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Cell-type-specific cDNA probes and the murine I region: The localization and orientation of A_α^d

(Ia gene/cell-type-specific probe/selected cDNA cloning)

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ABSTRACT **Labeled cDNA probes highly enriched for B-cell-specific (B*-T) and T-suppressor-cell-specific (T_s^*-B) sequences were used to screen a set of genomic cosmid clones spanning 230 kilobases of the murine immune response (I) region. With the B-cell derived probe, four I-region genes were detectable (A_β, E_β, $E_{\beta 2}$, and E_α), as well as an additional (fifth) region of hybridization. The T-cell probe, prepared from a putative I-J positive suppressor cell hybridoma, was negative in a parallel experiment. A genomic fragment corresponding to the new region of hybridization seen with the B-cell cDNA probe identified a discrete mRNA species in RNA blotting analysis that had a pattern of expression strikingly similar to E_α mRNA in a variety of lymphoid tumor lines. This fragment was also used to isolate cDNA clones from a library highly enriched (20–40 times) for B-cell-specific sequences (selected cDNA cloning). DNA sequence analysis of one of these cDNA clones indicates that this gene encodes the A_α molecule of the d haplotype (A_α^d). These findings establish that the order of the class II genes in the Ia complex is as follows: centromere-A_β-A_α-E_β-$E_{\beta 2}$-E_α.**

The classical experiments of McDevitt and Benacerraf and their co-workers first identified the major histocompatibility complex as an important modulator of the mammalian response to antigen (1). Differences between "high responders" and "low responders" to polypeptide antigens mapped to the MHC region in the mouse and to its equivalent in guinea pigs. The genes responsible for this modulation were named immune response (*Ir*) genes. Subsequent genetic analysis, coupled with sensitive cellular assays, localized the genes controlling this phenomenon in the proliferating or helper T-cell subset to the I region between the *K* and *D* loci of the mouse *H-2* complex (2, 3) and demonstrated that the group of molecules encoded by this region, called the Ia molecules, are the principal determinants of high or low responsiveness (4, 5). In addition, a wide variety of cellular and biochemical studies have demonstrated the importance of the Ia complex in the recognition of antigen by certain T cells (reviewed in ref. 6).

The I region has been divided by recombinational analysis into five subregions: I-A, I-B, I-J, I-E, and I-C (7). There are two well-defined Ia molecules, I-A and I-E, each with noncovalently associated α and β chains (8). The A_α, A_β, and E_β chains are encoded in the I-A subregion and the E_α chain are encoded in the I-E subregion (7–9). In mice, the I-A and I-E molecules are characteristic of B lymphocytes and macrophages but not T lymphocytes. The I-J determinant, characteristic of certain suppressor T cells, maps to the I-J subregion (10, 11). No cell-surface markers have been assigned to the I-B or I-C subregions.

Recently, 230 kilobases (kb) of the murine I region were isolated from a cosmid library, prepared from BALB/c strain DNA, using cross-hybridizing human probes and chromosomal walking procedures (12, 13). Three of the four known genes encoding Ia polypeptides were identified within these clones (A_β, E_β, and E_α). In addition, an apparent gene fragment, designated $E_{\beta 2}$, was also found within this region (12). Using cell-type-specific cDNA probes that represent a small subset (≈2%) of B- and T-cell gene expression (ref. 14; unpublished observation), we screened the cosmid clones for any additional genes that might be expressed in lymphocytes. We found this approach to be a rapid and efficient way of surveying large expanses of the chromosome, and it should be of general utility in "saturating" an expanse of cloned DNA for differentially expressed genes. In particular, we were able to localize and establish the orientation of the A_α^d gene.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. Bal 17, K46, L10A, X16C, and Bal·4 were obtained from J. Kim and R. Asofsky (15, 16) and are of the H-2^d haplotype. WEHI-231 was obtained from L. Lanier and N. Warner. The Abelson pre-B-cell lines 38B7 and 2043-1 (H-2^d) were obtained from N. Rosenberg. The suppressor T-cell hybridoma 34S-704 was obtained from M. Tanguichi (17). All lines were grown in RPMI 1640 medium containing glutamine/10% fetal bovine serum/2-mercaptoethanol (50 μM) in 5% CO_2/95% air.

