18. Y.-S. Lin and M. Green, *Cell* **64**, 971 (1991).
19. M. Ptashne and A. F. Gann, *Nature* **346**, 329 (1990); P. A. Sharp, *ibid.* **351**, 16 (1991); M. Carey, *Curr. Opin. Cell Biol.* **3**, 452 (1991); J. Greenblatt, *Cell* **66**, 1067 (1991); M. Horikoshi, M. Carey, H. Kakidani, M. R. Green, R. G. Roeder, *ibid.* **54**, 665 (1988); J. L. Workman, I. C. A. Taylor, R. E. Kingston, *ibid.* **64**, 533 (1991); G. E. Croston, L. A. Kerrigan, L. M. Lira, D. R. Marshak, J. T. Kadonaga, *Science* **251**, 643 (1991).
20. M. W. Van Dyke, R. G. Roeder, M. Sawadogo, *Science* **241**, 1335 (1988).
21. D. Sigman, *Biochemistry* **29**, 9097 (1990).
22. D. L. Popham, D. Szeto, J. Keener, S. Kustu, *Science* **243**, 629 (1989).
23. J. Gralla, *Cell* **66**, 415 (1991); J. Collado-Vides, B. Magasanik, J. D. Gralla, *Microbiol. Rev.* **55**, 371 (1991).
24. D. R. Herendeen, G. A. Kassavetis, J. Barry, B. M. Alberts, E. P. Geiduschek, *Science* **245**, 952 (1989).
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DNA Binding Activity of Recombinant SRY from Normal Males and XY Females

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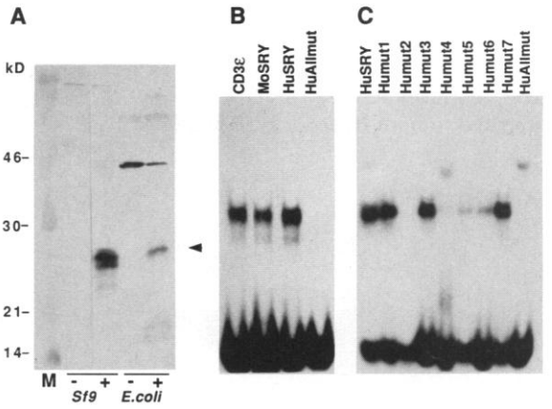
The protein encoded by the human testis determining gene, *SRY*, contains a high mobility group (HMG) box related to that present in the T cell–specific, DNA-binding protein TCF-1. Recombinant SRY protein was able to bind to the same core sequence AACAAAG recognized by TCF-1 in a sequence dependent manner. In five XY females point mutations were found in the region encoding the HMG box. In four cases DNA binding activity of mutant SRY protein was negligible; in the fifth case DNA binding was reduced. These results imply that the DNA binding activity of SRY is required for sex determination.

THE Y CHROMOSOME IS A DOMINANT inducer of male sex determination in mammals (*1*). In the absence of the Y chromosome, fetal genital ridges develop as ovaries; in the presence of the Y chromosome, the genital ridges develop as testes (*2*). The position of the Y-located gene responsible for inducing testis formation was deduced from the study of the genomes of individuals with two X chromosomes but with testicular development (*3*). In four cases, the sex-reversed individuals had inherited less than 40 kb of Y chromosomal DNA, and in this region we identified and cloned the gene *SRY* (sex-determining region Y gene) (*4*). *SRY* and the mouse homolog *Sry* (*5*) have many of the predicted properties of the testis-determining gene (*6–9*).

By analogy with the genetic control of other developmental processes, it would be predicted that *SRY/Sry* is part of a pathway of genes that control sex determination. The existence of this pathway can also be deduced directly: (i) the induction of *Sry* transcription at day 10.5 postcoitum implies the existence of "upstream" regulatory genes (*9*); and (ii) the testicular differentiation found in some XX males, in the absence of any Y-derived sequences, can best be explained by gain-of-function mutations in "downstream" genes (*4*). Similarly, many other sex-reversing mutations in both humans and mice must be affecting genes other than *SRY/Sry* in the sex determination pathway (*10*).

