A chicken repetitive DNA sequence that is highly sensitive to single-strand specific endonucleases

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ABSTRACT

A DNA sequence consisting of the 5-mer AGAGG repeated tandemly 32 times has been detected in a chicken genomic clone and found to be present in about 2000 copies per chicken genome. This sequence was highly susceptible to single-strand specific endonucleases isolated from *Aspergillus oryzae* (S1) and mung bean, but cleavage by a single-strand specific endonuclease isolated from *Neurospora crassa* occurred only at a pH below 5.5. Endonucleolytic cutting of the AGAGG sequence by the single-strand specific enzymes required a supercoiled substrate and was independent of ionic strength.

INTRODUCTION

Families of repetitive DNA sequences have been found in all eucaryotic genomes, but the function of most of these sequences has not been elucidated. Some highly repetitive DNA sequences, such as the Alu family found in human genomes, may be transposable DNA elements involved with regulation of gene expression (1-3). Another class of repetitive DNA sequences consist of short tandem repeats (5-10 bp) and evidence indicates that these sequences may be hotspots for DNA recombination (4-6). This family of sequences may also be involved with replication of DNA at telomeres (7).

We report here studies on a repetitive DNA sequence found in a chicken genomic clone. The clone was isolated while screening a genomic library for heat-shock genes. In the course of our studies with this clone, we discovered that it contained a moderately repetitive sequence in the chicken genome. Even more interesting was the observation that the sequence, which is 60% GC over a range of 160 base pairs, was unusually sensitive to single-strand specific endonucleases. Details of these results are described in this communication.

MATERIALS AND METHODS

Preparation of clones

Clone 11 is a genomic clone of chicken DNA in bacteriophage λ charon 4A that was chosen for study because it hybridized to a cDNA probe prepared from

an enriched population of chicken heat-shock mRNA (unpublished experiments). The location of any putative heat-shock gene in this clone is unknown. Clone pMT357 is a subclone of clone 11 containing a 5.7 kb EcoRI fragment inserted into the EcoRI site of pMT3, a derivative of pBR322. Clone 21H is a further subclone obtained as follows: a 1.7 kb PstI fragment, obtained from clone pMT357, was subcloned into pMT11, another derivative of pBR322, at the PstI site. The portion of inserted DNA located between a HincII site in the chicken DNA and a HincII site in the vector DNA was deleted by digestion with HincII and subsequent ligation. The resulting subclone, 21H, contains 850 bp of chicken DNA (Fig. 1).

The vectors pMT3 and pMT11 are derivatives of pBR322 containing the ampicillin resistance gene but not the tetracycline resistance gene. pMT3 contains 2290 bp and pMT11 contains 2275 bp. Both vectors have a large polylinker region containing cleavage sites for Bam HI, EcoRI, PstI, Hinc II, and many other restriction endonucleases. The polylinker region was used for insertion of DNA fragments for subcloning and for end labeling of DNA for sequencing. The vectors were a gift of Dr. Henry Huang (Washington University).

Restriction endonuclease cleavage mapping

DNA was digested with various restriction endonucleases using the conditions recommended by the vendor and analyzed by agarose or acrylamide gel electrophoresis.

Reverse hybridizations

Large DNA fragments were separated on agarose gels and blotted onto nitrocellulose filter paper by the method of Southern (8). Small DNA molecules were analyzed by acrylamide gel electrophoresis and transferred to activated APT paper (DPT paper) by the method of Stellwag and Dahlberg (9). The filters were prehybridized overnight in 50% formamide, 2 x SSC, 5 x Denhardt's solution, 0.1% SDS, and 100 µg/ml of denatured calf thymus DNA at 42°C. The probe, denatured by boiling for 5 min, was added and incubation at 42°C was continued overnight. The filters were rinsed in 2 x SSC, 0.1% SDS at room temperature for 30 minutes, and then rinsed at 68°C in 1 x SSC, 0.1% SDS for 1 hour. The filters or papers were dried and exposed to flashed film with one intensifying screen at -70°C.

