Eucaryotic Initiation Factor 4B of Wheat Germ Binds to the Translation Initiation Region of a Messenger Ribonucleic Acid†

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ABSTRACT: Purified preparations of eucaryotic initiation factor 4B (eIF4B) from wheat germ bind the monocistronic, uncapped, mRNA satellite tobacco necrosis virus RNA (STNV RNA) in nitrocellulose-mediated binding assays. This reaction is mRNA specific and yields dissociation constants (K_d) in the 10^{-7}–10^{-8} M range, depending upon the particular enzyme preparation tested. Purified wheat germ eIF4A, in the presence or absence of ATP, does not bind STNV RNA efficiently, but added eIF4A and ATP do enhance the efficiency of the eIF4B-dependent binding of STNV RNA. Wheat germ eIF4B binds the oligonucleotide containing the 5′-terminal 52 nucleotides of STNV RNA (designated 1–52) with the same affinity as intact STNV RNA. This binding affinity is less with the 1–44 oligonucleotide of STNV RNA and does not occur with the 1–33 oligonucleotide of STNV RNA that contains the 5′-terminal untranslated region and the initiator AUG codon at positions 30–32 of this mRNA. Wheat germ eIF4B therefore binds the translation initiation region of STNV RNA, and this binding requires up to 20 nucleotides on the 3′ side of the initiator AUG codon of this mRNA. Wheat germ eIF4B also efficiently binds an oligonucleotide containing nucleotides from positions 13–52 in from the 5′ terminus of STNV RNA, thereby establishing that the postulated 5′-terminal stem and loop secondary structure of STNV RNA [Leung, D. W., Browning, K. S., Heckman, J. E., RajBhandary, U. L., & Clark, J. M., Jr. (1979) *Biochemistry 18*, 1361–1366] is not functional or essential for this specific binding reaction. Wheat germ ribosomes show these same specificities toward binding of 5′-terminal fragments of STNV RNA. It follows that this specific interaction of eIF4B with the translation initiation region of STNV RNA is an essential step in the initiation of translation of this mRNA.

Two general approaches have been used to investigate how eucaryotes select correct initiator AUG codons on their mRNAs. First, various workers have studied the mechanistic steps associated with the formation of translation initiation complexes. These studies have led to the identification of a wide variety of eucaryotic initiation factors (eIFs) and to the characterization of general steps where these specific eIFs act in the initiation of eucaryotic mRNA translation [see Maitra et al. (1982) for a recent review of this area]. However, these studies have not revealed exact mechanistic roles for specific eIFs in the selection of initiator codons on eucaryotic mRNAs. Other workers have used studies of structural features of eucaryotic mRNAs as the basis for a second approach to detect potential mechanisms of initiator AUG selection in eucaryotes. These structural studies have characterized the 5′-terminal $m^7G(5')ppp(5')$... capping groups essential for the initiation of the translation of most eucaryotic mRNAs (Shatkin, 1976). Structural studies with mRNAs have also prompted "scanning models" which suggest that 40S ribosome migration in from the 5′ termini of eucaryotic mRNAs to the first AUG governs the selection of correct initiator AUG codons on eucaryotic mRNAs (Kozak, 1978, 1981, 1982). Yet, several eucaryotic mRNAs lack structural features required by these scanning models (Leung et al., 1976; Dhruva et al., 1980; Kozak, 1981; Hendy et al., 1981; Jay et al., 1981; Bos et al., 1981). Further, recent genetic studies designed to test these scanning models establish that structures around specific AUGs are more important during initiator AUG selection than is the position of an AUG relative to the 5′ terminus of the mRNA (Lomedico & Andrew, 1982). In short, there are limits to the current approaches being used to study the mechanism of initiator AUG selection in eucaryotes, and our general knowledge of these processes can benefit from new experimental approaches.

This paper reports a new experimental approach to initiator AUG selection in eucaryotes. This approach combines studies of a specific translation initiation step with specific structural features of a eucaryotic mRNA. Specifically, we report that the eucaryotic initiation factor eIF4B binds to specific nucleotides of the translation initiation region of the mRNA satellite tobacco necrosis virus (STNV)[1] RNA and that this specific interaction with eIF4B is a likely intermediate step in the uptake of this mRNA into translation initiation complexes on ribosomes.

Experimental Procedures

Materials. STNV RNA was prepared from germinating mung beans infected with the B strain of TNV and the SV1 strain of STNV (Clark & Klein, 1974). STNV [C-^{125}I]RNA was prepared from STNV RNA by the in vitro radioiodination procedure of Commerford (1971) as previously described (Browning et al., 1980). STNV [5′-^{32}P]RNA was prepared from STNV RNA by dephosphorylation and subsequent T_4 polynucleotide kinase mediated 5′-terminal phosphorylation with [γ-^{32}P]ATP as previously described (Leung et al., 1979). Both of these radiolabeled STNV RNAs were purified by gel electrophoresis and subsequent electroelution prior to assay

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[1] Abbreviations: STNV, satellite tobacco necrosis virus; TNV, tobacco necrosis virus; STNV [C-^{125}I]RNA, STNV RNA containing ^{125}I label in 5-[^{125}I]iodocytidylate as a result of radioiodination by the procedure of Commerford (1971); PMSF, phenylmethanesulfonyl fluoride; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; B-50, -100, -120, -150, -250, and -500 buffer, 20 mM Hepes potassium salt, pH 7.6, 0.1 mM EDTA, 1 mM dithiothreitol, and 10% glycerol containing 50, 100, 120, 150, 250, and 500 mM KCl, respectively; binding buffer, 20 mM Hepes potassium salt, pH 7.6, 100 mM KCl, 2 mM $Mg(OAc)_2$, 0.1 mM Na_2EDTA, and 1 mM dithiothreitol; BSA, bovine serum albumin.

