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Homology of Cytokine Synthesis Inhibitory Factor (IL-10) to the Epstein-Barr Virus Gene BCRFI

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Complementary DNA clones encoding mouse cytokine synthesis inhibitory factor (CSIF; interleukin-10), which inhibits cytokine synthesis by T_H1 helper T cells, were isolated and expressed. The predicted protein sequence shows extensive homology with an uncharacterized open reading frame, BCRFI, in the Epstein-Barr virus genome, suggesting the possibility that this herpes virus exploits the biological activity of a captured cytokine gene to enhance its survival in the host.

IMMUNE RESPONSES ARE SPECIFIC NOT only for a particular foreign antigen, but also for the class of effector response directed to that antigen. The antibody-mediated (humoral), and delayed-type hypersensitivity (DTH) responses are often mutually exclusive (*1*). Which response is mounted after exposure to antigen can determine the survival of the organism: BALB/c mice respond to infection by *Leishmania major* with a predominantly humoral response that is ineffective and leads to death, whereas C57Bl/6 mice have a DTH response that ultimately cures the infection (*2*). An explanation for these different immune responses is suggested by studies of two types of long-term mouse T cell clones (T_H1 and T_H2) (*3*) that differ in their patterns of cytokine synthesis in response to antigen or lectin stimulation. The functions of these types of T cell clones differ; T_H2 clones, which produce interleukin-4 and -5 (IL-4 and IL-5), provide help for B cell (antibody) responses (*4*), whereas T_H1 clones secrete IL-2 and interferon-γ (IFN-γ) and preferentially induce DTH and macrophage activation (*5*). If these functions reflect the mutually exclusive classes of immune response observed in vivo, then T_H1 and T_H2 cells may be mutually inhibitory. The T_H1 product IFN-γ inhibits T_H2 clone proliferation in vitro (*6*), and proliferation of T_H1 clones is inhibited by an unknown product of T_H2 cells (*7*). A product of T_H2 clones, cytokine synthesis inhibitory factor (CSIF), inhibits synthesis of IFN-γ and other cytokines produced by stimulated T_H1 clones (*8*). We now characterize cDNA clones encoding mouse CSIF, a novel product of T_H2 cells that shares nucleic acid and amino acid sequence homology with an uncharacterized gene in the Epstein-Barr virus genome, BCRFI (*9*).

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Mouse CSIF cDNA clones were isolated from a cDNA library prepared in the pcDSRα expression vector (*10*) from polyadenylated RNA isolated from the T cell clone D10 (*11*) after stimulation for eight hours with concanavalin A (Con A). The CSIF in COS7 transfection (*12, 13*) supernatants was assayed by its ability to inhibit IFN-γ synthesis by the T cell clone HDK.1 (T_H1) in response to stimulation by antigen (TNP-conjugated keyhole limpet hemocyanin; TNP-KLH) with syngeneic, irradiated (BALB/c) spleen cells as antigen-presenting cells (*8*). Mock transfections lacking plasmid DNA or with an irrelevant cDNA typically yielded little CSIF (<13 unit/ml), whereas randomly selected pools of 10^4 clones gave titers of 20 to 50 unit/ml. Two pools of 10^4 cDNA clones (pools 4.5 and 4.6), which expressed CSIF activity at levels of 70 to 90 unit/ml, were combined and serially subdivided for subsequent transfections, with CSIF-expressing pools being identified at each step. Two pools of 500 cDNA clones were thus identified, each of which generated transfection supernatants with CSIF activity of approximately 600 unit/ml. Finally, two single clones were isolated that expressed CSIF activity, F115 (Fig. 1A) and 0.5J. The titration curve for recombinant CSIF (rCSIF) was qualitatively similar to that of the D10-derived factor. CSIF activity in the transfection supernatants from either CSIF cDNA clone was generally 3×10^3 to 8×10^3 unit/ml, a level similar to that in supernatants of Con A–stimulated D10 cells (*8*). The F115 cDNA hybridized to 0.2 to 0.4% of cDNAs in the D10 library, indicating that CSIF mRNA is a moderately abundant species in these cells.