DNA isolated from chicken embryo fibroblasts was labeled by standard nick-translation procedures (10) for use as hybridization probes. The DNA was isolated as follows: chicken embryo fibroblasts, grown to confluence on a P150 tissue culture dish, were lysed with 1% SDS in the presence of 0.5 mg/ml of

proteinase K for 3 hr at 37°C. The viscous lysate was scrapped off the dish and extracted 2 times with phenol, 2 times with chloroform, and 2 times with ether. Ether was removed by bubbling with nitrogen. The material was then digested with 10 µg/ml RNase A for 3 hr at 37°C, and the RNase A was removed by extraction as above. After extensive dialysis against TE buffer (10 mM Tris-Cl pH 8.0, 1 mM EDTA), the DNA was readily cleaved by restriction endonuclease and was suitable for labeling by nick-translation.

Library Probing

Bacteriophage Charon 4A containing a chicken genome library (a gift from Richard Axel, Columbia University) was plated in soft agar overlays with E. coli DP50 SupF cells onto NZYDT plates (1.1% agar, 0.5% NaCl, 0.2% MgCl₂, 1% NA amino acid mix, 0.5% yeast extract (Difco), 0.04% thymidine, and 0.01% diaminopimelic acid). Plating conditions were such that about 3000 plaques/plate were obtained after 12 hours incubation at 37°C. A dry nitrocellulose filter was placed on top of the overlay for 30 seconds to transfer phage to the filter paper. The phage DNA was denatured in 0.5 N NaOH for 30 seconds and neutralized in 1.5 M NaCl, 0.5 M Tris-Cl, pH 8.0 for 5 minutes. The filter was then rinsed in 2X SSPE (360 mM NaCl, 20 mM NaH₂PO₄ (pH 7.4), and 2 mM EDTA) and dried on Whatman 3 MM paper. Filters were baked in a vacuum oven at 80°C for 2 hours. Hybridization conditions were the same as those described above except that 5 x SSPE was used in place of 2 x SSC.

DNA Sequencing

Clone 21H DNA was sequenced from a BamHI site in the polylinker region of the vector immediately adjacent to the insert (see below) by the method of Maxam and Gilbert (11). The 5' strand was labeled using T4 polynucleotide kinase (11), or the 3' strand was labeled by filling in with reverse transcriptase (12). After labeling, the DNA was cut with Hae III (see Fig. 1), the appropriate band was eluted from a polyacrylamide gel, and the DNA was further purified on a DE-52 spin column.

Single-strand specific endonuclease digestions

DNA was digested with S1 nuclease (P-L Biochemicals) at 37°C for 30 minutes in buffer containing 200 mM NaCl, 50 mM Na acetate pH 4.5, 1 mM ZnSO₄, and 0.5% glycerol. The DNA was then phenol extracted two times, ether extracted, and precipitated in ethanol with 5 µg of tRNA carrier before any subsequent enzyme digestions.

DNA was digested with mung bean single-strand specific endonuclease (P-L Biochemicals) at 37°C for 30 minutes in buffer containing 30 mM Na acetate pH

4.6, 50 mM NaCl, 1 mM ZnCl₂, and 5% glycerol. The DNA was extracted and prepared for subsequent enzyme digestion as described above for the S1 enzyme.

DNA was digested with Neurospora crassa single-strand specific endonuclease (P-L Biochemicals) at 37°C for 30 minutes using a variety of buffers. After digestion with the enzyme, the DNA was phenol extracted 2 times and ether extracted. The DNA was then precipitated with 5 µg of tRNA carrier in 2 M ammonium acetate using 2 volumes of ethanol. After pelleting, the DNA was precipitated in 0.3 M Na acetate using 3 volumes of ethanol. After centrifuging and resuspending the resulting pellet in the appropriate buffer, the DNA was ready for subsequent enzyme digestions.

RESULTS

Demonstration and localization of a chicken repetitive DNA sequence

Southern hybridization experiments using clone pMT357 to probe the chicken genome indicated that pMT357 contained a repetitive DNA sequence since the clone hybridized to a large number of chicken DNA sequences using stringent hybridization conditions (data not shown). A restriction endonuclease cleavage map of the pMT357 clone is given in Fig. 1. The presence of a chicken repetitive DNA sequence was verified and the location of the repetitive DNA sequence was determined by reverse hybridization experiments; that is, restriction endonuclease digested pMT357 DNA was size-separated on an agarose gel, transferred to nitrocellulose filter paper, and probed with nick-translated total chicken DNA. The chicken DNA strongly hybridized to a 850 bp HincII-PstI fragment (Fig. 2) demonstrating that this fragment contained a DNA sequence present in high copy number in the chicken genome. A similar reverse hybridization experiment demonstrated that the repetitive DNA sequence was within the 450 bp HincII-HaeIII fragment (data not shown).

