Table 3 Antibody response in mice immunized with the B-cell epitope $(NANP)_3$ linked to the T-cell epitope CS.T3

Mouse strain	H-2	Anti-$(NANP)_{50}$	Anti-sporozoite
C57BL/6	b	2,343	160
BALB/c	d	5,860	1,280
B10.MOla	f	938	40
C3H.HeJ	k	2,343	160
C3H.Q	q	3,705	320
B10.RIII	r	14,647	1,280
B10.SOla	s	1,482	1,280

Peptide $(NANP)_3$-CS.T3 was synthesized by the solid-phase technique using *N*-fluorenylmethoxy carbonyl (Fmoc)-amino acids, t-butyl-based side-chain protecting groups and a p-benzyloxybenzylalcohol polystyrene resin[24]. The N^{α}-unprotected CS.T3 peptide resin was elongated by three repeated couplings of Fmoc-$(NANP)_3$ by the O-benzotriazolyl-*N,N,N,N*-tetramethyluronium hexafluorophosphate procedure[25] (Knorr *et al.* manuscript in preparation). The $(NANP)_3$-CS.T3 peptide was cleaved with trifluoroacetic acid/methylene chloride/anisol and purified by HPLC. Mice (two per group) were immunized at the base of the tail with 50 μg of $(NANP)_3$-CS.T3 in incomplete Freund's adjuvant. Eight weeks later, they were boosted with 25 μg of the immunogen in complete Freund's adjuvant. Plasma were taken between two and six weeks later and tested individually by enzyme-linked immunosorbent assay for the presence of anti-$(NANP)_{50}$ antibody, and by indirect immunofluorescence for antibodies to sporozoites[26,27]. Enzyme-linked immunosorbent assay titres are geometric means of the last dilution of plasma with $A_{455} > 0.1$ and >twice A_{455} of plasma from mice injected with saline. The antigen used to coat the enzyme-linked immunosorbent assay plates was $(NANP)_{50}$. All pre-immune titres to $(NANP)_{50}$ were <150 and to sporozoites <40.

index[20]; however, when the peptide is modelled as a regular α-helix, clusters of hydrophobic residues in the N- and C-terminal halves of the peptide are found on opposite sides of the helix (K. Muller, unpublished results). It will require more detailed information on the three-dimensional structure of MHC antigens[21] to clarify the nature of the peptide binding.

Clones recognizing CS.T3 in association with DR2 and DR9 also recognize the parasite-derived CS protein[5,22] as well as the cysteine-substituted CS.T3 peptide, whose sequence corresponds exactly to that of the CS protein. The same situation holds for at least four other DR antigens (DR1, 5, w6 and 7; ref. 28 and F. Sinigaglia *et al.*, unpublished results). Thus, this determinant might be an excellent candidate for a vaccine inducing cellular immunity to sporozoites. In addition to inducing potentially protective T-cell effector functions, the CS.T3 peptide can be used as a carrier for $(NANP)_n$. A vaccine consisting of the invariant CS.T3 T-cell epitope linked to an appropriate B-cell epitope might well induce both humoral and cellular parasite-specific immunity in the genetically diverse human population.

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Sequences homologous to *ZFY*, a candidate human sex-determining gene, are autosomal in marsupials

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Sexual differentiation in placental mammals results from the action of a testis-determining gene encoded by the Y chromosome. This gene causes the indifferent gonad to develop as a testis, thereby initiating a hormonal cascade which produces a male phenotype[1,2]. Recently, a candidate for the testis-determining gene (*ZFY*, Y-borne zinc-finger protein) has been cloned[3,4]. The *ZFY* probe detects a male-specific (Y-linked) sequence in DNA from a range of eutherian mammals, as well as an X-linked sequence (*ZFX*) which maps to the human X chromosome[3]. In marsupials it is also the Y chromosome that seems to determine the fate of the gonad, but not all sexual dimorphisms[5]. Using the *ZFY* probe we find, surprisingly, that the *ZFY* homologous sequences are not on either the X or the Y chromosome in marsupials, but map to the autosomes. This implies *ZFY* is not the primary sex-determining gene in marsupials. Either the genetic pathways of sex determination in marsupials and eutherians differ, or they are identical and *ZFY* is not the primary signal in human sex determination.