COS7 cells transfected with the CSIF cDNAs were cultured in vitro with [^{35}S]methionine, and aliquots of supernatants were subjected to electrophoresis in reducing SDS-polyacrylamide gels (SDS-PAGE). Supernatants from either CSIF cDNA clone contained three labeled polypeptides (~21, 19, and 17 kD; Fig. 1B) that were absent in mock supernatants. These three species of rCSIF, identical to D10-derived CSIF (Fig. 1B), were immunoprecipitated by six monoclonal antibodies (SXC1-6) to mouse CSIF that define at least three antigenic determinants (*12*). Thus, CSIF cDNA clones express rCSIF with the biological activity, antigenic determinants, and molecular sizes of CSIF derived from the D10 T cell clone. Proteins immunoprecipitated from cultures with or without tunicamycin B2 (170 ng/ml) were digested with N-glycanase. Only the 17-kD species was detected in cultures containing tunicamycin (Fig. 1C). Similarly, N-glycanase digestion reduced the three polypeptides

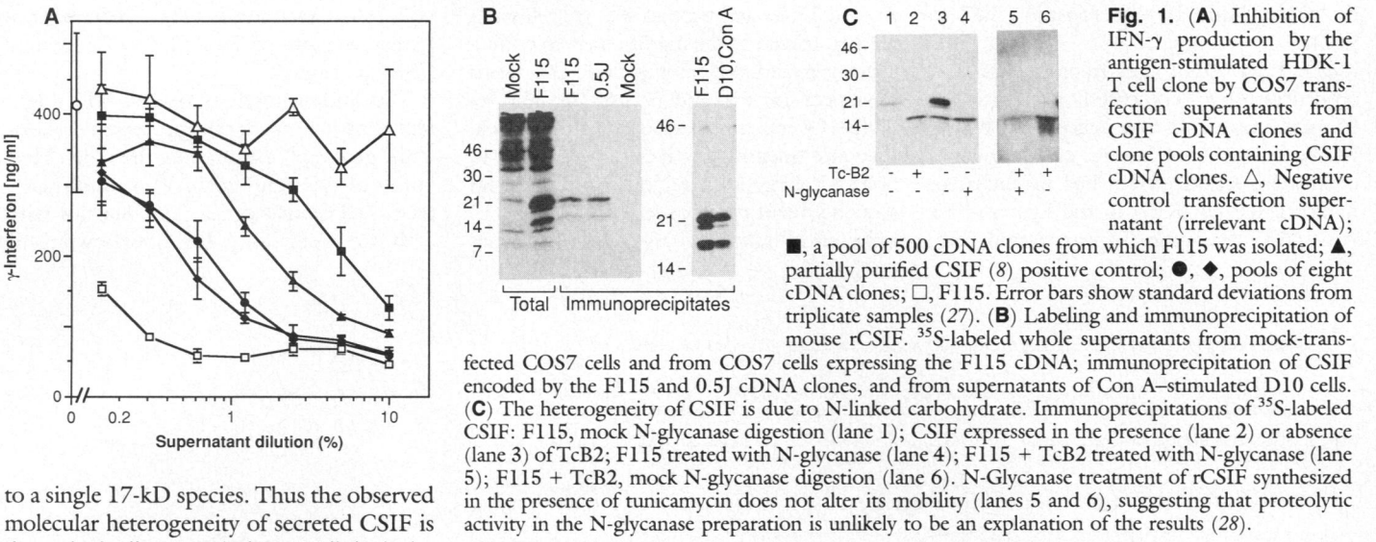

Fig. 1. (**A**) Inhibition of IFN-γ production by the antigen-stimulated HDK-1 T cell clone by COS7 transfection supernatants from CSIF cDNA clones and clone pools containing CSIF cDNA clones. △, Negative control transfection supernatant (irrelevant cDNA); ■, a pool of 500 cDNA clones from which F115 was isolated; ▲, partially purified CSIF (*8*) positive control; ●, ◆, pools of eight cDNA clones; □, F115. Error bars show standard deviations from triplicate samples (*27*). (**B**) Labeling and immunoprecipitation of mouse rCSIF. ^{35}S-labeled whole supernatants from mock-transfected COS7 cells and from COS7 cells expressing the F115 cDNA; immunoprecipitation of CSIF encoded by the F115 and 0.5J cDNA clones, and from supernatants of Con A–stimulated D10 cells. (**C**) The heterogeneity of CSIF is due to N-linked carbohydrate. Immunoprecipitations of ^{35}S-labeled CSIF: F115, mock N-glycanase digestion (lane 1); CSIF expressed in the presence (lane 2) or absence (lane 3) of TcB2; F115 treated with N-glycanase (lane 4); F115 + TcB2 treated with N-glycanase (lane 5); F115 + TcB2, mock N-glycanase digestion (lane 6). N-Glycanase treatment of rCSIF synthesized in the presence of tunicamycin does not alter its mobility (lanes 5 and 6), suggesting that proteolytic activity in the N-glycanase preparation is unlikely to be an explanation of the results (*28*).

to a single 17-kD species. Thus the observed molecular heterogeneity of secreted CSIF is due principally or entirely to N-linked glycosylation.

