Human major histocompatibility complex contains a new cluster of genes between the HLA-D and complement C4 loci

E.Kendall, C.A.Sargent[1] and R.D.Campbell*
MRC Immunochemistry Unit, Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU and [1]Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QP, UK

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ABSTRACT

A new cluster of genes has been defined in the human Major Histocompatibility Complex class III region. The seven novel genes, G12 to G18, are localised in a 160 kb segment of DNA extending from the complement gene cluster towards HLA-DR. The genes were identified by isolation of cDNA clones using cosmid genomic inserts as hybridisation probes, and by the detection of the corresponding transcripts in Northern blot analysis. Characterisation of the cosmid genomic DNA inserts, in conjunction with pulsed field gel electrophoresis analysis of uncloned DNA, for the presence of clustered sites for infrequently cutting restriction endonucleases has revealed that at least 5 of the 7 genes are associated with HTF-islands. These unmethylated CpG-rich sequences are frequently found at the 5′ ends of ubiquitously expressed genes. Together with previously published data 36 genes have now been defined in a 680kb stretch of DNA within the MHC. With one gene approximately every 20kb of DNA this represents the most densely packed region of the human genome so far characterised, and is of major significance in relation to the mapping and sequence analysis of the rest of the genome.

INTRODUCTION

The human Major Histocompatibility Complex (MHC) spans ~3500kb of DNA on the short arm of chromosome 6 (1). The polymorphic histocompatibility antigens involved in the genetic regulation of immune responsiveness are encoded by the class I and class II genes (2–4). These lie within 1500kb and 850kb segments of DNA at the telomeric and centromeric ends of the MHC, respectively (5, 6). The 1100kb of DNA between the class I and II regions, though generally termed the class III region, is known to contain genes that encode proteins of diverse function, unrelated to each other as well as to both types of histocompatibility antigen. These include genes encoding the complement proteins C2, Factor B, C4A and C4B (7), the microsomal enzyme steroid 21-hydroxylase (CYP21) (8, 9), the cytokines tumour necrosis factor (TNF) A and B (5, 10), and three members of the major heat shock protein HSP70 family (11, 12). Linkage of these genes with the flanking class I and class II genes has been established by pulsed field gel electrophoresis (PFGE) (5, 13). The C4B gene lies ~390kb from the most telomeric class II gene, DRA, while the C2 gene lies ~600kb from the class I gene HLA-B. Lying 220kb centromeric of HLA-B are the TNFA and B genes. Recently 800kb of the class III region has been cloned in overlapping cosmids extending centromeric from the HLA-B gene (11, 14–16). A detailed characterisation of the cloned DNA has led to the discovery of 17 novel genes, encoding proteins of unknown function, between HLA-B and the C4 gene. However, apart from the CYP21B gene and the Opposite Strand Gene (OSG) (17), which lie immediately 3′ of the C4B gene, no other genes have been assigned to the region between the HLA-D and C4 loci.

Susceptibility to a large number of autoimmune diseases has been linked to the MHC (18–22). Although the polymorphic class I, and more importantly, class II alleles may account for some of these associations, in many cases the role of the MHC in disease susceptibility is far from clear. Rather than being associated with a single locus, some diseases seem to be associated more strongly with allelic combinations of class I and class II genes, referred to as extended haplotypes. Thus the observed HLA associations in some diseases may be related to the presence of tightly linked loci between the class I and class II regions. As part of our continuing effort to define genes in the class III region which could be of pathogenic significance in disease susceptibility, we have extended our initial chromosome walk (16) by isolating further overlapping cosmids extending from the complement gene cluster towards the class II region. The cosmid DNA inserts covering ~160kb of DNA have been characterised for the presence of HTF (HpaII Tiny Fragment) islands (23–26). These unmethylated CpG-rich sequences are frequently associated with the 5′ ends of housekeeping, and also some tissue specific, genes (24, 27). Clustered sites for certain infrequently cutting restriction endonucleases with CpG

* To whom correspondence should be addressed

dinucleotides in their recognition sequence have been established and this has suggested the presence of 7 potential HTF-islands. The isolation of cDNA clones using whole cosmid inserts as hybridisation probes, and the detection of the corresponding mRNAs in Northern blot analysis has resulted in the mapping of 7 novel genes, at least 5 of which lie close to an HTF-island. These results, together with previously published data (15, 16), reveal that the MHC class III region contains a surprisingly large number of genes, with 36 genes having now been defined in a 680kb segment of DNA.