The number of copies of the repetitive DNA sequence per chicken genome was roughly determined by probing a chicken genomic library with clone 21H (a subclone containing the 850 bp HincII-PstI fragment as an insert). The genomic library, in bacteriophage lambda, was plated onto lawns of E. coli to give about 3000 plaques per petri dish. The plaques were transferred to nitrocellulose filter paper and probed with clone 21H. About 30 plaques per dish hybridized to the probe (data not shown). Assuming a chicken genome size of 3×10^9 bp, an average insert of about 17 kb of chicken DNA per recombinant phage, and a single sequence per insert, the number of copies of the repetitive DNA sequence per chicken genome is at least 2×10^3 .

The nature of the repetitive DNA sequence was revealed by sequencing the

Figure 1. Restriction endonuclease cleavage maps. Map of the 5.7 kb EcoRI insert in pM1357 is at the top; The 820 bp PstI-HincII insert in clone 21H is shown below (this insert is the same DNA denoted by the box in the upper map). In clone 21H, a HaeIII site is about 450 bp from the HincII site. The repetitive DNA sequence is found within the hatched area.

DNA from the HincII site in clone 21H toward the HaeIII site (Fig. 3). The pentamer AGAGG began at base number 84 from the HincII site and was repeated 32 times with no interruptions and no base pair changes (Fig. 4). There were a total of 176 base pairs that were exclusively purines on one strand and pyrimidines on the other. Bases 1-83 on the purine strand were also purine rich (76%). On the other side of the AGAGG repeat, bases 245-291, the strands were about 50% purine and 50% pyrimidine.

It was presumed that the AGAGG repetitive sequence was the high copy number sequence detected by reverse hybridization. To test this, clone 21H DNA was digested with Mnl I, analyzed by electrophoresis on a 4% acrylamide, 0.5% agarose composite gel, transferred to activated APT paper, and probed with chicken DNA. Since Mnl I recognizes the sequence GAGG, digestion of the AGAGG repeat with this enzyme should cleave the repeat into small fragments. Digestion with Mnl I produced fragments that did not hybridize with the chicken DNA probe (data not shown). The blot was reprobed with a mixture of pBR322 and 21H DNAs to determine whether small DNA fragments were successfully bound to the APT paper. The 63 and 57 base pair doublet of Alu I digested pBR322 DNA was detected (data not shown). Therefore, Mnl I cleaved the high copy number repeat DNA segment in 21H into sequences smaller than 60 bp. These data support our hypothesis that the AGAGG repeat sequence is the high copy number repetitive sequence in clone 21H.

Sensitivity of the AGAGG repeat sequence to single-strand specific endonucleases

Clone 21H was tested for sensitivity to the single-strand specific

Figure 2. Localization of the repetitive DNA sequence within pMT357 by reverse hybridization. A) Ethidium bromide stained, 1% agarose gel of pMT357 DNA digested with (1) PstI, or (2) PstI and HincII. B) Autoradiogram of the gel after blotting to nitrocellulose filter paper and probing with nick-translated, total chicken DNA. The probe hybridizes to a 1750 bp PstI fragment of clone pMT357 (lane 1). HincII cleaves the 1750 bp PstI fragment into a 930 bp fragment and a 820 bp fragment. The probe hybridizes to the 820 bp PstI-HincII fragment (lane 2).

endonuclease S1 since it has been reported that homocopolymer sequences may be S1 sensitive (13). It was found that restriction endonuclease cleavage fragments containing the AGAGG repetitive sequence were sensitive to the S1 enzyme (Fig. 5A). However, the vector itself contains sequences that constitute a major, a minor, and a subminor S1 cleavage site in pBR322 (14). These three S1 cleavage sites are all located in a 458 bp Hae III fragment in pMT11 that migrates just above the fragment containing the repetitive DNA sequence in Fig. 5A. This 458 bp fragment was not preferentially cleaved under the conditions used here, even at S1 concentrations 50-fold higher than those required to cleave the fragment containing the AGAGG repetitive sequence. In striking contrast, however, the fragment containing repetitive

Figure 3. DNA sequence of the repetitive DNA.
Autoradiogram of the repetitive DNA sequence. The 5' end of the DNA was labeled at the BamH1 site in clone 21H with T4 polynucleotide kinase. The labeled DNA was digested with HaeIII and the appropriate fragment was purified on a preparative acrylamide gel. The other strand was sequenced by labeling the 3' end at the BamH1 site by filling in with reverse transcriptase (data not shown).

