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.

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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

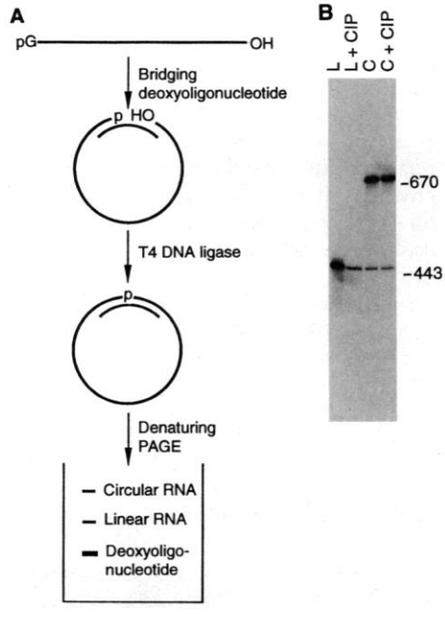

Fig. 1. In vitro production of circular RNAs. (**A**) Schematic outline of the synthesis of circular RNAs with the use of linear RNAs synthesized in vitro (p, phosphate), bridging deoxyoligonucleotides complementary to both the 5′ and 3′ ends of the RNA, and T4 DNA ligase. RNA molecules were transcribed 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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circular RNA was translated somewhat less efficiently than the linear RNA; this reproducible result may indicate that the circle imposes spatial constraints on the IRES that lead to less efficient use of the IRES. In contrast, circular RNA without an IRES did not direct the synthesis of a detectable protein product (Fig. 2C). Even the randomly cleaved linear species always present in preparations of circular RNAs without an IRES did not direct the synthesis of a discrete protein product (Fig. 2C).

We next explored the possibility that IRES-containing circular RNAs contain a preferential nuclease-sensitive site upstream of the initiator AUG codon (Fig. 2A). Such a finding would indicate that broken RNAs can direct the synthesis of the 23-kD protein from an uncapped 5′ end generated by nuclease cleavage. To test for IRES-specific nuclease cleavage, we monitored the translation of IRES-containing circles that contained a continuous reading frame. First, an EMCV-IRES element with an open reading frame (EMCV-ORF IRES) was constructed by eliminating several stop codons present in the wild-type EMCV-IRES (*10*, *11*). Although the nucleotides engaged in predicted secondary structures were not changed (*10*), the possibility remained that unknown secondary structure, unknown tertiary structure, or unknown protein binding sites were affected by the introduced mutations. The function of the EMCV-ORF IRES was tested by placing it between two cistrons of a dicistronic mRNA (*12*, *13*). This dicistronic mRNA contained a chloramphenicol acetyltransferase (CAT) coding region followed by the EMCV-ORF IRES and a luciferase coding region. The translation products synthesized by such dicistronic mRNAs are shown (Fig. 3). As expected, all dicistronic mRNAs directed the synthesis of CAT, the product of the first cistron (Fig. 3). In addition, both wild-type and EMCV-ORF IRES elements directed the synthesis of luciferase, whereas the EMCV-ΔIRES, which contains a 70-nucleotide (nt) deletion near the 5′ end of the IRES (Fig. 3), did not direct the synthesis of the second cistron. Thus, the EMCV-ORF IRES can direct internal initiation in linear RNA molecules.

To test the function of the EMCV-ORF IRES element in a circular RNA molecule, we analyzed the translation products of linear and circular RNAs containing EMCV-ORF IRES (EMCV-ORF IRES RNAs) (*14*). According to our predictions, linear EMCV-ORF IRES RNAs should direct the synthesis of two products, 35 and 20 kD in size (Fig. 4A). The 35-kD product should be synthesized by 5′ end–dependent scanning, where the second AUG codon in the EMCV-ORF IRES [which is the first AUG codon in a favorable context for translational initiation (*15*)] is used as the start codon; the 20-kD product should be synthesized by ribosomes recruited onto the mRNA by the EMCV-ORF IRES (Fig. 4A).

