Initiation of Reovirus Transcription by Inosine 5′-Triphosphate and Properties of 7-Methylinosine-Capped, Inosine-Substituted Messenger Ribonucleic Acids†

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ABSTRACT: Inosine 5′-triphosphate (ITP) can be utilized in place of guanosine 5′-triphosphate (GTP) for both the initiation and the elongation steps of reovirus transcription, resulting in the synthesis of mRNAs containing 5′-terminal m^7IpppI^m and internal pI. The apparent molecular weights of the I-substituted products were altered as a consequence of the absence of G–C base pairs and accompanying loss of ordered structure. The migration of I-substituted RNAs in agarose gels and glycerol gradients was similar to glyoxal-treated transcripts; i.e., it decreased 2-fold as compared to the corresponding untreated G-containing mRNAs. 7-Methylinosine-capped (m^7I-capped), I-substituted transcripts readily attached to wheat germ 80S ribosomes. Unlike native G-containing mRNAs, they also formed heavier complexes that sedimented faster than 80S complexes even in the presence of the nonhydrolyzable ATP analogue AMPP(NH)P and elongation inhibitor sparsomycin. I-substituted molecules that were capped posttranscriptionally to form m^7G-capped 5′ ends yielded mostly 80S monosomes, consistent with a strong influence of 5′-terminal structure on initiation of translation. Under limited conditions of initiation, I-substituted RNAs outcompeted G-containing transcripts for ribosome attachment. Although the results are consistent with enhanced binding and freer movement of ribosomes on unstructured templates, synthesis of acid-precipitable polypeptides in wheat germ extract directed by I-substituted RNAs was 15-fold less than with G-containing mRNAs.

Enzyme activities associated with purified reovirus catalyze the synthesis of viral mRNAs containing a blocked, methylated 5′-terminal structure, m^7GpppG^m (Furuichi et al., 1976), i.e., the same type of "cap" found in most viral and cellular mRNAs (Shatkin, 1976). Studies of reovirus and other eukaryotic systems indicate that the cap is formed during the initiation of transcription, and its presence increases mRNA stability and function. In cell-free protein synthesizing systems derived from wheat germ or mammalian cells, capped mRNAs are translated more effectively than the corresponding uncapped RNAs. This effect may be mediated at the level of initiation by proteins that recognize and bind to the cap (Sonenberg et al., 1979). A similar kind of interaction may be involved in another cap-dependent function, the priming of influenza virion transcriptase by reovirus mRNA and other heterologous capped mRNAs (Bouloy et al., 1979).

To explore the mechanisms of cap recognition, we have studied the effects of structural alterations in the cap on reovirus mRNA function. Viral transcription in the presence of *S*-adenosylethionine, rather than the homologous methyl donor, yielded mRNAs with ethylated caps (Furuichi et al., 1979). They were translated essentially as well as the methylated mRNAs, suggesting that the alkyl group induced conformation at the 5′ end of capped mRNA (Hickey et al., 1977) may be more important for function than the methyl groups per se. Substitution of the entire m^7G residue by another nucleoside was previously tested only indirectly by comparing cap analogues, including m^7GDP, m^7IDP, and m^7XDP, as inhibitors of capped mRNA binding to ribosomes (Adams et al., 1978). While the m^7G nucleotide strongly inhibited initiation of translation, the m^7I derivative was less effective, and m^7XDP was inactive. We have observed that the reovirus-associated "guanylyl" transferase and RNA polymerase activities, like the viral "methyl" transferases, are not absolutely substrate specific. Both activities utilize ITP in place of GTP, and, thus, reovirus mRNAs containing internal I and 5′-terminal m^7I or m^7G can be synthesized in vitro. We have used these I-substituted products to examine the effects of diminished secondary structure and altered 5′-terminal cap on mRNA function.

Experimental Procedures

Preparation of Reovirus Cores. Reovirus type 3 Dearing strain was purified from infected mouse L cells as described previously (Shatkin & LaFiandra, 1972). Virions (1.2 mg/mL) were digested with chymotrypsin (0.6 mg/mL) at 43 °C for 45 min in 70 mM Tris-HCl (pH 8) containing 90 mM KCl. The resulting virus cores were collected by centrifugation (13 000*g*, 15 min, 4 °C), washed, and resuspended in 50 mM Tris-HCl (pH 8) containing 50 mM KCl.

Synthesis of Viral mRNAs. [^{3}H]Methyl-labeled reovirus mRNAs were synthesized by incubating virus cores at 43 °C for 2 h in a 0.5-mL reaction mixture that included 70 mM Tris-HCl (pH 8), 18 mM $Mg(OAc)_2$, 4 mM each of ATP, CTP, UTP, and either GTP or ITP, 0.05 units of inorganic pyrophosphatase (Boehringer-Mannheim, West Germany), 5 mM phosphoenolpyruvate, 17 μg of pyruvate kinase, 200 μg of Macaloid, 100 μCi of *S*-adenosyl[*methyl*-^{3}H]methionine (specific activity 73 Ci/mmol), and reovirus cores (equivalent to 0.6 mg of virion protein). For the preparation of ^{32}P-labeled mRNA, reaction mixtures contained 0.2 mM nonradioactive *S*-adenosyl-L-methionine and 50 μCi of [α-^{32}P]UTP. After a 2-h incubation at 43 °C, the viral cores were removed by centrifugation, and the mRNA products in the supernatant fraction were purified by phenol extraction, Sephadex G-100 gel filtration, and ethanol precipitation.