Cytoplasmic RNA Preparation. Cells were grown to a high density (1–2 × 10^6 per ml), refreshed with new medium for 2–4 hr, chilled with phosphate-buffered saline, and cytoplasmic RNA was prepared as described (18). Poly(A)$^+$ RNA was made by selecting once over oligo(dT) cellulose.

Cell-Type-Specific Probes and Blot Hybridization. ^{32}P-labeled cDNA was synthesized as described (19) from 1–5 μg of template poly(A)$^+$ RNA in 50 mM Tris·HCl, pH 8.3/6 mM $MgCl_2$/60 mM NaCl/1 mM dATP/1 mM dGTP/1 mM dTTP/500 μCi of [α-^{32}P]dCTP (800 Ci/mmol; 1 Ci = 37 GBq) (6 μM final concentration)/30 μM dCTP/oligo(dT) (10 μg/ml)/20 mM dithiothreitol/actinomycin D (100 μg/ml) in a 100-μl reaction mixture. Ten units of avian myoblastosis virus reverse transcriptase (from J. Beard) was added per μg of poly(A)$^+$ RNA and incubated for 2 hr at 42°C. At the end of this time, an equal volume of 0.2 M NaOH was added, and the reaction was incubated at 70°C for 20 min to hydrolyze the RNA. The mixture was cooled on ice, neutralized with 1 M HCl, and NaOAc (pH 6.5) was added to 0.2 M and Na-

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Abbreviations: T_s, suppressor T cell; kb, kilobase(s).

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$DodSO_4$ was added to 0.1%. After warming to room temperature, the labeled cDNA was excluded from G-50F Sephadex in a Pasteur pipette column with a running buffer of 100 mM NaCl/50 mM Tris·HCl, pH 7.5/1 mM EDTA/0.02% Na$DodSO_4$. Fifty micrograms of tRNA was added as carrier and the cDNA was precipitated in a silanized Eppendorf tube (1.5 ml). The precipitate was washed once with 70% ethanol, dried, and resuspended in water. The labeled cDNAs (1.8×10^8 cpm/μg) were allowed to decay for 2 weeks to fragment the material.

Hybridization reactions were carried out in sealed glass capillaries (20 μl, silanized) in 0.5 M phosphate buffer/5 mM EDTA/0.1% Na$DodSO_4$ with the relevant mRNA at 1–1.5 mg/ml. Sheared mouse genomic DNA was also included (1.2 mg/ml; 20 μg per reaction mixture) to remove repeated sequences. After boiling for 60 sec to denature the genomic DNA and disaggregate the RNA, the reactions were incubated for 16–20 hr at 68°C. This achieved a C_0t value of 1500 for the RNA and 10 for the DNA. Hydroxyapatite chromatography was then used to fractionate the material in 0.12 M phosphate buffer/0.1% Na$DodSO_4$ at 60°C; 91.5% of the B-cell cDNA and 90.5% of the suppressor T-cell (T_s) cDNA bound to the column (double-stranded fraction). The single-stranded fraction was concentrated with *sec*-butanol, extracted with $CHCl_3$ to remove the butanol, and then aerated to remove the $CHCl_3$. It was then hybridized to Southern blots as described (20). Washing was with 5× SSPE for 1 hr at 65°C and then 1× SSPE for 2 hr at 65°C.

