MOLECULAR AND CELLULAR BIOLOGY, Mar. 1990, p. 1134–1144 Vol. 10, No. 3
0270-7306/90/031134-11$02.00/0
Copyright © 1990, American Society for Microbiology

Bidirectional RNA Helicase Activity of Eucaryotic Translation Initiation Factors 4A and 4F

FLORENCE ROZEN,[1] ISAAC EDERY,[1†] KAREN MEEROVITCH,[1] THOMAS E. DEVER,[2] WILLIAM C. MERRICK,[2] AND NAHUM SONENBERG[1,3]*

Department of Biochemistry[1] and McGill Cancer Center,[3] McGill University, Montreal, Quebec, Canada H3G 1Y6, and Department of Biochemistry, School of Medicine, Case Western Reserve University, Cleveland, Ohio 44106[2]

Received 25 September 1989/Accepted 22 November 1989

The mechanism of ribosome binding to eucaryotic mRNAs is not well understood, but it requires the participation of eucaryotic initiation factors eIF-4A, eIF-4B, and eIF-4F and the hydrolysis of ATP. Evidence has accumulated in support of a model in which these initiation factors function to unwind the 5′-proximal secondary structure in mRNA to facilitate ribosome binding. To obtain direct evidence for initiation factor-mediated RNA unwinding, we developed a simple assay to determine RNA helicase activity, and we show that eIF-4A or eIF-4F, in combination with eIF-4B, exhibits helicase activity. A striking and unprecedented feature of this activity is that it functions in a bidirectional manner. Thus, unwinding can occur either in the 5′-to-3′ or 3′-to-5′ direction. Unwinding in the 5′-to-3′ direction by eIF-4F (the cap-binding protein complex), in conjunction with eIF-4B, was stimulated by the presence of the RNA 5′ cap structure, whereas unwinding in the 3′-to-5′ direction was completely cap independent. These results are discussed with respect to cap-dependent versus cap-independent mechanisms of ribosome binding to eucaryotic mRNAs.

A critical step in eucaryotic protein biosynthesis is binding of the small (40S) ribosomal subunit to mRNA (36, 39). This step is rate limiting in translation initiation (25) and is a key target for regulation (reviewed in reference 53), but the mechanism of this process is poorly understood. Two pathways for the binding of 40S ribosomal subunits to mRNA have been described, which differ in their requirement for the cap structure. The 5′ cap structure, m^7GpppX (where X is any nucleotide) is a nearly ubiquitous feature of all eucaryotic mRNAs (49). Evidence indicates that translation initiation of the majority of eucaryotic mRNAs is accomplished in a cap-enhanced manner, whereby 40S ribosomal binding to mRNA is facilitated by the cap structure (reviewed in references 3 and 50). Recently, it has been shown that poliovirus (42) and encephalomyocarditis virus (26) mRNAs, which are naturally uncapped (13, 21, 38), initiate translation by a different mechanism. In this case, the 40S subunit binds directly to an internal element on the picornavirus 5′ untranslated region, by-passing upstream sequences and the requirement for the cap structure.

Although the two initiation pathways are mechanistically distinguishable, they nevertheless require a similar set of initiation factors (eIFs [57]) to bind 40S ribosomal subunits to mRNA. Cap-stimulated mRNA binding to the small ribosomal subunit requires at least three initiation factors, eIF-4A, eIF-4B, and eIF-4F, in addition to the hydrolysis of ATP (reviewed in references 10, 44, and 53). eIF-4F is a multisubunit complex consisting of three major polypeptides of 24, 50, and 220 kilodaltons (kDa) (8, 20, 59). The 24-kDa polypeptide is the cap-binding subunit, which also exists in a free form, termed eIF-4E (55). The 50-kDa polypeptide is a structural variant of free eIF-4A (8, 20). Although eIF-4F contains an eIF-4A subunit which is almost identical to free eIF-4A, both factors are required for maximal translation in vitro (20), indicating different roles. The 220-kDa component of eIF-4F (p220) has not been well characterized, except that its structural integrity is required for eIF-4F activity (11). eIF-4B is an 80-kDa polypeptide whose mechanism of action is not clear, but it is required for mRNA binding to 40S ribosomes and stimulates eIF-4A and eIF-4F activities. Consistent with their cap-independent translation, picornaviruses do not require eIF-4F but are dependent on eIF-4A and eIF-4B for translation (5, 57).

It was proposed that cap function is mediated by factors that interact with the cap structure and subsequently use the energy generated from ATP hydrolysis to unwind 5′-proximal mRNA secondary structure, thereby facilitating 40S subunit attachment (9, 52, 54). Numerous lines of evidence support this proposal (reviewed in references 10 and 53). The best evidence was provided by Ray et al. (43), who showed that eIF-4F and, to a lesser extent, eIF-4A induce conformational changes in mRNA structure as monitored by a nuclease-sensitivity assay. eIF-4A has been characterized as an ATP-dependent single-stranded RNA-binding protein, whose ATPase activity is stimulated by the presence of single-stranded RNA (1, 19). The involvement of an RNA-unwinding activity during translation initiation is consistent with the inhibitory effect of excessive mRNA 5′-proximal secondary structure on translation (41).

To provide direct evidence for an RNA duplex melting activity of eIF-4A and eIF-4F and to investigate the mechanism of unwinding, we developed a novel and simple RNA-unwinding assay. This assay should greatly facilitate the functional characterization of an interesting family of recently described genes with high homology to eIF-4A (32). These gene products are suggested to be RNA helicases involved in regulating gene expression in the cytoplasm, nucleus, and mitochondria. In addition, some of them play important roles during development.

MATERIALS AND METHODS

Oligonucleotide templates. Oligodeoxynucleotide templates for transcription were synthesized on an Applied Biosystems DNA Synthesizer, gel purified on denaturing

* Corresponding author.
† Present address: Department of Biology, Brandeis University, Waltham, MA 02254.

polyacrylamide–8 M urea gels, eluted in 0.5 M ammonium acetate overnight at 37°C, and desalted by adsorption on a C18 reverse-phase SepPak minicolumn. Oligodeoxynucleotides were annealed by heating equimolar amounts of the 18-mer oligodeoxynucleotide and the 57-mer template oligodeoxynucleotide to 100°C in 0.1 M sodium chloride and slowly cooling the mixture to room temperature. Conditions for annealing of oligodeoxynucleotides used in Fig. 8 are described in the legend.

In vitro transcription. Transcriptions by T7 RNA polymerase were carried out essentially as described previously (35). Reaction mixtures, except for the transcription in Fig. 8, contained 6 mM $MgCl_2$; 350 nM DNA template; 150 μM CTP, UTP, and ATP; 75 μM GTP and [α-^{32}P]GTP (50 μCi, 3,000 Ci/mmol); and 200 U of T7 RNA polymerase (New England BioLabs, Inc.) in a final volume of 100 μl. To obtain capped transcripts, we included 375 μM m^{7}GpppG in the reaction mixture. The efficiency of capping was determined to be approximately 50% for all types of RNA, as determined by two-dimensional thin-layer chromatography analysis by the method of Konarska et al. (27). Transcripts were gel purified on 8% polyacrylamide–8 M urea gels, eluted, and recovered as described previously (18). RNA duplexes (A and B) formed spontaneously during the purification steps.

In vitro unwinding assay. The unwinding activity of rabbit reticulocyte eucaryotic initiation factors (eIF-4A, eIF-4B, and eIF-4F were purified as described by Grifo et al. [20]; the purity of the factors as determined by Coomassie blue staining was 95% for eIF-4A and 80 to 85% for eIF-4F; eIF-4B was 90% pure and contained <3% eIF-4E) was assayed in vitro by measuring the conversion of the 40-mer RNA duplex to its monomer species (conditions for Fig. 8 are described in the legend). Initiation factors were incubated with 200 to 400 pg (1,000 to 2,000 cpm) of [^{32}P]RNA (capped RNAs were used throughout unless otherwise indicated) in a 10-μl reaction for 10 min at 37°C in buffer containing 20 mM HEPES (pH 7.5), 70 mM KCl, 2 mM dithiothreitol, 1 mM ATP, 0.5 mM magnesium acetate, 0.1 mM GTP (we found that this component can be omitted without affecting unwinding activity: it was added initially because it is present in translation reactions), 40 U of RNasin (Promega-Biotec), and 1 μg of tRNA. The reaction was terminated by the addition of 2.5 μl of 50% glycerol–2% sodium dodecyl sulfate–20 mM EDTA containing xylene cyanol-bromophenol blue dye. Products of unwinding were analyzed directly by electrophoresis on native 8% polyacrylamide gels (acrylamide/bisacrylamide ratio, 50:1 for RNA A and 15:1 for RNA B) containing 5% glycerol in 0.5× TBE, followed by autoradiography.