I-substituted transcripts containing 5′-terminal ppI were similarly prepared except that the ITP concentration was lowered to 0.4 mM and *S*-adenosylmethionine and inorganic pyrophosphatase were replaced by 0.5 mM *S*-adenosyl-L-homocysteine and 1 mM sodium pyrophosphate, respectively

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(Furuichi & Shatkin, 1976, 1977). To cap and methylate these transcripts, 7 μg of RNA was incubated with reovirus cores (0.6 mg of virion protein) in 0.3 mL of 60 mM Tris-HCl (pH 8) containing 5 mM $Mg(OAc)_2$, 4 mM GTP, 2.5 mM phosphoenolpyruvate, 8.5 μg of pyruvate kinase, 0.03 unit of inorganic pyrophosphatase, and 75 μCi of *S*-adenosyl[*methyl*-^{3}H]methionine (specific activity 22 Ci/mmol). After incubation for 90 min at 43 °C, cores were pelleted, and phenol-extracted mRNA was purified by Sephadex G-100 gel filtration. The first six gel-excluded fractions (total of 12) were pooled, and the ethanol-precipitated mRNA was used for ribosome binding.

Preparation of [^{3}*H*]*ITP.* [2-^{3}H]ATP (10 μL, 0.2 mCi, specific activity 20 Ci/mmol) was mixed with 29 μL of 2 M $NaNO_2$ and 70 μL of 3.1 N acetic acid–sodium acetate buffer (pH 3.8), incubated for 5 h at 37 °C, and analyzed by high-voltage paper electrophoresis at pH 3.5 in pyridine acetate buffer (Shapiro & Pohl, 1968). Conversion of [2-^{3}H]ATP to [^{3}H]ITP was about 95% complete on the basis of electrophoretic mobility as compared to ATP and ITP marker compounds. [^{3}H]ITP was eluted in H_2O from the dried paper and lyophilized.

Enzymatic Digestion and Analytical Methods. Conditions for digestion with P1 nuclease, bacterial alkaline phosphatase (BAP), and nucleotide pyrophosphatase, subsequent analyses of the digests by high-voltage paper electrophoresis in pyridine acetate buffer (pH 3.5), and paper chromatography in isobutyric acid–0.5 M NH_4OH (10:6) have been described (Furuichi et al., 1975a,b). For complete RNase T_2 digestion, ~2 μg of mRNA was incubated for 1 h at 37 °C in 0.1 mL of 50 mM sodium acetate (pH 4.5) containing 25 units of RNase T_2 (Calbiochem). Separation of m^{7}G from m^{7}I was accomplished by Whatman 3MM paper chromatography in H_2O; unmethylated and 2′-O-methylated nucleosides were resolved in 1-butanol–pyridine–H_2O (1:1:1).

Ribosome Binding and mRNA Translation in Wheat Germ Extract. Reovirus mRNA (0.5 μg) was incubated for 10 min at 25° C in 50-μL reaction mixtures containing 25 μL of S23 wheat germ extract and 0.2 mM sparsomycin except where indicated, and the resulting initiation complexes were resolved by centrifugation in 10–30% glycerol gradients. Translation assays were based on [^{35}S]methionine incorporation into acid-precipitable products. Both procedures have been described in detail previously (Furuichi et al., 1979).

Glyoxal Treatment and Agarose Gel Electrophoresis. Reovirus mRNAs were treated with glyoxal as described by McMaster & Carmichael (1977). Approximately 100 000 cpm of each ^{32}P-labeled mRNA was incubated for 1 h at 50 °C in 25 mM sodium citrate (pH 3.5), 50% dimethyl sulfoxide, and 1 M glyoxal. Controls were similarly incubated in the same solvent but without glyoxal. Samples were cooled to room temperature and analyzed by centrifugation in 5–30% glycerol gradients and by electrophoresis for 14 h at 25 mA and 4 °C in 1.75% agarose gels containing 7 M urea in 0.025 M citrate buffer (pH 3.5) (Smith & Furuichi, 1980). Radiolabeled bands were visualized by autoradiography with Kodak XR-5 X-ray film.

Results

Utilization of ITP in Place of GTP for Capped mRNA Synthesis. Reovirus contains RNA polymerase and mRNA-modifying activities (Furuichi et al., 1976). Consequently, viral cores incubated in reaction mixtures containing GTP, the other three ribonucleoside triphosphates, including [α-^{32}P]UTP, and *S*-adenosyl[*methyl*-^{3}H]methionine incorporated both radiolabels into transcripts of the viral genome RNA (Figure 1A).

