for such coupling. Analysis of other seven spanning receptors has suggested that basic, amphipathic helixes at either end of the third cytoplasmic loop are involved in G-protein coupling and appropriate residues are found in these positions both in the CX5 sequence and in the brain receptor, which has been shown to be G-protein coupled[6,22].

There are many reports of cannabinoids exerting suppressive effects on various cells of the immune system, including macrophages[23–25], although the significance of some of these observations has been questioned because of the high doses of drug used[26]. But the location of the CX5 receptor, and its distinct structure from the brain receptor, strongly suggest that the endogenous ligand for these receptors will have an immuno-modulatory role in addition to its neuronal function. Anandamide has been recently identified as a candidate ligand for the cannabinoid receptor[16] and this compound also binds to the CX5 receptor, although with an apparent affinity 30-fold less than that reported for the brain receptor. Anandamide is able to cross the blood brain barrier rapidly[27] but worthwhile speculation as to its function, and possible interactions between the neural and immunological systems, will require the identification of all the sources of this intriguing molecule. Furthermore, the question of further potential ligands from brain remains to the resolved[16]. Even so the existence of the CX5 receptor does have further implications. G-protein-coupled receptors are highly conserved throughout evolution[12], and yet the sequence of CX5 is considerably divergent from that of CB-R. Of the 162 residues in transmembrane sections of the human CB-R, three are different in rat CB-R, but 68 are different in human CX5. This suggests that the two receptors did not diverge recently and furthermore it suggests that it should be possible to identify receptor-specific cannabinoids. The fact that cannabinol appears to have a higher relative affinity for the CX5 receptor than for the brain receptor, may provide the basis for identifying such a ligand for the CX5 receptor. We suggest that in future the two receptors be distinguished by calling the brain receptor CB1 and the CX5 receptor CB2. It has been proposed that the peripheral effects of cannabinoids are either indirect effects of central actions, or reflect interactions with non-receptor proteins such as lipoxygenases[1,8]. It is clearly possible that some of these peripheral effects are in fact mediated through the CB2 receptor and it will be interesting to determine the activities of any cannabinoids specific for this receptor. □

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Cloning and gene defects in microsomal triglyceride transfer protein associated with abetalipoproteinaemia

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THE microsomal triglyceride transfer protein (MTP), which catalyses the transport of triglyceride, cholesteryl ester and phospholipid between phospholipid surfaces, is a heterodimer composed of the multifunctional protein, protein disulphide isomerase, and a unique large subunit with an apparent M_r of 88K (refs 1–3). It is isolated as a soluble protein from the lumen of the microsomal fraction of liver and intestine[4]. The large subunit of MTP was not detectable in four unrelated subjects with abetalipoproteinaemia[5], a rare autosomal recessive disease characterized by a defect in the assembly or secretion of plasma lipoproteins that contain apolipoprotein B (ref. 6). We report here the isolation and sequencing of complementary DNA encoding the large subunit of MTP. A comparison of this sequence to corresponding genomic sequences from two abetalipoproteinaemic subjects revealed a homozygous frameshift mutation in one subject and a homozygous nonsense mutation in the other. The results indicate that a defect in the gene for the large subunit of MTP is the proximal cause of abetalipoproteinaemia in these two subjects, and that MTP is required for the secretion of plasma lipoproteins that contain apolipoprotein B.

Based on the sequence of 1 of 10 peptides isolated from the large subunit of MTP (Fig. 1), a 20-base, 32-fold degenerate oligonucleotide probe was designed and used to screen a λgt10, bovine small intestine cDNA library. Overlapping bovine clones

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contained 2,580 bases of open reading frame followed by a stop codon, 298 bases of 3′ untranslated sequence, and a stretch of 18 adenine residues. All 10 MTP peptides were encoded by the cDNA (Fig. 1). The composite cDNA did not encode the amino terminus of the protein. After characterization of human clones, the 5′ bases corresponding to the N terminus of the mature bovine protein and a portion of the signal peptide were obtained by polymerase chain reaction (PCR; Fig. 1).

