Purification, chain separation and sequence of the MRC OX-8 antigen, a marker of rat cytotoxic T lymphocytes

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The MRC OX-8 antigen is a marker of the rat cytotoxic T lymphocytes that consists of disulphide-linked chains of mol. wts. 37 and 32 kd. It is thought to be equivalent to the human T8 and mouse Lyt2,3 antigens (all now called CD8 antigens). MRC OX-8 antigen was purified from thymocytes using a monoclonal antibody column and because antigenicity was retained after reduction and alkylation the two polypeptide chains could be separated by a subsequent affinity chromatography step. Peptides were isolated from each chain and their sequences determined. A cDNA probe coding for the mouse CD8 antigen (pLY2C-1 provided by Dr L.A. Herzenberg) was used to obtain rat cDNA clones from which the sequence of the equivalent rat molecule was determined. Peptides from the 32-kd chain were identified in this translated sequence whereas peptides from the 37-kd chain were not. The 32-kd polypeptide sequence consisted of 210 amino acids and had one possible N-linked glycosylation site. The N-terminal part of the sequence was surprisingly different from both its mouse and human counterparts but, as in the other two species, it showed a clear relationship to Ig V domains. Key words: MRC OX-8 antigen/rat cytotoxic T lymphocyte marker/lymphocyte glycoprotein/CD-8 antigen/immunoglobulin superfamily

Introduction

The CD8 antigen of lymphocytes is of considerable interest because it distinguishes cytotoxic T lymphocytes from T helper cells (Cantor and Boyse, 1975; Ledbetter *et al.*, 1980) and appears to play an important role in the function of cytotoxic T cells (Nakayama *et al.*, 1979; Fan *et al.*, 1980). The current hypothesis for this role is that the CD8 antigen interacts with class I major histocompatibility antigens (MHC) of target cells and restricts killing to those targets that present foreign antigen along with MHC class I antigen (Engleman *et al.*, 1981; Maconald *et al.*, 1982; Swain, 1983).

The CD8 antigen was first identified in the mouse when the Lyt-2 and Lyt-3 alloantigens were described (Boyse *et al.*, 1968). The Lyt-2 and Lyt-3 loci were found to be closely linked to each other (Itakura *et al.*, 1972) and to the immunoglobulin kappa light chain loci (Gottlieb, 1974). Using the alloantisera it was shown that Lyt2/3 antigen on thymocytes consisted of disulphide-linked chains of ~35 kd (Durda and Gottlieb, 1976).

With monoclonal antibodies a more comprehensive characterisation of the mouse antigen was possible (Ledbetter and Herzenberg, 1979) and antigens that appear equivalent to Lyt2/3

were identified on human (Terhorst *et al.*, 1980; Ledbetter *et al.*, 1981a) and rat lymphocytes (Brideau *et al.*, 1980). In all three species the antigens occur as dimers and higher multimers but differ in their chain composition both between species and between the thymocyte and mature T cell forms. On peripheral T cells the mouse antigen consists of two distinct subunits of mol. wts. 38 and 30 kd which carry the Lyt-2 and Lyt-3 antigenic determinants, respectively (Ledbetter *et al.*, 1981b; Walker *et al.*, 1984). The thymocyte antigen has an additional 34-kd chain which may be a variant of the 38-kd structure since both chains carry the Lyt-2 determinants and give similar peptide maps (Naim *et al.*, 1984). In humans the peripheral T cell complexes contain only one chain of mol. wt. 34 kd but in thymocytes an additional structure of 45 kd can be found in the complexes (Ledbetter *et al.*, 1981a; Snow and Terhorst, 1983). The 34-kd chain (T8) from human T cells has recently been cloned and sequenced (Kavathas *et al.*, 1984; Littman *et al.*, 1985; Sukhatme *et al.*, 1985) and shown to be equivalent to the mouse 34- and 38-kd chains that express the Lyt-2 determinant (Nakauchi *et al.*, 1985).

The rat MRC OX-8 antigen from thymocytes has previously been shown by immunoprecipitation to consist of two broad bands of mol. wts. 34 and 38 kd (Thomas and Green, 1983). In this paper we describe the purification of this antigen from thymocytes and the separation of the individual chains from which partial peptide sequences were obtained. The chain equivalent to Lyt-2 was then cloned in the rat using a mouse cDNA probe (pLY2C-1, kindly provided by Dr L.A. Herzenberg) and was found to correspond to the lower mol. wt. chain of the heterodimer.

