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Isolation of cDNA clones encoding T cell-specific membrane-associated proteins

Stephen M. Hedrick^{*†}, David I. Cohen^{*†}, Ellen A. Nielsen^{*} & Mark M. Davis^{*†‡}

^{*} Laboratory of Immunology, NIAID, National Institutes of Health, Bethesda, Maryland 20205, USA

[†] Department of Medical Microbiology, Stanford University School of Medicine, Stanford, California 94305, USA

Of 10 distinct cloned DNA copies of mRNAs expressed in T lymphocytes but not in B lymphocytes and associated with membrane-bound polysomes, one hybridizes to a region of the genome that has rearranged in a T-cell lymphoma and several T-cell hybridomas. These characteristics suggest that it encodes one chain of the elusive antigen receptor on the surface of T lymphocytes.

T LYMPHOCYTES, like B lymphocytes, are capable of recognizing a wide range of different antigens¹⁻³. As with B cells, the ability to recognize a given antigen is fixed in any particular clonal line of T cells. However, unlike B cells, T cells appear to recognize antigens in combination with self major histocompatibility (MHC) determinants⁴⁻⁶. In view of the similarities to B cells, early ideas as to how T cells recognize antigens centred on the use of either entire antibody molecules⁷ or at least some of the separately encoded (in the germ-line genome) segments that make up the antigen-binding sites of immunoglobulin heavy and light chains⁸⁻¹². But despite early reports that antibodies against immunoglobulin antigen-binding sites can react with T cells¹³⁻¹⁵ and recognize a target closely linked to the immunoglobulin heavy-chain locus^{16,17}, attempts to demonstrate an involvement of immunoglobulins in T-cell antigen recognition have proved consistently negative⁸⁻¹².

More recent investigations have taken a route largely independent of the antibody models and have succeeded in raising at first antisera¹⁸ and then monoclonal antibodies¹⁹⁻²² that specifically recognize particular T-cell lines or hybridomas. Some of these antibodies have been shown either to inhibit²⁰⁻²² or stimulate¹⁸ the response of a cell in a clone-specific fashion. Several groups have used these antibodies to immunoprecipitate disulphide-linked heterodimers, composed of two distinct glycoproteins of molecular weights 37,000-50,000 (refs 19-22), both of which appear to have variable and constant regions^{23,24}.

We have taken a different approach to this problem and have attempted to use the techniques of molecular genetics to isolate from a series of antigen-specific, MHC-restricted T helper hybridomas^{25,26} the genes that encode their antigen receptors. Our approach was based on the following assumptions about the nature of the T-cell receptor genes: (1) That they should be expressed in T cells but not in B cells. (2) That the mRNAs for the T-cell receptor proteins should be found on membrane-bound polysomes, as one would expect the nascent receptor

polypeptides to attach to the endoplasmic reticulum by a leader peptide, or signal sequence²⁷. (3) That like immunoglobulin genes those that encode the T-cell receptor, proteins should be rearranged in T cells as a mechanism of generating diversity and consequently increasing the antigen-recognition repertoire. (4) That like immunoglobulin genes they should have constant regions (as they presumably share at least some functions) and variable regions, would confer the antigen-binding specificity.

An experimental strategy could be developed on the basis of these assumptions, as B and T cells differ in only a small fraction of their gene expression (~2%, or 200-300 different sequences²⁸; M.M.D. and W. E. Paul, manuscript in preparation) and only a small proportion of lymphocyte mRNAs appear to be in the membrane-bound polysomal fraction (~3%)²⁹. Thus, by synthesizing ³²P-labelled DNA copies (cDNAs) of the membrane-bound polysomal RNA of antigen-specific T cells and removing by RNA hybridization those sequences also expressed in B cells, one should be left with a specific probe representing a very small fraction of total T-cell gene expression and likely to include copies of the mRNAs that encode the T-cell receptor. This probe can then be used to screen a library of cloned cDNAs³⁰, and the clones thus identified could be used to look for somatic gene rearrangements in T cells. Further restriction mapping and sequence analysis of the cDNA clones derived from different T cells might reveal variable and constant regions. We have carried through this strategy, and have managed to obtain a cDNA clone (TM86) representing mRNA that is expressed specifically in T cells, found in the membrane-bound polysomal fraction and encoded by genomic DNA that has rearranged in T cells.