RNase T_2 digestion. RNA was incubated with 30 U of RNase T_2 (Bethesda Research Laboratories, Inc.) in 50 mM sodium acetate (pH 5.5)–100 mM NaCl–2 mM EDTA for 2 h at 30°C. The RNA was extracted three times with phenol-chloroform and ethanol precipitated with 0.3 M sodium acetate and 10 μg of tRNA.

Quantitation of efficiency of unwinding. Unwinding efficiency is defined as a percentage based on the ratio of final unwound monomer RNA relative to the input duplex RNA. Quantitation was performed by scanning autoradiograms in the linear range with a soft laser-scanning densitometer (LKB Instruments, Inc.).

RESULTS

Duplex RNA substrates for helicase activity. To test directly for RNA helicase activity of eIFs required for 40S binding to mRNA, we designed RNA molecules with alternating G and C nucleotides that are predicted to form stable duplexes via base pairing, flanked by single-stranded regions of 30 nucleotides (Fig. 1). Single-stranded regions were included because in most cases helicases require single-stranded regions for initial attachment (reviewed in reference 15). In addition, the ATPase activity of eIF-4A and eIF-4F is maximally activated in the presence of single-stranded RNAs (1). To determine the direction of the putative helicase activity, we designed two types of molecules: (i) a 40-nucleotide RNA that contains an alternating G-C stretch of 10 nucleotides which, upon dimerization, is expected to produce a base-paired region at the 5′ end (ΔG = −35 kcal/mol [−146 kJ/mol]) and 3′-terminal extensions of single-stranded tails of 30 nucleotides (referred to as RNA A [Fig. 1A]); and (ii) a 40-nucleotide RNA containing a similar sequence to A, but with the G-C sequence located at its 3′ end, to yield upon dimerization an RNA duplex with 5′-terminal extensions of 30 nucleotides of single-stranded RNA (referred to as RNA B [Fig. 1B]). Note that the nucleotide sequence in the single-stranded RNA extensions in RNAs A and B is similar (when reading from the duplex in the 3′-to-5′ direction, the single-stranded RNAs in both dimers contain 21 identical nucleotides, and the rest of the nucleotides are also identical but inverted in position). Since the putative helicase factor is postulated to bind the single-stranded region prior to strand separation, it is expected that unwinding of RNA A would proceed in the 3′-to-5′ direction, whereas unwinding of structure B would proceed in 5′-to-3′ direction. (The direction of unwinding is defined relative to the strand which is initially bound by the helicase.)

Oligoribonucleotides were synthesized by the method of Milligan et al. (35), whereby RNAs are transcribed by using the T7 promoter (Fig. 1). To assess the importance of the cap structure for the unwinding activity, we synthesized capped or uncapped mRNAs by adding or omitting the cap analog m^{7}GpppG in the transcription reaction.

Characterization of helicase activity of initiation factors. Synthesized RNAs were subjected to electrophoresis on denaturing polyacrylamide gels, and full-length transcripts were purified for use in the unwinding reactions. First, we ensured that the RNAs synthesized could indeed form duplexes; heating the duplexed RNA to 90°C caused it to migrate faster on a nondenaturing polyacrylamide gel, as expected if the RNA duplex had fallen apart (Fig. 2A, compare lane 2 with lane 1). The denaturing effect shown here for RNA A occurred also with RNA B (see Fig. 7). Evidence that the structure of RNA duplexes is indeed that described in Fig. 1 was provided by digestion of the RNAs with RNase T_2. This treatment resulted in the removal of the single-stranded tails of the RNA duplex, resulting in a G+C-containing double-stranded RNA. The size and nucleotide composition of the RNase-resistant RNA were verified to be those expected from the G-C portion of the RNA only (see Fig. 6B; data not shown).

To test for factor-mediated unwinding, we incubated the RNA duplexes with highly purified initiation factors and stopped the reaction by adding 0.4% sodium dodecyl sulfate and 4 mM EDTA. It was necessary to add these components to disrupt formed high-molecular-weight RNA-protein complexes which complicate the determination of unwinding efficiency. Following unwinding, protein-RNA interactions are not necessary to keep the RNA duplexes unwound, since heating the RNA duplex to 90°C and then incubating it for 1 h at 37°C in the unwinding buffer is not sufficient for RNA duplex reformation (data not shown). However, the un-

A

T7 promoter

5' TAATACGACTCACTATAG 3'
3' ATTATGCTGAGTGATATCGCGCGCGCGTTGTTGTAATGTTAAATGTTAGGTGGTACC 5'

5' m7GpppG≡CAACAACAUUACAAUUUACAAUCCACCAUGG 3'
C≡G
G≡C
C≡G
G≡C
C≡G
G≡C
C≡G
G≡C
3' GGUACCACCUAACAUUUAACAUUACAACAAC≡GpppG7m 5'

B

T7 promoter

5' TAATACGACTCACTATAG 3'
3' ATTATGCTGAGTGATATCCTGGTACCTTGTTGTAATGTTAAATGTTAGCGCGCGCGC 5'

3' G≡CUAACAUUUAACAUUACAACAAGGUACCAGGpppG7m 5'
C≡G
G≡C
C≡G
G≡C
C≡G
G≡C
C≡G
G≡C
5' m7GpppGGACCAUGGAACAACAUUACAAUUUACAAUC≡G 3'

FIG. 1. DNA templates and duplex RNAs generated for unwinding assays. (A) DNA template used for T7 in vitro transcription and its corresponding capped RNA hybridized to itself to form RNA A. ↱, Site for transcription initiation. (B) DNA template used for T7 in vitro transcription and its corresponding capped RNA hybridized to itself to form RNA B. ↱, Site for transcription initiation.

wound RNAs can reform duplexes after heating if the conditions are made more favorable by increasing the salt concentration and incubation time (data not shown). Thus, the dissociation of RNA-protein complexes at the end of the incubation did not affect the magnitude of unwinding.

In the first set of experiments, we tested for unwinding activity of RNA A by the three initiation factors involved in mRNA binding to ribosomes: eIF-4A (Fig. 2A, lane 3), eIF-4F (lane 4), and eIF-4B (lane 5). None of these initiation factors, at the concentrations indicated, could bring about RNA unwinding, but increasing the concentration of eIF-4B by itself led to some unwinding (see, for example, Fig. 2B). A likely reason for the latter finding is addressed below. A combination of eIF-4A and eIF-4F marginally increased unwinding over the background level (less than 2%; Fig. 2A, lane 6), but a combination of eIF-4A and eIF-4B resulted in ca. 50% unwinding (lane 7), and a combination of eIF-4F and eIF-4B resulted in 100% unwinding (lane 8). This result demonstrates that in the presence of eIF-4B, eIF-4F is more efficient than eIF-4A in unwinding (see below).

To further characterize the unwinding reactions and to compare the unwinding efficiency of eIF-4A and eIF-4F, we performed titration experiments. Figure 2B shows a titration of eIF-4A unwinding activity in the presence of a constant amount of eIF-4B. (In this and subsequent experiments we used 1 μg of eIF-4B, since we found that this amount led to much more efficient unwinding than did the 0.5 μg used for Fig. 1A. Some unwinding occurs in the presence of eIF-4B alone [ca. 15%], and is most probably due to low levels of eIF-4F present in the eIF-4B preparation [<3% as determined by gel analysis and immunoblotting of the eIF-4E subunit of eIF-4F; data not shown]; this association has been previously reported [20, 55].) About 70% unwinding was achieved with 0.25 μg of eIF-4A (Fig. 2B). A similar degree

