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Antibody–Nucleic Acid Complexes. Inhibition of Translation of Silkmoth Chorion Messenger Ribonucleic Acid with Antibodies Specific for 7-Methylguanosine†

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ABSTRACT: Antibodies specific for 7-methylguanosine (m^7G) were evaluated for their ability to inhibit the translation of chorion mRNA in a wheat germ, cell-free amino acid incorporating system. Results obtained with antibody concentrations of 0.5–1.5 μM revealed dose-dependent inhibition of [^{3}H]-labeled amino acid incorporation into acid-insoluble radioactivity. Inhibition of translation was attributed to the interaction of anti-m^7G antibodies with the 5′ termini of chorion mRNAs on the basis that (a) anti-m^7G antibodies coupled to Sepharose (anti-m^7G–Sepharose) immunospecifically retained 5′-terminal cap structures of chorion mRNAs, i.e., m^7G (5′)ppp(5′)N^m, (b) significant inhibition of translation required a 2-h preincubation of anti-m^7G antibodies with mRNA, and (c) similar preincubation periods with anti-m^7G antibodies in the presence of the competing nucleoside hapten (m^7G) obviated the inhibitory effect of the antibody. The nature of the anti-m^7G antibody–mRNA complex was examined by digesting chorion mRNA with nuclease P_1 before (predigested) and after (postdigested) immunospecific adsorption to anti-m^7G–Sepharose adsorbent. Whereas predigested preparations yielded a single cap structure of the type m^7G(5′)ppp(5′)N, the predominating cap in the postdigested sample was m^7G(5′)ppp(5′)NpNpN. These latter data revealed that the nucleotide sequence adjacent to the cap was not significantly masked by the antibody and suggest the utility of anti-m^7G antibody as a site-specific probe.

The research described herein was prompted by numerous observations which have revealed the necessity of m^7G-containing[1] 5′-terminal oligonucleotide "caps" [m^7G(5′)-ppp(5′)N^m or m^7G(5′)ppp(5′)N^mpN^m] for the efficient in vitro translation of mRNAs (Shatkin, 1976; Revel & Groner, 1978). Initially, Both et al. (1975a,b) observed that the in vitro translation (wheat germ) of unmethylated, yet capped viral mRNA was significantly impaired when contrasted to control, m^7G-capped mRNA preparations. Decreased translational efficiencies were also observed with other mRNA preparations which had been chemically (Muthukrisnan et al., 1975; Kemper, 1976) or enzymatically (Shimotohno et al., 1977) treated for specific removal of the m^7G residue in their cap structures. The inability of these unmethylated or decapped mRNAs to interact with 40S ribosomal subunits further suggested that 5′-terminal caps provide a recognition signal for subsequent binding of mRNA to the ribosome (Both et al., 1975b), presumably by interacting with one or more initiation factors (Revel & Groner, 1978). Additional support for such a translational function has been provided by the finding that cap analogues inhibited the translation of capped mRNAs as well as their binding to ribosomes (Hickey et al., 1976; Weber et al., 1978). Controversy, however, persists as to the relative importance of the cap in translation. Thus, the degree of inhibition observed with uncapped mRNA or with cap analogues was found to be influenced by the concentrations of potassium ions (Weber et al., 1978; Chu & Rhoads, 1978) as well as the type of in vitro translational system employed (Lodish & Rose, 1977).

In view of the above and other findings which indicate the ability of selected antibody populations to immunospecifically recognize methylated nucleosides (Erlanger & Beiser, 1964; Levine et al., 1971; Munns et al., 1977a), we became interested in examining if antibodies specific for 7-methylguanosine (i.e., anti-m^7G antibodies) would inhibit the in vitro translation of chorion mRNA. Besides investigating the effects of anti-m^7G antibodies on in vitro translation, we conducted additional experiments to evaluate (a) the ability of anti-m^7G–Sepharose adsorbents to retain m^7G-containing caps derived from RNase T_2 digests of chorion mRNA and (b) the nature of the

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[1] Abbreviations used: 7MeGua, m^7G, and pm^7G represent the base, nucleoside, and 5′-phosphate of 7-methylguanosine, respectively; 6MeAde, m^6A, and pm^6A represent the base, nucleoside, and 5′-phosphate of N^6-methyladenosine, respectively; 6,6Me_2Ade, N^6,N^6-dimethyladenine; N, nucleoside; N^m, 2′-*O*-methyl nucleoside; anti-m^7G antibody, anti-7-methylguanosine antibody; anti-m^7G–Sepharose, anti-m^7G antibody coupled to Sepharose; Cl_3AcOH, trichloroacetic acid; RNase, ribonuclease; $NaDodSO_4$, sodium dodecyl sulfate.

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anti-m^{7}G antibody–mRNA complex. These investigations suggest the feasibility of an immunochemical approach for assessing the functional significance of m^{7}G-containing caps present in eucaryotic mRNA preparations as well as other methylated nucleosides present in various nucleic acid populations.

