# Masking of the CBF1/RBPJ$_\kappa$ Transcriptional Repression Domain by Epstein-Barr Virus EBNA2

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Epstein-Barr virus (EBV) nuclear antigen 2 (EBNA2) is a transcriptional activator that is essential for EBV-driven B cell immortalization. EBNA2 is targeted to responsive promoters through interaction with a cellular DNA binding protein, C promoter binding factor 1 (CBF1). A transcriptional repression domain has been identified within CBF1. This domain also interacts with EBNA2, and repression is masked by EBNA2 binding. Thus, EBNA2 acts by countering transcriptional repression. Mutation at amino acid 233 of CBF1 abolishes repression and correlates with a loss-of-function mutation in the *Drosophila* homolog *Su(H)*.

Epstein-Barr virus is associated with several human malignancies including Burkitt's lymphoma, posttransplant lymphoma, Hodgkin's disease, and nasopharyngeal carcinoma (*1*). EBV establishes a latent infection in B lymphocytes and immortalizes them. One of the first viral genes expressed after EBV infection is EBNA2, which is essential for B cell immortalization (*2*). EBNA2 regulates the expression of genes necessary for viral latency and contributes to the changes in surface expression of B cell activation antigens that are induced by EBV infection (*1*, *3*). EBNA2 is a transactivator that contains a negatively charged activation domain but, unlike most transcription factors, EBNA2 does not bind directly to DNA. Instead, EBNA2 is targeted to responsive promoters through interaction with a cellular DNA binding protein, CBF1 (*4*–*6*). CBF1 was previously designated recombination binding protein J$_\kappa$ (RBP J$_\kappa$) (*7*, *8*), because it was thought to be a DNA binding protein involved in immunoglobulin V(D)J rearrangement. However, this assignment proved to be an artifact. CBF1 is ubiquitously expressed and binds to a consensus DNA sequence GTGGGAA (*9*, *10*). CBF1 exhibits transcriptional repression activity when its binding site is adjacent to the TATA box of the adenovirus *pIX* gene (*11*). However, CBF1 binding sites are located in distal settings in both EBNA2-responsive cellular promoters and responsive EBV latency promoters (for example -368 in the EBV latency C promoter). We therefore sought to evaluate CBF1 function with the use of distal binding sites that were nonoverlapping with other transcription factor recognition sequences.

Because CBF1 is ubiquitous in available cell lines, the binding specificity of CBF1 was altered by expression of a CBF1 protein as a fusion with the DNA binding domain of yeast GAL4 (GAL4 amino acids 1 to 147). Cotransfection of GAL4-CBF1 into HeLa cells led to downregulation of chloramphenicol acetyltransferase (CAT) expression directed by a herpes simplex virus thymidine kinase (TK) promoter that contains five GAL4 binding sites located 150 base pairs upstream of the TATA box (5xGAL4TKCAT). A dose response assay is shown in Fig. 1. Expression from a cotransfected TK promoter lacking the GAL4 binding sites (TKLuc) was not affected by CBF1, which indicates that repression was dependent on tethering of CBF1 to the target promoter.

Transcription is regulated by the interplay of positive and negative regulatory factors. Although transactivation has been the subject of intense scrutiny, the mechanisms of transcriptional repression are less well understood. The interaction between CBF1 and EBNA2 offers a model for dissection of these processes. To locate the domain within CBF1 that mediates repression, segments of CBF1 were expressed as fusions with the GAL4 DNA binding domain (Fig. 2A). Transfection of these constructions into HeLa cells with the 5xGAL4TKCAT target identified a repression domain between residues 179 and 361 (Fig. 2B). Unlike previously described repression domains, this region is not rich in alanine, glutamine, or proline (*12*–*14*). Cotransfection of EBNA2 with 5xGAL4TKCAT and GAL4-CBF1 revealed that EBNA2 retained the ability to interact with intact CBF1 when it was fused to GAL4(1–147) and produced a dramatic increase in CAT activity (Fig. 2C). Cotransfection of EBNA2 with the set of GAL4-CBF1 deletion constructions resulted in efficient transactivation by EBNA2 in the presence of GAL4-CBF1 fusion proteins containing CBF1 residues 179 to 500, 179 to 475, and 179 to 361. Thus, the minimal interaction domain for EBNA2 is located within the same 183–amino acid region of CBF1 as the repression domain (amino acids 179 to 361).

