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Photochemical Cross-Linking of Cap Binding Proteins to Eucaryotic mRNAs: Effect of mRNA 5′ Secondary Structure

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We used UV light-induced cross-linking to study the interactions of cap binding proteins with the 5′ cap structure of eucaryotic mRNAs. Thymidine kinase gene (herpes simplex virus type 1) transcripts prepared in vitro using the SP6 RNA polymerase transcription system were capped and methylated posttranscriptionally with [α-^{32}P]GTP and *S*-adenosyl-L-methionine to yield cap-labeled transcripts. Irradiation of capped transcripts with crude rabbit reticulocyte initiation factors in the presence of ATP-Mg^{2+} resulted in the cap-specific cross-linking of two polypeptides with molecular masses of 24 and 80 kilodaltons (kDa). The cross-linking characteristics of these polypeptides resemble those of the cap-binding proteins previously detected by a chemical cross-linking assay (N. Sonenberg, D. Guertin, D. Cleveland, and H. Trachsel, Cell 27:563–572, 1981). However, the relative efficiency of the cross-linking of these two polypeptides to the cap structure was different from that in previous studies, and there was no detectable cross-linking of the previously described 50-kDa polypeptide. In addition, we present data indicating that the insertion of secondary structure into the 5′ noncoding region of *tk* mRNA, 6 nucleotides from the cap structure, decreases the cap-specific cross-linking of the 80-kDa but not the 24-kDa polypeptide. In contrast, the insertion of secondary structure 37 nucleotides from the cap structure had no significant effect on the cross-linking of either the 24- or the 80-kDa cap-specific polypeptide. These results demonstrate that the position of mRNA 5′-proximal secondary structure relative to the cap structure can influence the cap-specific interaction between the mRNA and a translation initiation factor.

All cellular eucaryotic mRNAs analyzed to date are blocked at their 5′ end by the cap structure m^{7}GpppN (1, 6, 27). Numerous studies have indicated that the cap structure facilitates translation initiation complex formation (see reference 1 for a review), protects the mRNA against 5′ exonucleolytic degradation (7), and more recently, might be involved in pre-mRNA processing (13). To identify protein factors which recognize the cap structure and may be involved in translation, Sonenberg and Shatkin (33) developed an assay which involves the covalent cross-linking of mRNA with an oxidized cap structure to polypeptides that bind at or near the cap structure. Using this assay, Sonenberg et al. (32) initially identified a 24-kilodalton (kDa) polypeptide (termed 24K-CBP [cap binding protein]) in the high-salt wash of rabbit reticulocyte ribosomes which could be specifically cross-linked to the oxidized cap structure of reovirus mRNA. A polypeptide of similar mobility on sodium dodecyl sulfate (SDS)-polyacrylamide gels and with identical cross-linking characteristics has been detected in initiation factors (IFs) from mouse Ehrlich ascites cells (32) and human HeLa cells (12, 17). It was subsequently demonstrated that additional polypeptides of 28, 50, and 80 kDa can be specifically cross-linked to the oxidized cap structure in an ATP-Mg^{2+}-dependent manner (29, 31). Using purified IFs, Grifo et al. (10) suggested that the 50- and 80-kDa polypeptides correspond to eIF-4A (eucaryotic IF-4A) and eIF-4B, respectively. This has been confirmed for eIF-4A by Edery et al. (4) and is probably true for eIF-4B (based on several indirect observations; 4, 11). Hence, we will refer to the 50- and 80-kDa polypeptides as eIF-4A and eIF-4B, respectively.

The molecular mechanism by which cap recognition factors act in facilitating mRNA-ribosome interactions is not well characterized. Several studies have implicated eIF-4A, eIF-4B, and, more recently, eIF-4F (also referred to as CBP II [35] or CBP complex [4, 5]) in the ATP-dependent binding of mRNA to 43S preinitiation complexes (2, 36). Sonenberg (29) postulated that cap recognition factors facilitate ribosome binding by melting mRNA secondary structure in an ATP-Mg^{2+}-dependent process. Subsequently, Lee et al. (16) showed that cap-specific cross-linking of eIF-4A (50-kDa polypeptide) and eIF-4B (80-kDa polypeptide) in crude IF preparations has a reduced dependence on ATP, if the mRNA has less stable secondary structure. This suggested that eIF-4A and eIF-4B can be cross-linked to the cap structure only after secondary structure has been melted. Edery et al. (4) reported that the eIF-4A in the CBP complex (comprising three major polypeptides of ~24 [24K-CBP], 50 [eIF-4A], and 220 kDa) cannot be cross-linked to mRNA unless eIF-4B is present. This implies that any putative melting activity is not present solely in the CBP complex but is dependent on eIF-4B or, alternatively, that eIF-4B mediates cap recognition by eIF-4A after denaturation of the mRNA. These data suggest a model by which the CBP complex binds to the 5′ cap structure and mediates the melting of 5′ secondary structure of the mRNA, possibly in conjunction with eIF-4B, followed by 40S ribosome binding (4, 5, 29–31). This model is consistent with our recent studies showing that the introduction of secondary structure into the 5′ noncoding region of herpes simplex virus type 1 *tk* mRNA acts to decrease translational efficiency in vivo and in vitro (22). We concluded from these studies that the 5′ mRNA secondary structure blocks translation by preventing 80S initiation complex formation.

In an attempt to analyze the step at which the block in initiation complex formation occurred, we studied here the interaction of the cap-specific polypeptides with the cap structure of mRNAs differing in secondary structure within

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the 5′ noncoding region. We adopted the UV light-induced cross-linking technique used by Greenberg (9) and others (26, 37) and noticed differences in the cross-linked protein profiles obtained by using this technique and the chemical cross-linking method introduced by Sonenberg and Shatkin (33). In addition, we found differences in the degree of cross-linking of eIF-4B when we compared *tk* mRNAs differing in the position of 5′ noncoding secondary structure relative to the cap structure. The results demonstrate that secondary structure near the mRNA 5′ terminus can influence the interaction of eIF-4B with the cap structure, whereas the presence of secondary structure farther downstream in the 5′ noncoding region does not affect this interaction.

MATERIALS AND METHODS

Materials and general methods. Restriction endonucleases and RNase A were purchased from Boehringer Mannheim Biochemicals. RNase-free DNase I and vaccinia virus guanylyltransferase were from Bethesda Research Laboratories, Inc. Human placenta RNase inhibitor was from Promega Biotec. m^7GpppG, GpppG, GDP, and 7-methyl-GDP (m^7GDP) were purchased from P-L Biochemicals, Inc. [5-^{3}H]CTP (23.7 Ci/mmol), [α-^{32}P]GTP (>3,000 Ci/mmol), and SP6 RNA polymerase were obtained from New England Nuclear Corp. Preparation of plasmid DNA, restriction enzyme digestion, and agarose gel electrophoresis of DNA were performed by standard methods (18). Preparation of high-salt wash of ribosomes from rabbit reticulocytes and HeLa cells as the source of IF was by the method of Schreier and Staehelin (25) and Lee and Sonenberg (17), respectively. Poliovirus type 1 (Mahoney strain) infection of HeLa cells was as described by Lee and Sonenberg (17), and IF preparations were made 3 h after poliovirus infection.

