Modulation of the Helicase Activity of eIF4A by eIF4B, eIF4H, and eIF4F*

Eukaryotic initiation factor (eIF) 4A is a DEAD box RNA helicase that works in conjunction with eIF4B, eIF4H, or as a subunit of eIF4F to unwind secondary structure in the 5′-untranslated region of mRNA, which facilitates binding of the mRNA to the 40 S ribosomal subunit. This study demonstrates how the helicase activity of eIF4A is modulated by eIF4B, eIF4H, or as a subunit of eIF4F. Results indicate that a linear relationship exists between the initial rate or amplitude of unwinding and duplex stability for all factor combinations tested. eIF4F, like eIF4A, behaves as a non-processive helicase. Either eIF4B or eIF4H stimulated the initial rate and amplitude of eIF4A-dependent duplex unwinding, and the magnitude of stimulation is dependent on duplex stability. Furthermore, eIF4A (or eIF4F) becomes a slightly processive helicase in the presence of eIF4B or eIF4H. All combinations of factors tested indicate that the rate of duplex unwinding is equivalent in the 5′ → 3′ and 3′ → 5′ directions. However, the optimal rate of unwinding was dependent on the length of the single-stranded region of the substrate when different combinations of factors were used. The combinations of eIF4A, eIF4B, eIF4H, and eIF4F showed differences in their ability to unwind chemically modified duplexes. A simple model of how eIF4B or eIF4H affects the duplex unwinding mechanism of eIF4A is proposed.

Eukaryotic initiation factor (eIF) 4A is the prototypic member of the DEAD box family of ATP-dependent RNA helicases (1). DEAD box (and related DEXH box) proteins share eight highly conserved amino acid sequence motifs and are involved in almost all aspects of RNA metabolism, including transcription, ribosomal biogenesis, pre-mRNA splicing, RNA export, translation, and RNA degradation (2, 3). The RNA binding, RNA-dependent ATPase, and RNA unwinding activities of eIF4A have been studied in great detail. Analyses of the RNA-activated ATPase activity of eIF4A have demonstrated that the binding of ATP and RNA to eIF4A are coupled and that eIF4A undergoes a sequence of conformational changes as it binds substrates (RNA and ATP), hydrolyzes ATP, and releases products (4, 5). A comprehensive investigation of the unwinding activity has demonstrated that eIF4A is able to function alone as an RNA helicase and that a quantitative relationship exists between the initial rate of unwinding and the stability of the duplex (6). A simple kinetic framework for the helicase activity of eIF4A was established and indicates that duplex unwinding is non-processive (6).

Recently, the helicase activity of eIF4A has been studied with respect to substrate specificity. Results confirmed that the degree of unwinding short (10–15 base pairs) RNA duplexes by eIF4A is dependent only on the stability (rather than the length) of the duplex, and that the amplitude of unwinding may also be correlated with duplex stability (7). eIF4A was also shown to be capable of unwinding blunt-ended (no single-stranded region) RNA/DNA, DNA/RNA, and RNA/DNA-PS (PS is phosphorothioate backbone) duplexes as well as acting in either a 5′ → 3′ or 3′ → 5′ (bidirectional) manner (7). These results, taken together with competitive inhibition experiments, suggest that eIF4A is capable of interacting directly with double-stranded RNA (7).

eIF4A is proposed to function in the m7G cap-dependent initiation of protein synthesis by unwinding secondary structure in the 5′-untranslated region of the mRNA, which facilitates the binding of the 40 S ribosomal subunit to the mRNA and allows for subsequent scanning of the 40 S subunit to the initiator AUG codon (see Refs. 8–10 for reviews on translation initiation). It has been demonstrated via in vitro model assays and fractionated lysate systems that eIF4A works in conjunction with eIF4B, eIF4H, and as a subunit of eIF4F in this important regulatory step of translation initiation (8, 11). Furthermore, eIF4A, eIF4B, and eIF4F have also been implicated in internal ribosome entry site-mediated (m7G cap-independent) initiation (12).

eIF4F is a heterotrimeric protein complex of ~250 kDa composed of eIF4A, eIF4E, and eIF4G (13). eIF4E (25 kDa) interacts directly with the m7G cap of mRNA and is a key protein involved in the regulation of protein synthesis (8, 14). eIF4G (171 kDa) acts as a scaffolding/adapter molecule to coordinate the interactions of eIF4A, eIF4E, eIF3, poly(A)-binding protein, and RNA (8). The RNA binding, ATPase, and helicase activities of eIF4A are all enhanced as part of the eIF4F complex (15–18).