Experimental Procedures

Processing of Follicular Epithelial Cells: mRNA Purification. Chorionating follicles from pupae of *Antheraea polyphemus* were dissected and staged according to established procedures (Paul et al., 1972). Initially, whole follicles (120–170) were disrupted in 2–3 mL of homogenizing buffer (HB; 500 mM KCl, 50 mM $MgCl_2$, and 50 mM Tris, pH 7.6, containing 2% Triton X-100 and 1 mg/mL of sodium heparin) with a mortar and pestle. This homogenate was subsequently diluted with HB (20 follicles/mL) and further disrupted in a Dounce homogenizer by five strokes with a loose-fitting pestle and by two strokes with a tight-fitting pestle. Polysomes in the above homogenates were isolated by centrifugation (SW41; 180 000*g*; 200 min) through a discontinuous sucrose gradient containing 2 mL of 20% sucrose (w/v) layered above 3 mL of 40% sucrose (sucrose solutions contained 100 mM KCl, 10 mM $MgCl_2$, and 10 mM Tris, pH 7.5). All of the above procedures were conducted at 0–4 °C.

Polysomal pellets were solubilized with TES buffer (100 mM NaCl, 1 mM EDTA, and 10 mM Tris, pH 7.5) containing 2% $NaDodSO_4$ and layered over linear sucrose gradients (15–30%) buffered in TES (plus 0.5% $NaDodSO_4$). Conditions of centrifugation and gradient fractionation for isolation of chorion mRNA sedimenting between 4S and 18S RNA are described in the legend of Figure 1.

To obtain [*methyl*-^{3}H]methionine-labeled chorion mRNA, we incubated chorionating follicles in Grace's medium (Grace, 1962) without hemolymph, antibiotics, and unlabeled methionine for 2 h at 24 °C in the presence of 5 mCi of [*methyl*-^{3}H]methionine (9 Ci/mmol). Yields of chorion mRNA approximated 5–8 μg/150 follicles and after 2 h of in vitro labeling possessed specific activities of 4000–6000 cpm/μg.

Methylated Nucleotide Analysis. Identification of radioactive methylated nucleotides has been described in detail by Munns et al. (1974). Briefly, these methods consisted of hydrolyzing oligonucleotide and mRNA preparations with 88% formic acid for 2 h at temperatures of 100 and 185 °C and separating the resulting methylated bases via single- or two-dimensional thin-layer chromatography on precoated cellulose plates (Munns et al., 1973). The employment of both low (100 °C) and high (185 °C) temperatures for hydrolysis also permitted a collective estimate of 2′-*O*-methylribose constituents (designated N^m). The basis of this estimate was derived from previous findings which indicated that the 2′-*O*-[*methyl*-^{3}H]methyl group of both purine and pyrimidine nucleotides was converted into a volatile product (methanol) during formic acid hydrolysis at 185 °C. At a temperature of 100 °C, however, it remains attached to nonvolatile, ribose phosphate products which do not migrate from the origin during subsequent thin-layer chromatographic development (Munns et al., 1974; also see Figure 2). High-temperature formic acid hydrolysis was conducted in sealed Pyrex tubes (Munns et al., 1974). Unlabeled carrier tRNA (25–50 μg) and various methylated nucleoside standards were hydrolyzed with radioactive RNA samples.

Chorion mRNA Translation. Wheat germ extracts were prepared by published procedures (Marcu & Dudock, 1974). The in vitro translational system contained the following in 50 μL: 15 μL of wheat germ extract; 1.0 mM ATP; 20 μM GTP; 8 mM creatine phosphate; 17.5 μg of creatine phosphokinase; 20 mM potassium *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonate, pH 7.0; 3 mM magnesium acetate; KCl to give 100 mM K^+; 2 mM dithiothreitol; 50 μM unlabeled amino acids minus valine, leucine, and glycine. The latter amino acids were added at 8.9 μM [2,3-^{3}H]valine (11.2 Ci/mmol), 6.7 μM [4,5-^{3}H]leucine (59.5 Ci/mmol), and 26.6 μM [2-^{3}H]glycine (15.5 Ci/mmol). Chorion mRNA and antibodies were added at the concentrations listed in the legend of Figure 3. Except where noted, antibodies were preincubated in the presence of chorion mRNA for 2 h at 0 °C in 15 μL of 15 mM NaCl and 10 mM phosphate, pH 7.5. This mixture was added to the remainder of the wheat germ system at 0 °C, and 5 μL-aliquots were taken before incubating at 25 °C for an additional 1 h. Additional 5-μL aliquots were withdrawn at selected times throughout the entire incubation period and processed for determination of trichloroacetic acid (Cl_3AcOH) precipitable radioactivity.

Immunochemistry. The production, purification, and characterization of rabbit anti-m^{7}G antibodies have been described in detail (Munns et al., 1977a) as has the isolation of m^{7}G-containing oligonucleotides by affinity chromatographic techniques (Munns et al., 1977b) employing anti-m^{7}G antibody coupled Sepharose adsorbents (anti-m^{7}G–Sepharose). To assess immunospecific retention of m^{7}G-containing caps, [*methyl*-^{3}H]methionine-labeled chorion mRNA was digested with ribonuclease T_2 (RNase T_2; Sigma), and aliquots of this digest (0.2 mL) were diluted to 2.0 mL in phosphate-buffered saline (PBS; 150 mM NaCl and 10 mM phosphate, pH 7.4). These aliquots were incubated in the presence of 0.1 mL of anti-m^{7}G–Sepharose adsorbent (wet-weight volume) with gentle rotation for 30 min at 24 °C. For removal of nonretained radioactivity, the immunoadsorbent was repeatedly washed by low-speed centrifugation (200*g*) and resuspended in 1.0–2.0 mL of PBS. For removal of immunospecifically retained radioactivity, the adsorbent was washed once in 10 mM phosphate, pH 7.4, and then resuspended and incubated in 1.0 mL of 7 M urea containing 1 mg/mL m^{7}G for 30 min at 24 °C prior to centrifugation and recovery of the supernatant (retained fraction). RNase T_2 digests (both unfractionated and immunospecifically retained) were characterized by DEAE-Sephacel chromatography (Figure 3).