Regulatory genes can exert their control by different mechanisms. In *Drosophila*, although the sex determination pathway is set by transcriptional regulation, the mechanism for transferring information between genes in the pathway is posttranscriptional; in nematode sex determination, transcriptional regulation is the main method used (*11*). The sequence of *SRY/Sry* suggests that it may encode a DNA binding protein, and this would be consistent with a role as a transcription factor. The protein product of *SRY/Sry* contains an 80–amino acid sequence motif, which is found in several proteins with known or suspected DNA



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Element	Probe sequence	DNA binding
CD3ε	ACACTGAG AACAAAG CGCTCTCACAC	+++
MoSRY	GTGCTTGT AACAAAG ATTTTAT	+++
HuSRY	GTTAACGT AACAAAG AATCTGGTAGA	+++
Humut1	..... C.....	+++
Humut3	....G....	+++
Humut7	....T	+++
Humut2	..... .C.....	+/−
Humut4	....C...	+/−
Humut5	....G..	+/−
Humut6	....G.	+/−
HuAllmut	..... CCGCGGT	−

Fig. 1. Identification of SRY as a sequence-specific DNA binding protein. (**A**) Western blot analysis of human SRY expressed in *E. coli* and in insect cells (*19*). Soluble extracts from Sf9 insect cells infected with baculovirus expressing β-galactosidase (−) or human SRY (+) and from induced *E. coli* cultures transformed with plasmid pJLA503 (−) and pJLA503-huSRY (+) were analyzed by Western blotting after SDS–polyacrylamide gel electrophoresis and probed with an anti-SRY rabbit serum. M: Prestained markers with molecular weights in kilodaltons as indicated. The arrow indicates the position of SRY, which migrates as a 24/25-kD doublet (predicted molecular weight, 23.9) from baculovirus-infected Sf9 cells and as a single band at 25 kD from bacteria. The doublet appears to be caused by an insect cell modification of the SRY protein. (**B**) DNA binding of SRY to the sequence AACAAAG as measured by electrophoretic mobility shift assay (*20*). *Escherichia coli* extracts containing SRY were incubated with 29-bp ^{32}P-labeled oligonucleotides probes containing the sequence AACAAAG or with a negative control, HuAllmut, where the AACAAAG in the human *SRY* probe has been replaced with CCGCGGT. (**C**) Electrophoretic mobility shift assay of SRY and probes with single point mutations across the AACAAAG sequence. (**D**) Summary of DNA-binding specificity of SRY to the DNA elements used as probes for DNA binding assays shown in (B) and (C). Dots indicate sequence homology to the human *SRY* probe. Binding of at least 30% of the DNA added to the reaction was judged as strongly positive (+++); weak, but detectable binding was recorded as +/−.

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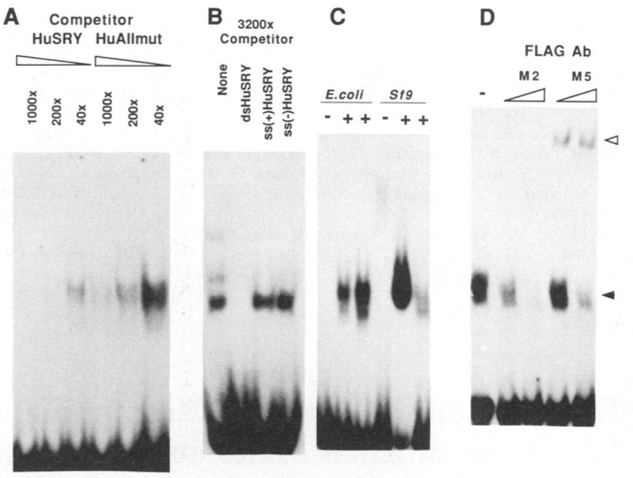
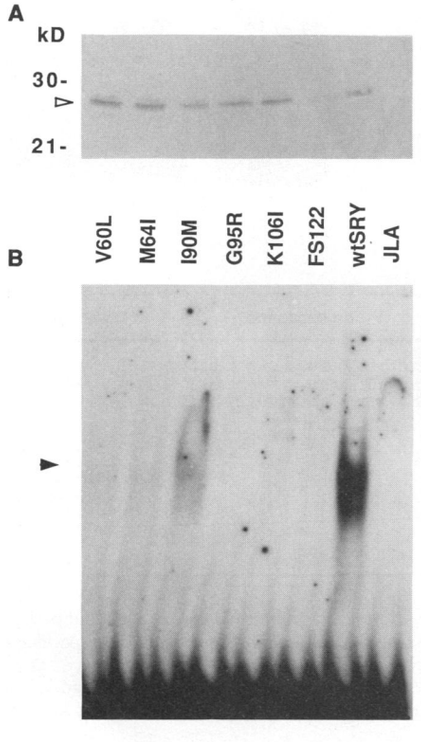