MATERIALS AND METHODS

Cosmid libraries

All details of library preparation, chromosome walking and characterisation of cosmid DNA inserts have been described (16). Where necessary walking probes which contained repetitive sequences were preannealed with human placental DNA as described under Probes.

Pulsed field gel electrophoresis

Southern blots for the linkage of walking probes, and for the analysis of HTF-island distribution, were prepared from 1.5% agarose gels (Sigma Type 1) run using the crossed-field gel electrophoresis method of Southern *et al.* (28). Fractionated DNA was transferred onto nylon membranes (Hybond N, Amersham) as described previously (6).

Southern and Northern blots

Southern and Northern blotting was carried out as described previously (16).

cDNA library screening

A cDNA library of 5×10^5 recombinants constructed from mRNA of the monocytic cell line U937 (29) in the expression vector CDM8 (30) (gift of D. Simmons) was screened using whole cosmid inserts as hybridisation probes. Before hybridisation a pre-annealing procedure was used to prevent cross hybridisation of repetitive elements as described under Probes.

Hybridisation of blots and libraries

All hybridisations were carried out at 42°C (16→40 hr) in 50% formamide, 5×Denhardts, 10% dextran sulphate, 1 M NaCl, 50 mM Tris (pH 7.4), 0.1% SDS (0.5% SDS for pulsed field gels). High stringency washes were performed in 0.2×SSC,

