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Genetics

Base sequence of a cloned snake W-chromosome DNA fragment and identification of a male-specific putative mRNA in the mouse

(interspersed simple repeats/W- and Y-chromosomal sequences/*Elaphe radiata*/evolutionarily conserved sequences)

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ABSTRACT A 2.5-kilobase fragment of a sex-specific satellite DNA from the Colubrid snake species *Elaphe radiata* has been cloned, and its sequence has been determined. It contains 26 and 12 copies, respectively, of two base quadruplets, G-A-T-A and G-A-C-A, as its sole highly repetitious elements. Southern hybridization experiments with genomic DNA of the chicken, the mouse, and man indicated male sex-specific conservation of at least parts of this cloned DNA. *In situ* hybridization experiments with metaphase chromosomes of the mouse showed that elements that can cross-hybridize with parts of the cloned snake DNA are concentrated in the pericentric region of the Y chromosome. In blot hybridization experiments with liver poly(A)$^+$polysomal RNAs of male and female mice, a probe consisting of the first 1,224 bases of the cloned snake DNA singled out a male-specific RNA of 1,250–1,400 bases. Inasmuch as the proximal end of this probe contained an open reading frame (44 consecutive amino acid-specifying codons), the *male-specific* putative mRNA so detected may specify H-Y antigen. By contrast, a probe consisting of bases 1,480–1,906, containing the simple repeats of the quadruplets, singled out a shorter (≈1,000-base) RNA from males and females alike. Although this RNA is poly(A)$^+$, we have yet to establish its attachment to ribosomes.

In the XX/XY chromosomal determining mechanism of mammals, males are the heterogametic sex. On the contrary, female heterogamety based on the ZZ/ZW scheme operates in several species of snakes and birds. The relationship between the two forms of sex-determining mechanisms has been poorly understood. Nevertheless, the recently demonstrated heterogametic sex-specific occurrence of H-Y antigen may indicate the two to be variations of the same theme (1).

Singh *et al.* (2) have described sex-specific satellite DNAs from the snake family Colubridae that hybridized preferentially to the W chromosome of the heterogametic female sex in these species and also seem to be conserved in the heterogametic sex of other vertebrate species. We have previously reported the isolation and cloning of one such satellite DNA from a female specimen of the Colubrid snake species *Elaphe radiata* (3). Initially, we used the isolated satellite DNA to probe Southern blots of restriction enzyme-digested genomic DNA of various vertebrate species. In all cases, male-specific hybridization patterns were observed. We then constructed a library of the satellite DNA in phage Charon 4A and subsequently isolated those phages whose DNAs hybridized specifically with fractions of male, but not the corresponding female, mouse DNA (4). Here we describe the sequence analysis and general characterization of one such cross-hybridizing snake satellite DNA fragment. Based on the sequence data, the fragment was split into appropriate probes and used to challenge (*i*) DNA blots of various vertebrate species, (*ii*) RNA blots of male and female mice, and (*iii*) mouse metaphase chromosome spreads in *in situ* hybridization experiments. The results suggest a remarkable evolutionary conservation of the different subfragments of this satellite DNA fragment, a preferential Y-chromosomal location, and differential sex-specific expression at the poly(A)$^+$RNA level.

MATERIALS AND METHODS

DNA and Satellite DNA Preparations, Electrophoresis, Blotting, and Hybridization Procedures. DNA was isolated from human placentas, C57BL/B6 mouse and *E. radiata* snake tissues, snake and inbred and panmictic chicken erythrocyte nuclei, and human leukocyte nuclei according to the procedure of Blin and Stafford (5). The size of the DNA obtained was estimated to be >50 kilobases (kb) by electrophoresis on neutral horizontal 0.3% or 0.4% agarose gels, using Charon 4A DNA (6) and its *Eco*RI fragments as molecular weight standards.