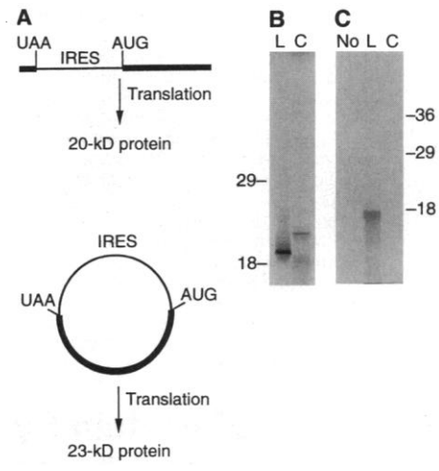

Fig. 2. Translation of linear and circular RNAs. (**A**) Predicted sizes of protein products translated in vitro from linear or circular RNAs containing the IRES element of EMCV. A segment of the capsid VP3 gene of poliovirus (nucleotides 1957 to 2471) and an additional 13 base pairs of polylinker sequence constituted the coding region for an approximately 20-kD protein. After circularization, the coding region was predicted to extend into the EMCV 5′ noncoding region, resulting in an ORF encoding a 23-kD protein. Initiator AUG codons, terminator UAA codons, and the location of the IRES are indicated. (**B**) Analysis of translation products of IRES-containing linear and circular mRNAs. Linear (L) and circular (C) RNA templates were translated in a rabbit reticulocyte lysate and the translation products were analyzed by SDS-PAGE. The migration of proteins of known molecular weights is indicated (in kilodaltons). (**C**) Analysis of translation products of linear and circular 600-nt RNAs lacking IRES elements. Reactions without added RNA (No) or with added linear (L) or circular (C) RNA templates were translated in a rabbit reticulocyte lysate and the translation products were analyzed by SDS-PAGE. The migration of proteins of known molecular weights is indicated.

The linear EMCV-ORF IRES RNA directed the synthesis of both proteins in vitro (Fig. 4B). Incubation of circular EMCV-ORF IRES RNAs, on the other hand, directed the synthesis of extremely long protein species, many of which were larger than 106 kD in size (Fig. 4B). Most of these products were larger than 40 kD, the largest protein that could be synthesized from monomeric linear RNA templates. If these products were the result of an unusual ligation activity in the translation lysate, they would have been expected to accumulate in the translation reaction containing the linear EMCV-ORF IRES mRNA as well. Thus, these proteins were likely synthesized by ribosomes traversing the circular RNA templates for multiple rounds. If these high molecular weight products were covalently linked multimers of the 40-kD polypeptide, rather than fortuitous aggregates of smaller proteins that resisted denaturation, they should resolve to discrete products after limited proteolysis. To test this prediction, sequences that encode a predicted cleavage site for the sequence-specific protease thrombin (*16*) were introduced upstream of the EMCV-ORF IRES. Proteins synthesized from ribosomes that had traversed the circular RNAs several times should consist of a repeating polypeptide chain with multiple thrombin cleavage sites. Incubation of the translation reaction with thrombin should yield 40-kD polypeptides (the predicted size of a product encoded by the circular RNA). We found that most of the high molecular weight products were cleaved into 40-kD polypeptides after incubation with thrombin (Fig. 4B). The

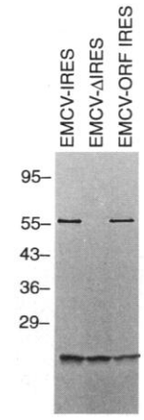

Fig. 3. Translation of dicistronic mRNAs bearing wild-type or mutated EMCV-IRES elements. The CAT gene is the first cistron and the luciferase gene is the second cistron in the dicistronic gene. The two cistrons are separated by the following intercistronic sequences: full-length EMCV-IRES, partially deleted EMCV-IRES (EMCV-ΔIRES), or EMCV-ORF IRES. Translation reactions were performed as described (Fig. 2) and the translation products were analyzed by SDS-PAGE. The migration of known protein markers is indicated at the left; CAT protein is 27 kD and luciferase protein is 62 kD.

synthesis of the long multimeric protein molecules could have occurred only on circular RNA templates. Thus, the eukaryotic translational apparatus can initiate and elongate protein synthesis on IRES-containing circular RNAs in the absence of a free 5′ end.