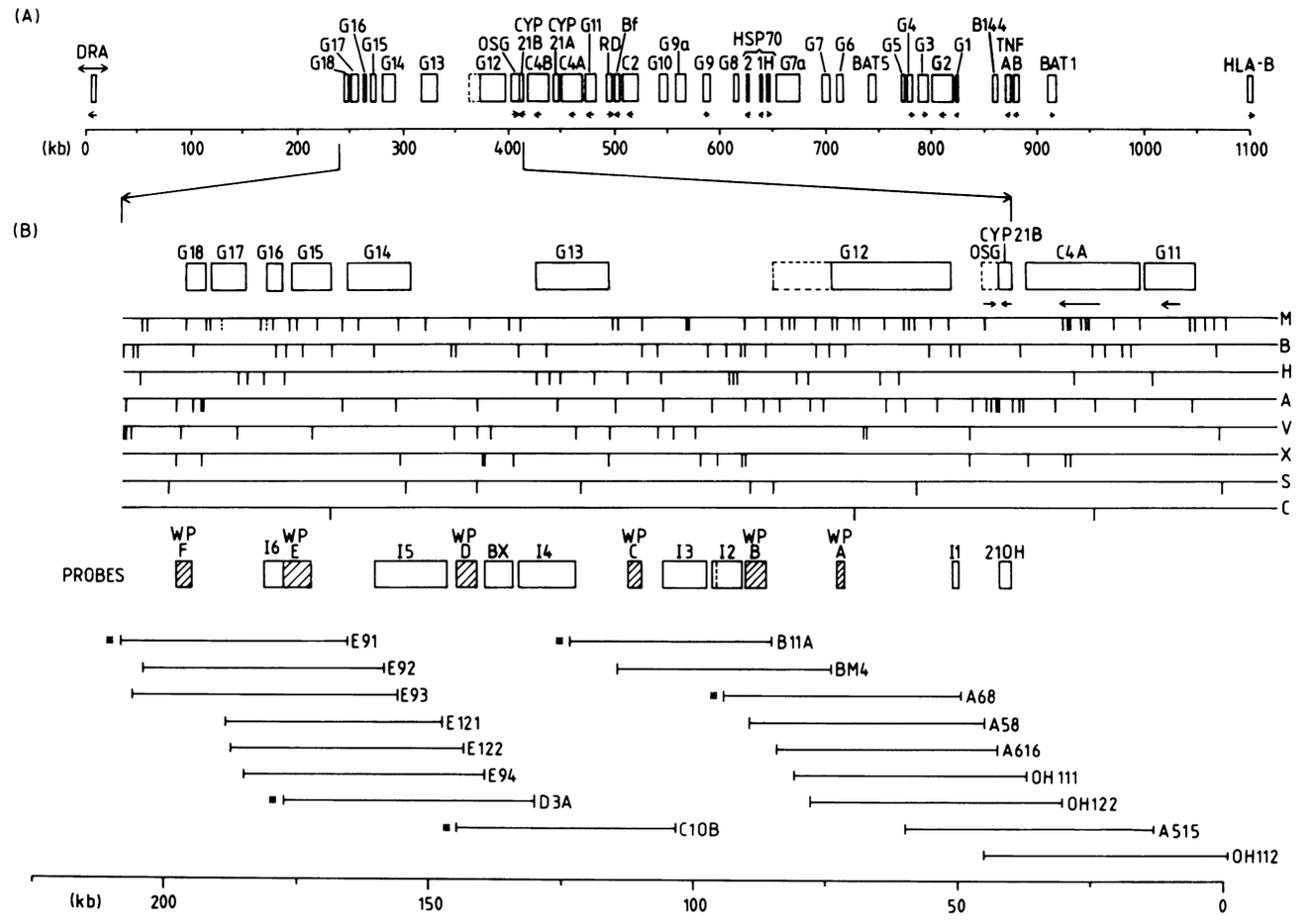

Figure 1. Molecular map of the MHC class III region. **(A)** The location of genes in the cloned segment of the class III region is shown by open boxes. The direction of transcription of the genes is indicated by arrows. This map is derived from information in refs 11, 12, 14–16, and 41. OSG represents the opposite strand gene defined by Morel *et al.* (17). **(B)** A detailed restriction map of the 17 overlapping cosmid clones extending centromeric of the C4A gene towards DRA, isolated by successive rounds of screening using the 210H probe and the walking probes WPA to WPE. The probe used to isolate each cosmid is indicated by the initial letter(s) in the designation of the cosmid. The genomic inserts were mapped with the enzymes *Bam*HI (M), *Bgl*II (B), *Hin*dIII (H), *Asp*718 (A), *Eco*RV (V), *Xho*I (X), *Sal*I (S) and *Cla*I (C). Open boxes above the restriction map indicate the location of genes, arrows beneath them indicating direction of transcription. The location of probes is shown beneath the map. The cosmids used to screen the cDNA library are indicated by a solid square to the left of the cosmid. Dotted lines indicate incomplete mapping data.

0.1% SDS at 65°C for 1 hr prior to autoradiography at −70°C between 2 intensifying screens. Between successive hybridisations, blots were stripped in 10 mM Tris (pH 8.0), 0.1% SDS, 1 mM EDTA at 80°C for 30→45 mins. Library filters were not stripped between hybridisations.

Probes

Genomic probes shown in Figure 1 were prepared from cosmid DNA inserts using the appropriate restriction enzyme digests and are as follows:
WPA, 1.4kb *Bam*HI-*Bgl*II; WPB, 4kb *Bam*HI; WPC, 2.2kb *Nco*I-*Bgl*II; WPD, 4kb *Eco*RV-*Bgl*II; WPE, 5kb *Hin*dIII-*Eco*RV: WPF, 3kb *Asp* 718; I1, 1.6kb *Bgl*II; I2, 4.8kb *Xho*I plus 0.9kb *Xho*I-*Asp* 718; I3, 9.5kb *Bgl*II; I4,10.5kb *Bam*HI-*Eco*RV; I5, 14kb *Bgl*II; I6, 4kb *Hin*dIII; BX, 5kb *Xho*I; DRA, 700bp *Pst*I cDNA (6); 21OH, 1.1 kb *Pvu*II-*Cla*I genomic fragment (5). All fragments were recovered from LGT agarose (Sea Plaque, FMC) gels and were radiolabelled using the random hexanucleotide priming method of Feinberg and Vogelstein (31). Where the probes were not unique they were heat-denatured and repetitive elements were preannealed with human placental DNA (10−250 μg/ml of hybridisation buffer) for 60→90 mins at 42°C prior to hybridisation.

RESULTS

Chromosome walking

Successive rounds of screening of the cosmid library with the walking probes WPA, WPB and WPC had resulted in the isolation of cosmid clones containing inserts which extend ~102kb from the 3′ end of the CYP21B gene (16). This walk was extended a further 63kb using walking probes WPD and WPE. In total the 17 overlapping cosmids encompassed ~165kb of DNA centromeric to CYP21B (Figure 1). The walking probes derived from the cosmid inserts were linked to each other and to CYP21B and DRA by PFGE. Each probe was hybridised to a single blot of genomic DNA digested with *Kpn*I, *Bss*HII, *Eag*I, *Mlu*I and *Pvu*I. Hybridisation to at least one common fragment by adjacent walking probes showed that the cosmid inserts were originally derived from a contiguous region of genomic DNA (Figure 2).