DNA was cleaved to smaller fragments (lanes 1-4). The S1 sensitivity of this sequence required a supercoiled substrate; if clone 21H DNA was first digested with HaeIII to break the DNA into linear fragments and subsequently digested with S1 enzyme, the fragment containing the AGAGG repeat sequence was not preferentially cleaved by the S1 enzyme (Fig. 5B). The requirement of a

EcoRI   BamHI HincII
GAATTCCTCCGGGATCCGTCACAGGAAAGGAAAAAGGAGAACACAGAATTAAGAGAATAACCT
CTACATACTGAAGGTATACTGAGAGAGAAAAGGAGGAGAGGAGAGGAGAGGAGAGGAGAGG
AGAGGAGAGGAGAGGAGAGGAGAGGAGAGGAGAGGAGAGGAGAGGAGAGGAGAGGAGAGGAGG
GAGGAGAGGAGAGGAGAGGAGAGGAGAGGAGAGGAGAGGAGAGGAGAGGAGAGGAGAGGCA
TGTGTGTAGGGAACAACATGTTCTCTCAATGCCTAATATGCTACATGT

Figure 4. Summary of DNA sequence data. Vector sequences are in small letters and chicken sequences are in large letters. The repeated 5-mer, AGAGG, is underlined. The boxed in sequence, TCTCTC, is a possible S1 cleavage site (see Fig. 6).

Figure 5(A). S1 sensitivity of clone 21H. Clone 21H DNA (0.5 μ g) was digested with S1 enzyme and subsequently digested with HaeIII. The figure is an ethidium bromide stained, 4% acrylamide, 0.5% agarose, composite gel. The arrow refers to the HaeIII fragment in lane 5 containing the AGAGG repeat sequence. Lanes 1-5, 21H DNA digested with 56, 40, 24, 8, 0 units of S1, respectively. Lanes 6 and 7, vector DNA, pMT11, digested with 40 and 0 units of S1, respectively. Lane 8, pBR322 DNA digested with AluI as a marker. Material at the bottom of lanes 1-7 is carrier tRNA. B) Superhelical dependence of S1 sensitivity. Clone 21H DNA (0.6 μ g) was analyzed as in (A). Lane 1, digested with Hae III only. Lane 2, digested first with S1 (0.8 units) and subsequently digested with Hae III. Lanes 3-5, digested with HaeIII first and subsequently digested with 0.8 units of S1 with NaCl concentrations of 500, 200, and 100 mM, respectively.

Figure 6. Mapping of the S1 cleavage site in clone 21H DNA. An ethidium bromide stained, composite gel containing 4% acrylamide and 0.5% agarose is shown. Lane 1, clone 21H DNA digested with S1. Lane 2, clone 21H DNA digested with S1 followed by *Eco*R1 digestion (see Fig. 3B). The bracket refers to the broad band mapping to the AGAGG repeat sequence. The arrow points to a faint, secondary, S1 cleavage site. The numbers refer to size of markers in base pairs.

supercoiled substrate for S1 sensitivity of the repeat sequence AGAGG extended over a range of sodium chloride concentration from 100 to 500 mM. Additional experiments demonstrated that the requirement for a supercoiled substrate extended down to 0 mM NaCl (data not shown).

The S1 cleavage site in clone 21H was mapped by digesting the DNA with S1 and subsequently digesting the DNA with *Eco*R1 (Fig. 6). The *Eco*R1 cleavage site was 18 bp to the 5' side of the *Hinc*II site shown in Fig. 4. The AGAGG repeat sequence begins 102 base pairs from this *Eco*R1 site. The S1 mapping experiment resulted in a broad band that was sized as extending from 180 to 220 bp from the *Eco*R1 site, confirming that the AGAGG sequence is S1 sensitive. These mapping experiments showed a second S1 cleavage site located about 290 bp from the *Eco*R1 site, which is probably the TCTCTC sequence box in Fig. 4. Thus, clone 21H has two preferred S1 cleavage sites: one was of large target size corresponding to the AGAGG repeat sequence, and the other was of small target size mapping near the vicinity of the sequence TCTCTC. Neither of the 2 faster migrating bands in Fig. 6, (lane 2) was an artifact of S1 digestion since S1 digestion alone did not produce these bands (lane 1).