The co-localization of the EBNA2 interaction and transcriptional repression domains within CBF1 suggested that EBNA2 might mask the repression domain, perhaps competing with a repression mediator for CBF1 interaction. To address this question, we examined the ability of the EBNA2 variants illustrated in Fig. 3A to modify GAL4-CBF1–mediated repression. Co-

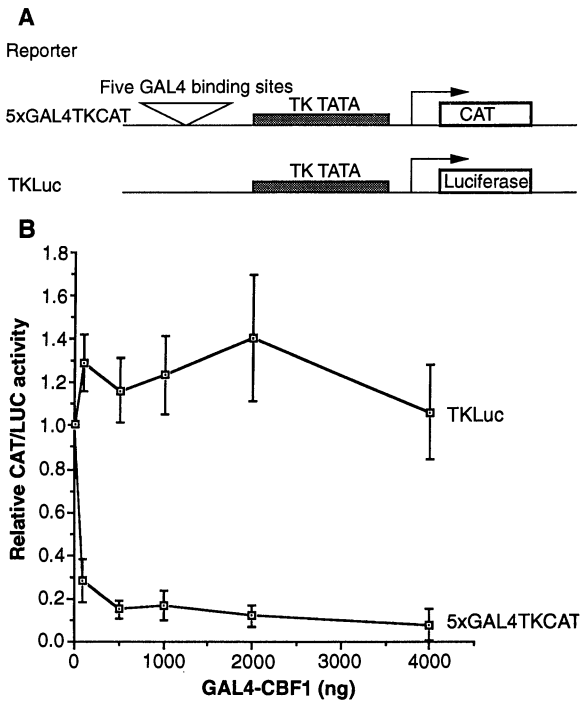


**Fig. 1.** GAL4-CBF1 represses expression from a target containing upstream GAL4 binding sites. (**A**) Reporter constructions used. 5xGAL4TKCAT has been described (*13*). TKLuc contains the TK promoter from 5xGAL4TKCAT cloned into the luciferase reporter pGL2BA (Promega) (**B**) Cotransfection assay in HeLa cells that received 5 μg of 5xGAL4TKCAT, 1 μg of TKLuc, and increasing amounts of GAL4-CBF1, which contains the human complementary DNA for CBF1 (*7*) expressed as a fusion with the GAL4 DNA binding domain (amino acids 1 to 147). Cells were harvested 40 hours after transfection. CAT and luciferase activities were measured as described (*9*). The results shown are an average of three experiments with standard deviation indicated. In all experiments, extracts were equalized for total protein. All plasmids expressing GAL4-CBF1 fusion proteins were sequenced and shown to be expressing comparable amounts of the appropriately sized proteins by immunoblot analyses with antibody to GAL4 (Upstate Biotechnology).

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transfection of wild-type EBNA2 with GAL4-CBF1 resulted in more than 75-fold activation of CAT expression from the 5xGAL4TKCAT target (Fig. 3B). A mutant EBNA2 (WW323SR) (*5*, *15*) that is unable to bind CBF1 did not transactivate and had no effect on GAL4-CBF1–mediated repression. However, a truncated EBNA2(1–425) that lacks the activation domain but retains the CBF1 interaction domain (*4*) alleviated CBF1-mediated repression, and CAT activity returned to the level seen in the absence of CBF1 (Fig. 3B). Therefore, EBNA2 has a dual strategy for modifying B cell gene expression: bringing an activation domain to responsive promoters and masking the repression domain of CBF1.