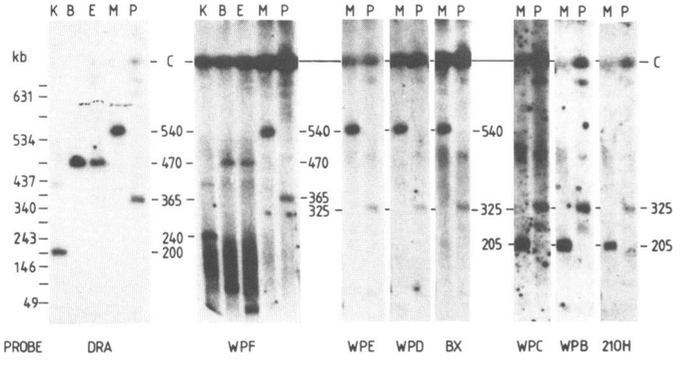

Figure 2. Linkage of walking probes to CYP21B and DRA. The various probes (indicated below each panel) were hybridised sequentially to a Southern blot of genomic DNA digested with *Kpn*I (K), *Bss*HII (B), *Eag*I (E), *Mlu*I (M) and *Pvu*I (P) and separated by crossed field gel electrophoresis using a 35s switching interval. The location of the probes on the molecular map can be found in Figures 1 and 4. Probe BX was used to position a *Mlu*I site. The position of the λ concatemer markers are given to the left, while the other numbers represent the size (in kb) of the fragments which were detected. C represents the zone of compression.

Mapping of CpG rich islands

In order to establish possible positions for novel genes in the cloned region, PFGE was used to define CpG-rich/HTF islands since these have been shown to be associated with most housekeeping, and some tissue-specific, genes (24, 27). In addition this approach proved successful in defining 14 HTF-islands in the region between the C4 and TNFA genes (16), all of which are associated with genes. The cosmids were mapped for the rarely cutting endonucleases *Bss*HII, *Kpn*I, *Eag*I, *Mlu*I, *Nru*I and *Pvu*I. Clustered sites for these enzymes, with two or more sites occurring over a region of 1−2kb of DNA, were found suggesting the presence of a number of CpG-rich sequences. The methylation status of these sites in uncloned genomic DNA was determined by sequential hybridisation of DNA probes to Southern blots from PFGE (Figure 3).

The most telomeric CpG-rich island has sites for *Kpn*I and *Eag*I, and lies ~8kb from the 3′ end of the CYP21B gene (Figure 4). There are two closely spaced CpG rich islands mapping ~8kb apart, each consisting of sites for *Kpn*I, *Bss*HII and *Eag*I, and which lie ~48kb and ~56kb from the CYP21B gene, respectively (Figure 4). The next potential CpG-rich island is characterised by sites for *Mlu*I and *Bss*HI (Figures 2 and 4). The remaining three CpG-rich islands lie 120kb, 145kb, and 160kb from the CYP21B gene (Figure 4) and are characterised by clustered sites for *Eag*I and *Kpn*I (island 5), *Kpn*I, *Nru*I and *Pvu*I (island 6), and *Bss*HII and *Eag*I, (island 7) all of which cleave in uncloned DNA (Figure 3). The remaining sites which cleave in uncloned DNA represent a single site for that enzyme in the cloned DNA. These could correspond to yet more CpG-rich sequences, but which contain multiple sites for that enzyme lying so close together as not to be easily mapped in the cloned DNA. No recognition sites for *Bss*HII, *Eag*I, *Pvu*I, *Nru*I, *Mlu*I were found to cleave in the 230kb between island 7 and DRA suggesting that this segment of DNA may not contain any further HTF-islands.

Genes and transcripts

Initial evidence that these putative HTF-islands were associated with novel genes came from the use of 'Zoo Blots' (32), with most of the HTF-island probes cross-hybridising to mouse, rat, cat, and sheep DNA, but not to chicken and shark DNA. This phylogenetic conservation of DNA sequence was suggestive that the HTF-island probes may contain at least partial coding regions of genes.

In order to try and detect associated transcripts via the isolation of cDNA clones we used the approach of hybridising whole cosmid genomic inserts onto a cDNA library using a preannealing procedure to prevent cross hybridisation by repetitive DNA elements. This approach enables the detection of HTF-island (and also non HTF-island) associated transcripts with the minimum number of probings of the library. Five cosmid inserts (E91, D3A, C10B, B11A, A68), that together spanned the whole of the cloned region (Figure 1), were used to screen a cDNA library constructed using mRNA from the monocytic cell line U937 (in which all of the novel genes detected telomeric of C4 are expressed).