Satellites III and IV from the DNA of a female specimen of *E. radiata* (7) were prepared in Cs_2SO_4/Ag^+ gradients by the procedure of Jensen and Davidson (8). The Ag^+/DNA ratio for optimum separation of the satellites from the bulk of the DNA was 0.28:1. After isolation, the satellites were centrifuged in an SW 40 rotor (Beckman) at 39,000 rpm (20°C) for 72 hr. Since the DNAs of satellites III and IV were not completely separated, they are hereafter referred to collectively simply as "satellite DNA."

For analytical purposes, DNAs were restriction enzyme-digested to completion, and samples were subjected to electrophoresis in 0.4% horizontal agarose gels, 0.8–1.5% vertical agarose gels, or 7% polyacrylamide gels in Tris borate/EDTA (depending on the fragment size being investigated) and then blotted onto nitrocellulose filters according to Southern (9). Blots were hybridized for 31 ± 2 hr with fragments of DNA labeled by nick-translation with [α-^{32}P]NTP (New England Nuclear) to a specific activity of $\approx 2 \times 10^8$ cpm/μg of DNA according to the method of Weinstock *et al.* (10).

RNA Preparations and Electrophoresis. Total cellular RNA was prepared according to Green *et al.* (11) and Shore and Tata (12) and cytoplasmic RNA was prepared according to Shore and Tata (12); polysomes were prepared according to Schutz *et al.* (13) and, from these polysomes, poly(A)$^+$RNA fractions were prepared (11). The denatured RNA samples were subjected to electrophoresis in 1% agarose gels [6% formaldehyde in 3-(*N*-morpholino)propanesulfonic acid (Sigma)/NaOAc/EDTA] us-

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Abbreviations: kb, kilobase(s); bp, base pair(s).

ing 5S, 18S, and 28S RNAs from mouse polysomes as size markers. RNA was blotted according to Southern (9) and hybridized as described above for restriction enzyme-digested DNA.

Construction of a Satellite DNA Library from *E. radiata*, Screening of the Library, and Subcloning of DNA Fragments in pBR322. After the preparation of satellites III and IV from the DNA of *E. radiata*, the DNA fragment length was estimated by horizontal agarose gel electrophoresis to be 10–20 kb. The internal *Eco*RI sites of the DNA fragments were methylated by using *Eco*RI methylase (14). Blunt ends were generated by a polymerase I (*Escherichia coli*) reaction in the presence of NTP. A blunt-ended *Eco*RI linker (C-A-T-G-A-A-T-T-C-A-T-G / G-T-A-C-T-T-A-A-G-T-A-C; a gift from R. Bruce Wallace) was ligated onto the blunt ends of the satellite DNA fragments, which were then digested with *Eco*RI. After stripping the oligonucleotides by passage over a Sephadex G-50 column, the digestion products were ligated with the cloning fragments of Charon 4A (6) and *in vitro* packaged (15), and the resulting library was amplified (14).

The libraries were screened according to Benton and Davis (16) by using nick-translated DNA fragments as probes (see *Results and Discussion*). Ten positive plaques (here designated CH4AErs 1–10) were purified (three cycles) and used to prepare DNA (6).

DNA fragments of interest were subcloned into pBR322 (17) and designated pErs1–10. Transformants were screened according to Grunstein and Hogness (18), except that Whatman 541 filter paper was used instead of nitrocellulose filters.

Restriction Mapping and Nucleotide Sequence Analysis. To construct a restriction map, single and double restriction enzyme digestions were carried out and the resulting fragments were analyzed by electrophoresis on agarose or polyacrylamide gels. DNA fragments used for sequence determinations were isolated from preparative 7% polyacrylamide gels by electroelution of the excised DNA band into a dialysis bag containing electrophoresis buffer at 1:2.5 dilution. After 3′ ("kinetion") or 5′ ("repair") labeling and either strand separation or digestion with an appropriate restriction enzyme, the desired fragment was isolated. Sequence determination of the isolated fragments was accomplished by using the G + A, A, C + T, T, and A > C cleavages described by Maxam and Gilbert (19) on 4%, 5%, 12%, or 15% polyacrylamide gels. The data presented were generally obtained by analyzing both strands of the DNA (>90%). Where this was not feasible, at least two different restriction fragments of one DNA strand were analyzed.