Marsupials (infraclass Metatheria) diverged from placental mammals (infraclass Eutheria) at least 130 million years ago[6], so comparisons of gene arrangements between these groups may provide information about the evolution of sex chromosomes and sex determination. Within the marsupials, the two orders Diprotodonta (represented in this work by *Macropus eugenii*, the tammar wallaby, and *M. robustus*, the wallaroo) and the Polyprotodonta (*Dasykaluta rosamondae* and *Sminthopsis crassicaudata*) diverged about 45 million years ago[7].

In both these orders the marsupial X chromosome bears the genes *HPRT*, *G6PD*, *PGK* and *GLA*[8] (symbols as in Fig. 3 legend), which are also X-linked in all eutherian mammals tested, confirming that at least part of the mammalian X is highly conserved in evolution, as predicted by Ohno[1]. *STS* has been excluded from the marsupial X (ref. 9), however, and the recent localization of *DMD* (refs 10, 11) to one autosome (5p in *M. eugenii* and 3q in *D. rosamondae*) and *OTC* (ref. 12) and *SYN1* (refs 10, 11) to another (1p in *M. eugenii* and 3p in *D. rosamondae* (symbols as in Fig. 3 legend), demonstrates that the region containing these genes is not present on the marsupial X. In humans these genes map to Xp21.2, Xp21.1 and Xp11.2,

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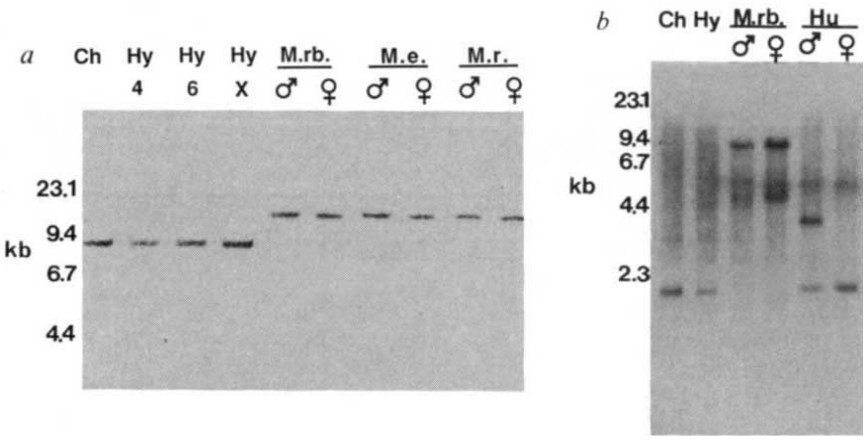

Fig. 1 (*a*), Southern blot[22,23] of *Pst*I-digested DNA samples probed with the human *ZFY* probe (pDP1007) and washed with 2×SSC at 60 °C. DNA from male and female *M. robustus* (M.rb.), *M. eugenii* (M.e), and *M. rufus* (M.r.) show a single band at ~12 kb, which is absent from cell hybrids (Hy) containing the *M. robustus* chromosomes 4, 6 and X on a Chinese hamster background. The bands at ~8 kb are the Chinese hamster (Ch) controls. *b*, Southern blot of *Eco*RI-digested DNA samples tested with human *ZFY* probe (pDP1007) and washed at low stringency (3×SSC at 50 °C). After overnight exposure at this stringency, one strongly hybridizing (~9.8 kb) band and a second weaker (~4.8 kb) band are revealed in DNA from *M. robustus* (M.rb); a third (~1.8 kb) band is evident after longer exposure. All three M.rb. bands are present in both sexes and absent in DNA from a hamster-M.rb. cell hybrid (Hy) carrying M.rb. chromosomes 4, 6 and X. Ch is the hamster control, and Hu DNA is from a human male and female, showing a strong male-specific band, and a second band whose intensity in female is double that of male DNA.

respectively[13], close to *ZFX*. Marsupial sex chromosomes are smaller than those of eutherians and apparently lack a pairing region[14,15]. One possible explanation is that the smaller X and Y chromosomes of marsupials may have been derived by translocation of a large pseudoautosomal region from the partially differentiated X and Y chromosomes of a common ancestor to marsupial autosomes[10,16].

