TABLE 2 Selective cell death in germinal-centre B cells

Tonsil B-cell fraction	% cells recovered from cultures after: 16 h	40 h	72 h	96 h
High-density resting B cells	96–99	60–81	54–58	30–39
Low- and medium-density CD39 and sIgD −ve	22–26	0–0	0–0	0–0
Low- and medium-density CD39 and/or sIgD +ve	64–73	48–56	41–50	20–30
Low- and medium-density CD38 +ve	38–42	19–21	10–12	2–8
Low- and medium-density CD38 −ve	87–95	68–90	68–90	50–85

The first three tonsil B-cell fractions were prepared as described in Table 1, the CD38 positive (+ve) and CD38 negative (−ve) fractions were prepared in a similar way using CD38-coated sheep red cells. The CD38 +ve fraction contains all germinal-centre B-lineage cells but does include some cells from outside germinal centres (Table 1). Cell fractions were cultured for the periods indicated in flat-bottomed microtitre wells at 37 °C in a moist atmosphere of 5% CO_2 in air in 200 μl of RPMI 1640 medium enriched with 10% fetal bovine serum. The starting cell concentration was 2×10^5 cells per ml. The number of viable cells recovered in two separate experiments from wells after the periods of culture indicated is shown. Cell viability was assessed by trypan-blue exclusion.

cytolysis[12,13]. The macrophage content of the germinal-centre cell fraction (Table 1) is similar to that in the fraction positive for CD39 or IgD, or both. It is difficult to understand why there should be selective macrophage-mediated lysis of the germinal-centre cells and why this should be inhibited by CD40. If the germinal-centre cells are not being killed, they must be killing themselves. The high physiological cell death rate among these cells *in vivo*[14,15], together with the finding that other tonsil B cell populations do not destroy themselves by apoptosis on culture (Table 2), seems to argue against this being an artefact of the culture system.

Affinity maturation of antibodies in responses to T-cell-dependent antigens is mainly attributable to alteration of the structure of the immunoglobulin variable region by somatic mutation[4]. The time of onset of this somatic mutation[16] correlates with the start of the germinal-centre reaction[15,17]. Other features consistent with a link between these processes have been described previously[5]. Mutations can increase, decrease or fail to modify the affinity of antibodies. The experiments reported here indicate how centrocytes with low affinity for antigen can be eliminated. The germinal-centre cells with high affinity are most likely to be those which survive to give rise to memory B cells[18,19].

TABLE 3 Ability of antibodies to prevent cell death

a, Soluble CD40 antibody and anti-human immunoglobulin

Culture additive	% Viable cells (mean ± s.e.m. of 5 expts) recovered after: 16 h	40 h	72 h	96 h
None	20 ± 6	0	0	0
Anti-Ig-coated sheep red cells	76 ± 6	23 ± 4	6 ± 8	0
Soluble CD40*	75 ± 12	39 ± 16	17 ± 4	0
Anti-Ig-coated sheep red cells plus soluble CD40*	86 ± 6	70 ± 12	40 ± 18	40 ± 24

b, Antibodies against different human immunoglobulin classes

Antibody added to coat sheep red blood cells	% Viable cells (mean ± s.e.m. of 5 expts) recovered after 16-h culture
None	20 ± 10
Polyvalent anti-Ig	70 ± 8
anti-IgG	54 ± 8
anti-IgM	24 ± 10
anti-IgD	26 ± 8
anti-IgA	23 ± 3

a, The prevention of cell death in cultures of germinal-centre cells by the addition of soluble CD40 antibody and sheep red cells coated with anti-human immunoglobulin. *b*, The relative ability of antibodies against different human immunoglobulin classes to prevent cell death in isolated germinal-centre cells. The germinal-centre cells used in these experiments were isolated as described in Table 1. They were cultured and their viability assessed as described in Table 2. The anti-immunoglobulins (Ig) used and the method used for their conjugation to sheep red cells is specified in Table 1.