CSIF is produced by induced T_H2 helper T cell clones, but not detectably by T_H1 clones (*8, 12*). RNA blot hybridization revealed CSIF mRNAs of 1.5 and 1.0 kb in mRNA from Con A–stimulated T_H2 clones D10 and CDC35 (Fig. 2A). The F115 and 0.5J cDNAs might represent the 1.5- and 1.0-kb species, respectively. Although the CSIF mRNA expressed by CDC35 is induction-specific, uninduced D10 cells do express CSIF mRNA at reduced levels. Low levels of CSIF activity were also detected in supernatants of uninduced D10 cells, consistent with our observation that this D10 subline proliferates and secretes cytokines at a detectable level even without stimulation by antigen or mitogen. In contrast, T_H1 clones generally express little or no detectable CSIF mRNA (Fig. 2A). CSIF mRNA was also detected in the $CD4^+$ HT2 cell line and the $Ly1^+$ B cell lymphoma CH12 (*14*). Low levels of CSIF mRNA were detected by PCR (Fig. 2B) in two mast cell lines (MC/9 and MM3), which is consistent with the ability of these cell lines to make "T_H2" cytokines such as IL-4 and IL-5 (*15*). All other cell lines tested, including NIH 3T3 (fibroblast), NFS60 (myeloid), and P388D1 (macrophage) were negative by both RNA blot and PCR analysis.

The complete CSIF cDNA (F115) was sequenced (Fig. 3A): it contains a 178–amino acid open reading frame (ORF) with an NH_2-terminal hydrophobic signal sequence predicted (*16*) to be 18 amino acids long. The resulting mature polypeptide is 160 amino acids in length, with a calculated molecular size of 18.7 kD and calculated *p*I of 8.1. The protein contains two potential sites for N-linked glycosylation, five Cys residues, and nine Met residues (Fig. 3A). The 3′-untranslated sequence also contains a 104-codon ORF, beginning with a Met codon at nucleotides 913 to 915 and ending at nucleotide 1227. However, this ORF lacks a hydrophobic signal sequence and is truncated in the 0.5J cDNA clone, which also expresses CSIF. The 3′-untranslated sequence contains several AT-rich regions similar to sequences that have been associated with regulation of mRNA stability (*17*).

Restriction digests of genomic DNA isolated from the livers and sperm of several strains of laboratory mice were analyzed by DNA blot hybridization (Fig. 3B). Hybridization to a mouse IL-3 cDNA (*18*) was a control for the amount of genomic DNA loaded on the gel. Identical Southern blot band patterns of relatively few bands were detected with similar intensities in DNAs from both liver and sperm, suggesting that CSIF may be a single-copy gene.

Three overlapping λ-phage clones that hybridized to the CSIF cDNA probe were isolated from a genomic library of B10.AJ1 mouse liver DNA. A restriction map of one clone (λ4) is shown in Fig. 3C. The genomic DNA clones account for a majority of the bands in the genomic blots (compare Fig. 3, B and C): the 5.7-kb Eco RI fragment is the smaller Eco RI band in the genomic blot; the 3.7-kb Eco RI fragment is apparently the truncated ~7-kb band. The λ4 clone contains mouse repetitive DNA sequence elements as indicated. Preliminary DNA sequence analysis shows that the mouse CSIF gene contains at least three intervening sequences. The sequence of one complete exon is shown in Fig. 3C. This exon is ~150 nucleotides long and contains an Eco RI site (nucleotides 402 to 407) that is present in the cDNA clone (Fig. 3, A and C). A second exon begins at position 518 of the cDNA sequence and extends at least as