Similar adsorption studies with anti-m^{7}G–Sepharose were conducted with intact and nuclease P_1 digested chorion mRNA preparations. The buffer employed in these latter adsorptions was 150 mM NaCl and 40 mM sodium acetate, pH 6.0 (see legends of Figures 5 and 6 for additional details).

For removal of residual nuclease activity, all antibody preparations were subjected to a final chromatographic step (DEAE- and CM-cellulose) as outlined by Palacios et al. (1972). Antibody preparations were judged free of nuclease contamination as evaluated by $NaDodSO_4$–sucrose gradient sedimentation analysis of [5-^{3}H]uridine-labeled 28S rRNA (isolated from mammalian KB cells) after incubation of this rRNA (1–5 μg) with antibody (10–500 μg) for 2 h at 0 °C. Additional evidence for the exclusion of nucleases was provided by the finding that the in vitro translation of chorion mRNA was unaffected by a 2-h preincubation of mRNA with anti-m^{7}G antibody and the competing nucleoside hapten m^{7}G (1 μg/assay; see Table II).

Results

Prior to assessment of the ability of anti-m^{7}G antibodies to inhibit immunospecifically the in vitro translation of chorion mRNA, various criteria had to be established. First, it was

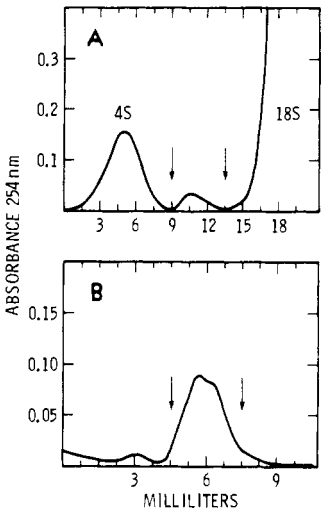

FIGURE 1: Purification of chorion mRNA via sucrose gradient centrifugation. Chorion mRNA, solubilized by treatment of polysomes with 2% $NaDodSO_4$, was separated from the bulk of tRNA (4 S) and rRNA (18 S) by sedimentation in linear sucrose gradients (15–30%). Panel A depicts the resulting absorbance profile (upper half of gradient) after centrifugation (SW27; 27 000 rpm; 21 h at 24 °C). Arrows designate that fraction of the gradient used to recover (ethanol precipitation) chorion mRNA. To remove traces of tRNA and/or rRNA contaminants, chorion mRNA was resedimented in a second linear sucrose gradient (15–30% sucrose; SW41; 40 000 rpm; 12 h at 24 °C) as depicted by the absorbance profile in panel B.

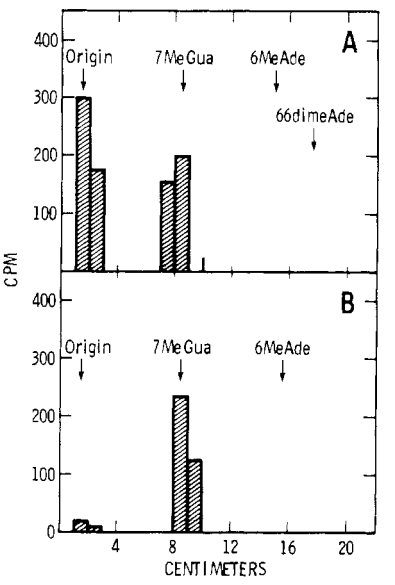

FIGURE 2: Identification of the [*methyl*-^{3}H]methylated nucleotides present in chorion mRNA previously isolated from follicles labeled in organ cultured with [*methyl*-^{3}H]methionine. Chorion mRNA was hydrolyzed in 88% formic acid at 100 and 185 °C, and the resulting products were chromatographed on cellulose thin-layer plates (solvent system: acetonitrile–ethyl acetate–2-propanol–1-butanol–ammonium hydroxide, 40:30:20:10:27). After development, plates were air-dried and the radioactivity in 1-cm sections was determined. Arrows mark the position of some of the standard bases cochromatographed with mRNA digests hydrolyzed at 100 (A) and 185 °C (B).

necessary for chorion mRNA to be characterized with regard to its content of [*methyl*-^{3}H]methylated nucleotide constituents to determine the presence of m^7G-containing, 5′-terminal cap structures. Second, immunochemical procedures had to be implemented to determine if anti-m^7G antibodies would immunospecifically interact with m^7G-containing caps. Third, the design of in vitro experiments would have to distinguish between the inhibition of mRNA translation that resulted from (a) the specific interaction of anti-m^7G antibodies with 5′-terminal caps of mRNA and (b) the potential interaction of anti-m^7G antibodies with other m^7G-containing RNAs endogenous to the wheat germ system, e.g., tRNAs and rRNAs. Lastly, if antibody-dependent inhibition was observed, the nature of the antibody–mRNA complex would have to be evaluated to determine whether the antibody (molecular weight 150 000) was masking other nucleotide sequences essential for translation, i.e., was the antibody performing as a site-specific probe.