* CD40 antibody G28-5 was added to cultures at a final concentration of 5 μg ml^{-1} (ref. 7).

Defects in the selection system identified in this study could be involved in the pathogenesis of low-grade follicular lymphomas. These lymphomas have a (14; 18) translocation involving apposition of the proto-oncogene *bcl-2* and the immunoglobulin heavy-chain locus[20,21]. Vaux *et al.*[22] showed that expression of *bcl-2* transfected into IL-3-dependent cell lines prevented cell death on withdrawal of IL-3. The introduction of constructs representing the *bcl-2*–immunoglobulin gene fusion into the germ line of mice leads to the accumulation of mature B cells in the adult mice[23]. It is an intriguing possibility that the *bcl-2* gene product may be involved in overriding the apoptotic tendency of germinal-centre cells. □

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Blockage of αβ T-cell development by TCR γδ transgenes

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T LYMPHOCYTES recognize antigens by means of T-cell receptors (TCR) composed of αβ (refs 1–3) or γδ heterodimers[4–6]. The mechanism governing the development of αβ- and γδ-bearing T cells from a common precursor T cell is so far unknown. It has been proposed that T-cell precursors rearrange their γ- and δ-chain genes first, and αβ T cells are generated only from those cells that fail to rearrange productively both γ- and δ-chain genes[7,8]. Our recent study on γδ-transgenic mice contradicted this

hypothesis, however, and indicated that repression of γ-chain gene expression mediated by a transcriptional silencer element has a critical role in the generation of αβ T cells[9]. Here we report that the generation of αβ T cells is severely blocked in transgenic mice carrying γ- and δ-chain transgenes without the associated silencer, thereby strengthening the validity of the silencer model of T-cell development.

Our recent analysis of γδ-transgenic mice (called KN6 transgenic mice) constructed with relatively long (~40 kilobases (kb) each) $V_4J_1C_1\gamma$ and $V_5DJ_1C\delta$ genomic DNA clones indicated that, in contrast to the Pardoll-Allison model[7,8], introduction of productively rearranged γ- and δ-chain genes into the germline does not prevent the rearrangement of the endogenous α- and β-chain genes and their cell-surface expression[9]. In these transgenic mice, transcription of the γ-chain transgene is repressed in αβ T cells apparently through a *cis*-acting DNA element (silencer) present in the flanking region(s) of the γ-chain gene. This led us to propose a model of T-cell development in which commitment of a given T-cell precursor to the αβ-lineage depends on the activation of the machinery acting on the silencer[9]. We have now constructed transgenic mice with γ- and δ-chain genes carrying no silencer element so as to perform the crucial test of the silencer model. The model predicts that the transgenes will be transcribed in all T cells in these mice, as opposed to the KN6 transgenic mice, and that their subsequent surface expression will prevent rearrangement and expression of TCR αβ-chain genes, in analogy to the immunoglobulin system[10].

As transgenes we used 15-kb and 21-kb genomic DNA fragments (Fig. 1) containing productively rearranged $V_5J_1C_1\gamma$ and $V_1D_2J_2C\delta$ genes, respectively, known to encode the γδ TCR expressed on the surface of most early fetal thymocytes[11,12] and dendritic epidermal γδ T cells (DEC)[11,13]. According to our previous study it is probable that the 15-kb γ-chain gene clone lacks the putative silencer element, because the transcription of a γ-chain transgene containing similar flanking regions is not repressed in αβ T cells[9]. We analysed the progeny of two transgenic founders (henceforth called DEC transgenic mice).

