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Isolation and characterization of a mouse interleukin cDNA clone that expresses B-cell stimulatory factor 1 activities and T-cell- and mast-cell-stimulating activities

(gene cloning/Ia induction/interleukin 2/interleukin 3/T-cell lymphokine)

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ABSTRACT **A cDNA sequence coding for a unique mouse interleukin that expresses B-cell-, T-cell-, and mast-cell-stimulating activities has been isolated from a mouse helper T-cell cDNA library. The library, constructed in the pcD expression vector, was screened by transfecting COS monkey cells with DNA pools to express the products encoded by full-length cDNA inserts. By assaying the transfected cell supernatants, we identified clones encoding a factor that stimulates T-cell and mast cell lines. This factor also induces Ia expression on resting B cells and enhances IgG1 and IgE production by B cells, two properties of B-cell-stimulatory factor 1. The DNA sequence codes for a polypeptide of 140 amino acid residues including a putative signal peptide. These results demonstrate that a single cDNA clone distinct from interleukin 2 and interleukin 3 encodes a polypeptide with multiple biological activities.**

The mouse T-cell clone Cl.Ly1$^+$2$^-$/9 was initially shown to produce several biological activities, including (*i*) stimulation of mast cell proliferation, (*ii*) stimulation of T-cell proliferation, (*iii*) activation of B cells to secrete immunoglobulin, and (*iv*) formation of hematopoietic colonies of various types (1–3). We previously described the isolation of cDNA clones coding for interleukin 3 (IL-3) from a cDNA library made with mRNA from activated Cl.Ly1$^+$2$^-$/9 cells (4). These cDNA clones express mast cell growth factor (MCGF) activity in transfected mammalian cells, but even saturating concentrations of IL-3 do not stimulate a cloned mast cell line to the same extent as supernatant derived from the original T-cell clone (5).

Recent experiments with Cl.Ly1$^+$2$^-$/9 cell supernatants have demonstrated the existence of a factor distinct from IL-3 that has MCGF activity and the ability to enhance the MCGF activity of IL-3 (6). Despite multiple biochemical fractionations, the MCGF activity copurifies with a T-cell growth factor (TCGF) activity that is distinct from interleukin 2 (IL-2) (6). These results are consistent with RNA blotting analysis showing that activated Cl.Ly1$^+$2$^-$/9 cells do not produce IL-2 mRNA (unpublished). Furthermore, the TCGF activity in cell supernatants is not blocked by a monoclonal antibody that completely inhibits the activity of mouse IL-2 (unpublished). These results demonstrate that the Cl.Ly1$^+$2$^-$/9 cells produce a factor, which is distinct from IL-3 and IL-2, with both MCGF and TCGF activities (MCGFII/TCGFII).

Cl.Ly1$^+$2$^-$/9 cells also produce high levels of three B-cell-stimulating activities. These include costimulation of anti-IgM-activated B cells (7), induction of Ia antigen on resting B cells (7), and enhancement of IgE and IgG1 production (8). Recent studies show that anti-IgM costimulation (9, 10), Ia induction (11, 12), and IgE (unpublished) and IgG1 (13) enhancement are all properties of partially purified B-cell stimulatory factor 1 (BSF-1). All of these activities in Cl.Ly1$^+$2$^-$/9 supernatants elute following gel filtration with an apparent M_r of ≈20,000 (ref. 7; unpublished results), the same size reported for BSF-1 from EL-4 cells (10). Together, these results suggest that Cl.Ly1$^+$2$^-$/9 cells produce high levels of a factor functionally identical to BSF-1.

Based on our data suggesting that Cl.Ly1$^+$2$^-$/9 cells produce BSF-1 and a factor with MCGF and TCGF activities, we undertook the isolation of cDNA clones for each of these factors. During the course of our work, however, results from other studies suggested that BSF-1 is identical to the factor having MCGFII/TCGFII activity. When the MCGFII/TCGFII activity was highly purified from Cl.Ly1$^+$2$^-$/9 supernatants, it was found to have Ia-inducing activity for B cells (unpublished). Another line of experiments demonstrated that BSF-1 purified from EL-4 cells also possesses MCGFII/TCGFII activity and that anti-BSF-1 antibody (14) can block the MCGFII/TCGFII activity produced by T cells (unpublished). In this paper we describe the isolation and characterization of cDNA clones that encode a protein with all of these activities. The expression of the functional product in mammalian cells provides final confirmation for the existence of this lymphokine and its ability to stimulate multiple cell types.