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in order to remove unreacted radiolabeling reagents and radiolabeled fragments of STNV RNA. [γ-^{32}P]ATP was prepared by the method of Johnson & Walseth (1979). 16S rRNA was prepared by phenol extraction of 30S ribosomes from *Escherichia coli*. T_4 polynucleotide kinase was the generous gift of Dr. O. C. Uhlenbeck. Poly(A), RNase A, and sequencing-grade RNase T_1 were purchased from P-L Biochemicals, Inc. Cell-free, 30000*g* supernatant (S-30) extracts of wheat germ were prepared by the method of Roberts & Patterson (1973) and then stored (without previous incubation) at −80 °C. [^{125}I]I^- and a mixture of 15 ^{14}C-labeled amino acids were purchased from Amersham Corp. [^{32}P]P_i was purchased from New England Nuclear Corp. DEAE-cellulose (DE52) and phosphocellulose (P11) were obtained from Whatman Chemical Separations Ltd. Sephadex CM-C50, Sepharose 6B, and Sephacryl S-200 were purchased from Pharmacia Corp. Blue Sepharose CL 6B was prepared from Sepharose 6B by the methods of Porath et al. (1971) and Heyns & DeMoor (1974).

Purification of Initiation Factors (All Operations at 1–4 °C). Initiation factor eIF4B was purified from 300 g of wheat germ by scaling up the procedure for purification of fractions "1A and 2A" described by Walthall et al. (1979) with the exception that all solutions used to prepare fraction 1A also contained 0.5 mM PMSF and 0.5 mg of soybean trypsin inhibitor/mL. The resultant fraction "2A" was dialysis concentrated against B-100 buffer to 0.5 mL in a Micro-ProDiCon Model MPDC-115A dialysis concentrator with a PA-10 membrane (Bio-Molecular Dynamics, Beaverton, OR). The concentrated enzyme solution was then further purified by passage onto a 1.3 × 4.5 cm column of Sephadex CM-C50 in B-100 buffer, by elution (1 mL/min) with B-100 buffer until no further A_{280}-absorbing material passed through the column, and by final elution (1 mL/min) of 1-mL fractions with B-250 buffer. All A_{280}-absorbing fractions eluted with the B-250 buffer were pooled, concentrated by dialysis concentration to 0.5 mL against B-500 buffer, and then loaded onto a 1.5 × 90 cm column of Sephacryl S-200 in B-500 buffer and then resolved by elution (0.5 mL/min) with B-500 buffer. Fractions eluted with B-500 buffer that revealed Coomassie Brilliant Blue R stainable 80000-dalton material when 25-μL aliquots were analyzed by $NaDodSO_4$–10% polyacrylamide gel electrophoresis (Laemmli, 1970) were pooled and concentrated to 0.5 mL by dialysis concentration against B-100 buffer before storage at −80 °C and eventual assay.

Initiation factor eIF4A was purified from the 40–60% ammonium sulfate fraction from the 120 mM KCl postribosomal supernate obtained from 300 g of wheat germ by scaling up the procedure of Walthall et al. (1979) with the exception that all solutions contained 0.5 mM PMSF and 0.5 mg of soybean trypsin inhibitor/mL. This dialyzed preparation was loaded onto a 2 × 20 cm column of phosphocellulose in B-50 buffer and then eluted (1 mL/min) with B-50 buffer. The first 80 mL of eluate containing $A_{280} > 0.2$ was pooled and loaded onto a 2 × 45 cm column of DEAE-cellulose in B-50 buffer. This column was then washed (1 mL/min) with B-50 buffer and then with B-120 buffer, each time until the A_{280} of the eluate dropped below 0.2. This column was finally washed (1 mL/min) with B-150 buffer, and all fractions (5 mL) containing $A_{280} > 0.2$ were pooled and loaded (0.5 mL/min) onto a 1.3 × 4 cm column of Blue Sepharose CL 6B in B-150 buffer. This last column was washed (0.5 mL/min) with B-150 buffer until the eluate was free of A_{280}-absorbing material and then further eluted (0.5 mL/min) with B-500 buffer. The two or three fractions (2 mL each) containing all of the small quantity of detectable A_{280}-absorbing material eluted from this column with B-500 buffer were pooled and concentrated to 0.5 mL by dialysis concentration against B-100 buffer prior to storage at −80 °C and eventual assay.

The concentrations of the approximately 80000- and 48000-dalton components present in the final preparations of eIF4B and eIF4A described above were determined by a two-step procedure. First the percent purity of these components in their respective preparations was determined from quantitative gel scanning of electrophoretically resolved (Laemmli, 1977) and Coomassie Brilliant Blue R stained protein samples. Typical preparations of eIF4B and eIF4A prepared as described here were 70–80% pure and 80–90% pure, respectively. Second, these percent purity values were applied to the total protein concentrations in the preparations determined by the Folin–Ciocalteu procedure of Lowry et al. (1951) to obtain the actual concentrations of the 80000- and 48000-dalton components.

Assays. The binding of initiation factors to STNV [C-^{125}I]RNA, STNV [5′-^{32}P]RNA, or radiolabeled fragments of these RNAs was carried out in 20-μL reactions containing binding buffer [20 mM Hepes potassium salt, pH 7.6, 100 mM KCl, 2 mM Mg(OAc), 0.1 mM Na_2EDTA, and 1 mM dithiothreitol] containing 10 μg of BSA/mL, 0.01–0.1 pmol of radiolabeled STNV RNA or RNA fragment containing 10^3–10^4 cpm of radioactivity, and the indicated levels of the 80000- and 48000-dalton components in our preparations of eIF4B and eIF4A, respectively. When eIF4A and eIF4B were assayed together, equimolar levels of the 80000- and 48000-dalton components were employed. ATP, when added, was 1 mM ATP. After 25 °C incubation for the indicated times, reaction mixtures were transferred onto moist (i.e., presoaked in binding buffer for at least 20 min) 25-mm diameter Millipore-type HAWP filters mounted in a vacuum filtration apparatus, and a 0.5 atm vacuum was applied to the filter *just as each reaction mixture was applied.* Immediately after each separate reaction mixture was applied to its filter, and while still maintaining a 0.5 atm vacuum, each filter was washed once with a 1-mL aliquot of cold (1–4 °C) binding buffer. The filters were then removed from the filter apparatus, dried (90 °C, 5 min), and analyzed for radioisotope by γ or scintillation counting. All assays were run in duplicate, and all data points represent the average of duplicate assays.