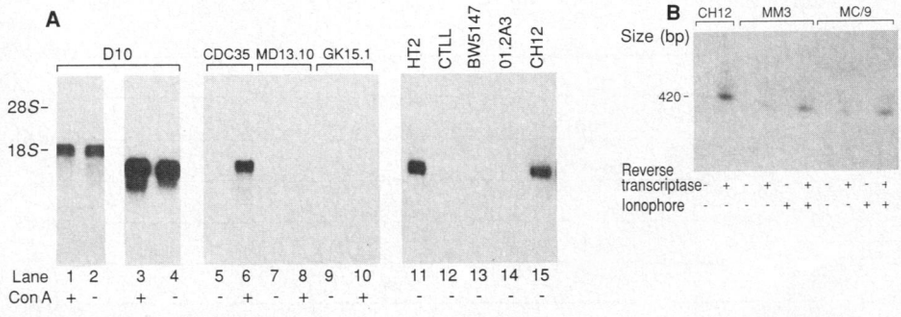

Fig. 2. (**A**) Detection of CSIF mRNA in mouse cell lines by RNA blot hybridization. Lanes 1 and 2 were probed with an overlapping pair of γ-actin synthetic oligonucleotides (*3, 8*); the remaining lanes all show hybridization of CSIF probes. CDC35 is a T_H2 clone; MD13.10 and GK15.1 are T_H1 clones. Low levels of CSIF mRNA were detected in induced GK15.1 cells by polymerase chain reaction (PCR). CTLL is a $CD8^+$ cytotoxic cell line (provided by W. Farrar). O1.2A3 is a mouse $\mu^+\delta^+CD23^+$ B cell hybridoma. (**B**) Detection of mouse CSIF mRNA by PCR in two mast cell lines; MM3 and MC/9 cultured in the presence or absence of ionomycin. Hybridization of the PCR product with a synthetic oligonucleotide specific for the amplified sequence is shown. Reverse transcriptase-negative controls are indicated (*29*).

far as the Hind III site (position 947 to 952).

The CSIF DNA and translated protein sequences were compared to other sequences in available sequence data banks. No homology with known cytokine genes or proteins was detected, but an uncharacterized ORF (BCRFI) in the Epstein-Barr virus (EBV) genome (*9*) was related (Fig. 4). The DNA and protein sequence similarity is restricted to the mature protein coding sequences and does not include the signal sequences or 5′- and 3′-untranslated regions. Overall nucleotide and amino acid sequence identity is approximately 70%; however, large local regions of amino acid sequence identity are observed (*19*). BCRFI lacks one N-linked glycosylation site and has only four Cys residues. The predicted molecular weights of BCRFI (~17 kD) and CSIF are similar.

This finding suggests that BCRFI might represent an ancestral, processed cellular CSIF gene (*19*) captured by the virus. Herpes viruses contain genes that are distantly related to cellular genes (*20*), but not with such strong similarity. It is not known if the