***In Situ* Hybridizations.** Metaphase chromosome spreads were prepared from bone marrow cells of mice injected with colchicine (20). *In situ* hybridizations were carried out according to Harper *et al.* (21) using DNA fragments labeled by nick-translation (10) with ^{125}I-labeled CTP, with minor modifications among experiments.

Enzymes. Restriction and DNA modification enzymes were purchased from Bethesda Research Laboratories, Boehringer Mannheim, and New England BioLabs. Digestions and other reactions were carried out as specified by the supplier.

RESULTS AND DISCUSSION

Organization of an *Eco*RI Fragment from Ch4AErs6 Subcloned into pBR322 (pErs5) Generating Sex-Specific Hybridization Patterns in Vertebrate DNAs. A map of the insert of Ch4AErs6 is shown in Fig. 1. This fragment was selected because of its unique hybridization behavior with different vertebrate DNAs. The most interesting were the sex-specific hybridization patterns generated when this fragment was used to probe *Alu* I/*Hae* III-digested genomic mouse DNA. The DNA stretch actually responsible for the observed sex-specificity was located in a 422-base-pair (bp) long *Hin*fI/*Sau*3AI fragment. Subsequent sequence analysis of the total 2.5-kb fragment (Fig. 2) showed that this 422 bp subfragment (probe 3) contained 26 and 12 copies, respectively, of the simple quadruplets 5′G-A-T-A 3′ / 3′C-T-A-T 5 and 5′ G-A-C-A 3′ / 3′ C-T-G-T 5′, flanked by sequences of a low or single copy nature (Fig. 2*B*). When the *Hin*dIII subfragment of probe 1 (positions 641–1,224) was hybridized to *Eco*RI-digested genomic DNA of *E. radiata*, a clear band was observed in the heterogametic female sex only (Fig. 3*A*, black arrow). The length of the DNA in this band corresponds to that of the cloned pErs5 insert. Consequently, the naturally occurring *Eco*RI sites in the satellite DNA, instead of the added *Eco*RI linkers, served as ligation points. This was confirmed by sequence analysis (Fig. 2*A*). Because of the similarity in the lengths of the bands from the genomic and cloned DNAs, it seems unlikely that any gross cloning artifacts (22) involving the simple repeats were generated.

The sequences flanking the simple repeat structure contain two significantly long open reading frames, T-A-T-A box-like sequences (23) (e.g., positions 900, 2,346), and potential poly(A) addition signals (e.g., positions 225, 1,223, 1,829, 2,251). Two longer potential translation products are shown in Fig. 2. Particularly noteworthy is the proximal region of the sequence shown in Fig. 2*A*, which consists of 44 consecutive amino acid-specifying codons followed by the chain-terminating codon TGA and then by the putative poly(A) attachment signal A-A-T-A-A-