MATERIALS AND METHODS

Cell Lines and Isolation of mRNA. Cl.Ly1$^+$2$^-$/9, a cloned T-cell line derived from a C57BL/6 mouse, was grown as described (1) and stimulated with Con A at 2 μg/ml for preparation of induced mRNA. Total cellular RNA was extracted from the cells by using the guanidium thiocyanate method (15), and poly(A)$^+$ RNA was selected by oligo(dT)-cellulose chromatography. The HT-2 T-cell line and the MC/9 mast cell line were cultured as described (5).

cDNA Library and Screening by Transfection. A pcD cDNA library was constructed with mRNA from Con A-induced Cl.Ly1$^+$2$^-$/9 cells by using a modified pcDV1 plasmid containing an *Nsi* I site at the previous location of the *Kpn* I site (16, 17). Transfection of plasmid DNA prepared from pooled cultures of 48 clones into COS monkey cells was performed as described (17).

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Abbreviations: IL-2, interleukin 2; IL-3, interleukin 3; MCGF, mast cell growth factor; TCGF, T-cell growth factor; BSF, B-cell stimulatory factor; LPS, lipopolysaccharide; GM-CSF, granulocyte-macrophage colony-stimulating factor; bp, base pair(s).

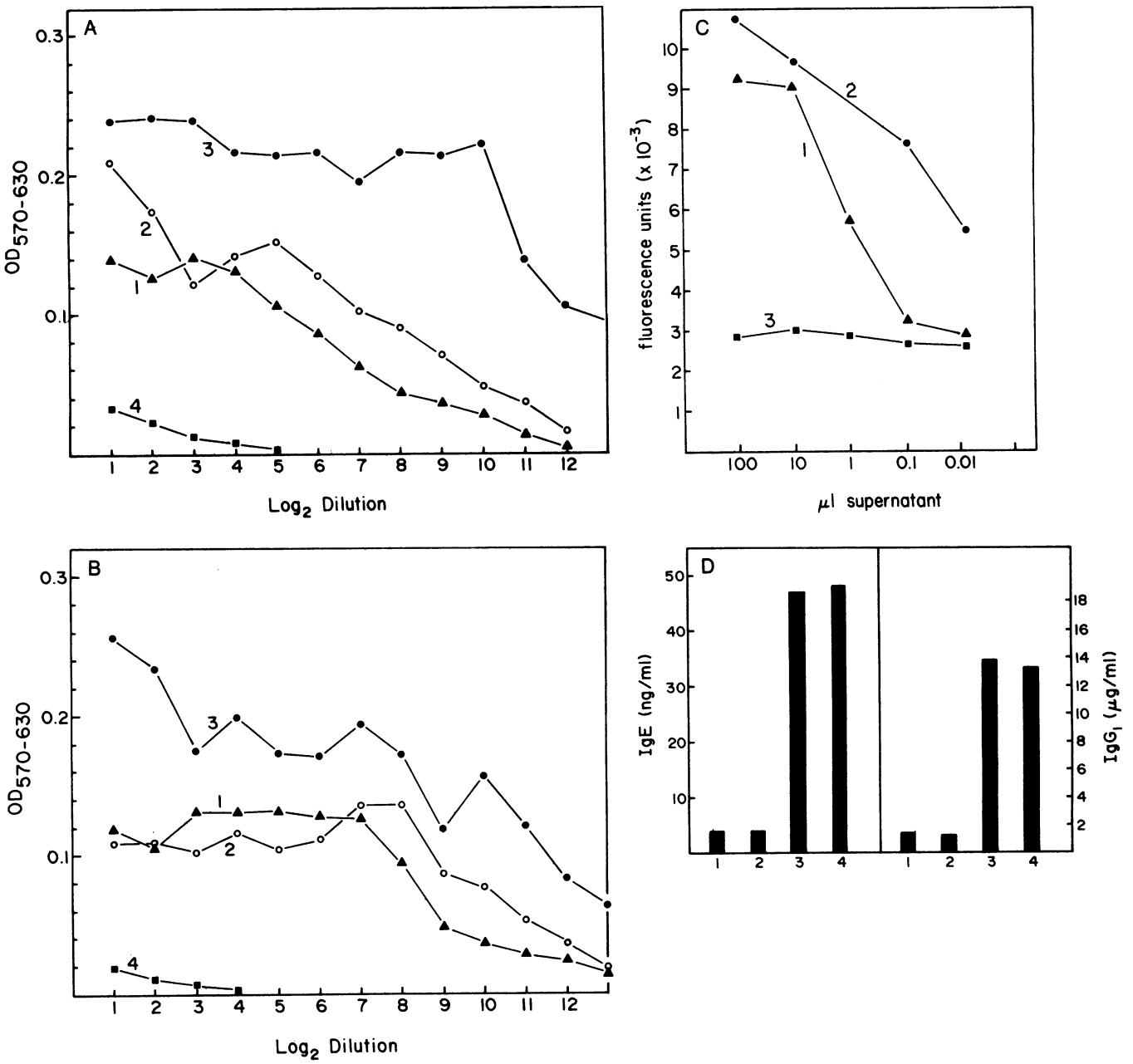

FIG. 1. Biological activities of supernatant from COS monkey cells transfected with clone 2A-E3. (*A*) TCGF activity was determined with HT-2 cells by using the colorimetric assay (18). Samples: 1, 2A-E3 COS supernatant; 2, Cl.Ly1$^+$2$^-$/9 cell supernatant; 3, COS-IL-2; 4, mock-transfected COS supernatant. (*B*) MCGF activity was determined by using MC/9 mast cells and a colorimetric assay (18). Samples: 1, 2A-E3 COS supernatant; 2, COS-IL-3; 3, Cl.Ly1$^+$2$^-$/9 cell supernatant; 4, mock-transfected COS