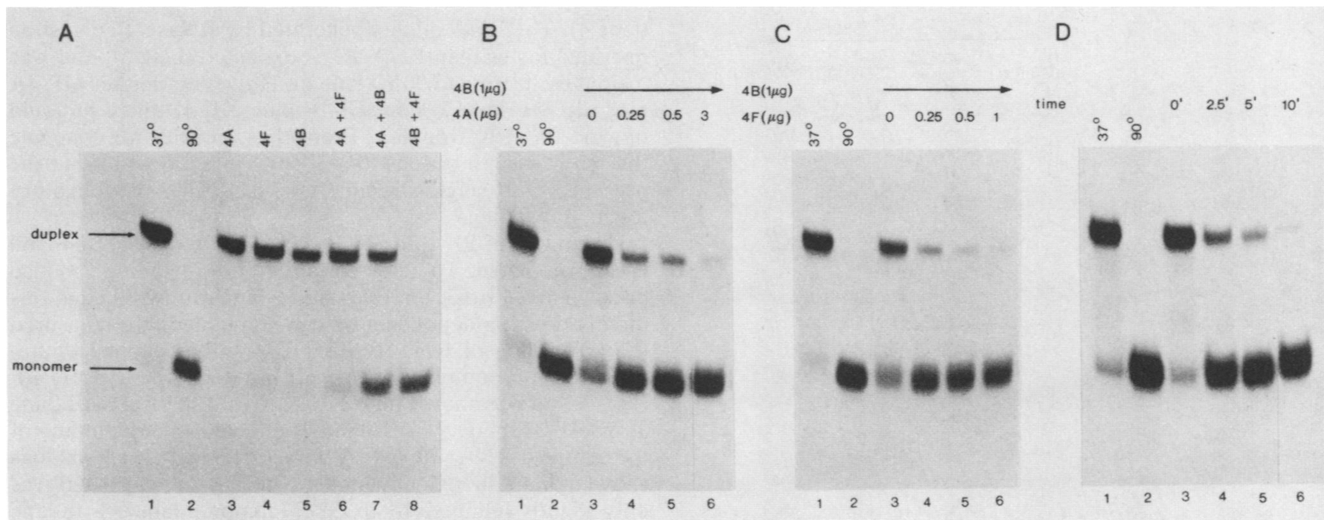

FIG. 2. Characterization of unwinding reaction of RNA A by eIF-4A, eIF-4B and eIF-4F. (A) Unwinding of capped RNA A using different combinations of eIF-4A (3 μg), eIF-4B (0.5 μg), and eIF-4F (1 μg) as indicated in the figure. For details concerning the in vitro unwinding assay, see Materials and Methods. (B) Titration of eIF-4A in the unwinding of RNA A. Unwinding was performed with 1 μg of eIF-4B and increasing amounts of eIF-4A as indicated in the figure. (C) Titrations of eIF-4F in the unwinding of RNA A. Unwinding was performed with 1 μg of eIF-4B and increasing amounts of eIF-4F as indicated in the figure. (D) Kinetics of unwinding of RNA A. Unwinding was performed at 37°C by using eIF-4A (3 μg) and eIF-4B (1 μg) for the times indicated in the figure.

of unwinding was obtained with 0.25 μg of eIF-4F in the presence of eIF-4B (Fig. 2C). However, on a molar basis, eIF-4F (M_r ca. 300,000) is approximately six times more efficient than eIF-4A in unwinding. A time course of the unwinding reaction with eIF-4A and eIF-4B shows that the reaction is relatively fast; by 2.5 min, more than 70% unwinding occurred (Fig. 2D).

Nucleotide and initiation factor specificity of helicase activity. A DNA or RNA helicase activity is defined as an activity that unwinds duplex polynucleotides by using a nucleoside triphosphate as an energy source (15). To determine whether the unwinding activity that we were studying is a helicase, we tested the requirement for nucleoside triphosphate hydrolysis in the unwinding reaction. We found that 0.5 mM ATP is sufficient for almost complete unwinding (Fig. 3, compare lane 5 with lane 3). dATP can substitute for ATP (lane 8), as is the case for several DNA helicases (16, 66). A nonhydrolyzable analog of ATP, AMPP-CP (lane 9), GTP (lane 10), CTP (lane 11), or UTP (lane 12) did not substitute for ATP in the unwinding reaction. This is consistent with the inability of eIF-4A and eIF-4F to bind nucleotides other than ATP and dATP (46). Thus, the unwinding reaction of the RNA duplex is mediated by a helicase activity. Similar results were also obtained with eIF-4F and eIF-4B (data not shown).

We also tested other initiation and elongation factors for their ability to unwind RNA A in conjunction with eIF-4B. eIF-2 (Fig. 4, lane 4), eIF-3 (lane 5), eIF-5 (lane 6), elongation factor 1α (EF-1α; lane 7), and EF-2 (lane 8) did not exhibit unwinding activity either in the presence or in the absence of eIF-4B (data not shown).

eIF-4A and eIF-4B are the essential components of the helicase activity. Both eIF-4A and eIF-4F can cooperate with eIF-4B to unwind the RNA duplex, but eIF-4F is more active than eIF-4A. It is expected that the active component in eIF-4F responsible for the helicase activity is the eIF-4A subunit. To examine this, we performed the unwinding reaction with an eIF-4F preparation from which the eIF-4A was ablated by phosphocellulose chromatography (43). This preparation of eIF-4F had no unwinding activity when tested in conjunction with eIF-4B (Fig. 5, compare lane 4 with lane 3). However, addition of exogenous eIF-4A restored activity. These results rule out the presence of inhibitors in the preparations of eIF-4F free of eIF-4A and demonstrate that the eIF-4A subunit of eIF-4F is most probably the component responsible for the combined eIF-4F–eIF-4B helicase activity.

Unwinding of RNA A is in a 3′-to-5′ direction and is cap independent. An important aspect of a translationally associated helicase activity is the role of the cap structure. In

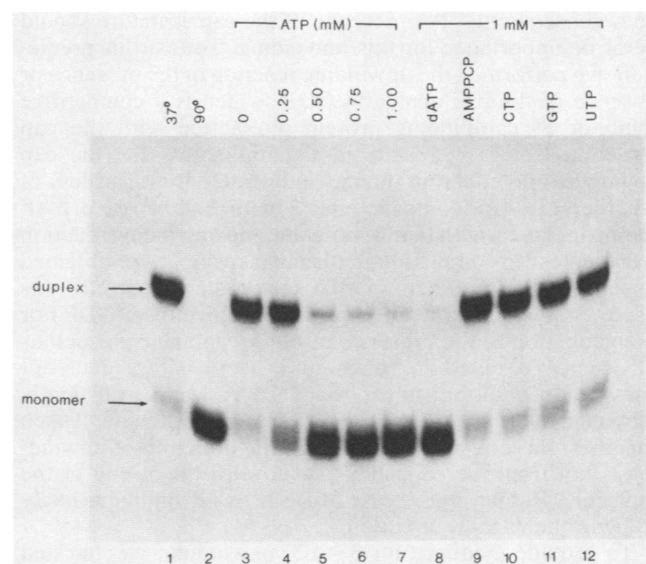

FIG. 3. Nucleotide-dependent unwinding of RNA A. Unwinding by eIF-4A (3 μg) and eIF-4B (1 μg) was performed with increasing amounts of ATP (lanes 3 to 7). dATP, AMPPCP, and other nucleoside triphosphates, at a concentration of 1 mM, were substituted for ATP as indicated in the figure.

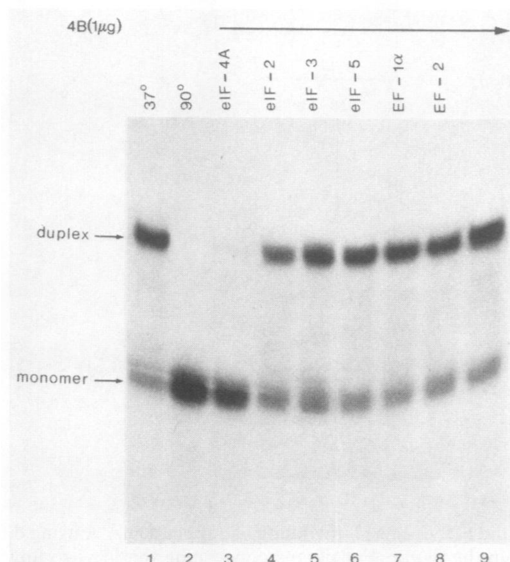

FIG. 4. Lack of unwinding activity by several other initiation and elongation factors. Unwinding of RNA A was performed with initiation and elongation factors (EF) indicated in the figure. Lanes 3 to 9 each contained 1 μg of eIF-4B. Lane 3 contained 3 μg of eIF-4A. All other factors were present at 1 μg.

RNA A the cap structure is most probably sequestered, because of the proximity to the base-paired region of the duplex, and might not be available for interaction with the cap-binding protein complex, eIF-4F (29). In addition, a minimum amount of single-stranded region is likely to be required for unwinding, which in RNA A is not available at the 5′ end. In this regard, Abramson et al. (1) have shown that the minimal size of an oligo(A) required for optimal RNA-dependent ATPase activity is 12 to 18 bases, and the "site size" for eIF-4A binding to a fluorescent derivative of poly(A) was 13 ± 2 nucleotides (17). Therefore, we do not expect that unwinding will proceed from the 5′ capped ends, and, consequently, the presence of the cap structure should be of no importance for this unwinding. To test this prediction, we performed the unwinding reaction in the presence or absence of the cap analog m^7GDP, which is a competitive inhibitor of cap-binding protein interaction with the cap structure (55). The results in Fig. 6A show that the cap analog had no effect on the unwinding activity regardless of whether eIF-4A (compare lane 2 with lane 1) or eIF-4F (compare lane 4 with lane 3; note that the unwinding reaction went almost to completion, but similar results were obtained under conditions where partial unwinding occurred) was used. Neither preincubation in the presence of m^7GDP nor preincubation in the presence of other cap analogs, such as m^7GpppG, resulted in inhibition of unwinding (data not shown). In addition, uncapped RNA A was as efficiently unwound as was capped RNA (data not shown). Taken together, these results suggest that the direction of unwinding is not from the capped 5′ end toward the 3′ end of the molecule. Rather, in the case of RNA A, unwinding is likely to be in the 3′-to-5′ direction.