eIF4B is a dimeric protein (70-kDa subunit) that has no independent catalytic activity and functions to stimulate the RNA binding, ATPase, and helicase activities of eIF4A and eIF4F, as well as overall globin synthesis in fractionated lysate systems (13, 16–19). eIF4B contains a canonical RNA recognition motif (RRM), an RNA binding domain, and a DRYG do-

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main that have been implicated in mRNA binding, self-association, interaction with eIF3, and stimulation of eIF4A helicase activity (20–23). Whereas the precise mechanisms of the stimulatory effects of eIF4B on eIF4A and eIF4F are unclear, investigation shows that eIF4B increases the binding affinity of eIF4A for RNA and ATP (16, 17, 24).

eIF4H is a small protein (25 kDa) that enhances the RNA helicase activity of eIF4A, as well as stimulating overall globin synthesis (6, 25, 26). eIF4H also contains an RRm and shows sequence homology to eIF4B in this region (25). It is postulated that eIF4H may act via protein-protein interactions to stabilize conformational changes that occur in eIF4A during RNA binding, ATP hydrolysis, and RNA duplex unwinding (26).

Whereas a simple kinetic framework for duplex unwinding has been established for eIF4A alone, it is unknown how this mechanism is affected by the addition of eIF4B and/or eIF4H or when eIF4A is a subunit of eIF4F. Furthermore, it was of interest to investigate how these factors affected the helicase activity of eIF4A with respect to processivity, duplex recognition, substrate specificity, and directionality. Therefore, similar experiments to those performed previously in our laboratory (6, 7, 26) were conducted with various combinations of eIF4A, eIF4B, eIF4H, and eIF4F in an attempt to elucidate further the mechanism of eIF4A-dependent duplex unwinding and the role of all these factors in the initiation of protein synthesis.

**EXPERIMENTAL PROCEDURES**

**Materials**

Reagents were purchased from the following suppliers: rabbit reticulocyte lysate from Green Hectares, Oregon, WI; ATP and bovine serum albumin from Promega; and [γ-32P]ATP from Perkin Elmer Life Sciences; DNA oligonucleotides PTT-23, PTB-44, D-14, D-15, D-16, D-17, D-20, and D-20C from the Molecular Biology Core Laboratory of Case Western Reserve University; DNA oligonucleotide D-44 from IDT, Inc., Coralville, IA; RNA oligonucleotides R-11, R-12, R-13, R-14, and R-15 from Cybersyn, Lenni, Pennsylvania; RNA oligonucleotides R-17–5’, R-19–5’, R-21–5’, R-23–5’, R-28–5’, R-38–5’, R-17–3’, R-19–3’, R-21–3’, R-23–3’, R-28–3’, R-38–3’–R-17C, and R-13C from DHARMACON Inc., Boulder, CO; Megashortscript™ In Vitro transcription kit from Ambion; and T4 polynucleotide kinase from New England Biolabs. DNA-PS, 2’-MOE, and 2’-MOE/PS oligonucleotides were supplied by Isis Pharmaceuticals, Carlsbad, CA.

**Methods**

**Purification of eIF4A, eIF4B, eIF4F, and eIF4H from Rabbit Reticulocyte Lysate**—Purification of eIF4A, eIF4B, eIF4F, and eIF4H follows the standard procedure used to purify protein translation initiation factors that have been previously published by this laboratory (13, 25, 27). Purification of recombinant eIF4H is described (26).

**DNA and RNA Oligonucleotides**—The sequences of all oligonucleotides used in this report are listed in Table 1 of Ref. 7. The DNA oligonucleotides synthesized by the Molecular Biology Core Laboratory of Case Western Reserve University were oligonucleotide purification cartridge-purified and stored in distilled H2O. DNA oligonucleotides synthesized by IDT, Inc., were polyacrylamide gel electrophoresis-purified, lyophilized, and resuspended in distilled H2O. RNA oligonucleotides synthesized by Cybersyn were polyacrylamide gel electrophoresis-purified, lyophilized, and resuspended in distilled H2O. RNA oligonucleotides synthesized by Dharmacon were deprotected per the manufacturer’s instructions, lyophilized, and resuspended in distilled H2O. Quantitation of each oligonucleotide was performed by UV spectroscopy, and a value of 33 μg per 1 A260 was used in determining concentration. Integrity and proper size of each oligonucleotide were assessed by 32P-end labeling each oligonucleotide (described below) and analyzed on a denaturing (7 M urea) 20% polyacrylamide gel with known size standards.