Analysis of T-cell mRNA

The number of different species in a given mRNA population can be estimated from an analysis of the kinetics of cDNA-mRNA hybridization reactions³¹. In this case, labelled cDNA was synthesized from cytoplasmic poly(A)⁺ RNA of the T helper hybridoma M12 (ref. 26), the RNA template was removed and the cDNA hybridized to the original RNA. The resulting C₀t

[‡] Present addresses: Department of Biology, University of California at San Diego, La Jolla, California 92093, USA (S.M.H.); Stanford University School of Medicine (M.M.D.).

Table 1 Analysis of C_0t curves

Labelled cDNAs	Component	Fraction	$C_0t_{1/2}$	$C_0t_{1/2}$ Pure	No. of species	mRNAs per cell
Cytoplasmic	1	48.6%	0.52	0.18	130	1,050
	2	51.4%	47.4	17.7	12,900	12
Free	1	56.8%	0.12	0.048	—	—
	2	43.2%	25.6	7.77	—	—
Membrane-bound	1	67.6%	0.12	0.058	—	—
	2	32.4%	15.7	3.8	—	—
MB $T_H^* - B$	1	41.7%	1.25	0.25	1	—
	2	58.3%	92.8	26.0	70	—
MB T_H^* subtraction						
Reaction	Double-stranded	Single-stranded				
1	91.3%	8.7%	$(T_{M12}^* - B_{L10A})$			
2	32.5%	67.7%	$(T_{M12}^* - B_{L10A})$			
3	44.4%	55.6%	$(T_{M12}^* + T_{M12})$			

Least-squares fit analysis³² of the reactions shown in Fig. 1. The curves were fitted to two components with root mean square (r.m.s.) errors of 0.03–0.05. The fractions were normalized to 100% and both uncorrected and 'pure' $C_0t_{1/2}$ values are shown. The number of species was determined using the corrected $C_0t_{1/2}$ values and an average mRNA size of 2,000 nucleotides²⁹. Purified ovalbumin mRNA (given by G. Stanley McKnight) was used as a kinetic standard. The calculation of number of mRNAs per cell is based on the average of 300,000 cytoplasmic poly(A)⁺ RNAs we observe in most T hybridomas in log phase growth. The MB $T_H^* - B$ analysis draws on the data in Fig. 1b. As we reproducibly find that the remaining B cell-reactive material consists largely of small, very slowly reacting cDNA which introduces a physiologically irrelevant slow component into the C_0t analysis (M. M. D. and W. E. Paul, manuscript in preparation), we subtracted the background curve directly from the homologous curve and computer fitted the difference. In this case, the L10A reaction with the MB $T_H^* - B$ is subtracted from the M12 reaction and fitted as shown in this table. The second part of the table summarizes the cDNA subtraction, with two successive subtractive hybridizations with B-cell (L10A) RNA followed by a back reaction with homologous (M12) RNA to remove unreactable sequences. The repetitive yield is 50–65% for each round of reaction and it is not selective for single-stranded or double-stranded nucleic acid (M. M. D. and W. E. Paul, manuscript in preparation). The single-stranded fraction is carried through to the next step from both reactions 1 and 2, and the double-stranded fraction of reaction 3 is the material analysed in Fig. 1b and which represents 2.6% of input membrane-bound polysomal cDNA, normalizing for yield losses. As the analytical reactions (Fig. 1b) indicate that 61% of this material represents T cell-specific sequences, 1.6% (2.6×0.61) of the initial cDNA is unique to T cells. As the membrane-bound RNA represents only 15% of the total, this value is further reduced by this fraction ($1.6\% \times 0.15$) to yield 0.24% of the mass of the cytoplasmic mRNA. As perhaps an equal amount of these same sequences are found in the free polysomal fraction (see text), the value of 0.24% must be revised upwards to 0.5%. This number is then divided into abundant and rare fractions as indicated in the table and the number of sequences of average size derived (~ 1 for component 1 and 70 for component 2).