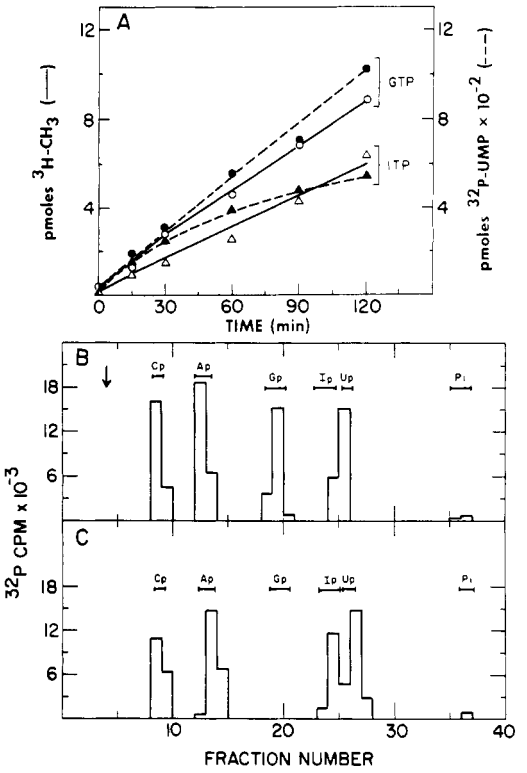

FIGURE 1: Time course of synthesis and nearest-neighbor analysis of reovirus G-containing and I-substituted mRNAs. (A) Transcription conditions were as described under Experimental Procedures with *S*-adenosyl[*methyl*-^{3}H]methionine or [α-^{32}P]UTP as labeled precursors in separate reaction mixtures and ITP substituted for GTP as indicated. At the times shown, 20- and 5-μL aliquots were removed to assay [^{3}H]methyl and [^{32}P]UMP incorporation into RNA, respectively. After 30 min at 0 °C in 5 mL of 10% trichloroacetic acid–sodium pyrophosphate, the samples were filtered through nitrocellulose filters, washed three times with 5% trichloroacetic acid, and counted in toluene-based scintillant. (B and C) RNase T_2 digests of [α-^{32}P]-UMP-labeled products were analyzed by high-voltage paper electrophoresis together with the indicated marker mononucleotides: (B) G-containing mRNA; (C) I-substituted mRNA. Arrow indicates origin.

Incubation mixtures containing ITP in place of GTP supported both RNA chain polymerization and methylation as measured by [^{32}P]UMP and [^{3}H]methyl incorporation, respectively.

The results in Figure 1A suggested that ITP could be used for both the initiation and the elongation steps of transcription catalyzed by reovirus-associated enzyme activities. To rule out the possibility that contaminating GTP present in the ribonucleoside triphosphates was responsible for the synthesis observed in reactions containing ITP, nearest-neighbor analyses were performed on the [^{32}P]UMP-labeled mRNAs. RNase T_2 treatment of the control and G-containing products and electrophoresis of the digest yielded radioactivity distributed among Cp (24%), Ap (29%), Gp (23%), and Up (24%) (Figure 1B). The presumptive I-substituted mRNA digest contained radioactivity migrating in the positions of Cp (23%), Ap (29%), Ip (~21%), and Up (~27%) but none comigrating with Gp (Figure 1C).

The absence of G in the ^{32}P-labeled mRNA synthesized in the presence of ITP indicated that I substitution was essentially complete. Furthermore, since the G-containing and I-substituted polymerase products were methylated to a similar extent (Figure 1A), it seemed likely that ITP was also used by reovirus cap-forming enzyme activities to produce transcripts containing 5′-terminal m^{7}I. Analysis of mRNAs synthesized in the presence of ITP and *S*-adenosyl[*methyl*-^{3}H]methionine supported this conclusion. As shown in Figure

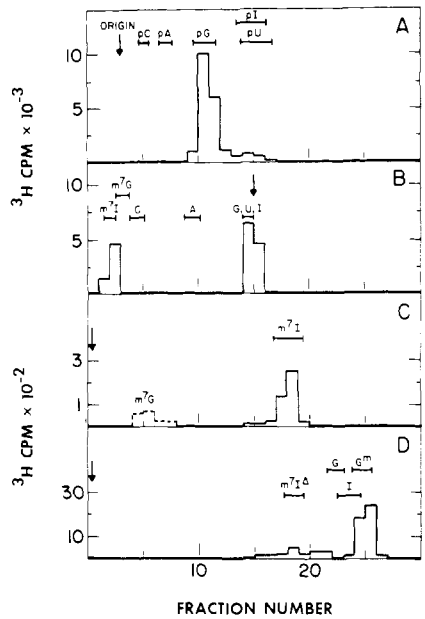

FIGURE 2: Analysis of 5′-[^{3}H]methyl-labeled reovirus mRNA synthesized with ITP in place of GTP. (A) RNA was digested with P1 nuclease and BAP and analyzed by high-voltage paper electrophoresis. (B) Material in fractions 11 and 12 of panel A was eluted, digested with nucleotide pyrophosphatase and BAP, and reanalyzed by electrophoresis (Furuichi et al., 1975). (C) Fractions 2 and 3 of panel B were eluted and separated by ascending chromatography in H_2O. ^{3}H-Labeled m^{7}G (---) and nonradioactive m^{7}I were used as markers. (D) Fractions 15 and 16 of panel B were eluted and analyzed by ascending chromatography in 1-butanol–pyridine–H_2O. Marker compounds including ring-opened m^{7}I (m^{7}I$^\Delta$) were located under UV light. After electrophoresis, 1-cm paper strips were cut and counted directly in toluene-based scintillant; after paper chromatography, the strips were soaked in 0.5 mL of H_2O for 0.5 h before counting in 10 mL of Hydrofluor (National Diagnostics).

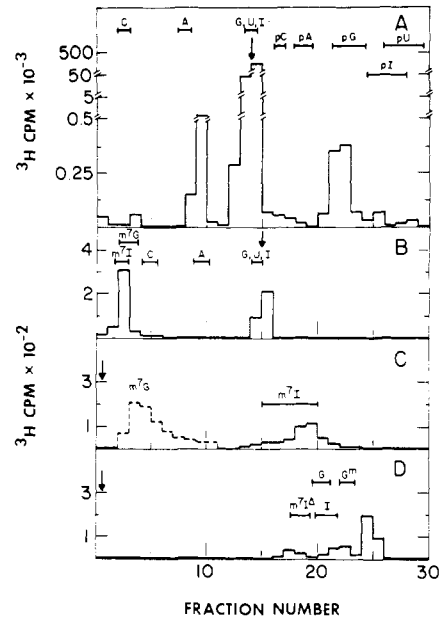

FIGURE 3: Analysis of ^{3}H-labeled I-substituted mRNA. Products were synthesized in 0.5-mL reaction mixtures as described under Experimental Procedures except that they contained [2-^{3}H]ITP (1 mM, 90 μCi) in place of GTP. (A) Paper electrophoresis of mRNA treated with P1 nuclease and BAP. (B) Material eluted from fractions 21–24 of panel A was digested with nucleotide pyrophosphatase and BAP and reanalyzed. (C) Fractions 2 and 3 of panel B were eluted and separated by paper chromatography in H_2O. (D) Fractions 15 and 16 from panel B were reanalyzed by chromatography in 1-butanol–pyridine–H_2O.