Complementary DNA libraries generated from human liver and human small intestine messenger RNA were screened with a bovine cDNA probe. The entire coding sequence for the large subunit of MTP was obtained independently from each library

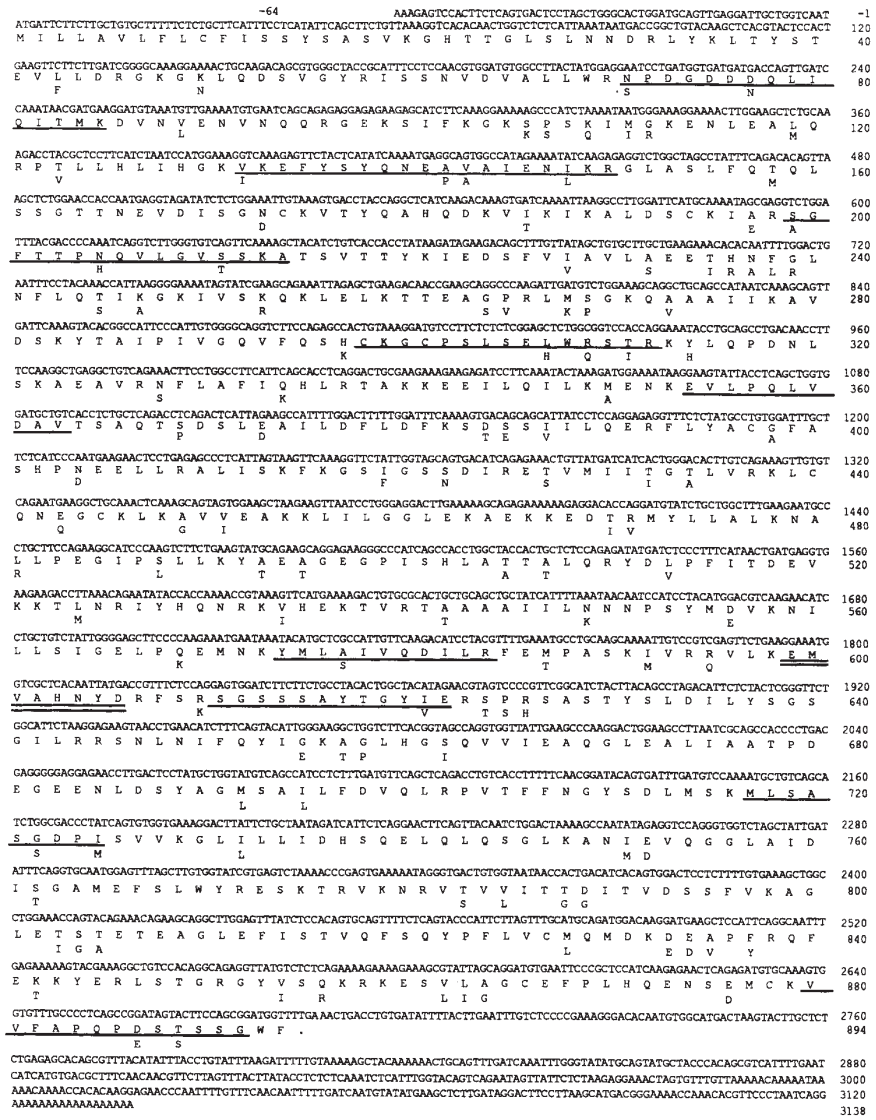


FIG. 1 Nucleotide sequence of the large subunit of human MTP cDNA and comparison of the predicted amino-acid sequence (single-letter code) with that of bovine MTP. The nucleotide and amino-acid sequences are numbered separately, with position 1 assigned to the first nucleotide or amino-acid residue of the coding sequence. The bovine protein sequence is aligned and mismatched amino acids are shown below the human protein sequence. The bovine sequence corresponding to the first seven amino acids of the human signal peptide were not characterized. The 10 underlined sequences show the position of the bovine MTP peptides that were sequenced.