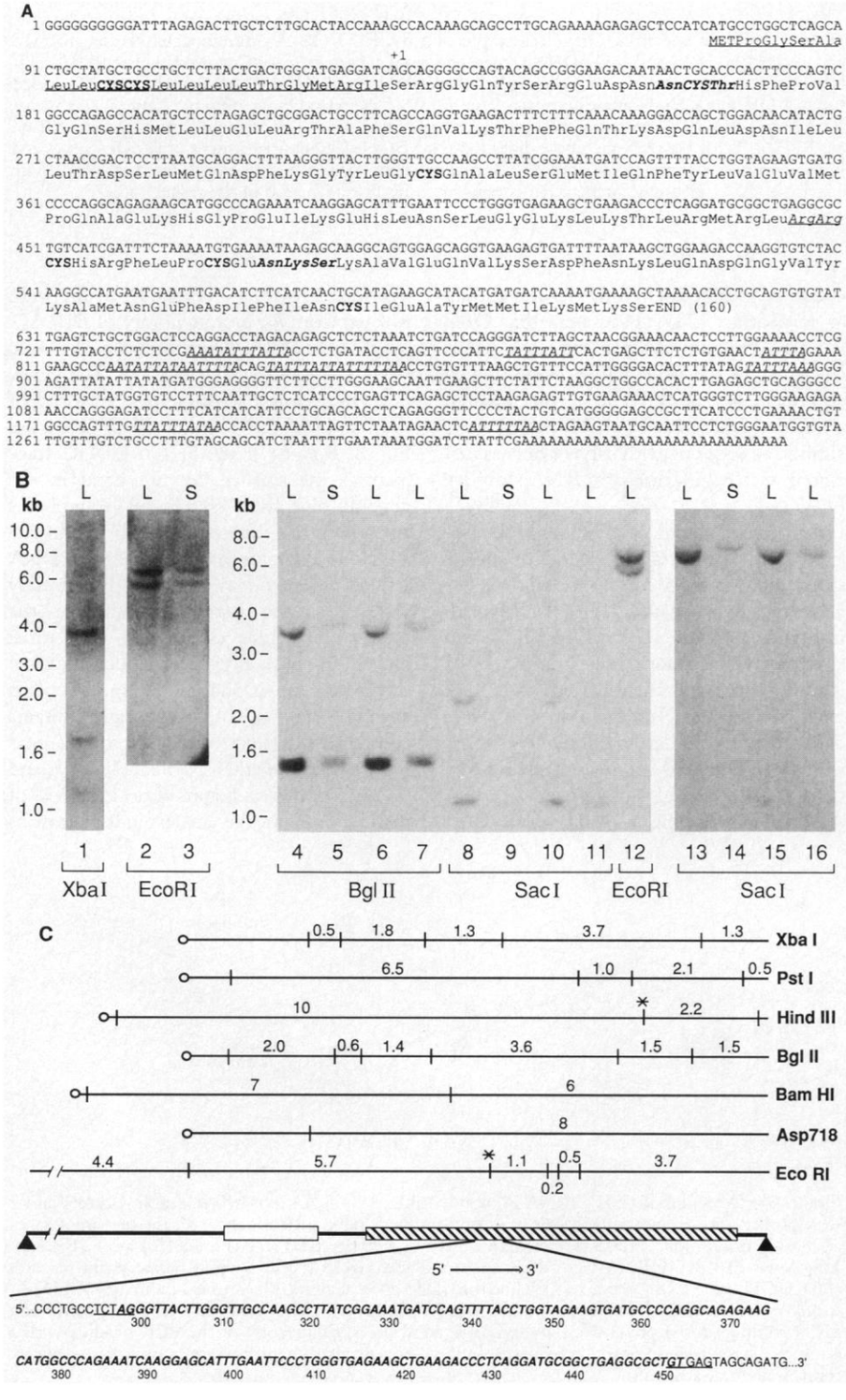

Fig. 3. (**A**) Nucleotide and predicted protein sequences of CSIF cDNA clone F115. Potential N-linked glycosylation sites are in boldface italic; cysteine residues are in boldface; the hydrophobic signal sequence is underlined; the predicted NH_2-terminal amino acid (*16*) is marked +1. A tandem ArgArg sequence, a possible site of proteolytic cleavage, is underlined. AT-rich regions that may regulate mRNA stability (*17*) are in underlined italic (*30*). (**B**) Detection of the mouse CSIF gene in mouse liver and sperm genomic DNA. Lanes 13 to 16 were probed with a mouse IL-3 cDNA (*18*); the other lanes show hybridization to the entire F115 cDNA insert. Lanes 1 to 3, 6, 10, and 15: BALB/c DNA; lanes 4, 5, 8, 9, and 12 to 14: AKR DNA; lanes 7, 11, and 16: C57BL/6 DNA. No restriction fragment length polymorphisms were detected with the combinations of restriction enzymes (Eco RI, Bgl II, Sac I, and Xba I) and mouse strains (BALB/c, C57BL/6, and AKR) used (*30*). (**C**) Restriction map and partial sequence of the mouse CSIF gene. Black triangles show boundaries (Eco RI linker sites) of the genomic DNA insert in the λ4 clone. Approximate sizes of restriction fragments are indicated in kilobases. For the indicated enzymes, sites to the left of the open circles were not mapped. The relative order of the adjacent 0.5-kb and 0.2-kb Eco RI fragments is uncertain. The region hybridizing to the F115 cDNA probe is indicated by the hatched bar. Restriction sites marked by an asterisk are also present in the F115 cDNA. The location of mouse repetitive DNA sequence elements is indicated by the open bar. The 5′ to 3′ orientation of the gene as suggested by partial DNA sequence analysis (*30*) is shown by the arrow. Genomic DNA sequence identical to the cDNA sequence (boldface italic) is numbered as in (**A**); splice signals at exon-intron boundaries are underlined (*31*).

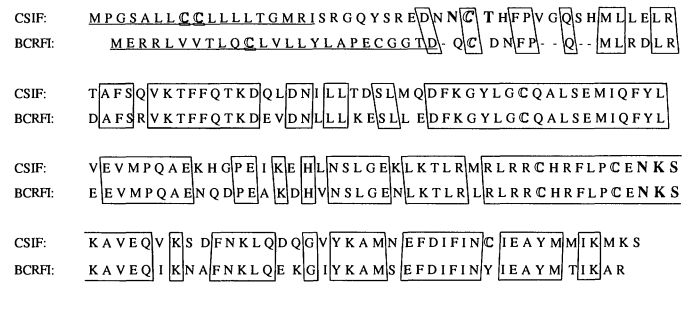

Fig. 4. Amino acid sequence homology shared by CSIF and EBV BCRFI (*9*). Boxed residues indicate identity between the two sequences. Putative N-terminal signal sequences are underlined. Potential N-linked glycosylation sites are in large bold face, and Cys residues are highlighted (*32*).