Fig. 2. Detailed analysis of SRY/DNA interaction. (**A**) Competition of SRY/DNA complex with human *SRY* probe. *Escherichia coli* extracts containing SRY were incubated with ^{32}P-labeled human *SRY* probe in the presence of unlabeled wild type (HuSRY) or mutant (HuAllmut) human *SRY* probes at 40-fold, 200-fold, and 1000-fold molar excess. (**B**) Competition of SRY/DNA complex with single-stranded DNA. *Escherichia coli* extracts containing SRY were incubated with labeled HuSRY probe then competed with 80 pmol (3200-fold molar excess) unlabeled HuSRY probe or with the + or − sense single strands of the HuSRY probe. (**C**) Recombinant SRY from insect cells binds specifically to AACAAAG. ^{32}P-labeled HuSRY probe was incubated with *E. coli* extracts from induced cultures harboring plasmid pJLA (−) or two cultures of pJLA-huSRY (+) or with baculovirus-infected Sf9 insect cell extracts expressing β-galactosidase (−) or SRY from two viral stocks (+). (**D**) *Escherichia coli* extracts containing FLAG-SRY fusion protein were incubated with labeled HuSRY probe in the absence of antibody and with M2 FLAG and M5 FLAG antibodies at 0.24 μg and at 2.4 μg. The closed arrow indicates the position of the FLAG-SRY/DNA complex, and the open arrow indicates the complex further retarded by monoclonal antibody. Retardation by the M2 antibody was much less effective than that by M5 and forms an internal control for nonspecific effects due to protein concentration. When present at 0.24 μg, M2 inhibits SRY-DNA complex formation, whereas at 2.4 μg both antibodies show inhibition.

binding activity. The motif was first recognized in high mobility group (HMG) proteins and is called the HMG box (*12*). HMG proteins bind to DNA without overt sequence specificity; however, sequence-specific transcription factors have variants of the same motif (*13*).

Among known sequence-specific, DNA-binding proteins, SRY is most closely related to two T lymphocyte proteins, TCF-1 and TCF-1α. TCF-1 binds to the sequence AACAAAG present in the CD3ε enhancer and to related sites in several other T cell specific genes. TCF-1α binds to the sequence CANAG present in some T cell receptor enhancers (*13*). SRY protein was produced in both *Escherichia coli* and insect cells (Fig. 1A) (*14*). The recombinant protein was used to test for binding to the TCF-1 target sequence AACAAAG and variants of this sequence. Radiolabeled double-stranded oligonucleotides were incubated with SRY and the resulting complexes were analyzed by acrylamide gel electrophoresis in a mobility shift assay. SRY protein forms a complex with the oligonucleotide probe containing the sequence AACAAAG (Fig. 1B, lane 1). Copies of the AACAAAG sequence are also found upstream of both the mouse *Sry* and human *SRY* genes (*15*). Oligonucleotide probes encoding these sequences were retarded to the same extent as the CD3ε probe (Fig. 1B, lanes 2 and 3). Because the CD3ε, *SRY*, and *Sry* oligonucleotide probes have different sequences flanking their AACAAAG core, binding by SRY protein is largely context-independent. SRY did not bind to the *SRY* oligonucleotide when AACAAAG was replaced with CCGCGGT (Fig. 1B, lane 4), and binding was drastically reduced by mutations at the second, fourth, fifth, and sixth positions (Fig. 1C).