METHODS. MTP from bovine liver was isolated as previously described[7]. The two components of the complex were fractionated by SDS–PAGE and the large subunit of MTP was isolated by electroelution. The subunit was dialysed into 8 M urea, diluted to 2 M urea, and immediately digested with trypsin. Ten different peptides were isolated by reversed phase HPLC and sequenced using gas-phase sequencing technology[2]. One million plaques from a λgt10 bovine small-intestine cDNA library (Clontech) were screened with a 32-fold degenerate oligonucleotide probe 5′-CT (C/T) TACCA (G/C) CG (A/G) GT (A/G) TT (A/G) AT-3′ designed from a peptide sequence (underlined twice). The oligonucleotide probes were 5′ end-labelled using T4 polynucleotide kinase and γ-^{32}P-labelled ATP (~6,000 Ci mmol^{-1}). Oligonucleotide hybridizations were at 37 °C in 6 × SSC, 20 mM $NaPO_4$, 2 × Denhardt's, 0.1% SDS, 0.1 mg ml^{-1} denatured salmon sperm DNA and washes were done at the same temperature in 2 × SSC, 0.1% SDS. To select clones of interest, washes were performed at progressively higher temperatures. The four clones with the highest T_m were isolated. The library was rescreened with oligonucleotide probes designed from sequence obtained from isolated MTP cDNA clones. A total of seven overlapping cDNAs were characterized, subcloned into the Bluescript plasmid (Stratagene), and sequenced on an Applied Biosystems 373 Automated DNA Sequencer using either dye-labelled primers or dye-labelled dideoxynucleotides. A 2.4-kb *Eco*RI fragment from a bovine clone, corresponding to bases 180 to 2,600 of the coding region, was gel purified and ^{32}P-labelled using a Multiprime labelling kit from Amersham. The probe was used to screen one million plaques from both a UniZap XR human liver cDNA library (Stratagene) and a λgt10 human small intestine cDNA library (Clontech). Hybridization was done under the same conditions as described for the oligonucleotide probes, except the hybridization and washes were done at 65 °C and washes were in 1 × SSC, 0.1% SDS. Positive clones were isolated and characterized. Inserts from three liver clones were excised from the λDNA as Bluescript plasmids and the DNA sequence determined. Contiguous sequence from the 5′ untranslated sequence through the entire coding region was obtained from these inserts. In addition, two inserts from the intestinal library that also contained untranslated sequences and the entire coding region were subcloned into the Bluescript vector and sequenced. The 5′ end of the bovine cDNA was extended by 83 bases (corresponding to base 20 through 102 of the human cDNA) by PCR of DNA prepared from the bovine λgt10 library. The forward primer was designed from human cDNA sequence and the reverse primer was designed from bovine cDNA sequence (5′-GCCTCGATACTATTTTGCCTGCT-3′, corresponding to bases 738 to 760 of the human cDNA). The PCR reaction contained 0.15 μM forward and reverse primers, 2 mM $MgCl_2$, 0.2 mM each dNTP and 2.5 U *Taq* polymerase in 1.25 × buffer (Perkin-Elmer-Cetus). The PCR was conducted for 35 cycles consisting of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min. Amplified DNA was gel purified using GeneClean (Bio 101), subcloned into pUC18 plasmid (Pharmacia), and sequenced.