**Transcription of RNA**—R-44 was synthesized by in vitro transcription and gel-purified as described (7). Quantitation of the purified transcript was performed by UV spectroscopy as described above.

**32P-End Labeling of Oligonucleotides**—The RNA, DNA, DNA-PS, 2’-MOE, and 2’-MOE/PS oligonucleotides were 5’-end-labeled using [γ-32P]ATP and T4 polynucleotide kinase as described (7).
RNA, and RNA/DNA-PS duplexes (7). Thus, in order to compare the efficiency of unwinding of these modified duplexes by different combinations of initiation factors, the degree of unwinding must be corrected to account for slight differences in duplex stability. This allows for comparisons among modified duplex unwindings independent of their stabilities, and thus, only differences in the chemical composition of the duplex substrates that may affect the efficiency of unwinding are compared. Therefore, the normalized amplitude given for each duplex in Fig. 4 is a ratio of the amplitude of unwinding measured experimentally versus the amplitude of unwinding that would be predicted for each duplex based on its stability (ΔG value). The predicted amplitude values for each factor combination are calculated from a standard curve generated by plotting the amplitude of unwinding versus duplex stability for the RNA/RNA substrates listed in Table I (data not shown and Ref. 7). Thus, the normalized amplitude of unwinding presented in Fig. 4 represents the percentage of unwinding observed for a chemically modified duplex, when compared directly to a RNA/RNA duplex of the identical stability, and shows differences in the efficiency of unwinding based only on differences in chemical composition.

RESULTS

Helicase Activity of eIF4A in the Presence of eIF4B and/or eIF4H or as a Subunit of eIF4F—Previous studies have demonstrated that the helicase activities of eIF4A and eIF4F are stimulated by eIF4B and/or eIF4H (6, 18, 26, 35). However, it is unknown to what degree eIF4B and/or eIF4H stimulate eIF4A or eIF4F and whether these factors are able to act in an additive or synergistic fashion with each other. Thus, it is unclear if eIF4A and eIF4F have similar mechanisms of unwinding and, furthermore, how eIF4B and eIF4H affect the mechanism of unwinding by eIF4A or eIF4F. In order to investigate how this family of initiation factors interact with each other with respect to unwinding activity, a comprehensive series of experiments using various combinations of eIF4A, eIF4B, eIF4F, and eIF4H was performed.

In order to determine the ratio of eIF4B relative to eIF4A for optimal unwinding activity, 0–0.4 μM eIF4B was titrated into reactions containing 0.2 μM eIF4A and 2.0 nM R-44/R-12 duplex substrate. Fig. 1A shows that half-maximal unwinding is achieved when the concentration of eIF4B (dimer) is ~0.10 μM. Thus, under conditions used in this assay, maximal stimulation of eIF4A helicase activity occurs when eIF4A and eIF4B are present in approximately equimolar amounts. This result is similar to that obtained when analogous experiments were performed with eIF4A and eIF4H (26), and comparable results were observed when 0–0.4 μM eIF4B or eIF4H was titrated against 0.2 μM eIF4F (data not shown).

Next, it was determined if eIF4B and eIF4H were able to act in an additive or synergistic fashion when combined together with eIF4A. eIF4B was titrated into reactions containing a fixed amount of eIF4A (0.2 μM) and a fixed “subsaturating” amount of eIF4H (either 0.025 or 0.05 μM). The results are presented in Fig. 1B and indicate that the stimulation of eIF4A helicase activity provided by eIF4B and eIF4H together is additive, not synergistic, when both eIF4B and eIF4H are present at subsaturating concentrations (compare initial rates when 0–0.1 μM eIF4B is used). Furthermore, using “saturating” amounts of eIF4B (0.2 μM) with respect to eIF4A in the helicase reactions minimizes any stimulatory effects of eIF4H when it is present in the reaction (compare initial rates when 0.2 μM eIF4B is used). Similar results were obtained in reciprocal experiments in which eIF4H was titrated into reactions containing eIF4A and subsaturating amounts of eIF4B (0.025 or 0.05 μM, data not shown) and again indicates that the stimulation provided by eIF4B and eIF4H is additive rather than synergistic.

