main of Igβ is sufficient to trigger two antigen-independent responses by a tyrosine-mediated mechanism establishes a direct link between mμ, Igβ, and tyrosine kinases in developing B cells.

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19. Bone marrow total RNA (5 μg) was subjected to electrophoresis in formaldehyde-containing agarose gels, transferred to nylon membranes, and blotted with antisense RNA probes. The μ probe covers the VD and J regions of the transgenic V region exon (*34*), and glyceraldehyde-3-phosphate dehydrogenase mRNA was used as a loading control [M. Piechaczyk *et al.*, *Nucleic Acids Res.* **12**, 6951 (1984)]. The starting construct for transgene production was a Hind III–Bam HI fragment of the human μ gene that contained constant and membrane exons modified to direct the synthesis of only the membrane form of human μ (*8*). A murine V region and enhancer (*34*) were added to increase the efficiency of transgene expression. In addition, Bam HI and Not I unique sites were placed around the membrane exons to allow for rapid replacement of the wild-type membrane coding sequence with specifically modified transmembrane domains (*17*). Transgenic mice were created by injection of purified DNA into *RAG-1*$^{-/-}$ × FVB/n F1 oocytes [R. R. Palmiter and R. L. Brinster, *Annu. Rev. Genet.* **20**, 465 (1986)], and founders were backcrossed to *RAG-1*$^{-/-}$ mice (*4, 5*).
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35. We thank J. Ravetch, R. M. Steinman, E. Spanopoulou, and members of our laboratory for helpful comments and suggestions. Supported by the Howard Huges Medical Institute and by NIH grants AI33890 and AI37526 to M.C.N.

2 November 1994; accepted 3 January 1995

FAP-1: A Protein Tyrosine Phosphatase That Associates with Fas

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Fas is a cell surface receptor that controls a poorly understood signal transduction pathway that leads to cell death by means of apoptosis. A protein tyrosine phosphatase, FAP-1, capable of interacting with the cytosolic domain of Fas, was identified. The carboxyl terminal 15 amino acids of Fas are necessary and sufficient for interaction with FAP-1. FAP-1 expression is highest in tissues and cell lines that are relatively resistant to Fas-mediated cytotoxicity. Gene transfer–mediated elevations in FAP-1 partially abolished Fas-induced apoptosis in a T cell line. These findings are consistent with an inhibitory effect of FAP-1 on Fas signal transduction.

Fas (also known as APO-1 and CD95) is a cell surface receptor that is expressed on a variety of normal and neoplastic cells. It shares significant amino acid sequence homology with several members of the tumor necrosis factor receptor (TNFR) family, including p55-TNFR, CD40, and the p75–nerve growth factor receptor (NGFR), which have been shown to act as either inhibitors or inducers of cell death (*1*). The ligand for Fas is expressed predominantly on cytolytic T cells (*2*), suggesting that Fas plays a role in the effector branch of cellular immune responses. Mutations in the genes encoding Fas or its ligand have been associated with lymphoproliferative and autoimmune disorders in mice (*3*). Furthermore, alterations in Fas production have been associated with autoimmune disease in humans and susceptibility to induction of apoptosis of T cells in human immunodeficiency virus–infected persons (*4*).

Monoclonal antibodies specific against Fas induce apoptosis in many types of cells (*5*). However, in some cases antibodies to Fas stimulate cell proliferation (*6, 7*), suggesting that the intracellular signal transduction pathways used by this receptor are subject to regulation. The cytosolic domain of Fas contains no similarity to known kinases or other enzymes that might transduce signals into cells. Deletion mapping analysis has identified a domain that is required for induction of apoptosis, which is called the "death domain." This domain shares homology with sequences located in the cytosolic domains of p55-TNFR1, CD40, and p75-NGFR (*8*). For some cells, apoptosis induced by antibodies to Fas is dependent on protein synthesis inhibitors such as cycloheximide (*8*). This implies either the existence of a labile protein that suppresses Fas-generated signals leading to cell death or the induction of proteins that inhibit the ability of Fas to trigger apoptosis. A negative regulatory domain has been mapped to the COOH-terminal 15 amino acids of Fas that is not homologous to other TNFR-like proteins; deletion of this domain can abrogate the dependence on protein synthesis inhibitors for apoptosis induced by antibody to Fas (*8*).

To identify complementary DNAs (cDNAs) encoding proteins that can potentially modulate the activity of Fas, we used a yeast two-hybrid system for cDNA library screening. We used the cytosolic domain of human Fas fused to a LexA DNA binding domain (*9, 10*) and random cDNAs fused in frame with a VP16 transactivation domain. Using a His synthetase gene (*HIS3*) under the control of LexA operators as a reporter, we identified 395 His^+ colonies from an initial screen of 3×10^8 transformants. Of these, 84 were also positive when a *lacZ* gene (β-galactosidase) under the control of a LexA operator was used as an alternative reporter. Mating tests were then performed (*11*); only 2 of the 84 candidate clones (numbers 31 and 43) reacted with the cytosolic domain of Fas (Ta-

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ble 1). These mouse cDNAs represented overlapping independent clones with insert sizes of 381 base pairs (bp) (clone 31) and 351 bp (clone 43); these clones share >95% homology with a human cDNA sequence encoding a protein tyrosine phosphatase (PTP) termed PTP-BAS (*12*).