Isolation and Characterization of Chorion mRNA. Sucrose gradient sedimentation techniques were employed for the isolation of chorion mRNA present in the polysomes of follicular epithelial cells. Isolated polysomes were solubilized with $NaDodSO_4$, and the chorion mRNAs (7 S–14 S) were separated from tRNAs (4 S) and rRNA (18 S) by sedimentation through linear sucrose gradients. The effectiveness of this gradient fractionation procedure is depicted in Figure 1. As indicated by these absorbance profiles, a second gradient centrifugation step (Figure 1B) was required to remove residual tRNA and rRNA contaminants. Similar profiles were obtained with follicles previously incubated in organ culture for 2 h with [*methyl*-^{3}H]methionine, thus indicating that the in vitro labeling conditions employed did not promote significant RNA degradation.

Aliquots of these latter mRNA preparations were used to determine the type and amount of [*methyl*-^{3}H]methylated nucleotide constituents present in chorion mRNA. Illustrated in Figure 2 are the radioactive profiles resulting from thin-layer chromatography of chorion mRNA preparations previously hydrolyzed in formic acid. Whereas two discrete peaks of radioactivity were observed after chromatography of the 100 °C hydrolysate (Figure 2A), only the radioactive peak coincident with the 7-methylguanine (7MeGua) standard was present in the 185 °C hydrolysate (Figure 2B). These data are consistent with the volatile nature of the 2′-*O*-methyl groups of nucleotides in formic acid at 185 °C (Munns et al., 1974) and indicate that [*methyl*-^{3}H]methionine-labeled chorion mRNA contains approximately equal quantities of [*methyl*-^{3}H]7MeGua (45%) and [*methyl*-^{3}H]N^m constituents (55%), where N^m reflects a collective estimate of 2′-*O*-methyl nucleosides. These data were substantiated by analysis of the radioactivity elution profiles derived from DEAE-cellulose chromatography of RNase-T_2 digested mRNA (see Figure 3A).

Immunochemical Characterization of 5′-Terminal Caps of Chorion mRNA. To determine whether anti-m^7G antibodies would immunospecifically interact with m^7G-containing caps, we immobilized antibodies on Sepharose (anti-m^7G–Sepharose), and the resulting immunoadsorbent was tested for its ability to retain [*methyl*-^{3}H]m^7G-containing cap structures of chorion mRNA. This was accomplished by digesting [*methyl*-^{3}H]methionine-labeled mRNA with RNase T_2 and incubating aliquots of this digest with anti-m^7G–Sepharose (30 min, 24 °C). At the conclusion of this incubation period the adsorbent was washed extensively before eluting the immunospecifically retained radioactivity with 7 M urea (containing 1 mg/mL m^7G). Results from these studies are presented in Table I and indicated that approximately 90% of the radioactive mRNA digest was retained by anti-m^7G–Sepharose. To determine whether the immunospecifically retained radioactivity was dependent upon the presence of m^7G, we conducted identical adsorption studies in the presence of two synthetic cap structures, i.e., $m^7G(5')ppp(5')A$ and $G(5')ppp(5')G$. These results are also presented in Table I and revealed that less than 1% of the radioactivity present in an RNase T_2 digest of chorion mRNA was retained when the

Table I: Immunospecific Retention of m^{7}G-Containing Oligonucleotides from RNase T_2 Digests of [*methyl*-^{3}H]mRNA by Anti-m^{7}G–Sepharose Adsorbent. Effects of Competing Haptens m^{7}G(5′)ppp(5′)A and G(5′)ppp(5′)G[a]

incubn mixture	radioact. incubated with adsorbent (cpm)	radioact. retained by adsorbent (cpm)	% retained
	Expt 1		
mRNA	2900	2540	87.6
mRNA + m^{7}G(5′)ppp(5′)A	2900	<25	<1.0
mRNA + G(5′)ppp(5′)G	2900	2630	90.7
	Expt 2		
mRNA	1200	1040	86.7
mRNA + m^{7}G(5′)ppp(5′)A	1200	<10	<1.0
mRNA + G(5′)ppp(5′)G	1200	1070	89.2

[a] Procedures employed for determination of these data are as described under Experimental Procedures. Values represent the average of duplicate determinations. The amount of hapten added to each incubation was 10 μg and represented a greater than 1000-fold molar excess of synthetic caps relative to the amount of caps present in chorion mRNA.

adsorption was conducted in the presence of 10 μg of m^{7}G(5′)ppp(5′)A. The presence of an equal amount of G(5′)ppp(5′)G, however, was ineffective as a competing hapten and thus indicated no significant cross-reactivity between anti-m^{7}G antibodies and G. These results are in accord with the specificity of anti-m^{7}G antibodies (Levine et al., 1971; Munns et al., 1977a).

Although the above investigation documented that the adsorption process was immunospecific (rather than nonspecific), it provided no information as to the nature of the radioactive products retained by anti-m^{7}G–Sepharose. This was accomplished by characterizing RNase T_2 digests of [*methyl*-^{3}H]mRNA via DEAE-cellulose chromatography. These results are presented in Figure 3 and revealed that both unfractionated (Figure 3A) and immunospecifically retained fractions (Figure 3B) possessed [*methyl*-^{3}H]-labeled cap structures as evidenced by the elution of radioactivity possessing a net charge of −6 and −7 (relative to adenosine nucleotide standards). Although not extensively characterized, treatment of these cap structures with pyrophosphatase has yielded [*methyl*-^{3}H]pm^{7}G and other [*methyl*-^{3}H]-labeled oligonucleotides as determined by thin-layer chromatographic