Several controls were performed to examine in more detail the specificity of the



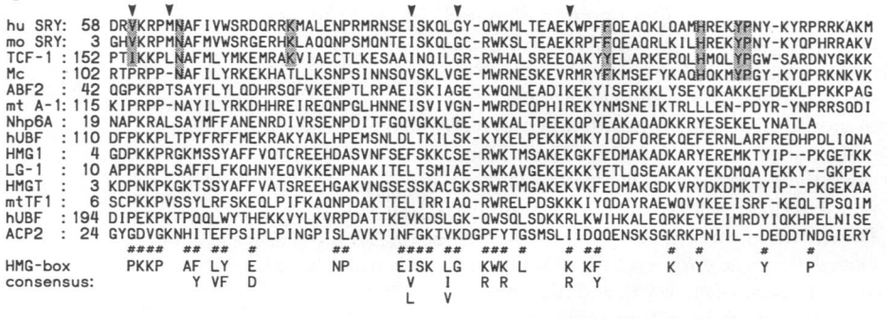


Fig. 3. Bacterial expression and DNA binding activity of recombinant SRY from XY females. (**A**) Expression of recombinant SRY from XY females shown on an immunoblot stained with anti-SRY rabbit serum. Extracts were prepared (*14*), assayed for total protein (*21*), then matched for staining intensity of SRY by immunoblot. Open arrow indicates the position of SRY. (**B**) Gel retardation analysis of the human *SRY* probe by extracts (10 to 15 μg of protein) containing recombinant SRY from XY females (*6*). Lane assignments as in (A). A closed arrow indicates the position of the SRY/DNA complex. (**C**) Protein sequence alignments of some members of the HMG-box family. Amino acid positions are indicated at the beginning of each line (*22*). Dashes were introduced to maintain optimal alignment. The crosshatches indicate residues identical or conserved in at least eight of the sequences, and a consensus is shown below. Residues identical or conserved in the SRY/Mc/TCF-1 subclass are shaded. Insertions occur in HMG1 at position 27 (KKKHP) and in HMGT at positions 27 (KKKHS) and 47 (SPRDSKAPK). Conserved amino acids are grouped as follows: Y/F, I/L/V, S/T, K/R, and D/E. The *SRY* mutations in the five XY females are shown above the arrows on the human *SRY* sequence. Abbreviations for the proteins aligned (*4, 5, 12, 13, 23*) are as follows: hu SRY, human SRY protein; mo SRY, mouse *Sry* protein; TCF-1, T cell factor 1; Mc, mating type protein of *S. pombe*; ABF2, *S. cerevisiae* ARS binding protein; mt A-1, mating-type protein of *N. crassa*; NHP6A, *S. cerevisiae* nonhistone protein A; hUBF, human RNA polymerase I upstream binding factor; HMG1, High mobility group protein 1; LG1, chromosome-associated protein from *Tetrahymena thermophilia*; HMGT, trout testis HMG protein; mtTF1, human mitochondrial transcription factor; ACP2, *S. cerevisiae* subunit of RNA polymerase C (III).

interaction of the SRY protein with the AACAAAG sequence present in the human *SRY* probe. Complex formation could be effectively competed by excess unlabeled *SRY* oligonucleotide, but only poorly by the *SRY* oligonucleotide with AACAAAG replaced by CCGCGGT (Fig. 2A). Single-stranded oligonucleotides were unable to compete for binding of SRY (Fig. 2B). Thus SRY, unlike HMG1 (*16*), has no apparent affinity for single-stranded DNA. We directly demonstrated the presence of SRY protein in the observed complexes by producing SRY protein tagged with a peptide epitope (*17*). The tagged SRY (called FLAG-SRY) formed complexes that were further retarded in their migration by the addition of epitope-specific antibody (Fig. 2D). SRY protein, produced in baculovirus-infected insect cells, also formed complexes with the *SRY* oligonucleotide probe (Fig. 2C). This suggests that ability to bind to DNA is an intrinsic property of the SRY protein and does not require *E. coli* or insect cell accessory factors.