The binding of radiolabeled STNV RNA, or radiolabeled fragments of STNV RNA, to wheat germ ribosomes was carried out in 100-μL reactions as described by Browning et al. (1980). The extent of binding of these radiolabeled RNAs by ribosomes was assayed by (3.5 h, 100000*g*, 2 °C) sucrose density gradient resolution of the reaction mixtures in 12-mL, 10–25% exponential sucrose gradients in 0.1 M KCl, 3 mM $Mg(OAc)_2$, 1.2 mM dithiothreitol, and 20 mM Hepes-K salt, pH 7.6, and subsequent analysis of A_{254} and radiolabel distribution in 0.5-mL fractions derived from the separate gradients.

Preparation of Radiolabeled Specific Oligonucleotide Fragments of STNV RNA. Specific ^{32}P-labeled 5′-terminal fragments of STNV RNA were prepared by treatment of 100 pmol of STNV [5′-^{32}P]RNA dissolved in 50 μL of H_2O (3 × 10^5 cpm/pmol) with 0.4 unit of RNase T_1 for 2 min at 25 °C. Specific ^{32}P-labeled 5′-terminal fragments of STNV [5′-^{32}P]RNA were then obtained by denaturation of all components, gel electrophoretic resolution on a 40 cm long, 13 cm wide, and 1.5 mm thick 20% polyacrylamide gel containing 7 M urea, 1 mM Na_2EDTA, and 50 mM Tris–borate, pH 8.3, and final extraction and reisolation of gel-resolved ^{32}P-labeled

materials (in the absence of carrier RNA) as described by Browning et al. (1980).

A collection of 5-^{32}P-labeled, 52-nucleotide-long fragments from random locations throughout STNV RNA was prepared as an experimental control. This involved initial treatment of 100 pmol of STNV RNA dissolved in 50 μL of H_2O with 0.4 unit of RNase T_1 for 2 min at 25 °C followed by phenol extraction and ethanol precipitation. The precipitated oligonucleotide products were then ^{32}P labeled on their 5′ termini by use of T4 polynucleotide kinase and [γ-^{32}P]ATP (Leung et al., 1979), resolved on polyacrylamide gels as described above for ^{32}P-labeled 5′-terminal oligonucleotides of STNV RNA, extracted, and finally isolated as described by Browning et al. (1980).

The 40-nucleotide-long oligonucleotide from positions 13–52 of STNV [C-^{125}I]RNA was obtained by 40S ribosome protection of STNV [C-^{125}I]RNA from RNase T_1 (Browning et al., 1980). Comparison of the primary sequence of STNV RNA (Leung et al., 1979) with fingerprint analyses (Browning et al., 1980) of the ^{125}I-labeled products produced from this oligonucleotide by exhaustive digestion with RNase A confirms that this oligonucleotide arises from positions 13–52 of STNV RNA.

Results

General Characteristics of eIF4B–STNV RNA Complex Formation. Characterization of the initiation of eucaryotic mRNA translation requires that one select out and characterize individual mechanistic steps of the overall process. We chose to characterize potential enzyme–mRNA complexes associated with the correct initiation of eucaryotic mRNA translation. We further chose cellulose nitrate filter-mediated mRNA binding assays to measure the formation of enzyme–mRNA complexes. These binding assays function because at physiological pHs, cellulose nitrate filters bind proteins but not RNAs. Accordingly, enzyme–mRNA complexes formed after mixing enzymes with radiolabeled mRNAs yield, upon filtration, cellulose nitrate filter-bound radiolabeled mRNA.

Such filter binding assays require selection of a specific enzyme and a specific radiolabeled mRNA. We selected the eucaryotic mRNA STNV RNA for these assays because STNV RNA is a readily available, efficient, and well-characterized mRNA that can be readily radiolabeled in vitro (Leung et al., 1979; Browning et al., 1980). STNV RNA also has the added advantage that translation of STNV RNA does not utilize a 5′-terminal capping group (Leung et al., 1976) so one can avoid interactions of a 5′-terminal capping group with known cap binding proteins (Sonenberg et al., 1978; Tahara et al., 1981). We further selected the eucaryotic intiation factors eIF4A and eIF4B of wheat germ for these binding assays because these enzymes, and ATP, are likely candidates for enzyme–mRNA interaction in that they are required for mRNA uptake onto eucaryotic 40S translation initiation complexes (Trachsel et al., 1977; Benne & Hershey, 1978). Lastly, we selected the eIF4A and eIF4B enzymes of wheat germ because wheat germ eIF4B has been extensively purified (Walthall et al., 1979) and enzymes from plants are most appropriate for studies with a plant viral mRNA.

Following the above reasoning, we modified existing procedures, and developed new procedures, to obtain extensively purified preparations of eIF4A and eIF4B of wheat germ (see Experimental Procedures). The resultant preparations of these enzymes contain, as major components, the approximately 48 000- and 80 000-dalton components characteristic of eIF4A and eIF4B, respectively (Figure 1), and demonstrate eIF4A and eIF4B activities when assayed in reconstituted in vitro protein synthesis assays deficient in eIF4A and eIF4B activities (Table I). These preparations may not be pure enough to characterize specific protein steps and protein–protein interactions associated with mRNA uptake into 40S translation initiation complexes; however, these preparations are sufficiently pure to characterize how a well-characterized mRNA interacts with these initiation factors. Accordingly, we utilized cellulose nitrate filter binding assays to evaluate how radiolabeled STNV RNA interacts with these purified enzyme preparations.