Results presented in Fig. 1 indicate that using 0.2 μM of each protein would allow for accurate measurement of unwinding activity for various combinations of initiation factors under these assay conditions. Therefore, the initial rates and amplitudes of unwinding duplexes of increasing length and stability for select combinations of eIF4A, eIF4B, eIF4F, and eIF4H were measured in the same manner as described previously (6, 7). Table I presents initial rate and amplitude data for unwinding the R-44/R-11, -12, -13, -14, and 15 duplexes by different factor combinations. It can be seen that the addition of eIF4B and/or eIF4H to eIF4A or eIF4F results in an increased degree of duplex unwinding, and the greatest degree of unwinding occurs when all four factors are present. Furthermore, it can be seen that both the initial rate and amplitude of unwinding decrease as the length and stability of the duplex increases for all combinations tested. It should be noted that neither eIF4B nor eIF4H exhibits any helicase activity in the absence of eIF4A or eIF4F, and no factor combination exhibits helicase activity in the absence of ATP or in the presence of ADPNP (data not shown and Refs. 6, 18, 26).

The results presented in Table I also indicate that the magnitude of stimulation of the initial rate of eIF4A helicase activity by eIF4B or eIF4H increases as the stability of the duplex increases (3.5–7.9 and 3.8–6.5×, respectively.) However, the
increase in the magnitude of duplex unwinding by eIF4F when compared with eIF4A is constant as the stability of the duplex increases (2.2-fold, Table I). These results suggest that eIF4A and eIF4F share a similar mechanism of unwinding, whereas the addition of eIF4B or eIF4H to eIF4A affects the helicase mechanism in some way.

To explore this hypothesis further, the ln(initial rates of unwinding) for selected factor combinations were plotted against the stability (ΔG value) of the duplexes in a similar manner reported previously for eIF4A alone and eIF4A + eIF4H (6, 7, 26). Fig. 2A shows increasing the concentration of eIF4A in the helicase reaction does not affect the value of the slope of the ln(initial rate of unwinding) versus duplex stability (i.e. the lines are parallel). The slopes of eIF4A-dependent unwinding for each concentration of eIF4A are equal to that of thermal melting and support the previously presented model that eIF4A-dependent unwinding is directly related to the degree of thermal melting of the duplex (6, 7). Fig. 2B shows the plot of the ln(initial rate of unwinding) versus duplex stability for eIF4F. When compared with the plots of eIF4A and thermal melting, the slopes have the same value (e.g. are parallel, Table I), which suggests that the mechanism for eIF4F-dependent unwinding is the same as for eIF4A (i.e. non-processive). Fig. 2C shows the plots of the ln(initial rates of unwinding) versus duplex stability for the combination of eIF4A + eIF4B, eIF4A + eIF4H, and eIF4A + eIF4B + eIF4F + eIF4H. Note that these lines are both shifted up and non-parallel (i.e. the value of the slopes decrease approximately 2-fold, Table I), when compared with eIF4A alone, eIF4F alone, or thermal melting, and are similar to the results obtained for eIF4A + eIF4H previously (26). The significance of these changes in terms of the helicase mechanism will be discussed below.

It should be noted that similar results to those presented in Fig. 2 are obtained when the ln(amplitude of unwinding) is plotted versus duplex stability for the various combinations of initiation factors tested (data not shown). Thus, there is a linear relationship between the ln(amplitude of unwinding) and duplex stability. Furthermore, the values of the slopes obtained when the ln(amplitude of unwinding) is plotted versus duplex stability for the various factor combinations used are the same as those presented in Fig. 2 and Table I (data not shown). This indicates that, like eIF4A alone (7), the relationship between the amplitude of unwinding and duplex stability is similar to that observed between the initial rate of unwinding and duplex stability for the different combinations of initiation factors investigated. Thus, since the amplitude of unwinding is linearly correlated with duplex stability, measuring the amplitude provides an efficient means for comparing how effectively various combinations of eIF4A, eIF4B, eIF4H, and eIF4F are able to unwind duplexes with different stabilities and/or physical and chemical modifications (see below).

### Effect of the Length and Direction of the Single-stranded Region on the Helicase Activity of Various Combinations of Initiation Factors

Recently, it was demonstrated (7) that the helicase activity of eIF4A is only minimally dependent on the length of the single-stranded region of a duplex substrate and that eIF4A is capable of unwinding a blunt-ended duplex. Also, it was shown (7) that rate of duplex unwinding by eIF4A is approximately equal for duplexes containing either 5'- or 3'-single-stranded regions, and thus bidirectional helicase activity is an inherent function of eIF4A. To investigate if eIF4A helicase activity would be altered by the presence of eIF4B, eIF4H, or as a subunit of eIF4F with respect to the length and directionality of the single-stranded region adjacent to the double-stranded region of a helicase substrate, the unwinding assay was performed using a series of 13-bp duplex substrates with either 5'- or 3'-single-stranded regions ranging from 0 to 25 nt. Fig. 3A is a schematic diagram representing the duplex substrates used. The sequences of both the single-stranded and double-stranded regions are exactly the same for the 5'- and 3'-substrates. Presented in Fig. 3B are the experimental data for eIF4A, eIF4A + eIF4B, eIF4A + eIF4H, and eIF4F unwinding duplexes with 0 (blunt) and 25 nucleotide 5'-single-stranded regions. The complete set of data is summarized in Fig. 3C.