2A, after digestion of I-substituted mRNA with P_1 nuclease and BAP followed by paper electrophoresis, all the ^{3}H radioactivity migrated as a negatively charged peak with pG, the position of the reovirus mRNA cap (Furuichi et al., 1975). The eluted peak was digested further with nucleotide pyrophosphatase and BAP and resolved by electrophoresis into two ^{3}H-labeled constituents (Figure 2B). One peak comprising ~35% of the counts migrated in the region of the positively charged markers, m^{7}G and m^{7}I, and the remainder stayed at the origin, the position of U, G, I, and their 2′-O-methylated derivatives. Paper chromatographic analysis indicated that the positively charged, labeled component was m^{7}I (Figure 2C). Most of the material from the origin migrated slightly faster than marker I by chromatography (Figure 2D). Although the authentic marker was not available, this is the position for I^m since 2′-O-methylation is known to increase nucleoside migration in this solvent as observed with G^m and other marker compounds. A small number of counts were also detected in the position of uncharged, ring-opened m^{7}I (m^{7}I$^\Delta$), presumably accounting for the higher amount of radioactivity at the origin relative to m^{7}I in Figure 2B.

To confirm that the I-substituted mRNAs contained 5′-terminal m^{7}IpppI^m, transcriptase products were synthesized in incubation mixtures that included unlabeled *S*-adenosylmethionine and ^{3}H-labeled ITP. Electrophoretic analysis of P_1 nuclease and BAP digests of this RNA resolved 0.3% of the total incorporated radioactivity in the position of negatively charged caps, consistent with an average chain length of about 1000 nucleotides (Figure 3A). Most of the labeled digested sample was converted to neutral nucleosides which remained at the origin with the marker I. The peak of positively charged radioactivity near the marker A presumably resulted from small amounts of residual [^{3}H]ATP in the [^{3}H]ITP since after elution this peak was shown to migrate with A by paper chromatography in isobutyric acid–0.5 M NH_4OH (10:6). The [^{3}H]I-labeled presumptive caps (fractions 21–23 in panel A) were eluted, digested with nucleotide pyrophosphatase and BAP, and reanalyzed. Again, two peaks of radioactive material were resolved, one comigrating with m^{7}I (57%) and the other in the position expected for I^m (43%) (Figure 3B). Chromatographic analyses (Figure 3C,D) were consistent with these assignments and the synthesis of I-substituted mRNA containing 5′-terminal m^{7}IpppI^m.

Loss of Secondary Structure and Change in the Apparent Molecular Weights of I-Substituted mRNAs. Reovirus mRNAs synthesized in vitro with [^{32}P]UTP and *S*-adenosyl[*methyl*-^{3}H]methionine as labeled precursors consist of three size classes of products, large (l), medium (m), and small (s), corresponding to average chain lengths of ~3500, 2000, and 1000 nucleotides (Joklik, 1974). They were readily resolved by velocity sedimentation in glycerol density gradients (Figure 4A). By contrast, the similarly radiolabeled but I-substituted transcripts sedimented considerably more slowly under the same conditions, and there was no resolution of the mRNA size classes (Figure 4B).

Further analyses demonstrated that the reduced sedimentation of the I-substituted RNAs was not due to a decrease in chain length but probably resulted from loss of intramolecular base pairing. Treatment of RNA with glyoxal renders the chains irreversibly denatured at neutral pH because glyoxal adduct formation with guanosine residues prevents G–C base pairing (McMaster & Carmichael, 1977). Glyoxal-derivatized reovirus mRNAs were no longer resolved into the three size classes by glycerol gradient centrifugation (compare Figure 5A,B). The glyoxalated products (Figure 5B) sedimented slowly as a single peak, very much like the profile of I-sub-

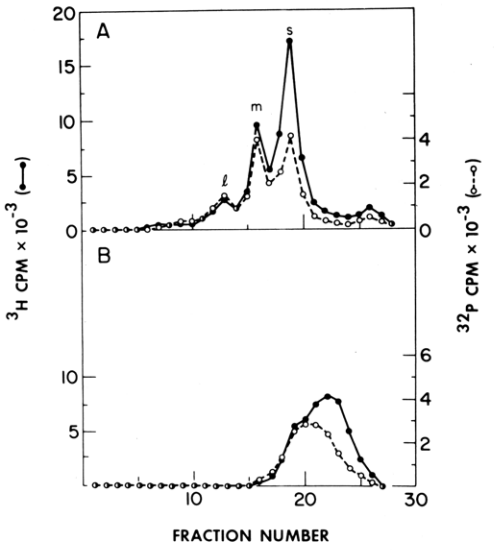

FIGURE 4: Sedimentation profiles of reovirus mRNAs. Products synthesized in reaction mixtures containing GTP (A) or ITP (B) in the presence of *S*-adenosyl[*methyl*-^{3}H]methionine and [α-^{32}P]UTP were purified and analyzed in 5–30% glycerol gradients (20 mM Tris-HCl, pH 8, 0.1 M KCl, 5 mM EDTA; SW41 26 000 rpm, 18 h). Fractions of 0.43 mL were collected and counted directly in 10 mL of Hydrofluor.