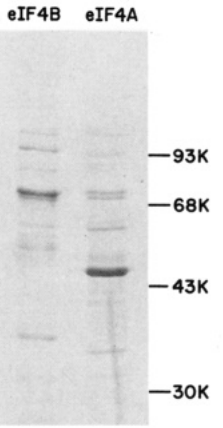

FIGURE 1: Gel electrophoretic resolution of the eIF4B and eIF4A preparations. Twenty-microliter aliquots of the concentrated and most purified preparations of eIF4B and eIF4A were resolved on 10% polyacrylamide gels after the method of Laemmli (1973) prior to being stained with Coomassie Brilliant Blue R.

Table I: Initiation Factor Activity of Isolated eIF4A and eIF4B[a]

Assay of eIF4A		
system	cpm of [^{14}C]Leu incorporated	sp act.
complete system	2330	1800
complete system minus eIF4A	1277	
Assay of eIF4B		
system	cpm of ^{14}C-labeled amino acid incorporated	sp act.
complete system	8244	7191
complete system minus eIF4B	2621	

[a] eIF4A was assayed in the laboratory of Joanne Ravel (Austin, TX) by using her eIF4A-deficient [^{14}C]leucine incorporation assay (unpublished results). eIF4B was assayed by a method patterned after Walthall et al. (1979). Specific activities represent differences (±added initiation factor) in terms of picomoles of [^{14}C]-leucine-increased incorporation per milligram of protein (for eIF4A) and picomoles of ^{14}C-labeled amino acid increased incorporation per milligram of protein (for eIF4B).

Known, physiologically significant, enzyme–RNA interactions demonstrate dissociation constants (K_d's) in the 10^{-7}–10^{-8} M range (Spierer et al., 1978; Schimmel & Soll, 1979; Schwarzbauer & Craven, 1981; Carey et al., 1983) where K_d is defined as

$$K_d = \frac{[\text{enzyme}][\text{RNA}]}{[\text{enzyme–RNA complex}]} \quad (1)$$

This equation dictates that if one wants to see more than half of an input level of radiolabeled RNA bound into enzyme–RNA complexes at equilibrium, one must employ enzyme levels greater than the estimated K_d of the reaction, but not so high as to facilitate non-time-dependent (i.e., nonspecific) enzyme–RNA interactions. Accordingly, we initally examine the time-dependent interaction of STNV [C-^{125}I]RNA with

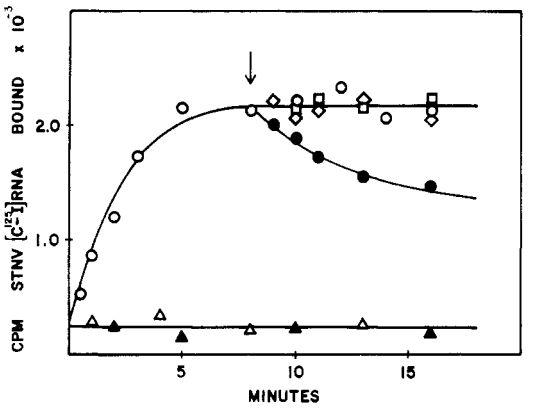

FIGURE 2: Time course of the binding of STNV [C-^{125}I]RNA by eIF4B and eIF4A. All points represent 20-μL assays of the binding of 0.2 pmol of STNV [C-^{125}I]RNA by 3.8 pmol of eIF4B (○) and eIF4A (△) and in the absence of added enzyme (▲). At equilibrium (8 min, indicated by the arrow), some of the assays with the eIF4B were supplemented with 0.2 pmol of unlabeled STNV RNA (●), poly(A) (average molecular weight 250 000) (□), or 16S rRNA (◇) and further incubated before assay at the indicated times.

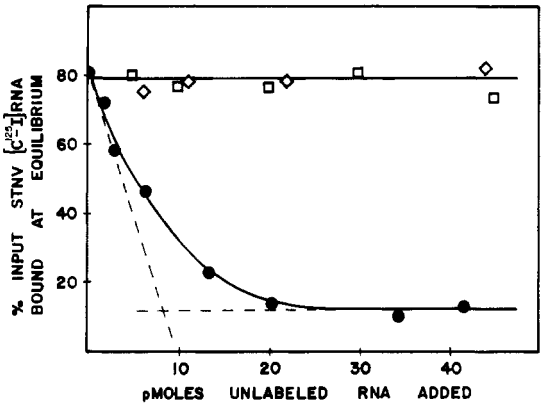

FIGURE 3: Influence of added unlabeled RNAs on the extent of eIF4B-dependent binding of STNV [C-^{125}I]RNA at equilibrium. 8.3 pmol of the 80 000-dalton component of eIF4B was mixed with 0.04 pmol of STNV [C-^{125}I]RNA (12 000 cpm) and the indicated quantities of unlabeled 16S rRNA (◇), poly(A) (average molecular weight 250 000) (□), and STNV RNA (●). All reactions were incubated for 8 min before assay.