The results indicate that eIF4H stimulates the ability of eIF4A to unwind a blunt-ended duplex by ~2.2-fold (Fig. 3, B and C, compare 0.2 μM eIF4A with 0.2 μM eIF4A + 0.2 μM eIF4H, 0-nt ss region). This stimulation increases to ~4-fold as the length of the single-stranded region is increased to 10 nt (Fig. 3C, compare 0.2 μM eIF4A with 0.2 μM eIF4A + 0.2 μM eIF4H, 10-nt ss region). There is no increase in stimulation beyond 10 nt, suggesting this is the minimal size of a single-stranded region needed for optimal unwinding activity by eIF4A + eIF4H. Note that this degree of stimulation (4-fold) is in close agreement with the data presented in Table I and previously for the combination of eIF4A + eIF4H using a duplex of similar stability (~21.4 kcal/mol, 5-fold (26)).

eIF4B was only able to stimulate the ability of eIF4A to unwind a blunt-ended duplex by ~1.7-fold (compare 0.2 μM eIF4A with 0.2 μM eIF4A + 0.2 μM eIF4B, 0-nt ss region). This
stimulation increases to 5-fold as the length of the single-stranded region is increased to 25 nt (Fig. 3, B and C, compare 0.2 μM eIF4A with 0.2 μM eIF4A/0.2 μM eIF4B, 25-nt ss region). Note that this degree of stimulation (5-fold) is in agreement with the data presented in Table I for the combination of eIF4A/eIF4B using a duplex of similar stability (ΔG = 21.4 kcal/mol, 5.3-fold).

In contrast to eIF4A alone, eIF4F helicase activity displayed the greatest dependence on the length of the single-stranded region, with almost no unwinding observed with the blunt-ended duplex (amplitude ~5%). eIF4F helicase activity increased dramatically as the length of the single-stranded region was increased, and maximal activity is shown between 15 and 25 nt (amplitude ~60%). This reflects an approximate 10-fold increase in the degree of unwinding relative to the
amplitude observed with the blunt-ended duplex (Fig. 3, B and C, 0-nt ss region versus 25-nt ss region).

Overall, results from this series of experiments also indicate that all combinations were able to unwind duplexes in both the 5′→3′ and 3′→5′ directions with approximately the same efficiency. However, like eIF4A alone, eIF4F appears to be slightly more active in the 5′→3′ direction for substrates with lengths of the single-stranded region shorter than that required for optimal helicase activity (4→6 nt for eIF4A and 15→25 nt for eIF4F, Fig. 3C).

Ability of Various Factor Combinations to Unwind Chemically Modified Duplexes—Previous results demonstrated that eIF4A unwinds RNA/DNA, DNA/RNA, and RNA/DNA-PS (PS is phosphorothioate backbone) duplexes with a similar efficiency as RNA/RNA duplexes and that eIF4A does not unwind DNA/DNA or RNA/2′-MOE (2′-MOE is 2′-methoxyethyl group) duplexes (7). Thus, it was of interest to examine if the addition of eIF4B or eIF4H to eIF4A, or if eIF4A was a subunit of eIF4F, affected the ability of eIF4A to unwind chemically modified duplexes. The combinations of eIF4A, eIF4A + eIF4B, eIF4A + eIF4H, and eIF4F were tested in the helicase assay using RNA/RNA, RNA/DNA, DNA/RNA, DNA/DNA, RNA/DNA-PS, and RNA/2′-MOE duplex substrates, and the results are presented in Fig. 4. Each factor combination unwound the RNA/RNA duplex with the expected amplitude for an RNA/RNA duplex having a stability of −21.4 kcal/mol (Table I). Thus, the amplitudes of unwinding the RNA/RNA duplex for each factor combination have been normalized to 100%, and unwinding of the modified duplexes (RNA/DNA, DNA/RNA, DNA/DNA, RNA/DNA-PS, and RNA/2′-MOE) have been corrected for slight differences in duplex stability and scaled to these values. Thus, the results presented in Fig. 4 are the normalized amplitudes of unwinding based on the stability of the duplex and therefore only reflect differences in the degree of unwinding due to the chemical composition of the duplexes (see “Materials and Methods” and Fig. 4 legend for further details on data treatment and normalization).