This finding has implications for the mechanism by which 40*S* subunits are recruited onto mRNAs. Contrary to earlier suggestions (*1*, *3*), 40*S* subunits do not necessarily thread onto mRNAs at their 5′ ends. IRES elements can recruit ribosomes onto IRES-containing mRNAs without assistance from the capped or uncapped 5′ ends in the mRNA or from their associated cap-binding protein factors (*17*–*19*). The mechanism by which 40*S* subunits are recruited onto the IRES-containing mRNA is unknown; they could be recruited by an interaction between IRES and ribosomal RNA sequences whose identity is still elusive (*20*–*22*), or they could be recruited by IRES-binding proteins such as the polypyri-

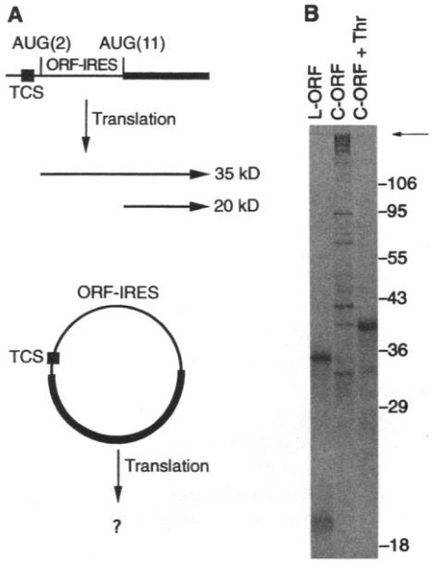

Fig. 4. Translation of linear and circular RNAs containing ORF-IRES elements. **(A)** Predicted sizes of protein products translated in vitro from linear or circular RNAs containing the EMCV-ORF IRES element. The positions of two initiator AUGs are indicated; AUG(2) is predicted to be the translational start codon for ribosomes entering the RNA at the 5′ end, and AUG(11) is predicted to be the start codon for ribosomes entering the RNA by the IRES. The predicted translation products, 35 and 20 kD in size, and the location of the thrombin cleavage site (TCS) are indicated. The VP3 coding region is indicated by the thick line. **(B)** Analysis of translation products of linear or circular 1083-nt RNAs containing ORF-IRES elements. Linear and circular RNA templates were translated in a rabbit reticulocyte lysate, and the translation products were analyzed by SDS-PAGE. The migration of proteins of known molecular weights is indicated. The arrow denotes the border between the stacking gel and the separating gel. Lane C-ORF + Thr displays the translation products directed from circular ORF RNAs (C-ORF) after incubation with thrombin.

midine-tract binding protein (*23*, *24*) or the La antigen (*25*). Because ribosomes can interact with certain RNA sequences in the absence of a free 5′ end, it is possible that the ribosome entry site in a capped mRNA without an IRES element is not necessarily the 5′ end of the mRNA, as has been postulated (*3*). For example, 40*S* subunits could be recruited onto mRNA sequences downstream of the capped 5′ terminal nucleotides by interacting with the cap-binding protein complex eIF-4F already in transit on the mRNA (*19*, *26*).

The spatial constraints imposed by circularization of IRES-containing RNA molecules did not interfere with IRES function. It may be interesting to examine whether naturally occurring circular RNAs, such as the RNA genome of certain viral pathogens (*27*), contain IRES elements. Circular transcripts have also been detected in the testis-determining *Sry* gene in the cytoplasm of adult mouse testes (*28*), and these transcripts are formed as by-products of certain splicing reactions (*29*–*31*). It has not yet been reported whether any of these circles can serve as substrates for eukaryotic ribosomes.