approximately 10^{-7} M levels of eIF4A and eIF4B (i.e., the 48 000- and 80 000-dalton components of the enzymes of Figure 1). Figure 2 shows that at this level of enzyme, eIF4B, but not eIF4A, catalyzes time-dependent enzyme–STNV [C-^{125}I]RNA complex formation. Heat-denatured eIF4B does not bind STNV [C-^{125}I]RNA in this reaction, and studies with eIF4B and STNV [5′-^{32}P]RNA yield data identical with those of Figure 2. Most importantly, this reaction is both reversible and specific since the addition of unlabeled STNV RNA at equilibrium yields a detectable off rate or back-exchange of radiolabel from enzyme–RNA complexes while the addition of the unlabeled non-mRNAs, 16S rRNA and poly(A), does not facilitate this back-exchange under these conditions. The specificity of this enzyme–RNA complex formation is more evident from the data of Figure 3 which show that one can initially add a quantity of unlabeled non-mRNA up to 5-fold in excess of the 8.3 pmol of the 80 000-dalton component of our eIF4B present and still not influence the extent of the enzyme–STNV [C-^{125}I]RNA formation at equilibrium. In contrast, the initial addition of unlabeled STNV RNA competes effectively with the formation of enzyme–STNV [C-^{125}I]RNA complexes at equilibrium. Significantly, the dashed lines of Figure 3 depict the quantity of total STNV RNA required to saturate the 8.3 pmol of the 80 000-dalton component of our eIF4B in this experiment and show an approximately 1:1 stoichiometry of the 80 000-dalton component to STNV RNA in the enzyme–STNV RNA complexes. Thus, the 80 000-dalton component of our eIF4B appears to be the functional enzyme of this enzyme–RNA complex formation, and we will therefore designate these complexes as eIF4B–STNV RNA complexes.

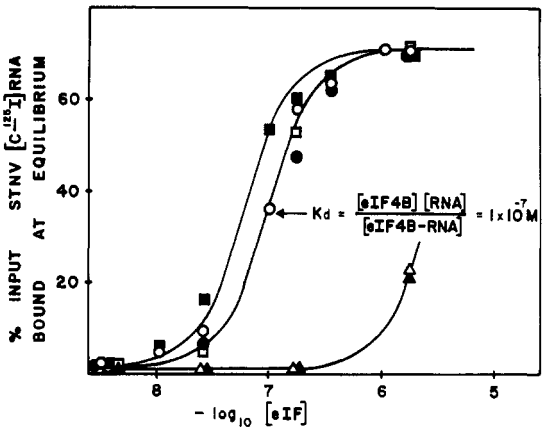

FIGURE 4: Binding constant (K_d) determinations with eIF4B and eIF4A and STNV [C-^{125}I]RNA. The indicated levels of eIF4B (○), eIF4B + ATP (●), eIF4B + eIF4A (□), eIF4B + eIF4A + ATP (■), eIF4A (△), and eIF4A + ATP (▲) were assayed. The molar levels represent levels of the 80 000- and 48 000-dalton components in eIF4B and eIF4A, respectively.

Determination of the K_d (eq 1) for this reaction provides further evidence of the specific nature of this interaction of eIF4B with mRNA. As seen in Figure 4, the enzyme level that yields half-maximal binding of STNV [C-^{125}I]RNA is equal to the K_d of the eIF4B–STNV [C-^{125}I]RNA complex, namely, 1×10^{-7} M. This K_d value varies with the specific preparation of eIF4B assayed and has ranged in our experiments from 3×10^{-7} to 3×10^{-8} M. This variation in K_d must reflect, in part, the accuracy of the assay and difficulties in measuring the concentration of the 80 000-dalton component present in the limited amounts of the low concentration enzyme available for these studies. However, experiments such as that of Figure 4 always show that added eIF4A and ATP, but not eIF4A alone, lower the observed K_d of the complex by a factor of approximately 0.5. This consistent lowering of the K_d for eIF4B–STNV RNA complex formation catalyzed by added eIF4A and ATP cannot be due to an ATP-dependent binding of STNV RNA by eIF4A alone for much higher levels of our preparation of eIF4A (±ATP) are needed to facilitate enzyme-dependent binding of STNV RNA (Figure 4). Therefore, eIF4A and ATP must aid or stabilize eIF4B–STNV RNA complex formation. This evaluation coincides with previous reports that eIF4A and eIF4B function in close proximity to each other (Grifo et al., 1982). Final characterization of enzymes that interact with eIF4B, or mRNA-bound eIF4B, will, however, require studies with purer enzyme preparations.

Binding of Specific Fragments of STNV RNA by Wheat Germ eIF4B. Characterization of the site on STNV RNA that interacts with eIF4B is essential to an understanding of the significance and the mechanism of formation of these eIF4B–STNV RNA complexes. We approached this necessary characterization by observing a general correlation between the intactness of STNV [C-^{125}I]RNA preparations and the maximum percent of input STNV [C-^{125}I]RNA bound in assays of K_d. This suggests that eIF4B binds a limited, yet specific region of STNV RNA. If this is so, then eIF4B should bind fragments of STNV RNA containing the specific binding

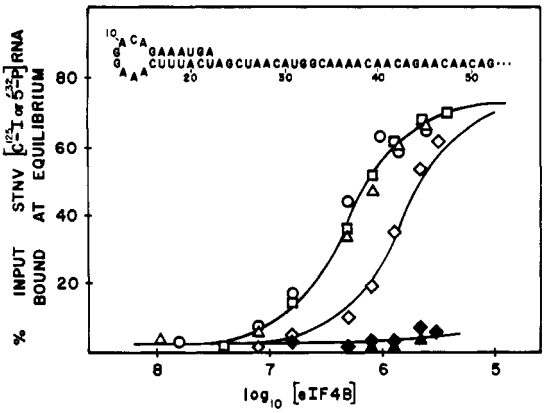

FIGURE 5: Binding constant (K_d) determinations with eIF4B and STNV [5′-^{32}P]RNA or specific radiolabeled fragments of STNV RNA. The indicated levels of eIF4B were incubated with intact STNV [5′-^{32}P]RNA (○), 5′-^{32}P-1–52 (□), 5′-^{32}P-1–44 (◇), 5′-^{32}P-1–33 or 5′-^{32}P-1–23 (▲), 5′-^{32}P-labeled random 52-nucleotide-long fragments of STNV RNA (◆), and C-^{125}I-13–52 oligonucleotides from STNV RNA [C-^{125}I]RNA (△).

site for eIF4B with the same affinity (K_d) as intact STNV RNA.