Sex reversal in XY females is caused by failure of the testis-determining or differentiation pathways. In about 15% of XY females (*6*), mutations have been found in *SRY* and these mutations all lie within the region that encodes the HMG box (Fig. 3C). The majority of mutations that have been tested are de novo and are not shared by the fathers and their XY daughters. In two cases, however, the fertile fathers and their daughters share the same *SRY* sequence. In these cases, it is not clear if the sequence alteration is contributing to the sex reversal. *SRY* sequences derived from six XY females were cloned in the *E. coli* expression vector (*14*). SRY protein was produced from five genes with point mutations, but it was not possible to produce protein corresponding to a frameshift mutant (Fig. 3A). We presume the frameshift protein is unstable in bacteria. Quantitation of SRY protein was made by varying wild-type and mutant SRY protein until comparable amounts were attained, as evaluated by immunoblot staining (Fig. 3A). Equal amounts of wild-type and mutant SRY protein were then used in electrophoretic mobility shift assays with the *SRY* oligonucleotide probe. No DNA binding activity was detected with the point mutants G95R and K106I, and negligible binding was associated with V60L and M65I (Fig. 3B). In contrast, the I90M mutant had retained DNA binding but at a reduced level. The mutants M64I and G95R are the result of de novo mutations, and the K106I mutation has not been tested; mutants from I90M and V60L are shared by fathers and daughters. The reduced binding activity of the I90M protein suggests an explanation for the partial penetrance of this mutation. The negligible binding associated with the V60L mutation is less expected and suggests that the in vitro assay is more stringent than the relevant in vivo conditions, which may involve other factors. For example, in *Saccharomyces cerevisiae*, MCM1 raises the affinity 500-fold of α2 for its operator (*18*). Nevertheless, in all the cases studied the results suggest that reduced DNA binding activity is contributing to sex reversal and that the HMG box is required for DNA binding.

The amino acid sequences of the HMG boxes from different proteins are aligned in Fig. 3C. In 28 positions the amino acids are highly conserved, and the substitutions in the V60L, I90M, G95R, and K106I mutants all occur at one of these positions. The M64I mutation, however, occurs at a position whose amino acid residue is variable between HMG box proteins. Inspection of sequences from the human SRY, mouse Sry, TCF-1, and the mating-type protein of *Schizosaccharomyces pombe* (Mc) reveal a further six conserved residues (shaded in Fig. 3C) confined to this subclass that may contribute to DNA sequence specificity of these regulatory proteins.

In summary, SRY binds specifically to the DNA sequence AACAAAG and bases at the second, fourth, fifth, and sixth positions appear critical for this interaction. Mutant SRY encoded by XY females loses this DNA binding ability, which implies that this activity is necessary for testicular development. At present, we do not know if the AACAAAG sequence upstream of the human *SRY* and mouse *Sry* genes is functionally important or coincidental. Moreover, further studies are required to test whether the AACAAAG sequence represents the optimal SRY binding site. SRY may bind another sequence more efficiently than AACAAAG, and other proteins in the genital ridge may contribute to SRY binding activity in vivo. Nevertheless, the demonstration that SRY binds to AACAAAG will help in defining the putative target sequences that mediate the actions of SRY in sex determination.