(or R_0t) curve was computer-fitted by the least-squares program of Pearson, Davidson and Britten³² and is shown in Fig. 1a. The analysis of this curve, presented in Table 1, indicates that there are about 13,000 different mRNA species in this cell line, most of which are in the rare abundance class (component 2 in Table 1) at about 12 molecules per cell. These findings are typical of a variety of *in vitro* cultured cell lines³³ although somewhat higher than the 8,000 different species seen in a murine plasma cell line²⁹. Also shown in Fig. 1a are the reactions of cytoplasmic poly(A)⁺ RNA with labelled cDNAs prepared from membrane-bound and free polysomal fractions of RNA, using the procedure of Mechler and Rabbits²⁹. Although a striking feature of that report was the detection of virtually no rare mRNA species in the membrane-bound fraction of a plasmacytoma, the analysis of the data presented here (Table 1)

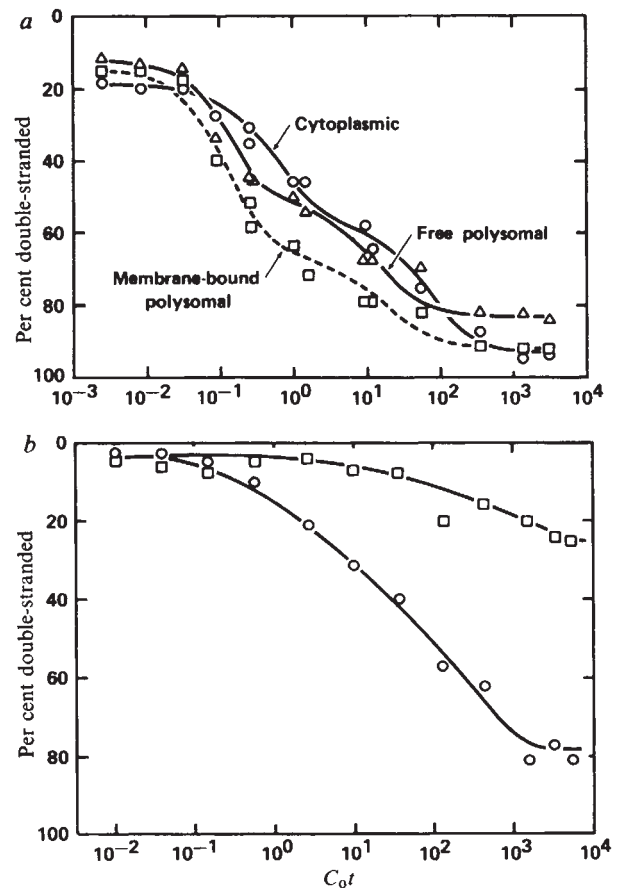


Fig. 1 Kinetic hybridization analysis of T-helper hybridoma mRNAs. *a*, Hybridization to an excess of cytoplasmic poly(A)⁺ RNA of: ○, total cytoplasmic cDNA; △, free polysomal cDNA; and □, membrane-bound cDNA. *b*, Hybridization of the MB $T_H^* - B_{L10A}$ probe to M12 cytoplasmic poly(A)⁺ RNA (○) and hybridization of the same probe to L10A poly(A)⁺ cytoplasmic RNA (□).

Methods: Cytoplasmic poly(A)⁺ RNA was prepared as described⁴⁶ from an *in vitro* cultured hybridoma line (M12). Membrane-bound and free polysomal poly(A)⁺ RNAs were prepared from these cells by the procedure of Melcher and Rabbits²⁹. ³²P-labelled cDNA was synthesized from these RNAs using oligo(dT) and reverse transcriptase³⁰. cDNAs were depleted of template RNA by base hydrolysis and reacted in 0.5 M phosphate buffer⁴⁷, 0.1% SDS, 5 mM EDTA in sealed glass capillaries with an excess of cytoplasmic poly(A)⁺ RNA. Reactions were assayed by hydroxyapatite chromatography and analysed as described in Table 1. C_0t values as given are normalized for the effect of the high salt⁴⁷.

clearly indicates the existence of a rare abundance class component of the equivalent fraction in M12 cells.

