We have identified and characterized CIPER, a novel protein containing a caspase recruitment domain (CARD) in its N terminus and a C-terminal region rich in serine and threonine residues. The CARD of CIPER showed striking similarity to E10, a product of the equine herpesvirus-2. CIPER formed homodimers via its CARD and interacted with viral E10 but not with several apoptosis regulators containing CARDs including ARC, RAIDD, RICK, caspase-2, caspase-9, or Apaf-1. Expression of CIPER induced NF-κB activation, which was inhibited by dominant-negative IκB and a nonphosphorylatable IκB-α mutant but not by dominant-negative RIP. Mutational analysis revealed that the N-terminal region of CIPER containing the CARD was sufficient and necessary for NF-κB-inducing activity. Point mutations in highly conserved residues in the CARD of CIPER disrupted the ability of CIPER to activate NF-κB and to form homodimers, indicating that the CARD is essential for NF-κB activation and dimerization. We propose that CIPER acts in a NIK-dependent pathway of NF-κB activation.

The Rel/NF-κB family of transcriptional factors plays an important role in immune and inflammatory responses, cell survival, and stress response by regulating the expression of numerous cellular and viral genes (1). In most cells, nuclear factor κB (NF-κB) is composed of homodimers or heterodimers of 50-kDa (p50) and 65-kDa (p65) subunits that are sequestered in the cytoplasm by a member of the IκB family of inhibitory proteins (1). In response to certain cellular, bacterial, and viral stimuli, NF-κB is activated through the phosphorylation and inactivation of IκB (1). Active NF-κB is released and translocated to the nucleus and binds to cognate DNA sequences. Signaling through the tumor necrosis factor receptor (TNFR) family and interleukin-1/Toll receptors induces NF-κB activation. Tumor necrosis factor family members bind to their cognate receptors, including TNFR1, TNFR2, CD95 (Fas/APO-1), TRAMP (DR3/WSL-1/AIR/LARD), CD27, CD30, and CD40, and regulate cell proliferation, apoptosis, and proinflammatory responses (2, 3). Some members of the TNFR family such as TNFR1, CD95, and TRAMP, so-called death receptors, contain a “death domain” in their cytosolic C termini that is responsible for signaling cell death (4, 5). Ligand-induced trimerization of the TNFR1 and CD95 results in the recruitment of the death domain adapter molecules TRADD and FADD, respectively, which are critical for the activation of the apoptotic response (6–9).

Structural and functional analyses of TNFR family members and interleukin-1/Toll receptors have revealed the existence of common intracellular mechanisms that are responsible for signal transduction and biological activities. The interleukin-1 receptor and Toll signaling pathways utilize the adaptor protein MyD88 to recruit the serine-threonine kinases IRAK1 and IRAK2 to mediate NF-κB activation (10, 11). Activation of NF-κB through TNFR family stimulation is mediated by TNFR-associated factors (TRAFs) (12). Several TRAFs directly interact with the cytoplasmic domain of TNFR family members, whereas these adaptor molecules associate with TNFR1 via the adaptor molecule TRADD and the Ser/Thr kinase RIP (12). TRAFs and RIP activate the NF-κB-inducing kinase (NIK), which in turn activates the IκB kinases (13). The IκB-α and IκB-β kinases phosphorylate IκB leading to its degradation by the ubiquitin pathway and subsequent NF-κB activation (13).

Components of signaling pathways are frequently connected by the interaction of proteins that contain homology domains. In the cell death pathway, three types of homology interaction domains have been identified including the death domain, the death effector domain, and the caspase recruitment domain (CARD). The death domain is also present as a protein module in components of the interleukin-1 receptor/Toll signaling pathways that mediate NF-κB activation, notably the adaptor protein MyD88 and the IRAK1 and IRAK2 kinases (10, 11). The CARD was originally identified as a conserved sequence of about 90 amino acids present in several proteins of the cell death pathway including RAIDD, CED-3, caspase-1, caspase-2, caspase-9, and CED-4 (14). CARDs have been proposed to mediate the binding between adaptor molecules and caspases, such as the RAIDD-caspase-2 interaction, which allows the
recruitment of this caspase to the TNFR1 complex (15). In addition, CED-3 and caspase-9 are similarly associated with their regulators CED-4 and Apaf-1, respectively, through CARD interactions (16, 17). To identify novel regulators of intracellular pathways, we searched public data bases for expressed sequence tag (EST) clones with homology to the CARD of caspase-2. In this study, we report the identification and characterization of a novel CARD-containing protein, CIPER, which positively regulates both apoptosis and NF-κB activation.