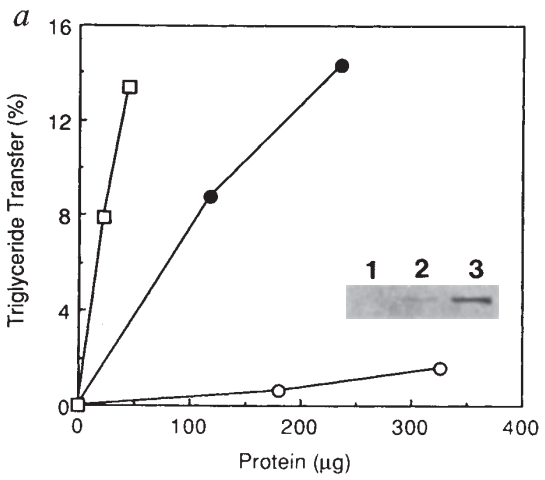
and no difference in the cDNA sequences was observed (Fig. 1). Comparison of the bovine and human cDNAs revealed 88% nucleotide identity in the coding region. Sequence analysis of the human gene (our manuscript in preparation) revealed that the 18 A residues located at the 3′ end of the human cDNA are encoded by genomic DNA. Polyadenylation of the message may occur at one of two AATAAA consensus signals[8] found 550 and 676 bases 3′ of the 18 base stretch of A in the human genomic DNA. The predicted translation product of the large subunit of human MTP contains 894 amino acids. Cleavage between the signal peptide and the mature protein is predicted to occur between amino acid 18 and 19 of the human protein[9]. The mature protein has a predicted M_r of 97K. There is 86% identity between the deduced amino-acid sequence of bovine and human MTP large subunit. Allowing for conservative amino-acid substitutions, there is 94% homology. There was no obvious homology between these sequences and that of any protein in available sequence data banks.

To confirm that the cDNA encodes the microsomal triglyceride transfer protein, the full coding sequence for the large subunit of MTP was subcloned into an expression vector and was transiently expressed in transformed human skin fibroblasts[10]. Extracts from the transfected cells showed substantial triglyceride transfer activity above background levels (Fig. 2). In addition, northern blot analysis of poly(A)$^+$ RNA from available human tissues (Fig. 2) revealed message levels that are consistent with the known tissue distribution of MTP activity[4].

An intestinal biopsy was obtained from a 39-year-old abetalipoproteinaemic female offspring of a consanguineous mating. We had previously demonstrated the absence of MTP activity and protein in this subject[5]. Total RNA was isolated and reverse transcribed into first-strand cDNA. The cDNA encoding the large subunit of MTP was amplified by PCR and sequenced directly. Three independent PCR products revealed a cytosine deletion at base 215 (Fig. 3). The frameshift mutation leads to a premature stop codon 21 bases downsteam and a predicted translation product of 78 amino acids. To determine whether both alleles of the gene encoding MTP contain the frameshift mutation, the 187 base-pair exon (our manuscript in preparation) that encodes the mutation was amplified from the subject's genomic DNA and sequenced directly. A single sequence that contained the frameshift mutation was observed, indicating that both alleles contain the defect. The mutation was also confirmed by sequencing eight plasmid clones of the PCR product.

A genomic library was constructed from the DNA of a 14-year-old abetalipoproteinaemic female who lacks MTP[5] and clones encoding the large subunit of MTP were isolated. Sequence analysis revealed a C to T mutation corresponding to base 1,783 of the cDNA. This changes an Arg codon to a premature stop codon. A truncated translation product of 594 amino acids is predicted. This nonsense mutation also eliminates a *Taq*I restriction endonuclease site. To determine if the subject is homozygous for this mutation, genomic DNA was digested by *Taq*I and analysed by Southern hybridization. The normal exon encoding base 1,783 was digested into two fragments by *Taq*I (Fig. 4). DNA from the abetalipoproteinaemic subject (proband) was not digested at this site, indicating both alleles contain the defect. The parents displayed the restriction pattern of both the normal and mutant alleles.