The probe pDP1007 (ref. 3) was hybridized to 'Noah's Ark' blots, containing DNA from male and female pairs of several species. The pDP1007 insert is a genomic sequence derived from the human Y chromosome, and encodes a putative 'zinc-finger' domain of *ZFY*. A single strong band was detected at high stringency, in a position identical for male and female, and identical across three *Macropus* species (Fig. 1*a*). Although there are clearly conserved marsupial sequences which show strong homology with human *ZFY*, these sequences are not male-specific (Y-linked) in any of these marsupial species. In addition, there was no difference in band intensity between male and female, as would be expected for X-linked sequences, suggesting that the sequences are autosomal. Single bands corresponding to different relative molecular masses were obtained with DNA from species belonging to other marsupial orders: *S. crassicaudata* and the American opossum, *Didelphis virginiana*. The absence of a *ZFX* homologue on the marsupial X was confirmed by probing Southern blots of DNA from hamster–marsupial cell hybrids which retained an intact *M. robustus* X chromosome[9]. DNA from the hybrid showed the band of ~8 kilobases (kb) detected in *Pst*I-digested hamster DNA controls, but not the band of ~12 kb detected in *M. robustus* DNA (Fig. 1*a*), confirming that a *ZFY* homologous sequence is not detected on the marsupial X, at least in this species. On Southern blots of *Eco*RI-digested marsupial DNA washed at low stringency, three *ZFY*-homologous bands were detected: one very strong, one weaker (Fig. 1*b*) and a third that could be detected only after prolonged exposure. Once again, these bands were of similar intensity and corresponded to the same molecular mass for male and female, and were absent from the cell hybrid.

These sequences were localized to *M. eugenii* and *S. crassicaudata* chromosomes by *in situ* hybridization with human *ZFY* probes pDP1007 (ref. 3) and pMF1. The plasmid pMF1 is a human testicular complementary DNA clone isolated using an oligomer derived from the published sequence of pDP1007; the 2.4-kb insert in pMF1 encodes the zinc-finger domain of pDP1007 and other coding sequences 5′ to the finger region. These extra sequences contain an apparently complete open reading frame encoding an acid-rich protein domain. In Southern blot hybridizations of human DNA, pMF1 recognizes the same sequences as pDP1007 and additional Y-derived bands.

Analysis of the grain distribution revealed no hybridization site on either the X or Y in male or female of either species with either probe. But a strong hybridization signal (presumably equivalent to the single band on Southern blots at high stringency) was detected with both probes on chromosome 5p of both male and female *M. eugenii* (Fig. 2*a*) and on chromosome 3q of male and female *S. crassicaudata* (Fig. 2*b*) at the sites corresponding to the positions of the *DMD* sequences in these species[10,11] (Fig. 3*a*, *b*). A secondary signal was consistently

Table 1 Analysis of *ZFY in situ* hybridization data for *M. eugenii* male

Chromosome	Deviance	Standardized residual
1	64	2.8
2	65	−2.2
3	63	−2.5
4	63	−2.4
5	29*	8.0*
6	66	−1.8
7	65	2.1
X	62	−2.4
Y	65	−1.8

A full account of this method of analysis is given elsewhere[12,17]. The GLIM computer program was written[17] to analyse the *in situ* hybridization data by comparing the observed distribution of grains with the distribution expected, omitting each chromosome in turn. The lowered deviance when the hybridized site is omitted provides a clear indication of the chromosome to which the gene has hybridized. An example of the output obtained in the present study (for *M. eugenii* male) is given in Table 1. Taking the data as a whole, the fit is adequate only for the model omitting chromosome 5 and the standardized residual is highly significant compared with the standard normal distribution. But from examination of the raw data we suspected that there could also be two weaker sites. To test whether these sites would hybridize significantly, we deleted chromosome 5 from the analysis and repeated the procedure with the remaining chromosomes. In the absence of chromosome 5, chromosome 1 has a deviance of 14 and a standardized residual of 5, and hence is a site of significant hybridization. Similarly, when both chromosomes 1 and 5 are deleted, then chromosome 7 shows a significant hybridization signal (deviance 4, standardized residual 5). Deletion of all three significant chromosomes from the analysis does not reveal any other significant sites of hybridization. Such analysis confirms chromosome 5 as the main site of hybridization, and chromosomes 1 and 7 as minor but significant sites in *M. eugenii*. Identical results were obtained for *M. eugenii* female cells. Similar analysis of the grain numbers of *S. crassicaudata* chromosomes confirm that 3q is the main site of hybridization and that 3p and 1p significant but minor sites in this species.