Selected cDNA Cloning. The first cDNA strand was synthesized and hybridized as described above for the probe except that 70 mM KCl was used instead of NaCl and 1 mM dCTP was used with only trace amounts of ^{32}P-dCTP (to give a first strand specific activity of 10^5 cpm/μg). After hydroxyapatite selection, the bulk of the single-stranded material was concentrated with *sec*-butanol and excluded from Sephadex G-50F (Pasteur pipette column) to remove the phosphate buffer (which would inhibit precipitation). Fifty micrograms of tRNA carrier was added and the single-stranded cDNA was precipitated. Because this cold cDNA is somewhat longer, the reactions were closer to completion with 95% and 96% of the B-cell cDNA hybridizing to the T-cell (Bal 4) mRNA in two separate experiments. The selected cDNA was resuspended in water and made double-stranded with DNA polymerase I under standard conditions with at least 75% of the first strand mass synthesized (the second strand specific activity was 10^6 cpm/μg). The double-stranded material was diluted with 4 vol of nuclease S1 buffer, and the hairpin loop was cleaved with nuclease S1. The extent of nuclease S1 cleavage was analyzed on a denaturing gel. G-C tailing with terminal transferase (P-L Biochemicals) was as described (21) with the modification that the nucleotide concentration was varied to control the rate of the tailing reaction. In this case, 100 μM dCTP was necessary for a 1.5-min reaction at 37°C, yielding 20–30 cytidines per 3′ end (average). With other lots of terminal transferase, as little as 2 μM dCTP was necessary.

RESULTS

Screening I-Region Genomic Clones with B-Cell and T-Cell Derived cDNA Probes. B- and T-cell tumors reproducibly differ by ≈2% (1.7–2.4%) of their cytoplasmic poly(A)$^+$ RNA

