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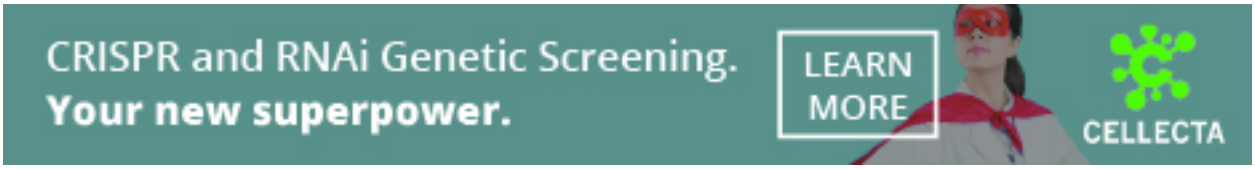

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PCR Amplification of *SRY*-related Gene Sequences Reveals Evolutionary Conservation of the *SRY*-box Motif

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***SRY* (sex-determining region of the Y chromosome) has recently been identified as a key regulatory gene in mammalian sex determination. The open reading frame of this gene contains an 80-amino-acid motif, the *SRY*-box, which shares a high degree of homology with a DNA-binding domain found in the high-mobility-group (HMG) proteins HMG1 and HMG2. The *SRY* box motif is highly conserved in several sequence-specific DNA-binding proteins that are known to act as transcription factors. Here we describe the use of degenerate PCR primers to identify *SRY*-related sequences containing the *SRY*-box motif from the genomic DNA of a variety of species. The results of this study suggest that in a diverse array of species *SRY*-related genes may serve as transcription factors that regulate a variety of developmental pathways, including sex determination.**

In mammals, the male phenotype is determined by the expression of a testis-determining factor (TDF) found on the Y chromosome. Recently, a gene was discovered within the sex-determining region of the Y chromosome in mice (*Sry*) and humans (*SRY*) that is expressed at the onset of testicular differentiation.[1] There is now substantial experimental evidence to support the correlation of *SRY* with TDF.[2–4] Furthermore, male-specific homologs to the human *SRY* gene have been detected in all eutherian mammals tested.[5]

Translation of the open reading frame of *SRY* produces a putative protein containing an 80-amino-acid motif that has strong homology to a DNA-binding domain (HMG-box) in the high-mobility-group (HMG) proteins, HMG1 and HMG 2.[5,6] This region of homology is referred to as the *SRY*-box, to distinguish it from the nonsequence-specific DNA-binding HMG-box proteins. A diverse array of proteins containing this *SRY*-box motif have been shown to have well-defined functions including: (1) Mc and mat-1, the cell-specialization regulators of the fission yeast *Schizosaccaromyces pombe*[7] and *Neurospora crassa*,[8] respectively.; (2) SteII, a yeast protein from *S. pombe* that regulates sexual development[9]; (3) cell differentiation factors TCF1 and TCF1a, human T-cell-specific transcription factors,[10] and LEF1 a human lymphoid-specific transcription factor[11]; (4) hUBF (human upstream binding factor), a general transcription factor for RNA polymerase I activity[12]; and (5) mouse protein IRE-ABP, which specifically binds the insulin response element A.[13]

In addition, four other genes related to *SRY*, *Sox*-1 to -4, have been identified in the mouse and mapped to autosomal sites.[14] These *SRY*-related genes contain the conserved *SRY*-box, but differ from the male-specific *SRY*-box domain at several critical amino acid sites.[14] The functions of *Sox* 1–4 are unknown, although expression patterns of these genes suggest a role in neuronal development.

Studies of the DNA-binding ability of the eukaryotic transcription factors have shown that the *SRY*-box is itself a sequence-specifc DNA-binding motif[11,13,15,16] and that the amino-terminal region of the box contains residues critical for DNA recognition.[16] It is evident that the male-specific *Sry/SRY* gene products constitute an important class of HMG-like proteins whose function in sex determination is dependent upon the DNA-binding ability of the *SRY*-box. Furthermore, the conservation of this DNA-binding domain in a number of *SRY*-related genes suggests that a similar genetic mechanism may control a variety of developmental processes, including sex determination.

