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Immunology

Isolation and initial characterization of multiple species of T-lymphocyte subset cDNA clones

(subset specificity/concanavalin A/interleukin 2/T-cell antigen receptor/unidentified T-cell mediators)

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ABSTRACT **A modified differential screening procedure was applied to analyze cDNA libraries of cloned helper T lymphocytes (Th) and cytolytic T lymphocytes (CTL). Negative and positive differential screening and RNA blot analysis were used to identify cDNA clones that were expressed preferentially in Th or CTL. Seven clones corresponded to previously described T-cell genes, and 16 additional types of cDNA clones were isolated, 9 from Th and 7 from CTL. Of these, 3 were expressed in both Th and CTL, 7 were expressed in only Th, and 6 only in CTL. These clones were analyzed for induction after stimulation by interleukin 2 or Con A or after stimulation of the T-cell antigen receptor (TCR). Three different patterns of expression were seen: (*i*) induction only by Con A, (*ii*) induction by Con A and interleukin 2, and (*iii*) induction by Con A and TCR stimulation. The approach is potentially useful for analyzing paths of T-cell differentiation and detecting cDNA clones encoding unrecognized cytokines.**

T lymphocytes play a central role in the immune network both as effectors and as regulators. They may be divided into subsets endowed with distinct properties such as helper, suppressor, and cytolytic activities. These activities may be mediated by subset-specific immune effectors, which are elaborated and secreted after stimulation with either lectin or specific antigen (1–3). Not all activities are correlated with cloned genes, and the regulatory mechanisms by which the same stimulus can activate different sets of genes in these closely related cell types are not known.

Analysis of subset-specific genes requires pure populations of T cells. Glasebrook and Fitch (4) have developed two T-cell clones from the same mouse spleen; L2 shows helper activity and L3 displays cytolytic properties. These clones are well characterized on a cellular level (5, 6).

The isolation of a broad representation of L2- and/or L3-specific transcripts is the object of this study. We have developed a protocol for a differential screening without prior selection, which allowed us to isolate even very rare mRNA species specific to a cell type, and applied the approach to the analysis of cDNA libraries from Con A-stimulated L2 and L3 cells. T-cell-specific cDNAs were analyzed to determine whether they were specific for L2 or L3 cells and whether they were inducible by Con A, interleukin 2 (IL-2), or stimulation of the T-cell antigen receptor (TCR).

MATERIALS AND METHODS

Cells. Methods for isolating and maintaining the cloned helper T lymphocytes (Th), L2, and the cloned cytolytic T lymphocytes (CTL), L3, have been described previously (7). To stimulate the cloned T cells, we resuspended them at 10^6–10^7 cells per ml and cultured them with Con A (Pharmacia) at 10 μg/ml for L2 cells or 2 μg/ml for L3 cells or human recombinant IL-2 (rIL-2; Cetus) at 10^2–10^3 units/ml. Immobilized clonotypic monoclonal antibody 384.5, which reacts with the TCR of L3 cells (8, 9), was used to stimulate L3 cells.

Mouse thymoma cells, EL4, and mouse B-cell lines, A20.2j and K46, were maintained in RPMI 1640 medium containing 5% fetal calf serum. EL4 cells were stimulated with phorbol 12-tetradecanoate 13-acetate (PTA; 10 ng/ml) for up to 20 hr, monitoring the stimulation by IL-2 assay (10).

cDNA Libraries. RNAs of L2 and L3 cells that were stimulated by Con A for 14 hr, were extracted (11) and poly(A)$^+$ mRNA was purified on an oligo(dT)-cellulose column (12). Double-stranded (ds) cDNA was synthesized from the poly(A)$^+$ mRNA (13). The cDNA was methylated at *Eco*RI sites, *Eco*RI linkers were ligated to cDNA, and then the cDNA was enriched for molecules larger than 250,000 daltons by passage over Bio-Gel A-150m columns. The cDNAs were inserted into the *Eco*RI site of λgt10 bacteriophage cloning vector (14).