Each set of homologous cDNAs isolated was mapped back to the relevant cosmid insert and had its genomic localisation identified by hybridisation to cosmid Southern blots (Figure 5A). In addition the cDNA inserts were hybridised to Southern blots of uncloned genomic DNA from the HLA homozygous cell line used to construct the cosmid library to determine whether the

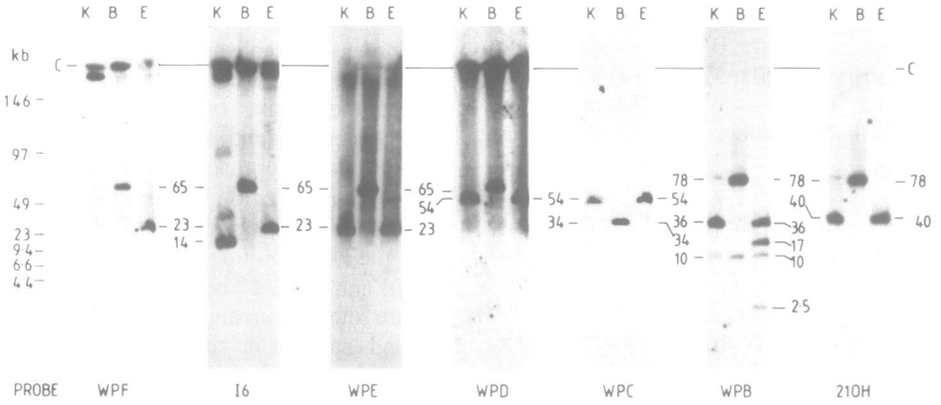

Figure 3, Mapping of HTF-islands. Genomic DNA digested with *Kspl* (K), *Bss*HII (B), and *Eag*I (E) was separated by crossed field gel electrophoresis using a 7.5s switching interval. The probes used are indicated below each panel. The position of the markers (concatemers of λ DNA and a HindIII digest of λ DNA) are shown at the left, while other numbers represent the size (in kb) of the fragments which were detected. C represents the zone of compression.

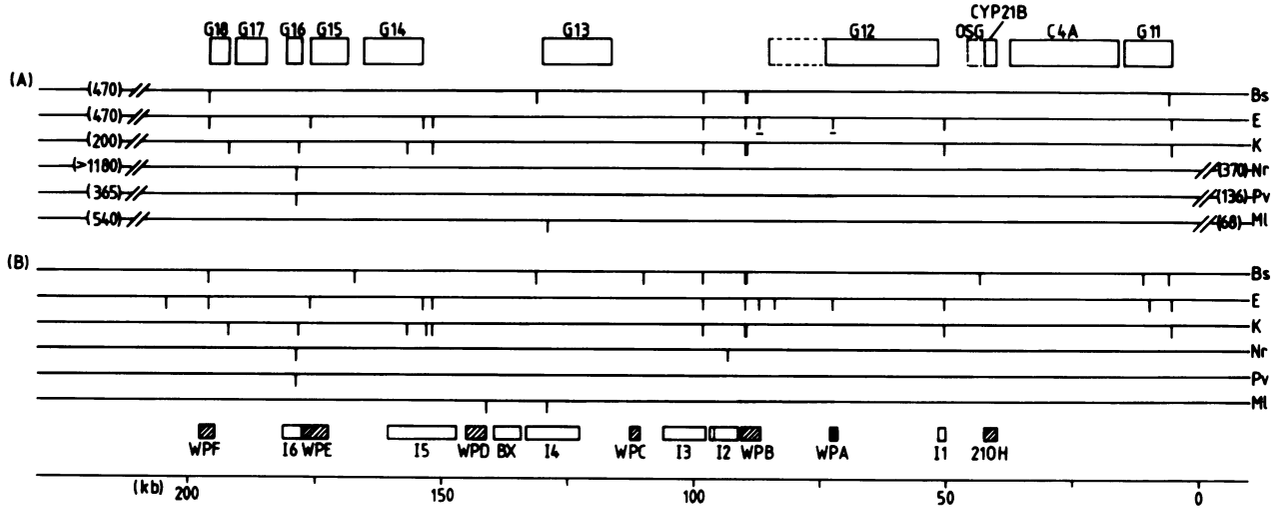

Figure 4. Molecular maps illustrating the locations of sites for the infrequently cutting restriction enzymes *Bss*HII (B), *Eag*I (E), *Kspl* (K), *Nru*I (Nr), *Pvu*I (Pv) and *Mlu*I (M) cleaved in **(A)** uncloned and **(B)** cloned genomic DNA. The data used to derive the map in (A) are shown in Figures 2 and 3. The position of the *Nru*I site which cleaves in uncloned genomic DNA is taken from ref 6. The numbers in brackets refer to the distances in kb to the next cleavable restriction site. Boxes above the maps show the location of genes, while those below the maps indicate the positions of probes.