The combination of eIF4A + eIF4H was able to unwind RNA/DNA and DNA/RNA substrates with slightly greater activities than predicted (113 and 111%, respectively), yet was slightly less active in unwinding the RNA/DNA-PS substrate (73%). In contrast, eIF4A + eIF4B was less active in unwinding RNA/DNA, DNA/RNA, and RNA/DNA-PS substrates (76, 61, and 71% of predicted amplitude, respectively) when compared with eIF4A alone. Surprisingly, eIF4F was unable to unwind the RNA/DNA, DNA/RNA, or RNA/DNA-PS substrates (3, 1, and 2%, respectively). No factor combination was able to unwind a DNA/DNA or RNA/2′-MOE duplex to any significant degree. These results indicate that the recognition of modified duplex substrates by eIF4A may be modulated by the presence of eIF4B or eIF4H or as a subunit of eIF4F. The basis for these differences will be discussed below.

DISCUSSION

In this study, the modulation of eIF4A helicase activity by eIF4B, eIF4H, or as a subunit of the eIF4F complex was investigated. Results indicate that optimal helicase activity is achieved when eIF4A and eIF4B exist in equimolar amounts. Similar results were observed previously with eIF4A and eIF4H (26). When eIF4B and eIF4H are included together with eIF4A, an additive stimulatory, rather than synergistic, effect is observed. Similar additive behavior between eIF4B and eIF4H was observed in the RNA-dependent ATPase and globin synthesis assays (25) and suggests that eIF4H and eIF4B may complement each other when either protein is limiting in vivo.

The results presented in Fig. 2A indicate that as the concentration of eIF4A is increased, greater initial rate values exist for each ΔG value. That these lines are parallel to each other and that of thermal melting indicate that the non-processive mechanism of unwinding by eIF4A is not altered by an increase of eIF4A concentration. This interpretation may be extended to eIF4F. Fig. 2B shows that a linear relationship between the ln(initial rate of unwinding) and duplex stability also exists for eIF4F, and that the value of slope is unchanged (is parallel, Table I) when compared with either thermal melting or eIF4A-dependent unwinding. Furthermore, Table I indicates that the magnitude of the increase in helicase activity when eIF4A is a subunit of eIF4F is independent of duplex stability (−2.2× at all ΔG values). This increase in activity is likely due to eIF4F having a greater binding affinity for RNA than eIF4A (−2.5-fold as measured by nitrocellulose filter binding assays) (16, 17). Thus, the mechanism of unwinding by eIF4A as a subunit...
of eIF4F appears similar to that of eIF4A alone (i.e. non-processive) and may be described by the models of unwinding proposed previously for eIF4A (6, 7).

It should be noted that none of the duplex substrates used in the experiments with eIF4F contained a 5′ m7G cap, and it would be expected that a capped duplex would serve as a slightly better substrate as demonstrated previously for eIF4F + eIF4B (18). By using a capped substrate, a direct comparison of eIF4A alone versus eIF4A as part of the eIF4F complex would be difficult due to the effects of eIF4F binding to the m7G cap. In addition, using non-capped substrates bears physiological significance as eIF4F, eIF4A, and eIF4B have been shown to be required for internal initiation of EMCV mRNA (12). This RNA does not have an m7G cap structure but rather possesses an internal ribosome entry site, and it has been postulated that rearrangement or unwinding of this structure by eIF4F (or eIF4A and eIF4B) may be required for mRNA and 40 S ribosomal subunit interactions (12).

The behavior of eIF4A or eIF4F is in contrast with the combinations of eIF4A + eIF4B, eIF4A + eIF4H, and eIF4A + eIF4B + eIF4H + eIF4F. The results indicate there is a change in the magnitude of stimulation with respect to duplex stability (i.e. addition of eIF4B and eIF4H shows a greater fold stimulation of eIF4A activity as the stability of the duplex is increased, Table I). This change translates into an approximate 2-fold decrease in the value of the slope of the ln(initial rate of unwinding) versus duplex stability for these factor combinations relative to those seen with thermal melting, eIF4A alone, or eIF4F alone (Fig. 2C and Table I). Thus, a decrease in the value of the slope is interpreted to mean that the mechanism of eIF4A (or eIF4F) helicase activity is altered by the presence of eIF4B or eIF4H. The increase in the stimulation of eIF4A helicase activity by eIF4B or eIF4H as the stability of the duplex increases suggests that either of these factors may allow eIF4A to unwind more base pairs before dissociating from the duplex substrate. This implies that the unwinding reaction is becoming slightly processive.