In vitro transcriptions and capping reactions. The construction of pX1/SP, pXJP15/SP, and pXJPB/SP has been described in detail elsewhere (22; the important features of these constructs are shown in Fig. 3). Before transcription with SP6 RNA polymerase, plasmids were linearized with *Eco*RI, phenol extracted, washed six times with ether, and passed over NACS Prepac columns (Bethesda Research Laboratories) to remove trace amounts of contaminating bacterial RNA. Uncapped *tk*/SP64 mRNA transcripts were synthesized from *Eco*RI-linearized DNA templates essentially as described by Green et al. (8), except that [5-^{3}H]CTP was used to monitor the level of RNA synthesis. RNA transcripts were capped and methylated as described by Monroy et al. (20) by using vaccinia virus guanylyltransferase in the presence of [α-^{32}P]GTP and *S*-adenosyl-L-methionine. For the preparation of capped but nonmethylated transcripts, *S*-adenosyl-L-methionine was omitted from the reaction mixture. *methyl*-^{3}H-labeled reovirus mRNA was synthesized with viral cores in the presence of *S*-[*methyl*-^{3}H]adenosyl-L-methionine (New England Nuclear), and oxidation of reovirus mRNA was performed as described by Muthukrishnan et al. (21) and Sonenberg and Shatkin (33).

Cross-linking of mRNA to IFs. Chemical cross-linking of oxidized reovirus mRNA to IFs was performed as described by Lee et al. (16). Photochemical cross-linking of reovirus mRNA and *tk*/SP64 transcripts was performed essentially as described by Ulmanen et al. (37). Briefly, 2×10^4 to 4×10^4 cpm of ^{32}P-mRNA (cap labeled) ($\sim 6 \times 10^4$ cpm/μg) was incubated in a total volume of 30 μl in 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.5)–0.5 mM magnesium acetate [Mg(OAc)$_2$]–2 mM dithiothreitol–3% glycerol–1 mM GTP (no changes were observed when this compound was omitted)–100 mM potassium acetate (KOAc) (unless otherwise indicated)–IF preparations (high-salt wash of ribosomes) at 30°C for 10 min. Reaction mixtures were irradiated at 254 nm at 4°C at a distance of 4 cm with a 15-W General Electric G15T8 germicidal lamp for 1 h. The samples were then digested for 30 min at 37°C with 20 μg of RNase A, boiled for 5 min in electrophoresis sample buffer, and subjected to electrophoresis on 10 to 15% gradient SDS-polyacrylamide gels (15), followed by autoradiography. Quantitation of labeled bands was performed by scanning autoradiograms with a model 1650 transmittance-reflectance scanning densitometer (Bio-Rad Laboratories).

RESULTS

Cross-linking of oxidized reovirus mRNA to crude IF preparations from rabbit reticulocytes and other sources has been used to identify proteins that interact with the cap structure of eucaryotic mRNAs (12, 29, 31, 32). We wished to extend this methodology to study the effect of the location (relative to the 5′ cap structure) of 5′-proximal mRNA secondary structure on the efficiency of mRNA cross-linking to the different cap-specific polypeptides.

In previous studies, reovirus mRNAs were synthesized by reovirus cores in the presence of *S*-[*methyl*-^{3}H]adenosyl-L-methionine as the methyl donor. As a consequence, the cap structure of reovirus mRNAs became exclusively labeled with a *methyl*-^{3}H group. The ribose moiety of the cap structure was then oxidized to yield a dialdehyde which is capable of Schiff base formation with primary amino groups of proteins. Polypeptides that interact with mRNA in a cap-specific manner were detected by incubating oxidized *methyl*-^{3}H-labeled reovirus mRNA with IF preparations followed by reductive stabilization of RNA-protein complexes with sodium cyanoborohydride. The reaction mixtures were treated with RNases A and T1 to degrade the mRNA portion of the complex, and the polypeptides that were covalently linked to the radioactive cap structure were resolved on SDS-polyacrylamide gels and detected by fluorography. Cap specificity was assessed by performing parallel experiments in the presence of the cap analog, m^7GDP. It was impractical to use reovirus mRNA to study the effect of mRNA secondary structure on the interaction of CBPs with the cap structure, because it is not possible to generate reovirus mRNAs in the standard in vitro transcription system with localized modifications in the nucleotide sequence. Consequently, we chose to use the SP6 expression system to synthesize mRNAs in vitro (8). In this system, the desired gene is inserted downstream from the SP6 promoter in pSP64 (described in reference 19), and the construct is linearized by using a restriction site downstream from the inserted gene followed by transcription with SP6 polymerase. mRNAs synthesized in this system are then capped with [α-^{32}P]GTP and methylated with *S*-adenosyl-L-methionine by using the enzyme guanylyltransferase from vaccinia virus. In this system, the gene in question can be mutated by different techniques, thus giving rise to mRNAs with altered primary or secondary structures. In addition, the mRNA is labeled exclusively in the cap structure with ^{32}P instead of ^{3}H, resulting in a much reduced exposure time for autoradiography. The amounts of RNA transcribed in the SP6 transcription system, although relatively large (up to 50 μg of uncapped RNA for 3 μg of DNA in a total reaction mixture of 100 μl), are smaller than the milligram amounts

produced by reovirus cores. Consequently, to minimize losses of mRNA that are likely to occur during the oxidation and subsequent purification of small amounts of mRNA, we circumvented these steps and adopted the photochemical technique for cross-linking of the mRNA to cap-specific polypeptides. This method of identifying cap-specific polypeptides has several other important advantages, as will be addressed in the discussion.