In an attempt to generate functional variants of CBF1, we engineered four mutations into the CBF1 repression domain (Fig. 4A). CBF1 proteins with mutations at amino acids 190 to 192, 306 to 308, and 327 to 330 retained the ability to repress expression from 5xGAL4TKCAT. Most dramatically, mutation at amino acids 233 to 235 (EEF233AAA) (*15*) resulted in a complete loss of repression function (Fig. 4B). Each of the mutant proteins retained the ability to mediate EBNA2 transactivation, which indicates that the mutations had not disturbed the overall conformation of this domain (Fig. 4C).

CBF1 contains a transferable repression domain that mediates repression when this domain is bound to sites 150 base pairs upstream of the RNA initiation site. Repression is also effective when binding sites are located as far as 500 base pairs upstream (*16*). Although repression may be mediated by DNA bending (*17*) or by competing with positive transcription factors for overlapping DNA binding sequences, neither of these mechanisms is likely here because the GAL4 binding sites were placed well upstream of the TATA box and did not impinge on other transcription factor binding sites, and DNA targeting was mediated by GAL4 binding and not by direct binding of CBF1 to DNA. Possible mechanisms include either direct interaction between the

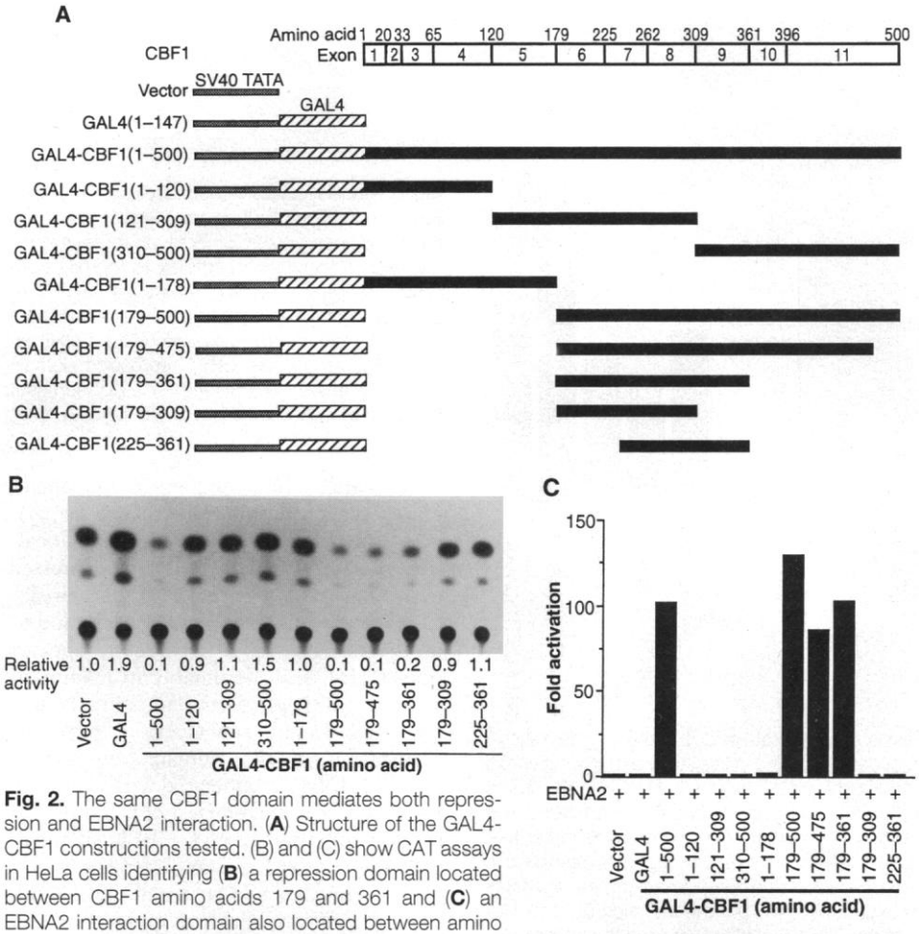
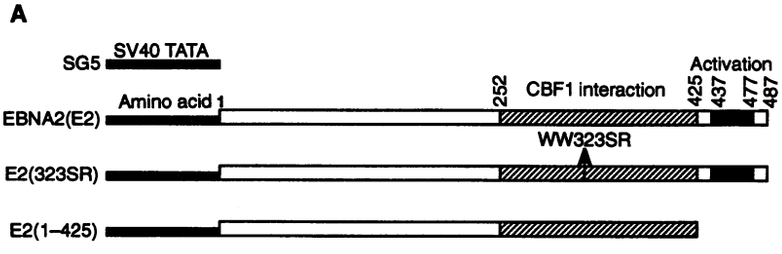