**EXPERIMENTAL PROCEDURES**

**Isolation of the CIPER cDNA**—The partial nucleotide sequence of cDNAs encoding peptides with homology to the CARD domain of human caspase-2 (U13021) were found in EST data bases of GenBank using the TBLASTN program. The entire nucleotide sequence of EST clones 703916 and 574273 was determined by dideoxy sequencing.

**Northern Blot Analysis and In Situ Hybridization**—The entire cDNA insert of EST clone 703916 was radiolabeled by random priming using a commercial kit (Boehringer Mannheim) and applied for analysis of human poly(A)⁺ RNA blots from various tissues (CLONTECH Laboratories) according to the manufacturer’s instructions. For in situ hybridization, frozen sections containing mouse embryo tissues were processed as described (18). Each specimen was hybridized with a digoxigenin-labeled antisense RNA probe synthesized from a full-length mouse CIPER cDNA using an in vitro transcription kit (Boehringer Mannheim). As a control, a sense CIPER RNA-labeled probe was synthesized and used for hybridization as above. Hybridization, development, and mounting of slides were performed as described (18).

**Construction of Expression Plasmids**—The entire cDNA insert (1.8 kilobase pairs) of EST clone 574273 was cloned into the EcoRI and NotI sites of pcDNA3 (Invitrogen) to produce pcDNA3-CIPER. The entire open reading frame of CIPER was inserted into the BamHI and XhoI sites of pcDNA3-Flag or pcDNA3-Myc (19) to produce C-terminal Flag- or Myc-tagged CIPER. The E10 gene was amplified by polymerase chain reaction using equine herpesvirus-2 DNA (a gift from A. Davison, University of Glasgow, UK). Deletion mutants of pcDNA3-CIPER-N (1–119)-Myc, pcDNA3-CIPER-C (120–233)-Myc, and pcDNA-E10-Myc were constructed by a two-step polymerase chain reaction method (20). pcDNA3-caspase-8-AU1 pcDNA-Apaf-1-Myc and pcDNA3-caspase-2 DNA (a gift from A. Davison, Zymed Laboratories Inc.) were provided by D. V. Goeddel (Tularik, South San Francisco, CA).

**Transfection, Expression, Immunoprecipitation, and Immunodetection of Tagged Proteins**—5 × 10⁵ human 293 or 293T cells were transfected with expression plasmids by a calcium phosphate method as described (21). The total amount of transfected plasmid DNA was adjusted with pcDNA3 plasmid to be the same within individual experiments. After transfection, cells were harvested at different times and lysed with 0.2% Nonidet P-40 isonicotinic acid. For immunoprecipitation, 1 mg of soluble protein was incubated with 1 μg/ml of polyclonal anti-Myc antibody (Santa Cruz) overnight at 4 °C, and tagged proteins were immunoprecipitated with protein A-Sepharose 4B (Pierce) and pcDNA3-Flag or pcDNA3-Myc antibodies. Immunoprecipitated proteins or total lysates were subjected to 12% SDS-polyacrylamide electrophoresis and immunoblotted with monoclonal antibody to Flag (Kodak) or polyclonal anti-Myc antibody. MCF7 and HeLa cells were co-transfected with pcDNA3-β-gal and pcDNA3-shRNA plasmids. At least 300 cells from three random fields were counted in each experiment, and the data show the mean and S.D. of triplicate cultures. Data shown are representative of at least three independent experiments. Statistical significance was determined by one-way analysis of variance followed by Student-Neuman-Keuls post-doc comparisons.