Initially, we wished to compare the efficiency and pattern of cross-linking obtained by the photolabeling technique with that obtained by the chemical cross-linking technique. To this end, we performed the chemical cross-linking assay using *methyl*-^{3}H-labeled reovirus mRNA that had been oxidized to convert the 5'-terminal m^{7}G to the reactive dialdehyde. The polypeptides that became labeled by the chemical cross-linking assay in the presence of ATP-Mg^{2+} are shown in Fig. 1 (lane 1). These include the 24-kDa CBP, the 50-kDa protein (eIF-4A), and the 80-kDa protein (eIF-4B), which are indicated by arrowheads, in addition to several other polypeptides. Cross-linking of the former polypeptides is cap specific, because 0.6 mM m^{7}GDP completely inhibited (>90%, as determined by scanning densitometry) their cross-linking (lane 2), whereas the same concentration of GDP had no effect on the cross-linking pattern (lane 3), in accordance with previous reports (29, 31). In the absence of ATP-Mg^{2+}, IFs 4A and 4B did not cross-link to mRNA, in agreement with previous results (4, 10, 29), whereas cross-linking of the 24K-CBP was reduced to some extent (~50%; lane 4). This cross-linking was inhibited by m^{7}GDP (>90%; lane 5) but not GDP (lane 6). The cross-linking pattern obtained by using the photochemical cross-linking method in the presence and absence of ATP-Mg^{2+} is shown in lanes 7 to 12. In the presence of ATP-Mg^{2+}, three major polypeptides of ~24, 65, and 80 kDa became cross-linked to the cap structure (lane 7). Cross-linking of the 24- and 80-kDa polypeptides was inhibited by 0.6 mM m^{7}GDP (lane 8) but not by the same concentration of GDP (lane 9), whereas cross-linking of the 65-kDa polypeptide was not inhibited by either nucleotide. In contrast to the pattern obtained by the chemical cross-linking method, eIF-4A (50-kDa polypeptide) did not become cross-linked to a significant extent by UV light irradiation. Photochemical cross-linking of the 24-kDa polypeptide occurred in the absence of ATP-Mg^{2+}, whereas that of the 80-kDa polypeptide requires ATP-Mg^{2+} (cf. lane 10 with lane 7). The effect of ATP-Mg^{2+} cannot be due to the effect of ATP on UV absorption, because a nonhydrolyzable analog, AMP-P(CH_2)P, could not substitute for ATP in the cross-linking reaction (data not shown). The ATP-Mg^{2+}-independent photochemical cross-linking of the 24 kDa polypeptide to mRNA was inhibited by m^{7}GDP (lane 11) but not by GDP (lane 12). Based on the photochemical cross-linking characteristics of the 24- and 80-kDa polypeptides with respect to inhibition by m^{7}GDP and dependence on ATP-Mg^{2+}, in addition to their comigration on SDS-polyacrylamide gels with the chemically cross-linked 24- and 80-kDa polypeptides, it is very likely that these polypeptides correspond to the 24K-CBP and eIF-4B. Moreover, we have recently obtained evidence, using polyclonal antibodies directed against eIF-4B, that the photoinduced cross-linked 80-kDa polypeptide is eIF-4B, thus reinforcing the belief that the 80-kDa polypeptide identified by the chemical cross-linking is indeed eIF-4B (S. Milburn, J. Pelletier, J. W. B. Hershey, and N. Sonenberg, manuscript in preparation). It is clear, however, that the relative cross-linking efficiencies of the 24K-CBP and eIF-4B are different by the two cross-linking techniques. Cross-

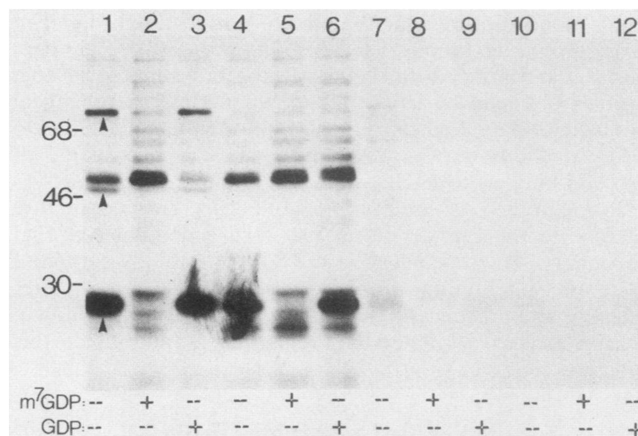

FIG. 1. Comparison of CBP cross-linking patterns obtained by the chemical and photochemical cross-linking techniques. Rabbit reticulocyte IF preparations (180 μg) were incubated with ~0.5 μg (40,000 cpm) of *methyl*-^{3}H-labeled oxidized reovirus mRNA (lanes 1 to 6) or *methyl*-^{3}H-labeled unoxidized reovirus mRNA (lanes 7 to 12) for 10 min at 30°C in a total volume of 30 μl under the conditions described in Materials and Methods. Chemical (lanes 1 to 6) or photochemical (lanes 7 to 12) cross-linking was performed, and samples were analyzed on a 10 to 15% SDS-polyacrylamide gel followed by autoradiography as described in Materials and Methods. Reaction mixtures included 1 mM ATP in lanes 1 to 3 and lanes 7 to 9, and m^{7}GDP or GDP at a concentration of 0.6 mM was added where indicated in the figure. The molecular masses of standard proteins are indicated in kilodaltons in the left margin, and the chemically cross-linked cap-specific polypeptides are indicated by arrowheads in lane 1.

linking of the 24K-CBP was approximately sevenfold more efficient than that of eIF-4B (as determined by scanning densitometry) when the chemical cross-linking method was used, whereas the photolabeling method resulted in an equal intensity of cross-linking of the 24K-CBP and eIF-4B in this experiment. As will be shown below, in other experiments cross-linking of eIF-4B was significantly greater than that of the 24K-CBP by the photochemical cross-linking method. In addition, the extent of cross-linking of eIF-4B and the 24K-CBP by UV light was ~10- and 70-fold, respectively, less than that observed with the chemical cross-linking assay (cf. lanes 1 and 7). Thus, the efficiency of UV light-induced cross-linking was significantly lower than that of chemical cross-linking. However, this shortcoming can be overcome by the use of high-specific-activity [α-^{32}P]GTP to label specifically the cap structure as shown below.

To further support the conclusion that the chemically and photochemically cross-linked 24- and 80-kDa polypeptides are the same, we analyzed the UV-induced cross-linking of cap-specific polypeptides in extracts from mock- or poliovirus-infected HeLa cells. Lee and Sonenberg (17) previously analyzed cap-specific polypeptides in crude IF from mock- and poliovirus-infected HeLa cells. They showed that the extent of cross-linking of several polypeptides (24, 28, 32, 50, and 80 kDa) is reduced in preparations from poliovirus-infected as compared with mock-infected cells. UV irradiation of IF preparations from mock-infected cells with *methyl*-^{3}H-labeled reovirus mRNA in the presence of ATP-Mg^{2+} resulted in the cross-linking of three major polypeptides of 24, 32, and 80 kDa (Fig. 2, lane 3). Cross-linking of these polypeptides was totally inhibited by m^{7}GDP (cf. lane 3 with lane 4). This cross-linking pattern is similar to that obtained by the chemical cross-linking