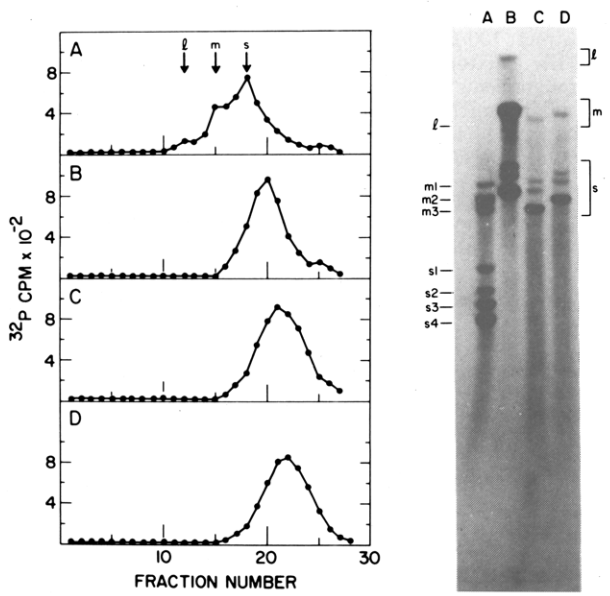

FIGURE 5: Effect of glyoxal treatment on apparent molecular weights of reovirus mRNAs. Glycerol gradient sedimentation profiles of [^{32}P]UMP-labeled mRNAs synthesized in the presence of GTP (panel A) and glyoxal treated (panel B) or with ITP before (panel C) and after (panel D) glyoxalation. Analyses were as in Figure 4. The autoradiograph shows the patterns obtained with the same mRNAs by electrophoresis in an agarose gel as described under Experimental Procedures. (Lanes A and B) G-containing RNAs before and after glyoxal treatment. (Lanes C and D) I-substituted RNA before and after glyoxalation. Reovirus mRNAs of large (l), medium (m), and small (s) size indicated on the right for lanes B–D and on the left for lane A.

stituted mRNAs (Figure 5C). Glyoxal treatment of the I-substituted mRNA had little additional effect on an already decreased sedimentation rate, consistent with the replacement of G by I residues in this RNA (Figure 5D). Aliquots of the same mRNA preparations were also analyzed by agarose gel electrophoresis (Figure 5). The gel patterns in lanes A–D were in agreement with the sedimentation profiles in panels A–D obtained with the same RNAs. The autoradiograph clearly demonstrated that the decreased sedimentation rate of gly-

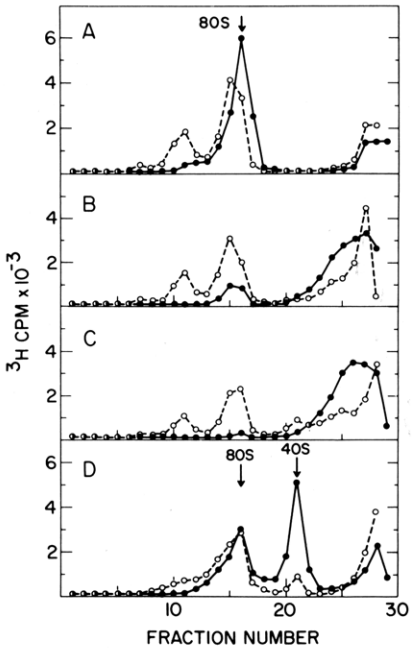

FIGURE 6: Effects of m^{7}GMP and nonhydrolyzable analogues of ATP and GTP on the binding of reovirus mRNAs to wheat germ ribosomes. G-containing mRNAs (5′-m^{7}GpppG^m, ●—●) and I-substituted mRNAs (5′-m^{7}IpppI^m, ○---○), both methyl-^{3}H-labeled in the 5′ termini, were incubated in wheat germ S23 extracts under initiation conditions and analyzed separately in 10–30% glycerol gradients (SW41 rotor, 39 000 rpm, 2.5 h). (A) Control incubations; (B) binding in the presence of 1 mM m^{7}GMP; (C) ATP replaced by 1 mM AMPP(NH)P; (D) GTP replaced by 0.8 mM GMPP(NH)P.

oxalated or I-substituted molecules was not a reflection of the presence of shorter molecules, arising, for example, by premature termination or by the action of contaminating RNase activity. As seen in lane A, untreated G-containing mRNA was resolved into the four s species and three m-class mRNAs. The small amount of large transcripts did not separate into the three constituent mRNAs. The same G-containing mRNAs after glyoxalation migrated at a 2-fold decreased rate, and the two smallest mRNAs, s3 and s4, as well as the m species were not resolved (lane B). The I-substituted mRNAs were similar in position to those of the glyoxalated, G-containing species (lane C), and glyoxal treatment resulted in only a small further decrease in their migration (lane D). The gradient and gel analyses taken together indicate that the I-substituted molecules are probably full-length but have altered apparent molecular weights due to loss of ordered structure.