**RESULTS AND DISCUSSION**

**Identification of CIPER**—To identify potential apoptosis regulatory genes, we searched public data bases of ESTs for clones with homology to the CARD of caspase-2/ICH-1 (14). Three human and two mouse ESTs containing overlapping nucleotide sequences with significant amino acid homology to the CARD of caspase-2 were identified. Sequence analysis of human and mouse cDNAs revealed open reading frames that encoded proteins of 233 amino acids (Fig. 1A). Both human and mouse proteins exhibited a high level of similarity (91% amino acid identity; Fig. 1B), suggesting that they represent the human and mouse orthologues. We have designated these proteins CIPER, αCasp3-3/ICH-1, E10-like regulator (see below). Analysis of the CIPER amino acid sequence revealed that it contained a N-terminal CARD with significant amino acid similarity to the CARD present in the prodomain of caspase-2/ICH-1, RAIDD, caspase-9, CED-3, CED-4, and Apaf-1 (Fig. 1C). Significantly, the CARD of CIPER was most homologous (51% identical) to E10, a protein encoded by the equine herpesvirus-2 gene (26), whose function remains unknown (Fig. 1C). Unlike caspases and other caspase regulators, the C-terminal region of CIPER had no significant similarity to any other protein in public data bases. Significantly, however, the C-terminal region of CIPER is rich in Ser/Thr residues (Fig. 1B).

**CIPER Is Expressed in Multiple Adult and Embryonic Tissues**—We performed Northern blot analysis to determine the distribution of CIPER RNA transcripts in various human tissues. CIPER was detected in all human tissues examined including spleen, lymph node, peripheral blood lymphocytes, heart, brain, placenta lung, skeletal muscle, and pancreas, as two transcripts of 2.5 and 1.3 kilobases (Fig. 2A). We also evaluated the distribution of CIPER mRNA at stage 15 of mouse embryonic development by in situ hybridization. In the mouse embryo, CIPER mRNA labeling was detected in the glangionic eminence and the ventricular zone of the developing brain, olfactory epithelium, tongue, whisker follicles, salivary gland, heart, lung, liver, and intestinal epithelia (Fig. 2B, a–c).

**CIPER Promotes Apoptosis**—To begin to elucidate the physiological function of CIPER, expression constructs producing Flag-tagged CIPER were introduced into 293T, HeLa, and MCF7 cells, which were subsequently observed for features of apoptosis. In these cells, expression of CIPER induced no or modest apoptotic activity when compared with control plasmid (Fig. 3A). We tested next whether CIPER could regulate apoptosis induced by caspase-8, a death protease coupled to death receptor pathways. Significantly, expression of CIPER augmented apoptosis induced by caspase-8 in all cell lines tested (Fig. 3A). Enhancement of caspase-8-mediated apoptosis induced by CIPER required a catalytic active caspase-8 because CIPER did not augment the level of apoptosis induced by a mutant caspase-8 protein with a single amino acid change (Cys to Ser) in the conserved active pentapeptide (2). Furthermore, caspase-8-induced apoptosis potentiated by CIPER was inhibited by the broad-based caspase inhibitors hactolovi-
rus p35 and zVAD-fmk (2). To further verify these observations, we transfected 293 cells with the CIPER cDNA and control plasmid and isolated stably transfected clones that expressed CIPER protein (see Fig. 5). Consistent with the results in transient transfection assays, CIPER expression enhanced apoptosis induced by TNFR1 and CD95 stimulation in stably transfected clones (Fig. 3, B and C). In addition, we also tested the ability of CIPER to modulate apoptosis induced by FADD, TRADD, and caspase-4, -9, and -10. CIPER enhanced apoptosis induced by all these pro-apoptotic proteins (2).

CIPER Induces NF-κB Activation—Signaling through the TNFR1 pathway activates both apoptosis and NF-κB activation (3, 27, 28). To test whether CIPER activates NF-κB, CIPER expression plasmids were co-transfected with the NF-κB reporter plasmid, pBIIx-Luc, or control plasmid lacking NF-κB binding sites in 293 cells. CIPER induced NF-κB activation in a dose-dependent manner (Fig. 4A). Induction of NF-κB activation by CIPER was specific in that transfection of 293 cells with expression plasmids producing ARC and RAIDD, two CARD-containing proteins did not promote NF-κB activation. Consistent with its high level of homology, expression of E10, a product of the equine herpesvirus-2, also induced NF-κB activation (Fig. 4A). To determine the region of CIPER required for NF-κB activation, we engineered two deletion mutants, CIPER-N (1–119) and CIPER-C (120–233), to express the N-terminal half including the CARD and C-terminal half that is rich in Ser/Thr amino acids, respectively. CIPER-N (1–119), but not CIPER-C (120–233), induced NF-κB activation (Fig. 4A), indicating that the N-terminal half of CIPER that includes the CARD is sufficient and essential for NF-κB activation (Fig. 4A). Immunoblotting analysis revealed that wt and mutant forms of CIPER were expressed at comparable levels, suggesting that the loss of function was not due to insufficient levels of expression (Fig. 4A). Interestingly, CIPER was detected as a doublet of 28 and 31 kDa (Fig. 4A). Treatment of lysates with