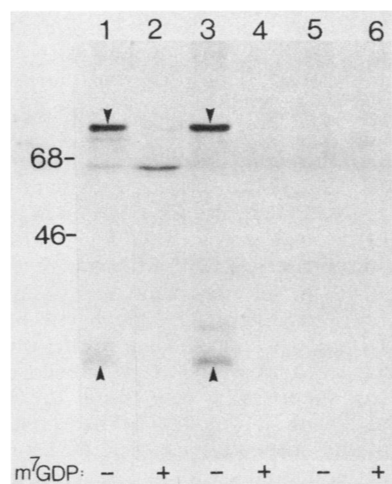

FIG. 2. Photochemical cross-linking pattern of CBPs from mock- and poliovirus-infected HeLa cells. *methyl*-^{3}H-labeled reovirus mRNA (40,000 cpm; ~0.5 μg) was incubated with 180 μg of rabbit reticulocyte IF or 100 μg of IF from HeLa cells in a total volume of 30 μl, followed by UV light irradiation as described in Materials and Methods. Samples were treated with RNase A and resolved on a 10 to 15% SDS-polyacrylamide gel that was treated with En^{3}Hance, dried, and exposed against XAR-5 film (Kodak) at −70°C for 2 months. Lanes 1 and 2, Rabbit reticulocyte IF; lanes 3 and 4, IF from mock-infected HeLa cells; lanes 5 and 6, IF from poliovirus-infected HeLa cells. m^{7}GDP (0.6 mM) was added where indicated in the figure. The molecular masses of standard proteins are indicated in kilodaltons in the left margin, and the positions of the 24- and 80-kDa cross-linked polypeptides are indicated by arrowheads in lanes 1 and 3.

method, with the exception of the 28-kDa polypeptide and eIF-4A, that became cross-linked only by the latter technique (17). Similar to the pattern of cross-linking observed with rabbit reticulocyte IF (lanes 1 and 2), the cross-linking of the 80-kDa polypeptide (eIF-4B) was the most pronounced and there was no apparent cross-linking of eIF-4A. The ability of the cap-specific polypeptides to interact with the cap structure is, however, impaired in poliovirus-infected IF preparations (cf. lanes 5 and 3). Thus, with the exception of the 50-kDa polypeptide (eIF-4A) and the 28-kDa polypeptide, the UV cross-linking assay gave results similar (qualitatively) to those of the chemical cross-linking assay in terms of cap specificity, ATP-Mg^{2+} requirement, and effects of poliovirus infection. However, whereas it is clear that in the case of the chemical cross-linking technique, cross-linking of 24-CBP and eIF-4B occurs via the oxidized ribose of the m^{7}G group, it is not clear that the photochemical cross-linking of these polypeptides also occurs to this residue. To establish that the actual site of the photochemical cross-linking is indeed to the m^{7}G group, as opposed to bases downstream from the cap structure, we performed the following experiment. Photochemical cross-linked mixtures were digested with the enzyme tobacco acid pyrophosphatase (Bethesda Research Laboratories) or with RNase A and analyzed on SDS-polyacrylamide gels. The extent of cap-specific cross-linking of eIF-4B was similar with both enzymes (data not shown). Because tobacco acid pyrophosphatase should cleave only the pyrophosphate bonds in the cap structure, we concluded that the photoaffinity cross-linking of eIF-4B occurs via the m^{7}G group of the cap structure.

To study the interaction of cap-specific polypeptides with mRNAs differing in 5′ secondary structure, we used the SP6 system to prepare mRNA from *tk* gene constructs differing in the number of *Bam*HI linkers inserted into the region of the gene corresponding to the 5′ noncoding portion of the mRNA. The various constructs chosen for this study are shown in Fig. 3. Recombinant plasmid pX1/SP contains a *Hin*cII-*Pvu*II fragment of the herpes simplex virus type 1 *tk* gene placed under the control of the SP6 promotor (see reference 22 for details). Plasmid pXJP15/SP is a derivative of pX1/SP that contains three *Bam*HI linkers (5′-CCGGATCCGG-3′) 42 nucleotides (nt) downstream from the cap site, whereas pXJPB/SP contains two *Bam*HI linkers 11 nt downstream from the cap site. In the cloning procedure, the staggered ends generated by *Bam*HI were filled in with the Klenow fragment of DNA polymerase I. This resulted in the addition of four extra nucleotides on each side of the inserted linker fragment. The introduced linkers and five additional nucleotides flanking the insert on each side have dyad symmetry; hence, when transcribed into mRNA in the SP6 system, they should form a stable stem-loop structure. We experimentally verified the presence of these structures by demonstrating that the region of the mRNAs (synthesized from pXJPB/SP and pXJP15/SP) containing the *Bam*HI linkers was resistant to cleavage by the single-stranded-specific S1 and T1 RNases and sensitive to the double-stranded-specific V1 nuclease (data not shown). We have previously shown that the translational efficiency of pXJP15/SP- and pXJPB/SP-derived mRNAs is greatly reduced as compared with pX1/SP-derived mRNA in both in vitro and in vivo assays (22). Furthermore, our studies indicated that the cause of the decreased translational efficiency of the pXJP15/SP transcript is reduced ribosome binding to this mRNA (22), and we have recently found that ribosome binding to the pXJPB/SP transcript is also inefficient as compared with pX1/SP transcript binding (data not shown). Ribosome binding is most probably dependent on prior interaction of CBPs with the mRNA cap structure (4, 5, 11, 27). Thus, it is plausible that the secondary structure of pXJP15/SP- and pXJPB/SP-derived mRNAs prevents interaction of cap recognition factors with the cap structure and consequently impedes ribosome binding. To test this possibility, we examined the interaction of cap-specific proteins

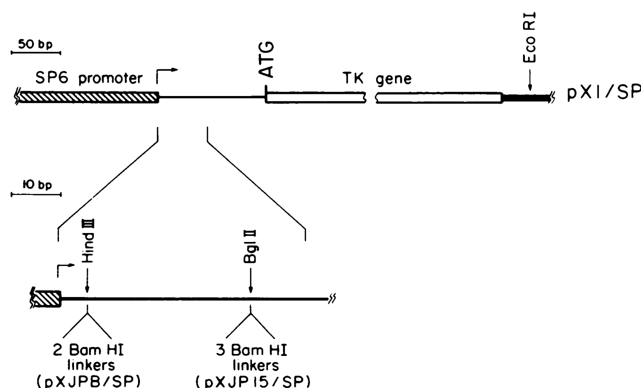

FIG. 3. Schematic representation of the structure of *tk*/SP64 derivatives. Constructions were performed as described in reference 22. The *Hin*cII-*Pvu*II fragment of the *tk* gene was inserted into the *Hin*cII restriction site of pSP64. The thick line at the right end of the pX1/SP construct represents sequences originating from pSP64. The right-angled arrow denotes the start and direction of transcription. In enlarged form are shown the restriction sites used to insert *Bam*HI linkers as described in Materials and Methods.