Binding of I-Substituted mRNAs to Wheat Germ Ribosomes. It was recently reported (Kozak, 1980) that not only can I-substituted reovirus mRNAs bind to wheat germ ribosomes but also ribosome "scanning" apparently continues through the 5′-proximal initiation site to form complexes larger than 80S monosomes even in the presence of sparsomycin which normally inhibits elongation. This effect is seen in Figure 6A where G-containing mRNAs formed mainly 80S initiation complexes with wheat germ ribosomes while the I-substituted RNAs yielded heavier complexes as well. I-substituted RNA had other ribosome binding properties distinct from those of G-containing mRNAs. Cap analogue m^{7}GMP at a concentration that inhibited normal initiation complex formation by 80% had a smaller effect on ribosome binding of I-substituted RNA, diminishing the yield of both 80S and heavier structures by ~30% (compare Figure 6A,B). Furthermore, the ATP requirement was markedly lower for ribosome binding of I-substituted RNA, addition of the non-

Table I: Translation of Reovirus mRNAs in Wheat Germ Extract[a]

mRNA	[35S]methionine incorporation (pmol)	
	30 min	60 min
none	0.002	0.004
G containing	0.507	0.834
I substituted	0.029	0.053

[a] Incubation mixtures consisting of 12.5 μL of wheat germ S23 extract, [35S]methionine (21 μCi, specific activity 715 Ci/mmol), 1 μg of the indicated reovirus mRNA, 1 mM ATP, 0.2 mM GTP, 5 mM creatine phosphate, 40 μg/mL creatine phosphokinase, 20 mM *N*-2-(hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid buffer (pH 7.5), 90 mM KCl, 3 mM magnesium acetate, 2 mM dithiothreitol, and 10 μM each of 19 amino acids (minus methionine) were incubated at 25 °C. Aliquots of 3 μL from a total of 25 μL were spotted on filter paper disks and processed through trichloroacetic acid and ethanol as described (Furuichi et al., 1979).

hydrolyzable analogue AMPP(NH)P decreasing initiation complexes by 30% as compared to >95% with G-containing mRNA (Figure 6C). Since GTP hydrolysis is normally required for 60S ribosomal subunit joining to 40S initiation complexes (Levin et al., 1973; Kozak & Shatkin, 1976), G-containing mRNA in the presence of the nonhydrolyzable analogue GMPP(NH)P formed 40S (39%) and 80S (37%) complexes. By contrast, I-substituted mRNA yielded few 40S complexes under these conditions (Figure 6D).

Although ribosomes readily attached to I-substituted mRNAs and progressed along the denatured templates in the presence of sparsomycin, there was a striking decrease in polypeptide synthesis in response to the I-substituted messengers as compared to native mRNAs. As shown in Table I, the yield of acid-precipitable products was 15-fold lower in wheat germ cell-free translating extracts directed by I-substituted templates.

The results in Figure 6 indicate that I-substituted RNAs have less stringent requirements for ribosome binding than normal mRNAs. This is supported by the finding that when a 1:1 mixture of [32P]UMP-labeled, I-substituted mRNA and [3H]methyl-labeled, G-containing mRNA was incubated under conditions of mRNA saturation, the former effectively outcompeted the latter for attachment to ribosomes (Figure 7A–C). The competitive effect was partially overcome when the input ratio of G- to I-containing RNAs was raised to 4:1 (Figure 7D). Competition was relieved essentially completely by adding 6–8-fold more wheat germ extract per μg of mRNA, and both types of mRNA in the mixture bound to the same extent as when they were incubated separately under the same conditions, i.e., 76% for [3H]methyl-labeled, G-containing RNA and 43% for [32P]UMP-labeled, I-substituted RNA (Figure 7E).

The ribosome binding properties of I-substituted mRNAs that contained 5′-terminal m7G were more similar to native mRNAs than to m7I-capped, I-substituted transcripts. As shown in Figure 8, in the presence of sparsomycin, m7G-capped, G-containing RNAs formed 80S complexes (panel A) while m7I-capped, I-substituted transcripts yielded heavier complexes and proportionately fewer monosomes (panel B). I-substituted mRNAs that contained a m7G cap formed mainly 80S complexes and lower amounts of heavier structures (panel C). The increase in 80S structures was apparently due to the presence of a m7G cap rather than an inability, for example, because of shorter chain length, to bind more than a single ribosome. m7G-capped, I-substituted mRNAs in the absence of sparsomycin formed fast sedimenting complexes (Figure 8C, open circles) similar to those obtained under the same

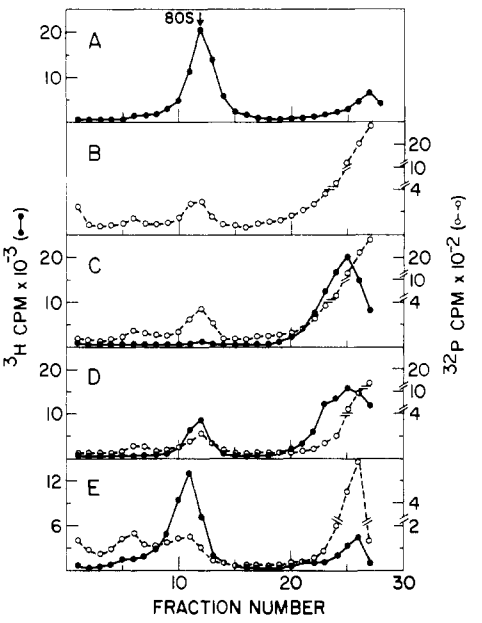

FIGURE 7: Competitive ribosome binding of capped G-containing and I-substituted mRNAs. [3H]Methyl-labeled G-containing mRNAs (5′-m7GpppGm, ●—●) and [α-32P]UMP-labeled I-substituted mRNAs (m7IpppIm, ○---○) were assayed for ribosome binding under competitive conditions (25-μL assay and mRNA concentrations of 40–80 μg/mL, panels A–D) or in the presence of excess wheat germ S23 extract (100-μL assay and mRNA concentration of 10 μg/mL, panel E). Mixtures were analyzed by centrifugation in 10–30% glycerol gradients as described (Furuichi et al., 1979). (A) 1 μg of G-containing mRNA; (B) 1 μg of I-substituted mRNA; (C) 1 μg of each RNA; (D) 1.2 μg of G-containing mRNA and 0.3 μg of I-substituted RNA; (E) 0.5 μg of each RNA.