**Fig. 2.** The same CBF1 domain mediates both repression and EBNA2 interaction. (**A**) Structure of the GAL4-CBF1 constructions tested. (B) and (C) show CAT assays in HeLa cells identifying (**B**) a repression domain located between CBF1 amino acids 179 and 361 and (**C**) an EBNA2 interaction domain also located between amino acids 179 and 361. Transfections were done as in Fig. 1, but with 1 μg of the indicated GAL4-CBF1 fusion constructions. TKluc served as an internal control for transfection efficiency. An EBNA2-expressing plasmid (pPDL151; 1 μg) was included in the assay shown in (C).



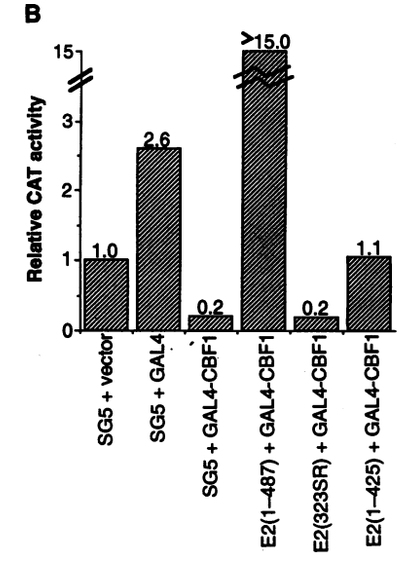




**Fig. 3.** Binding of EBNA2 to CBF1 masks repression. (**A**) Structure of the EBNA2 effector constructions used. (**B**) CAT assay in N18 neuroblastoma cells, showing that EBNA2(1–425), which lacks the COOH-terminal activation domain (*4*), abolishes CBF1-mediated repression. The mutant EBNA2(323SR) (*15*) is unable to bind to CBF1 (*5*) and does not affect repression. The indicated EBNA2 expression constructions were cotransfected with 5 μg of 5xGAL4TKCAT, 1 μg of TKluc, and 1 μg of GAL4-CBF1.