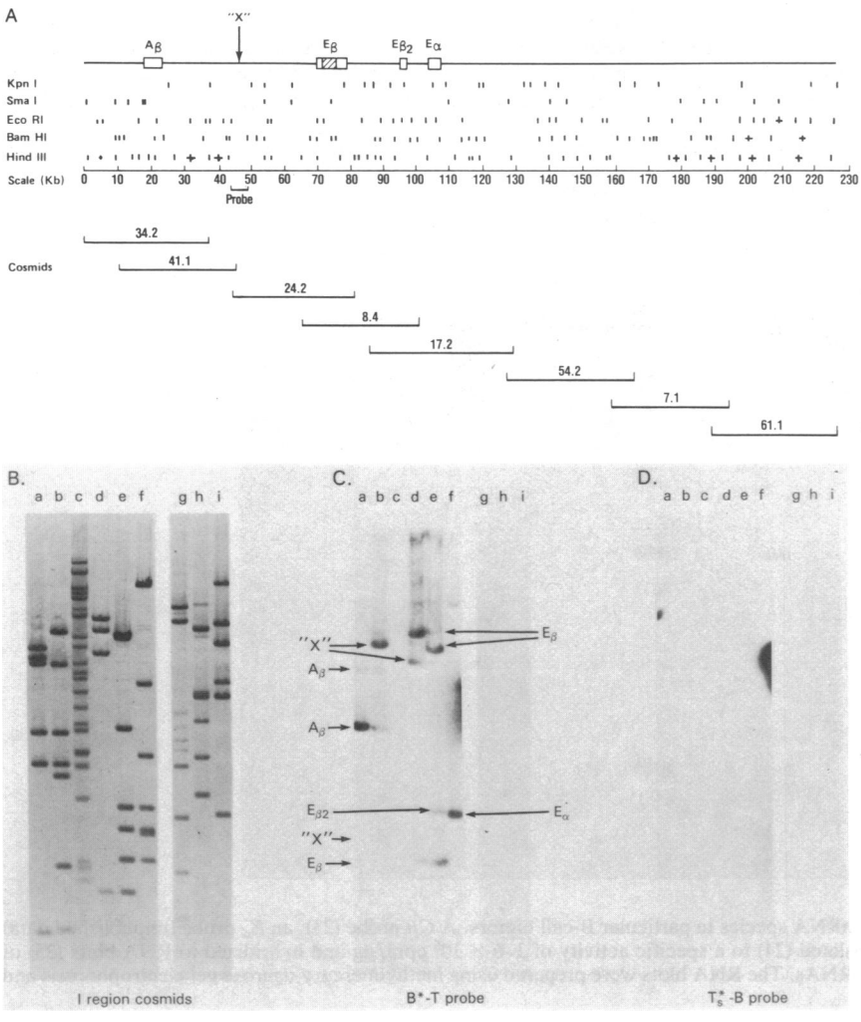

FIG. 1. Hybridization of B*-T and T_s^*-B cDNA probes to I-region genomic clones. (*A*) I-region genomic clones, relevant restriction sites, and the location of the A_β, E_β, $E_{\beta 2}$, E_α genes (12, 13). (*B*) Ethidium bromide-staining pattern of the cosmid clones shown in *A*. Lanes: a, cosmid 34.2; b, 41.1; c, size markers (the sizes, in kb are 48, 33, 23, 21.6, 19.7, 16.5, 15.5, 13.3, 10.6, 9.8, 8.6, 8.4, 7.7, 6.6, 5.9, 5.6, 4.8, 4.5, 3.6, 2.5, 2.4, and 2.2); d, 24.2; e, 8.4; f, 17.2; g, 54.2; h, 7.1; and i, 61.1. Lanes a, b, d, e, and f are *Eco*RI digests; lanes g, h, and i are *Kpn*/*Sma* digests. (*C*) Hybridization of the clones to a B*-T probe (Bal 17/Bal 4). The faint background seen in the marker lane (c) and other lanes seems due to nonspecific trapping of labeled cDNA by high molecular weight DNAs. Lane designations are as in *B*. (*D*) Hybridization of the I-region clones to a T_s^*-B probe (34S-504-L10A). Lane designations are as in *B*.

(mRNA) mass (16). Consequently, labeled cDNA probes corresponding to these differences can be enriched up to 50-fold and used to detect even very rare sequences in clone-screening experiments. We screened Southern blots of the I-region cosmid clones with two different selected cDNA probes, a B-cell-specific or a B*-T probe (B-cell cDNA minus T-cell mRNA) and a T_s probe made by subtracting the cDNA of a T_s hybridoma with B-cell mRNA (T_s^*-B). Fig. 1*A* shows the I-region cosmids, the position of the identified genes, and the relevant restriction maps (from refs. 12 and 13). Each of these clones were digested with *Eco*RI, or with *Kpn* I and *Sma* I, electrophoresed through an agarose gel and blotted onto nitrocellulose filters in duplicate. The ^{32}P-labeled cDNAs were synthesized to high specific activity (1.8×10^8 cpm/μg), and allowed to decay for 2 weeks to promote strand scissions (1–2 per molecule). This fragmentation allows shared domains or repeated sequences to be removed while cell-type-specific regions of a given mRNA would still be present. The B-cell line used was the B-cell lymphoma, Bal 17, which is typical of B-cell stage tumor lines (IgM^+ IgD^+ Ia^+) (15). The T-cell mRNA used to subtract it was isolated from Bal 4, which is a (Thy 1^+ $Lyt1^-$ $Lyt2^+$ TL^+) thymoma (16). The T_s line is a putative $I\text{-}J^+$, BW5147 fusion with keyhole limpet hemocyanin-specific suppressor activity (17) and its cDNA was subtracted with mRNA from another B lymphoma, L10A (15).

In addition to being subtracted once with mRNA, the cDNAs were also hybridized with total genomic DNA to a repetitive C_0t of 10 in the same reaction. This removes highly repeated sequences that might give false positives in the screen. Fig. 1*B* shows representative ethidium bromide-stained restriction fragments of the cosmid clones; Fig. 1*C* shows the hybridization with the B-cell probe (B*-T), and Fig. 1*D* shows the results with the T-cell probe (T_s^*-B). With the B-cell probe, one can clearly see all of the previously identified (ref. 12) gene regions hybridizing; namely A_β, E_β, $E_{\beta 2}$, and E_α. In addition, a fifth region of hybridization, labeled "X," can be seen in Fig. 1*C* (lanes b and d). From the restriction map, we can localize the X region to the area between A_β and E_β (see Fig. 1*A*). By contrast, in a parallel experiment, the T_s-cell probe was completely negative, with no detectable hybridization above background either in the putative I-J subregion (at the 3′ end of the E_β gene) or anywhere in the clones surveyed. Since the *Ia* genes appear to represent ≈0.01% of the mRNA of B-cell lines (unpublished results) and are clearly detectable in Fig. 1*C*, we estimate that the sensitivity of the T_s-B probe screen may be as low as 0.001–0.002% or 2–5 mRNAs per cell. Kronenberg *et al.* (22) have also been unable to detect an I-J mRNA using labeled genomic fragments to probe a variety of T_s hybridomas.