PTP-BAS was originally cloned from human basophils by a reverse transcriptase–polymerase chain reaction (RT-PCR) method that used degenerate primers targeted against conserved sequences found in PTPs. Three isoforms arising from alternative splicing have been identified by cDNA cloning, the longest of which is predicted to encode a 2485–amino acid protein (*12*). PTP-BAS lacks a transmembrane domain but contains a membrane-binding domain similar to that found in the cytoskeleton-associated proteins ezrin, radixin, moesin, and protein 4.1, as well as in the PTPs PTPH1, PTP-MEG, and PTPD1 (*12*, *13*). In addition to a catalytic domain located near its COOH-terminus, PTP-BAS contains six repeats (Gly-Leu-Gly-Phe; GLGF) (Fig. 1). These structures are thought to mediate intra- and intermolecular interactions among protein domains and may play a role in targeting proteins to the submembranous cytoskeleton or in regulating enzyme activity (*12*). Both Fas-interacting clones 31 and 43 correspond to the third GLGF repeat in PTP-BAS (Fig. 1), suggesting that this domain mediates specific interactions with the cytosolic domain of Fas and implying that PTP-BAS is a Fas-associated phos-

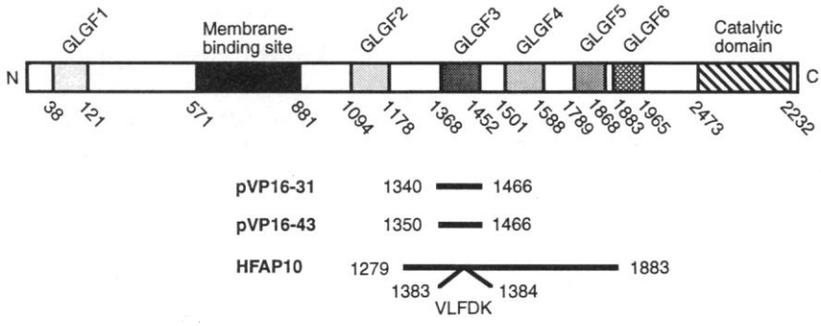

Fig. 1. Diagram of FAP-1 protein and cDNA clones. (**Top**) The structure of the 2485–amino acid human FAP-1 protein (also known as PTP-BAS type-1) (*12*), showing the locations of the catalytic, membrane-binding, and GLGF repeat domains (*12*, *13*). (**Bottom**) The cDNAs identified by two-hybrid screening of a mouse embryo cDNA library: pVP16-31 and pVP16-43 with encoded amino acids relative to the human FAP-1 protein. A human FAP-1 partial cDNA (encoding amino acids 1279 through 1883 of PTP-BAS type-1) from brain was cloned and found to contain a five–amino acid insert in the GLGF3 domain, as compared with the published sequence (*12*). V, Val; L, Leu; F, Phe; D, Asp; and K, Lys. GenBank accession numbers of pVP16-31, pVP16-43, and HFAP10 are L34581, L34582, and L34583, respectively. C, COOH-terminal; N, NH_2-terminal.

Table 1. Specific interaction of FAP-1 with the Fas cytosolic domain. L40 strain cells with pVP16-31 or pVP16-43 were cured of plasmid pBTM116-Fas and then mated with NA87-11A cells transformed with various pBTM116 plasmids as described (*24*). Growth was measured on His-deficient media, and color was measured by a β-galactosidase colorimetric filter assay (*22*).

Proteins	pVP16	Growth	Color
Fas(191–335)	31 and 43	+	Blue
Ras(V12)	31 and 43	–	White
Ras(L35R37)	31 and 43	–	White
CD40(216–277)	31 and 43	–	White
CD40(225–269)	31 and 43	–	White
Bcl-2(83–218)	31 and 43	–	White
Lamin	31 and 43	–	White
Ras(V12)	c-Raf	+	Blue
Lamin	c-Raf	–	White