Cell-surface expression of transgene-encoded TCR was investigated by flow cytometry, using monoclonal antibodies reactive with all γδ TCR (3A10)[14] or $V_{\gamma5}$-encoded γ-chains only (F536)[11]. Consistent with previous reports, few if any γδ T cells from nontransgenic adult mice expressed $V_{\gamma5}$-encoded γδ TCR on their surface (Fig. 2*a*)[11,12]. By contrast, more than 95% of γδ thymocytes or splenic T cells from the DEC transgenic mice were reactive with the F536 monoclonal antibody (Fig. 2*a*, and data not shown), indicating that, like the KN6 γδ transgenic mice (Fig. 2*a* and ref. 9), the transgenes dominated the γδ T cells for the expression of their TCR. The proportion of γδ T cells in the thymus from DEC transgenic mice (25–75%) was dramatically increased in comparison with nontransgenic mice (0.5%) or KN6 transgenic mice (4.5%) (Fig. 2*a*). In addition, unlike KN6 transgenic mice, the thymus size was severely reduced in DEC transgenic mice (data not shown), suggesting that the generation of the main thymocyte subpopulation, that is, $\alpha\beta TCR^+$ cells, was strongly hampered in these animals.

This supposition was confirmed by immunofluorescence analysis using the anti-αβTCR monoclonal antibody H57-597[15]; less than 3% of thymocytes from 7-day-old DEC transgenic mice were $\alpha\beta TCR^+$, whereas most (>60%) thymocytes from the age-matched control mice or KN6 transgenic mice carried αβ TCR (Fig. 2*b*). The proportion of $\alpha\beta TCR^+$ thymocytes increased with age to reach 50% in 10-week-old mice (Fig. 2*b*). But because the total number of thymocytes was greatly reduced in the DEC transgenic mice, the absolute number of αβ chain-bearing thymocytes never reached more than 5% of the level observed in nontransgenic mice (data not shown).

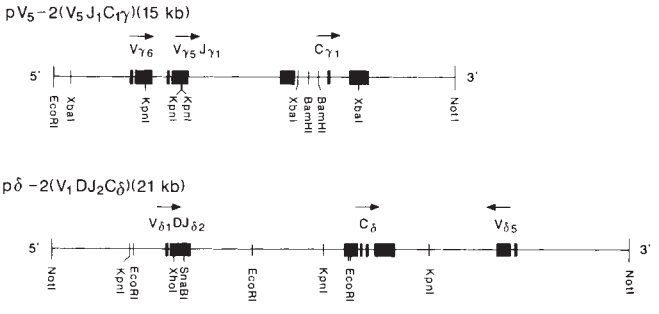

FIG. 1 Restriction maps of γ- and δ-chain genomic DNA clones used for the construction of transgenic mice. Filled boxes designate exons or gene segments; arrows above indicate transcriptional orientation.

METHODS. High-relative molecular mass DNA was isolated from a γδ T-cell hybridoma KI-129[12] as previously described[22] and was used to construct a cosmid library using the plasmid pWe15 vector[23]. The library was screened with ^{32}P-labelled $V_{\gamma5}$, C_γ, $V_{\delta1}$ or C_δ probes for the cloning of the γ- and δ-chain genes. The C_γ and C_δ probes have been previously described[24,25]. Screening led to the isolation of clones pV5-2 and pδ-2, containing the entire γ- and δ-chain genes expressed by the KI-129 hybridoma. The junctional sequences of clones pV5-2 and pδ-2 were identical to those published[13,26]. The $V_{\gamma5}$ gene was assigned according to ref. 27. The γδ-transgenic mice were generated after injection into fertilized mouse eggs of purified pV5-2 and pδ-2 DNA as previously described[5,28].