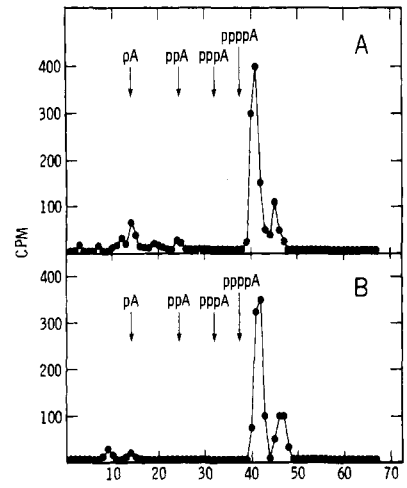

FIGURE 3: Characterization of [*methyl*-^{3}H]-labeled mono- and/or oligonucleotides derived from RNase T_2 digests of chorion mRNA and retained by anti-m^{7}G–Sepharose. Elution profiles of radioactivity resulting from DEAE-cellulose chromatography of unfractionated (A) and immunospecifically retained fractions (B) of RNase T_2 digest of [*methyl*-^{3}H]methionine-labeled mRNA. Gradient elution was achieved with 100 mL of 7 M urea, 0.03 M NaCl, and 0.025 M Tris, pH 7.8, in the mixing chamber and 100 mL of 7 M urea, 0.280 M NaCl, and 0.025 M Tris, pH 7.8, in the reservoir. Each fraction contained 3.0 mL of effluent, and upon conclusion of gradient elution the column (1 × 15 cm) was eluted with 20 mL of 1.0 M NaCl (no radioactivity was detected in this latter fraction). The mono- (−2), di- (−3), tri- (−4), and tetraphosphates (−5) of adenosine were included as the reference standards.

techniques (Munns, unpublished experiments). These results are consistent with previously published data regarding the structure of caps present in mammalian mRNA preparations. Also important was the observation that the immunoadsorbent quantitatively and exclusively retained m^{7}G-containing caps from RNase T_2 digests. This was provided by the finding that equal amounts of radioactivity were associated with the [*methyl*-^{3}H]-labeled caps of unfractionated as well as immunospecifically retained digests (each RNase T_2 digest represented 0.25 μg of chorion mRNA). The lack of appreciable quantities of radioactivity migrating as mononucleotides (−2; i.e., pA), as well as the inability to detect [*methyl*-^{3}H]6MeAde by formic acid hydrolysis procedures (Figure 2), indicated the absence of internal m^{6}A residues in these chorion mRNA preparations.

Inhibition of Chorion mRNA by Anti-m^{7}G Antibodies. In view of the above findings, studies were initiated to assess the ability of these antibodies to inhibit the in vitro translation

Table II: Inhibition of Translation of Chorion mRNA by Anti-m^{7}G Antibody. Effects of Antibody Preincubations with mRNA. Effects of the Competing Hapten m^{7}G[a]

preincubn mixture	preincubn time (h)	Cl_3AcOH-precipitable radioact. cpm (% of control) expt 1	Cl_3AcOH-precipitable radioact. cpm (% of control) expt 2	inhibn (%) expt 1	inhibn (%) expt 2
mRNA (control)	0	37 300 (100)	53 900 (100)		
mRNA	2	36 800 (98.6)	52 700 (97.8)	<5.0	<5.0
mRNA + antibody	0	31 900 (85.5)	48 200 (89.4)	14.5	10.6
mRNA + antibody	2	16 400 (44.0)	27 300 (50.6)	56.0	49.4
mRNA (control)	2	48 500 (100)	71 300 (100)		
mRNA + m^{7}G	2	46 100 (95.1)	72 900 (102)	<5.0	<5.0
mRNA + m^{7}G + antibody	2	47 600 (98.1)	68 800 (96.5)	<5.0	<5.0
mRNA + antibody	2	25 200 (52.0)	32 500 (45.6)	48.0	54.4

[a] Chorion mRNA (1.5 μg/assay) was preincubated alone, with anti-m^{7}G antibody (7.5 μg), and with anti-m^{7}G antibody plus m^{7}G (1.0 μg) for 0 and 2 h at 0 °C prior to their addition into a wheat germ translational system. Incubations were conducted at 25 °C for 60 min (50 μL/assay), and aliquots (5 μL) were withdrawn at selected time intervals and processed for determination of trichloroacetic acid (Cl_3AcOH) precipitable radioactivity (for additional details, see Experimental Procedures). Values presented were derived from two independent experiments with each value representing the mean of duplicate determinations after a 60-min translation period.

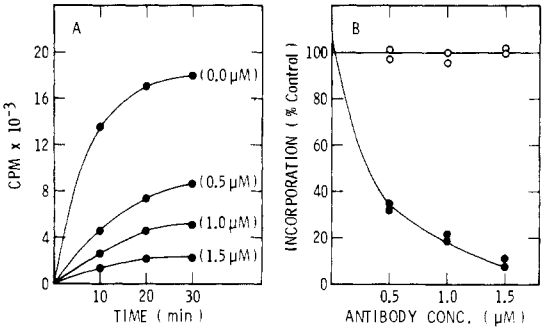

FIGURE 4: Effects of anti-m^{7}G antibodies on the in vitro translation of chorion mRNA. Kinetic and dose-response parameters. Chorion mRNA (0.2 μg) was preincubated in the presence of increasing amounts of antibody (1.5, 3.0, and 4.5 μg/20 μL or 0.5, 1.0, and 1.5 μM) for 2 h at 0 °C prior to addition into the wheat germ translational system. The kinetics of incorporation of [^{3}H]amino acids into Cl_3AcOH-precipitable protein is presented in panel A. Panel B depicts the ability of increasing concentrations of antibody to inhibit the incorporation of [^{3}H]-labeled amino acids into protein after a 10-min incubation. Open circles represent incorporation in the presence of 1 μg of competing nucleoside hapten (m^{7}G).