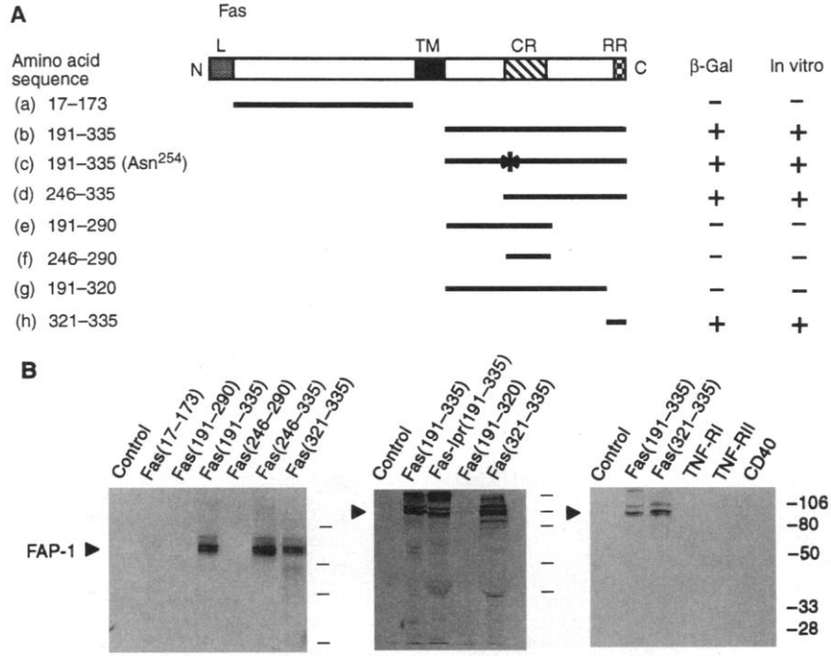

Fig. 2. Mapping of the site on Fas involved in binding to the GLGF3 domain of FAP-1. (**A**) The structure of the Fas protein is depicted with the relative locations of its leader sequence (L), transmembrane domain (TM), conserved cytosolic regions (CR), and unique regulatory region (RR) indicated. A series of Fas deletion mutants were generated (*14*) that contained (a) the extracellular domain of Fas minus its leader sequence (amino acids 17 through 173); (b) the complete cytosolic domain of Fas (191 through 335); (c) a cytosolic domain mutant containing a Val→Asn^{254} mutation (indicated by an asterisk), analogous to an identified mutant allele of Fas in the cg-strain of *lpr* autoimmune mice, which has been shown to be deficient in Fas-mediated induction of apoptosis (*8*); (d) a deletion mutant (246 through 335) lacking the sequences between the TM of Fas and the CR that are required for the induction of apoptosis (*8*); (e) a COOH-terminal deletion mutant (191 through 290) that contains sequences from the TM to the end of the CR; (f) a mutant containing only the CR of Fas (246 through 290); (g) a truncation mutant lacking the COOH-terminal 15 amino acids (191 through 320) that have been shown to constitute a negative RR in Fas (*8*); and (h) a mutant containing only the COOH-terminal 15 amino acids of Fas (321 through 335). They were expressed in L40 strain yeast cells as LexA DNA binding domain fusion proteins with VP16–FAP-1 (clone 31). Protein-protein interactions were detected through the use of a *lacZ* reporter gene under the control of LexA operators with β-galactosidase plate and filter assays (*22*) and scored as positive (blue, +) or negative (white, –). Essentially identical results were obtained in EGY48 strain yeast (*25*). (**B**) These same Fas cDNAs were also subcloned in frame into either pGEX–4T-1 or pGEX–2T-1 and expressed as GST fusion proteins in *E. coli* (*15*). The indicated affinity-purified GST-Fas fusion proteins immobilized on glutathione-Sepharose were incubated with ^{35}S-labeled human FAP-1 protein fragment (1323 through 1883), and specifically bound proteins were detected by SDS-PAGE analysis (*16*) and scored as either positive for binding (+) or negative (–) in (A). Examples of autoradiography results from an SDS-PAGE of samples from the in vitro binding assays are shown. GST nonfusion protein was used as a control. Molecular size markers are shown at right in kilodaltons.

phatase. We therefore called it FAP-1.

To determine the region in the cytosolic domain of Fas that is required for binding to the GLGF3 domain of FAP-1, we prepared a series of Fas deletion mutants that were expressed in yeast as fusion proteins with the LexA DNA binding domain (Fig. 2A) (*14*). When tested in the two-hybrid system, the polypeptide encoded by the longer of the FAP-1 cDNAs (pVP16-31) mediated interactions with LexA fusion proteins containing only the last 15 amino acids of Fas (amino acids 321 through 335) but not with Fas mutants lacking the COOH-terminal 15 amino acids. Thus, the 15–amino acid COOH-terminus of Fas that functionally represents a negative regulatory domain appears to be both necessary and sufficient for interactions with the GLGF3 domain of Fas.