RNA Blot Analysis. A 4.7-kb fragment from the genomic clone 24.2 (Fig. 1*A*, "probe"; probe 2 in ref. 12) contains most of the X region and was used in RNA blot analyses of lymphoid tumor mRNAs (Fig. 2). These data show that the X region encodes a discrete mRNA species of 1.25 kb and that the mRNA seems coordinately expressed with E_α. Both molecules are strikingly B-cell-stage specific in their lack of expression in either the two plasma cells or pre-B-cell lines. Also interesting is the decrease in these two messages in WEHI-231 cells after 24 hr of LPS stimulation, versus the relatively unchanged expression of the $C\mu$ gene in these cells.

Selected cDNA Cloning. Probe 2 was used to isolate a nearly full-length cDNA clone from a library enriched from B-cell-specific (B-T) sequences. The procedure for constructing this library is shown in Fig. 3. The cDNA was synthesized from Bal 17 (B) poly(A)$^+$ RNA in the presence of actinomycin D (to suppress foldback loop formation), then hybridized to a 10-fold excess of T-cell mRNA (Bal 4) and selected on hydroxyapatite. The single-stranded fraction was made double-stranded with DNA polymerase I, trimmed with nuclease S1 and cloned into the *Pst* site of pBR322 by

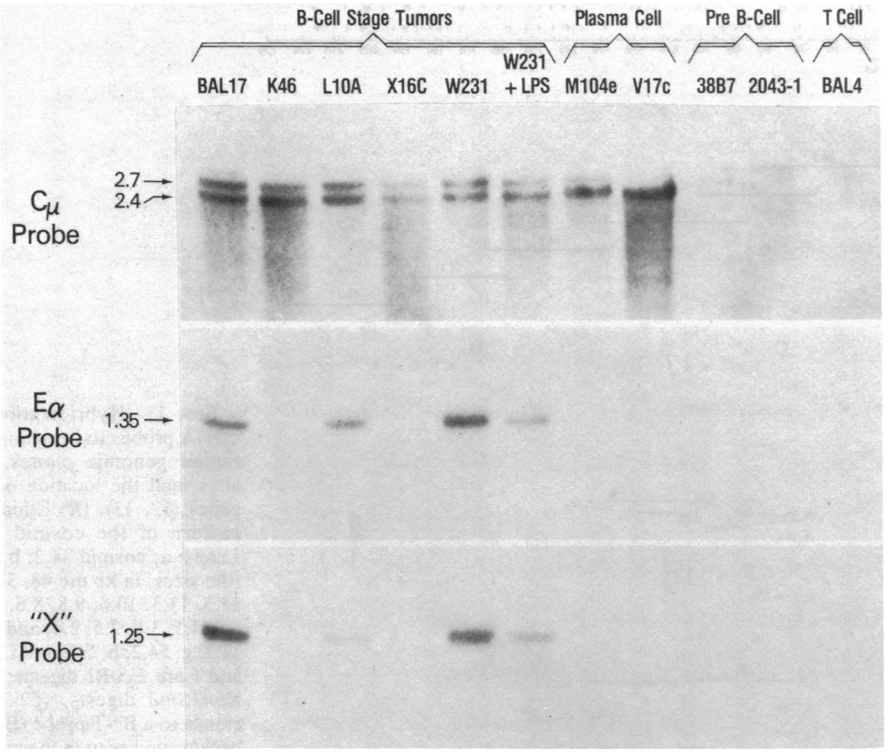

FIG. 2. Hybridization of probe X to a discrete mRNA species in particular B-cell tumors. A $C\mu$ probe (23), an E_α probe (unpublished data) and the X genomic probe (Fig. 1*A*) were nick-translated (24) to a specific activity of $3–6 \times 10^8$ cpm/μg and hybridized to RNA blots (25) of various lymphoid tumor line poly(A)$^+$ cytoplasmic RNAs. The RNA blots were prepared using methylmercury-agarose gel electrophoresis and APT paper (26). Size markers are in kb.