![Fig. 1. Deduced amino acid sequence, domain structure, and alignment of CIPER. A, schematic representation of CIPER. Numbers correspond to amino acid residues shown in B. The CARD is indicated by a closed box. B, amino acid sequences of human and mouse CIPER. Conserved identical and nonidentical residues are indicated by asterisks and dots, respectively. CARDs are indicated by boxes. Hydrophobic and aromatic amino acid residues are shown by shading; positive and negative charged residues are shown by dark and light gray shading, respectively. α helix and β strand breakers are shown by bold letters. The nucleotide sequences of human and mouse CIPER are available as accession numbers AF057700 and AF057701 in the GenBank™ database. C, alignment of CARDs of CIPER, E10 (U20824), RAIDD (U79115), caspase-2 (U13021), CED-3 (L29052), caspase-9 (U56390), CED-4 (X69016), and Apaf-1(AF013263). Conserved H1–8 α helices are shown according to the three-dimensional structure of RAIDD (37).](https://example.com/fig1.png)

![Fig. 2. Tissue distribution of CIPER. A, expression of CIPER in human tissues by Northern blot analysis. PBL, peripheral blood lymphocytes. B, expression of CIPER in mouse embryo at E15d. a, specific labeling is observed in ventricular zone (VZ), olfactory epithelium (OF), ganglionic eminence (GE), salivary gland (SG), tongue (T), whisker follicles (W), fat deposits (FD), heart (H), lung (LU), and intestinal epithelium (I). b and c, high magnification of salivary gland (b) and lung (c). d, labeling with sense CIPER probe.](https://example.com/fig2.png)
FIG. 3. Regulation of apoptosis by CIPER. A, CIPER potentiates apoptosis induced by caspase-8. 293T, HeLa, and MCF7 cells were transfected with pcDNA3 or pcDNA3-CIPER-Flag (1 μg) in the presence or absence of pcDNA3-caspase-8-AU1 (0.2 μg). The percentage of specific apoptosis was calculated as described under “Experimental Procedures” in triplicate cultures. B and C, CIPER enhances apoptosis induced by TNFR1 or CD95 in stably transfected 293 clones. Cell clones stably transfected with CIPER or control plasmid were transiently transfected with pcDNA3, pcDNA3-TNFR1 (0.5 μg), or pcDNA3-Fas (1 μg) as indicated. Apoptosis was determined as described under “Experimental Procedures” in triplicate cultures.

FIG. 4. CIPER induces NF-κB activation. A, NF-κB activation by CIPER in 293 cells. 293 cells were transfected with 0, 0.02, 0.05, 0.1, 0.2, and 0.5 μg of pcDNA3-CIPER-Myc (WT), 0.5 μg of pcDNA3-CIPER-N(1–119)-Myc (N), or pcDNA3-CIPER-C(120–233)-Myc (C) in the presence of pBIIx-Luc or control plasmid pf-Luc in indicated lanes. Cell lysates were prepared, and luciferase activity was measured as described under “Experimental Procedures.” CIPER-Myc proteins in the same lysates were subjected to 12% SDS-polyacrylamide gel electrophoresis and immunoblotting with polyclonal anti-Myc Ab (inset). B, NF-κB activation by CIPER in Jurkat-T cells. Jurkat-T cells were transiently transfected with pcDNA3 or pcDNA3-CIPER-Flag (3 μg) plus HIV-CAT and ΔεB-HIV-CAT as indicated. Cell lysates were prepared, and CAT activity was measured as described under “Experimental Procedures.”
Novel CARD-containing Protein CIPER Induces NF-κB Activation