Results

Protein purification and chain separation

The steps in the purification are given in Table I and after two cycles of affinity chromatography followed by reduction and alkylation and a gel filtration step, a preparation showing two broad bands after silver staining was obtained (Figure 1 'total'). The two chains had mol. wts. of ~32 and 37 kd. The upper band could be further sub-divided into three chains of 35, 37 and 38 kd. The basis of this heterogeneity is unknown.

Antigenicity was retained after reduction and alkylation and from an immunoblot (not shown) it was found that the MRC OX-8 antibody labelled only the 32-kd chain. Thus chain separation was possible using a further affinity chromatography step as shown in Figure 1. The 37-kd polypeptides passed straight through the column while the 32-kd chain was bound and specifically eluted at high pH. The OX-8 antigenic activity was assayed at all stages in the preparation and the final yield was 7% (see Table I) with a purification factor of 5800-fold to give 4.2 nmols of the 32-kd polypeptide. Assuming an antigenic site number per cell of 40 000 [28 000 molecules of IgG are bound (Brideau *et al.*, 1980) and we assume 50% is bivalently bound], then 4×10^{11} thymocytes should contain 27 nmols of antigen. The starting figure calculated from 7% yield of antigenic activity and 4.2 nmol of polypeptide recovered is 59 nmol. These figures are

Table I. Purification of the OX-8 antigen

Fraction	Protein (mg)	Yield of antigenic activity (%)	Relative specific activity	Purification factor
4×10^{11} cells in Tween-40	8400	100	0.012	1
Tween membrane	380	43	0.11	9
Tween membrane in deoxycholate	360	58	0.16	13
Antibody column eluate 1	4	46	11.5	966
Antibody column eluate 2	2	29	14.5	1218
After gel filtration	0.3	13	43	3612
Antibody column eluate after chain separation	0.1 (4.2 nmol)	7	70	5833

These results are an average from two preparations, except the last step which is an average from three. Relative specific activity equals % antigenic activity divided by protein content. For other details see Materials and methods.

Fig. 1. Silver stain of a 12% SDS-polyacrylamide gel. The purified material (reduced and alkylated) is shown in **track 1** (total). This material was then passed through a 1-ml MRC OX-8 monoclonal antibody column and the material that did not bind to the column is shown in **track 2**. The third track shows the material that was specifically eluted from the column at high pH.

in reasonable agreement given the uncertainties involved.

Peptide purification

The reduced and alkylated chains were succinylated after chain separation and then digested with trypsin followed by size fractionation on a Biogel P-30 column. The elution profiles of digests of the 37-kd and 32-kd chains were substantially different (see Figure 2) and in both cases a considerable amount of material ran at the column front. However, some retarded peaks of absorbance at 206 nm were seen and all but peak 2 from the 32-kd chain were further fractionated by reverse-phase h.p.l.c. All suitable peaks were sequenced on the gas phase sequencer to yield

Fig. 2. Peptide separation on a Biogel P-30 column. (a) and (b) show the elution profiles after succinylation and trypsin digestion of the 37- and 32-kd chains, respectively. Peptide pools were made according to the absorbance and are indicated by letters in (a) and numbers in (b). Pool A and pool 1 represent material that passed straight through the column in the excluded volume. The other pools (except pool 2) were further fractionated on reverse-phase h.p.l.c. and the peptide sequences obtained are shown in Table II.

Fig. 3. OX-8 mRNA structure and three clones used to derive the sequence. The hatched portion of the bar represents the signal sequence and the lines show sequenced 5' and 3'-untranslated sequences. B, H, R and P are restriction sites for *Bam*HI, *Hind*III, *Eco*RI and *Pst*I, respectively. The putative intron sequenced in pX8.6 is shown as a line and the splice sites are indicated by dotted lines. Only the portions sequenced of pX8.1 and pX8.17 are shown.

the peptide sequences given in Table II.