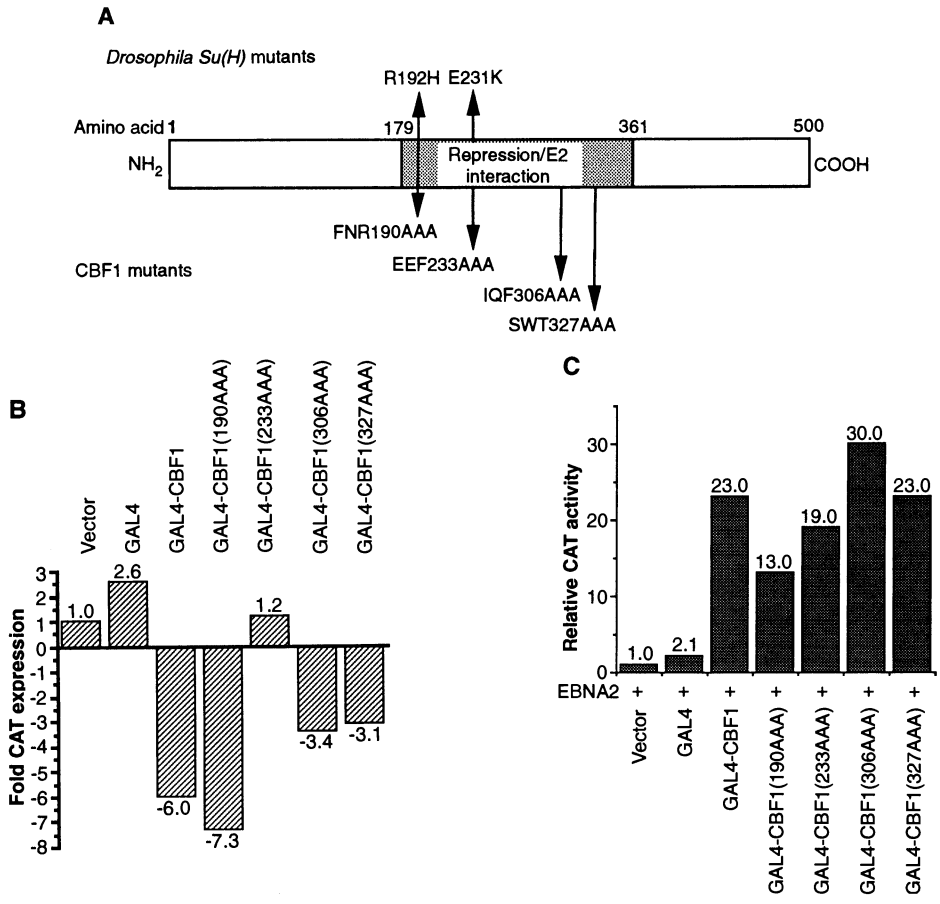



**Fig. 4.** Mutation at amino acid 233 abolishes CBF1 repression. (**A**) Diagram of CBF1, showing the relative locations of the repression domain and of mutations introduced into CBF1 in this study and in a published study (*18*) of the *Drosophila* homolog *Su(H)* (*15*). (B) and (C) show CAT assays in N18 neuroblastoma cells. *Su(H)* regulates sensory organ cell development (*19, 20*). Neuroblastoma cells were used in this assay and in Fig. 3 to provide a correlation with the *Drosophila* genetic data by demonstrating that repression by CBF1 also occurs in a neuronal cell background. Transfections were done as described in Fig. 3. (**B**) Three of the four mutations introduced into the CBF1 repression domain had only minor effects on CBF1-mediated repression of CAT expression from 5xGAL4TKCAT, but the mutation EEF233AAA (*15*) completely abolished repression function. (**C**) Each of the CBF1 mutations retained the ability to be transactivated by cotransfected wild-type EBNA2, which indicates that the mutations did not greatly alter CBF1 conformation and that the repression and EBNA2-interaction functions of CBF1 can be separated by mutation.

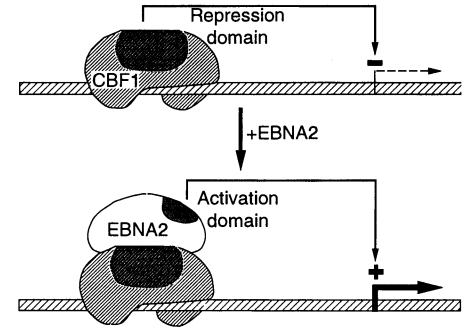



**Fig. 5.** Models for CBF1-mediated repression and EBNA2 counteractivation. As diagrammed, the repression domain of CBF1 directly interacts with the basal transcription complex to repress transcription. EBNA2 binding masks the CBF1 repression domain and introduces a powerful activation domain to positively upregulate transcription. Alternatively, the CBF1 repression domain may form a binding interface for a co-repressor protein that is displaced by EBNA2 binding.