cDNA Probe. Six micrograms of poly(A)$^+$ mRNA was denatured with 10 mM methylmercuric hydroxide and incubated in a buffer containing 100 mM Tris·HCl at pH 8.3, 50 mM KCl, actinomycin D at 50 μg/ml, 30 mM 2-mercaptoethanol, 10 mM $MgCl_2$, $(dT)_{12-18}$ at 5 μg/ml, 0.5 mM each of dATP, dCTP, and dGTP, 0.01 mM dTTP, 0.001 mM [α-^{32}P]dTTP (3000 Ci mmol^{-1}; 1 Ci = 37 GBq), and reverse transcriptase from avian myeloblastosis virus at 1000 units/ml at 46°C for 30 min. Single-stranded (ss) cDNA was freed from its template RNA by incubation in 200 mM NaOH/10 mM EDTA at 60°C for 30 min and passed over a 4-ml column of Sephadex G-100. The specific activity of the probe was usually ≈1.6–2.0 × 10^8 cpm/μg of cDNA.

Subtracted cDNA Probe. The ss cDNA prepared from L2 RNA was hybridized to a R_0t of 1200–1500 (mol of nucleotide per liter) × sec with poly(A)$^+$ mRNA of A20.2j in 0.41 M sodium phosphate buffer, pH 6.8, containing 0.1% NaDodSO$_4$ and 1 mM EDTA, in a volume of 25–50 μl. The ss cDNA fraction was collected by chromatography through a hydroxylapatite column as recommended by the vendor (Bio-Rad). Seven percent of input cDNA was recovered in the ss fraction and used for a second round of hybridization to A20.2j poly(A)$^+$ mRNA to an equivalent R_0t of 500 (mol/liter) × sec. Approximately 93% of initial input radioactivity was recov-

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Abbreviations: Th, helper T lymphocytes; CTL, cytolytic T lymphocytes; IL-2, interleukin 2; IL-3, interleukin 3; rIL-2, recombinant IL-2; CSF-GM, granulocyte/macrophage colony-stimulating factors; cRNA, complementary RNA; ss, single-stranded; ds, double-stranded; TCR, T-cell antigen receptor; PTA, phorbol 12-tetradecanoate 13-acetate; LGL, large granular lymphocytes.

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ered. Starting with 6 μg of poly(A)$^+$ mRNA, approximately 5.5×10^6 cpm was obtained as a probe.

DNA and RNA Blot Hybridization. Recombinant phage DNA was prepared (15) and digested with *Eco*RI. DNA fragments were transferred to GeneScreen*Plus* membranes (New England Nuclear) and hybridized with ss cDNA probes (16). RNA was run on 1.2% formaldehyde denaturing agarose gel (17) and transferred to GeneScreen*Plus*. Probes for RNA hybridization were prepared from gel-purified cDNA inserts by the random priming method (18).

RESULTS

Fig. 1 is flow sheets of our approach. The ^{32}P-labeled cDNA probe, prepared from poly(A)$^+$ mRNA of A20.2j, was used to screen the library. The total cDNA probe could detect a clone corresponding to 0.02–0.05% of the test mRNA (19). Of 18,000 plaques from the L2 cDNA library, 614 (3.4%) failed to hybridize to the B-cell cDNA probe. The subtracted L2 cDNA probe was hybridized to these 614 plaques and 114 (18%) gave a signal; 372 plaques gave no signal to the subtracted L2 cDNA probe or B-cell cDNA probe. Of those 372, 72 clones (19%) contained cDNA inserts. The 186 (114 + 72) clones from the L2 cDNA library were subjected to further analysis.