When cDNA from membrane-bound polysomal RNA is repeatedly hybridized to B-cell mRNA and fractionated on hydroxyapatite (Table 1), the non-hybridizing DNA consists of 2.6% of the input cDNA, and the reactions shown in Fig. 1b (and analysed in Table 1) indicate that 61% of this fraction is T cell-specific and divided as shown between rare and moderately abundant classes (components 2 and 1 respectively, the $C_0t_{1/2}$ 'pures' of which are almost indistinguishable from those of cytoplasmic cDNA). Although only 15% of the polyribosomal RNA was membrane-bound in these cells, the T cell-specific genes were found to be as prevalent in the free polysomal as in the membrane-bound fractions of RNA. We therefore estimate that the pool of membrane-bound RNA derives from $\sim 30\%$

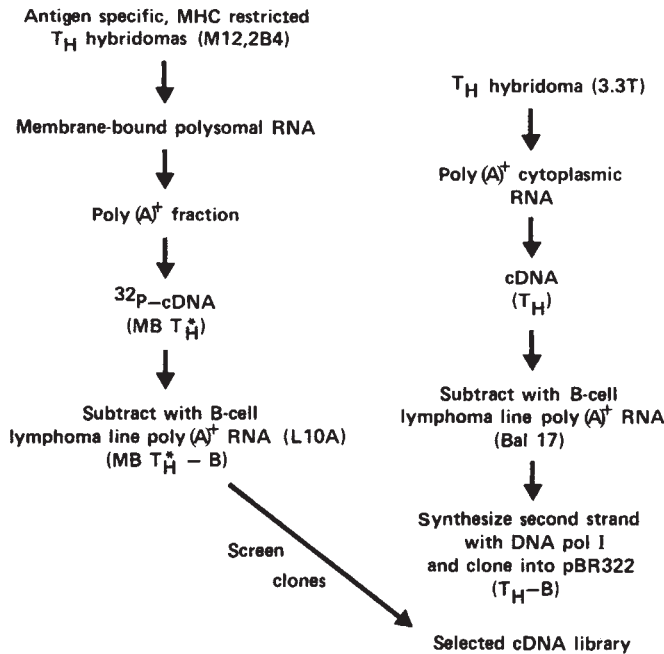


Fig. 2 Strategy of clone isolation. ³²P-labelled cDNA was synthesized from membrane-bound polysomal RNA of T_H hybridomas and subtracted with B-cell mRNA (L10A). These probes were then used to screen a selected cDNA library (T_H-B) constructed as described³⁰ from another T_H hybridoma/B cell combination.

of the total polysomal RNA. Thus, the subtracted cDNA (MBT_{M12}*-B_{L10A}) represents ~0.5% of the input cDNA and ~70 different species (see Table 1 legend). This latter number could actually be an overestimate for the following reasons: (1) Rare mRNAs have, in several cases^{34,35}, been shown to be larger than the average mRNA. (2) A certain fraction (0.2-0.4%) of the cDNA differences between any two mRNA populations are not reproducibly expressed in other cells of the same type, indicating a certain background level of random gene expression or loss of expression (M.M.D. and S.M.H., unpublished observation). (3) The use of total cytoplasmic RNA rather than polysomal may introduce a significant number of non-translated sequences into the estimates.

cDNA clone isolation

The strategy used to isolate cDNA clones representing membrane-bound mRNAs expressed in T but not in B cells is shown in Fig. 2. Membrane-bound T helper (T_H) cell cDNA probes were subtracted with B-cell mRNA and used to screen a cDNA library that was itself the product of a T_H-B cell reaction, and which was constructed as described previously^{30,36} for a B cell-specific library³⁰. The T_H-B library was 20-fold enriched for T cell-specific sequences as judged by the fact that 95% of the mass of the cDNA was removed in the subtraction (at the hydroxyapatite stage). As indicated in Fig. 2 the library of 5,000 selected clones was screened and rescreened by standard procedures^{37,38} using the MBT_{2B4}*-B_{L10A} probe. Thirty-five clones were screened and rescreened positive (TM clones). In order to determine which clones were derived from the same mRNA species and which were different, as well as to remove any false