eIF4B binds an oligonucleotide composed of the 5′-terminal 52 nucleotides of STNV [5′-^{32}P]RNA (designated 1–52) containing the translation initiation region of this mRNA (Leung et al., 1979) with the same affinity that it binds intact STNV [5′-^{32}P]RNA (Figure 5). The binding affinity is less with the 5′-terminal 1–44 oligonucleotide of STNV [5′-^{32}P]-RNA and does not occur with either the 5′-terminal 1–33 oligonucleotide of STNV [5′-^{32}P]RNA (i.e., the 5′-terminal untranslated region and the initiator AUG codon of this mRNA) or the 5′-terminal 1–23 oligonucleotide of STNV [5′-^{32}P]RNA. This interaction of eIF4B with fragments of STNV RNA is specific, that is, not just dependent upon the length of the radiolabeled oligonucleotide assayed, for as also shown in Figure 5, eIF4B does not bind a collection of 52-nucleotide-long 5′-^{32}P-labeled oligonucleotides randomly selected from STNV RNA. Wheat germ eIF4B therefore binds to the translation initiation region of STNV RNA, and this interaction requires a specific structure, or nucleotide sequence, characteristic of this site on the mRNA.

The binding studies described above define the limits on the 3′ side of the region of STNV RNA that interacts with eIF4B; namely, the binding of eIF4B to STNV RNA requires between 12 and 20 nucleotides on the 3′ side of the initiator AUG of this mRNA. Full characterization of this eIF4B–STNV RNA interaction requires definition of the limits on the 5′ side of the region of STNV RNA that interacts with eIF4B and evaluation of the role of the postulated 5′-terminal stem and loop secondary structure of STNV RNA in the binding of STNV RNA by eIF4B.

The 40-nucleotide-long oligonucleotide (positions 13–52) derived by protection of STNV [C-^{125}I]RNA from RNase T_1 with 40S ribosomes (Browning et al., 1980) provides a convenient and specific radiolabeled oligonucleotide to evaluate these limits and functions. As seen in Figure 5, eIF4B binds the oligonucleotides from positions 13–52 of the 5′ terminus of STNV [C-^{125}I]RNA as efficiently as it binds intact STNV [5′-^{32}P]RNA. The binding of eIF4B to STNV RNA therefore does not employ either the 5′-terminal stem and loop structure postulated for STNV RNA (Leung et al., 1979) or the 5′-terminal nucleotides from positions 1–12 of STNV RNA. Instead, eIF4B must recognize features of the translation initiation site region of STNV RNA 13–52 nucleotides from the 5′ terminus of this mRNA. The nucleotide sequence of

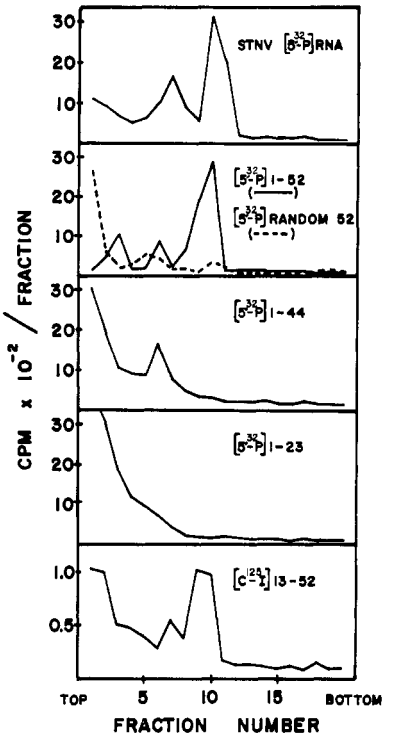

FIGURE 6: Binding of STNV [5′-^{32}P]RNA and specific radiolabeled fragments of STNV RNA to wheat germ ribosomes. The major and minor peaks of bound STNV [5′-^{32}P]RNA correspond to the locations of 80S and 40S ribosomes, respectively.

this limited region of STNV RNA does not contain any apparent secondary structure, suggesting that eIF4B recognizes, and binds to, some feature of the nucleotide sequence of the translation initiation site of STNV RNA.

Binding of Specific Fragments of STNV RNA by Wheat Germ Ribosomes. The data of Figure 5 establish that eIF4B of wheat germ interacts with the translation initiation region of STNV RNA. If this eIF4B–mRNA interaction is an obligate step in the initiation of translation of STNV RNA, then one would expect that translation initiation complex formations by wheat germ ribosomes will demonstrate the same specificities toward specific fragments of STNV RNA that one finds during the binding of these specific fragments by eIF4B. Accordingly, we investigated the ability of wheat germ ribosomes to bind to STNV [5′-^{32}P]RNA and radiolabeled specific fragments of STNV RNA. As seen in Figure 6, wheat germ ribosomes readily bind STNV [5′-^{32}P]RNA and the 5′-terminal 1–52 oligonucleotide of STNV [5′-^{32}P]RNA while these same ribosomes demonstrate a decreased binding efficiency toward the 5′-terminal 1–23 oligonucleotide of STNV [5′-^{32}P]RNA or a random selected population of 52-nucleotide-long oligonucleotides from STNV RNA. Most importantly, these wheat germ ribosomes also demonstrate efficient binding of the 40-nucleotide-long ^{125}I-labeled oligonucleotide 13–52 of the 5′ terminus of STNV [C-^{125}I]RNA. Therefore, wheat germ ribosomes do demonstrate the same specificities toward the binding of specific fragments of STNV RNA that one sees during STNV RNA fragment binding by eIF4B. This suggests that the interaction of eIF4B with the translation initiation region of STNV RNA is an obligate step in the initiation of translation of this mRNA by the wheat germ system.

Discussion

This paper reports a specific binding reaction between the eucaryotic initiation factor eIF4B and the eucaryotic mRNA STNV RNA. Four features of this protein–RNA interaction stand out and deserve further comment.