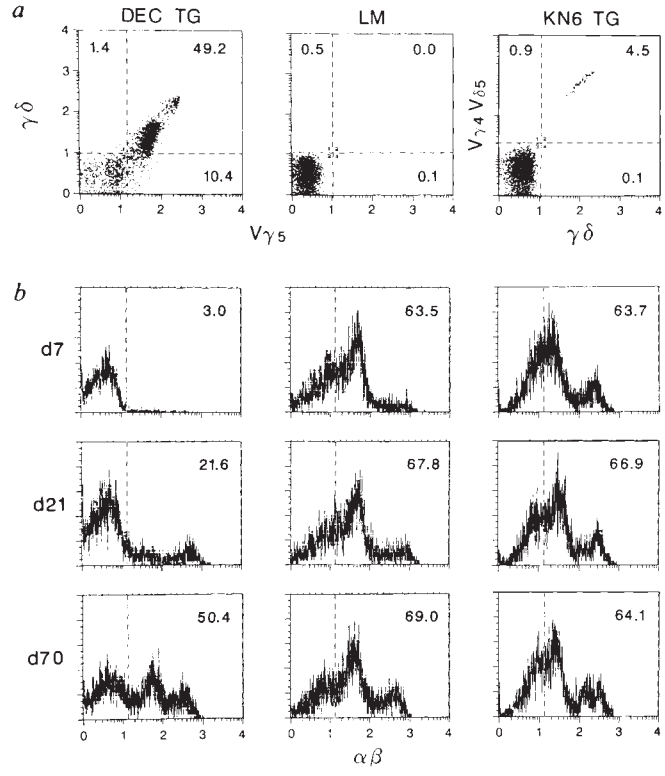

FIG. 2 Surface expression of γδ and αβ TCR by thymocytes from DEC transgenic, KN6 transgenic and nontransgenic mice. *a*, Thymocytes from 70-day-old DEC transgenic (left) and nontransgenic (middle) litter-mate mice were stained with anti-$V_{\gamma5}$ monoclonal antibody F536 followed by fluorescein isothiocyanate (FITC)-coupled goat anti-hamster IgG and with biotinylated anti-γδ-chain monoclonal antibody 3A10 followed by phycoerythrin-streptavidin. Shown are the dot-plot histograms (F536, horizontal axis; 3A10, vertical axis) obtained after flow cytometric analysis using a FACSCAN (Becton-Dickinson) and the percentage of cells in each quadrant. Fluorescence intensity is expressed in $\log_{10}$ units. For comparison is shown the dot-plot histogram (3A10, horizontal axis; 8D6 (anti-$V_{\gamma4}V_{\delta5}$)[14], vertical axis) of thymocytes from age-matched KN6 transgenic mice[9] (right). *b*, Thymocytes from 7-, 21- and 70-day-old DEC transgenic (left), nontransgenic (centre) and KN6 transgenic (right) mice were stained with biotinylated anti-αβ-chain monoclonal antibody H57-597 followed by phycoerythrin-streptavidin. The percentages of positive cells are indicated to the right of each histogram. Fluorescence intensity is expressed in $\log_{10}$ units.