If only the initial binding event (4A–ATP + RNA) is enhanced by eIF4B or eIF4H, it would be expected that the stimulation of eIF4A activity for all duplexes would be equal, regardless of stability, and that the slope of the ln(initial rate of unwinding) versus duplex stability would be parallel to that of eIF4A alone. Fig. 2C indicates that the slopes of factor combinations containing eIF4B and/or eIF4H are not parallel to that of eIF4A alone and implies that eIF4B or eIF4H may act at a kinetic step in the unwinding reaction after the initial substrate binding event.

In the case of eIF4B, previous studies have demonstrated that mammalian eIF4B stimulates the ATPase activity of eIF4A primarily by increasing the affinity of eIF4A for RNA (16, 17). This increase in binding affinity of eIF4A for RNA due to the presence of eIF4B is supported by previous results (16), and recent investigation (24) indicates that wheat germ eIF4B functions to enhance the affinity of eIF4A for both ATP and RNA. It is possible that eIF4B stimulates eIF4A helicase activity by increasing both the affinity of eIF4A for RNA and increasing the utilization of ATP by eIF4A. These increases would allow eIF4A to undergo more duplex unwinding events per substrate binding event. This possibility is in agreement with the conclusions drawn for wheat germ eIF4A and eIF4B (24).

It is also conceivable that eIF4B stimulates eIF4A helicase activity by affecting the duplex unwinding step directly, possibly by stabilizing one or more conformational changes that eIF4A cycles through during ATP hydrolysis (5). This possibility was suggested previously for eIF4H, which only slightly enhances the affinity of eIF4A for RNA and does not significantly increase the catalytic step of ATP hydrolysis by eIF4A (25, 26). Furthermore, since both eIF4B and eIF4H have RRMIs, it is also possible that either protein could help to destabilize the duplex substrate by binding to the newly formed single-stranded region after partial strand separation by eIF4A. Thus, although eIF4B and eIF4H both seem to stimulate eIF4A helicase activity by allowing eIF4A to unwind more base pairs per productive binding event, their mechanisms of action would appear to be dissimilar as judged by how they differentially affect the RNA binding and ATPase activities of eIF4A. It is also possible that eIF4H and/or eIF4B fulfill the role of additional domains found in the HCV NS3, PcrA, and Rep helicases (36–39) that are absent from the eIF4A structure (40–42).

It should be noted that the experimental methodology used here has limitations in that only complete duplexes or fully displaced single strands may be detected, and that ATPase activity cannot be monitored concomitantly with unwinding activity. Thus, whereas macroscopic differences in the overall rates and amplitudes of duplex unwinding by eIF4A and other initiation factors are detectable using different reaction conditions, the microscopic kinetic events of the unwinding reaction demonstrated for other helicases (38, 43–48) are difficult to investigate via this assay. We also wish to stress that the use of the ln(initial rate of unwinding) versus ΔG plots are used as a convenient means to illustrate the relationship between the initial rate (or amplitude) of unwinding and duplex stability for various types of duplex substrates. The rationale for this relationship is documented in a previous study (6), and the equation used is only an approximation of the overall macroscopic unwinding process. Furthermore, the significance of the changes of slopes in these plots are used only in a qualitative sense to indicate that the helicase mechanism of eIF4A and eIF4B (or eIF4H) is likely different from that of eIF4A or eIF4F alone, which appears to be dependent on the rate of thermal melting (6, 7).

Results from experiments designed to test the effect of the length and direction of the single-stranded region on helicase activity of various combinations of factors demonstrate that as the size of the protein complex is increased, the length of the single-stranded region required for optimal unwinding activity also increased. That eIF4F is unable to unwind a blunt-ended duplex and that the combination of eIF4A + eIF4B has a minimal ability to unwind a blunt-ended duplex is in agreement with previous experiments performed by Rozen et al. (18), and also with data showing that secondary structures placed proximal to the m7G cap are more inhibitory to translation initiation than those placed further downstream in the 5′-untranslated region of mRNAs (49).