supernatant. (*C*) Expression of Ia antigen on B cells cultured in test samples was determined by fluorescent staining. Samples: 1, 2A-E3 COS supernatant; 2, Cl.Ly1$^+$2$^-$/9 cell supernatant; 3, mock-transfected COS supernatant. The fluorescence units were calculated by multiplying the percentage of positive cells in each sample by the intensity of fluorescent staining. (*D*) IgE (left) and IgG1 (right) levels in supernatants of LPS-stimulated B cells cultured with test samples. Samples: 1, medium only; 2, 20% COS mock supernatant; 3, 10% Cl.Ly1$^+$2$^-$/9 plus 20% COS mock supernatant; 4, 20% 2A-E3 COS supernatant. Supernatant levels of IgE and IgG1 were determined by using an isotype-specific ELISA (8).

Bioassays. TCGF activity was determined by using the HT-2 T-cell line, and MCGF activity was determined by using the MC/9 mast cell line. Proliferation was determined either by incorporation of [^{3}H]thymidine or by a colorimetric assay as described (5, 18). The induction of Ia antigen on B cells was done as described (12). Briefly, the Ia-positive phenotype was determined by staining with anti-I-A^{bd} (D3.137.5.7.) or MK-d6 (anti-I-A^d) monoclonal antibodies and appropriate fluorescein-conjugated second antibodies. The analysis of stained cells was done with a fluorescence-activated cell sorter. The enhancement of IgE and IgG1 production in cultures of lipopolysaccharide (LPS)-stimulated T-cell-depleted spleen cells was measured by isotype-specific ELISA of culture supernatants (8).

DNA Sequence Analysis. Nucleotide sequences were determined by using a modified procedure of Maxam and Gilbert (19, 20) or a dideoxy chain-termination protocol (21) with supercoiled DNA templates (22).