By similar analysis of approximately 8000 L3 cDNA clones, 150 plaques (≈2.0%) that failed to hybridize to ^{32}P-labeled B-cell cDNA probe were selected. Instead of screening the 150 plaques with subtracted L2 cDNA probe, we digested recombinant phage DNA of each clone with *Eco*RI and immobilized the fragments on the filter. ^{35}S-labeled B-cell cDNA probe was used to hybridize to the filters. The use of ^{35}S for cDNA labeling and Southern analysis increased the sensitivity at least 5-fold. Fifty-six inserts (Fig. 1*b*) from L3 were identified, each of which failed to hybridize to the B-cell cDNA probe.

One hundred and eighty-six L2 cDNA inserts and 56 L3 cDNA inserts were hybridized to cDNAs of CSF-GM, IL-3, IL-2, TCR α-chain, TCR β-chain, c-*myc*, and c-*fos*. Twelve clones hybridized to cDNA, for IL-3, 6 to CSF-GM, 3 to IL-2, 2 to TCR β-chain, and 1 each to TCR α-chain and c-*myc* (Table 1). Twenty-nine clones whose cDNA inserts were less than 50 base pairs (bp) were eliminated from further study. The blots containing 132 L2 cDNA and 54 L3 cDNA inserts were hybridized to ^{35}S-labeled ss cDNA probe prepared from poly(A)$^+$ mRNA of unstimulated L3 or of unstimulated L2, respectively. Sixty-one inserts of L2 cDNA hybridized to the L3 cDNA probe and 14 inserts of L3 cDNA hybridized to the L2 cDNA probe.

The 71 (132 − 61) inserts from L2 and 40 (54 − 14) inserts from L3 were used as probes with blots of 10 μg of poly(A)$^+$ mRNA from K46, LGL (rat NK cell) (2), unstimulated or PTA-stimulated EL4, and 10 μg of total RNA from unstimulated or Con A-stimulated L2 or L3 cells.

Among these inserts, 29 (≈40%, 29/71) from L2 and 19 (≈47%, 19/40) from L3 hybridized to K46 or all lanes. Fourteen inserts (≈20%, 14/71) from L2 hybridized only to Con A-stimulated L2, or both to L2 and L3 RNA. Those cDNA inserts represented nine different cDNAs. From L3, 8 (20%, 8/40) were T-cell specific, representing seven different genes; one gene was inducible by Con A in both L2 and L3, three genes were expressed constitutively and inducible by Con A only in L3 cells; and the rest were inducible by Con