Isolation and characterisation of rat CD8 clones

Initial attempts to clone one of the rat CD8 chains by use of an oligonucleotide probe predicted from the sequence of peptide 2 (see Table II) were not successful and after the cloning of the human CD8 (T8) antigen was reported (Kavathas *et al.*, 1984) it was decided to clone one of the rat chains using pLY2C-1, the mouse *Lyt* 2 cDNA probe (Nakauchi *et al.*, 1985). After an initial screening of a rat thymocyte cDNA library (Thomas *et al.*, 1985), ~200 positive clones were obtained from 100 000 colonies. Eight clones were examined in detail and all contained an internal *Hind*III and *Eco*RI site. The largest clone pX8.6 had

Fig. 4. Nucleotide and corresponding amino acid sequence of thymocyte cDNA clones coding for the OX-8 antigen.

an insert of 1.8-kb and nucleic acid sequence was obtained from the coding region of this insert. An open reading frame of 744 bases was found which, when translated, gave a sequence that was similar to human T8 and mouse Lyt-2 sequences. However, there was no initiator codon or signal sequence in this reading frame although an initiator codon and potential signal sequence could be seen further upstream. Another clone (pX8.3) gave the same sequence but four other clones (pX8.1, 13, 17 and 18) sequenced in this region produced an open reading frame including an initiator codon and signal peptide. If 86 nucleotides were deleted from the pX8.3 and pX8.6 sequences then all the clones could be exactly aligned as shown in Figure 3. The points at which the 86 nucleotides are deleted form typical splicing sites (Breathnach and Chambon, 1981):

Thus it appears that clones pX8.3 and pX8.6 contain an unspliced intron transcribed into cDNA. In the human gene an intron is found in exactly this position (Littman *et al.*, 1985). However, the mouse protein sequence differs in this region and if the putative rat intron sequence is translated, four out of the first six residues are identical to the mouse signal sequence:

Mouse signal sequence

MASPLTRFLSLNLLLLGESIIILGSG---

Rat signal sequence/intron

MAS RVI CFLS LNL LLL/GEL MI LVE G---

This suggests that the mouse gene may have evolved to use an alternative splice site. In the rat many potential splice sites can be identified in the intron and N-terminal region but from the clones sequenced only one site appears to be used.

The nucleotide and protein sequence is shown in Figure 4 where the nucleotides around the initiator methionine (ACACCATGG) agree well with the consensus sequence (CAACCATGG) found for an efficient eukaryotic translational start site (Kozak, 1984). To the 5' side of the initiator methionine there are at least 50 nucleotides of untranslated sequence. From

Fig. 5. Northern blot analysis of OX-8 antigen mRNA. RNA was fractionated on a 1.5% agarose formaldehyde denaturing gel and blotted onto Gene Screen (New England Nuclear). pX8.6 was nick translated and used as the probe. 5µg of RNA was loaded in each track. 1: thymocyte RNA; 2: cortical thymocyte RNA; 3: T cell RNA; 4: B cell RNA. Eukaryotic rRNA was used as a marker.

the stop codon 720 bp were sequenced (data not shown) but no polyadenylation site was found.

Northern blot analysis

A message size of ~ 1.8 kb was determined by Northern blot analysis. mRNA was present in cortical thymocytes, thymocytes and T cells but not in B lymphocytes (Figure 5). The message size is similar to that found for Lyt-2 (1.8 kb) in mouse but considerably smaller than the message for human T8 (2.5 kb) (Nakauchi *et al.*, 1985; Littman *et al.*, 1985; Kavathas *et al.*, 1984).

Sequence of the OX-8 polypeptide chain

All the peptides from the 32-kd chain shown in Table II fitted the sequence in Figure 4 with only one discrepancy. In peptide 2, residue 7 is given as a Phe in Table II but an Ile is predicted from the cDNA sequence. The phenyl isothiocyanate derivatives of Ile and Phe run at very similar positions on h.p.l.c. and reinspection of the traces suggests that the Phe assignment was incorrect.

The peptides establish the protein sequence in Figure 4 as cor-

rect from residue 26 to 202 but an unambiguous assignment of the start of the protein cannot be made. One preparation of the intact 32-kd chain was sequenced but the sequence obtained was at a very low level (20 pmol *versus* 2.5 nmol loaded) and did not correspond to any sequence in Figure 4. This suggests that the N terminus is blocked and the most likely possibility is a pyroglutamic acid residue derived from glutamine. The Gln residue shown in Figure 4 was chosen on the basis of the guidelines for cleavage of signal sequences (Von Heijne, 1983).

The mature protein consists of 210 amino acids and contains

only one region likely to span the membrane, namely residues 162-184. The sequence C-terminal to this is likely to be inside the cell since it contains a cluster of basic residues as is typically found in this position for many cell surface molecules. The sequence N terminal to the transmembrane region has one possible N-linked glycosylation site and is likely to be externally orientated. This sequence can be divided into two domains, the N-terminal domain of 110 residues which shows similarities to variable regions of immunoglobulins (see below) and the membrane proximal domain of ~50 residues which is notable for a high content of proline (21%).