First, this reaction occurs with a high efficiency (low K_d and high percent of input RNA bound) while demonstrating an approximately 1:1 stoichiometry between STNV RNA and the 80000-dalton component characteristic of eIF4B (Figures 3 and 4). This efficiency and stoichiometry are both more favorable than other reports of the binding of initiation factors to mRNAs (Baglioni et al., 1978; Padilla et al., 1978; Sonenberg & Shatkin, 1978; Grifo et al., 1982, 1983). Our more favorable efficiency and stoichiometry reflect, in part, our consideration of the full kinetic features of both the enzymatic reaction and the cellulose nitrate mediated enzyme–RNA complex binding assay. Specifically, we have allowed the enzymatic reactions to proceed to true equilibrium prior to assay, and we have used a rapid filtration procedure that avoids dilution of the reaction systems prior to filtration. In this connection, it is important to recognize that the K_d equation (eq 1) dictates that any dilution of an enzyme-dependent RNA binding reaction followed by delay prior to filtration through the cellulose nitrate will decrease the amount of preformed enzyme–RNA complex.

Second, the observed 1:1 stoichiometry between the 80000-dalton component of eIF4B and STNV RNA (Figure 3) dictates that the active enzyme is this 80000-dalton protein. The data of Figure 4 support this contention by revealing that eIF4A ± ATP does not bind efficiently to STNV RNA. In fact, the low level of binding of STNV RNA by eIF4A (Figure 4) may well reflect low levels of eIF4B in our eIF4A preparations (Figure 1) rather than an eIF4A-specific phenomenon. Further characterization of the role of eIF4A and ATP in the eIF4B-dependent binding of STNV RNA is not possible with our data.

Third, the binding of eIF4B to STNV RNA appears to involve recognition of specific nucleotides in the translation initiation site of this mRNA (Figure 5). If the binding of eIF4B to STNV RNA is an essential step in the translation of this mRNA, the individual nucleotide or nucleotide sequence specific character of this reaction dictates that STNV RNA should demonstrate a class of translation initiation efficiency mutants involving nucleotide interchanges, deletions, etc., relative to the wild-type mRNA. Perhaps such mutations account for the observed mutation-dependent reduction of STNV yield observed when STNV and TNV are passed repetitively in an alternate host plant (Donis-Keller et al., 1981).

Fourth, the eIF4B–STNV RNA complex forming reaction is mRNA specific in that eIF4B does not bind non-mRNAs (Figures 2 and 3). This issue of the mRNA specificity of eIF4B is primary to the future significance of the observations reported here. On the one hand, STNV RNA lacks a 5′-terminal capping group and does not employ a 5′-terminal capping group during its translation (Leung et al., 1976). One can therefore argue that STNV RNA compensates for its lack of a 5′-terminal capping group through use of an eIF4B-mediated translation initiation site recognition process such as reported here and that this recognition process leads to a correct, yet unique, initiation of translation of this mRNA. Alternately, this paper may be the first to implicate eIF4B as a general translation initiation site recognition agent for eucaryotic mRNAs. Obviously, one must approach these separate hypotheses by examination of the role of eIF4B with other eucaryotic mRNAs. Such studies are currently in progress in our laboratory.

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Registry No. 5′-ATP, 56-65-5.

References

Baglioni, C., Simili, M., & Shafritz, D. A. (1978) *Nature (London) 275*, 240.

Benne, B., & Hershey, J. W. B. (1978) *J. Biol. Chem. 253*, 3078.

Bos, J. L., Polder, L. J., Bernards, R., Schrier, P. I., van den Elsen, P. J., van der Eb, A. J., & van Ormondt, H. (1981) *Cell (Cambridge, Mass.) 27*, 121.

Browning, K. S., & Clark, J. M., Jr. (1980) *Biochemistry 19*, 5922.

Browning, K. S., Leung, D. W., & Clark, J. M., Jr. (1980) *Biochemistry 19*, 2276.

Carey, J., Cameron, V., de Haseth, P. L., & Uhlenbeck, O. C. (1983) *Biochemistry 22*, 2601.

Clark, J. M., Jr., & Klein, W. J. (1974) *Methods Enzymol. 30*, 754.

Commerford, S. L. (1971) *Biochemistry 10*, 1993.

Dhruva, B. R., Shenk, T., & Subramanian, K. N. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 4514.

Donis-Keller, H., Browning, K. S., & Clark, J. M., Jr. (1981) *Virology 110*, 43.

Grifo, J. A., Tahara, S. M., Leis, J. P., Morgan, M. A., Shatkin, A. J., & Merrick, W. C. (1982) *J. Biol. Chem. 257*, 5246.

Grifo, J. A., Tahara, S. M., Morgan, M. A., Shatkin, A. J., & Merrick, W. C. (1983) *J. Biol. Chem. 258*, 5804.

Hendy, G. N., Kronenberg, H. M., Potts, J. T., Jr. & Rich, A. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 7365.

Heyns, W., & DeMoor, P. (1974) *Biochim. Biophys. Acta 358*, 1.

Jay, G., Nomura, S., Anderson, C. W., & Khoury, G. (1981) *Nature (London) 291*, 346.

Johnson, R. A., & Walseth, T. F. (1976) *Adv. Cyclic Nucleotide Res. 10*, 135.

Kozak, M. (1978) *Cell (Cambridge, Mass.) 15*, 1109.

Kozak, M. (1981) *Nucleic Acids Res. 9*, 5233.

Kozak, M. (1982) *J. Virol. 42*, 467.

Laemmli, U. K. (1970) *Nature (London) 227*, 680.

Leung, D. W., Gilbert, C. W., Smith, R. E., Sasavage, N. L., & Clark, J. M., Jr. (1976) *Biochemistry 15*, 4943.

Leung, D. W., Heckman, J. E., RajBhandary, U. L., & Clark, J. M., Jr. (1979) *Biochemistry 18*, 1361.

Lomedico, P. T., & McAndrew, S. J. (1982) *Nature (London) 299*, 221.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265.

Maitra, U., Stringer, E. A., & Chandhuri, A. (1982) *Annu. Rev. Biochem. 51*, 869.