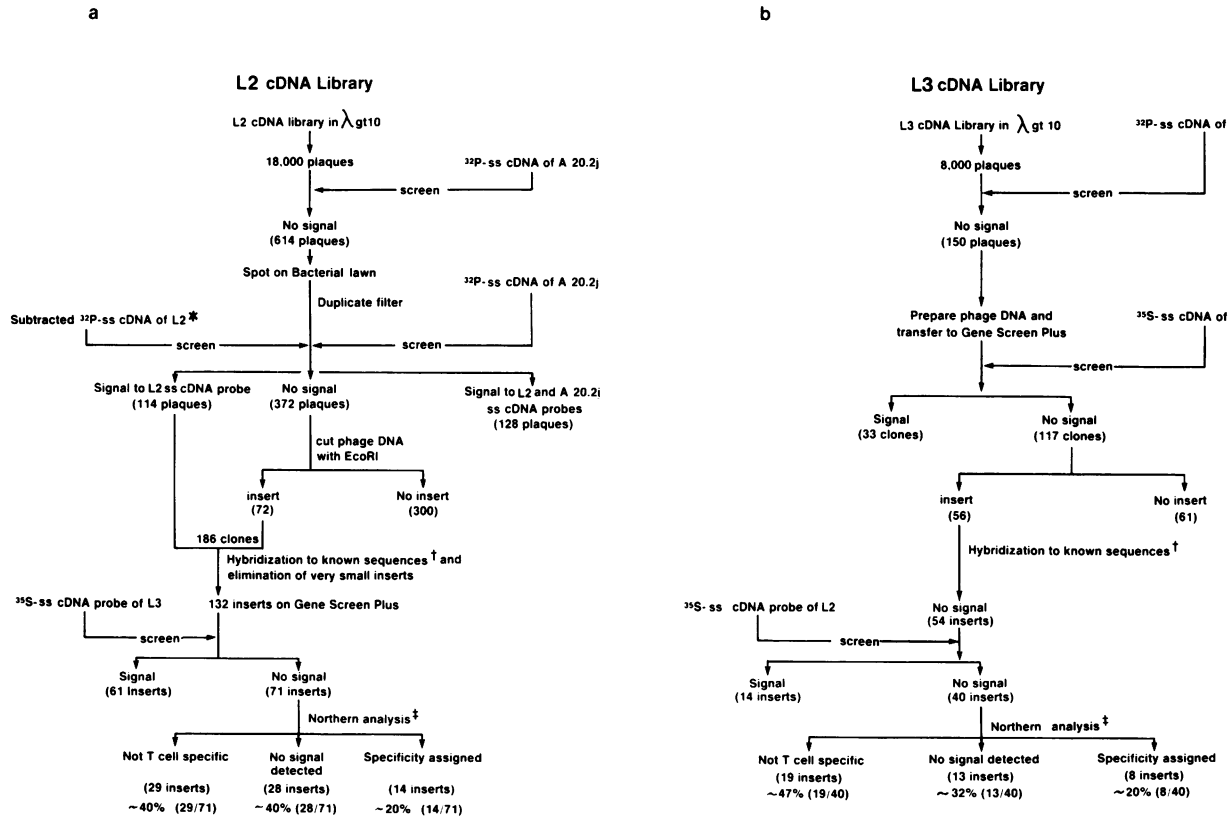

FIG. 1. Flow sheets of the approach to identify L2-specific (*a*) and L3-specific (*b*) cDNA clones.
*Preparation of subtracted L2 cDNA probe is described in *Materials and Methods*.
†Probes of known sequences were prepared from cDNAs of granulocyte/macrophage colony-stimulating factors (CSF-GM), interleukin 3 (IL-3), IL-2, TCR α-chain, TCR β-chain, c-*myc*, and c-*fos*.
‡Insert of each negative recombinant phage DNA was gel-purified and used as a probe for RNA blot hybridization ("Northern") analysis of K46, unstimulated or PTA-stimulated EL4, large granular lymphocytes (LGL), and unstimulated or Con A-stimulated L2 and L3.

Table 1. T-cell-specific cDNA clones isolated from L2 and L3 cDNA library

Origin	Group	cDNA clone	Number of times isolated
L2	1	CSF-GM	6
	2	IL-3*	12
	3	IL-2	3
	4	TCR α-chain	1
	5	TCR β-chain	2
	6	c-*myc*	1
	7	pBK791	4
	8	pBK642	1
	9	pBK671	1
	10	pBK631	3
	11	L2G53#3	1
	12	L2G95#3	1
	13	L2G95#4	1
	14	L2G25#4	1
	15	L2S35#3	1
			Total 39
L3	1	TCR β-chain	2
	2	L3G29#4	1
	3	L3G25#4	1
	4	L3G14#2	1
	5	L3G10#6	1
	6	L3G7#1	1
	7	L3G18#3	1
	8	L3G26#1	2
			Total 10

T-cell-specific cDNA clones were isolated from ≈18,000 clones of L2 library and ≈8000 clones of L3 library. After enrichment of T-cell-specific sequences, cDNA clones for CSF-GM, IL-3, IL-2, TCR α-chain, TCR β-chain, and c-*myc* were detected by hybridization with the corresponding full-length cDNA provided by other laboratories. By cross-hybridization, the other clones (14 from L2 and 8 from L3) turned out to represent 16 different genes (9 from L2 and 7 from L3). Those cDNA clones representing 16 different genes were subjected to further analysis.