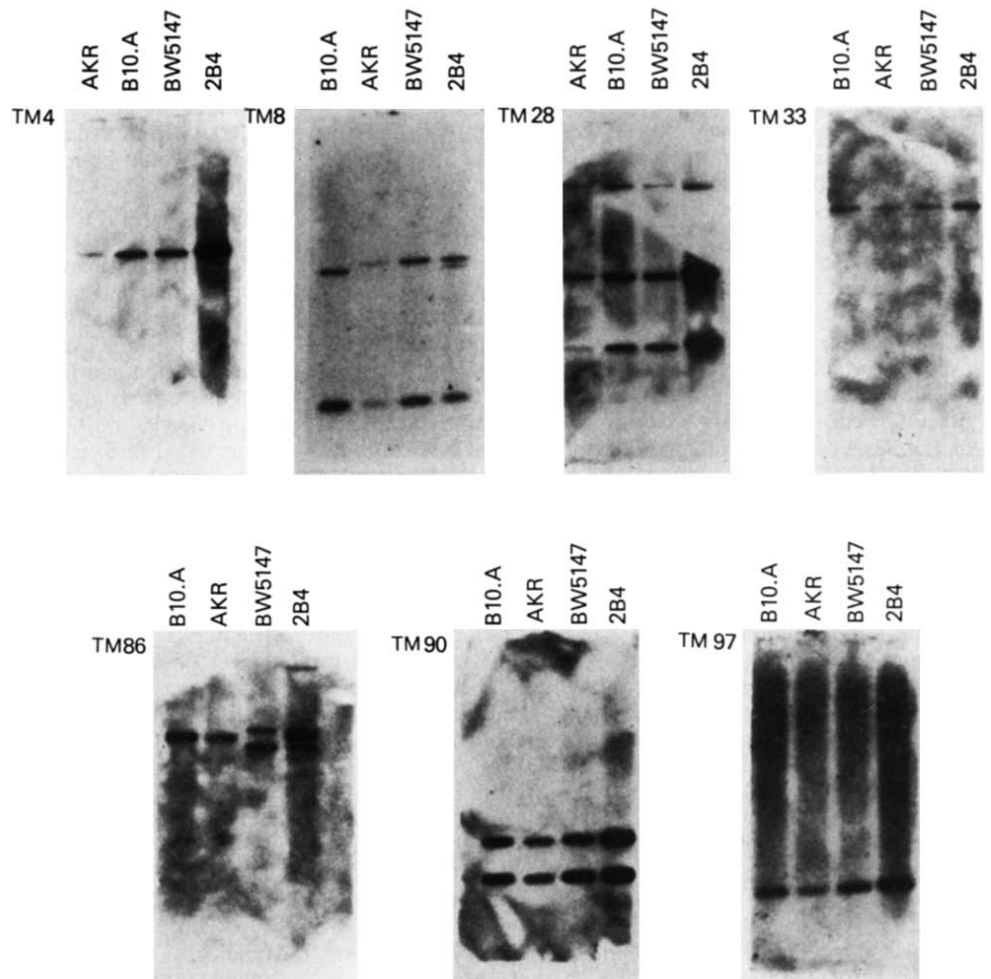


Fig. 3 Southern blot analysis of TM clones. ³²P-labelled inserts from the seven clones indicated were hybridized to Southern blots containing DNA from liver cells of strains B10.A and AKR, and DNA from BW5147 and the T_H hybridoma 2B4. The DNAs were prepared by standard methods⁴⁸, digested with the restriction enzyme PvuII, electrophoresed through 0.9% agarose and blotted onto nitrocellulose. Hybridization was in 50% formamide, 5 × SSPE⁴⁸ at 42 °C. Washing was done in 2 × SSPE at 55 °C.

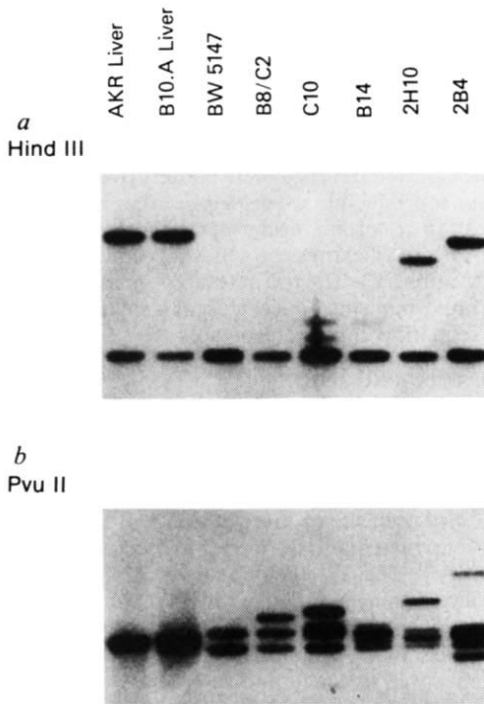


Fig. 4 Analysis of other T_H hybrids for rearrangement of TM86. In this case, four additional antigen-specific hybridoma lines were examined for rearrangement using the TM86 probe and the procedures outlined in Fig. 3 legend. B8/C2 and B14 are T_H hybrids specific for insulin and C10 is a T_H hybrid for lysozyme⁴⁹.