Discussion

Figure 6 shows the comparison of the OX-8 sequence with mouse Lyt-2 and human T8 sequences. The overall homology between species is surprisingly low (see Table III). The most highly conserved regions are the transmembrane and cytoplasmic sequences and the argument that the 32-kd chain of OX-8 is the rat homologue of Lyt-2 and T8 is most strongly made on the basis of these regions. The greatest divergence is seen in the external Ig-like segment where the level of identity between rat and human sequences is only 42% and between rat and mouse 60%. This compares with a figure of 87% identity for a comparison of rat and mouse Thy-1 antigen (Williams and Gagnon, 1982) and ~75% identity for mouse *versus* human α and β chains of Class II MHC antigen (Kaufman *et al.*, 1984). Thus the CD8 antigen sequences seem to be diverging more rapidly than those for other cell surface antigens. On the assumption that CD8 binds to Class I MHC antigen then it could be argued that the CD8 and MHC structures are co-evolving. However, the percent identity between the three external domains of human and mouse Class I MHC beginning with the N-terminal domain are 68%, 72% and 74% (HLA-B7 *versus* H-2K^b, Kabat *et al.*, 1983). These values are considerably higher than for CD8 comparisons and thus these two sets of molecules do not seem to be diverging at the same rate.

Table II. Amino acid sequence of OX-8 tryptic peptides

Peptide sequence	Amount of peptide at first cycle of sequencing	Position in cDNA sequence
37-kd chain		
B No sequence obtained		
C ELQDSNKNK	500 pmol	Not found
D FVKQF-K	400 pmol	
FVKQF	200 pmol	Not found
32-kd chain		
2 KENNKYFLT	300 pmol	71-79
G		
3 ₁ VCK*CPRLVK	200 pmol	193-202
3 ₂ SKLNDILDPNLF-A	300 pmol	56-69
5 DTSQG	50 pmol	26-30

The letters and numbers refer to the pools made after gel filtration and h.p.l.c. of the tryptic peptides. A dash indicates that no phenylthiohydantoin amino acid was determined at this position. In pools D and 2 two amino acids were found at some positions and the residue found in lower amounts is given below the predominant sequence. *At these positions the phenyl isothiocyanate derivative did not correspond to any standard amino acid but the residues were probably cysteines that had reacted with iodoacetamide added as proteolytic inhibitor during antigen purification (Sunderland *et al.*, 1979).

Fig. 6. Alignment of rat OX-8 sequence with mouse Lyt-2 (Nakauchi *et al.*, 1985) and human T8 (Littman *et al.*, 1985). Identical residues are boxed and dashes represent gaps in the sequences put in to maximise the alignments. The arrows define boundaries for the regions indicated in Table III, i.e., the signal sequence, N-terminal domain, membrane proximal domain, transmembrane region and cytoplasmic tail.

The potential N-glycosylation sites of CD8 are also strikingly different between the species with three such sites in the mouse compared with one equivalent site in the rat and none in the human CD8 chain (the human sequence has an Asn-Pro-Thr sequence but the Pro would debar glycosylation) (Bause and Hettkamp, 1979; Campbell *et al.*, 1981). This is in contrast to Thy-1 antigen and class II antigens where N-linked carbohydrate sites are totally conserved between mouse and rat and mouse and human, respectively (Williams and Gagnon, 1982; Kaufman *et al.*, 1984). In human it is known that the CD8 antigen has O-linked carbohydrate (Snow and Terhorst, 1983) and it seems likely that this will also occur in OX-8 since only 24 kd of the apparent 32 kd is accounted for by protein and the remaining 8 kd is unlikely to be all accounted for by a single N-linked carbohydrate structure.