Figure 7. Digestion of clone 21H DNA with *N. crassa* enzyme. The ethidium bromide gel is the same type as in figures 5 and 6. Lanes 1-2, single-stranded ϕ X174 DNA, undigested and digested with 1 unit of *N. crassa* enzyme, respectively. Lanes 3-7, 0.3 μ g of 21H DNA digested with 10, 1, 0.1, 0.01, and 0 units of *N. crassa* enzyme, respectively, prior to digestion with HaeIII. The arrow points to the fragment containing the repeat sequence. Material at the bottom of lanes 2-6 is tRNA carrier. The buffer for all *N. crassa* digestions is 50 mM Tris-Cl pH 8.0, 6 mM $MgCl_2$, and 150 mM NaCl.

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The sensitivity of the AGAGG sequence to single-strand specific endonucleases was not unique to S1. The sequence was also susceptible to a similar nuclease isolated from mung bean (data not shown). However, the sequence was resistant to the single-strand specific endonuclease isolated from *Neurospora crassa* using conditions that were optimal for that enzyme (pH 8) (Fig. 7). The *N. crassa* enzyme was functional in the buffer used for these digestions since the single-stranded ϕ X174 DNA (lane 2) was totally digested by the enzyme. However, the pH for digestion using the S1 or the mung bean enzyme was 4.5 to 4.8. The AGAGG repeat sequence could be cleaved by the *N. crassa* enzyme at pH 4.8 or below but not at pH 5.5 or above (Fig. 8). Sensitivity of the AGAGG repeat sequence to the *N. crassa* enzyme was independent of both divalent cations and of sodium chloride concentration

Figure 8. pH dependent sensitivity of clone 21H DNA to *N. crassa* enzyme. The *N. crassa* buffers used in each lane are: (1) 1 x S1 buffer (200 mM NaCl, 1 mM ZnSO₄, 50 mM Na acetate pH 4.5, 0.5% glycerol), (2) 50 mM Na acetate pH 4.8, 1 mM ZnSO₄, 150 mM NaCl, (3) 50 mM Na acetate pH 5.5, 1 mM ZnSO₄, 150 mM NaCl, (4) 50 mM Tris-Cl pH 6.8, 1 mM ZnSO₄, 150 mM NaCl, (5) 50 mM Tris-Cl pH 8.0, 1 mM ZnCl₂, 150 mM NaCl. In lane 6, the DNA was preincubated in 50 mM Na acetate pH 4.8, 1 mM ZnSO₄, 150 mM NaCl for 30' at 37°C. The pH was then neutralized with 50 mM Tris-Cl pH 8.0 before addition of *N. crassa* enzyme. Lane 7, control 21H DNA digested with the HaeIII enzyme without a previous *N. crassa* digestion. In lanes 1-6, 0.3 µg of 21H DNA was digested with 1 unit of *N. crassa* enzyme. All lanes contain single-stranded øX174 DNA as an internal control to be certain that the *N. crassa* enzyme was working in the given buffer conditions. Gel is similar to that described in Fig. 5, but is a composite from different experiments. Arrow shows fragment containing the repeat sequence.

(data not shown).

The reason for a pH dependent cleavage by the *N. crassa* enzyme preparation is unclear. One possibility is that the low pH digestions were creating apurinic sites on the AGAGG strand which are involved somehow with the sensitivity of this stretch of DNA to single-strand specific endonucleases. This possibility was tested by preincubating the DNA at pH 4.8 prior to digestion with the *N. crassa* enzyme at neutral pH; however, the AGAGG repetitive DNA sequence was not susceptible to *N. crassa* enzyme after preincubation at low pH (Fig. 8, lane 6). Another possibility is that the *N. crassa* enzyme preparation itself has an unusual pH-dependent activity. Purified preparations of enzyme have both endonucleolytic and exonucleolytic

activities and different specificities for single and double-stranded DNAs; these activities vary with ionic conditions (15,16).