REFERENCES AND NOTES

1. C. W. Ford *et al.*, *Lancet* i, 711 (1959); P. A. Jacobs and J. A. Strong, *Nature* **183**, 302 (1959).
2. A. Jost, *Recent Prog. Horm. Res.* **29**, 1 (1973).
3. G. Vergnaud *et al.*, *Am. J. Hum. Genet.* **38**, 172 (1986); G. Guellaen *et al.*, *Nature* **302**, 172 (1984).
4. M. S. Palmer *et al.*, *Nature* **342**, 937 (1990); A. H. Sinclair *et al.*, *ibid.* **346**, 240 (1990).
5. J. Gubbay *et al.*, *ibid.*, p. 245.
6. P. Berta *et al.*, *ibid.* **348**, 448 (1990); R. L. Jager, M. Anvret, K. Hall, G. Scherer, *ibid.*, p. 452; J. R. Hawkins *et al.*, *Hum. Genet.*, in press. XY female patients will be referred to in the text by the amino acid substitution their *SRY* mutation encodes. Patient J.N. has a valine to leucine substitution at amino acid position 60 of human SRY and is therefore called V60L; patient A.M. is M64I; patient Cal A. is G95R; patient S. has a 4-bp deletion causing a frameshift mutation is FS122. Patient 213 is I90M; patient 207 is K1061 (J. R. Hawkins *et al.*, unpublished data).
7. R. H. Lovell-Badge and E. Robertson, *Development* **109**, 635 (1990).
8. P. Koopman *et al.*, *Nature* **351**, 117 (1991); J. Gubbay, A. Economou, A. Sinclair, D. I. Jackson, unpublished data.
9. P. Koopman *et al.*, *Nature* **348**, 450 (1990).
10. A. de la Chapelle, *Development* **101**, 33 (1987); E. Eicher and L. Washburn, *Annu. Rev. Genet.* **20**, 327 (1986); M. Palmer *et al.*, *Nature* **342**, 937 (1989); M. A. Ferguson-Smith *et al.*, *Hum. Genet.* **84**, 198 (1990).
11. J. Hodgkin, *Nature* **344**, 721 (1990).
12. H.-M. Jantzen, A. Admon, S. P. Bell, R. Tjian, *ibid.*, p. 830.
13. M. van de Wetering *et al.*, *EMBO J.* **10**, 123 (1991); M. Oosterwegel *et al.*, *J. Exp. Med.* **173**, 1133 (1991); M. Waterman and K. Jones, *New Biol.* **2**, 621 (1990). Within the HMG box, SRY is closely related to TCF-1 (24% amino acid identity) and to *S. pombe* Mc protein (29%)—all three proteins contain only a single HMG box.
14. For expression of SRY in bacteria, DNA encoding the human *SRY* open reading frame was excised by polymerase chain reaction (PCR) from the genomic clone p53.3 (*4*) and from genomic DNA or PCR fragments derived from XY females (*6*) by the forward primer NDEIHUSRY (5′-TTGTTTTTG-CATATGCAATCTTATGCTT-3′) and reverse primer Y53.3 ECO (5′-AATGTTACCGAATTC-TCCTACAGC-3′). PCR fragments were cleaved with Nde I and Eco RI, cloned into the bacterial expression vector pJLA503 [B. Schauder *et al.*, *Gene* **52**, 279 (1987)] and transformed in *E. coli* strain DH5α. Cytoplasmic extracts were prepared (*13*) from induced cultures and stored in aliquots at −70°C. For expression of SRY in insect cells, human *SRY* coding sequence was excised by PCR from the genomic clone p53.3 (*4*) with the primers BGLIIHUSRYFOR 5′-CTCCTTGTTAGATCT-TATGGAATCA-3′ and Y53.3ECO, cleaved with Bgl II and Eco RI, and inserted into the baculovirus expression vector pVL1392 (Invitrogen) to give plasmid pVL-huSRY. *Spodoptera frugiperda* (Sf 9) cells were cotransfected with a mixture of Bsu 36I digested baculovirus genomic DNA, AcRP23.lacZ [R. D. Possee and S. C. Howard, *Nucleic Acids Res.* **15**, 10233 (1987); P. A. Kitts *et al.*, *ibid.* **18**, 5667 (1990)] and pVL-huSRY by means of lipofectin (Bethesda Research Laboratories). Recombinant viruses were purified by two sequential plaque assays and characterized by dot blot analysis and β-galactosidase assay [M. Summers and G. Smith, *Tex. Agric. Exp. Stn. Bull.* **1555** (1987)]. Sf 9 cells (3×10^6) in a 25-cm^3 flask were infected at a multiplicity of infection (MOI) between one and ten with SRY recombinant virus clones 11/1, 15/2, or β-galactosidase control virus. After 3 days, cytoplasmic extracts were prepared (*13*) and stored at −70°C. Typically, 50 μl of extracts were used for SDS–polyacrylamide gel electrophoresis/Western (RNA) blot analysis [E. Harlow and D. Lane, *Antibodies: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988)] and 1 to 5 μl for gel retardation analysis.
15. A search for the sequence AACAAAG was made in the 25 kb of sequence flanking the human *SRY* gene and the 15 kb of sequence flanking the mouse *Sry* gene. One stretch of ten nucleotides containing this sequence was identical between human *SRY* (94 nucleotides upstream of the initiating ATG) and mouse *Sry* (288 nucleotides upstream of putative initiating ATG; A. Sinclair, D. Jackson, J. Gubbay, unpublished data).
16. A. P. Butler *et al.*, *J. Biol. Chem.* **19**, 10613 (1985).
17. K. S. Prickett *et al.*, *BioTechniques* **7**, 580 (1989). The fusion protein FLAG-SRY was constructed by PCR with the forward primer 5′-CTGCATATGGACTA-CAAGACGACGACGACAAAATGCAATCTTAT-GCTTCT-3′ and Y53.3ECO as the reverse primer. This forward primer introduces the epitope sequence, DYKDDDDK, at the NH_2-terminus of SRY. This epitope is recognized by the monoclonal antibodies M2 and M5 (Immunex).