RESULTS

Construction and Screening of cDNA Library. As shown in previous studies (5, 6), Cl.Ly1$^+$2$^-$/9 cells produce MCGF and TCGF activities distinct from IL-2 and IL-3. Both activities have been attributed to a single protein, and we

refer to this entity here as MCGFII/TCGFII. To confirm the biological activity of mRNA isolated from Con A-induced Cl.Ly1$^+$2$^-$/9 cells, it was microinjected into *Xenopus leavis* oocytes. MCGF, TCGF, and B-cell Ia-inducing activities could be detected in the oocyte incubation medium (data not shown). This biologically active mRNA was used to construct a cDNA library in the pcD expression vector, and 10^4 clones were picked and grown individually in microtiter plates. To focus only on the novel MCGF activity, 53 IL-3 cDNA clones identified by hybridization with a ^{32}P-labeled IL-3 cDNA probe were eliminated. A single clone hybridizing to a labeled granulocyte-macrophage colony-stimulating factor (GM-CSF) cDNA probe was also removed from the set of cDNA clones.

Starting from this group of clones devoid of IL-3 and GM-CSF, we used a screening protocol involving transfection of plasmid DNA representing random pools of 48 cDNA clones into COS monkey cells. Expression of biological activity was then evaluated by testing of the COS cell medium in appropriate bioassays. This procedure has been used to identify functional cDNA clones for mouse IL-2 and human GM-CSF (17, 22). In this case, an initial set of plasmid pools was screened primarily by using proliferation assays with the HT-2 and MC/9 cell lines. Among the first 110 pools assayed on these two cell lines, 8 produced significant activity in the HT-2 assay. Several of these pools had weak but significant MCGF activity, but, because the MCGF activities were generally weaker and more variable, we did not rely on this assay for identifying positive pools.

Because several lines of evidence suggested the identity of the MCGFII/TCGFII factor and BSF-1, we assayed approximately half of the COS supernatants from the random pool transfections for Ia-inducing activity on mouse B cells to confirm this relationship. Among the pools tested, each pool shown to be active for TCGF activity was found also to have Ia-inducing activity. Thus, there is a perfect correlation between the TCGF activity and the Ia-inducing activity.

Isolation of Functional Mouse cDNA Clones that Express TCGF and MCGF Activities. One pool, 2A, which was reproducibly the most active in all assays, was subdivided into smaller subpools representing horizontal and vertical rows of the 48-well microtiter plate. One horizontal and one vertical subpool were positive for both MCGF and TCGF activities. The single clone 2A-E3, common to both subpools, was then grown individually and its plasmid DNA was transfected as before. The resulting COS supernatant was then assayed for the presence of MCGF, TCGF, Ia-inducing, and IgE/IgG1-enhancing activities.

A 366-base-pair (bp)-long *Pst* I fragment isolated from clone 2A-E3 (see Fig. 2) and labeled with ^{32}P was used as a probe to screen pools that had been positive for biological activity as well as other untested pools. Nine hybridizing clones were isolated and their DNA was analyzed by restriction mapping. All pools that exhibited biological activity contained at least one hybridizing clone that shared a common restriction cleavage map with clone 2A-E3. The frequency of hybridizing clones among the 10^4 that were picked suggests a frequency of ≈0.2% in the total library. Of the hybridizing clones that were tested, ≈90% expressed a functional protein.

Biological Activities of Clone 2A-E3. When supernatant from COS cells transfected with the single 2A-E3 clone was tested for TCGF activity on HT-2 cells, the dose–response curve reached the same maximal level as seen with supernatant from Cl.Ly1$^+$2$^-$/9 cells (Fig. 1*A*). Even at saturating levels, however, the COS supernatant did not achieve the same level of stimulation obtained with recombinant IL-2 (Fig. 1*A*). When the same COS supernatant was tested on MC/9 mast cells, the maximal stimulation was approximately the same as with recombinant IL-3 (Fig. 1*B*). These results, employing a colorimetric assay (18), were also confirmed by using incorporation of [^{3}H]thymidine (data not shown).

We also tested the COS-expressed material of clone 2A-E3 for two activities of BSF-1, induction of Ia expression on mouse B cells (11, 12) and enhancement of IgG1 (13) and IgE production (unpublished). The COS supernatant had significant Ia-inducing activity (Fig. 1*C*) and enhanced the production of IgE and IgG1 by LPS-stimulated B cells (Fig. 1*D*). Results with this cDNA clone clearly show that all of these activities are associated with a single gene product. An assay for stimulation of fibroblast growth using mouse 3T3 cells was negative (data not shown).