To provide evidence for 3′-to-5′ unwinding, we digested the 3′ protruding tails of the RNA duplex with RNase T_2 and tested the purified flush-ended duplex RNA, containing the G-C region only, for its ability to be unwound. Figure 6B shows the complete unwinding of RNA A by the combination of eIF-4A and eIF-4B (lane 3) or eIF-4F and eIF-4B (lane 4). The RNA duplex generated by RNase T_2 digestion migrated faster than RNA A, as expected (lane 5), and was melted by heating to 90°C (lane 6). However, neither eIF-4A plus eIF-4B (lane 7) nor eIF-4F plus eIF-4B (lane 8) could unwind this RNA duplex. From these results we conclude that unwinding proceeds in a 3′-to-5′ direction and that the presence of 3′ single-stranded tails in RNA A is obligatory for its unwinding.

Unwinding of RNA duplex B is in the 5′-to-3′ direction and is cap stimulated. To determine whether unwinding can also proceed in a 5′-to-3′ direction and whether unwinding in this direction is cap dependent or cap stimulated, we measured the unwinding of RNA B (Fig. 1B), either capped or uncapped, with combinations of eIF-4A, eIF-4B, and eIF-4F (Fig. 7). As was shown for RNA A, eIF-4A plus eIF-4B (lane 3) or eIF-4F plus eIF-4B (lane 4) effected the unwinding of the capped RNA duplex. Competition with cap analogs showed that although unwinding by eIF-4A plus eIF-4B was only slightly inhibited by m^7GDP (compare lane 5 with lane 3; this could be due to the low levels of eIF-4F present in the eIF-4B preparation), unwinding by eIF-4F plus eIF-4B was significantly inhibited by m^7GDP (ca. 50%; compare lane 6 with lane 4). This result indicates that unwinding of capped RNA B by eIF-4F is stimulated by the presence of the cap structure and that the higher efficiency of unwinding by eIF-4F is, at least in part, due to its ability to bind to the cap structure. To substantiate these results, we also performed the unwinding reaction with uncapped RNA B. In this case, there was no significant difference in the efficiency of unwinding by eIF-4A or eIF-4F when used in combination with eIF-4B (compare lane 10 with lane 9), and unwinding by

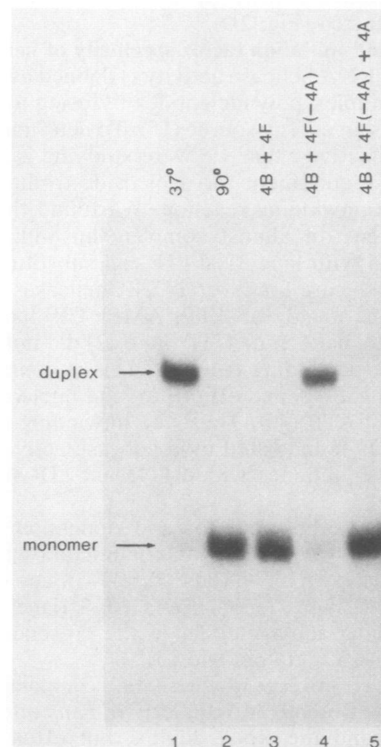

FIG. 5. Absolute requirement of eIF-4A for unwinding. eIF-4F was depleted of its eIF-4A component by phosphocellulose chromatography (43) and tested in the presence of eIF-4B for unwinding of RNA A (lane 4). The following amounts of factors were added: eIF-4A, 3 μg; eIF-4B, 1 μg; eIF-4F, 1 μg; eIF-4F (−4A), 1 μg.

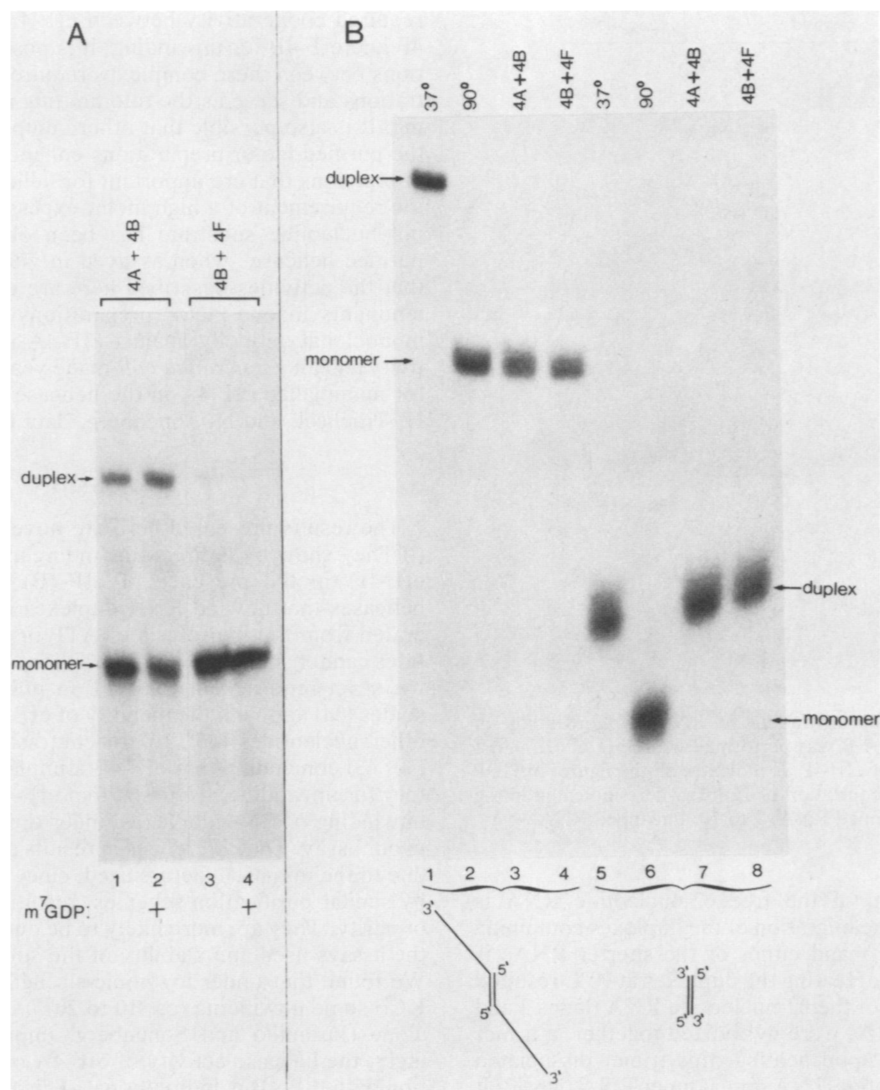

FIG. 6. Cap-independent and single-stranded RNA-dependent unwinding of RNA A. (A) Cap-independent unwinding of RNA A. Unwinding of RNA A was performed with 0.17 μg of eIF-4A and 1 μg of eIF-4F (3 pmol of each). Each lane contained 1 μg (12.5 pmol) of eIF-4B. The cap analog m^7GDP was added at a final concentration of 1 mM, where indicated below the figure. (B) Absolute requirement of single-stranded RNA region for unwinding. RNA A was digested with 30 U of RNase T_2 (see Materials and Methods) and used in unwinding with eIF-4A (3 μg), eIF-4B (1 μg), and eIF-4F (1 μg), as indicated in the figure. Lanes: 1 to 4, before RNase T_2 digestion; 5 to 8, after RNase T_2 digestion. The probable secondary structures of the RNAs are shown below the figure.

eIF-4F was reduced to the level observed with the capped RNA B in the presence of m^7GDP (compare lane 10 with lane 6). Thus, efficient unwinding of RNA B is enhanced by the presence of the cap structure when eIF-4F is used. This is consistent with a 5'-to-3' direction of unwinding of RNA B. In an experiment similar to that described for RNA A (Fig. 6B), we showed that the single-stranded regions at the 5' ends of RNA B were required for the unwinding, since their removal by RNase T_2 totally abolished the ability of the duplex to be unwound by initiation factors (data not shown). In addition, experiments similar to those described above (Fig. 2 to 5) gave similar results with RNA B as with RNA A (data not shown). We conclude, therefore, that eIF-4A or eIF-4F in combination with eIF-4B can unwind an RNA duplex in the 5'-to-3' direction in addition to the 3'-to-5' direction. Significantly, in the case of eIF-4F, the unwinding in the 5'-to-3' direction is enhanced by the presence of the cap structure.