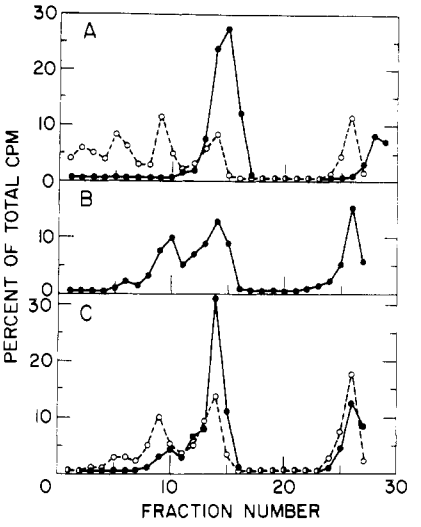

FIGURE 8: Comparative binding of m7G-capped, I-substituted mRNA. Ribosome binding was done as in Figure 6 with 5′-[3H]methyl-labeled mRNAs that were (A) G containing, (B) I substituted, or (C) I substituted but containing 5′-terminal m7GpppIm in >90% of the molecules as determined by analyses like those in Figure 2. (●) profiles obtained with 0.2 mM sparsomycin in the incubation mixtures and in the absence (○) of the inhibitor. Total cpm analyzed in the five gradients ranged from 6200 to 8114.

conditions with native mRNAs (Figure 8A, open circles) or with fully I-substituted mRNAs even in the presence (or absence) of sparsomycin (Figure 8B). These results provide additional evidence for recognition of the 5′-terminal structure of eukaryotic mRNAs during initiation of protein synthesis.

Discussion

Previous studies have shown that ITP can be utilized in place

of GTP by various RNA-synthesizing enzymes, including *E. coli* DNA-dependent RNA polymerase (Lee & Yanofsky, 1977), Qβ bacteriophage replicase (Mills & Kramer, 1979), and vesicular stomatitis virion RNA-dependent RNA polymerase (Testa & Banerjee, 1978). The findings in Figure 1 and those in another recent report (Kozak, 1980) indicate that reovirus-associated transcriptase, an enzyme that copies one strand of each of the ten double-stranded viral genome RNA segments (Joklik, 1974), also is capable of synthesizing I-substituted mRNAs. Substitution of I for G in the reovirus transcripts markedly decreased their electrophoretic migration in agarose gels and sedimentation in glycerol density gradients (Figure 4 and 5). With consideration of the lower stability of I–C (two hydrogen bonds) as compared to G–C base pairs (three hydrogen bonds), this is the result predicted for mRNAs that have been reported previously to contain extensive regions of intramolecular secondary structure (Warrington et al., 1973). It is of interest in this regard that treatment of denatured reovirus mRNAs with glyoxal, a dialdehyde that forms guanosine adducts and prevents G–C base pairing by steric hindrance (McMaster & Carmichael, 1977), caused the same relative change in apparent molecular weight as I substitution. Similarly, the mobilities of I-substituted Qβ RNA fragments in polyacrylamide gels were inversely related to their molecular weights, indicative of structureless, random coils (Mills & Kramer, 1979).

As a consequence of altering RNA structure, I substitution has striking functional effects on transcription and translation. Termination of transcription at bacterial attenuator sites was suppressed, and read-through products were synthesized almost exclusively by *E. coli* DNA-dependent RNA polymerase when ITP was used in place of GTP in vitro (Lee & Yanofsky, 1977). Similarly, Qβ replicase "pausing", resulting from the formation of hairpin structures at G–C-rich 3′ termini of nascent strands (Mills et al., 1978), was partially overcome by I substitution (D. Mills, unpublished experiments), and VSV transcription in vitro in the presence of ITP in place of GTP yielded full-length 42S plus strands (P. Chanda and A. K. Banerjee, unpublished experiments). In addition, priming of the influenza virion transcriptase in vitro was more effective with I-substituted reovirus mRNAs as compared to the corresponding G-containing mRNAs (Krug et al., 1980). As observed for ribosome binding (Figure 8C), the 5′-terminal residue also influenced flu priming, and I-substituted reovirus transcripts with m^7G caps were preferred over the corresponding RNAs with m^7I caps.

Although ITP can replace GTP for RNA chain elongation by Qβ replicase, *E. coli* DNA-dependent RNA polymerase, or VSV transcriptase in vitro, the analogue is not utilized by the phage enzyme at the level of initiation (Feix & Hake, 1975). In a previous study (Kozak, 1980), synthesis of I-substituted reovirus mRNA necessitated a two-step incubation, implying that the virion-associated transcriptase resembles the Qβ enzyme in requiring GTP for initiation. Although we also observed some of the properties reported for ITP-supported reovirus transcription, i.e., a decrease in overall rate and a preferential synthesis of s-size class products (Kozak, 1980), our findings indicate that the reovirus transcriptase can utilize ITP for initiation as well as elongation. They demonstrate further that the GTP analogue is used by other reovirion-associated activities, including guanylyl and methyl transferases, that are responsible for the formation of the mRNA 5′-terminal cap. Consequently, under appropriate conditions, m^7I-capped, I-substituted reovirus transcripts were synthesized (Figures 2 and 3). The mRNA guanylyl transferase recently purified from HeLa cell nuclei was also found to use ITP in place of GTP as donor in the capping reaction (Venkatesan & Moss, 1980).