To confirm these two-hybrid results, we performed in vitro binding assays. The Fas deletion mutants were expressed in *Escherichia coli* as glutathione-S-transferase (GST) fusion proteins, affinity-purified on glutathione-Sepharose (*15*), and tested for binding to a ^{35}S-L-Met–labeled fragment of the human FAP-1 protein (amino acids 1323 through 1883) prepared by translation in vitro (*16*). The human FAP-1 partial cDNA used for preparation of in vitro–translated protein was obtained by hybridization screening of a human fetal brain cDNA library (*17*). It contained a five–amino acid insert in the GLGF3 domain, relative to the published PTP-BAS sequence, and presumably therefore represents a different isoform of this PTP that is expressed in brain (Fig. 1). Using this in vitro binding assay approach, we obtained results identical to those with the two-hybrid system: The last 15 amino acids of Fas were found to be both necessary and sufficient for binding to the FAP-1 fragment (Fig. 2B). Moreover, no interaction was detected with GST fusion proteins containing the cytosolic domains of p55-TNFRI, p75-TNFRII, or CD40 (*18*).

The finding that FAP-1 interacts with

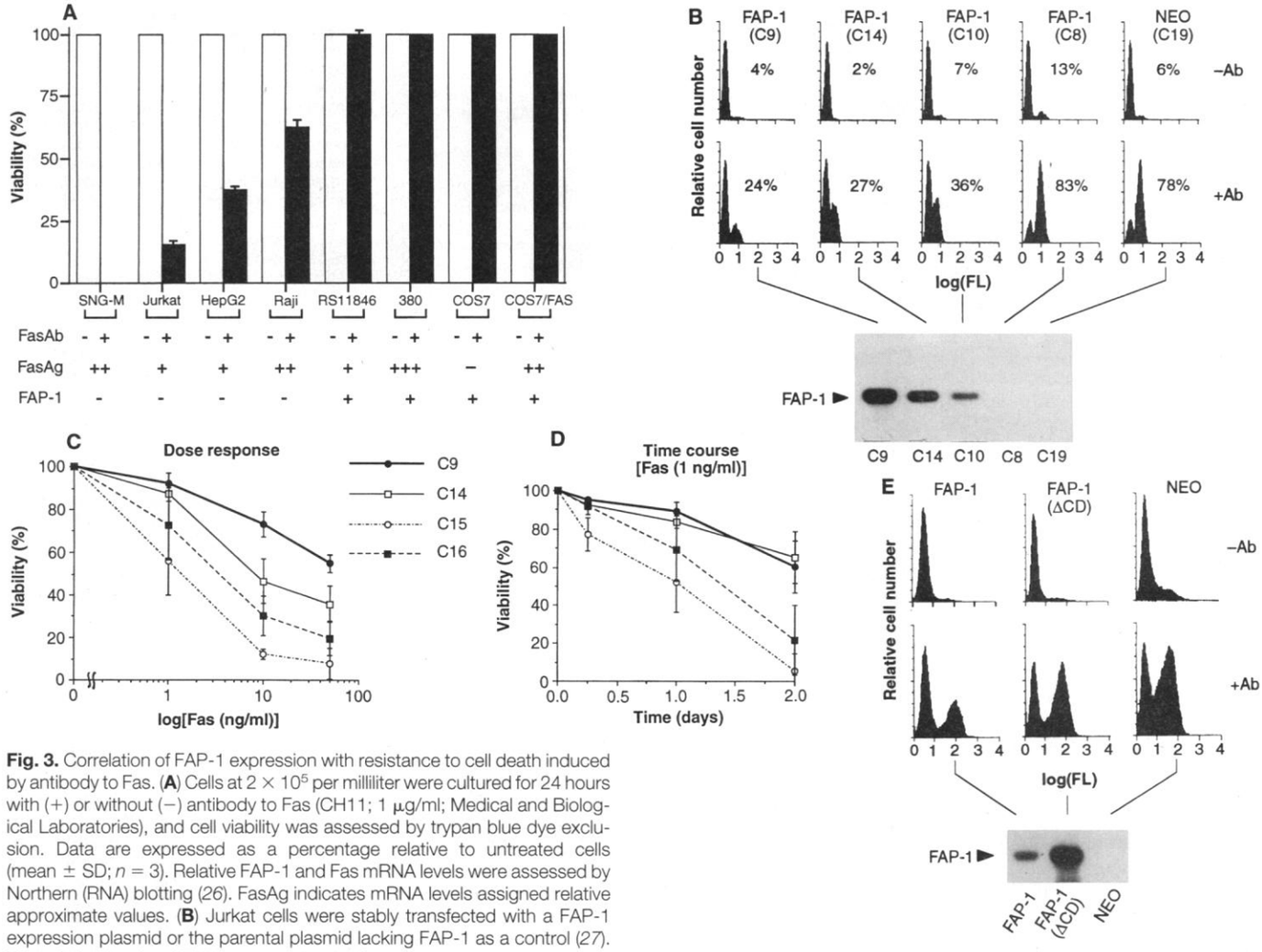

Fig. 3. Correlation of FAP-1 expression with resistance to cell death induced by antibody to Fas. (**A**) Cells at 2×10^5 per milliliter were cultured for 24 hours with (+) or without (−) antibody to Fas (CH11; 1 μg/ml; Medical and Biological Laboratories), and cell viability was assessed by trypan blue dye exclusion. Data are expressed as a percentage relative to untreated cells (mean ± SD; $n = 3$). Relative FAP-1 and Fas mRNA levels were assessed by Northern (RNA) blotting (*26*). FasAg indicates mRNA levels assigned relative approximate values. (**B**) Jurkat cells were stably transfected with a FAP-1 expression plasmid or the parental plasmid lacking FAP-1 as a control (*27*). The relative levels of FAP-1 expression in G418-resistant subclones were then determined by a RT-PCR assay (bottom) (*28*). Data shown are derived from a single exposure of the same blot (lanes reordered for clarity of presentation). Transfected clones were incubated with or without (Ab) CH11 (50 ng/ml), and the TUNEL assay was performed 4 hours later (*19*). Representative histograms are shown (top). Clones C8, C9, C10, and C14 received the FAP-1 plasmid pRc/CMV–FAP-1, whereas clone C19 was transfected with the parental pRc/CMV plasmid (NEO). Data are representative of multiple clones. A control immunoglobin M (IgM) antibody (MOPC-104E; Cappel) did not induce DNA fragmentation and apoptosis in Jurkat cells (*23*). FL, fluorescence. (**C**) FAP-1 transfectant clones C9 and C14 were compared with Neo-transfected control clones C15 and C16 with regards to survival [determined by trypan blue dye exclusion (mean ± SD; $n = 3$)] when cultured for ~1 day with various concentrations of CH11 antibody (zero equals no antibody) (C) or for various times with CH11 antibody (1 ng/ml) (**D**). Cell viability was >95% for cells treated with an IgM control antibody (*23*). (**E**) TUNEL assays and RT-PCR analyses were performed as described in (B) with untransfected Jurkat cells and bulk transfectants that expressed either full-length FAP-1 or a truncated FAP lacking the catalytic domain (ΔCD) (*27*).