Bidirectional unwinding. The previous results indicate that eIF-4A or eIF-4F in combination with eIF-4B could unwind duplex RNA in a bidirectional manner. To further substantiate this conclusion, we wanted to demonstrate bidirectional unwinding on a single molecule. To this end, we prepared a trimer RNA that contained duplexes at each end of a central single-stranded region of 30 nucleotides (Fig. 8A). The free energy of each duplex was −35 kcal/mol (similar to the duplexes used above [Fig. 1]). Dissociation of the short RNAs could be initiated only from the middle single-stranded region, since the ends are flush double stranded (based on the results in Fig. 6). In this experiment the only RNA labeled to high specific activity was the long RNA (62 nucleotides), and dissociation was followed by a shift in the migration of this RNA. Figure 8B shows the helicase activity of eIF-4A plus eIF-4B or eIF-4F plus eIF-4B on this RNA. Lanes 1 to 7 show the positions of the different possible hybrids that can be formed between the three different

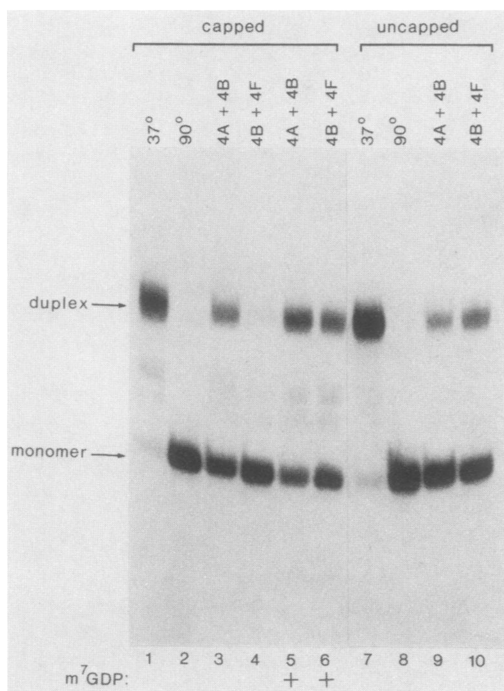

FIG. 7. Cap-dependent unwinding of RNA B. Unwinding of capped or uncapped RNA B was performed with 3 μg of eIF-4A, 1 μg of eIF-4B and 1 μg of eIF-4F as indicated in the figure. m^7GDP was added at a final concentration of 1 mM, where indicated in the figure. Lanes: 1 to 6, capped RNA; 7 to 10, uncapped RNA.

RNAs. The migration of the free 62-nucleotide RNA is shown in lane 1, and the migration of the duplexes containing the 62-nucleotide RNA and either of the shorter RNAs is shown in lanes 2 and 4. Heating the duplexes at 90°C resulted in the release of most of the 62-nucleotide RNA (lanes 3 and 5). When all three RNAs were hybridized together, a trimer was formed (lane 6); upon heating, this trimer dissociated into the free 62-nucleotide RNA and a dimer RNA (lane 7; it is possible that heating did not cause the complete dissociation of the trimer RNA components, because of excess of the short RNAs that resulted in partial rehybridization; these conditions were different from those used for formation of the duplexes described above). Incubation of the trimer RNA with eIF-4B plus eIF-4F or eIF-4B plus eIF-4A yielded a similar pattern to that obtained upon heating of the RNA to 90°C (compare lanes 8 and 9 with lane 7). Importantly, the factor-mediated dissociation is completely dependent on ATP (compare lane 10 with lane 9). The appearance of the 62-nucleotide RNA in the presence of protein factors in an ATP-dependent manner is evidence for the dissociation of both short RNAs from the 62-nucleotide RNA and strongly indicates that the helicase can function in a bidirectional fashion. Furthermore, the helicase is not limited to binding single-stranded ends, but can access the RNA by binding to an internal region devoid of secondary structure.

Efficient unwinding of all RNAs requires a high molar excess of initiation factors over RNA (e.g., a 200-fold excess of eIF-4F). Consequently, we cannot distinguish between a mechanism that involves RNA-binding activity that displaces a duplex versus one that acts catalytically in the displacement reaction. Attempts to achieve unwinding by reducing the amounts of initiation factors and extending incubation times were unsuccessful. However, in light of the required cooperativity between eIF-4A and eIF-4B or eIF-4F and eIF-4B for unwinding, it is possible that the interactions between these complexes require high protein concentrations and serve as the rate-limiting step in RNA unwinding. It is also possible that other components not present in the purified-factor preparations enhance or stabilize protein interactions that are important for helicase activity. Finally, the requirement of a high molar excess of protein factors to polynucleotide substrate has been observed with several purified helicases when assayed in vitro (47). It is unlikely that the activities described here are due to unknown contaminants in our factor preparations, since addition of a monoclonal antibody against eIF-4A inhibited helicase activity and an *Escherichia coli*-made yeast eIF-4A substituted for mammalian eIF-4A in the helicase assay (M. Jaramillo, H. Trachsel, and N. Sonenberg, data not shown).

DISCUSSION

The results presented here are novel in several respects. (i) They show by a direct-unwinding assay that eIF-4A and eIF-4F (in the presence of eIF-4B) are bona fide RNA helicases that unwind RNA duplexes by using energy generated from the hydrolysis of ATP or dATP; other nucleotides cannot substitute in this reaction. This is in accord with the strict requirement for ATP in mRNA binding to ribosomes (34) and with the inability of eIF-4A or eIF-4F to bind other nucleotides (46). (ii) In contrast to previous reports (30, 43) concluding that eIF-4B stimulates but is not obligatory for unwinding, we found that eIF-4B is required for the unwinding of RNA duplexes under the salt conditions used in our assay. The differences in results are most probably not due to the initiation factors used, since factors were purified by similar purification schemes, resulting in similar degrees of purity. They are more likely to be due to the differences in the assays used and stability of the substrates being tested. We found that under low-ionic-strength conditions (40 mM KCl) some unwinding (ca. 10 to 20%) occurred with eIF-4F alone (Jaramillo and Sonenberg, unpublished). (iii) Strikingly, the helicase activity of eIF-4A or eIF-4F in combination with eIF-4B is bidirectional. This mode of unwinding is without precedent, since all DNA helicases characterized to date are unidirectional (see, for example reference 66; a recent report [31] showed that the primosome DNA helicase activity can be manifested in either direction along the DNA strand. However, different components of the primosome are responsible for the opposite direction of helicase activities. The DNA B protein acts in the 5′-to-3′ direction, whereas factor Y acts in the 3′-to-5′ direction). Likewise, the *E. coli* transcription termination factor rho is a 5′-to-3′ RNA-DNA helicase (4). RNA-RNA helicase activities of p68 (a member of the eIF-4A family) and simian virus 40 T antigen have been recently reported (24, 47). Although the directionality of p68 was not investigated, it was found that T-antigen RNA unwinding occurs in a 3′-to-5′ direction (47), as was reported for its DNA unwinding (58). In agreement with these results, we have found (unpublished observations) that simian virus 40 T antigen unwound RNA A (i.e., in the 3′-to-5′ direction) but was unable to unwind RNA B (i.e., in the 5′-to-3′ direction). This suggests that bidirectionality is not an intrinsic property of RNA helicases. (iv) Either eIF-4F or eIF-4A in the presence of eIF-4B can bind to an internal region of RNA devoid of secondary structure (Fig. 8). These results have certain intriguing implications and are discussed below.

Roles for eIF-4F and eIF-4A during translation initiation.