BCRFI protein is expressed or if it functions in either the life cycle of EBV or in its interaction with the immune system. However, primary EBV infection in adults (generally leading to acute infectious mononucleosis) is associated with substantial dysfunction in both T and B cell compartments of the immune system (*21*). Interestingly, IFN-γ inhibits the generation and outgrowth of EBV-transformed B cells in vitro (*22*). Since both CSIF and BCRFI (*23*) inhibit IFN-γ synthesis, an intriguing possibility is that EBV has exploited the biological activity of the product of a captured cytokine gene to manipulate the immune response against virally infected cells, thereby promoting survival of the virus. The expression of CSIF mRNA (Fig. 2A) and protein by the CH12 lymphoma and a family of related B lymphomas (*24*) is also of considerable interest in view of this cytokine's strong relationship to a protein encoded by a virus that immortalizes B cells. Greater insight into the CSIF-BCRFI relationship will develop as the biological activities of the two cytokines are further characterized. Conceivably, mimicry of host cytokines could be a defense mechanism also utilized by other pathogens or parasites.

That CSIF [IL-10; (*24*)] inhibits the effector functions of T_H1 cells suggests that it may play a role in determining the class of immune response directed against a particular antigen or foreign organism. CSIF therefore appears to be a suppressor factor for T_H1 immune responses. In this light, a human analog of CSIF (*19*) may have clinical significance in situations where inhibition of T_H1-like responses or IFN-γ synthesis is desirable, for example, autoimmunity or transplant rejection. CSIF might also be a useful adjuvant for immunization, since its suppression of T_H1-like effector function would likely result in a strong antibody response to the immunogen. Conversely, CSIF antagonists—blocking antibodies, soluble CSIF receptors, or the modified cytokine—might be useful in regulating T_H2-like responses. For example, synthesis of immunoglobin E is regulated principally by the cytokine IL-4 (*25*), a product of T_H2 cells, and IL-5 is a mediator of the growth of eosinophils (*26*), which play an important role in diseases such as asthma.

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19. Analysis of human CSIF cDNA clones isolated from a human T cell clone (P. Vieira *et al.*, in preparation) revealed that the human cytokine cDNA is likewise related to BCRFI only in the mature protein coding sequence, but is homologous to its murine counterpart throughout the coding sequence and 3′-untranslated region.
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24. Considering the expression of CSIF mRNA and protein by several cell types (Fig. 2; A. O'Garra *et al.*, in preparation), we and our colleagues speculated that CSIF might be pleiotropic. CSIF is a cofactor for proliferation of mast cell lines (L.-A. Thompson-Snipes *et al.*, in preparation) and thymocytes (I. MacNeil *et al.*, in preparation). We requested that the Nomenclature Committee of the International Union of Immunological Societies assign an interleukin designation for CSIF. The Committee declined to specify a name, but an inquiry to the editors of *Nature, Science, Proceedings of the National Academy of Sciences U.S.A., International Immunology, Journal of Experimental Medicine, Journal of Immunology*, and *Cell* revealed no known conflict with the designation IL-10. Thus we provisionally assign the name IL-10 to CSIF.
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27. COS7 cells were transfected by electroporation (*12, 13*). On day 4 after transfection supernatants were harvested and debris removed by centrifugation. CSIF-containing COS7 supernatants could be stored at 4°C for at least 1 week, or subjected to at least two freeze (−80°C)-thaw cycles without significant loss of activity. Supernatants were concentrated 10- to 20-fold on centricon 10 membranes (Amicon, Danvers, MA), then diluted to their original volumes with RPMI 1640 medium. Following a subsequent tenfold concentration, supernatants were tested in the CSIF bioassay (*8*) at concentrations of 10% or less of the concentrate. IFN-γ was assayed by ELISA (*8*) after 24 hours of culture. Units are defined by reference to a partially purified CSIF standard (*8*). In general, 1 unit of CSIF produces half the maximum inhibition of IFN-γ synthesis in a 0.1-ml culture in 24 hours.
28. COS7 cells were cultured with [^{35}S]Met with or without TcB2 (170 ng/ml) (*13*). CSIF was immunoprecipitated with monoclonal antibodies to CSIF as described (*12*). N-glycanase digestion of immunoprecipitated CSIF was done in the presence of EDTA, SDS, NP-40, and phenylmethylsulfonyl fluoride [G. Peltz, K. Frederick, C. L. Anderson, B. M. Peterlin, *Mol. Immunol.* **25**, 243 (1988)], for 18 to 24 hours at 37°C with N-glycanase (8 unit/ml; GENZYME, Boston, MA). SDS-PAGE (15 to 16% gels) was followed by treatment with Amplify (Amersham, Arlington Heights, IL) as recommended by the manufacturer and autoradiography for 24 to 96 hours.
29. RNA blots: Isolation of RNA, electrophoresis of polyadenylated RNA (I to 2 μg) or total RNA (10 to 15 μg) in formaldehyde–1% agarose gels, and hybridization conditions were as described [S. O. Gollnick, M. L. Trounstine, L. C. Yamashita, M. R. Kehry, K. W. Moore, *J. Immunol.* **144**, 1974 (1990)]. The CSIF probe was the 700-bp Pst I fragment at the 5′ end of F115 (Fig. 3). O1.2A3 cells were provided by D. Conrad [W. T. Lee and D. H. Conrad, *J. Immunol.* **136**, 4573 (1986)]. PCR assays: Mast cells were cultured overnight at 10^5 cells per milliliter in the presence of 1.2 μM ionomycin (Calbiochem, San Diego, CA) (*15*). Ionomycin stock solution was 1.5 mM in DMSO; controls were cells cultured alone or with only DMSO added. PCR was done for 40 cycles on reverse-transcribed total RNA with synthetic primers from the CSIF sequence that allowed detection of both the 1.5-kb and 1.0-kb mRNA species (primer 1: nucleotides 240 to 270, sense strand; primer 2: nucleotides 630 to 660, antisense strand). Control reactions in which reverse transcriptase was omitted were done for each RNA sample. Amplified fragments were detected initially by ethidium bromide staining in 1.5% agarose gels, and then by hybridization to an oligonucleotide probe (nucleotides 431 to 457, Fig. 3A) to verify specificity of amplification.
30. DNA sequence analysis was accomplished by the chain-termination method in either M13mp18/19 or Bluescript KS+ (Stratagene, La Jolla, CA) vectors with the Sequenase 2.0 kit (US Biochemical,