As has been reported for the human and mouse CD8 sequences, the N-terminal part of the OX-8 sequence shows similarities to Ig V-domains and the other sequences that are like Ig V-domains, namely the T cell receptor V domains (Patten *et al.*, 1984), Thy-1 (Williams and Gagnon, 1982) the Poly Ig receptor domains (Mostov *et al.*, 1984) and domain I of MRC OX-2 antigen (Clark *et al.*, 1985). Comparison of the human CD8 (T8) sequence with the sequences related to V-domains leads to the conclusion that T8 is most like Ig V_L domains and in particular V_{kappa} (Littman *et al.*, 1985; Sukhatme *et al.*, 1985). In the mouse similarities to V_{kappa} were also noted as was the fact that the CD8 sequence was like V_H in terms of length of sequence

between the conserved Cys residues (Nakauchi *et al.*, 1985). However, with the rat sequence the V_{kappa} relationship does not stand out so clearly. The OX-8 sequence seems equally like V_{kappa}, V_{lambda} and Thy-1 and this is shown in Figure 7 where 100 residues of the OX-8 sequence are aligned with the other sequences. The V_{kappa} sequence shown gives 25 identities but the average number of identities for four human V_{kappa} (one of each sub-group) plus four mouse V_{kappa} sequences was 21 (V domain sequences from Kabat *et al.*, 1983). With the V_{lambda} sequence shown there are 25 identities and the average for one of each of the six human V_{lambda} sub-groups plus one mouse V_{lambda} was 23. For rat and mouse Thy-1 26 and 24 identities can be obtained with no more breaks in the sequence than for the V_L sequences. For the other V-like sequences the average numbers of identities were: V_H (seven human sequences): 20; poly Ig R (domains I-V): 17; T cell receptor β chains (seven sequences): 20; MRC OX-2 domain I (one sequence): 14.

One chain of the CD8 complex has been sequenced in human, mouse and now rat. In humans the CD8 complex consists of only this chain on peripheral T cells or in association with a 45-kd chain in the thymus. However, in rodents a second glycoprotein chain of similar mol. wt. to the chain already sequenced forms an integral part of the CD8 complex. In this paper the peptide and nucleic acid sequences together unequivocally prove the distinction between the two chains. The second chain is yet to be sequenced and its relationship to the other rodent chain and to the human CD8 sequence will be of considerable interest in furthering the understanding of a set of molecules that are surprisingly different between these species given that they appear to have an important function in the triggering of cytotoxic T lymphocytes.

Materials and methods

Protein purification

Membrane preparation and affinity chromatography was essentially as in Sunderland *et al.* (1979). A typical preparation of 4×10^{11} thymocytes was obtained from 200 Sprague-Dawley rats, 4-8 weeks old, purchased from Olac Limited, Bicester or Department of Psychology, University of Oxford. Membrane was prepared using the Tween-40 method and then solubilised in sodium deoxycholate. The extract was then applied to a 10 ml MRC OX-8 monoclonal antibody column (10 mg IgG/ml) and eluted at pH 11.5. The eluted material was neutralised, pooled and re-applied to the column. The eluate was then concentrated and dialysed before being reduced and alkylated in 0.4 M Tris-HCl pH 8, 50 mM EDTA, 2% w/v SDS. Dithiothreitol (DTT) was added to a concentration of 2.5 mM and incubated at 37°C for 1 h. A 2-fold excess over DTT of

Table III. Percentage identities of the rat OX-8 sequence with mouse and human CD8 sequences

Region	Identities with mouse		Identities with human	
	No.	%	No.	%
Signal sequence	13/27	48	7/26	27
N-terminal domain	73/122	60	49/118	42
Membrane proximal domain	31/48	65	25/48	52
Transmembrane region	21/25	84	21/25	84
Cytoplasmic tail	21/28	75	18/28	64
Mature polypeptide	146/223	65	113/219	52

Boundaries for the regions are shown by arrows in Figure 6. When sequence lengths differed the number of residues from the longer sequence was used as the denominator in calculating percent identities.

Fig. 7. Alignment of the first 100 amino acids of OX-8 sequence with sequences of V_{kappa} (107B'CL), V_{lambda} (NIG, subgroup 6) (Kabat *et al.*, 1983) and rat Thy-1 (Campbell *et al.*, 1981). Residues identical to OX-8 are boxed and dashes show gaps in the sequence inserted to maximise the alignment. The bars above the sequences show the position of β -strands in the Ig-fold determined by comparison of the V-domain sequences with known structures for V_{lambda} sequences (Edmundson *et al.*, 1975; Amzel and Poljak, 1979).

¹⁴C-labelled iodoacetamide was added for 15 min in the dark at room temperature. A second aliquot of DTT at 8 mM was added and incubated at 37°C for 4 h followed by addition of cold iodoacetamide in 3-fold excess over DTT and incubated as before. This was then applied to a Sephacryl S-400 gel filtration column (150 × 1 cm) (Pharmacia, Uppsala) equilibrated in 0.2% w/v SDS, 0.1 M ammonium bicarbonate. The column effluent was monitored by absorbance at 280 nm, radioactivity, SDS-PAGE and by assaying the fractions for antigenic activity. SDS-PAGE was on 12% gels (Laemmli, 1970) which were stained using the silver method (Morrisey, 1981) or in some cases by immunoblotting (Towbin *et al.*, 1979).