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6. Plasmid DNAs were linearized with Sma I and then transcribed in vitro with T7 RNA polymerase as described (*5*), except that guanosine monophosphate (GMP) was included in the reactions [10:1 ratio of GMP to guanosine triphosphate (GTP)]. Transcripts were annealed with DNA oligodeoxynucleotides complementary to both the 5′ and 3′ ends of RNA molecules. The mixture was heated to 90°C and cooled slowly to room temperature (1 hour) in a solution containing 10 mM tris-HCl (pH 7.5), 100 mM NaCl, and 0.1 mM EDTA. Ligation buffer and T4 DNA ligase (100 units per nanomole of transcript) were then added to the hybridization solution, and the reaction proceeded for 8 to 16 hours at room temperature. The ligated products were separated by electrophoresis in 4% polyacrylamide–8 M urea gels, visualized by ultraviolet (UV) shadowing, eluted overnight [in a solution of 10 mM tris-HCl (pH 7.5), 250 mM KCl, and 1 mM EDTA], and precipitated with ethanol. The H_2O used in this study was purified photocatalytically and was free of organic contaminants (Photo Catalytics) (*32*).
7. To identify the putative RNA circle in Fig. 1B, we synthesized the linear RNA transcripts to be circularized in the presence of guanosine nucleoside and labeled the 5′ ends of the RNA molecules with ^{32}P by T4 polynucleotide kinase. Purified RNA species were treated with CIP at 37°C for 30 min and were analyzed on a 4% polyacrylamide–8 M urea gel.
8. C. Chen and P. Sarnow, data not shown.
9. The Nco I–Nhe I DNA fragment containing base pairs 1957 to 2471 of the polioviral genome was ligated into plasmid p5′NC-LUC (*33*), which was digested with Nco I and Xba I to yield plasmid p5′NC-VP3. A Pvu II–Nco I DNA fragment that contained the T7 RNA polymerase promoter and the EMCV-IRES (R strain) was cleaved from pCITE-1 (Novagen) and ligated to plasmid p5′NC-VP3, which was digested with Pvu II and Nco I to yield plasmid pEMCV-IRES/VP3. The plasmid lacking an IRES element was constructed by replacing the EMCV-IRES in pEMCV-IRES/VP3 with base pairs 1 to 70 of a poliovirus complementary DNA (cDNA) to yield pΔ5′NC/VP3. Plasmid DNAs were linearized with Sma I and transcribed by T7 RNA polymerase as described (*5*). Translation reactions were performed at 30°C for 45 min in a 12.5-μl reaction volume containing 8.5 μl of rabbit reticulocyte lysate (Flexi-system, Promega), 0.5 μg of polyacrylamide gel–purified linear or circular RNAs, and 0.75 μl of [^{35}S]methionine (Amersham, 1000 Ci/mmol). The KCl concentration was 120 mM in the reaction mixtures in which IRES-containing mRNAs were translated. A 4-μl aliquot of each reaction mixture was analyzed on 15% SDS-polyacrylamide gels.
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11. A Pvu II–Nco I fragment from pCITE-1 was cloned into p5′NC-LUC (*33*), which was digested with Pvu II–Nco I to yield plasmid pEMCV-IRES/LUC. Next, pEMCV-IRES/LUC was partially digested with Eco RI and then digested to completion with Sma I. The fragment containing the EMCV-IRES/LUC gene was isolated, and the ends of the fragment were repaired by the Klenow enzyme. In parallel, plasmid pCV (*34*) was digested with Xba I and Sal I, the CAT containing fragment was isolated, and the ends of the fragment were repaired with Klenow. Both fragments were ligated together to yield plasmid pCAT/EMCV-IRES/LUC, which contains a promoter for T7 RNA polymerase upstream of the CAT gene. To construct pCAT/ΔEMCV-IRES/LUC, plasmid pCAT/EMCV-IRES/LUC was digested with Avr II and Hind III, releasing a 69-base pair fragment. The ends of the large fragment were repaired with Klenow and religated; the result was the deletion of base pairs 415 to 484 in the IRES. Plasmid pCAT/EMCV-ORF IRES/LUC is identical to pCAT/EMCV-IRES/LUC except for the introduction of the following base pair changes in the EMCV-IRES (*10*) and the presence of sequences encoding a thrombin cleavage site (*16*): nucleotides 493 (G was changed to C), 585 (A to C), 759 (U to C), and 780 (U to C). These changes were introduced with the use of the Transformer site-directed mutagenesis method (Clontech). The polycytidine sequences present at the 5′ end of the EMCV-IRES (*10*) were replaced with sequences encoding a thrombin cleavage site (*16*). This step was performed by polymerase chain reaction (PCR) amplification of the EMCV-IRES with the use of primer 1 [containing sequences encoding a thrombin cleavage site (*16*) at its distal 5′ end, hybridized to sequences 309 to 337 in the EMCV-IRES (*10*)] and primer 2 [annealed to sequences 409 to 427 in the EMCV-IRES (*10*)]. The presence of all expected mutations was confirmed by sequencing.
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14. Production of circular RNAs and in vitro translation reactions were carried out as described (Fig. 2). After completion of the translation reaction, ribonuclease A (100 μg/ml) and ribonuclease T1 (100 units/ml) were added to digest RNA templates. One-tenth of the reaction mixture was then incubated with 1 NIH unit of thrombin (Sigma) at 25°C for 8 hours in 20 μl of a solution of 50 mM tris-HCl (pH 8.0), 150 mM NaCl, 2.5 mM CaCl, and 0.1% β-mercaptoethanol. Reaction products were analyzed on a 15% SDS-polyacrylamide gel.
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