose paper and subsequently hybridized to the corresponding Eco RI fragments as labelled probes. The autoradiographs are shown in figure 7. Radioactivities were subsequently determined in a liquid szintillation counter. By comparison of the radioactivity values at a linear range of increase the relative amounts of the three sequences in genomic DNA were calculated to be 0.4 % in case of REM 1, 0.08 % in case of REM 2 and 0.02 % in case of REM 3. Since the haploid genome of Xenopus laevis consists of $3 \times 10^9$ nucleotides (30) these percentages are equivalent to approximately 25000 copies of REM 1, 5000 copies of REM 2 and about 1200 copies of REM 3. The proportion of these values roughly corresponds to the relative contribution of the three sequences to the 500 bp band, which is reflected by the number of clones belonging to each of the three families (see Fig. 2). However, since the three REM sequences are released at different ex-

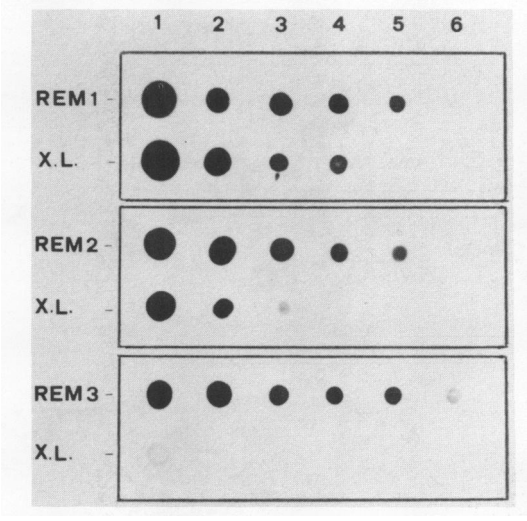


Figure 7: Quantitation of REM sequences in genomic DNA. 0.1 µg plasmid DNA (REM 1, 2 and 3) and 1 µg Xenopus DNA (1) were diluted 1:3 (2), 1:10 (3), 1:30 (4), 1:100 (5), 1:300 (6), denatured and fixed to nitrocellulose paper. Autoradiographs after hybridization with corresponding $^{32}$P-labelled 500 bp REM sequences are shown. Filters were finally counted in a liquid szintillation counter and quantitation of REM sequences was done by taking into consideration the REM contents of plasmid DNAs (1/10 $\hat{=}$ 500/4361 + 500).

tents during Eco RI digestion to 500 bp monomers (Fig. 6), this might be taken as an random coincidence. According to the copy numbers REM 1 represents a highly and REM 2 as well as REM 3 a moderately repetitive sequence family.

In situ hybridization to mitotic chromosomes

To investigate the chromosomal location of the three different repeat families we have hybridized $^{3}$H nick translated REM sequences in situ to mitotic chromosome preparations. The results are displayed in figures 8 and 9. In case of REM 1 we observed silver grains at most chromosomes. After short exposure they seem to be preferentially located at the telomeric regions and often also near the centromeres. Longer exposure times reveal additional locations also at other sites. By in situ hybridization with REM 2 we found clusters of grains distributed over nearly all chromosomes without being attached to any dominant chromosomal loci. The hybridization with REM 3 resulted almost exclusively in labelling two homo-