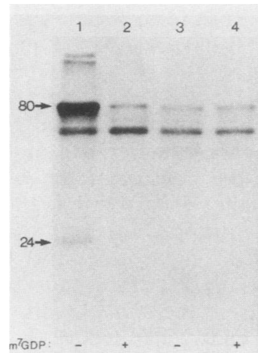

FIG. 4. UV light-induced cross-linking of ^{32}P-labeled $G\overset{*}{p}ppG$. . .- and $m^7G\overset{*}{p}ppG$. . .-terminated pX1/SP mRNA to rabbit reticulocyte CBPs. IF (~100 μg) was incubated with [^{32}P]$m^7G\overset{*}{p}ppG$. . .-terminated mRNA (4 × 10^4 cpm; ~0.7 μg; lanes 1 and 2) or [^{32}P]$G\overset{*}{p}ppG$. . .-terminated mRNA (4 × 10^4 cpm; ~0.7 μg; lanes 3 and 4) in the presence of 1 mM ATP, followed by UV light irradiation as described in Materials and Methods. Samples were resolved on a 10 to 15% gradient SDS-polyacrylamide gel that was dried and exposed against Fuji X-ray film for 12 h at −70°C. m^{7}GDP (0.6 mM) was added as indicated in the figure. The positions of the 24- and 80-kDa cross-linked polypeptides are indicated in the left margin.

with the various *tk* mRNA derivatives by using the UV cross-linking technique.

Transcription of *Eco*RI-linearized pX1/SP and derived plasmids yields runoff transcripts that have a 5′-terminal pppG structure (22). For studies of UV-induced cross-linking, mRNAs were capped by using vaccinia virus guanylyltransferase and labeled exclusively at their 5′ terminus by using [α-^{32}P]GTP in the reaction mixture (8, 20). We have found that although only 10 to 15% of the mRNAs are capped, more than 95% of these capped mRNAs are methylated (data not shown). This results in the production of mRNAs with a ^{32}P label in the α phosphate of the cap structure ($m^7G\overset{*}{p}ppG$. . ., in which * denotes the ^{32}P label). We investigated the specificity of the photochemical cross-linking reaction of the *tk* transcripts by using $m^7G\overset{*}{p}ppG$. . .- and $G\overset{*}{p}ppG$. . .-terminated pX1/SP mRNAs in the presence and absence of the cap analog, m^{7}GDP. UV irradiation of $m^7G\overset{*}{p}ppG$. . .-terminated pX1/SP mRNA with rabbit reticulocyte IF resulted in the cap-specific cross-linking of the 24K-CBP and eIF-4B (Fig. 4, cf. lane 1 with lane 2 which includes m^{7}GDP), consistent with the results obtained with *methyl*-^{3}H-labeled reovirus mRNA. However, no proteins were found to specifically cross-link to $G\overset{*}{p}ppG$. . .-terminated pX1/SP mRNA, as m^{7}GDP had no effect on the cross-linking of any of the polypeptides (cf. lanes 3 and 4; a polypeptide that comigrates with eIF-4B cross-links to this mRNA, but this cross-linking is not affected by the addition of m^{7}GDP; the identity of this polypeptide is not known, but it is possible that it is eIF-4B, because it can cross-link to some extent in a cap-independent manner; 32).

In light of the observations showing that salt concentration influences the degree of cap dependency exhibited by capped mRNAs for translation (3, 5, 38) and for interaction with cap-specific polypeptides (18), we examined the cap-specific cross-linking pattern obtained with various *tk* constructs at different salt concentrations. In these experiments, decreased amounts of reticulocyte IF were used with the intention of limiting the components that might be involved in melting mRNA 5′ secondary structure (29–31). The cross-linking pattern obtained with mRNA prepared from pX1/SP, pXJP15/SP, and pXJPB/SP constructs after irradiation with rabbit reticulocyte IF at different salt concentrations (50,

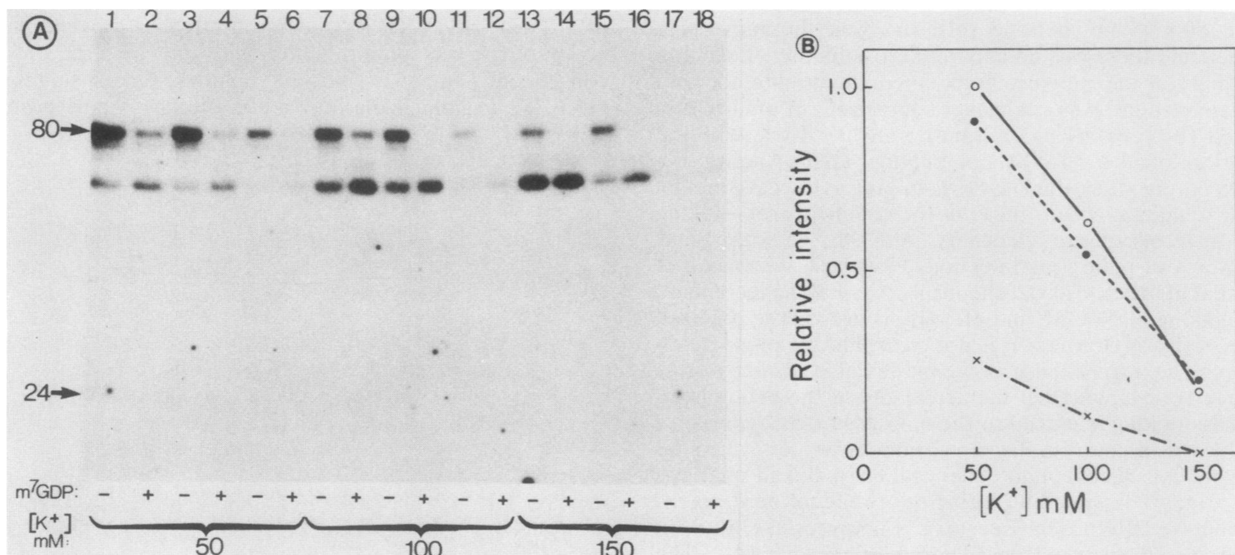

FIG. 5. Effect of K$^+$ concentration on UV light-induced cross-linking of rabbit reticulocyte CBPs to mRNA prepared from pX1/SP, pXJP15/SP, and pXJPB/SP. ^{32}P-mRNA (cap labeled) (2 × 10^4 cpm; ~0.3 μg) was incubated with 24 μg of rabbit reticulocyte IF preparation in the presence of 1 mM ATP for 10 min at 30°C, UV irradiated, and processed for autoradiography as described in Materials and Methods. The autoradiograph is shown in panel A. KOAc was added to give the final concentrations indicated in the figure. The mRNAs used were as follows. Lanes 1, 2, 7, 8, 13, and 14, pX1/SP; lanes 3, 4, 9, 10, 15, and 16, pXJP15/SP; lanes 5, 6, 11, 12, 17, and 18, pXJPB/SP. m^{7}GDP (0.6 mM) was added as indicated in the figure, and the positions of the 24- and 80-kDa cross-linked polypeptides are indicated in the left margin. (B) Quantitative analysis of the labeled 80-kDa polypeptide band from panel A. The value obtained for lane 1 was set as a relative intensity of 1. Symbols: ○, pX1/SP; ●, pXJP15/SP; ×, pXJPB/SP.