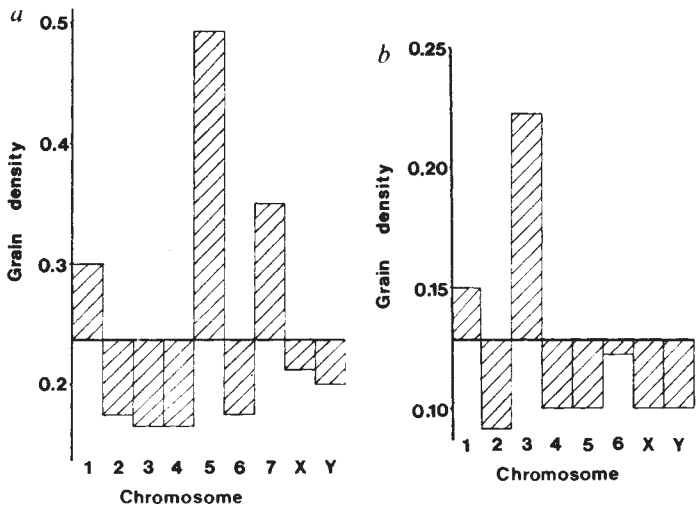

Fig. 2 Histograms showing results of *in situ* hybridization experiments. The line representing the expected number of grains per chromosome is calculated by dividing the total number of grains present over chromosomes by the relative proportion of the genome represented by each chromosome. *a*, Hybridization of pMF1 0.05 $\mu g\ ml^{-1}$) to metaphase chromosomes from *M. eugenii* male. *b*, Hybridization of pDP1007 to metaphase chromosomes from *S. crassicaudata* male. The *in situ* hybridization method used for mapping heterologous probes has been reported elsewhere[12]. Two independent experiments were carried out on each species, cells of both sexes were used, and slides were scored by two independent investigators, identifying chromosomes by size and morphology.

demonstrated on the *M. eugenii* chromosome 1p and the *S. crassicaudata* 3p, the location of the *OTC* and *SYN1* genes in these species[10-12]. A third, weaker signal was consistently detected on *M. eugenii* chromosome 7 and *S. crassicaudata* 1p. Statistical analysis of the data with the GLIM program[17] confirmed that these chromosomes contained one major and two minor sites of hybridization (Table 1). Proof of correlation between *in situ* hybridization data and Southern bands awaits cloning and localization of each sequence.

We conclude that *ZFY*-homologous sequences are autosomal in marsupials. The *ZFY* probe in marsupials maps to the same location as *DMD*, with a secondary site near the *OTC* and *SYN1* genes. All four of these genes are located close to *ZFX* on the human X chromosome. There is no evidence for either X or Y borne *ZFY*-homologous sequences in marsupials. Thus, none of the sequences can be the primary testis-determining gene, because this must logically be on the Y. Furthermore, the exclusion of these sequences from the marsupial X chromosome excludes the possibility that differences in their dosage between males and females can control other sexual dimorphisms.

The differences in the location of *ZFY* homologous sequences in eutherian and metatherian mammals must mean either that the primary sex-determination signal is accomplished by unrelated genes in the two mammalian infraclasses, or that *ZFY* is not the primary sex-determining gene in eutherians. The latter hypothesis is hard to reconcile with genetic and molecular analysis of the sex-determining region of the human Y.