*A partial sequence analysis revealed that the IL-3-related clones contained two different species.

A in L3 cells but not found in unstimulated L3 cells (Table 2). Twenty-eight inserts (≈40%, 28/71) from the L2 cDNA library and 13 inserts (≈32%, 13/40) from the L3 library did not hybridize to any of the RNAs. Because less L2 or L3 RNA was available for blot hybridization analysis, we have not been able to eliminate the possibility that those inserts not expressed in K46, EL4, or LGL could still be expressed in L2 or L3 at a low level.

The 16 cDNA inserts listed in Table 2 were cloned in M13 vector mp8. Their partial nucleotide sequence was determined and compared with sequences in GenBank‖ and of cDNAs from CTL or Th that were published recently (20–23). L3G10#6 was homologous (>95%) to serine esterase gene sequence (20), and L2 pBK671 proved to be identical to proenkephalin cDNA.

The expression patterns of selected clones were investigated after stimulation of L2 or L3 by Con A, through the IL-2 receptor, or through the TCR. rIL-2 and Con A were chosen as stimulants of L2 because L2 cells produce lymphokines when stimulated by Con A and they proliferate in response to IL-2 alone (24). Three L2-specific clones (pBK791, pBK642, and pBK671) (Fig. 2) were tested for induction by Con A or rIL-2. pBK791 and pBK642 cRNAs were detectable at 1 hr after Con A stimulation, reached peaks at 8 hr, and decreased thereafter. pBK791 cRNA accumulation was more prominent after IL-2 stimulation than after Con A stimulation. The pattern of pBK642 cRNA expression was essentially the same as that of pBK791. pBK671 was inducible only by Con A, not by rIL-2. Accumulation of pBK671 (proenkephalin) transcript reached maximal level at 8 hr after Con A stimulation and then decreased. IL-3 cRNA (Fig. 2*d*) was inducible only by Con A stimulation, not by rIL-2.

As stimulants of L3 cells, rIL-2 and clonotype-specific monoclonal antibody directed to TCR were chosen because L3 cells proliferated after TCR stimulation without IL-2 (9), although an earlier study (25) using a different CTL clone suggested IL-2 was needed. The inducibility of three L3-specific cDNA clones was tested after TCR stimulation by

‖National Institutes of Health (1986) Genetic Sequence Databank: GenBank (Research Systems Div., Bolt, Beranek, and Newman, Cambridge, MA), Tape Release 46.

Table 2. Characteristics of T-cell-specific clones from L2 and L3 cDNA libraries

Specificity	cDNA clone	Origin	Size of mRNA, S	Inducibility			Expression in EL4
				Con A	IL-2	TCR*	
L2 and L3	L3G29#4	L3	23	+	−	+	−
	L2G95#4	L2	14	+	ND	ND	−
	L2G25#4	L2	12	+	ND	ND	−
L2 only	L2G95#3	L2	12	+	ND	ND	−
	L2S35#3	L2	16	+	ND	ND	−
	L2G53#3	L2	12	+	ND	ND	−
	pBK671	L2	20	+	−	ND	−
	pBK791	L2	18	+	+	ND	+†
	pBK642	L2	22	+	+	ND	+†
	pBK631	L2	18	+	ND	ND	+†
L3 only	L3G10#6	L3	12	+‡	−	−	−
	L3G25#4	L3	20	+§	−	+	−
	L3G14#2	L3	21/12¶	+§	−	+	−
	L3G7#1	L3	18	+	−	ND	+†
	L3G18#3	L3	16	+	−	ND	+†
	L3G26#1	L3	24	+	ND	ND	+†

ND, not done.

*L3 cells were stimulated with immobilized clonotypic monoclonal antibody 384.5, directed against the antigen receptor of L3 (8, 9).