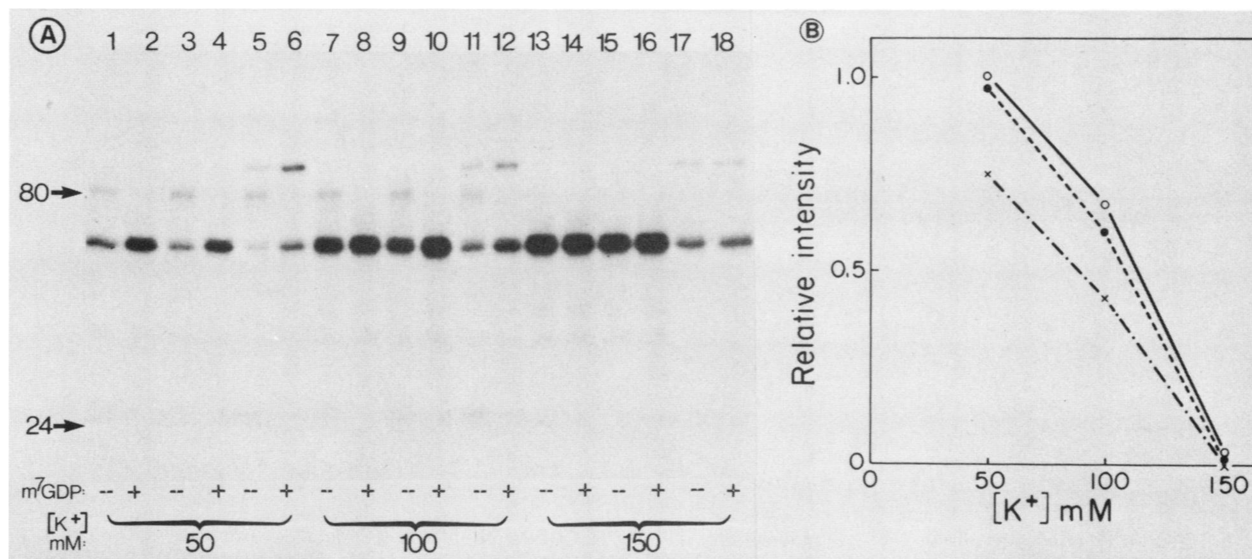

FIG. 6. Photochemical cross-linking pattern of CBPs to inosine-substituted mRNA prepared from pX1/SP, pXJP15/SP, and pXJPB/SP as a function of K^+ concentration. ^{32}P-mRNA (cap labeled) (2×10^4 cpm; ~0.3 μg) was incubated with 24 μg of rabbit reticulocyte IF preparation for 10 min at 30°C, UV irradiated, and processed for autoradiography as described in Materials and Methods. The autoradiograph is shown in panel A. KOAc was added to give the final concentrations indicated in the figure. The mRNAs used were as follows. Lanes 1, 2, 7, 8, 13, and 14, pX1/SP; lanes 3, 4, 9, 10, 15, and 16, pXJP15/SP; lanes 5, 6, 11, 12, 17, and 18, pXJPB/SP. m^7GDP (0.6 mM) was added as indicated in the figure, and the positions of the 24- and 80-kDa cross-linked polypeptides are indicated in the left margin. (B) Quantitative analysis of the labeled 80-kDa polypeptide band from panel A. The value obtained for lane 1 was set as a relative intensity of 1. Symbols: ○, pX1/SP; ●, pXJP15/SP; ×, pXJPB/SP.

100, and 150 mM K^+) is shown in Fig. 5A. Cap-specific cross-linking in this experiment occurred primarily to eIF-4B (80-kDa polypeptide), cross-linking of the 24K-CBP being less pronounced than in previous experiments (see, for example, Fig. 4). The cross-linking of eIF-4B decreased with increasing concentrations of K^+ from 50 to 150 mM with all three mRNAs (cf., for example, lane 13 with lane 1 or lane 17 with lane 5), as might be expected if secondary structure is increased at higher salt concentrations. However, the significant result in this experiment is that the extent of cross-linking of pX1/SP and pXJP15/SP mRNAs to eIF-4B was similar under all salt concentrations (Fig. 5A; cf. lanes 3, 9, and 15 with lanes 1, 7, and 13, respectively; see Fig. 5B for quantitative analysis). This similarity is in sharp contrast to the low efficiency of pXJPB/SP mRNA cross-linking to eIF-4B at all salt concentrations as compared with pX1/SP mRNA (cf. lanes 5, 11, and 17 with lanes 1, 7, and 13, respectively; see Fig. 5B for quantitative analysis). The results also indicate that the deleterious effect of the increase of salt concentration on eIF-4B cross-linking is more pronounced with pXJPB/SP mRNA as compared with the two other mRNAs. Increasing the K^+ concentration from 50 to 100 mM resulted in a 40% inhibition of eIF-4B cross-linking to pX1/SP and pXJP15/SP mRNA, whereas the inhibition was 70% for pXJPB/SP mRNA cross-linking. This is consistent with the idea that the increase of secondary structure at higher salt concentration is responsible for the reduced extent of cross-linking. In contrast to the differential pattern of eIF-4B cross-linking with regard to the mRNA used, there was no difference in the degree of 24K-CBP cross-linking to the different mRNAs at any given salt concentration (this could be clearly seen with longer exposure of the X-ray film than that used in the experiment shown in Fig. 5A [data not shown]). This reinforced previous findings that the cross-linking of the 24K-CBP is independent of ATP and mRNA secondary structure, whereas the cross-linking of eIF-4B is dependent on ATP and the degree of mRNA secondary structure (16, 29). On the basis of these results, we conclude that the ability of eIF-4B to cross-link to mRNA is influenced by secondary structure near the 5′ terminus (6 nt from the cap structure) but is not affected by secondary structure farther downstream (37 nt from the cap structure).