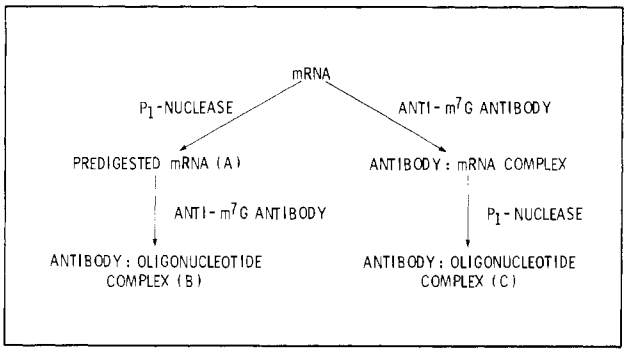

FIGURE 5: Schematic of the protocol employed to assess the nature of the anti-m^{7}G antibody–chorion mRNA complex.

of chorion mRNA. To reduce the potential interaction of anti-m^{7}G antibodies with endogenous m^{7}G-containing RNAs (tRNAs and rRNA) and to enhance antibody interaction with chorion mRNA, we preincubated antibodies with chorion mRNA for 2 h as well as added them directly to the wheat germ system at the onset of translation, i.e., a 0-h preincubation period. Similar assays employing identical preincubation periods with chorion mRNA alone, with m^{7}G, or with anti-m^{7}G antibody plus m^{7}G provided the appropriate controls. The results of these experiments are presented in Table II and revealed that the translation of chorion mRNA was inhibited significantly by anti-m^{7}G antibody only after a 2-h preincubation period; i.e., translation was inhibited to an extent of 50–55% with a 2-h preincubation period, yet only to an extent of 10–15% after a 0-h preincubation. Whereas the inhibition observed in the latter instance could be attributed to the interaction of anti-m^{7}G antibodies with any m^{7}G-containing RNAs in the wheat germ system (including both endogenous tRNAs and rRNA and exogenous chorion mRNA), the *difference* in inhibition observed between the 2- and 0-h preincubation (approximately 40%) was attributed to the specific interaction of anti-m^{7}G antibody with chorion mRNA.

Similar preincubation periods of mRNA with m^{7}G or with anti-m^{7}G antibody plus m^{7}G (1 μg/assay) had little or no effect on subsequent translation. These results were expected in view of the findings reported by Hickey et al. (1976) indicating that m^{7}G did not inhibit the in vitro translation of globin mRNA. However, the introduction of this competing hapten (m^{7}G) into these experiments served several purposes. First, it confirmed the absence of nuclease contamination in these antibody preparations, and second, it provided additional evidence that the antibody-dependent inhibition of chorion mRNA translation was immunospecific.

That the translation of chorion mRNA was inhibited throughout a 30-min incubation period was provided by the findings presented in Figure 4A. These data demonstrated that preincubation (2 h) of anti-m^{7}G antibody (1.5 μM) with mRNA (0.2 μg) resulted in 93, 90, and 89% inhibition of translation after 10-, 20-, and 30-min incubation periods, respectively. By use of increasing amounts of anti-m^{7}G antibody (0.50–1.5 μM) during the preincubation period, it was further observed that the inhibition of translation of chorion mRNA was dose dependent. These results are presented in Figure 4B and revealed that antibody concentrations of 0.5, 1.0, and 1.5 μM inhibited translation to an extent of 67, 80, and 90%, respectively. Additional control experiments indicated that translation of chorion mRNA (0.2 μg) was inhibited to an extent of 10% or less when equivalent amounts of antibody (1.5–4.5 μg) were added at the onset of translation (i.e., 0-h preincubation controls; see Table II). Noteworthy was the finding that the concentration of antibody required to achieve a 50% inhibition of chorion mRNA translation (ca. 0.3–1.0 μM, depending upon various conditions) was significantly less than that observed with the 5′-phosphate of m^{7}G (100–150 μM; Morrow and Hunsley, unpublished experiments). Since the average molecular weight of chorion mRNAs has been estimated to be approximately 150 000 (Gelinas & Kafatos, 1973), the data in Figure 4B indicate that approximately six antibody molecules were required per mRNA to inhibit the translation by 50%, i.e., a 6:1 molar ratio.

Anti-m^{7}G Antibodies as Site-Specific Probes. Although the above studies revealed the ability of anti-m^{7}G antibodies to interact with m^{7}G-containing caps of chorion mRNA, the distinct possibility existed that these antibodies were masking additional nucleotide sequences adjacent to 5′-terminal caps. To examine the nature of the antibody–mRNA complex, chorion mRNA was digested with nuclease P_1 prior to (predigested) and after (postdigested) its adsorption to anti-m^{7}G–Sepharose. This protocol assumed (illustrated in Figure 5) that if significant antibody-dependent masking was occurring, then an antibody–mRNA complex should inhibit or obviate the hydrolytic activity of nuclease P_1 (molecular weight 44 000) at or near the 5′ end of the mRNA. Furthermore, if masking did exist, the oligonucleotide obtained by immunospecific adsorption of predigested mRNA (complex B, 5) would be considerably smaller in size than the oligonucleotide(s) derived from digesting mRNA previously complexed with antibody (complex C, Figure 5).