Identification of a key regulatory gene in mammalian sex determination provides an opportunity to investigate the evolution of this developmental pathway, using species with either chromosomal (*Drosophila* and chicken) or temperature-dependent (alligators and geckos) sex determination. Here we describe the use of degenerate PCR primers

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to amplify a family of *SRY*-box–containing sequences from these species. Identification and characterization of this gene family will provide information concerning their evolutionary conservation and, ultimately, their functional homologies.

MATERIALS AND METHODS

Genomic DNA was prepared using whole body tissue from adult alligator, gecko, and *Drosophila*[17] and whole blood from chicken.[18] Three degenerate oligonucleotide primers [RG4-L, 5′-GAATTCGGTCAAGCGACCCATGAA(C/T)GCNTT-3′; RG5-L, 5′-AAGCTTAGGTCGGTACTT(G/A)TA(G/A)T(C/T)NGG(A/G)TA-3′; 8S, 5′-AAGCTTATGGCCC(A/T)GGA(G/A)AACCCCAAGATG-3′] directed against conserved regions of the *SRY*-box of human, mouse, and rabbit *SRY* and *Sry*-related mouse autosomal genes, were synthesized for PCR amplification and cloning of *SRY*-related sequences. Nucleotides in parentheses indicate partial degeneracy and an N signifies total degeneracy. The primers RG4-L and RG5-L were adapted from those used to clone *SRY*-related sequences from the lesser black-backed gull.[19] Primer 8S was designed specifically to amplify sequences containing the amino acid motif MA(Q/L)EN, present in mouse *Sry*-related genes expressed in the developing nervous system.[20]

PCR reactions were performed in a final volume of 100 µl containing 50 mM KCl, 10 mM Tris·HCl (pH 9.0), 1.5 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X-100, 200 M each dNTP, 20 pmoles each primer (gel purified), 4.5 units of *Taq* polymerase (Promega), and 0.5–1.0 µg of genomic DNA. The thermal profile for each reaction was: 94°C for 2 min, followed by 35 cycles of annealing at 55°C for 1 min, extending at 72°C for 1 min and denaturing at 94°C for 45 sec, followed by a final extension at 72°C for 5 min (using a TECHE PHC2). For the primer pair, 8S/RG5-L annealing was performed at 60°C.

Several PCR reactions of each DNA were pooled for cloning. Amplified

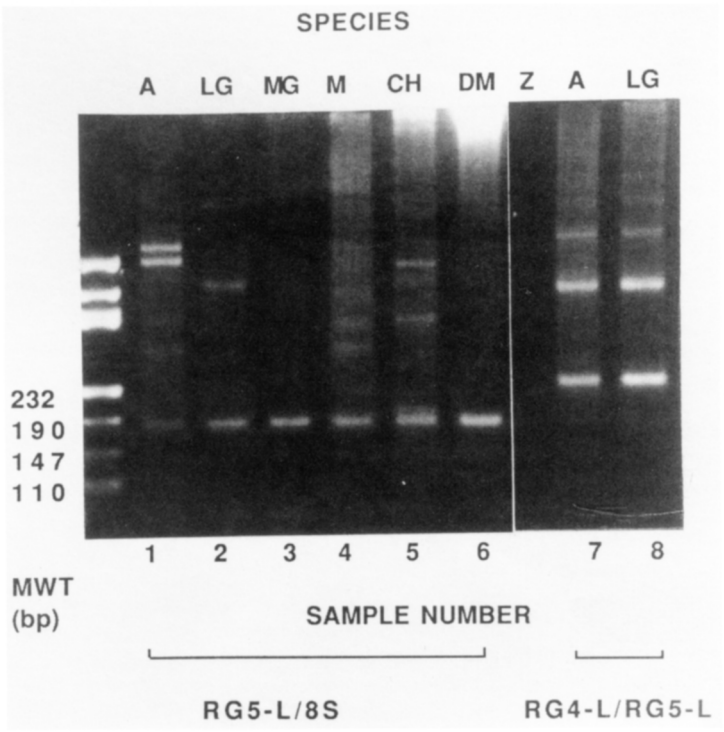

FIGURE 1 PCR-amplified DNA from species with either CSD or TSD. NuSieve 3:1 gel (4%) (FMC, 1990), showing PCR-amplified products from genomic DNA: (Lane *A*) American alligator; (lane *LG*) leopard gecko; (lane *MG*) Mediterranean gecko; (lane *M*) mouse; (lane *CH*) chicken; (lane *DM*) *Drosophila melanogaster*; (lane *Z*) zero control (no DNA). Samples 1–6 were amplified using primers RG5-L/8S and samples 7 and 8 were amplified using the primer pair RG4-L/RG5-L.