†Expression of corresponding transcripts was detectable only in PTA-treated EL4 cells, not in unstimulated cells.

‡L3G10#6 transcripts were expressed constitutively and were slightly inducible by Con A.

§Transcripts of L3G25#4 and L3G14#2 were expressed constitutively at a low level and were markedly inducible by Con A.

¶Two different bands were hybridized to ^{32}P-labeled L3G14#2 (see Fig. 3 *b* and *e*).

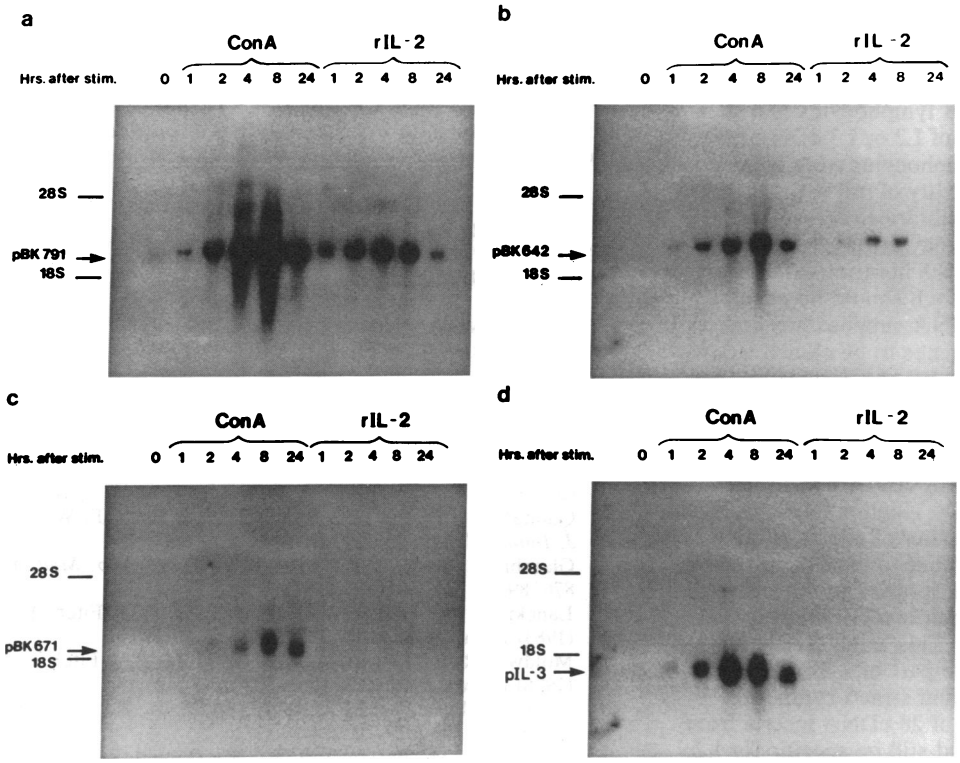

FIG. 2. Kinetic analysis of complementary RNA (cRNA) accumulation of L2-specific cDNA clones after Con A or IL-2 stimulation. L2 cells were stimulated with Con A at 10 μg/ml or rIL-2 at 100 units/ml for 0, 1, 2, 4, 8, or 24 hr and RNA was extracted at each time. Ten micrograms of total RNA was fractionated, immobilized, and hybridized successively with ^{32}P-labeled pBK791 (*a*), pBK642 (*b*), pBK671 (*c*), and pIL-3 (*d*). Previous probe was stripped off before hybridizing with the next probe. Expression of pBK791 (*a*) and pBK642 transcripts (*b*) was inducible either by Con A or by rIL-2, while expression of pBK671 (*c*) was inducible only by Con A, not by rIL-2. Expression of pIL-3 (*d*), an IL-3-related cDNA clone, was also inducible by Con A, not by rIL-2. Positions of 18 and 28S rRNA markers are indicated on the left.