Thus, two aliquots of [*methyl*-^{3}H]methionine-labeled chorion mRNA (one intact, the other digested with nuclease P_1; see Figure 5) were incubated with anti-m^{7}G–Sepharose (60 min; 0 °C) and the adsorbent was subsequently washed in a stepwise manner for removal of nonretained radioactivity. The elution of radioactivity from these adsorbents is illustrated in Figure 6 and indicated that 80 and 70% of the radioactivity present in nuclease P_1 digested mRNA (6A) and intact mRNA (6B), respectively, were retained by anti-m^{7}G–Sepharose. Nuclease P_1 digestion of immobilized mRNA (i.e., antibody–mRNA complex) released an additional 10–20% of this retained radioactivity (Figure 6B). Characterization of the oligonucleotide(s) retained by the immunoadsorbents via DEAE-cellulose chromatography (Figure 7) revealed that predigested preparations coeluted with the m^{7}G(5′)ppp(5′)A standard while the elution pattern obtained from postdigested

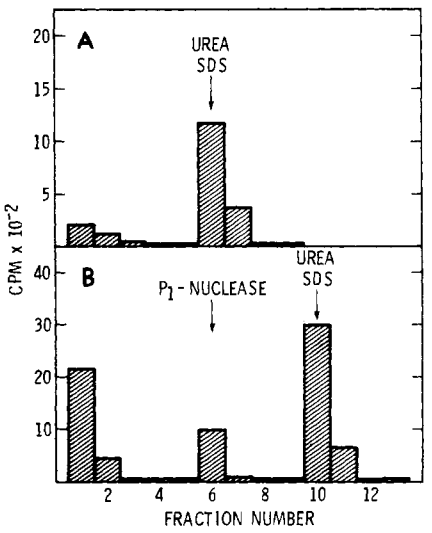

FIGURE 6: The elution of radioactivity from anti-m^{7}G–Sepharose adsorbent previously incubated (60 min at 0 °C) with nuclease P_1 digested (A) and intact [*methyl*-^{3}H]methionine-labeled chorion mRNA (B). Procedures for adsorption and elution of nuclease P_1 digested mRNA (panel A) are described under Experimental Procedures. Incubations and initial wash steps (fractions 1–5) were conducted with acetate-buffered saline (150 mM NaCl and 40 mM sodium acetate, pH 6.0). Panel B, fractions 1–5, represent the radioactivity eluted from anti-m^{7}G–Sepharose (1.0-mL washes with acetate-buffered saline) after incubating intact chorion mRNA with immunoadsorbent for 60 min at 0 °C. The mRNA immobilized by the adsorbent was digested with nuclease P_1 by resuspending the anti-m^{7}G–Sepharose in 0.5 mL of acetate-buffered saline containing 100 μg of nuclease P_1 (30 min at 24 °C). After additional wash steps (fractions 6–9), the immunospecifically retained radioactivity was removed by reincubating the adsorbent in the presence of urea and $NaDodSO_4$ (fractions 10–12, panel B).

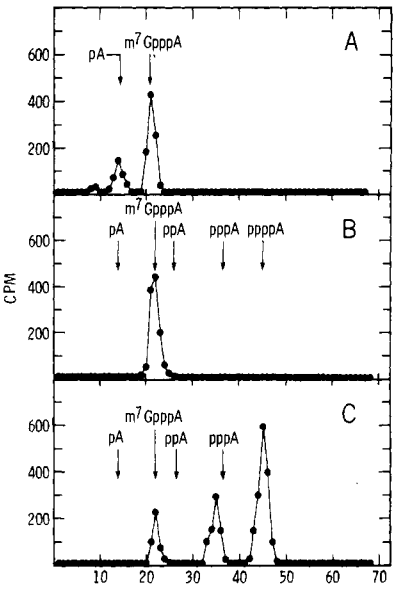

FIGURE 7: Characterization of [*methyl*-^{3}H]-labeled caps of chorion mRNA digested with nuclease P_1 before (predigested) and after (postdigested) adsorption to anti-m^{7}G–Sepharose. Elution profiles of radioactivity obtained by DEAE-cellulose chromatography of nuclease P_1 digested mRNA. Chromatography was conducted as described in the legend of Figure 3 with a linear salt gradient of 0.05–0.15 M NaCl. Panel A, unfractionated nuclease P_1 digested mRNA. Panel B, nuclease P_1 digested mRNA immunospecifically retained by anti-m^{7}G–Sepharose (material eluted from fractions 6 and 7 of Figure 6A, i.e., predigested preparation). Panel C, mRNA immunospecifically retained by anti-m^{7}G–Sepharose prior to nuclease P_1 treatment (material eluted from fractions 10 and 11 of Figure 6B, i.e., postdigested preparation).

samples (Figure 7C) indicated structures of the type m^{7}G(5′)ppp(5′)N (fractions 21–23), m^{7}G(5′)ppp(5′)NpN (fractions 33–36), and m^{7}G(5′)ppp(5′)NpNpN (fractions 43–47). The assignment of these structures was based upon their elution relative to adenosine nucleotide standards and a knowledge of the specificity of nuclease P_1.