Cleveland, OH). The Intelligenetics Suite programs (Intelligenetics, Mountain View, CA) were used for data analysis. DNA blots: 10 to 15 μg of genomic DNA from either sperm [B. Hogan, F. Constantini, E. Lacy, in *Manipulating the Mouse Embryo* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1986), p. 107] or liver was digested with the indicated restriction enzyme, fractionated by electrophoresis through 0.9% agarose gels, blotted, and hybridized (*29*).

31. The restriction map of the λ4 clone was deduced from agarose gel band patterns of digests of the clone with individual restriction enzymes or pairs of enzymes. Plasmid subclones of the 5.7-kb and 3.7-kb Eco RI fragments and the 3.6-kb Bam HI–Hind III fragment were also analyzed. Mapping gels were blotted and hybridized to the entire F115 cDNA probe. Mouse repetitive DNA sequence elements were detected [M. Steinmetz *et al.*, *Cell* **24**, 125 (1981)] in a ~1.7-kb region between the Bgl II and Xba I sites as indicated by probing restriction digests of λ-phage clone DNA (1 μg per lane) with ^{32}P-labeled whole genomic DNA. A mouse CD23 cDNA [S. O. Gollnick *et al.* in (*29*)] containing repetitive elements in its 3′-untranslated region was used as a control.
32. Databases screened were the EMBL nucleic acid sequence bank release 20, Genbank release 61, Swiss Prot protein sequence database release 12, and Protein Identification Resource release 21. No other herpes virus sequences in the databases had any sequence with such a marked relationship to CSIF, although a weakly homologous sequence was found in the varicella zoster virus genome [A. J. Davison and J. E. Scott, *J. Gen. Virol.* **67**, 1759 (1986); nucleotides 31965 to 32133; 24% identity over 59 amino acids]. The search did not reveal any proteins with significant homology to the ORF in the 3′-untranslated sequence of F115, although we noted an isolated seven amino acid identity between this ORF (nucleotides 1144 to 1164; Fig. 3A) and amino acids 62 to 68 of a mouse T cell receptor Vβ sequence (D. L. McElligott, S. B. Sorger, L. A. Matis, S. M. Hedrick, *J. Immunol.* **140**, 4123 (1988).
33. The authors thank E. Mocarski, C. Martens, A. O'Garra, R. Kastelein, H. Rugo, and P. O'Hanley for helpful discussions, F. Vega for oligonucleotides, L. Thompson-Snipes, A. Zlotnik, and V. Dhar for providing cells for RNA preparation, Y.-H. Chien for providing the mouse genomic DNA library, and D. Nomura for assistance in homology searches. P.V. was supported by a DNAX Research Institute postdoctoral fellowship. DNAX Research Institute is supported by Schering-Plough Corporation.