Structure of the cDNA Insert for Clone 2A-E3. The cDNA insert was initially analyzed by restriction endonuclease digestion; a restriction cleavage map of the cDNA insert and the structure of the plasmid vector are shown in Fig. 2. The DNA sequence of the entire cDNA insert (Fig. 3) was then determined by using a combination of Maxam–Gilbert chemical cleavage and dideoxy chain-termination methods (19–21). The cDNA insert is 585 bp long excluding the poly(A) tail. There is a single long open reading frame, with the first ATG codon located 56 nucleotides from the 5′ end followed by 140 codons ending with the termination codon TAG at nucleotide positions 476–478. The NH_2-terminal segment of the predicted polypeptide is hydrophobic, as would be expected for a secreted protein.

Expression of BSF-1/MCGFII/TCGFII in T Cells. Assays of cell supernatants had previously indicated that the expression of this gene product is inducible by Con A in Cl.Ly1$^+$2$^-$/9 cells. Inducible expression of this gene was confirmed by analysis of mRNA isolated from cells treated or untreated with Con A. Fig. 4*A* shows an autoradiograph of an RNA blot analyzed with a ^{32}P-labeled probe derived from clone 2A-E3. The results show that a single prominent

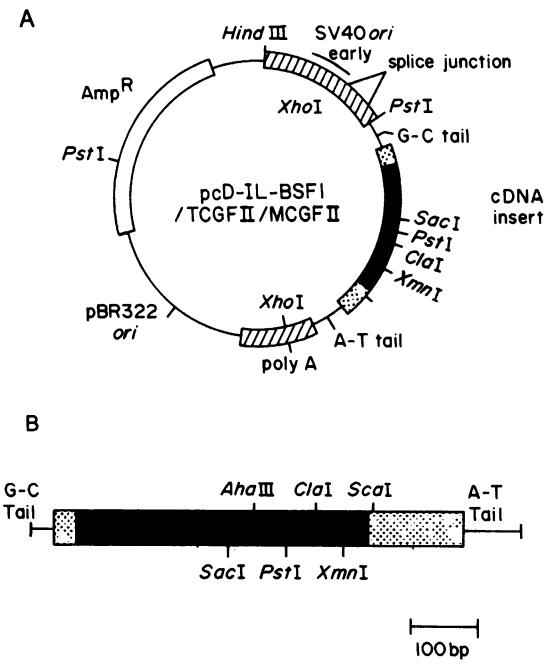

FIG. 2. Map of expression vector and 2A-E3 insert. (*A*) General diagram of pcD 2A-E3, the plasmid carrying the functional mouse interleukin cDNA insert. The cDNA insert extends from the G·C tail to the A·T tail and contains the indicated restriction endonuclease cleavage site; the coding region is heavily shaded, and the noncoding regions are lightly shaded. The direction of transcription from the simian virus 40 (SV40) promoter is indicated by the arrow. The structure of the remainder of the plasmid is as described in ref. 16. (*B*) Restriction endonuclease cleavage map of the insert in clone 2A-E3.

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          10          20          30          40          50
TTAGCATCTC TTGATAAACT TAATTGTCTC TCGTCACTGA CGCACAGAGC TATTG ATG GGT CTC
                                                          MET Gly Leu

     70                  85                  100                 115
AAC CCC CAG CTA GTT GTC ATC CTG CTC TTC TTT CTC GAA TGT ACC AGG AGC CAT
Asn Pro Gln Leu Val Val Ile Leu Leu Phe Phe Leu Glu Cys Thr Arg Ser His

            130                 145                 160
ATC CAC GGA TGC GAC AAA AAT CAC TTG AGA GAG ATC ATC GGC ATT TTG AAC GAG
Ile His Gly Cys Asp Lys Asn His Leu Arg Glu Ile Ile Gly Ile Leu Asn Glu

175                 190                 205                 220
GTC ACA GGA GAA GGG ACG CCA TGC ACG GAG ATG GAT GTG CCA AAC GTC CTC ACA
Val Thr Gly Glu Gly Thr Pro Cys Thr Glu MET Asp Val Pro Asn Val Leu Thr