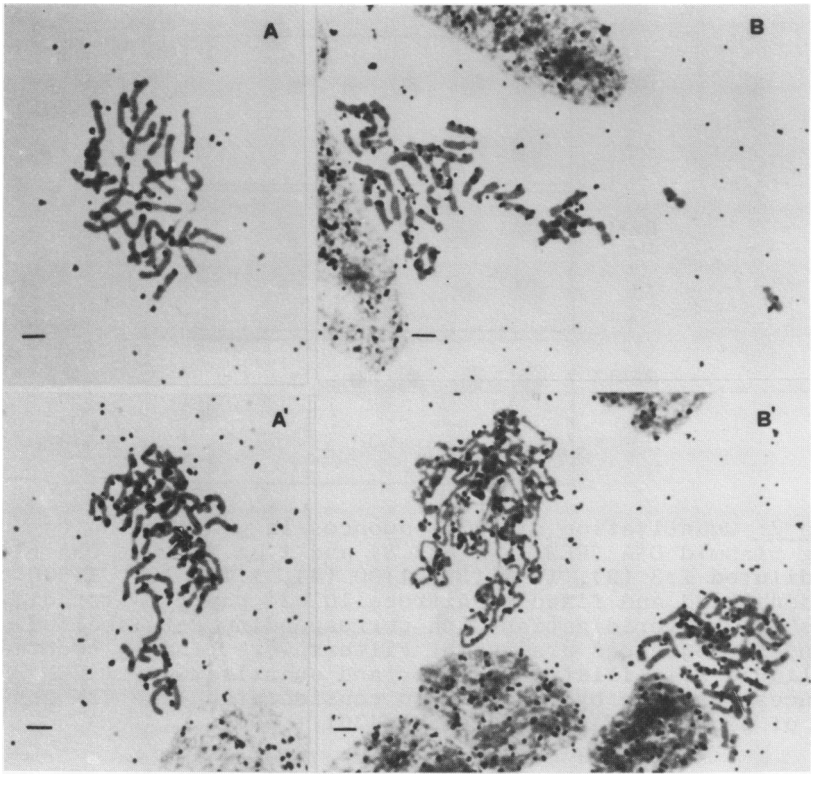


Figure 8: In situ hybridization of $^{3}$H-labelled REM 1 (A and A') and REM 2 sequences (B and B') to mitotic chromosomes of Xenopus laevis (metaphases; B' shows additionally a prophase). Specific activities of the probes were 2 x $10^{7}$ dpm/µg; exposure was for 10 days (A and B) or 16 days (A' and B'), respectively. Chromosome squashes are Giemsa stained. Bars represent 10 µm.

logous chromosomes on the long arms near the centromeres. These chromosomes presumably represent pair number 1 according to the karyotype analysis of Tymowska and Kobel (31), because they are the largest, submetacentric chromosomes and the two homologs do always slightly differ in size.

Transcription of REM sequences

Since we were particularly interested whether the REM sequences are transcribed during embryonic development, we assayed the transcriptional behaviour by analysis of RNA from unfertilized eggs and from various embryonic stages. The autoradio-

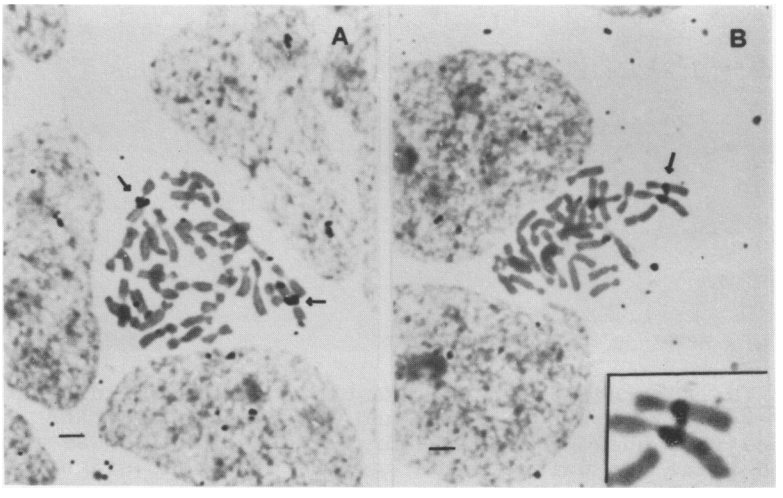


Figure 9: In situ hybridization of $^{3}H$-labelled REM 3 sequence to two sets of metaphase chromosomes of Xenopus laevis (A+B). One submetacentric chromosome pair shows specific hybridization on the long arm near the centromere region (arrows). Magnified chromosomes are shown in insert. Specific activity of the probe was 3 x $10^{7}$ dpm/µg, exposure was for 10 days. Chromosome squashes are Giemsa stained. Bars represent 10 µm.