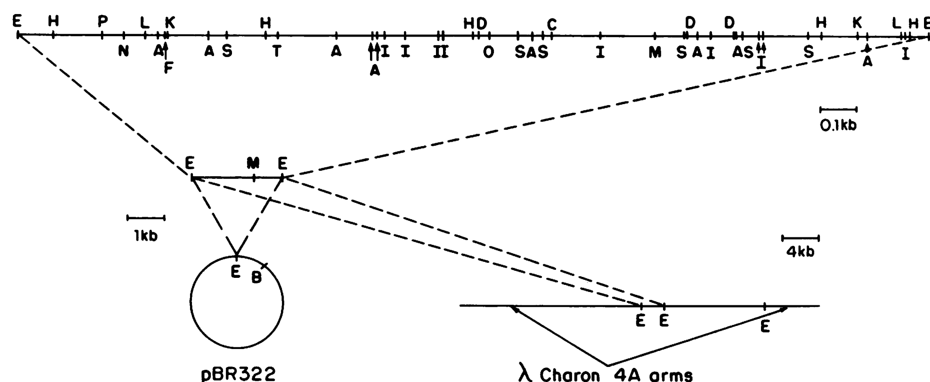

FIG. 1. Schematic diagram of the structure of Ch4AErs6 and pErs5 including a physical map of the 2.5-kb *E. radiata* satellite DNA insert. This satellite DNA component contains sequences that cross-hybridize in sex-specific patterns with vertebrate genomic DNAs. Sites: A, *Alu* I; B, *Bam*HI; C, *Eco*RV; D, *Hin*dIII; E, *Eco*RI; F, *Fnu*4HI; H, *Hae* III; I, *Hin*fI; K, *Hha* I; L, *Hpa* II; M, *Mbo* II; N, *Kpn* I; O, *Eco*RII; P, *Hpa* I; S, *Sau*3AI; T, *Taq* I.