Table 1. Characterisation of genes and transcripts

Gene	No. of cDNA clones detected	Largest cDNA insert (kb)	Limits of gene (kb)	Single copy	mRNA[a] (kb) 1	2	3	4
G12	2	1.0	23–34	Yes[b]	–	–	–	–
G13	40	2.0	15.0	Yes	2.7	2.7	2.7	2.7
G14	23	2.1	11.0	Yes	2.0	2.0	2.0[c]	2.0[c]
G15	22	2.1	7.5	Yes	2.1	2.1	2.1	2.1
G16	14	1.2	4.0	No	1.2	1.2	1.2	1.2
G17	15	1.6	7.5	No	3.3	3.3	3.3	3.3
					2.4	2.4	2.4[c]	2.4[c]
					1.6	1.6	1.6	1.6
G18	15	1.5	4.5	Yes	1.5	1.5	1.5	1.5
					1.4	1.4	1.4	1.4

[a]: Northern blots used RNA from the cell lines: 1, U937; 2, U937 + PMA; 3, Molt4; 4, Raji.
[b]: cDNA probe hybridised faintly to 7kb *Bam*HI and 12kb *Hin*dIII fragments not accounted for in the cosmid digests.
[c]: present in low abundance.

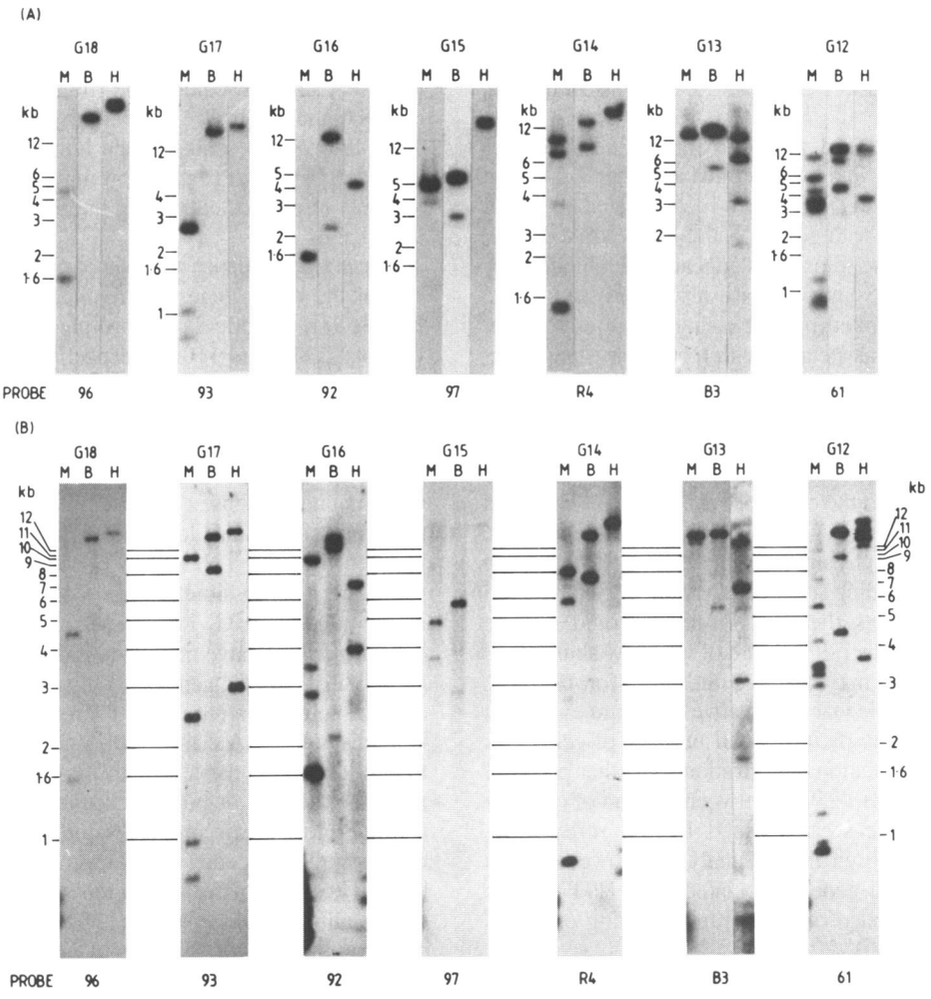

Figure 5. Cosmid **(A)** and genomic **(B)** Southern blot analysis of cDNA clones corresponding to the novel genes G12 to G18. The cDNA inserts were hybridised to Southern blots of cosmid and genomic DNA digested with *Bam*HI (M), *Bgl*II (B) and *Hin*dIII (H). The genomic DNA was prepared from the HLA homozygous consanguineous cell line (HLA type: A2, Cw7, B7, C2C, BfS, C4A3, C4BQO, DR2) used to construct the cosmid library. Gene and probe names are given above and below each panel, respectively. The location of the genes in the molecular map is shown in Figure 1. Numbers indicate the position of DNA markers in kb.

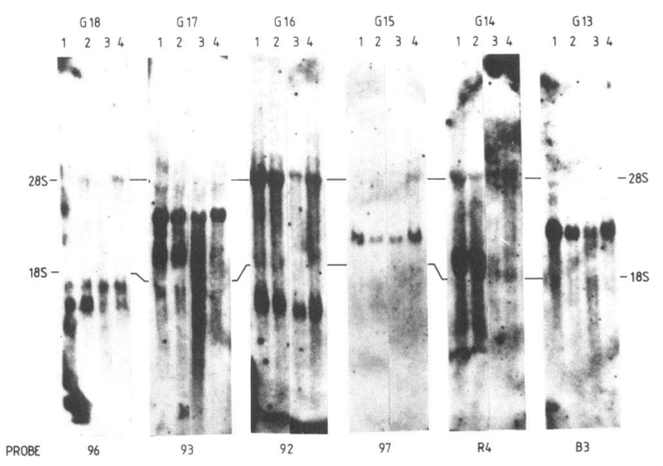