G-C tailing (27). The transformation efficiency was high (50,000–200,000 clones per μg of insert) and the average insert size was 500 base pairs. Less than 5% of the clones are capable of hybridizing to labeled T-cell cDNA. We have also made a selected cDNA library of T-cell-specific sequences and T. Sargent and his colleagues have made a *Xenopus*-stage-specific library (28). In cases where small differences exist between mRNA populations, these selected libraries can be an important source of clones of cell-type-specific cDNAs. In the case examined here, probe X hybridized with 46 colonies out of ≈9000 total, whereas an unselected library consisting of the same number of clones would be expected to yield between 1 and 2 positives. Therefore, we estimate a 20- to 40-fold enrichment for the sequences of interest.

Sequence of the A^d_α cDNA Clone. One of the largest cDNA clones, pBal17 105, with an insert size of 956 nucleotides, was sequenced by the procedure of Maxam and Gilbert (29). An open reading frame extends for 768 nucleotides, beginning with a methionine initiator codon, including a probable leader peptide, and terminating with a TGA stop codon. By comparing this sequence to that determined for other class II molecules, we were able to identify this gene as encoding A^d_α (30, 31). There is perfect homology (10/10 amino acid residues) between the amino acid residues predicted by this clone to that established for A^d_α by protein sequence analysis (30). Furthermore, the coding region sequence in pBal17 105 is identical to that of the A^d_α cDNA clone described by Benoist *et al.* (31).

The *Ia* Gene Cluster. The data presented in this paper now allow us to position all of the known *Ia* genes within the I region of the MHC (Fig. 4). Recombination analyses using a peptide map assay had previously suggested an order of A_α-A_β-E_β-E_α (32), but the data presented here clearly indicate an order of A_β-A_α-E_β-E_α. The difference cannot be explained by postulating an inversion in gene order between the k and d strains because genomic clones from the k haplotype have been shown to have an identical arrangement (13). Partial genomic sequence analysis of A_α has determined the orientation of the gene (Fig. 4), showing that both of the α genes are transcribed in the same direction, opposite to that of the β genes. This suggests that the putative ancestral gene complex ($\beta\alpha$) was duplicated as a unit; that is, $\beta\alpha \rightarrow \beta\alpha\beta\alpha$.

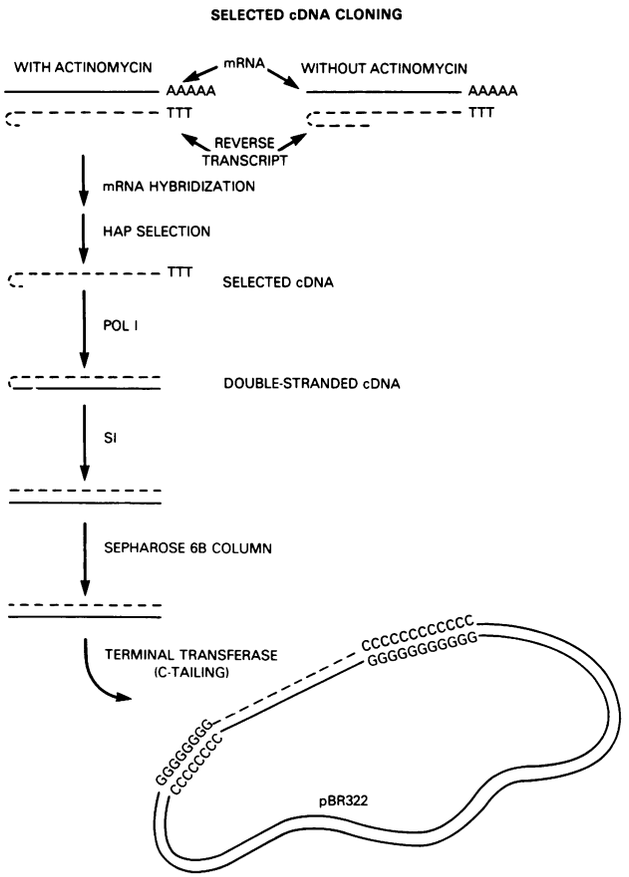

FIG. 3. Selected cDNA cloning. cDNA was synthesized in the presence of actinomycin to suppress hairpin-loop formation. It is postulated that a small foldback loop is synthesized and serves as a primer for DNA polymerase I but it is possible that this is a very transient structure. The remainder of the procedure is similar to those in the literature (see *Materials and Methods*).