anti-L3 TCR monoclonal antibody, 384.5, or by rIL-2 (Fig. 3). The sizes of the RNAs to which the clones were hybridized were 12S (L3G10#6), 21S and 12S (L3G14#2), and 20S (L3G25#4). L3G10#6 (the serine esterase) was expressed constitutively at a very high level and slightly inducible by Con A in L3 cells. The expression of these sequences was tested in another CTL clone (CTLLAII), which contains granules carrying cytolytic proteins (26). L3G10#6 and L3G25#4 were expressed constitutively in CTLLAII but L3G14#2 was not expressed constitutively in CTLLAII (data not shown). As shown in Fig. 3, the expression of L3G10#6 was not elevated after TCR or IL-2 stimulation. In contrast, L3G25#4 and L3G14#2 were inducible by TCR but not by IL-2 stimulation. These results indicate that pathway for gene activation mediated by TCR stimulation is distinct from that mediated by IL-2.

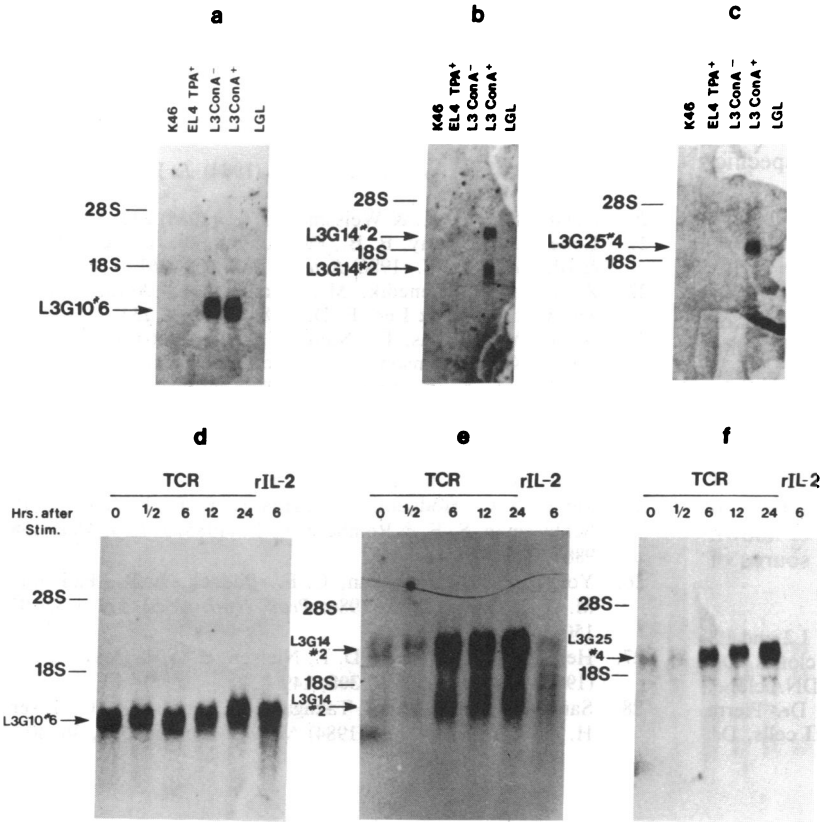

FIG. 3. Pattern of expression of L3-specific cDNA clones. *a*, *b*, and *c* show the specificity pattern of L3G10#6, L3G14#2, and L3G25#4, respectively. Poly(A)$^+$ mRNA was prepared from K46, from EL4 after stimulation with PTA at 10 ng/ml for 20 hr, and from LGL (rat NK cells), and total RNA was prepared from unstimulated L3 (L3ConA$^-$) or from L3 after stimulation with Con A at 2 μg/ml for 6 hr (L3ConA$^+$). Ten micrograms of total RNA or poly(A)$^+$ mRNA was fractionated on a formaldehyde/agarose gel, transferred, and hybridized with ^{32}P-labeled insert. L3G10#6 and L3G25#4 were also expressed in another CTL clone, CTLLAII, but not expressed in L2 cells. *d*, *e*, and *f* show the pattern of cRNA accumulation of L3G10#6, L3G14#2, and L3G25#4, respectively. L3 cells were stimulated with clonotypic monoclonal antibody 384.5, directed against TCR, for 0, ½, 6, 12, or 24 hr or with rIL-2 at 100 units/ml for 6 hr. L3G10#6 (*d*) was expressed constitutively and its expression was not elevated after TCR or rIL-2 stimulation. L3G14#2 (*e*) and L3G25#4 (*f*) were inducible by TCR stimulation but not by rIL-2.

DISCUSSION

One strategy in collecting L2- or L3-specific clones would have been to prepare cDNA libraries by using ss cDNA subtracted by mRNA common to B lymphocytes and then screening the libraries with a probe of L2 or L3 cDNA from which sequences expressed in B lymphocytes were removed (27, 28). However, when the availability of mRNA is limited and it is necessary to have cDNA libraries representing all mRNA, the preselection procedure may be undesirable. Our approach started with extensive cDNA libraries without any preselection procedures. The approach should be generally applicable to situations in which RNA supplies are limited and the level of expression of the genes to be cloned is low or unknown.