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Induction of CD4$^+$ Human Cytolytic T Cells Specific for HIV-Infected Cells by a gp160 Subunit Vaccine

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Cytolytic T lymphocyte (CTL) responses were evaluated in humans immunized with recombinant human immunodeficiency virus type 1 (HIV) envelope glycoprotein gp160. Some vaccinees had gp160-specific CTLs that were shown by cloning to be CD4$^+$. Although induced by exogenous antigen, most gp160-specific CTL clones also recognized gp160 synthesized endogenously in target cells. These clones lysed autologous CD4$^+$ T lymphoblasts infected with HIV. Of particular interest were certain vaccine-induced clones that lysed HIV-infected cells, recognized gp160 from diverse HIV isolates, and did not participate in "innocent bystander" killing of noninfected CD4$^+$ T cells that had bound gp120.

THE HOST RESPONSE TO VIRAL INfection depends on the lysis of infected host cells by virus-specific CTLs (*1*). Several candidate HIV vaccines are soluble recombinant forms of the HIV envelope glycoproteins gp160 and gp120 (*2*). Soluble protein antigens do not normally elicit a CD8$^+$ CTL response because the processing pathways for exogenous protein antigens do not allow association of these antigens with class I major histocompatibility complex (MHC) molecules (*3, 4*). Soluble protein vaccines, therefore, probably will not elicit CD8$^+$, class I–restricted CTLs like infection with HIV does (*5, 6*). Instead, exogenous protein antigens associate with class II MHC and are recognized by CD4$^+$ T cells (*7*). Because some virus-specific CD4$^+$ T cell clones have cytolytic activity (*8*), soluble subunit vaccines may also elicit specific CTLs. However, the usefulness of such CD4$^+$ CTLs in limiting the spread of viral infection will depend on whether the processing of endogenously synthesized viral protein in infected cells permits association of viral antigen with class II MHC and subsequent recognition by CD4$^+$ CTLs induced with soluble exogenous forms of the same viral protein. In other viral systems, the processing of endogenously synthesized viral proteins for recognition by CD4$^+$ T cells has been documented in some (*9*), but not other (*3, 10*), cases.

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We analyzed the CTL response in human volunteers immunized with an HIV subunit vaccine that consisted of a purified recombinant form of the envelope glycoprotein precursor, gp160 (*11*). Healthy HIV-seronegative volunteers received intramuscular injections of 40 or 80 μg of recombinant gp160 (MicroGeneSys, Inc., West Haven, Connecticut) with alum at 0, 1, 6, and 18 months (*12*). Other volunteers received alum alone or hepatitis B vaccine according to the same immunization schedule. CTL responses were evaluated immediately before and 2 weeks after the 18-month boost. Peripheral blood mononuclear cells (PBMC) from vaccinees and controls were stimulated in vitro with gp160 and then tested 7 days later for cytolytic activity against autologous Epstein-Barr virus–transformed B lymphoblastoid cell lines (B-LCL) pulsed with gp160 (Fig. 1). Cultures from normal, nonimmune, HIV-seronegative donors (n = 10) and from control vaccinees immunized with alum (n = 2) or with a hepatitis B vaccine (n = 3) lacked antigen-specific CTL activity. The culture from one of eight gp160 vaccinees was positive for gp160-specific cytolytic activity immediately before the 18-month boost, even though this volunteer received only 40 μg of antigen and it had been over 1 year since the last boost. When retested 2 weeks after the boost, three of eight volunteers had gp160-specific CTL activity, including the volunteer who was positive before the boost. Thus repeated immunization with low doses of soluble HIV envelope protein induced a CTL response in a significant fraction of those immunized, and in some cases activity persisted for over 1 year.

To characterize the cells responsible for the gp160-specific cytolytic activity, positive cultures were restimulated with antigen and then cloned by limiting dilution; all positive cultures were successfully cloned (Table 1). All of the clones had the CD4$^+$CD8$^-$ phenotype. The isolation of CD4$^+$ clones was not due to selection against CD8$^+$ cells during the cloning procedure because positive bulk cultures consisted almost exclusively of CD4$^+$ lymphoblasts (*14*). The cytolytic activity of vaccine-induced gp160-specific T cell clones was class II MHC-restricted.

In infected cells, the envelope protein precursor gp160 is cleaved by a cellular protease into an NH_2-terminal fragment, gp120, that contains the CD4 binding site and a COOH-terminal fragment, gp41, that contains the hydrophobic fusion and transmembrane anchor domains (*15*). These subunits remain noncovalently associated on the surface of infected cells and on the envelope of HIV virions. HIV isolates have sequence variability in both subunits, but it