The mutations identified in the two abetalipoproteinaemic



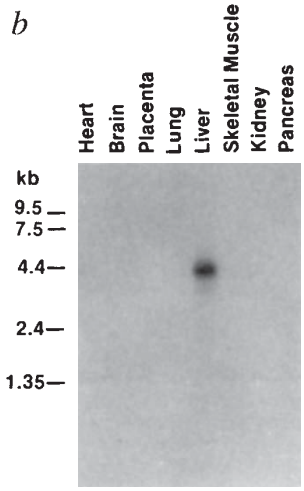
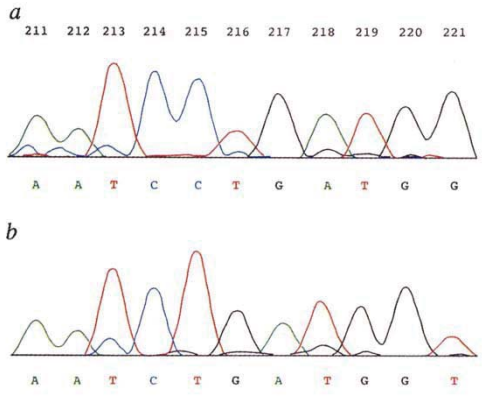


FIG. 2 *a*, Expression of human MTP in transformed human skin fibroblasts (1508T)[10]. Transfer activity was measured in extracts of 1508T cells transfected with either pcDNA/Neo (negative control, open circles) or pcDNA/MTP (solid circles). For comparison, transfer activity was also measured in extracts of HepG2 cells (squares). Activity is expressed as the percent of radiolabelled triglyceride transferred from donor vesicles to acceptor vesicles as a function of cell protein. Aliquots (20 μg cell protein) of the same cell extracts used for activity measurements were analysed for expression of MTP by western blot (inset) with an antibody directed against the large subunit of MTP. Lanes 1–3 are extracts from 1508T cells transfected with pcDNA/Neo, 1508T cells transfected with pcDNA/MTP, and HepG2 cells, respectively. *b*, A northern blot containing 2 μg poly(A)$^+$ RNA from human heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas was hybridized to a cDNA probe for the large subunit of MTP. A 4.4-kb band in the liver lane was apparent. RNA from human intestine was not available for northern blot analysis, however, the presence of MTP mRNA in intestine was evident from the cDNA clones obtained from the human intestinal library. In addition, MTP mRNA was readily detectable in hamster intestine (not shown).

METHODS. *a*, A 3.2-kb fragment containing the entire coding sequence for human MTP, extending from nucleotide −64 to 3,138 was subcloned into plasmid pcDNA/Neo (Invitrogen) to yield plasmid pcDNA/MTP. Transfection mixtures were made by dissolving 50 μg plasmid in 1.5 ml serum-free DMEM. This was added dropwise to a solution of 120 μl lipofectin reagent (Bethesda Research Laboratories) dissolved in 1.5 ml serum-free DMEM. After a 15-min incubation at room temperature, the transfection mixtures were added to 100-mm dishes of 1508T cultures (plated at 25% confluency 24 h before transfection) containing 7 ml serum-free DMEM. Twenty-four h later the transfection mixtures were removed and 10 ml fresh DMEM containing 10% fetal bovine serum was added for an additional 24 h. Cells were scraped from the dishes and washed with phosphate-buffered saline. Cell extracts, MTP activity measurements, and western blot analyses were as described previously[5]. *b*, A prepared northern blot was purchased from Clontech. Each lane contained 2 μg poly(A)$^+$ from the indicated tissue. The blot was hybridized to a 2.4-kb *Eco*RI bovine cDNA probe for the large subunit of MTP as described in Fig. 1.