18. C. A. Keleher *et al.*, *Mol. Cell. Biol.* **9**, 5228 (1989).
19. SDS-polyacrylamide gel electrophoresis and immunoblotting was as described in Fig. 1. The polypeptide MHREKYPNYKYRP corresponding to amino acids 119 and 131 of human SRY were synthesized. Rabbit serum raised against this peptide was diluted 1:50 in phosphate-buffered saline (PBS)/0.05% Tween 20, and blots were probed overnight at 4°C. Detection of anti-SRY antibodies was accomplished by 2-hour incubation of the immunoblot with horseradish peroxidase–conjugated second antibody (Dako).
20. Gel retardation analysis was modified from M. Fried and D. M. Crothers, *J. Mol. Biol.* **172**, 263 (1984). For consistent specific activities, GGG was synthesized at the 5′ end of oligonucleotides shown in Fig. 1D and annealed to their complement and then labeled by Klenow DNA polymerase in a fill-in reaction with [α-^{32}P]dCTP. All probes were purified by 8% polyacrylamide gel electrophoresis. In a typical binding reaction, extract (5 to 10 μg of protein), 1 μg of poly(dI-dC) and 50 ng of sonicated salmon sperm DNA were incubated in a final volume of 16 μl containing 10 mM Hepes, pH 7.9, 60 mM KCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), and 12% glycerol. After 5 min incubation at room temperature, about 20 fmol of probe (10,000 to 20,000 cpm) was added and the mixture was incubated for an additional 5 min. In competition experiments, nonlabeled competitor DNA was added with the poly(dI-dC). The samples were then separated by electrophoresis through a nondenaturing 4% polyacrylamide gel run in 0.25× TBE at room temperature.
21. M. M. Bradford, *Anal. Biochem.* **72**, 248 (1976).
22. Single-letter abbreviations for the amino acids are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
23. M. Kelly *et al.*, *EMBO J.* **7**, 1537 (1988); J. F. X. Diffley and B. Stillman, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 7864 (1991); C. Staben and C. Yanofsky, *ibid.* **87**, 4917 (1990); D. Kolodrubetz *et al.*, *J. Biol. Chem.* **265**, 3234 (1990); L. Wen *et al.*, *Nucleic Acids Res.* **17**, 1197 (1989); S. Y. Roth *et al.*, *ibid.* **15**, 8112 (1987); B. Pentecost *et al.*, *ibid.* **13**, 4871 (1985); M. A. Parisi and D. A. Clayton, *Science* **252**, 965 (1991); W. Haggren and D. Kolodrubetz, *Mol. Cell. Biol.* **8**, 1282 (1988); C. Mosrin *et al.*, *ibid.* **10**, 4737 (1990).
24. We thank B. Griffiths for producing and analyzing the anti-SRY antisera and I. Goldsmith for synthesis of oligonucleotides. We also thank R. Possee for technical advice and for providing AcRP23.*lacZ* baculovirus DNA. We are grateful to N. Jones, R. Treisman, R. Marais, D. Bentley, and A. Sinclair for critical reviews of the manuscript. V.R.H. is the recipient of a Commonwealth Scientific and Industrial Research Organization (CSIRO) (Australia) Postdoctoral Fellowship. We also thank Immunex Corporation for providing the FLAG antibodies M2 and M5 for evaluation. Supported by the Medical Research Council of Great Britain and the Imperial Cancer Research Fund.