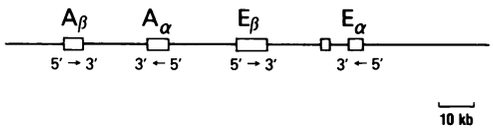

FIG. 4. The organization of the *Ia* gene cluster. Partial sequence analysis of the genomic A_α clone established its transcriptional orientation within the Ia complex. This sequence was determined from the *Eco*RI site of the X genomic subclone (Fig. 1*A*) located in the 3′-untranslated region of the A^d_α gene and from a *Pst* I site in the first external domain. The small box to the left of E_α is $E_{\beta 2}$, a possible pseudogene (12).

DISCUSSION

Selected Probes and Libraries. The data presented in this paper demonstrate the utility of using selected probes to screen specific subsets of the genome. A selected cDNA probe screening, such as we have described here, may be the method of choice for identifying all of the cell-type-specific genes in a given genomic subregion. The I region is particularly interesting in this regard as there are a number of functions and molecules mapped to this region for which genes have not yet been isolated (33–35). Additional genomic clones, especially toward the K region proximal to A_β (Fig. 1*A*), and probe combinations other than those used here, may be fruitful in detecting these genes. This combination of a cell-type selection with a genomic-subregion selection can also be extended to libraries derived from mini-chromosomal DNA or specific chromosomes. In addition, we have found selected cDNA libraries to be an important resource for deriving cell-type-specific clones because it considerably decreases the number of clones to be screened.

One major caveat in using cDNA selection is the possible loss of sequences of interest because of the presence of homologous mRNA in the absorbing population due to aberrant or lower level expression, translational regulation, or the differential expression of members of a related gene family. In fact, on examination with a partial A_β cDNA probe (36) we have found that the mRNA from the T-cell line Bal 4 used here for absorption contains small but detectable quantities of A_β mRNA (unpublished observation). Since A_β is clearly detectable on the screen of the I region clones (Fig. 1*C*) it would seem that the 10:1 mass excess of Bal 4 mRNA to Bal 17 cDNA was not sufficient to obscure the signal seen in Fig. 1*B*. This appears to be the exception rather than the rule, however, since most T cells do not express A_β and in general we find that 6/7 B-cell markers (κ, λ, A_β, A_α, E_β, E_α, but not μ) and 1/1 T-cell markers (Thy 1; ref. 37) are in the relevant absorbed B*-T and T*-B cDNA probes, respectively. Previously, Mather *et al.* (38) obtained a J-chain gene isolate using a cDNA subtraction based on protein expression data. Thus, in general it seems appropriate to use subtracted probes to screen for a wide variety of different genes.

Identification of A^d_α. We have used a B-cell-specific probe to screen directly genomic clones and to isolate the class II MHC gene for A^d_α. In the course of this work we have shown

that A^d_α maps between A^d_β and E^d_β, in contrast to the gene order predicted from previous work (32). The B-cell-specific probe was sensitive enough to screen, in addition, each of the previously identified class II genes (A_β, E_β, $E_{\beta 2}$, E_α). The screen depends on the observation that most murine T-cell lymphomas that we have examined (unpublished observations) do not actively transcribe the *I-A* or *I-E* locus.

Using a similar approach, a T_s-specific probe was found not to hybridize with the I region cosmids. Such a result could be explained if *I-J* gene products were quite closely related to products expressed by B lymphomas, if *I-J* were a transcriptional variant of such a product, or if *I-J* were encoded outside the region probed. The failure to find *I-J* in these genomic clones is consistent with other reported work (22).

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