        235                 250                 265                 280
GCA ACG AAG AAC ACC ACA GAG AGT GAG CTC GTC TGT AGG GCT TCC AAG GTG CTT
Ala Thr Lys Asn Thr Thr Glu Ser Glu Leu Val Cys Arg Ala Ser Lys Val Leu

                295                 310                 325
CGT ATA TTT TAT TTA AAA CAT GGG AAA ACT CCA TGC TTG AAG AAG AAC TCT AGT
Arg Ile Phe Tyr Leu Lys His Gly Lys Thr Pro Cys Leu Lys Lys Asn Ser Ser

    340                 355                 370                 385
GTT CTC ATG GAG CTG CAG AGA CTC TTT CGG GCT TTT CGA TGC CTG GAT TCA TCG
Val Leu MET Glu Leu Gln Arg Leu Phe Arg Ala Phe Arg Cys Leu Asp Ser Ser

            400                 415                 430
ATA AGC TGC ACC ATG AAT GAG TCC AAG TCC ACA TCA CTG AAA GAC TTC CTG GAA
Ile Ser Cys Thr MET Asn Glu Ser Lys Ser Thr Ser Leu Lys Asp Phe Leu Glu

445                 460                 475             488         498
AGC CTA AAG AGC ATC ATG CAA ATG GAT TAC TCG TAG TACTGAGCCA CCATGCTTTA
Ser Leu Lys Ser Ile MET Gln MET Asp Tyr Ser

        508         518         528         538         548         558         568
ACTTATGAAT TTTTAATGGT TTTATTTTTA ATATTTATAT ATTTATAATT CATAAAATAA AATATTTGTA