Indirect binding assay

Dilutions of material at each step in the purification were made into 0.5% BSA, 0.15 M NaCl, 10 mM Tris-HCl pH 8 and incubated with tissue culture supernatant containing MRC OX-8 antibody at 1/25. Residual antibody activity was assayed by incubating 50 µl aliquots of the mixture with 2.5 × 10⁶ glutaraldehyde-fixed rat thymocytes, washing and then incubating with [¹²⁵I]rabbit F(ab')₂ anti-mouse IgG antibody (Williams *et al.*, 1977). A graph was plotted of radioactivity (remaining bound to the target cells after washing) against dilution of antigen. This produces an inhibition curve where 1 unit of antigenic activity is defined as the amount of antigen that produces 50% inhibition of antibody binding to 2.5 × 10⁶ target cells. To obtain yield of antigenic activity, values were related to the starting activity put at 100%.

Chain separation

Material from gel filtration was made to 0.5% w/v with deoxycholate and applied to the 1 ml MRC OX-8 affinity column and eluted as before. The final amount of protein in each chain was determined by amino acid analysis (Campbell *et al.*, 1981).

Preparation of tryptic peptides

Material from three preparations was pooled and after exhaustive dialysis, lyophilised. The two chains were then succinylated in 6M guanidinium chloride, 1 M Tris pH 10 using 200-fold excess succinic anhydride over the estimated lysine residues. Five aliquots of solid succinic anhydride were added over 2.5 h at room temperature. After dialysis, the material was trypsinised with three aliquots of 1% w/w TPCK-treated trypsin at 37°C over 24 h in 2 M urea, 0.1 M CaCl₂, 0.1 M ammonium bicarbonate. Tryptic peptides were then separated on a Biogel P-30 column (1 × 150 cm) equilibrated in 0.1 M ammonium bicarbonate. Column effluent was monitored by absorbance at 206 nm and pooled accordingly. These were concentrated using a Speedvac (Savant Inc.) and equilibrated with 10 mM ammonium acetate before being applied to a reverse-phase h.p.l.c. C18µ bondapak column (Waters Associates Limited). This was equilibrated in 10 mM ammonium acetate, pH 6.8 (buffer A) and 5% buffer B (10 mM ammonium acetate, 70% v/v acetonitrile). Peptides were eluted with a linear gradient to 60% acetonitrile over 1 h at a flow-rate of 1 ml/min. Peptides were sequenced on an Applied Biosystem Gas Phase Sequencer using the 01tfav programme.

Screening of rat thymocyte cDNA library

A rat thymocyte cDNA library prepared by the 'loopback' method (Thomas *et al.*, 1985) was screened with a 600 bp Lyt-2 cDNA insert obtained by *EcoRI* digestion of the pLY2C-1 clone (Nakauchi *et al.*, 1985). The library was screened on Whatman 541 paper (Gergen *et al.*, 1979) with ³²P end-labelled Lyt-2 cDNA insert in the following hybridisation solution: 50% deionised formamide, 2 × Denhardt's, 5 × SSC, 1% SDS, 500 µg/ml denatured, sonicated herring sperm DNA and 20 µg/ml denatured, sonicated crude DNA prepared from *Escherichia coli* transformed with pAT153/PvuII/8 (Anson *et al.*, 1984) at 42°C overnight. The filters were washed twice in 2 × SSC, 0.5% SDS at 65°C for 30 min and then once in 0.2 × SSC, at 65°C.

DNA sequencing

A positive clone was digested with restriction endonucleases and cloned into the *SmaI* site of M13 mp8. M13 plaques were screened by transfer to nitrocellulose and hybridised with the ³²P-labelled pLY2C-1 insert (Benton and Davis, 1977). Positive plaques were sequenced by the dideoxy method (Sanger *et al.*, 1977; Messing, 1983). Each nucleotide was sequenced on average six times. Restriction fragments from other clones were cloned into M13 mp8 or M13 mp9 and also sequenced by the dideoxy method. Computer analysis was carried out using the programmes described by Staden (1982).

Northern blot analysis

The preparation of RNA from various lymphoid cell types was as described in Thomas *et al.* (1985).

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