CBF1 repression domain and components of the basal transcription complex (Fig. 5) or an indirect interaction mediated by tethering of a co-repressor to the CBF1 repression domain. EBNA2 binding masks the CBF1 repression domain and relieves repression. In the co-repressor scenario, EBNA2 binding would displace the co-repressor. The adenovirus E1A transactivator relieves repression by the cellular transcription factor YY1, which is a member of the Krüppel family of proteins (*13*). However, it is unclear whether masking of a repression domain occurs in that case.

CBF1 is highly conserved among humans, mice, and *Drosophila* (*18*). The *Drosophila* homolog of CBF1 is *Suppressor of Hairless* [*Su(H)*], a gene whose function is important for the appropriate development of sensory organ cells in the peripheral nervous system (*19, 20*). Knockout mutations in *Su(H)* cause lethality in the first day of pupal development. Mutation of amino acid 231 from glutamic acid to lysine in *Su(H)* causes a hypomorphic hairless phenotype, whereas mutation of amino acid 192 from arginine to histidine enhances the hairless phenotype (*19*). In our experiments, mutation at amino acids 190 to 192 (FNR190AAA) (*15*) showed a 20% increase in repressive activity, whereas mutation at amino acids 233 to 235 (EEF233AAA) (*15*) completely ablated repression. In light of these results, the Su(H) phenotypes are explicable as a loss of repression activity (amino acid 231) and an increase in repressive ability (amino acid 192). This focuses attention on the region around amino acids 192 to 233 as a core repression domain and potentially as the region for interaction with a co-repressor protein.

Recently, *Su(H)* has been shown to bind to the product of the *Drosophila* *Notch* gene, which encodes a transmembrane receptor. In transfection experiments in *Drosophila cells*, *Su(H)* was localized to the cytoplasm when coexpressed with *Notch* but was released and translocated to the nucleus when *Notch* bound to its ligand *Delta* (*21*). It therefore appears that *Su(H)*, and presumably in mammalian cells CBF1, are participants in a *Notch*-regulated signal transduction pathway. Truncation of the human homolog of *Notch*, *TAN-1*, is associated with human T lymphoblastic leukemias (*22*). Thus, disruption of the *TAN-1*–CBF1 signaling pathway can lead to tumorigenesis.

The prevalence of potential CBF1 binding sites in DNA sequences in the databases suggests that CBF1 may play an important role in downregulation of transcription in the resting B cell. Two pathways can be envisaged for transmission of B cell proliferation signals. Release of a bound protein from *TAN-1* would permit a *TAN-1*–CBF1 interaction that would retain CBF1 in the cytoplasm and relieve CBF1-mediated repression of that subset of genes containing CBF1 binding sites. In this scenario, EBNA2 is able to initiate B cell activation by directly blocking CBF1-mediated transcriptional repression and circumventing this signaling pathway. A second pathway could involve induced expression of a cellular antagonist of CBF1 whose function is mimicked by EBNA2.

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# Expression Cloning of a Protective *Leishmania* Antigen

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Parasite-specific $CD4^+$ T cells have been shown to transfer protection against *Leishmania major* in susceptible BALB/c mice. An epitope-tagged expression library was used to identify the antigen recognized by a protective $CD4^+$ T cell clone. The expression library allowed recombinant proteins made in bacteria to be captured by macrophages for presentation to T cells restricted to major histocompatibility complex class II. A conserved 36-kilodalton member of the tryptophan–aspartic acid repeat family of proteins was identified that was expressed in both stages of the parasite life cycle. A 24-kilodalton portion of this antigen protected susceptible mice when administered as a vaccine with interleukin-12 before infection.