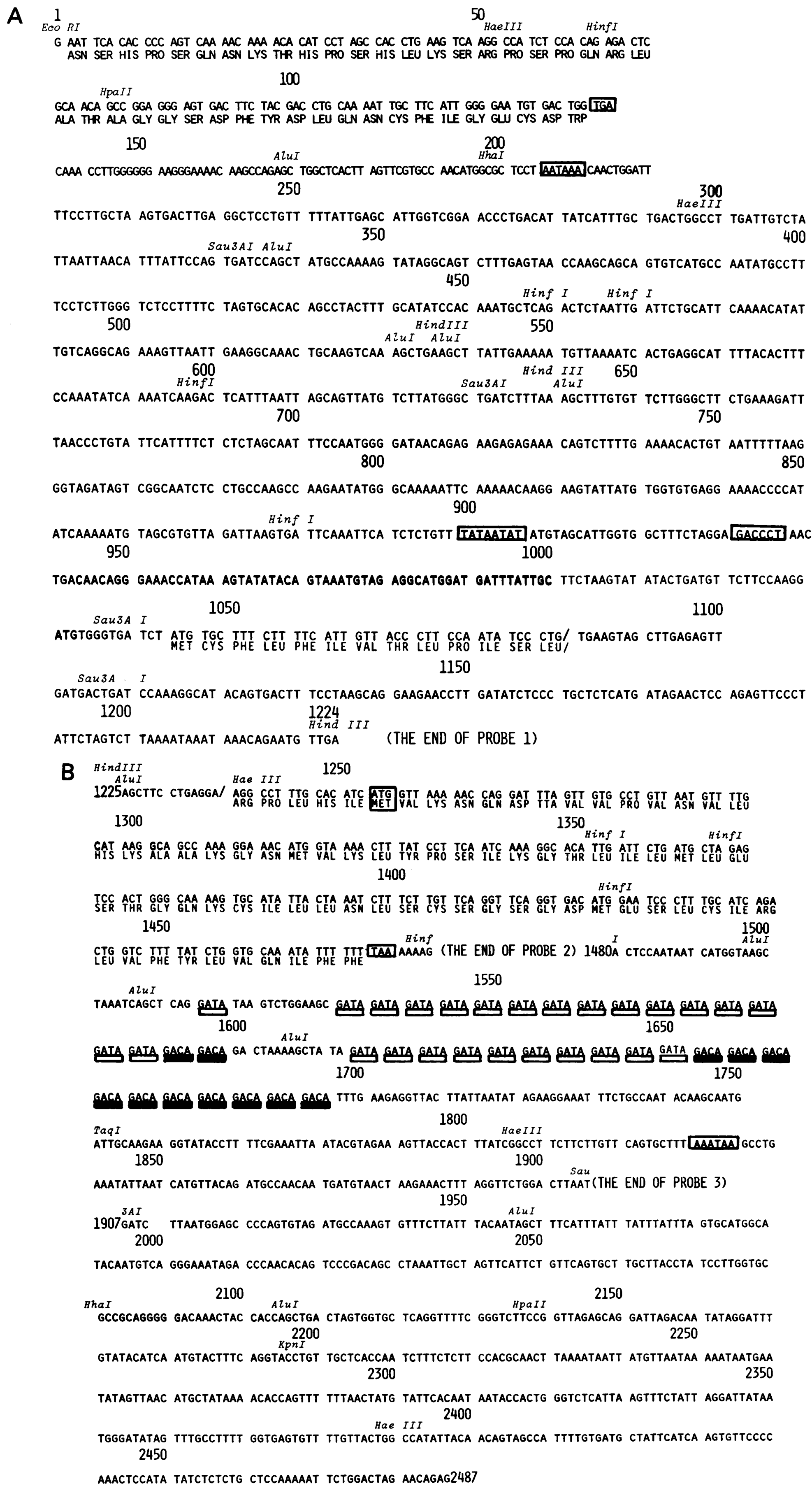

FIG. 2. (*Legend appears at the bottom of the next page.*)

A (row 3). It is thus probable that this proximal region represents a 3′ portion of a particular structural gene. It should be further noted that the base sequences occupying rows 6–11 are rich in oligo(T) and oligo(A). Such (A + T)-rich "linker" zones are the distinctive characteristic of the intergenic spacer region immediately adjacent to a structural gene (24).

A peculiar phenomenon was observed with one *Hae* III site (position 1,807) and a few *Alu* I sites, which were confirmed by sequence analysis of several different restriction fragments of both strands. In the cloned DNA, these sites could not be digested by *Hae* III or *Alu* I, respectively, even in the presence of excess enzyme. A similar situation was observed in bovine satellite DNA (25).

Evolutionary Conservation of Subfragments of pErs5. When the complete nick-translated insert of pErs5 was used as a hybridization probe to challenge *Hae* III/*Alu* I-digested human DNA on blots from two unrelated individuals, the pattern shown in Fig. 3 (lanes G and H) was observed. With the DNA of several unrelated healthy individuals, a plethora of different patterns was observed. Family investigations established the heritability of the restriction fragment polymorphisms found. In this context, a recent report on a highly polymorphic locus closely linked to the human insulin gene is of interest (27). This polymorphism is generated by variation in the number of copies of short simple repeats, while the flanking single-copy sequences are essentially identical in different individuals.

Using the complete pErs5 probe to challenge blots of panmictic chicken DNA, we observed patterns similar to those obtained with the blots from unrelated humans—i.e., hybridization to restriction fragments having polymorphic lengths in different individuals (results not shown). However when this probe was hybridized to blots of *Hae* III digests of inbred chicken DNA, the results shown in Fig. 3 (lanes C and D) were obtained: The DNAs from both inbred male and inbred female chickens show similar patterns of hybridizing bands, but only the male DNA shows a considerable smear in the long DNA fragment range. This finding corresponds to our results with inbred mice, in which we observed essentially the same situation (3)—i.e., a male-specific smear in the long DNA range after *Hae* III/*Alu* I digestion.

Since we found two longer open reading frames in the insert of pErs5, we split it into appropriate subfragments and repeated the hybridization experiments. The isolated and labeled *Sau*3AI/*Hha* I fragment (position 1,907–2,081) containing a shorter open reading frame yielded single bands from *Eco*RI-digested mouse DNA of both sexes (Fig. 3, lanes I and J). The hybrids between cloned snake and genomic mouse DNA exhibited high thermal stability, indicating strict evolutionary conservation of at least parts of this sequence. However to date our limited blot hybridizations have produced no evidence for the presence of any mRNA species in the mouse that cross-hybridizes to this specific subfragment.