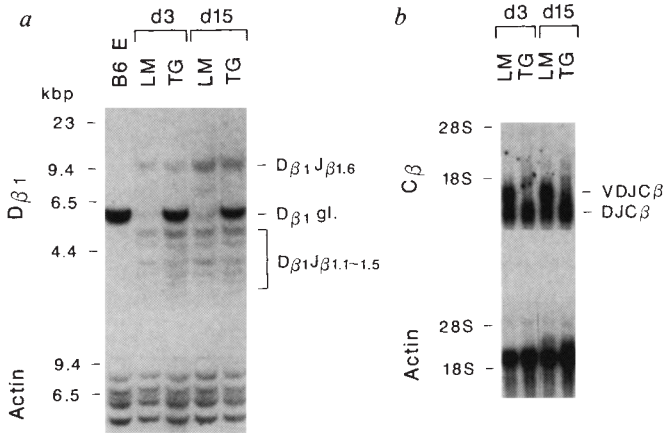

FIG. 3 Southern and northern blot analysis of DNA and RNA, respectively, from total thymocytes of DEC transgenic mice and nontransgenic littermates. *a*, Genomic DNA of thymocytes from 3- and 15-day-old DEC transgenic (TG) and nontransgenic (LM) mice and from C57BL/6 embryo (B/6 e) were digested with *Pvu*II, separated and transferred to nitrocellulose membrane. The Southern blot was hybridized with $D_{\beta 1}$ or actin probes. gl, Germ line DNA. DJ_β 1.1–1.6 bands were assigned according to ref. 29. *b*, RNA of thymocytes from 3- and 15-day-old DEC transgenic (TG) and nontransgenic (LM) mice was electrophoresed, transferred onto nitrocellulose membrane and hybridized to C_β or actin probes. The position of the 18S and 28S ribosomal RNA and that of $VDJC_\beta$ and DJC_β transcripts are indicated.
METHODS. Genomic DNA from thymocytes was purified as previously described[22], digested with *Pvu*II restriction endonuclease, separated (5 μg lane^{-1}) on a 0.6% agarose gel and transferred to GeneScreen Plus membrane (NEN) according to ref. 30. Hybridization with ^{32}P-labelled $D_{\beta 1}$ probe (*Pst*I fragment (1.06kb) from the clone encoding germ-line $D_{\beta 1}$ DNA[31]) and actin (*Pst*I fragment (0.8 kb) from a complementary DNA clone[32]) probes was carried out as previously described[30]. DNA probes were labelled by a random priming method[33]. The autoradiographic bands were detected and intensities quantitated using a FUJI Bioimage Analyzer BA100[34]. Total RNA was extracted using the guanidium thiocyanate–CsCl gradient method[35] and electrophoresed (10 μg lane^{-1}) through formaldehyde containing 1.2% agarose gel[22]. The RNA was transferred to GeneScreen Plus membrane and baked at 80 °C for 2 h. Northern blot hybridization with ^{32}P-labelled probes was performed under the same conditions as that of the Southern blot.

The above results indicate that introduction into the germ line of productively rearranged 'short', in contrast to 'long'[9], γ- and δ-chain transgenes severely hampers the development of αβ T cells. To further investigate the mechanism by which αβ T-cell differentiation is blocked, we analysed the DNA from thymocytes of young (3- and 15-day-old) DEC transgenic mice by the Southern blot method using a $D_{\beta 1}$ probe (Fig. 3*a*). In nontransgenic thymocytes, the germ-line $D_{\beta 1}$ band was barely detectable, reflecting the fact that incomplete *DJ* or complete *VDJ* rearrangements had taken place in most cells. By contrast, most $D_{\beta 1}$ gene segments remained in the germ-line configuration and the rest in the *DJ* state in the young transgenic mice (Fig. 3*a*). Consistent with the results of the Southern blot analysis, only low levels of full-size TCR β-chain messenger RNA (1.6 kb) were detected in transgenic thymocytes in comparison with nontransgenic thymocytes (Fig. 3*b*). Because *VJα* rearrangements were only partly inhibited in DEC transgenic mice (data not shown), these results strongly indicate that the primary mechanism by which the DEC γδ transgenes prevent αβ T-cell development is the blockage of complete *VDJβ* gene rearrangements. If this is indeed the case, why then do αβ T cells develop in older DEC transgenic mice? Northern blot analysis of αβ and γδ T-cell hybridomas from DEC transgenic mice shows that the levels of γ- and δ-chain transgene transcripts are lower in the former than in the latter (data not shown), suggesting that the level of γδ-TCR transcripts must be above a certain threshold to efficiently inhibit rearrangements of endogenous TCR genes. The αβ T cells observed in older DEC transgenic mice, then, are probably due to the accumulation of rare T cells in which the level of the transgene expression was below this threshold.

The drastic reduction in the number and the retardation of the appearance of αβ T cells in the DEC transgenic mice is perfectly consistent with the silencer model of T-cell development. The possibility must be considered, however, that αβ T cells are actually produced in these mice but are deleted in the thymus because of their coexpression of the transgene-encoded γδ TCR. Although nothing is known about the ligand specificity of the transgene ($V_5J_1C_1\gamma$ and $V_1D_2J_2C\delta$)-encoded TCR, it is conceivable that its expression in the αβ-cell lineage leads to negative selection of αβ T cells. To investigate this possibility, we crossed the DEC transgenic mice with αβ-transgenic mice[16]. If the silencer model is correct, then the introduction of functional α- and β-chain genes into DEC γδ-transgenic mice would be expected to restore αβ T cells. As shown in Fig. 4, ~80% of thymocytes from quadruple αβγδ-transgenic mice expressed αβ