TABLE 1 Percentage Conservation of a 38-Amino-acid Stretch from Within the *SRY*-box, Identified for Species with Either CSD or TSD

	AES	AMA	LG	MG	DM	CH	SOX-4	SOX-3	SOX-2	SOX-1	m-y	h-y
ADW	79–84	58–66	71–89	84–95	58–68	66–71	84–87	63–66	66	61–63	53	45–47
AES		63–68	63–79	76–82	66–71	61–71	92–95	68	71	66	55	45–47
AMA			45–61	61–66	79–95	89–97	58–66	87–95	89–95	89–95	60–63	63
LG				66–87	61–68	47–63	60–76	53–63	53–66	47–51	47–51	42–53
MG					61–68	55–66	74–79	63–66	66–68	53–63	50–53	45
DM						79–97	63–68	87–95	84–95	84–92	58–66	50–55
CH							66–71	92–97	89–95	89–92	58–71	68–71
SOX-4								66	66	61	55	50
SOX-3									95	97	58	61
SOX-2										95	63	61
SOX-1											63	58
m-y												76

The conservative amino acid substitutions (Ser/Thr, Glu/Asp, Ile/Val/Leu) were considered homologous.[25] Clones with incomplete sequence data in this region have not been included in the calculations. Maximum and minimum homologies have been quoted for comparison between groups of clones.

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bands were purified, blunted, and concatamerized.[18,21] Resultant concatamers were linearized and cloned into either Bluescript (RG4-L/RG5-L) or pUC 18 (8S/RG5-L) vectors. Recombinant clones were identified by blue/white color selection (X-Gal/IPTG), or by colony screening, using radiolabeled primers. Clones were subsequently sequenced in both directions.

RESULTS

PCR methodology facilitated the rapid cloning of 17 American alligator (A), 2 leopard gecko (LG), 3 Mediterranean gecko (MG), 12 *Drosophila melanogaster* (DM), and 18 chicken (CH) *SRY*-related sequences. Using the primer pair RG4-L/RG5-L, a fragment of 228 bp was found in A, LG, and MG species that comigrated with an amplified mouse band (Fig. 1). Sequences containing the MAQ/LEN motif, found in the autosomal mouse *Sry*-related genes, were not identified using these primers. To facilitate the cloning of these specific sequences, the primer 8S was designed and tested for some species (A, DM, and CH) giving a fragment of 174 bp (Fig. 1).

Various estimations of the fidelity of *Taq* DNA polymerase have been made.[22,23] For the nucleotide and magnesium concentrations used for these experiments, we can assume a maximum cumulative error frequency of 10^{-5} (i.e., one wrong nucleotide per fragment or possibly only one wrong nucleotide per 10 fragments).