positives, we ³²P-labelled by nick-translation each positive clone to make labelled probes which were then hybridized to representative Northern blots. Five of the positive clones were reactive with B-cell mRNA, and the remaining 30 fell into one of 10 distinct patterns of mRNA size and expression. One of these 10 different clones cross-hybridized strongly with and gave a Northern blot pattern superimposable on a rat Thy-1 cDNA clone isolated by Silver and colleagues³⁹. As Thy-1 is a classical T-cell membrane antigen not expressed on B cells, the fact that it is one of the genes isolated in this small set is an important internal control.

Our procedure for obtaining cDNAs of membrane-bound, T cell-specific mRNAs should have enriched for particular sequences to such an extent that even very low abundance mRNAs are represented at levels comparable to those of abundant mRNAs in the original population. This should overcome the difficulties earlier workers have found in detecting low abundance mRNAs^{40,41}. In fact, hybridization of the inserts of the less abundant TM clones to the 3.3T-selected cDNA library in at least two cases indicates that we have been able to isolate DNA copies of mRNAs represented by only 1 in 100,000 clones in the unselected library.

Somatic gene rearrangements

To determine whether any of the seven TM clones which, on Northern blotting, were shown to represent mRNAs large enough to encode T-cell receptor proteins¹⁹⁻²², had been copied from mRNAs encoded by genes that, like immunoglobulin genes, rearrange somatically, we prepared labelled probes from them which we hybridized to Southern blots of genomic DNA from the thymoma BW5147 (from the mouse strain AKR), AKR liver cells, the antigen-specific T-cell line 2B4 (a fusion of T cells from B10.A mice with BW5147) and B10.A liver cells. The autoradiograms from Southern blots are shown in Fig. 3. Except for the restriction polymorphism between AKR and B10.A seen with TM8 (Thy-1), the patterns of hybridization with each clone were identical for all the sources of DNA except in the case of TM86 (Fig. 3). Clearly, there was a strikingly

different pattern of *Pvu*II fragments that hybridized to the clone from either BW5147 or 2B4 compared with liver DNA from either of the parental strains. The clones surveyed here were also hybridized to *Eco*RI and *Hind*III digests of genomic DNA, and in each case only TM86 showed a significant difference between the T-cell DNAs and liver DNAs.

It would be expected that genomic rearrangements of a receptor gene should be different for T cells of different antigen specificities. To test this possibility, genomic blots consisting of DNA from five antigen-specific T-cell hybridomas were hybridized with a nick-translated insert from clone TM86. The results, shown in Fig. 4, demonstrate that DNA from each of the antigen-specific T cells gives a different pattern. Three different B-cell lymphoma DNAs give patterns identical to that of the liver (data not shown), indicating that this type of rearrangement may only take place in T cells. It is also significant that no restriction fragment length polymorphisms can be detected with TM86 between the two strains for which data are shown here or in the three other strains that we have examined, either with the *Pvu*II enzyme or several others. This diminishes the possibility that the rearrangements could be due to the presence of a viral sequence since one would expect a highly unstable integrated viral sequence to be changing at least as dramatically in evolutionary time.

Discussion

As previously demonstrated^{43,44} the techniques used in this report provide a general means of isolating sets of genes that are particularly important to the differentiated state of a cell. This set should include at least a portion of the genes required for the specialized functions of the cell, as well as genes involved in regulation and cellular differentiation. As the set of DNA clones obtained is very small, elaborate screening procedures can be used to further define them. In cases where the target population is very small (~0.5% for the set of genes examined here) the enrichment of the relevant probe (~×200) is sufficient to allow the isolation of very rare genes. For the studies reported here, we were able to screen a small set of cDNA clones for possible somatic rearrangement of the genes encoding the mRNAs from which they were derived. Such a screening procedure would be much too laborious if used to screen a large number of cDNA clones. An extensive characterization of TM86 and related cDNA clones is presented in the accompanying report⁴².