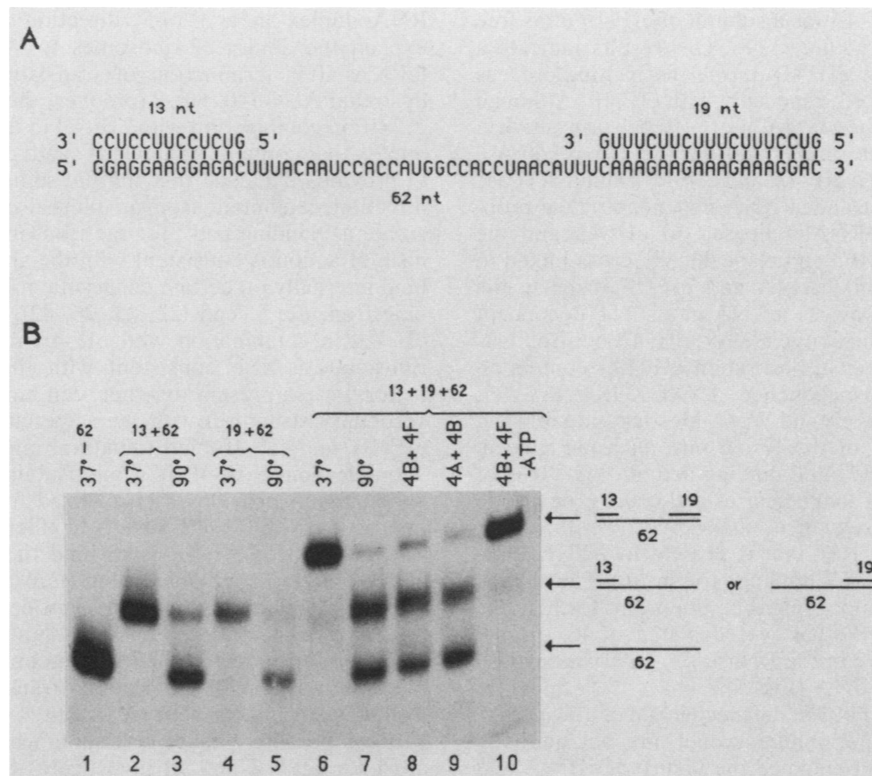

FIG. 8. Bidirectional unwinding from a trimer RNA. (A) This panel shows nucleotide sequence of RNA templates and predicted base pairing to generate a trimer RNA. (B) Transcription of the RNA templates was done as described in Materials and Methods with the following modifications. Reaction mixtures contained 20 mM $MgCl_2$, 150 nM DNA template, and 1 mM each ATP, CTP, and GTP. The concentration of UTP varied for the different DNA templates as follows: 2 mM for the 30-mer, 2.4 mM for the 36-mer, and 1 mM for the 79-mer. The amount of labeled [α-^{32}P]UTP added was 150 μCi (3,000 Ci/mmol) for the 79-mer and 10 μCi for the 30- and 36-mer oligodeoxynucleotides. Uncapped duplex or trimer RNA templates were generated by mixing 0.08 pmol of a 62-nucleotide RNA (30,000 cpm/pmol) (lane 1) with 0.2 pmol of either a 13-nucleotide RNA (1,650 cpm/pmol) (lanes 2 and 3) or a 19-nucleotide RNA (1,650 cpm/pmol) (lanes 4 and 5) or both (lanes 6 to 10) in 0.4 M NaCl–3 mM magnesium acetate–20 mM Tris–7.5 and 1 mM dithiothreitol, heating the mixture at 90°C for 5 min, and slowly cooling it to 40°C. Unwinding of the trimer RNA was performed essentially as described in Materials and Methods, with 2 μg of eIF-4B plus 2 μg of eIF-4F (lanes 8 and 10) or 2 μg of eIF-4B plus 6 μg of eIF-4A (lane 9) in the presence (lanes 8 and 9) or absence (lane 10) of 1.3 mM ATP and 0.6 mM magnesium acetate for 2 h at 37°C.

Although we show that both eIF-4F and eIF-4A in the presence of eIF-4B have helicase activity, it is likely that they do not perform equivalent functions in the cell. Both components are required for maximal translation in vitro (20). Also, eIF-4A is the most abundant initiation factor (three molecules per ribosome), whereas the eIF-4E subunit of eIF-4F is present in limiting amounts (0.2 to 0.3 molecule per ribosome [7]). In addition, protein-synthesizing extracts prepared from poliovirus-infected cells in which eIF-4F activity is impaired are not stimulated by the addition of eIF-4A, but are stimulated by eIF-4F (59). Since all cellular mRNAs are capped, and eIF-4A in the presence of eIF-4B has a high affinity for single-stranded RNA (1), we favor the following general model. eIF-4F binds the cap structure, and, in combination with eIF-4B, the eIF-4A component hydrolyzes ATP and initiates the unwinding process followed by binding of free eIF-4A molecules (in an eIF-4B- and ATP-dependent mechanism) to the newly formed unstructured RNA, preventing it from refolding. This may continue until an AUG is encountered or when sufficient RNA is unwound that the 40S ribosome can attach to the mRNA. Once the 40S ribosome binds, the initiation factors dissociate from the mRNA, thus allowing the restructuring of the upstream RNA, preserving its influence on the next round of 40S subunit binding.

There are several possible explanations for the enhanced helicase activity of eIF-4F relative to eIF-4A. The enhanced helicase activity in the 5′-to-3′ direction is probably due, in part, to the affinity of eIF-4F to the mRNA 5′ cap structure. When it is part of eIF-4F, eIF-4A is in close proximity to the proper RNA activator, which activates ATP hydrolysis and subsequent unwinding. Therefore, the role of eIF-4F would be to enhance the rate with which eIF-4A binds RNA, a necessary prerequisite for helicase activity. This mechanism may explain why mRNAs with regions devoid of secondary structure in the immediate vicinity of the cap are translated very efficiently (see, for example, references 14 and 29). Also consistent with this scenario is the fact that introducing stretches of single-stranded RNA 5′ to a stem-loop structure negated the inhibitory effects of the secondary structure on translation (28). The enhanced activity of eIF-4F that is independent of the cap structure, which is manifested in the cap-independent 3′-to-5′ or 5′-to-3′ unwinding, might be due to the greater ability of eIF-4F than eIF-4A to bind eIF-4B, thus more readily forming an active helicase complex.

Role of eIF-4B. In most studies, the role of eIF-4B has been suggested to be facilitative, enhancing already existing (but weak) activities of eIF-4A and eIF-4F (1, 2, 19). The strong requirement for eIF-4B in the RNA helicase assay described here is consistent with its absolute requirement for

the cross-linking of eIF-4A (as a subunit of eIF-4F or as free eIF-4A) to the cap structure (1, 8). Our results indicate a more dominant role for eIF-4B during the melting process than previously suggested, especially with eIF-4F. Although eIF-4B is required for the eIF-4A or eIF-4F helicase activity, there is ample evidence to indicate that the active helicase component is eIF-4A. (i) eIF-4A and eIF-4F exhibit ATPase activity that is single-stranded RNA dependent (1), a property expected for an RNA helicase. (ii) eIF-4A and the eIF-4A subunit in eIF-4F can be specifically cross-linked to ATP or dATP (45). (iii) eIF-4A and eIF-4F increase the RNase sensitivity of reovirus mRNA in an ATP-dependent manner (43). In all of the above assays, eIF-4B by itself was inactive. (iv) Mouse, yeast, and rabbit eIF-4As contain an ATP-binding consensus sequence AXXGXGKT (33, 37; S. C. Conroy, T. E. Dever, and W. C. Merrick, unpublished results), and mutation of the lysine into an asparagine in mouse eIF-4A abolished ATP-binding activity (45). It is of interest that the eIF-4A-like helicases and many procaryotic helicases contain the sequence AXXGXGKT, rather than the sequence GXXXXGKT that is present in ATP-binding proteins (63) and most GTP-binding proteins (6). The alanine upstream of the GKT motif might be important for helicase activity (12). (v) eIF-4F that was depleted of its eIF-4A component was inactive in the RNase sensitivity assay (43) and had no helicase activity (Fig. 5).

What is the role of eIF-4B in the mechanism of unwinding? There are two likely possibilities which are not mutually exclusive: eIF-4B could enhance the ability of eIF-4A and eIF-4F to bind RNA or stimulate the necessary ATPase activity. It is possible that eIF-4B serves as an RNA-binding protein, which facilitates eIF-4A and eIF-4F binding to the mRNA. Consistent with this possibility is the finding that eIF-4B could bind nonspecifically to RNA by using a filter-binding assay, under conditions where eIF-4A did not bind (56). Additionally, eIF-4B elutes from phosphocellulose at greater than 300 mM KCl, at pH 8.0 (20). It should be noted, however, that eIF-4F can bind capped mRNAs via its eIF-4E component in the absence of eIF-4B (8). Therefore, the requirement for eIF-4B during eIF-4F-mediated unwinding is likely not to be entirely due to an eIF-4B-promoted increase in affinity of eIF-4F for RNA.

The function of eIF-4B might be analogous to DnaC protein of *Escherichia coli*, which, by forming a complex with DnaB protein (64), facilitates binding of the latter to single-stranded DNA, which is a prerequisite for its ATPase, helicase, and priming activities (62). Moreover, several proteins that stimulate DNA helicase activity but do not by themselves have ATPase or unwinding activity, have recently been purified. These include the replication protein A and Rep helicase stimulatory potein, required for unwinding mediated by large T antigen (65) and *E. coli* Rep protein (51), respectively. In both cases it was suggested that the stimulatory factors enhanced helicase activity by binding to single-stranded DNA. Perhaps helicases require an eIF-4B-like activity, implying high conservation not only in helicase structure-function relationships (32, 48), but also in the molecular mechanism of unwinding.