◀ FIG. 3 Frameshift mutation in the gene encoding the large subunit of MTP in an abetalipoproteinaemic subject. Normal (*a*) and mutated (*b*) sequences from base 211 to base 221 are compared. The sequence obtained from the abetalipoproteinaemic subject exhibits a cytosine deletion at base 215. Identical sequence was obtained from PCR-amplified RNA or DNA from the subject.
METHODS. Total RNA was isolated from an intestinal biopsy by the RNAzolB method (CinnaBiotex Labs). RNA (50 ng) was reverse-transcribed into first-strand cDNA using random hexamer primers and reagents supplied by Perkin-Elmer-Cetus. PCR reactions were as described in the legend to Fig. 1 except 50 cycles were conducted. Primer sets amplified DNA between bases −8 and 605, −8 and 767, and 14 and 960 (see Fig. 1). Amplified DNA was gel purified using GeneClean. A portion of the DNA was sequenced directly. The remaining DNA was subcloned into pUC18 plasmid and multiple clones were sequenced. Genomic DNA, isolated from a second biopsy, was heated to 95 °C for 5 min and added to a PCR as described above using primers designed from the sequences of introns flanking the exon (from bp 62 to 249 of the cDNA) containing the mutation. The forward and reverse primers were 5′-CCCTTACAATGAAAACTGG-3′ and 5′-GGTAC-ACTTCTCCAAAAACTT-3′, respectively. Amplification was at 97 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min for 3 cycles. The denaturation temperature was lowered to 94 °C for 32 additional cycles. The PCR products were purified and sequenced as described above.

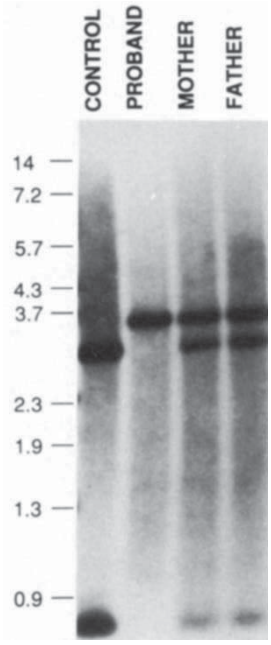


FIG. 4 The loss of *Taq*I restriction endonuclease site in an abetalipoproteinaemic patient. Genomic DNA was isolated from a control subject, the proband, and the proband's mother and father, digested with *Taq*I, and Southern blotted. The filter was hybridized to a PCR probe of the exon containing the mutation. The presence of the restriction site in the control DNA is indicated by the two hybridizing fragments at 3.0 and 0.6 kb. The deletion of the *Taq*I site in both alleles of the abetalipoproteinaemic subject's genomic DNA is demonstrated by the absence of these bands and the presence of a single larger hybridizing band at 3.6 kb. The presence of all three hybridizing bands in the proband's parents' DNA confirms that both parents are heterozygous for the mutation.
METHODS. Blood (6 ml) from a control subject, the proband, and the proband's parents was drawn into heparin tubes and genomic DNA was prepared from each sample with a Qiagen Blood DNA kit following the manufacturer's protocol. Each DNA (10 μg) was digested with *Taq*I, electrophoresed on a 1.0% agarose gel and blotted to nitrocellulose. A 302-bp probe comprising the exon (from bp 1,770 to 1,867 of the cDNA) which contains the mutation and flanking intron sequences, was generated by PCR of DNA with primers designed from intron sequences (5′-ATTTGGCTTCCTCTTTTT-3′ and 5′-GGGACTACCTGATGACACA-3′). The filter was probed at 65 °C using conditions described in Fig. 1. The filter was washed at 65 °C in 1 × SSC, 0.1% SDS.

subjects explain the absence of MTP activity and protein, and indicate that a defect in the gene for the large subunit of MTP is the proximal cause of abetalipoproteinaemia in these subjects. These results also demonstrate that MTP is required for the assembly of plasma lipoproteins that contain apolipoprotein B (apoB). The mechanism by which apoB and lipid associate to form a mature lipoprotein particle in the liver or intestine is not fully understood. Newly synthesized apoB may be degraded or secreted depending upon the availability of cholesteryl ester[11] or triglyceride[12,13]. In the presence of neutral lipid, apoB is protected from proteolytic degradation and forms a mature lipoprotein particle which is secreted. When cholesteryl ester or triglyceride availability is limited, apoB is degraded in a pre-Golgi compartment[14–16]. The results of this study indicate that MTP is necessary for the assembly of apoB particles which are protected from proteolytic degradation and are destined for secretion. An MTP-mediated addition of lipid to apoB early in the assembly process may play a key role in the stabilization of apoB. MTP may also participate in the transfer of additional lipid to apoB particles at stages distal to the sorting point between degradation and secretion.