graphs of Northern blots after hybridization to REM 1, REM 2 and REM 3 are shown in figure 10. Although the RNA had been purified by CsCl-gradient centrifugation, the high molecular weight band observed after hybridization to all three REM sequences is still due to a contamination with DNA. This can already be deduced by the facts that the band is observed at identical positions in all three cases, that even trace amounts of DNA would result in an autoradiographic signal and that signal intensities increase with RNA of developing stages which in turn can be correlated to increasing amounts of genomic DNA/embryo during the early development. Final proof was achieved by RNAse incubation of another Northern blot before hybridization to REM 1 probe. This treatment did selectively not affect the high molecular weight band (not shown). However, additional bands are detected which show varying signal intensities for RNAs from different developmental stages and which differ in size according to the three REM sequences having been used as hybridization probes. Hybridization with REM 1 reveals a band

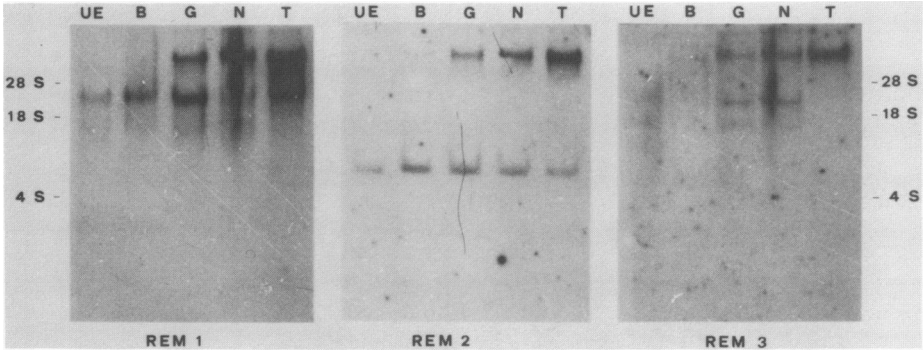


Figure 10: Autoradiographs of RNA blots after hybridization with $^{32}$P-labelled REM 1, REM 2 and REM 3 sequences. RNA isolated from one egg or one embryo, respectively, was run on each lane. UE: unfertilized egg, B: blastula, stages 7-9, G: gastrula, stages 10-12, N: neurula, stages 15-18, T: tadpole, stages 39-41. Stage numbers are according to Nieuwkoop and Faber (27). Positions of 18 S rRNA, 28 S rRNA and 4 S RNA are indicated.

which corresponds to an RNA species migrating between 18 S and 28 S ribosomal RNA. This RNA is continuously accumulated during the different stages investigated, because the signal intensities drastically increase whereas the total amount of RNA/embryo remains nearly constant during embryogenesis (from 4.4 µg in fertilized egg to 7.3 µg in tadpole (32)). The REM 2 sequence hybridized to RNA molecules of roughly about 200 - 300 nucleotides which obviously show the highest copy numbers/embryo at blastula and gastrula stages but which decrease till the tadpole stage. In contrast to REM 1 and REM 2, hybridization with the REM 3 sequence yielded signals only with the RNA of gastrula and neurula stages. We observed one band migrating between 18 and 28 S ribosomal RNA but an additional band running faster than 18 S RNA which is only faintly visible in figure 10 was clearly identified after longer exposure to X-ray films (not shown). From the results of the three Northern blot hybridization experiments we conclude that all three REM sequences or at least parts of them are transcribed and that transcription of the corresponding genes is regulated under developmental control.