We performed experiments to examine the idea that the secondary structure and not the mere extra nucleotides introduced downstream from the cap structure prevents the interaction of eIF-4B with the cap structure. We prepared in vitro transcripts from pX1/SP, pXJP15/SP, and pXJPB/SP in which inosine was substituted for guanosine, resulting in a less structured mRNA (14). If our interpretation of the results is correct, then inosine-substituted mRNA derived from pXJPB/SP might, in the absence of ATP-Mg^{2+}, cross-link to eIF-4B with an efficiency similar to that of pX1/SP mRNA. We have previously shown that inosine-substituted reovirus mRNA is capable of cross-linking to eIF-4B (identified as an 80-kDa polypeptide) in the absence of ATP-Mg^{2+}, in contrast to the inability of native reovirus mRNA to do so (16). We interpreted our results to indicate that the requirement of ATP-Mg^{2+} for the cross-linking of eIF-4B to native reovirus mRNA reflects the requirement for melting the 5′-proximal secondary structure of the mRNA. The cross-linking profiles obtained with pX1/SP, pXJPB/SP, and pXJP15/SP inosine-substituted mRNAs under three different salt concentrations, in the absence of ATP-Mg^{2+} are shown in Fig. 6A. Cap-specific cross-linking occurred to eIF-4B, consistent with previous results (16) and to a doublet in the region of the 24K-CBP (cf. lane 1 with lane 2 which contains m^7GDP). There is an ~96-kDa polypeptide that cross-links only to pXJPB/SP in a cap-independent manner (for example, see lanes 5 and 6). We have no explanation for this, although it may be due to the base content of the mRNA, because a similar polypeptide is not seen with guanosine-containing mRNA (Fig. 5, lanes 5 and 6). Significantly, the

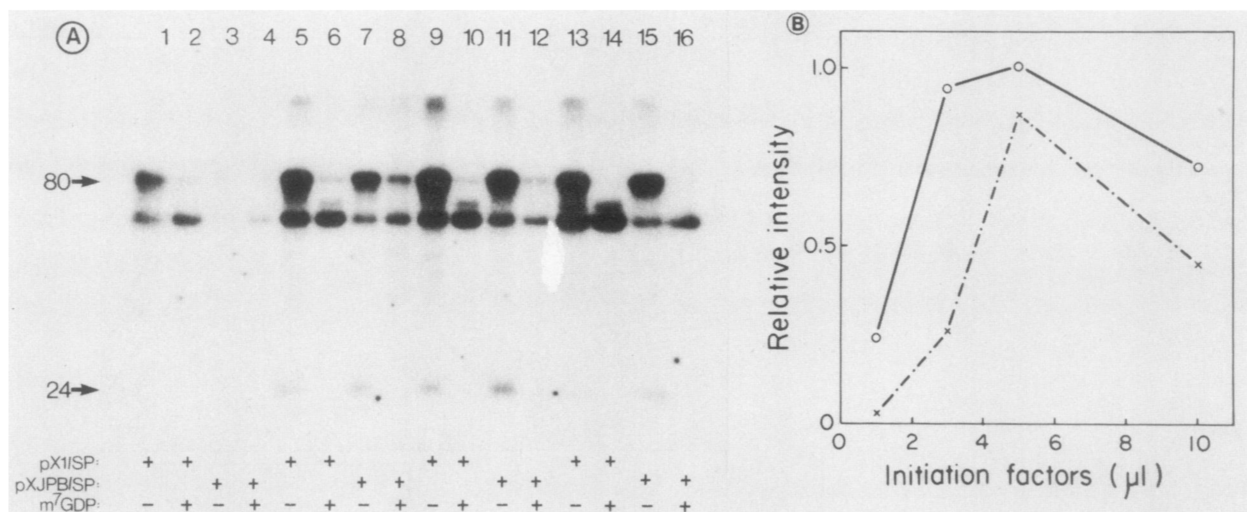

FIG. 7. Effect of IF concentration on UV light-induced cross-linking of CBP to ^{32}P-mRNA (cap labeled) from pX1/SP and pXJPB/SP constructs. ^{32}P-mRNA (cap labeled) (2×10^4 cpm; ~0.3 μg) was incubated with increasing amounts of rabbit reticulocyte IF preparations (12 mg/ml) in the presence of 1 mM ATP, followed by UV irradiation and SDS-polyacrylamide gel analysis of the cross-linked products as described in Materials and Methods. The autoradiograph is shown in panel A). The amounts of IF added were as follows. Lanes 1 to 4, 12 μg; lanes 5 to 8, 36 μg; lanes 9 to 12, 60 μg; lanes 13 to 16, 120 μg. mRNAs used are indicated in the figure. m^{7}GDP (0.6 mM) was added where indicated, and the positions of the 24- and 80-kDa cross-linked polypeptides are indicated in the left margin. (B) Quantitative analysis of the labeled 80-kDa polypeptide band from panel A. The value obtained for lane 9 in panel A was set as a relative intensity of 1. Symbols: ○, pX1/SP; ×, pXJPB/SP.

extent of cross-linking of the cap-specific polypeptides to the three mRNAs was very similar at all salt concentrations. (At 50 and 100 mM KCl, cross-linking was equal, but then it dropped sharply at 150 mM KCl, as is evident from the quantitation of the eIF-4B bands on the autoradiograph [Fig. 6B]). This behavior was in sharp contrast to the lower efficiency of native pXJPB/SP mRNA cross-linking to eIF-4B in the presence of ATP-Mg^{2+} (cf. Fig. 6 with Fig. 5). Similar results were obtained when the experiments shown in Fig. 6 were performed in the presence of ATP-Mg^{2+} (data not shown). Thus, the reduction of secondary structure of pXJPB/SP mRNA abolished the differential cross-linking of this mRNA to eIF-4B relative to pX1/SP mRNA.

In light of the hypothesis that the CBP complex, possibly in conjunction with eIF-4B, is involved in the melting of 5′ mRNA secondary structure (5, 30) and that this complex is limiting in the cell (23, 24), it is conceivable that the addition of this factor to the cross-linking reactions containing pX1/SP or pXJPB/SP mRNAs will abolish the differential cross-linking of these mRNAs to eIF-4B (i.e., the CBP complex will preferentially stimulate cross-linking of eIF-4B to pXJPB/SP mRNA). Unfortunately, we could not test this prediction directly, because our latest preparations of the CBP complex contained an ~80-kDa polypeptide (possibly a contaminant; 11) that cross-linked nonspecifically to the cap structure and consequently interfered with the interpretation of the data. We alternatively used total IF preparation from rabbit reticulocytes. Albeit less satisfactory than that obtained for the purified CBP complex, the data obtained should indicate whether IF preparations contain a factor which is required more for mRNAs having increased secondary structure. We cross-linked increasing amounts of total IF preparations to pX1/SP and pXJPB/SP mRNAs, and the analysis of the cross-linked polypeptides on an SDS-polyacrylamide gel is shown in Fig. 7A. When 12 μg of IF preparation was used, the major cap-specific cross-linked protein was eIF-4B, whereas cross-linking of the 24K-CBP is not evident at this exposure (lane 1). In addition, cross-linking of pX1/SP mRNA was more efficient than of pXJPB/SP mRNA (cf. lane 3 with lane 1), as shown before (Fig. 5). The addition of increasing amounts of IF increased the extent of cross-linking of both mRNAs to eIF-4B and the 24K-CBP, but the efficiency of cross-linking of pX1/SP mRNA to eIF-4B reached a plateau after the addition of 36 μg of IF (lane 5), whereas the efficiency of cross-linking of pXJPB/SP mRNA to eIF-4B reached a similar level only when 60 μg of IF was added (lane 11; the quantitation of cross-linking is shown in Fig. 7B). The results also show that there was no significant differential cross-linking of the 24K-CBP to the two mRNAs under any of the conditions (cf., for example, lanes 7 and 5 or lanes 11 and 9), consistent with the contention that cross-linking of the 24K-CBP to the 5′ end of the mRNA is independent of the degree of mRNA 5′ secondary structure, whereas cross-linking of eIF-4B is dependent on this structure.