the COOH-terminal negative regulatory domain of Fas suggests that this PTP may somehow inhibit Fas-generated signals that lead to apoptosis. To explore this possibility, we correlated the presence or absence of FAP-1 expression with relative sensitivity to apoptosis induced by antibody to Fas in a variety of cell lines that express Fas. Four of four tumor cell lines (SNG-M, Jurkat, HepG2, and Raji) that lacked FAP-1 mRNA, as determined by Northern (RNA) blotting, were sensitive to variable extents to induced cell death caused by antibody to Fas (Fig. 3A). In contrast, all three tumor lines tested which expressed FAP-1 (RS11846, 380, and COS-Fas) were completely resistant to antibody to Fas. This resistance could not be explained by differences in the relative levels of Fas antigen expressed on the surface of the cells, as determined by immunofluorescence flow cytometric analysis.

Next, a cDNA encoding the full-length FAP-1 protein was expressed in a Fas-sensitive clone from the T cell leukemia line Jurkat. Analysis of several independent transfected clones revealed a correlation between the levels of FAP-1 expression and relative resistance to Fas-mediated cytotoxicity. Some representative clones were treated with antibody to Fas for 4 hours and DNA fragmentation indicative of apoptosis was detected by TUNEL assay (*19*); resistance to Fas-mediated apoptosis was demonstrated in clones with higher levels of FAP-1 expression (Fig. 3B). Transfected Jurkat clones with high levels of FAP-1 expression withstood higher concentrations of antibody than control cells did (Fig. 3C) and remained viable for longer periods of time when cultured with antibody to Fas (Fig. 3D). In contrast to Jurkat cells which expressed full-length FAP-1, transfectants expressing a truncated version of FAP-1 lacking the catalytic domain were not protected from Fas-induced DNA degradation (Fig. 3E). Relative levels of Fas expression were equivalent for all subclones shown, on the basis of immunofluorescence flow cytometric assays.

Although other factors besides FAP-1 may contribute to the inhibition of Fas signal transduction events involved in the induction of apoptosis (*7*, *20*), the data presented here support the idea that FAP-1 is a negative regulator of Fas-induced pathways that lead to cell death. This finding therefore implies the involvement of a protein tyrosine kinase (PTK) in some aspect of Fas-mediated cytotoxicity. It has been reported that Fas-induced apoptosis is accompanied by rapid tyrosine phosphorylation of proteins in T cells and can be blocked by pharmacological inhibitors of PTKs (*21*). Presumably, therefore, an antagonistic relation between this unknown PTK and FAP-1 influences the relative sensitivity of cells to apoptosis induced by antibody to Fas.