The striking characteristic of TM86 is that the genes undergo somatic rearrangement in T cells but not B cells. The pattern of *Pvu*II fragments from liver DNA which hybridize to TM86 consists of three closely spaced bands of 5.2–6.5 kilobases (kb). The T lymphoma BW5147 has retained none of the germ-line fragments, and instead shows a pattern of two bands different from any in the liver DNA. We conclude that BW5147 has rearranged one or both of its gene copies; if it has rearranged only one, then it must have lost the other. Consistent with the notion that TM86 represents one chain of the T-cell receptor, each of the different antigen-specific T-cell hybridomas has one or more new homologous restriction fragments. Interestingly, each of the antigen-specific T-cell hybridomas has the same two fragments as BW5147, but has lost various of the germ-line fragments. Considering that immunoglobulin genes are the target of oncogene translocations in B lymphomas and plasmacytomas⁴⁵, it is attractive to speculate that the maintenance of the rearranged chromosomes of BW5147 reflects the translocation of an oncogene to TM86.

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Sequence relationships between putative T-cell receptor polypeptides and immunoglobulins

Stephen M. Hedrick^{*‡}, Ellen A. Nielsen^{*}, Joshua Kaveler[†],
David I. Cohen^{*†} & Mark M. Davis^{*†‡}

^{*} Laboratory of Immunology, NIAID, National Institutes of Health, Bethesda, Maryland 20205, USA

[†] Department of Medical Microbiology, Stanford University School of Medicine, Stanford, California 94305, USA

Comparison of the sequence of a cloned T cell-specific cDNA with those of cross-reacting cloned cDNAs isolated from a thymocyte library indicates the presence of variable, constant and joining regions remarkably similar in size and sequence to those encoding immunoglobulin proteins. Together with the evidence for somatic gene rearrangements reported in the accompanying paper, this strongly suggests that the TM86 cDNA clone encodes one chain of the T-cell receptor for antigen.

IN THE preceding paper¹ we reported the isolation of a cDNA clone, TM86, which represents a species of mRNA that is expressed in T cells but not in B cells, encodes a membrane-associated protein, and is rearranged in T cells. We report here our further analysis by nucleotide sequencing of clone TM86, and also of several cross-reacting clones isolated from a thymocyte cDNA library. Our data reveal a remarkable resemblance between the amino acid sequences of the proteins encoded by the mRNAs represented by the TM86 and related cDNA clones and the immunoglobulin proteins of B cells.

Immunoglobulin proteins have a characteristic primary and secondary structure made up of a series of intrachain disulphide-bonded domains (as reviewed in ref. 2). Both heavy and light immunoglobulin chains begin with a leader peptide of 17-29 residues, followed by a variable region of 94-97 residues then a joining region of 13-17 residues (in heavy chains the variable and joining regions are separated by short 'diversity' elements of 1-14 residues). These elements are encoded separately in the germ-line genome by gene segments that are brought together during B-cell ontogeny³⁻⁶ to generate an enormous range of different antibody genes. The domains of the constant regions

of immunoglobulins (of variable number depending on polypeptide chain type and class) are encoded by separate exons⁷, but they do not appear to rearrange during development. The sequences of our T cell-specific cDNA clones has revealed a similar arrangement of constant and variable regions, strongly suggesting that the mRNAs that they represent encode polypeptide components of the T-cell antigen receptor.

Characterization of cDNA clones

In view of the evidence presented in the preceding paper that the region of the genome represented by cDNA clone TM86 is rearranged in T cells, we sought to isolate cDNAs representative of independent rearrangement events by screening a thymocyte cDNA library (provided by C. Benoist) with TM86 using standard conditions⁸. Positive clones were subcloned into the *EcoRI* site of plasmid pUC9 (ref. 9). Partial restriction maps and the sequencing strategies¹⁰ are shown in Fig. 1 for TM86 and the three thymus-derived clones 86T1, 86T3 and 86T5. The 3' termini of the 86T series of clones are all identical, as each clone has an internal *EcoRI* site in that region, and the variation in position of the 5' termini is presumably due to random chain termination during library construction.

As the largest mRNA detected on Northern blots with TM86 has a size of ~1,300 bases¹, by subtracting a poly(A) tail of

[‡] Present address: Department of Biology, University of California at San Diego, La Jolla, California 92093, USA.