As a result of the well established relationship between elevated plasma cholesterol levels and premature coronary heart disease[17], considerable research has focused on identifying new approaches to lower plasma lipid levels. The results of this and previous investigations indicate that: (1) plasma chylomicrons, very low-density, and low-density lipoproteins are absent in abetalipoproteinaemic subjects[6]; (2) the defect in abetalipoproteinaemia is specific for lipoprotein assembly in that complications of this disease are secondary to deficiencies of fat soluble vitamins which are transported on apoB-containing lipoproteins[6]; and (3) defects in MTP can cause abetalipoproteinaemia. Thus, we propose that inhibition of MTP will provide a specific mechanism for lowering plasma cholesterol and triglyceride levels. □

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A mouse homologue of the *Drosophila* tumour-suppressor gene *l(2)gl* controlled by Hox-C8 *in vivo*

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THE homeobox is a 183-base-pair DNA sequence originally found in *Drosophila* segmentation and homeotic genes[1,2]. In *Drosophila*, homeotic genes are clustered in the *Antennapedia* and *Bithorax* complexes, collectively called the homeotic gene complex (HOM-C)[3]. In the mouse genome, about 40 homeobox genes (*Hox*) are clustered in four chromosomal regions (*Hox* A to D). The *Hox* genes are arranged in the same order and have the same antero-posterior pattern of expression as their structural homologue in the HOM-C[4,5], suggesting that they control mouse pattern formation in the same way that HOM-C members do in *Drosophila*. Homeobox gene products are believed to be transcription factors that regulate expression of target genes. A few candidate target genes have been identified in *Drosophila* by various approaches[6] but the *Hox* gene targets are poorly understood, mostly because of limitations in the available approaches. Here we identify several candidate *Hox* gene targets, including a mouse homologue of the *Drosophila* tumour-suppressor gene *l(2)gl*, by immunopurification of DNA sequences bound to a Hox protein in native chromatin.

To identify *Hox* gene targets we first isolated the DNA sequences bound by a Hox protein in native chromatin by immunochemical means, and then identified the gene in the isolated sequence. The antibodies used were raised against a bacterially synthesized Hox-C8 (Hox 3.1) protein, and gave immunohistochemical staining patterns on sections of mouse embryos almost identical to those previously reported[7].

To isolate Hox-C8 target sequences from spinal cord, soluble nucleoprotein complexes were prepared from paraformaldehyde-fixed mouse spinal cords by sonication, passed through the anti-Hox-C8 antibody affinity column, and treated with protease. After the immunoenriched DNA had been digested with *Hae*III and cloned into λgt10 vector, about 2×10^4 independent clones were obtained. Cloning efficiency from the immunoenriched DNA was about one-tenth that of the unfixed genomic DNA. To assess the enrichment of the Hox-C8-bound sequences, we asked whether the *Hox-A7* (*Hox 1.1*) 5′ flanking sequence is significantly represented in the library, because homeobox genes themselves are potential targets of homeobox gene products[8–10]. A 5.0 kilobase (kb) restriction fragment corresponding to the 5′ flanking region of *Hox-A7* hybridized to 80 independent clones among the 2×10^4 library clones. Because only one out of 5×10^5 random genomic clones is expected to hybridize with a 5.0 kb unique genomic sequence, the 5′ flanking sequence of the *Hox-A7* gene in mouse spinal cord and that *Hox-A7* is a candidate target gene of *Hox-C8*. *Hox-A7* 5′ flanking sequence was