To further verify the NF-κB-activating ability of CIPER, we developed 293 cell clones by stable transfection with expression plasmids producing wt, CIPER-N (1–119), CIPER-C (120–233), and CIPER(L41Q). Immunoblotting analysis of two independently derived clones transfected with each construct showed that they expressed wt and mutant CIPER protein (Fig. 5C). In agreement with the transient assay shown in Fig. 5B, clones expressing wt or mutant CIPER containing the CARD (residues 1–119) expressed significantly more NF-κB activity (15–70-fold) than clones transfected with empty vector (Fig. 5C). Significantly, clones expressing the point mutant (L41Q) or CIPER-C (120–233), a mutant with deletion of the CARD, did not express significant NF-κB activity (Fig. 5C), confirming that the CARD is essential for the ability of CIPER to activate NF-κB. We also tested the ability of CIPER mutants to promote apoptosis. These experiments showed that CIPER-N (1–119) and CIPER(S231A) promoted TNFR1- and CED-4 mediated apoptosis, but the CARD mutants, CIPER(L41Q), CIPER(G79R), and CIPER(L41Q/G79R) did not.

NF-κB Activation Induced by CIPER Is Inhibited by Dominant Repressor Forms of NIK or IκBα but Not by Dominant-negative RIP—To determine the molecular ordering of CIPER relative to other components of the tumor necrosis factor signaling pathway, CIPER was co-expressed with dominant repressor forms of IκB and RIP, and NIK. The double mutation S32A/S36A of IκBα cannot be phosphorylated or degraded and therefore blocks the nuclear translocation of NF-κB and transactivation of NF-κB-responsive genes (30). The RIP (558–671) and NIK(KK429–430AA) mutants act as dominant inhibitors of the endogenous kinases whose activities are required for NF-κB activation (8, 31). NF-κB activation induced by CIPER was blocked by overexpression of dominant repressor forms of CARDs of CED-3 and RAIDD are critical for heterodimerization with CED-4 and caspase-2, respectively (15, 29). In addition, we constructed another point mutation, CIPER(S231A), outside the CARD as a control (Fig. 5A). Immunoblotting experiments showed that these mutants expressed CIPER protein (Fig. 5). We used transient co-transfection of plasmids expressing CIPER mutants and a NF-κB reporter plasmid into 293 cells to determine the regions of CIPER required for NF-κB activation. The analysis revealed that wt, CIPER-N (1–119), and CIPER(S231A) promoted NF-κB activation, whereas the CARD point mutants CIPER(L41Q), CIPER(G79R), CIPER(L41Q/G79R), and CIPER-C (120–233) did not (Fig. 5B).

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IκB and NIK but not by dominant-negative RIP (Fig. 6). The RIP mutant was functional, because it inhibited NF-κB activation induced by TNFR1 stimulation (Fig. 6). These results suggest that CIPER functions upstream of NIK and IκB, and downstream of or in parallel to RIP in a NF-κB activation pathway. These results are compatible with published reports showing that TRAFs and RIP activate NIK, which in turn activates the IκB kinases (13).

CIPER Self-associates and Interacts with Viral E10—The CARD has been proposed to mediate signaling through homophilic interactions (14). We therefore tested the ability of CIPER to associate with other CARD-containing proteins. Expression constructs producing several HA- and Flag-tagged apoptosis regulatory proteins and Myc-tagged CIPER were transiently co-transfected in 293T cells. Cell lysates were immunoprecipitated with anti-Myc Ab, and co-immunoprecipitated proteins were analyzed by immunoblotting with anti-Flag Ab. The analysis shown in Fig. 7A revealed that CIPER co-immunoprecipitates with viral E10, the molecule to which it associates in the CARD. 293T cells were transiently transfected with pcDNA3-CIPER-Flag (4 μg) and pcDNA3-Apa1-1-Myc (9 μg), pcDNA3-E10-Myc (3 μg), or pcDNA3-CIPER-Myc (3 μg) plasmids in indicated lanes. CIPER-Myc proteins were immunoprecipitated with anti-Myc polyclonal Ab, and co-immunoprecipitated proteins were detected by immunoblotting with anti-Flag (top panel). CIPER-Flag and Myc-tagged proteins in cell lysates were detected by polyclonal Ab to Myc (middle panel) and monoclonal Ab to Flag (bottom panel), respectively. WT, wild type; WB, Western blot; IP, immunoprecipitation.