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10. Two-hybrid screens were performed essentially as described (*9*) in L40 strain cells [*MATa, trp1, leu2, his3, ade2, LYS2::(lexAop)*^{4}-HIS3, URA3::(lexAop)^8-lacZ] with plasmid pBTM116 containing a human Fas cDNA (amino acids 191 through 335) subcloned in frame with the LexA open reading frame (ORF) and a mouse embryo cDNA library cloned into pVP16. Clones that formed on His-deficient media (His$^+$) were transferred to plates containing X-gal (40 μg/ml), resulting in 84 clones that produced a blue reaction product (β-Gal$^+$) in plate and filter assays (*22*).
11. We cured the 84 His$^+$, β-Gal$^+$ clones of the LexA-Fas plasmid by growing cells in Trp-containing medium and then mating them against a panel of α type yeast, strain NA87-11A (*MATα, leu2, his3, trp1, pho3, pho5*) containing plasmid pBTM116 that produced LexA DNA binding domain fusion protein containing Fas (amino acids 191 through 335), portions of the CD40 cytosolic domain, Bcl-2 protein, lamin, and mutant Ha-Ras proteins (*22*). Mated cells were selected for growth in medium that lacked Trp (pBTM116 plasmid) and Leu (pVP16 plasmid) and tested for the ability to trans-activate a *lacZ* reporter gene by a β-Gal colorimetric filter assay (*22*).
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13. The original description of PTP-BAS (*12*) indicated only three GLGF repeat domains, but our analysis with the DNA Strider program (version 1.2) (CEA, France) suggests the presence of six GLGF repeat domains in this PTP.
14. The cDNA sequences encoding various fragments of human Fas were generated by PCR with the following forward (F) and reverse (R) primers containing Eco RI (underlined) and Bcl 1 (italic) sites (bold indicates the stop codon, TCA); F1, 5′-GGAATTCAGATTATCGTCCAAAAGTG-3′; F2, 5′-GGAATTCAAGAGAAAGGAAGTACA-3′; F3, 5′-GGAATTCAAAGGCTTTGTTCGAAAG-3′; R1, 5′-GTGA**TCA**GTTAGATCTGGATCCTTC-3′; R2, 5′-GTGA**TCA**CGCTTCTTTCTTTCCATG-3′; R3, 5′-GTGA**TCA**CTAGACCAAGCTTTGGAT-3′; HFAS-*lpr* (Asn^{254}, 5′-TTCGAAAGAATGGTAACAATGAAGCCAAA-3′; HFAS-15F, 5′-AATTCGACTCAGAAAATTCAAACTTCAGAAATGAAATCCAAAGCTTGGTCTAG-3′; and HFAS-15R, 5′-TCGACTAGACCAAGCTTTGGATTTCATTTCTGAAGTTTGAATTTTCTGAGTCG-3′. We used the following combinations of primers to produce the indicated human Fas cDNA fragments: (a) F1 and R1, amino acids 17 through 173; (b) F2 and R3, 191 through 335; (c) F2 and R3 and Fas-*lpr* (Asn^{254}); (d) F3 and R3, 246 through 335; (e) F2 and R2, 191 through 290; (f) F3 and R2, 246 through 290; (g) Fas/APO-1 (191 through 320), which was generated by restriction endonuclease digestion with Spe I, followed by treatment with T4 DNA polymerase to generate a stop codon; and (h) Fas-15F and Fas-15R, 321 through 335. These Fas deletion mutant cDNAs were subcloned into the Eco RI and Bam HI sites of pEG202 [A. S. Zervous, J. Gyuris, R. Brent, *Cell* **72**, 223 (1993)] in frame with the LexA ORF, the results were confirmed by DNA sequencing, and then the cDNAs were excised with Eco RI and Sal I and subcloned into pBTM116 in frame with LexA.
15. The Fas cDNAs described in (*14*) were subcloned into the Eco RI and Xho I sites of either pGEX-2T-1 or pGEX-4T-1 in frame with the ORF of GST and expressed in DH5αF′ or HB101 cells (BRL/Gibco) by induction with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 8 to 16 hours at 30°C. GST fusion proteins were purified from bacterial lysates with glutathione–Sepharose 4B (Pharmacia, Piscataway, NJ).
16. The HFAP10 cDNA was subcloned into the Eco RV site of Bluescript pSK-II and in vitro translated from an internal Meth (amino acid 1323) in the presence of [^{35}S]-L-Meth with a coupled transcription-translation system (TNT lysate, Promega) and T7 RNA polymerase, resulting in the production of a human FAP-1/β-Gal fusion protein that was incubated with GST-Fas fusion proteins immobilized on glutathione-Sepharose in 50 mM tris (pH 8.0), 150 mM NaCl, 5 mM dithiothreitol (DTT), 2 mM EDTA, 0.1% NP-40, 1 mM phenylmethylsufonyl fluoride, and leupeptin (1 μg/ml) for 16 hours at 4°C. The beads were then vigorously washed five times in the same solution, pelleted by centrifugation, and boiled in Laemmli sample buffer before analysis by SDS–polyacrylamide gel electrophoresis (PAGE) and fluorography.
17. A human fetal brain cDNA library in λgt11 was screened with the ^{32}P-labeled insert from pVP16-31 used as a hybridization probe, resulting in four independent human Fas partial cDNA clones; HFAP10 is the longest.
18. The cDNA sequences encoding the human CD40 were generated by PCR with the following F and R primers containing Eco RI (underlined) and Bcl I (italic) sites (bold indicates the stop codon, TCA): CD40 (amino acids 216 through 277), 5′-GGAATTCAAAAAGGTGGCCAAG-3′ (F2) and 5′-TGATCA**TCA**CTGTCTCTCCTGCAC-3′ (R2); CD40 (225 through 269), 5′-GGAATTCAAGGCCCCCCACCCCAAG-3′ (F1) and 5′-TGA**TCA**ACTCTCTTTGCCATCCTC-3′ (R1). The PCR products were digested with Eco RI and Bcl I, then directly cloned into the Eco RI and Bam HI site of pBTM116. The Eco RI and Sal I fragments from pBTM116-CD40 were also subcloned into the Eco RI and Sal I sites of pGEX4T-1 (Pharmacia) for the expression of GST-CD40 proteins.
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24. L40 strain cells containing pVP16-31 or pVP16-43 were cured of plasmid pBTM116-Fas (amino acids 191 through 335) by growth on Trp and then mated with NA87-11A cells transformed with various pBTM116 plasmids producing LexA DNA binding domain fusion proteins containing portions of the Fas, CD40, Bcl-2, lamin, or mutant Ras proteins as indicated (*11*). Interactions of LexA and VP16 fusion proteins were detected by growth on His-deficient media and by a β-galactosidase colorimetric filter assay (*22*) based on the ability to trans-activate HIS3 and *lacZ* reporter genes containing LexA operators. L40 cells producing a VP16-Raf fusion protein served as a positive control when mated with NA87-11A cells containing pBTM116-Ras(V12) (*9*).
25. The Fas cDNAs described in (*14*) in pEG202 was cotransformed with pVP16-31 into EGY48 cells (*MATα trp1 ura3 his3 LEU2::pLexAop6-LEU2*) containing pSH18-34 (Gal1 promoter–*lacZ* with eight LexA operators), and β-galactosidase activity