      578
TAATGTAACA GAAAAAA
```

FIG. 3. Nucleotide sequence and deduced amino acid sequence for the cDNA insert of clone 2A-E3. The nucleotide sequence begins with position 1 at the first nucleotide following the oligo(dG) segment. The amino acid sequence begins with the first in-phase ATG codon for the single long open reading frame. The underlined amino acids indicate the locations of potential N-glycosylation sequences (Asn-Xaa-Thr or Asn-Xaa-Ser).

mRNA species was detected only in mRNA isolated from induced T cells. mRNA samples from several mouse cell lines were then analyzed with the same radiolabeled probe. Hybridization could be detected only in mRNA from the EL-4 cell line treated with the phorbol ester phorbol 12-myristate 13-acetate. EL-4 is known to produce BSF-1 under these conditions. The GK15-1 and LB2-1 T-cell clones represent a subset of T cells that do not produce MCGFII/TCGFII activity (unpublished), and no hybridization with the labeled probe was observed with the GK15-1 or LB2-1 mRNA samples. These results show that there is good correlation between production of biological activities and expression of the mRNA.

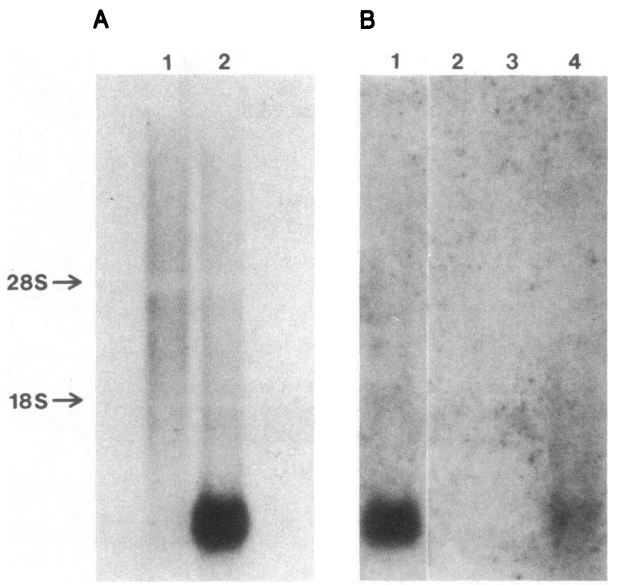

FIG. 4. (*A*) RNA blot analysis of 5 μg of poly(A)$^+$ mRNA isolated from uninduced (lane 1) or ConA-induced (lane 2) Cl.Ly1$^+$2$^-$/9 cells. RNA was separated on a 0.8% agarose/formaldehyde gel and then transferred to a Nytran filter. The filter was hybridized with a ^{32}P labeled 366-bp *Pst* I–*Pst* I fragment isolated from clone 2A-E3. (*B*) RNA blot of mRNA samples isolated from mouse T-cell lines. Lanes: 1, Con A-induced Cl.Ly1$^+$2$^-$/9, 5 μg; 2, Con A-induced T-cell clone LB2-1 (23), 5 μg; 3, Con A-induced T-cell clone GK15-1 (24), 5 μg; 4, phorbol 12-myristate 13-acetate-induced EL-4 lymphoma cells, 10 μg.

DISCUSSION

We describe here the isolation of a cDNA clone encoding a T-cell product that not only stimulates the proliferation of certain T cells and mast cells but also induces the expression of Ia antigens on resting B cells and enhances IgE and IgG1 production. BSF-1 is presently the best characterized of the lymphokines regulating the function of B cells. It was first thought to act only on B cells stimulated by agents such as anti-IgM antibodies (9, 10), but its other properties include the induction of Ia antigens on resting B cells, an early event in B-cell activation (11, 12), and the isotype-specific enhancement of IgG1 (13) and IgE (unpublished) production by LPS-activated B cells. The multiple activities expressed by this cDNA clone confirm and extend the known properties of BSF-1 and are consistent with recent studies on the protein isolated from Cl.Ly1$^+$2$^-$/9 cells or from EL-4 cells (unpublished).

The single long open reading frame in the mouse 2A-E3 cDNA clone consists of 140 amino acid residues. Because this lymphokine is a secreted protein, a hydrophobic leader sequence would be expected to precede the sequence for the

mature secreted form of the protein. Analysis of the hydrophobicity of the polypeptide and comparison with a proposed consensus sequence for the processing of signal peptides (23) suggest that cleavage of the precursor polypeptide would occur following the serine residue at position 20 (Fig. 3). We therefore predict that the mature polypeptide would be 120 amino acid residues long and begin with a histidine residue. The deduced M_r of the mature secreted protein is ≈14,000. This predicted molecular weight does not take into account potential posttranslational glycosylation of the polypeptide, which is predicted by the presence of three potential N-glycosylation sequences (Asn-Xaa-Thr or Asn-Xaa-Ser at positions 61–63, 91–93, and 117–119, respectively).

Despite the biological activities of this interleukin that are similar to activities of IL-2 and IL-3, there is no significant nucleotide sequence homology between the cDNA clone described here and either the IL-2 or IL-3 cDNA sequences. At the amino acid sequence level, however, there are two regions that can be discerned to have homology with these two gene products. Amino acid residues 32–39 are 70% homologous to residues 49–56 of the IL-3 precursor polypeptide (4). Amino acids 95–103 are 60% homologous with residues 52–61 of mouse IL-2 (17). The significance of these sequence homologies is at present unknown. There are no other homologies that could be detected with other cloned lymphokines such as interferon γ or interleukin 1.

The biological activity data and analysis of mRNA levels suggest that Cl.Lyl$^+$2$^-$/9 cells produce high levels of this interleukin. Analysis of various T-cell clones suggests that only certain T cells express this gene product, and this subset does not synthesize IL-2 or interferon γ (unpublished). Cl.Lyl$^+$2$^-$/9 is typical of this type of T cell. A second subset of helper T-cell clones does make IL-2 and interferon γ but does not make BSF-1/MCGFII/TCGFII (unpublished). The availability of the cDNA clones we have isolated should aid studies on the expression and regulation of this gene in different subsets of T cells as well as other cell types. Sufficient quantities of purified recombinant protein can now be prepared that will permit detailed studies on the properties of this multifunctional interleukin and its possible interaction with other growth or differentiation signals.

Note Added in Proof. Honjo and co-workers have independently isolated cDNA clones similar to those described here (25). We have agreed to jointly propose that the lymphokine encoded by these cDNA clones be designated "interleukin 4."

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