Initiation of translation as measured by attachment to wheat germ ribosomes was effectively carried out by I-substituted transcripts (Kozak, 1980; Figure 6). However, polypeptide chain synthesis was 15-fold lower with I-substituted as compared to G-containing mRNAs (Table I). Furthermore, while normal, G-containing mRNAs bound one 40S ribosomal subunit per molecule of messenger, complexes formed by I-substituted RNA contained as many as 5–6 40S subunits per transcript (Kozak, 1980). Similarly, in wheat germ S23 extracts that contained both 40S and 60S ribosomal subunits, structures larger than 80S initiation complexes were also detected with I-substituted RNAs. These results were interpreted on the basis of a "scanning" mechanism of initiation (Kozak, 1978). That is, due to lack of secondary structure in I-substituted RNA, the initial 40S subunit or ribosome failed to stop at the 5′-proximal initiation site, allowing others to attach sequentially and scan or to form polysomes even in the presence of sparsomycin (Kozak, 1980). The enhanced ease of ribosome attachment to I-substituted RNAs is also indicated by a diminished sensitivity to the inhibitory effects of m^7GMP, GMPP(NH)P, and AMPP(NH)P (Figure 6) and an ability to outcompete G-substituted mRNAs in binding assays (Figure 7). The diminished dependence of denatured RNAs on the m^7G cap and on ATP hydrolysis for ribosome attachment suggests that an early step in initiation may be an unfolding of the mRNA 5′-terminal region, i.e., the ribosome binding site, possibly mediated by an ATP-dependent activity of a cap binding protein (Sonenberg et al., 1979, 1980).

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Purification, Subunit Structure, and Immunological Properties of Chromatin-Bound Ribonucleic Acid Polymerase I from Cauliflower Inflorescence†

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ABSTRACT: The large-scale purification of deoxyribonucleic acid (DNA) dependent ribonucleic acid (RNA) polymerase I from cauliflower inflorescence has been achieved following solubilization of the enzyme from the chromatin template. The initial step of purification of the enzyme by heparin–Sepharose chromatography at high ionic strength prevents reaggregation of chromatin proteins which can occur if salt concentrations are reduced. The enzyme was further purified by phosphocellulose, diethylaminoethyl (DEAE)-Sephadex, and DEAE-cellulose chromatography and glycerol density gradient centrifugation. The purified RNA polymerase I has a specific activity of 400–450 nmol of UMP incorporated into RNA in 20 min per mg of protein. From 1 kg of tissue, ~300 μg of purified enzyme is obtained with an overall yield of >30%. The purification of the enzyme to homogeneity takes about 4–5 days. The overall purification is ~10000-fold from total tissue and ~250-fold from isolated chromatin. Cauliflower RNA polymerase I purified by this procedure consists of seven polypeptides of 190000, 170000, 125000, 38000, 25000, 22000, and 17500 daltons as determined by one- (dodecyl sulfate) and two- (8 M urea, pH 8.7, followed by dodecyl sulfate) dimensional polyacrylamide gel electrophoresis. Polypeptides of 125000, 38000, and 22000 have stoichiometries of ~1, the 25000 and 17500 subunits have stoichiometries of 1.5–2, and the 190000 plus 170000 polypeptides sum to a stoichiometry of 1. The 170000 polypeptide probably arises from the 190000 polypeptide by proteolysis during purification of the enzyme. Comparison of the subunit structure of cauliflower RNA polymerase I with cauliflower RNA polymerases II and III by one- and two-dimensional polyacrylamide gel electrophoresis indicates that three polypeptides of 25000, 22000, and 17500 daltons associated with each class of enzyme have identical mobilities, and this suggests that there is a common pool of low molecular weight subunits in RNA polymerases I, II, and III. By use of immunological methods [Renart, J., Reiser, J., & Stark, G. R. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3116–3120], it is shown that the three putative common subunits as well as the 38000 subunit of RNA polymerase I cross-react with antibodies raised against cauliflower RNA polymerase II.

Eukaryotic cells contain three distinct classes of nuclear DNA[1]-dependent RNA polymerases which are generally referred to as RNA polymerases I, II, and III. Each class of RNA polymerase has characteristic chromatographic and catalytic properties, transcriptional functions, intracellular localizations, and subunit structures [reviewed by Roeder (1979)]. RNA polymerase I is localized in the nucleolus and transcribes ribosomal DNA. RNA polymerase II is found in the nucleoplasm and catalyzes the synthesis of precursors to messenger RNA. RNA polymerase III is also localized in the nucleoplasm and transcribes sequences coding for 5S ribosomal RNA, precursors to transfer RNA, and some other small RNAs. Each class of enzyme has a characteristic sensitivity to inhibition by the fungal toxin, α-amanitin. While RNA polymerase I in higher eukaryotes is refractory to α-amanitin, RNA polymerases II and III are inhibited by 50% at about 0.01–0.05 μg/mL and 10–20 μg/mL α-amanitin, respectively. In some cases, however, RNA polymerase III is inhibited only at concentrations in the range of 1–2 mg/mL α-amanitin (Guilfoyle, 1976; Renart & Sebastian, 1976) or is refractory to the toxin (Sklar et al., 1976), and in yeast, RNA polymerase I is inhibited by α-amanitin (Schultz & Hall, 1976; Valenzuela et al., 1976b). Although each class of RNA polymerase has a distinct subunit structure, certain of the subunits appear to be common to RNA polymerases I, II, and III (Buhler et al., 1976; Valenzuela et al., 1976a; Roeder, 1976; D'Alessio et al., 1979) as judged by one- and two-dimensional polyacrylamide gel electrophoresis. In yeast, genetic data (Thonart et al.,

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[1] Abbreviations used: ABM, aminobenzyloxymethyl; DBM, diazobenzyloxymethyl; Tris, tris(hydroxymethyl)aminomethane; $NaDodSO_4$, sodium dodecyl sulfate; DNA, deoxyribonucleic acid; RNA, ribonucleic acid; UMP, uridine 5′-phosphate.

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