was assessed by colorimetric plate and filter assays (*22*).

26. Total RNA (20 μg) isolated from various cell lines was subjected to Northern blot assay with ^{32}P-labeled HFAP10 probe, and the results were scored as detectable or undetectable. All cell lines expressed Fas antigen as determined by both flow cytometric immunofluorescence assay with antibody to Fas DX2 (*23*) and by Northern blotting with a ^{32}P-labeled Fas cDNA probe.
27. A cDNA encoding the full-length FAP-1 protein was constructed with a series of four overlapping PCR reactions and DNA derived from a λgt11 fetal brain cDNA library. The 5′- and 3′-flanking primers contained Not I sites that were used for subcloning downstream of the cytomegalovirus (CMV) promoter in pRc/CMV, an expression plasmid that contains a G418 resistance gene (Invitrogen). Jurkat cells were electroporated with 25 μg of pRc/CMV or pRc/CMV–FAP-1, and stable transfectants were obtained by selection in G418 (0.8 mg/ml). Independent clones were obtained by limiting dilution. In addition, a cDNA encoding a COOH-terminal–truncated FAP-1 protein was created by introduction of a stop codon after position 2225. The cDNAs encoding the full-length and truncated FAP-1 proteins were subcloned into pREP-9 (Invitrogen) and expressed in Jurkat cells.
28. Total RNA was isolated from individual transfectant clones and 3 μg was reverse transcribed with a FAP-1–specific primer (5′-AGGTCTGCAGAGAAGCAAGAATAC-3′). PCR amplification was then performed for 25 cycles with the same R and a F primer (5′-GAATACGAGTGTCAGACATGG-3′). The resulting PCR products (607 bp) were subjected to agarose gel electrophoresis and analyzed by Southern (DNA) blotting with a ^{32}P–end-labeled internal FAP-1 oligonucleotide probe (5′-CTAACTCCATTGACAGCTAGGA-3′).
29. We thank P. Krammer for Fas cDNA; C. Ware for GST-TNFRI and TNFRII (University of California at Riverside); S. Hollenberg and E. Golemis for the two-hybrid systems; N. Iwama, H. Kobayashi, and L. Fong for technical assistance; and the Council for Tobacco Research (CTR-3113) for its support. T.S. is fellow of the U.S. Army Medical Research and Development Command, Breast Cancer Research Program. J.C.R. is a Scholar of the Leukemia Society of America.