DNA sequences isolated from the species used in this study show stronger homologies to mouse autosomal *Sry*-related genes than to the human or mouse Y-specific loci (Table 1). The alligator sequences constitute three major protein classes and have been designated ADW, AES, and AMA, where the second and third letters denote amino acids that have been conserved specifically within the *SRY*-box at positions 23 and 25 (ADW and AES) or 21 and 22 (AMA) of the 72 amino acids shown (Fig. 2). The ADW and AES groups have greatest similarity with *Sox*-4 (84–87% and 92–95%, respectively), whereas the AMA sequences show the highest degree of homology with *Sox*-1, *Sox*-2, and *Sox*-3 (87–95%) (Fig. 2; Table 1). The levels of amino acid conservation between specific alligator and mouse *SRY*-related sequences are greater than, or equivalent to, the level

```
ma-4      HN  AEISKRLGKR    WKLLKDSDKI    PFIQEAERLR    LKHMAD
ma-3      --  S-------AD    ----T-AE-R    ---D--K---    AV--KE
ma-2      --  S-------AE    ----SETE-R    ---D--K---    AL--KE
ma-1      --  S-------AE    --VMSEAE-R    ---D--K---    AL--KE

sox 5     --  SN---I--S-    --AMTNLE-Q    -YYE-QA--S    KQ-LEK
xsox5     --  SN---I--S-    --SMTNLE--    -YYE-QA--S    KQ-LEK
hsox5     --  SN---I--S-    --AMTNLE-Q    -YYE-QA--S    KQ-LEK

sox6      --  SN---I--S-    --SMSNQE-Q    -YYE-QA--S    KI-LEK
hsox6     --  SN---I--S-    --SMSNQE-Q    -YYE-QA--S    KI-LEK

SOX 7     --  ---L-M---S    --A-TL-Q-R    -YVD------    -Q--Q-
sox 8     --  -VL--M---A    --E-NAAE-R    --VE-A----    VQ-LR-
sox 9     --  S-------AE    ----T-AE-R    ---D--K---    AV--K-
sox 10    --  S-------AE    ----TESE-R    ---D--K---    AM--KE

m-y       Q-  T----Q--C-    --S-TEAE-R    --F---Q--K    IL-REK
h-y       R-  S----Q--YQ    --M-TEAE-W    --F---QK-Q    AM-REK

ADW-2     --  --------R-    -Q--Q--E--    --VGK-----    ------
ADW-4     --  --------R-    -Q--Q--E--    --VK--DGLL    ------
ADW-5     --  --------R-    -Q--Q--E--    --VK--GG--    ------

AES-1     --  ----------    -----G----    ---R------    ------
AES-2     --  ----------    -----G----    ---R------    I-----
AES-4     --  ------R---    ----------    ---RA-----    ------

AMA1      --  SV------AE    ----SEAE-R    --SD--K---    AM--KE
AMA2      --  S-------AE    ----SEAE-R    ---D--K---    AM--KE

LG-27     --  --------R-    -Q--Q--ERY    LSRRR*SV-     ------
LG-28     --  --------R-    -Q--Q--E--    --VK-*SV-     ------
MG-42     --  --------RP    VQ--Q--E--    --VK--G---    V-----
MG-43     --  ----E---R-    -Q--Q--E--    --VK--G---    ------
MG-44     --  -----S--RS    SQ--Q--E--    --VK--G---    ------

D10       --  S-------AE    ----A--E-R    ---D--K---    AL--KE
D16       --  P---------    ----SEAE-R    -Y-D--K---    AQ--KE
D17       --  S-------AE    ----SEAE-R    -Y-D--K---    AQ--KE
D23       --  S-------AE    ----AE-E-R    ---D--K---    AL--KA
D33       --  S-------AE    ----AE-E-R    -L-D--K---    AL--NH
D36       Q-  SH------AE    ----AA-E-R    ---D--K---    AL--KE
D63       --  S-------AE    ----AE-E-R    ---D--K---    AL--KE
D64       --  S-------AE    --D-SE-E-R    ---D--K---     V--KE

CH-4      --  S-------AE    ----SEAE-R    ---DDS----    AM--KE
CH-7      --  S-S-----AE    ----SEAE-R    ---D--K---    AM--KE
CH-46     --  S-------AE    ----SEAE-R    ---D--K---    AM--KE
CH-1      --  S-------AE    --Q-SEAE-R    ---D--K-H-    AM--KE
CH-61     --  S-------AE    ----SEAE-R    ---D--K-P-    AM--KE
CH-60     --  S-------AE    ----SEAE-R    -Y-D--K---    AQ--KE
CH-32     --  S---Q---AE    ----SEAE-R    -Y-D--K---    AQ--KE
CH-44     --  S-------AE    ----SEAE-R    -Y-D--K---    AQ--KE
CH-2      --  S-M-----AE    ----SEAE-R    ---D--K---    AS--KE
CH-3      --  S-------AD    ----S-AE-R    ---D--K---    AV--KE
```

FIGURE 2 Comparison of *SRY* and related amino acid sequences for seven species, representing 38 of the 80 amino acids constituting the *SRY*-box region of the *SRY* genes. Regions containing PCR primer sequence have not been considered when calculating homologies (see Table 1). Species are: American alligator (A), leopard gecko (LG), Mediterranean gecko (MG), *Drosophila melanogaster* (DM), and chicken (CH). Using the primer pair RG5-L/8S AMA, DM, and CH sequences were amplified; ADW, AES, LG, and MG sequences were amplified with primers RG4-L/RG5-L. These sequences include 38 of 80 amino acids that constitute the mammalian *SRY*-box region of *Sry* and *SRY*-related genes. Where one or more of the clones sequenced for any species were identical to each other, only one has been shown. Each amino acid sequence was compared against the sex-specific copies of *SRY* (m-y/mouse, h-y/human) and *Sry*-related mouse autosomal genes *sox*-1 to 4. Dashed lines (–) represent sequence identity with respect to *sox*-4. Amino acids outlined for h-y and m-y indicate sex-specific motifs. An asterisk is inserted in LG sequences for alignment. GenBank accession numbers for sequences listed are M86310–M86339 inclusive.