Evidence to indicate that the latter two oligonucleotides were not the result of incomplete nuclease P_1 digestion was provided by the finding that chorion mRNA digested in an identical manner (yet not immunospecifically adsorbed) was completely degraded (Figure 7A). Additionally, DEAE-cellulose characterization (0.05–0.4 M NaCl) of the mRNA bound to anti-m^{7}G–Sepharose (subsequently eluted with 7 M urea containing 1% $NaDodSO_4$) indicated that it was not degraded during immunospecific adsorption; i.e., no radioactivity was eluted throughout this salt gradient range. Although not unequivocal, these results imply that the antibodies employed in the present investigation were behaving as site-specific probes; i.e., their ability to mask nucleotide sequences appears to be limited to a trinucleotide sequence adjacent to the triphosphate bridge. An almost identical pattern of 5′-terminal oligonucleotides has been observed by nuclease P_1 digestion of the adenovirus mRNA–anti-m^{7}G antibody complex (Munns, unpublished experiments).

Discussion and Conclusion

From the experimental results described herein it was determined that preincubation of anti-m^{7}G antibodies with chorion mRNA significantly inhibited mRNA translation. It was further concluded that this inhibition resulted from the interaction of anti-m^{7}G antibody with m^{7}G-containing cap structures present in chorion mRNA (Figures 3 and 7). This conclusion was based upon the ability of anti-m^{7}G–Sepharose adsorbents to immunospecifically retain [*methyl*-^{3}H]-labeled cap structures (Table I and Figure 3), to inhibit the translation of chorion mRNA only after a suitable preincubation period (Table II and Figure 4A), and to inhibit the translation of chorion mRNA in a dose-dependent manner (Figure 4B). Since other m^{7}G-containing RNA species participate in the translation of mRNA (e.g., tRNAs and rRNA), it was essential to discriminate between the interaction of anti-m^{7}G antibodies with these species and that of chorion mRNA. This was accomplished by assessing the degree to which chorion mRNA could be inhibited by various preincubation periods (Table II). Lastly, characterization of the 5′-terminal oligonucleotides resulting from nuclease P_1 digestion of chorion mRNA before and after adsorption to anti-m^{7}G–Sepharose revealed that the antibodies employed throughout this investigation were performing as site-specific probes (Figure 5–7).

Immunochemical Approaches for Assessing Structure–Function Relationships. Although these studies confirmed the importance of an m^{7}G-containing, 5′-terminal cap structure for the in vitro translation of mRNA in wheat germ extracts, the major emphasis of this research was directed toward assessing the use of such antibodies as immunochemical probes to (a) *detect* the presence of minor hapten constituents in various RNA and DNA populations, (b) *isolate* specific oligonucleotides possessing the corresponding haptens, and (c) *establish* their potential functional role(s) in nucleic acid metabolism. In regard to the latter, if one assumes that various cellular processes (replication, transcription, posttranscriptional processing, and translation) are dependent upon the presence of modified constituents in nucleic acids, it would seem logical to conclude that the interaction between these constituents and their corresponding antibodies would inhibit or negate such processes. Thus, the immunospecific interaction between an

antibody (anti-m^{7}G) and a corresponding hapten-containing nucleic acid (chorion mRNA) should provide interesting and relevant data regarding the biological function (translation) of the hapten component.

Studies are now in progress to assess the in vitro translation of m^{6}A-containing mRNA in the presence of anti-m^{6}A antibodies. It is anticipated that these results may provide some insight into the location of m^{6}A within these mRNAs.

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High-Yield Cleavage of Tryptophanyl Peptide Bonds by *o*-Iodosobenzoic Acid†

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ABSTRACT: A new procedure to cleave tryptophanyl peptide bonds in high yield is reported. The method involves treatment of the S-alkylated protein with *o*-iodosobenzoic acid. The procedure is highly selective for tryptophan and does not modify tyrosine or histidine, but may convert methionine to its sulfoxide derivative. The yields in the cleavage are 70–100%. Tryptophanyl bonds to alanine, glycine, serine, threonine, glutamine, arginine, and *S*-(pyridylethyl)cysteine are split in nearly quantitative yield, while those preceding isoleucine or valine are split in ~70% yield in the proteins examined in this work. The chemical mechanism for tryptophanyl bond cleavage has not been defined, but it is likely that oxidation of the indole ring occurs during the reaction with *o*-iodosobenzoic acid. Some problems with the quality of commercial preparations of the reagent are discussed.

Automatic amino acid sequence analysis depends upon efficient procedures for the selective chemical or enzymatic cleavage of polypeptide chains in order to generate a limited number of large fragments. Many procedures have been devised to effect protein cleavage at various amino acid residues [see review by Spande et al. (1970)]. However, the number of such procedures that are useful in protein structure work is quite limited since many procedures suffer from lack of specificity, low yields of cleavage, or incompatibility of the reaction conditions with polypeptide solubility. Frequently, results obtained on model compounds and small peptides do not extend to proteins.

Residues that are infrequent in most proteins (e.g., Trp, Met, Arg) are the best candidates for cleavage sites. The digestion of methionyl bonds with cyanogen bromide provides the best example of a near quantitative and highly selective procedure. Several methods have been proposed to cleave polypeptide chains at tryptophanyl bonds (Konigsberg & Steinman, 1977; Shechter et al., 1976; Ozols & Gerard, 1977; Sakiyama, 1977; Savige & Fontana, 1977b). Most of these procedures have involved either oxidative bromination or oxidative chlorination. However, nonselective oxidation of many residues and low yields of cleavage have caused these methods to be of limited utility.

The best available method and the one most widely used for cleaving at tryptophan in polypeptides utilizes BNPS-skatole[1] (Omenn et al., 1970). After cysteine is reduced and

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