In marsupials, as in eutherian mammals, the Y chromosome appears to be testis-determining; the few XXY animals described possess testes, whereas X0 animals lack testes[5]. But it is clear that other sexual dimorphisms are a function of X-linked and/or autosomal genes. The phenotypes of marsupials with aneuploid sex chromosomes, unlike human XXY and X0, are not unambiguously male or female, suggesting that, whereas the Y chromosome determines testicular differentiation, the dosage of X chromosomes may influence the differentiation of scrotum, pouch and mammary glands[5]. Recent observations of early development show that the determination of scrotum, pouch, mammary glands, gubernaculum and processus vaginalis precedes gonadal differentiation[18] and is not androgen-dependent, unlike that of eutherian mammals[19-21]. The differences in sex determination between marsupials and eutherians may conceivably relate to a more dominant role of *ZFY* in eutherians.

If testis determination turns out to be accomplished by unrelated genes in the two therian subclasses, it will be of interest to determine which was the ancestral system. The observation of a single band on Southern blots of DNA from male and female chickens[3] implies that *ZFY*-homologous sequences may be autosomal in birds. This supports the proposition that the testis-determining function of *ZFY* has evolved since the divergence of the eutherian lineage 130 million years ago. The same proposition is indicated by the conservation between eutherian and marsupials of the close proximity of *ZFY* homologous sequences to *DMD* and *OTC*, *SYN1*, suggesting that the

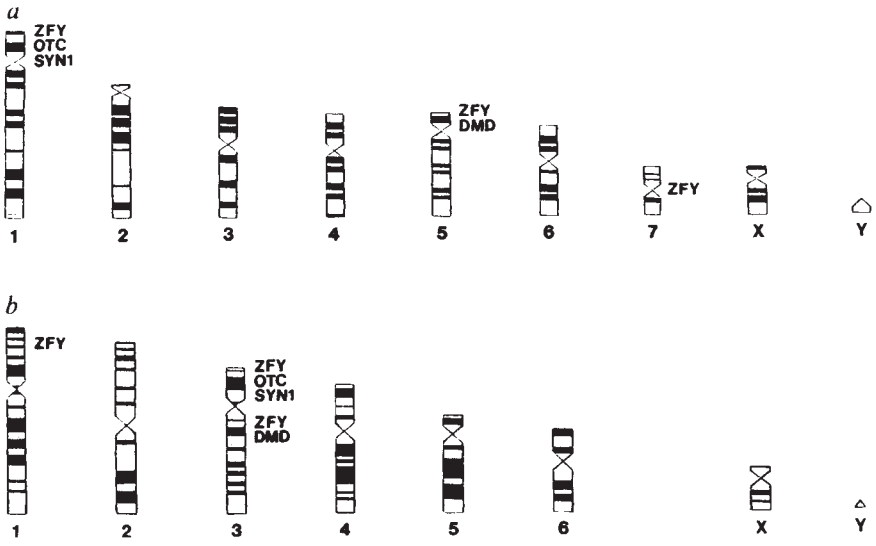

Fig. 3 Ideograms of *a*, *M. eugenii* and *b*, *S. crassicaudata* showing the sites of mapped genes. Gene symbols: *ZFY*, Y-linked zinc-finger protein; *ZFX*, X-linked zinc-finger protein; *DMD*, Duchenne muscular dystrophy; *OTC*, ornithine transcarbamylase; *SYN1*, synapsin I; *G6PD*, glucose-6-phosphate dehydrogenase; *HPRT*, hypoxanthine phosphoribosyl transferase; *PGK*, phosphoglycerate kinase; *GLA*, galactosidase-α; *STS*, steroid sulphatase.

ancestral zinc-finger protein sequences must have been located near these genes in a common therian ancestor in a region that was pseudoautosomal or autosomal, and not subject to X-inactivation[10,16]. If *ZFY*-homologous genes have a secondary role in sex determination in vertebrates other than eutherians, then a secondary step in a sex-determining pathway may have taken on a primary function in eutherians. The identification and isolation of the primary testis-determining gene, which must be borne on the Y in marsupials, now assumes great importance.