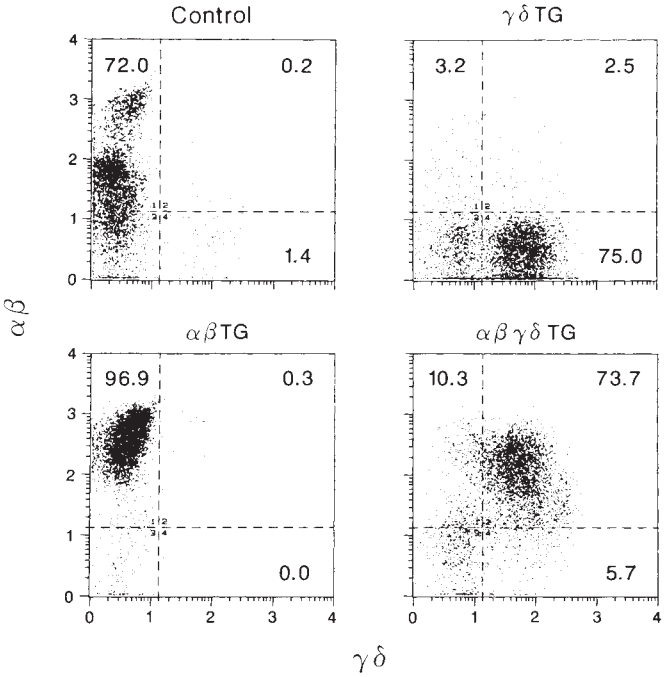

FIG. 4 Surface expression of γδ and αβ TCR by thymocytes from female γδ-, αβ- and αβγδ-transgenic mice. Thymocytes from 3-day-old DEC transgenic (γδ-TG), αβ-transgenic (αβ-TG), αβγδ-transgenic (αβγδ-TG) and nontransgenic (LM) mice were stained with FITC-coupled anti-γδ-chain monoclonal antibody 3A10 and biotinylated anti-αβ-chain monoclonal antibody H57-597 followed by phycoerythrin–streptavidin. The dot-plot histograms (3A10, horizontal axis; H57-597; vertical axis) obtained after cytofluorometric analysis are shown as well as the percentage of cells in each quadrant. Fluorescence intensity is expressed in $\log_{10}$ units.
METHODS. DEC transgenic females (H-2^b) were mated with αβ-transgenic male mice[16] (H-2^b) and their female progeny analysed 3 days after birth. Transgene expression was screened by staining thymocytes with anti-$V_{\beta 8}$ monoclonal antibody F23.1[36] and anti-αβ monoclonal antibody H57-597, or with anti-$V_{\gamma 5}$ monoclonal antibody F536 and anti-γδ monoclonal antibody 3A10. More than 95% of αβ T cells from αβ- and αβγδ-transgenic mice were $V^+_{\beta 8}$ and more than 98% of γδ T cells from γδ- and αβγδ-transgenic mice were $V^+_{\gamma 5}$.

TCR, whereas such cells were barely detectable in the thymus from DEC transgenic littermates. Furthermore, total numbers of $\alpha\beta$ thymocytes in $\alpha\beta$- and $\alpha\beta\gamma\delta$-transgenic mice were identical. Therefore we conclude that the drastic reduction in the number of $\alpha\beta$ T cells in the DEC transgenic mice is not due to intrathymic deletion of immature $\alpha\beta$ T cells.