Figure 6. Northern blot analysis. cDNA inserts corresponding to G13 to G18 were hybridised to Northern blots containing ~20 μg total RNA from the cell lines (1) U937, (2) U937 stimulated with PMA, (3) Molt4 and (4) Raji. Gene and probe names are given above and below each panel, respectively. The locations of the genes and probes are shown in Figure 1. The position of migration of 28S and 18S RNA is indicated.

genes were single copy (Figure 5B), and to Northern blots of total RNA from a panel of cell lines representing monocyte (U937), macrophage (U937 stimulated with phorbol 12-myristate 13-acetate), T lymphocyte (Molt4), and B lymphocyte (Raji) lineages to detect the corresponding transcripts (Figure 6). Altogether 7 novel genes, designated G12–G18, details of which are summarised in Table 1, were identified. For two of the genes (G17 and G18) multiple transcripts were detected. In addition, a comparison of the cosmid and genomic DNA fragments hybridising with the cDNA probes obtained for G16 and G17 revealed strong cross-hybridisation to genomic fragments not accounted for in the cosmid restriction map. This is likely to be due to the presence of closely related sequences elsewhere in the genome, since the same cross-hybridising fragments were detected by other independent cDNA clones.

DISCUSSION

The mapping of HTF-islands is an important starting point when trying to locate genes in a large cloned region of DNA. These CpG-rich sequences are invariably found at the 5′ ends of genes which are ubiquitously expressed (24, 27). This was certainly true in the segment of DNA between the C4 and TNFA genes,

where 14 potential HTF-islands were mapped (16). Further analysis revealed that DNA fragments derived from these HTF-islands detected transcripts in Northern blot analysis in the cell lines studied.