This study attempted to analyze L2 or L3 cDNA species that failed to hybridize to the cDNA probes prepared from developmentally closely related cells (T cell vs. B cell, Th vs. CTL). As shown in Fig. 1, 40–50% of cDNA pools selected as putative subset-specific were not T-cell subset-specific. This could be due to the limited number of copies of mRNA or failure of ss cDNA probe to hybridize to the cloned cDNA fragments. Since the sizes of the transcripts corresponding to most of the clones are large and their level of expression is significant, we suspect the latter. This might come about when cloned cDNA fails to include part of a 3′ region of a mRNA because of incomplete second strand synthesis.

We could not detect expression of 28 cDNA inserts from L2 and L3 in this study. They could still be specific for L2, L3, or both because (*i*) those inserts did not detect homologous RNA from 10 μg of poly(A)$^+$ mRNA of EL 4, B cells, or LGL, (*ii*) most cDNA inserts whose specificity could be assigned were Con A inducible, so L2 and L3 RNA loads (10 μg of total RNA) possibly were not high enough to detect a basal level of RNA. For example, L3G25#4 and L3G14#2 (Fig. 3) hybridized only to Con A-stimulated L3 RNA in the screening assay but were found to be expressed constitutively when blot hybridization analysis was done with 10 μg of poly(A)$^+$ RNA of L3 or CTLLAII. Combining the numbers with cDNA inserts whose specificities were assigned (Table 1, 39 for L2 and 10 for L3), the upper limit of subset-specific cDNA inserts is 67 for L2 and 23 for L3. These were approximately 0.37% (67/18,000) for L2 and 0.30% (23/8000) for L3 of the initial number of plaques screened and are in good agreement with the estimate obtained from kinetic hybridization analysis (28). A large fraction of T-cell-specific clones were also specific for either CTL or Th.

The L2 cDNA library was prepared from L2 mRNA stimulated by Con A for 14 hr when amounts of mRNA corresponding to most cDNA clones tested were in a decreasing phase. When we rescreened cDNA libraries with L2G53#3, L2S35#3, L3G25#4, or L3G14#2, only one out of 35,000 clones (≈0.003%) was present, confirming that the screening method we describe here can isolate cDNA clones corresponding to very scarce mRNAs.

Identification of the functions of both inducible and noninducible CTL-specific molecules may be important to understand the mechanisms involved in the killing process of cloned CTL cells. The majority of clones are inducible and their cRNAs are expressed at levels in the range of known lymphokines. These clones represent a potential source of as-yet-unidentified mediators of T-cell function.

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