Biological significance of bidirectional unwinding. The finding that eIF-4A and eIF-4F possess bidirectional helicase activity is unexpected. This is because of the unidirectional nature of all known helicases and the general belief that the ribosome accesses the initiator AUG from the 5′ end, promoted by melting of mRNA secondary structure by the processive, cap-dependent 5′-to-3′ action of initiation factors. However, the ability of initiation factors to unwind an RNA duplex in a 3′-to-5′ direction could, for example, explain the ability of ribosomes to "reach back" on the mRNA after termination of translation to initiate at an upstream AUG (40, 60). Moreover, the ability of eIF-4A or eIF-4F in combination with eIF-4B to unwind a partial RNA duplex that contains an internal single-stranded region (Fig. 8) provides evidence that the unwinding process, in either direction, can initiate from an internal site on the mRNA and create a "landing pad" for the ribosome (42). This mechanism of action is consistent with the ability of ribosomes to bind internally on certain eucaryotic mRNAs, without scanning from the 5′ end (22, 23, 26, 42). The involvement of eIF-4A in combination with eIF-4B in internal binding of ribosomes is also consistent with the finding that these factors, when present together, can bind to mRNA (lacking secondary structure) with the same efficiency that eIF-4A, eIF-4B, and eIF-4F bind to natural mRNAs (2).

Implications. eIF-4F is a multisubunit helicase in which each subunit provides a specialized function which, when interacting with eIF-4B, results in efficient helicase activity. The function of eIF-4E is to bind the cap; eIF-4A is the active helicase component; and p220 most probably aligns the individual components in a proper configuration such that maximal activity of eIF-4A is obtained. Because eIF-4E is present in the cell in limiting amounts and functions in a rate-limiting step of translation, translation may be controlled by regulating helicase activity.

It will be of interest to determine whether other helicases are bidirectional and to investigate the structure-function relationships underlying this novel property. With the recent discovery of a family of putative RNA helicases (see, e.g., references 32 and 48) and the discovery that p68 and simian virus 40 T antigen function as RNA helicases (24, 47), it becomes increasingly clear that the ability to induce controlled changes in RNA conformation is likely to be a crucial and paramount regulatory phenomenon in the cell.

ACKNOWLEDGMENTS

Florence Rozen and Isaac Edery contributed equally to the work.

We thank J. Hurwitz, J. Pelletier, and members of the laboratory for critical comments on the manuscript.

This research was supported by a grant from the Medical Research Council of Canada (to N.S.) and by Public Health Service grant GM26796 (to W.C.M.) from the National Institutes of Health. N.S. is the recipient of a Medical Research Council Scientist Award from the Medical Research Council of Canada; F.R. is a recipient of a predoctoral studentship from Fonds de la Recherche en Santé du Québec Fellowship; I.E. and K.M. are recipients of predoctoral fellowships from the Cancer Research Society (Montreal); and T.E.D. was supported by National Institutes of Health training grant AM07319 in metabolism.

LITERATURE CITED

1. **Abramson, R. D., T. E. Dever, T. G. Lawson, B. K. Ray, R. E. Thach, and W. C. Merrick.** 1987. The ATP-dependent interaction of eukaryotic initiation factors with mRNA. J. Biol. Chem. **262:**3826–3832.
2. **Abramson, R. D., T. E. Dever, and W. C. Merrick.** 1988. Biochemical evidence supporting a mechanism for cap-independent and internal initiation. J. Biol. Chem. **263:**6016–6019.
3. **Banerjee, A. K.** 1980. 5′-Terminal cap structure in eucaryotic messenger ribonucleic acids. Microbiol. Rev. **44:**175–205.
4. **Brennan, C. A., A. J. Dombroski, and T. Platt.** 1987. Transcription termination factor rho is an RNA-DNA helicase. Cell **49:**945–952.
5. **Daniels-McQueen, S., B. M. Detjen, A. J. Grifo, W. C. Merrick, and R. E. Thach.** 1983. Unusual requirements for optimal

translation of polioviral RNA in vitro. J. Biol. Chem. **258:** 7195–7199.

6. **Dever, T. E., M. J. Glynias, and W. C. Merrick.** 1987. GTP binding domain: three consensus sequence elements with distinct spacing. Proc. Natl. Acad. Sci. USA **84:**1814–1818.
7. **Duncan, R., S. C. Milburn, and J. W. B. Hershey.** 1987. Regulated phosphorylation and low abundance of HeLa cell initiation factor eIF-4F suggest a role in translational control. J. Biol. Chem. **262:**380–388.
8. **Edery, I., M. Humbelin, A. Darveau, K. A. W. Lee, S. Milburn, J. W. B. Hershey, H. Trachsel, and N. Sonenberg.** 1983. Involvement of eIF-4A in the cap recognition process. J. Biol. Chem. **258:**11398–11403.
9. **Edery, I., K. A. W. Lee, and N. Sonenberg.** 1984. Functional characterization of eukaryotic mRNA cap binding protein complex: effects on translation of capped and naturally uncapped RNAs. Biochemistry **23:**2456–2462.
10. **Edery, I., J. Pelletier, and N. Sonenberg.** 1987. Role of eukaryotic RNA cap-binding protein in regulation of translation, p. 335–366. *In* J. Ilan (ed.), Translational regulation of gene expression. Plenum Publishing Corp., New York.
11. **Etchison, D., J. Hansen, E. Ehrenfeld, I. Edery, N. Sonenberg, S. Milburn, and J. W. B. Hershey.** 1984. Demonstration in vitro that eucaryotic initiation factor 3 is active but that a cap-binding protein complex is inactive in poliovirus-infected HeLa cells. J. Virol. **51:**832–837.
12. **Ford, M. J., I. A. Anton, and D. P. Lane.** 1988. Nuclear protein with sequence homology to translation initiation factor eIF-4A. Nature (London) **332:**736–738.
13. **Frisby, D., M. Eaton, and P. Fellner.** 1976. Absence of 5′ terminal capping in encephalomyocarditis virus RNA. Nucleic Acids Res. **3:**2771–2787.
14. **Gehrke, L., P. E. Auron, G. J. Quigley, A. Rich, and N. Sonenberg.** 1983. 5′ conformation of capped alfalfa mosaic ribonucleic acid 4 may reflect its independence of the cap structure or of cap binding protein for efficient translation. Biochemistry **22:**5157–5164.
15. **Geider, K., and H. Hoffman-Berling.** 1981. Proteins controlling the helical structure of DNA. Annu. Rev. Biochem. **50:**233–260.
16. **Goetz, G. S., F. B. Dean, J. Hurwitz, and S. W. Matson.** 1988. The unwinding of duplex regions in DNA by the simian virus 40 large tumor antigen-associated DNA helicase activity. J. Biol. Chem. **263:**383–392.
17. **Goss, D. J., C. Woodley, and A. J. Wahba.** 1987. A fluorescence study of the binding of eucaryotic initiation factors to messenger RNA and messenger RNA analogs. Biochemistry **26:**1551–1556.
18. **Grabowski, P. J., R. A. Padgett, and P. A. Sharp.** 1984. Messenger RNA splicing *in vitro*: an excised intervening sequence and potential intermediate. Cell **37:**415–427.
19. **Grifo, J. A., R. D. Abramson, C. A. Satler, and W. C. Merrick.** 1984. RNA-stimulated ATPase activity of eukaryotic initiation factors. J. Biol. Chem. **259:**8648–8654.
20. **Grifo, J. A., S. M. Tahara, M. A. Morgan, A. J. Shatkin, and W. C. Merrick.** 1983. New initiation factor activity required for globin mRNA translation. J. Biol. Chem. **258:**5804–5810.
21. **Hewlett, M. J., J. K. Rose, and D. Baltimore.** 1976. 5′-Terminal structure of poliovirus polyribosomal RNA is pUp. Proc. Natl. Acad. Sci. USA **73:**327–330.
22. **Hassin, D., R. Korn, and M. S. Horwitz.** 1986. A major internal site for the in vitro translation of the adenovirus DNA polymerase. Virology **155:**797–804.
23. **Herman, R. C.** 1986. Internal initiation of translation on the vesicular stomatitis virus phosphoprotein mRNA yields a second protein. J. Virol. **58:**797–804.
24. **Hirling, H., M. Scheffner, T. Restle, and H. Stahl.** 1989. RNA helicase activity associated with human p68 protein. Nature (London) **339:**562–564.
25. **Jagus, R., W. F. Anderson, and B. Safer.** 1981. The regulation of initiation of mammalian protein synthesis. Prog. Nucleic Acid Res. Mol. Biol. **25:**127–185.
26. **Jang, S. K., H. G. Kraüsslich, M. J. H. Nicklin, G. M. Duke, A. C. Palmenberg, and E. Wimmer.** 1988. A segment of the 5′ nontranslated region of encephalomyocarditis virus RNA directs internal entry of ribosomes during in vitro translation. J. Virol. **62:**2636–2643.
27. **Konarska, M. M., R. A. Padgett, and P. A. Sharp.** 1984. Recognition of cap structure in splicing *in vitro* of mRNA precursors. Cell **38:**731–736.
28. **Kozak, M.** 1987. Leader length and secondary structure modulate mRNA function under conditions of stress. Mol. Cell. Biol. **8:**2737–2744.
29. **Lawson, T. G., M. H. Cladaras, B. K. Ray, A. K. Lee, R. D. Abramson, W. C. Merrick, and R. E. Thach.** 1988. Discriminatory interaction of purified eukaryotic initiation factors 4F plus 4A with the 5′ ends of reovirus messenger RNAs. J. Biol. Chem. **263:**7266–7276.
30. **Lawson, T. G., K. A. Lee, M. M. Maimone, R. D. Abramson, T. E. Dever, W. C. Merrick, and R. E. Thach.** 1989. ATP-dependent dissociation of helical structures in mRNA molecules by eukaryotic initiation factors −4F, −4A and −4B. Biochemistry **28:**4729–4734.
31. **Lee, M. S., and K. J. Marians.** 1989. The *Escherichia coli* primosome can translocate actively in either direction along a DNA strand. J. Biol. Chem. **264:**14531–14542.
32. **Linder, P., P. F. Lasko, P. Leroy, P. J. Nielsen, K. Nishi, J. Schnier, and P. P. Slonimski.** 1989. Birth of the D-E-A-D box. Nature (London) **337:**121–122.
33. **Linder, P., and P. P. Slonimski.** 1989. An essential yeast protein, encoded by duplicated genes TIF1 and TIF2 and homologous to the mammalian translation initiation factor eIF-4A, can suppress a mitochondrial missense mutation. Proc. Natl. Acad. Sci. USA **86:**2286–2290.
34. **Marcus, A.** 1970. Tobacco mosaic virus acid-dependent amino acid incorporation in a wheat embryo system *in vitro*. J. Biol. Chem. **245:**955–961.
35. **Milligan, J. F., D. R. Groebe, G. W. Witherell, and O. C. Uhlenbeck.** 1987. Oligoribonucleotide synthesis using T7 polymerase and synthetic DNA templates. Nucleic Acids Res. **15:**8783–8798.
36. **Moldave, K.** 1985. Eukaryotic protein synthesis. Annu. Rev. Biochem. **54:**1109–1149.
37. **Nielsen, P. J., G. K. McMaster, and H. Trachsel.** 1985. Cloning of eukaryotic protein synthesis initiation factor genes: isolation and characterization of cDNA clones encoding factor eIF-4A. Nucleic Acids Res. **13:**6867–6880.
38. **Nomoto, A., Y. F. Lee, and E. Wimmer.** 1976. The 5′ end of poliovirus mRNA is not capped with $m^7G(5')ppp(5')Np$. Proc. Natl. Acad. Sci. USA **73:**375–380.
39. **Pain, V. M.** 1986. Initiation of protein synthesis in mammalian cells. Biochem. J. **235:**625–637.
40. **Peabody, D. S., S. Subramani, and P. Berg.** 1986. Effect of upstream reading frames on translation efficiency in simian virus 40 recombinants. Mol. Cell. Biol. **6:**2704–2711.
41. **Pelletier, J., and N. Sonenberg.** 1985. Insertion mutagenesis to increase secondary structure within the 5′ noncoding region of a eukaryotic mRNA reduces translational efficiency. Cell **40:** 515–526.
42. **Pelletier, J., and N. Sonenberg.** 1988. Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA. Nature (London) **334:**320–325.
43. **Ray, B. K., T. G. Lawson, J. C. Kramer, M. H. Cladaras, J. A. Grifo, R. D. Abramson, W. C. Merrick, and R. E. Thach.** 1985. ATP-dependent unwinding of messenger RNA structure by eukaryotic initiation factors. J. Biol. Chem. **260:**7651–7658.
44. **Rhoads, R. E.** 1988. Cap recognition and the entry of mRNA into the protein synthesis initiation cycle. Trends Biochem. Sci. **13:**52–56.
45. **Rozen, F., J. Pelletier, H. Trachsel, and N. Sonenberg.** 1989. A lysine substitution in the ATP binding site of eucaryotic initiation factor 4A abrogates nucleotide-binding activity. Mol. Cell. Biol. **9:**4061–4063.
46. **Sarkar, G., I. Edery, and N. Sonenberg.** 1985. Photoaffinity labeling of the cap-binding protein complex with ATP/dATP. J. Biol. Chem. **260:**13831–13837.
47. **Scheffner, M., R. Knippers, and H. Stahl.** 1989. RNA unwinding activity of SV40 large T antigen. Cell **57:**955–963.