## DISCUSSION

Colony hybridization of cloned fragments has shown, that the 500 bp band being visible in Eco RI digests of Xenopus DNA mainly consists of three different types of repetitive sequences. These sequences, which are present at 25000 (REM 1), 5000 (REM 2) and 1200 (REM 3) copies per haploid genome, show different genomic organizations. REM 1 and REM 2 sequences belong to repeat families, members of which are dispersed through the genome, and by in situ hybridization it could be demonstrated that they are located on the majority of chromosomes. However, the REM 2 sequence exhibits a more complex genomic organization since members of this family are also found in tandem like arrangement. The REM 3 sequence is the monomeric unit of a tandem repeat and this repeat family is located on only one chromosome pair (presumably number 1). Although there might be no correlation, this location as well as transcriptional activity (see below) remind to recent findings from Triturus cristatus carniflex, where a satellite DNA located on the long arm of chromosome 1 is transcribed during oogenesis (33). Nucleotide sequence analysis of REM sequences has shown, that the sequences contain direct repeats, inverted repeats and palindromes. The observation of a long inverted repeat in REM 1 which also covers the Eco RI cleavage sites makes it likely, that this sequence is part of a larger repeat element. Screening of a cosmid library to search for this element in Xenopus DNA is in progress. The analysis of this sequence implies some interesting aspects. From the nucleotide sequence of REM 1 it can already be followed that this long inverted repeat allows the formation of foldback structures. Moreover it has been postulated that foldback DNA may contain movable elements of the Xenopus genome (34). The 1723 element (4), the structure of which is otherwise different from that of the REM 1 sequence, also exhibits terminal inverted repeats and has been suggested to be a transposable element. Transposable elements from Drosophila with terminal inverted repeats are well characterized (35). We do not yet know, whether the inverted repeat is flanked by a direct repeat, another feature of transposition, but the widespread distribution of the

REM 1 sequence over most chromosomes makes it likely, that this sequence may be transposable. An interesting phenomenon of the REM 2 sequence is the interference of a direct repeat and a palindromic element. Direct repeats are thought to be generated by target site duplications during insertion of movable elements (36). Although the nucleotide sequence framed by the two direct repeats does not resemble any described movable element, transposition cannot be excluded. In this case it would be tempting to speculate that the formation of the palindrome would have facilitated the integration or that an already preexisting palindrome would have served as target site.

A rather unexpected result was the observation of transcription of all three REM sequences, which is obviously under developmental control. It would be interesting to know about the biological role of these transcripts but neither sequence analysis nor comparison by aid of a computer program to more than 50 published Xenopus nucleic acid sequences revealed any functional significance. The size of RNA molecules detected after hybridization with REM 1 and REM 3 sequences would suggest a cotranscription with other types of sequences, whereas the REM 2 transcripts may be exclusively transcribed from the investigated sequence itself. Analysis of polyadenylated RNA for its contents of REM sequences, their presence in nuclear and cytoplasmic RNA as well as injection of cloned sequences in oocyte nuclei will clearly help to elucidate by which type of polymerase these elements are transcribed, and whether transcripts are confined to nuclei. The sequences do not contain the split polymerase III promotor (37), but since the transcripts of REM 1 and REM 3 are of higher molecular weight, the promotor may lie outside of the cloned regions. In case of polymerase II transcription the REM sequences may either represent intervening sequences or they are located at the untranslated regions, presumably at the 3' ends, of mature mRNA. We would at least predict by the frequent occurrence of translational stop codons that the REM sequences are not translated. Repetitive elements residing in polyadenylated RNA have been reported for various organisms, like Dictyostelium

discoideum (38), mouse (39) and Xenopus (3, 6), and functional roles of these sequences for the regulation of transcription have been postulated. Although at present a random distribution within stage specifically expressed genes cannot be excluded, the observation that REM sequences are transcribed under developmental control should make them particulary interesting with respect to the Britten and Davidson model of gene regulation (40).

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