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Protection of Macaques Against SIV Infection by Subunit Vaccines of SIV Envelope Glycoprotein gp160

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Simian immunodeficiency virus (SIV) is a primate lentivirus related to human immunodeficiency viruses and is an etiologic agent for acquired immunodeficiency syndrome (AIDS)–like diseases in macaques. To date, only inactivated whole virus vaccines have been shown to protect macaques against SIV infection. Protective immunity was elicited by recombinant subunit vaccines. Four *Macaca fascicularis* were immunized with recombinant vaccinia virus expressing SIV*mne* gp160 and were boosted with gp160 produced in baculovirus-infected cells. All four animals were protected against an intravenous challenge of the homologous virus at one to nine animal-infectious doses. These results indicate that immunization with viral envelope antigens alone is sufficient to elicit protective immunity against a primate immunodeficiency virus. The combination immunization regimen, similar to one now being evaluated in humans as candidate human immunodeficiency virus (HIV)–1 vaccines, appears to be an effective way to elicit such immune responses.

THE SPREAD OF AIDS AND HIV INFECTION has become a global concern (*1*). Development of a safe and efficacious vaccine against HIV is an important component in control of this disease. Major advances made in recent years include the demonstration that inactivated whole virus vaccines protect macaques from infection by SIV (*2–4*), which is a lentivirus closely related to HIV (*5*). However, concerns about insufficient inactivation and inadequate animal models for safety testing confound the use of whole inactivated HIV in seronegative humans. Most efforts in HIV vaccine development to date therefore have been focused on subunit vaccines (*6*). Recently, Stott *et al.* (*7*) reported that protection against SIV infection appears to correlate with antibodies against cellular components, rather than viral antigens. These findings not only complicate interpretations of earlier vaccine studies but also raise questions about subunit approaches to vaccine development. In the present study, we sought to demonstrate protective immunity in the SIV-macaque model by immunization with recombinant viral subunit vaccines.

The approach we undertook was a combination immunization regimen that included the use of a live recombinant vaccinia virus for priming and a subunit immunogen for boosting. We have shown that rodents immunized with this combination regimen generated greater HIV-specific antibody responses than those that received either live recombinant virus or HIV-1 gp160 alone (*8*). The target antigen we chose for the present work is the envelope glycoprotein gp160 of SIV. Other studies have indicated partial protection in macaques immunized with either an envelope-enriched virion protein preparation (*3, 9*) or fusion proteins containing SIV envelope peptide sequences (*10*).

Using described methods (*11, 12*), we inserted the entire SIV *env* into the genome of either vaccinia virus (New York City Board of Health strain) (*13, 14*) or baculovirus (*Autographa californica*) (*15*). The *env* we used was derived from an infectious molecular clone of SIV*mne* (clone 8), which was originally isolated from a pig-tailed macaque suffering from lymphoma (*16*). This gene encodes a full-length surface glycoprotein, gp120, and a truncated form of the transmembrane protein, gp32 (*17*). Both forms of the glycoproteins, as well as the precursor gp160, were expressed by the recombinant vaccinia virus, v-SE5, under the control of the early-late 7.5K promoter (*18*). On the other hand, the SIV gp160 expressed in recombinant baculovirus (Ac-SE5) infected insect cells (*Spodoptera frugiperda*, Sf 9) was not efficiently processed (*19*), as was observed previously in HIV-1 gp160 expressed by recombinant baculovirus (*12*). The SIV*mne* gp160 expressed by Ac-SE5 was partially enriched by lentil lectin affinity chromatography to approximately 25 to 30% purity as determined by Coomassie staining of an SDS-polyacrylamide gel following electrophoresis (*19*).

Four macaques (*M. fascicularis*) were first immunized with v-SE5 by skin scarification in two inoculations 12 weeks apart; each inoculation was 1×10^8 plaque-forming units per animal. After the primary immuni-

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