The Isolated Interspersed Simple Repeat Hybridizes *in Situ* Predominantly to the Mouse Y Chromosome. To obtain further information on the chromosomal distribution in mammals of sequences cross-hybridizing with the cloned snake DNA, we carried out *in situ* hybridization experiments with [^{131}I]CTP-labeled probes. These experiments included metaphase chromosome spreads from bone marrow cells of male mice hybridized with a DNA fragment containing the simple repeat segment. In this case, the distribution of the silver grains was nearly homogeneous over the entire *autosomal* chromosome complement. A χ^2 test showed that the intensity of labeling is not significantly increased on any autosome. The *in situ* hybridization data from metaphase chromosomes of female mice confirmed these observations. Yet we found nearly 13 times the number of grains expected on the mouse Y chromosome, corresponding to 20% of the total grains observed. This result is consistent with

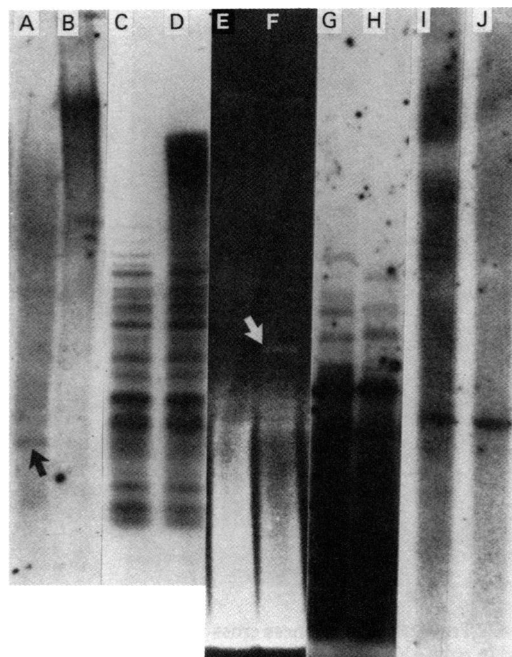

FIG. 3. Evolutionary conservation of the pErs5 insert and its subfragments in different vertebrate species. Three- or four-microgram samples of DNA digested to completion with restriction enzyme were separated on 0.4% agarose gels in Tris/borate/EDTA buffers. Lanes A, B, E, F, G, H, I, and J are blots obtained after Southern transfer (9) and hybridization to nick-translated restriction fragments of pErs5 (specific activity, usually 2×10^8 cpm/μg of DNA). Lanes: A and B, hybridization of the nick-translated *Hin*dIII subfragment of probe 1 (position 641–1,224 in Fig. 2*A*) to *Eco*RI-digested female and male, respectively, *E. radiata* genomic DNA; arrow, ≈2.5-kb-long single band (determined relative to markers run in the same gel); E and F, electrophoretic separation of *Hae* III/*Alu* I-digested female and male, respectively, human DNA as visualized by ethidium bromide staining; arrow, prominent band of 3.4-kb-long repeated DNA [this corresponds to the band originally observed by Cooke (26) and subsequently shown to be predominantly associated with the Y chromosome]; G and H, hybridization pattern of the labeled total insert of pErs5 to *Hae* III/*Alu* I-digested genomic DNA from unrelated healthy female and male, respectively, humans (this is a blot of the stained gel shown in lanes C and D; note that pErs5 does not cross hybridize with the 3.4-kb repeated sequence); C and D, hybridization pattern of the labeled complete insert of pErs5 to *Hae* III-digested genomic DNA of female and male, respectively, chickens from a highly inbred line; note the prominent smear in the higher molecular weight region of the male lane; I and J, hybridization of *Eco*RI-digested female and male, respectively, C57BL/B6 mouse genomic DNA with a labeled 175-bp-long subfragment of pErs5 (positions 1,907–2,071); a single band is apparent in both lanes.