48. **Seraphin, B., M. Simon, A. Boulet, and G. Faye.** 1989. Mitochondrial splicing requires a protein from a novel helicase family. Nature (London) **337:**84–87.
49. **Shatkin, A. J.** 1976. Capping of eukaryotic mRNAs. Cell **9:** 645–653.
50. **Shatkin, A. J.** 1985. mRNA cap binding proteins: essential factors for initiating translation. Cell **40:**223–224.
51. **Smith, K. R., Y. E. Yancey, and S. W. Matson.** 1989. Identification and purification of a protein that stimulates the helicase activity of the Escherichia coli Rep protein. J. Biol. Chem. **264:**6119–6126.
52. **Sonenberg, N.** 1981. ATP/Mg^{++}-dependent crosslinking of cap binding proteins to the 5′ end of eukaryotic mRNA. Nucleic Acids Res. **9:**1643–1656.
53. **Sonenberg, N.** 1988. Cap binding proteins of eukaryotic messenger RNA: functions in initiation and control of translation. Prog. Nucleic Acid Res. Mol. Biol. **35:**174–207.
54. **Sonenberg, N., D. Guertin, D. Cleveland, and H. Trachsel.** 1981. Probing the function of the eukaryotic 5′ cap structure by using a monoclonal antibody directed against cap-binding proteins. Cell **27:**563–572.
55. **Sonenberg, N., M. A. Morgan, W. C. Merrick, and A. J. Shatkin.** 1978. A polypeptide in eukaryotic initiation factors that crosslinks specifically to the 5′-terminal cap in mRNA. Proc. Natl. Acad. Sci. USA **75:**4843–4847.
56. **Sonenberg, N., and A. J. Shatkin.** 1978. Non-specific effect of m^7GMP on protein-RNA interactions. J. Biol. Chem. **253:** 6630–6632.
57. **Staehelin, T., H. Trachsel, B. Erni, A. Boschetti, and M. H. Schreier.** 1975. Initiation of mammalian protein synthesis, p. 309–323. *In* Proceedings of the Tenth FEBS Meeting, vol. 39. North-Holland/American Elsevier.
58. **Stahl, H., P. Droge, and R. Knippers.** 1986. DNA helicase activity of SV40 large tumor antigen. EMBO J. **5:**1939–1944.
59. **Tahara, S. M., M. A. Morgan, and A. J. Shatkin.** 1981. Two forms of purified m^7G-cap binding protein with different effects on capped mRNA translation in extracts of uninfected and poliovirus-infected HeLa cells. J. Biol. Chem. **256:**7691–7694.
60. **Thomas, K. R., and M. R. Capecchi.** 1986. Introduction of homologous DNA sequences into mammalian cells induces mutations in the cognate gene. Nature (London) **324:**34–38.
61. **Trachsel, H., N. Sonenberg, A. J. Shatkin, J. K. Rose, K. Leong, J. E. Bergmann, J. Gordon, and D. Baltimore.** 1980. Purification of a factor that restores translation of vesicular stomatitis virus mRNA in extracts from poliovirus-infected HeLa cells. Proc. Natl. Acad. Sci. USA **77:**770–774.
62. **Wahle, E., R. S. Lasken, and A. Kornberg.** 1989. The dnaB-dnaC replication protein complex of *Escherichia coli*. II. Role of the complex in mobilizing dnaB functions. J. Biol. Chem. **264:**2469–2475.
63. **Walker, J. E., M. Saraste, M. J. Runwick, and N. J. Gay.** 1982. Distantly related sequences in the α- and β-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. EMBO J. **1:**945–951.
64. **Wickner, S., and J. Hurwitz.** 1975. Interaction of Escherichia coli dnaB and dnaC(D) gene products in vitro. Proc. Natl. Acad. Sci. USA **72:**921–925.
65. **Wold, M. S., and T. Kelly.** 1988. Purification and characterization of replication protein A, a cellular protein required for *in vitro* replication of simian virus 40 DNA. Proc. Natl. Acad. Sci. USA **85:**2523–2527.
66. **Wood, E. R., and S. W. Matson.** 1987. Purification and characterization of a new DNA-dependent ATPase with helicase activity from Escherichia coli. J. Biol. Chem. **262:**15269–15276.