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of homology found among the different mouse (*Sox*-1, *Sox*-2, *Sox*-3, and *Sox*-4; 61–97%) and alligator (ADW, AES, and AMA; 58–84%) protein classes (Table 1). The conserved sequence homology observed between alligator and mouse sequences for this region of the *SRY*-box may reflect functional conservation, suggesting that these proteins have similar roles in reptiles and mammals.

For sequences differing in only one amino acid, e.g., AES-1 and AES-2, CH-7 and CH-46, CH-32 and CH-44, nucleotide sequences varied by a single base of the amino acid codon. This may suggest that these clones are identical and that the difference observed is a consequence of polymerase infidelity.

A comparison of *SRY*-related sequences derived from other species (Fig. 2) shows that both LG and MG *SRY*-box sequences share the conserved amino acid region of ADW, while the DM and CH sequences are more closely related to the AMA group (Fig. 2; Table 1). The degree of *SRY*-box homology between species with temperature-dependent sex determination (e.g., ADW and LG; 71–89%), or species with chromosomal sex determination (e.g., DM and CH; 79–97%), is not significantly different from the level of amino acid conservation among species with alternative modes of sex determination (e.g., ADW and MG, 84–95%; AMA and DM, 79–95%; AMA and CH, 89–97%; Table 1).

Preliminary work in our laboratory, using the PCR-amplified sequences as probes, has identified a large number of different *SRY*-box–containing cDNAs from alligator and chicken embryonic cDNA libraries (unpublished results). These findings suggest that PCR-derived sequences represent genes that are developmentally expressed and that the large number of these closely related sequences is not due to PCR artifacts.

Different primer pairs have been used to amplify selectively different groups of *SRY*-box sequences, revealing highly conserved sequences in invertebrates (*D. melanogaster*) and a number of vertebrates (reptiles and birds). None of the sequences isolated so far show sequence characteristics unique to *SRY/Sry*, but the size of this growing gene family suggests that such sequences have yet to be discovered.

The use of PCR to identify members of this gene family has provided the first step for assessing the significance and degree of evolutionary conservation of these highly related genes.

DISCUSSION

Mammalian Y-specific *SRY* and autosomal *SRY*-related gene sequences contain a highly conserved *SRY*-box motif.[5,14] The distinction between these *SRY*-box sequences is the invariant site specificity of critical amino acids in the putative *SRY* gene product.[2,3] The *SRY*-box domains discovered for other species[19] (Fig. 2) show greater homology with the mouse *Sox* genes than with the male-specific *SRY* (Table 1). These preliminary data suggest that *Sry/SRY* may act as a major testis-determining gene only in mammals, although it is possible that we failed to amplify sex-specific *SRY* sequences in the species examined. The high degree of conservation among *SRY*-box sequences presents difficulties when assessing their evolutionary relationships to *Sry/SRY*. Adequate comparison requires sequence information from outside the *SRY*-box motifs.

Mouse autosomal copies of *Sry* have been shown to be expressed predominantly in neural tissue and in other ectoderm derivatives of the developing mouse embryo.[20] The strong homology that exists between the *SRY*-box of mouse autosomal *Sry*-related genes and *SRY*-related genes of other species (Table 1) suggests that their functions may be similar. It is reasonable to suggest that the alligator *SRY*-related gene products do act as transcriptional regulators in view of the putative DNA-binding domain they share with a number of known transcription factors. For other families of transcription factors, e.g., homeobox genes, paired box genes, and zinc finger genes,[24] the conservation of DNA-binding domains has been shown to reflect functional homology for the resulting gene products. Further analyses, including developmental expression and cellular localization studies (using cDNA sequences derived from the American alligator and chicken), will help to define the role of *SRY*-related genes in vertebrate development.

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