FIG. 2 (*on preceding page*). Nucleotide sequence of the 2.5-kb *Eco*RI insert of pErs5. Position 1 is the guanosine of the pBR322 *Eco*RI site. The inserted sequence progresses from this point in a direction away from the pBR322 *Bam*HI site. Some key restriction sites are labeled. Significantly long potential open reading frames are translated into the corresponding protein sequences. Possible candidates for T-A-T-A boxes and poly(A) addition sites are boxed, as are some stop codons in potentially important locations. Two potential splice sites are indicated (slash marks) as are the tetramers of the simple repeat components (white and black bars). The nucleotide sequence was determined according to Maxam and Gilbert (19). Only one strand is shown, although confirmation was obtained by determining the sequence of >90% of the complementary strand. (*A*) Positions 1–1,224. (*B*) Positions 1,225–2,487.

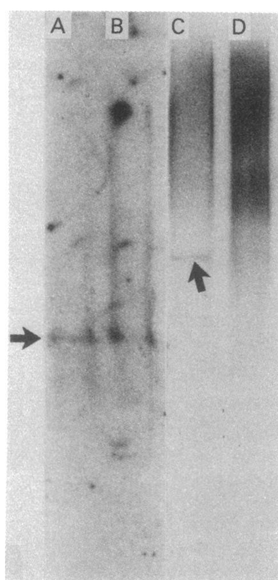

FIG. 4. Transcribed sequences cross-hybridizing to pErs5 are represented in poly(A)$^+$ RNA preparations from mouse liver. Five-microgram samples of poly(A)$^+$RNA from male (lanes A and C) and female (lanes B and D) mice were subjected to electrophoresis in 1% denaturing agarose gels [in 3-(*N*-morpholino)propanesulfonic acid/NaOAc/EDTA], blotted according to Southern (9), and hybridized to labeled subfragments of pErs5. Lanes: A and B, a poly(A)$^+$ preparation from *total cellular* liver RNA hybridized with a pErs5 subfragment (positions 1,480–1,906, probe 3 in Fig. 2*B*) yields one weak band with both sexes (arrow); C and D, a *polysomal* poly(A)$^+$RNA preparation probed with a different subfragment (probes 1 and 2; positions 1–1,224 in Fig. 2*A*) shows a cross-hybridizing band only in the male RNA (arrow).

data from Singh *et al.* (2) in which total banded krait minor satellite DNA or unpurified cross-hybridizing sequences were used. In our case with the cloned and purified segment, >80% of the Y chromosomal silver grains were located over the pericentric region. These results substantiate our initial hypothesis of a ubiquitously repeated element that is distributed in at least a partially sex-specific pattern in mouse chromosomes.

Sequences Cross-Hybridizing to Subfragments of pErs5 Are Expressed in Different Fractions of Mouse Liver RNA. The presence of open reading frames in the insert of pErs5, the conservation of subfragments thereof in several vertebrate species, and their specific chromosomal locations prompted us to ask questions concerning their possible expression on the RNA level. Using the *Sau* 3AI/*Eco*RI fragment (position 1,907–2,487) to probe blots of the poly(A)$^+$ fraction of total cellular RNA of mouse liver of both sexes, we observed no specific hybridization. Yet probe 3 (position 1,480–1,906), containing the simple repeat elements, bound in a single weak band in both sexes (Fig. 4, lanes A and B). The respective RNA species is ≈1,000 bases long when judged relative to the markers. If the strand complementary to that shown in Fig. 2 is the one actually used as a template *in vivo*, then it is quite possible that probe 3 cross hybridizes with a nontranslated segment of a poly(A)$^+$ message.

When polysomal poly(A)$^+$RNA fractions from male and female mouse liver were challenged with probe 1, a single band, 1,250–1,400 bases long, appeared only in the lane containing male RNA.

At this point, we do not know to what extent this poly(A)$^+$RNA sequence is translated *in vivo*. The identification of any protein product will have to follow isolation and cloning of the corresponding cDNA and genomic genes.

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