Padilla, M., Canaani, D., Groner, Y., Weinstein, J. A., Bar-Joseph, M., Merrick, W., & Schafritz, D. A. (1978) *J. Biol. Chem. 253*, 5939.

Porath, J., Janson, J. C., & Laas, T. (1971) *J. Chromatogr. 60*, 167.

Schimmel, P. R., & Soll, D. (1979) *Annu. Rev. Biochem. 48*, 601.

Schwarzbauer, J., & Craven, G. R. (1981) *Nucleic Acids Res. 9*, 2223.

Shatkin, A. J. (1976) *Cell (Cambridge, Mass.) 9*, 645.

Sonenberg, N., & Shatkin, A. J. (1978) *J. Biol. Chem. 253*, 6630.

Sonenberg, N., Morgan, M. A., Merrick, W. C., & Shatkin, A. J. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4843.

Spierer, P., Bogdanov, A. A., & Zimmerman, R. A. (1978) *Biochemistry 17*, 5394.
Tahara, S. M., Morgan, M. A., & Shatkin, A. J. (1981) *J. Biol. Chem. 256*, 7691.
Trachel, H., Erni, B., Schreier, M. H., & Staehelin, T. (1977) *J. Mol. Biol. 116*, 755.
Walthall, B. J., Spremulli, L. L., Lax, S. R., & Ravel, J. M. (1979) *Methods Enzymol. 60*, 193.

Structural and Chemical Characterization of a Homogeneous Peptide *N*-Glycosidase from Almond†

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ABSTRACT: A peptide *N*-glycosidase that catalyzes the hydrolysis of N-linked oligosaccharide chains from glycopeptides and glycoproteins has been purified to homogeneity from almond emulsin and from almond meal. Purification from almond emulsin using ion-exchange chromatography, gel filtration chromatography, and preparative polyacrylamide gel electrophoresis gave an enzyme which was purified more than 700-fold and with a yield of 63%. An alternative procedure, more suitable for efficient large scale purification, used ion-exchange, affinity, and gel filtration chromatography. When purification began with almond emulsin, the enzyme was purified 1200-fold with a 37% yield, while when purification began with almond powder, the enzyme was purified 9000-fold with a yield of 45%. The homogeneous enzyme is stable at 4 °C for several months in 10 mM sodium acetate, pH 5.0, buffer. The peptide *N*-glycosidase is itself shown to be a glycoprotein consisting of a single polypeptide chain with a molecular weight of 66 800 on sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis. Circular dichroism spectra of the native molecule indicate the presence of a high (approximately 80%) α-helix content. The amino acid and carbohydrate contents of the enzyme are presented. When a convenient new assay with a turkey ovomucoid glycopeptide as a substrate is used, the enzyme preparation exhibits a broad pH optimum centered between pH 4 and pH 6. The enzyme is readily inactivated by SDS and guanidine hydrochloride, but it is stable in the presence of moderate concentrations of several other protein denaturants. Several divalent metal ions, e.g., Mg^{2+}, Zn^{2+}, Co^{2+}, and Cu^{2+}, increase the enzyme activity by as much as 50%, but 10 mM ethylenediaminetetraacetic acid does not inhibit the enzyme activity. Although the enzyme cleaves the carbohydrate from a variety of glycopeptides used in this study (albeit at different rates), it does not hydrolyze the carbohydrate from most of the corresponding partially denatured glycoproteins under otherwise comparable conditions. However, the carbohydrates from denatured ovomucoid and ribonuclease B were completely removed. Following purification by affinity chromatography, the homogeneous endoglycosidase exhibits a negligible β-hexosaminidase activity, measured by using a synthetic substrate for assay.

Numerous endoglycosidases have been purified from bacterial cultures and have been found to be of great help in elucidating the structures of the oligosaccharides of glycoproteins (Tarentino et al., 1974; Tarentino & Maley, 1974; Koide & Muramatsu, 1974; Elder & Alexander, 1982). A related enzyme activity has also been reported in mammalian tissues (Tachibana et al., 1982). Endoglycosidases such as endo H, endo D, and endo F cleave high mannose, complex glycans, and both types of glycans from the native or partially denatured glycoproteins, respectively (Tarentino et al., 1974; Tarentino & Maley, 1974). However, they do not completely remove the carbohydrate residues attached to the peptide or protein chain.

Recently, a peptide *N*-glycosidase from almond emulsin has been partially purified and characterized (Takahashi, 1977; Takahashi & Nishibe, 1978; Takahashi & Nishibe, 1981; Plummer & Tarentino, 1981; Tarentino & Plummer, 1982). Almond endoglycosidase has been shown to completely remove oligosaccharides from several glycoproteins and glycopeptides (Takahashi & Nishibe, 1981). It appears that the removal of oligosaccharides from native glycoproteins is typically much slower than their removal from derived glycopeptides, at least in the cases of ovalbumin, bromelain, and desialotransferrin. By use of an enzyme immobilized on Sepharose, Takahashi et al. (1982) were able to completely deglycosylate the native enzyme takaamylase A. In contrast, Tarentino & Plummer (1982) have shown that the release of oligosaccharides from native glycoproteins is very difficult, and they were able to remove oligosaccharides from denatured glycoproteins only in the presence of chaotropic salts and disulfide bond reducing agents. To date, the properties of a homogeneous peptide *N*-glycosidase have not been reported.

Considering the contradictory facts in the literature and the great importance of having such an enzyme available in a pure state for use in studies of the role of oligosaccharides in glycoprotein structure and function, we have purified one of the endoglycopeptidases to homogeneity and have carefully studied some of its physical and structural properties as well as some of the conditions necessary for its action on glycoprotein and glycopeptide substrates. As will be evident from our study, the enzyme is itself a glycoprotein, and this important property was used to develop an affinity chromatography procedure for convenient purification of the enzyme. In addition, we also utilized a rapid and convenient assay procedure which will also

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