23 November 1994; accepted 10 January 1995

Initiation of Protein Synthesis by the Eukaryotic Translational Apparatus on Circular RNAs

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The ribosome scanning model predicts that eukaryotic ribosomal 40S subunits enter all messenger RNAs at their 5′ ends. Here, it is reported that eukaryotic ribosomes can initiate translation on circular RNAs, but only if the RNAs contain internal ribosome entry site elements. Long-repeating polypeptide chains were synthesized from RNA circles with continuous open reading frames. These results indicate that ribosomes can translate such RNA circles for multiple consecutive rounds and that the free 5′ end of a messenger RNA is not necessarily the entry point for 40S subunits.

Fifteen years ago, it was reported that circular RNAs could bind to prokaryotic but not to eukaryotic ribosomes (*1*, *2*). This finding provided the basis for work on the mechanism of translational initiation on eukaryotic mRNAs and suggested that eukaryotic mRNAs are functionally monocistronic and that ribosomes enter these mRNAs by threading onto their free 5′ ends, like beads on a string (*1*, *3*). However, this model has been challenged with the recent discovery that internal ribosome entry site (IRES) elements, present in the sequences of certain viral and cellular mRNAs, can mediate initiation of translation without prior scanning from the 5′ end of the IRES-containing mRNA by 40S subunits (*4*). Thus, 40S subunits enter IRES-containing mRNAs either by direct binding to the IRES element or by binding at the 5′ end of the mRNA and subsequent transfer to the IRES.

To test whether eukaryotic 40S subunits enter template mRNAs exclusively at free 5′ ends, we examined the ability of circular RNA substrates, with or without an IRES element, to direct the synthesis of defined protein products. Circular RNAs were produced by a modified method (*5*, *6*) (Fig. 1A). Putative RNA circles that migrated more slowly than their linear forms were isolated and characterized. The results from one such assay, a dephosphorylation test, are shown (Fig. 1B) (*7*). Linear 5′-radiolabeled RNA could be dephosphorylated after incubation with calf intestine phosphatase (CIP) (Fig. 1B). In contrast, ligation of linear 5′-radiolabeled RNA resulted in a more slowly migrating RNA species that was resistant to phosphatase treatment (Fig. 1B); this finding indicates that the low-mobility RNA species represents circular RNA. The linear species formed by degradation of the purified circular RNAs were also resistant to phosphatase treatment, indicating that degradation had occurred at random sites in the circle. Comparison of RNA fragments obtained after ribonuclease digestion of radiolabeled linear and low-mobility RNA species provided further evidence that the low-mobility RNA represents RNA circles (*8*).

To test whether RNA circles could direct the synthesis of a defined protein, we analyzed the translation products synthesized after incubation of linear and circular RNAs in a rabbit reticulocyte lysate (*9*). The predicted sizes of protein products made by linear and circular RNAs containing the IRES element of encephalomyocarditis virus (EMCV) are shown in Fig. 2A. Translation of linear IRES-containing RNA produced a 20-kD product. However, after circularization, the same RNA directed the synthesis of the predicted 23-kD product (Fig. 2B). The IRES-containing

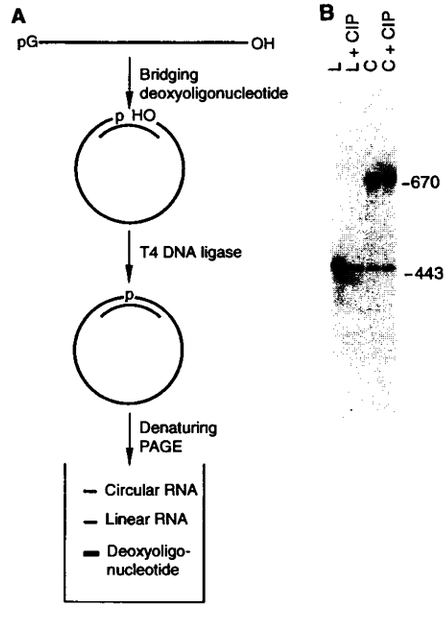

Fig. 1. In vitro production of circular RNAs. (**A**) Schematic outline of the synthesis of circular RNAs with the use of linear RNAs synthesized in vitro (p, phosphate), bridging deoxyoligonucleotides complementary to both the 5′ and 3′ ends of the RNA, and T4 DNA ligase. RNA molecules were transcribed by T7 RNA polymerase with the use of linearized DNA templates. After annealing of RNA transcripts to complementary bridging DNA oligodeoxynucleotides and subsequent incubation with T4 DNA ligase, circular RNAs were purified after separation by denaturing polyacrylamide gel electrophoresis (PAGE); the bottom of the figure is a representation of the resulting gel. This protocol is a modification of a method described in (*5*). (**B**) Dephosphorylation assay to identify RNA circles. RNA transcripts, 453 nt in length, were labeled with ^{32}P at their 5′ ends and circularized as shown in (A). Both linear (L) and putative circular (C) forms were incubated with CIP, and the reaction products were analyzed after electrophoresis on a urea-containing polyacrylamide gel. The positions of 443- and 670-nt RNA markers are indicated.

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