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Cloning and expression of a rat D_2 dopamine receptor cDNA

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Dopamine receptors are classified into D_1 and D_2 subtypes on the basis of their pharmacological and biochemical characteristics[1,2]. The D_2 dopamine receptor has been implicated in the pathophysiology and treatment of movement disorders[3], schizophrenia[4] and drug addiction[5]. The D_2 dopamine receptor interacts with guanine nucleotide-binding proteins to induce second messenger systems[6,7]. Other members of the family of receptors that are coupled to G proteins share a significant similarity in primary amino-acid sequence and exhibit an archetypical topology predicted to consist of seven putative transmembrane domains[8]. We have taken advantage of the expected nucleotide sequence similarities among members of this gene family to isolate genes coding for new receptors. Using the hamster β_2-adrenergic receptor gene as a hybridization probe we have isolated related genes including a cDNA encoding the rat D_2 dopamine receptor. This receptor has been characterized on the basis of three criteria: the deduced amino-acid sequence which reveals that it is a member of the family of G-protein-coupled receptors; the tissue distribution of the mRNA which parallels that of the D_2 dopamine receptor; and the pharmacological profile of mouse fibroblast cells transfected with the cDNA.

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Table 1 K_i values (nM) for L-RGB2Zem-1 and rat striatum

	RGB-2 transformed Ltk⁻ cells	Rat striatum
(+)Butaclamol	0.83	1.0
(−)Butaclamol	>1,000	>1,000
Haloperidol	3.0	5.3
Dopamine+GTP	17,000	6,300
Sulpiride		
high affinity	80	67 (87%)
low affinity	—	>10,000 (13%)
SCH 23390		
high affinity	—	35 (16%)
low affinity	1,000	780 (84%)
Ketanserin		
high affinity	—	27 (25%)
low affinity	>1,000	1,000 (75%)

The 50% inhibitory concentration values (IC_{50}) calculated in Fig. 3*b* were converted to K_i values as described[24]. Results are geometric means of three experiments in which 0.5 nM [^{3}H]spiperone was inhibited by various concentrations of unlabelled drug. This concentration of radioligand is more than 10 times its K_d value. This explains the discrepancies between the IC_{50} values in Fig. 3*b* and these K_i values. All binding data for L-RGB2Zem-1 membranes were fit best by assuming the presence of only one class of binding sites. On the other hand inhibition by several drugs of [^{3}H]spiperone binding to rat striatal membranes was fit best by assuming the presence of two classes of binding sites. The proportions of binding sites with high and low affinity for inhibitors are shown in parantheses. SCH 23390 and ketanserin inhibited 10–20% of [^{3}H]spiperone binding to rat striatal membranes with high affinity. In rat striatal membranes inhibition of radioligand binding by sulpiride was fit best by assuming one class of binding sites but 10–15% of the (+)butaclamol-displaceable binding was not inhibited by sulpiride at the concentrations used. It seems likely that the binding sites with high affinity for ketanserin and SCH 23390 and which are not displaced by sulpiride represent binding of [^{3}H]spiperone to 5-HT_2 serotonin receptors in rat striatal membranes. Binding of SCH 23390 to 5-HT_2 receptors has been described previously[25]. In rat striatal membranes the apparent affinity of drugs for D_2 dopamine receptors, the class of binding sites comprising 80–90% of [^{3}H]spiperone binding, was indistinguishable from the apparent affinity of drugs to membranes prepared from L-RGB2Zem-1 cells. K_i values for the class of binding sites representing 10–25% of specific binding were calculated by assuming that the radioligand was binding to serotonin receptors with a K_d value of 1 nM.

A rat genomic library was screened under low-stringency hybridization conditions with a nick-translated 1.3-kilobase (kb) *Hin*dIII fragment containing most of the coding region of the hamster β_2-adrenergic receptor (β_2AR) gene[9] (Fig. 1). Several clones were found to hybridize to the hamster probe. One clone, called RGB-2, contained a 0.8 kb *Eco*RI–*Pst*I fragment that hybridized to the hamster β_2AR probe in Southern blot analysis. This fragment was sequenced and shown to have a stretch of nucleotides with a high degree of identity (32 out of 40 bases) to the nucleotide sequence of transmembrane domain VII of the hamster β_2AR. One reading frame displayed a significant similarity to the amino-acid sequence of transmembrane domains VI and VII of the hamster β_2AR. This genomic fragment also contains a 3′ intron splice site[10] and 400 base pairs (bp) of putative intronic sequence (data not shown).