This series of experiments also indicates that the ability of eIF4A to unwind duplexes in either the 5′ → 3′ or 3′ → 5′ direction with equal efficiency is not affected by eIF4B or eIF4H (Fig. 3C). This is in agreement with previous studies performed (18). eIF4F shows some preference for unwinding in the 5′ → 3′ direction with shorter single-stranded regions (<15 nt, Fig. 3C). This may be due to the eIF4A subunit being asymetrically positioned in the eIF4F complex such that access of the eIF4A subunit to the double-stranded region is favored in the 5′ → 3′ orientation and is somewhat occluded in the 3′ → 5′ orientation with shorter single-stranded regions. This slight preference for 5′ → 3′ unwinding may be of biological significance, as scanning is proposed to occur in a 5′ → 3′ direction.

The ability of eIF4A to unwind RNA/DNA, DNA/RNA, RNA/ DNA-PS duplexes may be modulated by eIF4B, eIF4H, or when
eIF4A is a subunit of eIF4F. eIF4H stimulated eIF4A unwinding of RNA/DNA and DNA/RNA duplexes to the expected levels when compared with an RNA/RNA duplex of identical stability, whereas the activity of eIF4A + eIF4B was reduced. Surprisingly, eIF4F was only able to unwind RNA/RNA duplexes. These differences in behavior are likely explained by the chemical composition of single-stranded and double-stranded regions presented to the proteins, the differences in the helical conformations (A-form versus B-form) of the duplex substrates, as well as the conformational states available to eIF4A alone and with other initiation factors.

Recent structural studies of eIF4A show that the molecule is composed of two domains connected by a polypeptide linker (40, 42), indicating that the conformation of eIF4A may be flexible rather than rigid. Since eIF4H is a small protein and contains only one RRM, it may not impose a more rigid conformation on the structure of eIF4A. Thus eIF4A alone, or in the presence of eIF4H, is able to recognize both A-form (RNA/RNA) and intermediate form (RNA/DNA and DNA/RNA) helices, allowing the expected amplitudes of unwinding based on duplex stability (Fig. 4). In contrast, eIF4B is much larger than eIF4H and contains both an RRM and arginine-rich RNA-binding motif (21). This may impose a more rigid conformation on eIF4A during the unwinding process. Therefore, this combination of factors may have a lower tolerance for recognizing intermediate-form duplexes, as is observed with the less than expected amplitudes with RNA/DNA and DNA/RNA duplexes relative to RNA/RNA duplexes (Fig. 4). This reasoning may be extended to when eIF4A is a subunit of eIF4F. Like eIF4B, the eIF4G subunit contains an RNA binding domain plus two arginine-rich motifs (50), and since eIF4A is a subunit of the eIF4F complex, eIF4G and/or eIF4E may impose a more rigid conformation on eIF4A such that only A-form (RNA/RNA) helices are recognized by eIF4F in the unwinding process. Thus, eIF4F is unable to unwind either RNA/DNA or DNA/RNA duplexes.

That the inability of eIF4A to unwind DNA/DNA or RNA/2'-MOE duplexes could not be relieved by eIF4H, eIF4B, or as a subunit of eIF4F supports the idea that the helicase mechanism is primarily a function of eIF4A, and at least one RNA strand (a 2'-OH) is required for unwinding activity and/or any combination of factors is unable to recognize a B-helix (DNA/DNA). Furthermore, the presence of a bulky 2'-MOE group on one strand prevents eIF4A from interacting properly with the duplex substrate as discussed previously (7).

In conclusion, a simple model consistent with the results presented here and from previous investigations is presented in Fig. 5. This model illustrates how eIF4B and/or eIF4H may enhance the helicase activity of eIF4A. This model may also be applicable to the combination of eIF4A + eIF4H, although eIF4H does not bind to RNA well and may exert its influence more by direct protein-protein interactions with eIF4A. Note that the second round of unwinding is what allows the combination of eIF4A + eIF4B (or eIF4A + eIF4H) to efficiently unwind more stable duplexes than eIF4A alone, which is limited to a single round of unwinding per productive binding event.

It is stressed that this model illustrates only a subset of possible variations that may exist among eIF4A, eIF4B, eIF4H, ATP, ADP, and RNA. In addition, the presence of eIF4F increases the number of variations and complexity of the unwind-
ing reaction. Furthermore, the nature of the protein-protein interactions and the order of binding events among the different proteins and the duplex substrate are still unknown. A more detailed analysis of these complex interactions will be required to fully understand the distinct steps of the mechanism of unwinding by eIF4A, eIF4B, eIF4F, and eIF4H.

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