Taken together, these observations strongly support the hypothesis that T-cell precursors are committed to generating $\alpha\beta$ T cells, independently of the rearrangement status of their γ-chain genes, and that this commitment involves activation of factor(s) that repress γ-chain gene transcription through interaction with a *cis*-regulatory element. Recently, Winoto and Baltimore[17] found that most extrachromosomal circular DNA generated by V_α-J_α joinings retain the δ-chain gene segments in the germ-line configuration and proposed that the control of δ-chain gene rearrangement plays a key part in the separation of $\alpha\beta$ and $\gamma\delta$ T-cell lineages. But, Takeshita *et al.*[18] reported that some 'α-circles' do contain rearranged δ-chain gene segments, suggesting that the control at the level of δ-chain gene rearrangement is insufficient.

It has previously been shown that, in contrast to C_1-associated γ-chain genes, $V_2J_2C_2\gamma$ genes are transcribed in $\alpha\beta$ T-cell clones and hybridomas[4,19–21]. The protein products of these genes have not been detected on the surface of freshly isolated T cells, however. Thus, the silencer model seems to apply to the separation of most, if not all, $\alpha\beta$ and $\gamma\delta$ T-cell lineages. □

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A protein binding to the J_κ recombination sequence of immunoglobulin genes contains a sequence related to the integrase motif

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SITE-SPECIFIC recombination requires conserved DNA sequences specific to each system, and system-specific proteins that recognize specific DNA sequences. The site-specific recombinases seem to fall into at least two families, based on their protein structure and chemistry of strand breakage (reviewed in ref. 1). One of these is the resolvase–invertase family, members of which seem to form a serine–phosphate linkage with DNA. Members of the other family, called the integrase family, contain a conserved tyrosine residue that forms a covalent linkage with the 3′-phosphate of DNA at the site of recombination. Structural comparison of integrases shows that these proteins share a highly conserved 40-residue motif[2]. *V-(D)-J* recombination of the immunoglobulin gene requires conserved recombination signal sequences (RS) of a heptamer CACTGTG and a T-rich nonamer GGTTTTTGT, which are separated by a spacer sequence of either 12 or 23 bases (reviewed in refs 3 and 4). We have recently purified, almost to homogeneity, a protein that specifically binds to the immunoglobulin J_κ RS containing the 23-base-pair spacer sequence[5]. By synthesizing probes on the basis of partial amino-acid sequences of the purified protein, we have now isolated and characterized the complementary DNA of this protein. The amino-acid sequence deduced from the cDNA sequence reveals that the J_κ RS-binding protein has a sequence similar to the 40-residue motif of integrases of phages, bacteria and yeast, indicating that this protein could be involved in *V-(D)-J* recombination as a recombinase.

The J_κ RS-binding protein was purified almost to homogeneity from the murine pre-B-cell line 38B9 (Fig. 1*a*) by the procedure of Hamaguchi *et al.*[5]. The J_κ RS-binding protein with a relative molecular mass (M_r) of 60,000 (60K) was expressed in lymphoid cell lines but not in nonlymphoid cell lines. The protein binds to the J_κ RS with a dissociation constant (K_d) of 1 nM. DNase-I footprinting analysis and the binding kinetic assay with several mutant probes strongly indicate that the protein directly interacts with the heptamer sequence and that a mutation in the heptamer greatly reduces the affinity of the J_κ RS-binding protein for DNA. Our initial attempt to determine the N-terminal amino-acid sequence was unsuccessful, probably because of modification of the N-terminal residue. The purified preparation was then digested with trypsin, and the resulting peptides were fractionated by reverse-phase HPLC (Fig. 1*b*). Twenty-three peptides were re-chromatographed and their amino-acid sequences were determined. Two oligodeoxyribonucleotide probes were synthesized on the basis of partial amino-acid sequences of the tryptic peptide 21 and used to screen a cDNA library derived from 38B9 poly(A)$^+$RNA (Fig. 2*a*, *b*). The probes hybridized with 2 of 1×10^6 colonies of the cDNA library. The two cDNA clones were purified and isolated by repeated screening. The clones designated as RBP-1 and RBP-2 contained 3.3- and 4-kilobase (kb) inserts, respectively. Restriction-site map-