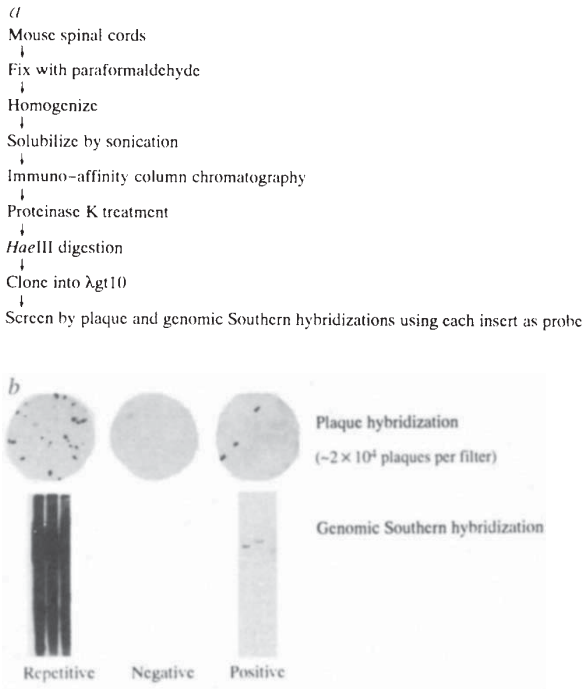

FIG. 1 The strategy for isolation of the *Hox-C8* target sequence, *a*, Procedure for immunoenrichment. *b*, Three typical examples of the plaque—and genomic Southern hybridizations. Left, Labelled insert hybridized with multiple plaques in a filter replica of the immunoenriched clones and also hybridized with a smear of restricted fragments on a genomic Southern blot which indicates inclusion of repetitive sequences in the insert. Middle, Labelled insert did not hybridize with any plaques of a filter replica. Right, Labelled insert hybridized with multiple plaques in a filter replica and hybridized with a unique restricted fragment on a genomic Southern blot.
METHODS. Antibodies: The *Sac*I–*Xho*I fragment of the first exon of *Hox-C8* was prepared from a mouse genomic clone mg51 and cloned into T7 expression plasmid pET3B[22]. Preparation of antisera and affinity purification of anti-Hox-C8 antibodies were as described[23]. Immunopurification: Spinal cords of five ICR mice were incubated in 10 mM Na–phosphate buffer pH 7.0, 150 mM NaCl containing 4% paraformaldehyde, at 0 °C, overnight. Fixed spinal cords were homogenized and washed with 10 mM Na–phosphate buffer pH 7.0, 150 mM NaCl. The nucleoprotein complexes were solubilized by sonication. After centrifugation at 10,000*g* for 15 min, the supernatant was loaded on the antibody-bound Sepharose CL-4B affinity column. The column was washed with 10 mM Na–phosphate buffer pH 7.0, 1 M NaCl followed by 0.1 mM Na–phosphate buffer pH 7.0. The washing with high- and low-salt solutions was repeated three times and the bound nucleoprotein complexes were eluted with 3 M NaSCN containing 10 mM Na–phosphate buffer pH 7.0 and dialysed against 0.1 mM Na–phosphate buffer pH 7.0 at room temperature for 24 h. The dialysed sample was incubated with 0.1 mg ml^{-1} proteinase K, 50 mM Tris–HCl buffer pH 8.0, 100 mM EDTA, 0.3% SDS at 37 °C overnight, treated with phenol–chloroform and precipitated with ethanol. The purified DNA was digested with *Hae*III, blunt-ended by Klenow fragment and cloned into λgt10 vector. *b*, The insert of each phage clones was labelled by PCR[24] with ^{32}P-dCTP and hybridized to a set of nitrocellulose replica filters, each representing $\sim 2 \times 10^4$ plaques of immunoenriched populations, and hybridized to restricted total mouse DNA on a Southern blot. Plaque and genomic Southern hybridizations were as described[25].

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