We have now characterised the segment extending 165kb centromeric of the C4 gene and have defined a further 7 potential HTF-islands. Isolation of cDNA clones has revealed that 5 of these islands are associated with genes. Within the restricted panel of cell line RNAs used these transcripts appear to be ubiquitously expressed. The genes G15 and G17 are also expressed in the four cell lines, but are flanked by just a single rare cutter site. These genes could also be associated with CpG-rich islands, but which contain sites for rarely cutting enzymes not used in the current analysis. For one of the genes, G12, although two cDNA clones were isolated no transcript was detected in any of the cell lines, even when blots with higher loadings of polyA$^+$ RNA were probed. This was also the case for two of the HTF-islands (islands 2 and 3). However, genomic probes containing the putative HTF-islands were phylogenetically conserved suggesting the presence of potential coding sequence. Although we have been unable to detect transcripts these HTF-islands could still be associated with genes, but the mRNAs are of very low abundance, or are very unstable (as may also be the case for the G12 transcript). Also it is possible that the putative genes are expressed in a tissue-specific manner, or in a small number of cell types in a single stage of differentiation or activation. No other potential HTF-islands map in the 220−250kb between the end of cos E91 and the DRA gene, at least as defined by the rare-cutting restriction endonucleases used in this analysis. However, it is possible that there may be genes associated with HTF-islands characterised by the presence of other rarely cutting enzymes such as NaeI and NarI (26).

A detailed molecular map of 890 kb of the MHC class III region is now available through the characterisation of overlapping cosmid clones (11, 14−16, this work). Altogether this segment contains at least 36 genes. A large number of these, 19, are associated with HTF-islands, and appear to be ubiquitously expressed. However, intermingled with these genes are genes which are expressed in a more tissue specific manner. For example the principal site of synthesis of the complement proteins C4, C2 and Factor B is the liver (33) while the CYP21B and OSG genes are expressed in the adrenal gland (17). In addition one of the novel genes, G1, defined by Sargent *et al.*, (16) appears to have a very restricted expression, the G1 mRNA being observed only in U937 and Molt 4 cells. Thus the cloned region and the remaining segment yet to be cloned may contain several more genes which are expressed in a tissue specific manner.

The presence of at least one gene per 20kb of DNA in this segment of the genome is of particular relevance to the mapping and sequence analysis of the rest of the human genome. The human MHC is located in the chromosome band 6p21.3 which is a Giemsa (G) negative or light band. A recent survey of gene localisations by in situ hybridisations indicates that the bulk (74%) are located in G-negative bands (34). Our analysis of the gene density in the class III region is compatible with the suggestion that genes, both housekeeping and tissue specific, may predominantly be located in G-negative bands. On the other hand those regions of chromosomes which stain positively with Giemsa, the G-dark bands, may be deficient in genes. PFGE analyses of different regions of the genome appear to indicate that HTF-islands, and therefore HTF-island associated genes, occur much less frequently in the G-dark bands compared to their frequency in G-light bands (34). An extreme example of this is the G-dark band p21 on the X chromosome. This band contains the Duchenne Muscular Dystrophy gene which spans ~2000kb of DNA and lies in a region largely devoid of HTF-islands (35). Thus in order to sequence the human genome, because of the considerable effort required, it may be more rewarding initially to direct efforts to sequencing the DNA corresponding to the G-light bands.

Nucleotide sequence analysis of cDNA clones has revealed that two of the novel genes in the class III region, BAT2(G2) and BAT3(G3), encode large proline-rich proteins of molecular weights 228kd and 110kd, respectively, which do not appear to be members of any known family of proteins (36). Although the functional properties of BAT2 and BAT3 and the proteins encoded by the other novel genes mapped in the class III region are yet unknown, some may be involved in different aspects of the immune response. For example a genetic locus controlling the expression of an alloantigen recognised by NK cells has been mapped between TNFA and H-2S in the mouse H-2 complex (37), and it is possible that the equivalent gene is also present in the human MHC. Recent work using HLA-deletion mutant cell lines which have a large homozygous deletion between the HLA-DPA2 and C4 genes (38) suggest that this segment of DNA contains a function required for presentation of intracellular viral antigens with HLA class I molecules(39). The phenotype of the mutant cells is consistent with the loss of transport of peptides from the cytosol, or possibly secondary to a defect in a peptide loading mechanism in the endoplasmic reticulum. Although retaining the C4 genes (40) the precise endpoint of the deletion has not been characterised and it is possible that it encompasses one or all of the novel genes defined in this analysis. Thus one or a combination of the protein products of these novel genes could be necessary for the presentation of intracellular antigens by HLA class I molecules.

As yet it is not possible to define whether the novel loci explain any of the disease susceptibilities which map to the MHC. However, it will be of major interest to look for variations in expression of the genes or allelic variation in the sequence of the genes to determine whether they relate in any way to the observed MHC associations with an individual's susceptibility to develop autoimmune disease.

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