The CARD was essential for CIPER function because mutations in residues that are conserved in the CARD destroyed the ability of CIPER to homodimerize, whereas the S231A mutation outside the CARD did not (Fig. 7B). Moreover, the CIPER-N (1–119) mutant that contains the CARD dimerized with wt CIPER, whereas the CIPER-C (120–233) mutant did not (Fig. 7B), indicating that the CARD mediates CIPER homodimerization. Immunoblotting analysis revealed that wt and mutant forms of CIPER were expressed at comparable levels, ruling out that the loss of function was due to insufficient levels of expression (Fig. 7B). These results indicate that the CARD is necessary and sufficient for CIPER homodimerization.

We have identified a novel CARD-containing protein, CIPER, that can activate NF-κB. The CARD of CIPER exhibits a high level of similarity to that of E10, a gene product of the equine herpesvirus-2. Like its cellular homologue, expression of E10 induced the activation of NF-κB. Although the mechanism by which E10 activates NF-κB remains to be elucidated, it is possible that E10 mediates NF-κB activation via its association with CIPER. This model would be compatible with the observation that CIPER self-associates via its CARD, a process that might be required for NF-κB activation. Alternatively, both CIPER and its viral homologue E10 might activate NF-κB through a common cellular target. The signaling pathway(s) in which CIPER acts to promote NF-κB activation remains unclear. NF-κB activation induced by CIPER was blocked by dominant-repressor forms of IκB or NIK but not by dominant-negative RIP. These results suggest that CIPER acts downstream of RIP and upstream of NIK and IκB in a RIP-dependent NF-κB activation pathway. The results are also compatible with a model in which CIPER activates a NIK-regulated pathway of NF-κB activation distinct from that signaled through RIP. Several signaling pathways that activate NF-κB including those induced by CD40, interleukin-1/Toll receptors are RIP-independent (10, 11, 32).

The CARD was essential for CIPER function because mutations in residues that are conserved in the CARD destroyed the ability of CIPER to promote NF-κB activation. The essential role of the CARD could reflect a requirement for CIPER to recruit additional protein(s) via the CARD. For example, CIPER self-association might mediate oligomerization of CIPER-binding factor(s), a process perhaps essential for signaling NF-κB activation. In this model, the CARD of CIPER might serve as a domain that would bring into close proximity effector
molecules that mediate NF-κB activation. This model would be reminiscent of the model proposed for Apaf-1 in the cell death pathway, in which oligomerization through the CARD would bring into proximity two catalytic domains of caspase-9, thus initiating proteolysis (17). CIPER did not bind to RICK/RIP2, NIK, and TRAF1, -2, -3, and -6, proteins that can activate NF-κB (12, 32–34). Identification of CIPER-interacting proteins might well reveal the physiological function of CIPER.

The CARD was first described as a peptide module present in the prodomains of upstream caspases and adaptor molecules such as CED-4/Apaf-1 and RAIDD that mediates the recruitment of caspases (14, 35). Indeed, RAIDD and Apaf-1 can recruit caspase-2 and caspase-9 via CARD interactions (15, 16, 19). However, we found that CIPER is unable to bind CARD-containing caspases and caspase regulators such as RAIDD, ARC, or Apaf-1. These results suggest that CARDs may mediate interactions not only between caspase regulators and caspases but also between cellular transducers of the NF-κB activation pathway. Similarly, another CARD-containing protein, RICK/RIP2, is a Ser/Thr kinase that interacts with and regulates the tumor necrosis factor family of receptors by binding to adaptor molecules devoid of caspase activity (32, 33). Like CIPER, RICK/RIP2 promotes both NF-κB activation and apoptosis (32, 33). These results suggest a broader role for CARD domains in cellular signaling events than previously appreciated. Activation of NF-κB has been shown to provide an anti-apoptotic signal in certain cells (27). It is likely, therefore, that the apoptosis regulatory function of CIPER may depend on the cellular context. While this manuscript was being reviewed, another group identified a gene, bel-10, at the breakpoint of the recurrent t(1;14)(p22;q32) chromosomal translocation of mucosa-associated lymphoid tissue (MALT lymphoma) (38). The sequence of CIPER is identical to that of bel-10.

Acknowledgments—We are grateful to M. Benedict, L. del Peso, and Y. Hu for critical review of the manuscript, C. Vincenz and M. Dyer for stimulating discussions, and A. Davison, V. Dixit and D. Goeddel for reagents.

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