DISCUSSION

The chemical cross-linking assay has been very useful in identifying polypeptides that bind at or near the cap structure of eucaryotic mRNAs and elucidating their possible roles in facilitating initiation complex formation (4, 10, 32). This assay, however, has several limitations which preclude its application to a range of experiments as detailed in Results. One of the disadvantages of the chemical cross-linking technique is that the interaction between the aldehyde groups of the oxidized ribose moiety of the cap structure occurs via ε-amino or α-terminal amino groups of proteins. These groups might not be present in the cap binding site of the different polypeptides that interact with the cap structure. In contrast, it has been shown that as many as 11 different amino acids can form covalent bonds with uracil upon UV irradiation (28). Thus, the probability of detecting CBPs by the photolabeling technique is a priori greater than with the chemical cross-linking technique. Furthermore, the application of the photochemical technique is not restricted to those few viral mRNAs which can be

purified to large amounts, this feature being particularly attractive with the advent of the SP6 transcription system. Finally, because [α-^{32}P]GTP is used to prepare the labeled cap structure, results are obtained with much shorter exposure of the autoradiograms to X-ray film as compared with the chemical cross-linking method in which tritium is used.

The high-energy intermediates that are formed during irradiation of mRNA have a relatively short half-life, and as a consequence, the site of covalent linkage in the protein must be very close to the cap structure. This decreases the probability of nonspecific cross-linking, and, indeed, the data in Fig. 1 demonstrate that there is less nonspecific cross-linking with the photolabeling technique as compared with the chemical technique.

We identified two polypeptides of 24 and 80 kDa that can be photochemically cross-linked to the cap structure of reovirus and *tk* mRNAs. These polypeptides are most probably the same as the 24-kDa (24K-CBP) and 80-kDa (eIF-4B) polypeptides observed in the chemical cross-linking assay, based on their similar behavior in terms of cap specificity, ATP-Mg^{2+} requirement for cross-linking, and the inability to interact with the cap structure after poliovirus infection of HeLa cells. It is significant, however, that there are qualitative and quantitative differences between the cross-linking patterns obtained by the two techniques. Unlike with the chemical cross-linking assay, we did not observe cap-specific cross-linking of eIF-4A in the photochemical assay. A simple explanation for this is that the position of eIF-4A relative to the mRNA is not within the reactive range of the high-energy and short-lived intermediate species produced by irradiation of mRNA. However, because it is cross-linked in the chemical assay, eIF-4A must be in proximity to the cap structure. In the chemical cross-linking assay, the 24K-CBP is cross-linked with the highest efficiency (Fig. 1), whereas in the photochemical assay eIF-4B cross-links with greater efficiency than the 24K-CBP. It is possible that this difference is due to the fact that the chemical cross-linking reaction proceeds through the ribose group of the m^7G moiety of the cap structure (33), whereas the covalent link between the cap structure and proteins, induced by UV light, is probably via the ring structure of the m^7G group. This is based on the studies of Steinmaus et al. (34) indicating that the 8-position of the guanine ring is the most reactive group and that a free radical is generated upon the photolysis of this compound. Thus, these results suggest that eIF-4B is positioned in closer proximity to the ring of the m^7G than to its ribose moiety, whereas the opposite applies for the 24K-CBP.

Photochemical cross-linking has been previously used to identify proteins that interact with eucaryotic mRNAs, but no attempt was made in these studies to determine the proteins that cross-link specifically to the cap structure (9, 26). It is of interest, however, that a polypeptide termed p78X was found to cross-link to mRNA by UV light irradiation and that this polypeptide was different from the protein that binds to the poly(A) tail of eucaryotic mRNAs (termed p78A; 26).

We found an inverse relationship between the efficiency of mRNA cross-linking to eIF-4B and K^+ concentration (Fig. 5). This is expected according to our model, because the degree of secondary structure is increased at higher salt concentrations. That the high salt concentration per se is affecting the activity of a factor(s) involved in the melting of the mRNA secondary structure is an unlikely possibility, because it cannot explain the differential cross-linking patterns obtained with mRNA from pX1/SP and pXJPB/SP constructs at the different salt concentrations used. These results are, however, different from those obtained by the chemical cross-linking technique (16). In the latter study, it was reported that the cross-linking efficiency of oxidized reovirus mRNA to the 80-kDa polypeptide (eIF-4B) increased with increasing K^+ concentrations (16). A possible explanation for these differences is that the mRNA cross-linking site in eIF-4B is different in the two techniques. This would seem a reasonable argument, considering the differences in the mechanism of chemical and photochemical cross-linking.

The results presented here indicate that eIF-4B is unable to interact with the cap structure if excessive secondary structure exists close to the cap structure (6 nt in pXJPB/SP) yet is able to do so if the stem-loop structure is farther downstream (37 nt in pXJP15/SP). The cross-linking of the 24K-CBP, however, does not seem to be affected by the insertion of *Bam*HI linkers at either position. This observation is consistent with a mechanism by which the 24K-CBP as part of the CBP complex binds to the mRNA in an ATP-independent fashion, followed by ATP-dependent melting of mRNA secondary structure and subsequent interaction of eIF-4B (4, 30).

In a recent report from this laboratory (22), we showed that increasing *tk* mRNA secondary structure within the 5′ noncoding region results in a decreased translational efficiency in in vivo and in vitro systems. mRNAs from both constructs pXJP15/SP and pXJPB/SP showed a drastic reduction in translational efficiency when compared with mRNA obtained from pX1/SP. Because the position of the hairpin loops is different in each of the constructs, the data presented here suggest that the block in translation of pXJPB/SP (but not pXJP15/SP) is at least partially the result of the inability of eIF-4B to interact with the 5′-terminal cap structure, with subsequent impediment of ribosome binding and protein synthesis. The impaired translation of pXJP15/SP is probably due to a step subsequent to cap recognition and eIF-4B binding, involving further melting of the mRNA 5′ noncoding region perhaps concomitantly with 40S ribosome binding or migration along the mRNA 5′ leader region.

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