The immunology of *Leishmania major* infection has been characterized in inbred strains of mice. The response is CD4-dependent, presumably reflecting the residence of the obligate intracellular amastigotes in macrophages within endolysosomal compartments that contain major histocompatibility complex (MHC) class II molecules (*1*). Thus, mice with disruption of the MHC class II or $\beta_2$-microglobulin genes are, respectively, susceptible or resistant to primary *L. major* infection, reflecting requirements for $CD4^+$ but not $CD8^+$ T cells (*2*). Most mice control an infection in association with the development of a type 1 T helper ($T_H1$) cell response that ensures production of the macrophage-activating cytokine interferon γ (IFN-γ), which is required for cure (*3*). In contrast, susceptible BALB/c mice develop a $T_H2$ cell response that is incapable of mediating parasite clearance and that interferes with the actions of $T_H1$-derived cytokines, primarily through the production of interleukin-4 (IL-4) (*4*).

The importance of $T_H1$ cells for the development of protective immunity against infection with *L. major* has been confirmed by means of clonally derived parasite-specific T cells. After adoptive transfer, a number of parasite-specific $CD4^+$ $T_H1$ cell clones and lines protected sublethally irradiated BALB/c mice (*5*, *6*). Although protective T cell clones belong to the $T_H1$ subset, some parasite-specific $T_H1$ T cell clones are not protective and even exacerbate the disease (*7*). Taken together, these results suggest that the ability of T cells to eliminate *L. major* from the infected host depends both on the cells' fine specificity and the type of cytokines that they secrete after antigen stimulation.

To identify antigens capable of eliciting this protective T cell response, we identified the parasite antigen recognized by the protective T cell clone 9.1-2, a $T_H1$ clone derived from spleen of BALB/c mice that had been vaccinated with an antigenic fraction separated from a soluble extract of *L. major* promastigotes (*6*). This T cell clone uses a heterodimeric $V_\beta4$, $V_\alpha8$ T cell antigen receptor (TCR) representative of the clonotypic, restricted TCR expansion that occurs early after the infection of mice with *L. major* (*8*). We used *L. major* promastigote mRNA to construct a complementary DNA (cDNA) library in prokaryotic expression vector pET3a-δ9 (*9*). This vector was designed for the high-level expression of cloned cDNA molecules in *Escherichia coli* as fusion proteins containing an epitope from influenza hemagglutinin (HA); this epitope is recognized by monoclonal antibody (mAb) 12CA5 (*10*). Pools of 5000 transformants were grown, and the expression of recombinant proteins was induced after infection with a recombinant λ bacteriophage carrying the T7 DNA polymerase gene. The recombinant proteins were purified from crude bacterial lysates by means of affinity columns and dialysis against isotonic buffer (*11*).

We incubated bone marrow–derived macrophages from BALB/c mice (*12*) in the presence of pools of recombinant proteins with mAb 12CA5 to facilitate targeting to MHC class II compartments through Fc receptor (FcR)–mediated internalization. Subsequently, LMR 16.2 T cell hybridoma cells, which were derived from the parasite-specific $T_H1$ clone 9.1-2 (*13*), were added, and the supernatants were screened for IL-2 secretion as an index of T cell activation. Three pools from ~150,000 cDNA clones induced IL-2 secretion that could be abolished by the addition of mAbs to $I\text{-}A^d$. Antigen-presenting cells from mismatched MHC class II mice (C57BL/6, CBA) did not stimulate IL-2 secretion from LMR 16.2 in this assay. One of the three pools was sequentially fractionated and rescreened until a single colony (clone 23.12.10.33) that gave rise to IL-2 secretion by hybridoma LMR 16.2 was identified.

The recombinant protein expressed by this clone had a molecular size of 24 kD. Incubation of the purified protein with LMR 16.2 or clone 9.1-2 resulted in the release of IL-2 or IFN-γ, respectively, in a dose-dependent manner (Fig. 1). Production of IFN-γ was inhibited by incubation with mAbs to either $I\text{-}A^d$ or CD4 (*14*). No IFN-γ was generated when cells were incubated with recombinant ovalbumin that had been fused with the HA epitope and produced in *E. coli* by the same method (*14*).

The 950–base pair (bp) cDNA insert from clone 23.12.10.33 hybridized to two *L. major* transcripts of 1500 and 1800 nucleotides as revealed by Northern (RNA) blot-

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