DISCUSSION

We have isolated a genomic clone of chicken DNA containing a repetitive DNA sequence estimated to be present in at least 2×10^3 copies per chicken genome. The sequence consists of the pentamer AGAGG repeated 32 times in succession. The AGAGG repeat sequence described in this paper has been recently reported and identified as an avian repetitive DNA sequence (17). In that work, the pentamer AGAGG was repeated 36 times with no base pair changes or interruption in intron C of the chicken "ovalbumin-X" gene. The sequence data reported here and the sequence data for the clone of the intron of the ovalbumin gene have other similarities besides the AGAGG repeat. In both clones, sequences flanking one side of the repeat are highly purine rich, and sequences flanking the other side of the repeat show no unusual preponderance of either purines or pyrimidines. Another report of the sequence AGAGG repeated 25 times in succession occurs upstream from the mouse C immunoglobulin gene (18), however, it is not known if the AGAGG repeat sequence is a mouse repetitive DNA sequence. The sequence GAAA, repeated 43 times, has been found 230 nucleotides upstream from a human β -actin-like pseudogene and is reported to be highly repetitive in the human genome (19).

We found that the AGAGG repetitive sequence present in a supercoiled molecule is highly sensitive to single-strand specific endonucleases. It was readily cleaved by S1 and a single strand endonuclease isolated from mung bean at pH 4.5 to 4.8. It was also sensitive to a similar enzyme isolated from N. crassa when tested at low pH, but not at pH values of 5.5 or above. The sensitivity of the AGAGG sequence to the N. crassa enzyme was independent of ionic strength, and other components of the buffers such as Cl^- , SO_4^- , acetate, and glycerol. Thus, it is possible that the secondary structure of the repetitive DNA sequence may be pH dependent. The sensitivity of the AGAGG repeat sequence to single-strand specific endonucleases was not a result of the creation of apurinic sites in the DNA sequence since preincubation of the DNA at pH 4.5-4.8 before N. crassa digestion did not render the sequence susceptible to the enzyme at higher pH.

Superhelical DNA molecules are generally sensitive to the S1 enzyme at sites containing either inverted repeat sequences that can form cruciform structures or at AT rich regions of DNA (14). The preferred S1 cleavage sites in clone 21H came somewhat as a surprise since the AGAGG repetitive DNA

sequence is a GC rich sequence that should not be able to form a hairpin structure. There is a S1-sensitive site in a sequence consisting of the doublet GA, repeated 16 times, which occurs in the spacer of a sea urchin histone gene repeat (13). S1 cleavage at this site, however, is different from that of the AGAGG repeat since sensitivity of the (GA)₁₆ sequence requires a low NaCl concentration (60 mM NaCl or less) and is not dependent on a supercoiled substrate. More Recently, a (GA)₁₀ sequence has been identified as an S1 nuclease site but sensitivity required that the DNA be supercoiled (20). This site was in the intergenic spacer region separating the 5' ends of D. melanogaster H3 and H4 histone genes.

It is not known why the AGAGG sequence may have unpaired bases, but out-of-register DNA slippage has been proposed as a model for S1 sensitivity of the (GA)₁₆ sequence. Simple sequences similar to the AGAGG repeat sequence are apparent recombinational hot spots (4-6) and altered secondary structure in the sequence may facilitate recombinational events. Experiments correlating S1 cleavage sites in purified, supercoiled plasmids to S1 cleavage sites in isolated chromatin (21) suggest that there may be a physiological significance to the type of S1 cleavage site described for clone 21H. In this regard, it is of interest that sequences similar to those described occur near Drosophila heat shock genes. Many direct repeats of 5'GAAGA were found in the 3' non-coding region of the hsp27 gene (22). Other Drosophila heat shock genes (hsp70, hsp22, and hsp26) have AG rich sequences in the 5' flanking region of the gene and these were recently shown to be sites in supercoiled plasmid DNA sensitive for S1 nuclease (23). Thus, repetitive AGAGG sequences may be involved in gene regulation; we have preliminary data, based on Northern gel analyses, indicating that some poly A⁺ mRNA species have sequences homologous to the repetitive DNA sequence.

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