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Antibody·Nucleic Acid Complexes. Immunospecific Retention of Globin Messenger Ribonucleic Acid with Antibodies Specific for 7-Methylguanosine†

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ABSTRACT: Antibodies specific for 7-methylguanosine (m^7G) were immobilized to Sepharose, and this adsorbent (anti-m^7G/Sepharose) was tested for its ability to select for globin mRNA on the basis of its 5′-terminal, m^7G-containing cap, i.e., $m^7G(5')ppp(5')N$. Preliminary studies with [^{3}H]m^7G indicated that the binding of ^{3}H-labeled hapten to immobilized antibody was (i) essentially complete within 30 min, (ii) not affected significantly by variations in pH (5.0–9.0), temperature (0–56 °C), or salt concentrations (0.01–1.0 M NaCl), and (iii) specific for m^7G-containing (oligo)nucleotides with no detectable cross-reactivity toward comparable unmethylated (oligo)nucleotides. Estimates based upon the amount of active antibody coupled to Sepharose (0.15 $\mu g/\mu L$) and its affinity constant ($K_a = 5 \times 10^7$ M^{-1}) revealed that 50 μL of anti-m^7G/Sepharose was sufficient to quantitatively bind the equivalent of 5 μg of globin mRNA, a value in excellent agreement with that experimentally obtained. In assessment of the immunospecific selection of mRNA, rabbit globin mRNA was specifically labeled at each terminus via periodate oxidation and NaB^3H_4 reduction. Upon recovery of oligo(dT)–cellulose, the ^{3}H-labeled mononucleotides derived from enzymatic digestion (P_1 nuclease and tobacco acid pyrophosphatase) of ^{3}H-labeled globin mRNA yielded a [^{3}H]-pm^7G:[^{3}H]pA ratio of 0.41. This result indicated the presence of 3′-terminal fragments of mRNA generated during the labeling reaction and enriched during recovery of oligo(dT)–cellulose chromatography. However, when incubated with anti-m^7G/Sepharose, 63% of the poly(A)-containing ^{3}H-labeled globin mRNA was immunospecifically retained and possessed a [^{3}H]pm^7G:[^{3}H]pA ratio of 0.95 in contrast to a 0.15 ratio for the nonretained preparation. Last, potential degradation of immunospecifically retained, unlabeled globin mRNA was not evident as evaluated by analysis of the resulting in vitro translational product, i.e., rabbit globin (ca. 15 000 molecular weight). Moreover, kinetic data from the latter experiments demonstrated that the translation of m^7G-selected mRNA was 30–50% more efficient than that of unfractionated mRNA preparations. As determined from sucrose gradient centrifugation data, increased translational efficiency was attributed to the removal of an 18S rRNA contaminant.

The unique poly(A) sequence at the 3′ terminus of most eukaryotic mRNAs provides an almost universal approach for isolating these mRNAs by oligo(dT)–cellulose (Edmonds et al., 1971; Aviv & Leder, 1972) or poly(U)–Sepharose (Lindberg & Persson, 1972) chromatography. Under conditions that promote base-pair formation [moderate to high salt concentrations, e.g., 0.1–0.5 M NaCl in 0.01 M tris(hydroxymethyl)aminomethane (Tris),[1] pH 7.4], these adsorbents select poly(A)-containing mRNAs yet exclude the bulk of tRNA and rRNA populations. While such chromatographic techniques have been used with considerable success, they are not amenable to the isolation of mRNAs lacking poly(A) sequences (Milcarek et al., 1974; Nemer et al., 1976). Further, it is probable that a fraction of the mRNAs selected on the basis of poly(A) content have undergone degradation either

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[1] Abbreviations: m^7G', pm^7G', and pA′, trialcohol (ribose ring-opened) derivatives of 7-methylguanosine, its 5′-phosphate, and adenosine 5′-phosphate, respectively; m^7G and pm^7G, 7-methylguanosine and its 5′-phosphate, respectively; m^7GpppN^m, m^7G linked by a 5′–5′-triphosphate to a 2′-O-methylated nucleoside; m_2^2G, N^2,N^2-dimethylguanosine; anti-m_2^2G/Sepharose and anti-m^7G/Sepharose, affinity-purified antibodies coupled to Sepharose; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

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in vivo or during isolation. This latter fraction would represent, therefore, only the 3′-terminal portion of the mRNA molecule.

The more recent findings indicating that most eukaryotic mRNAs possess a second unique structure at their 5′ terminus, i.e., an m^{7}G-containing cap structure of the type m^{7}G(5′)-ppp(5′)N... (Shatkin, 1976), prompted us to examine if antibodies specific for 7-methylguanosine (anti-m^{7}G antibodies) could be employed to isolate mRNAs on the basis of this 5′-terminal modification. This immunochemical approach appeared promising in view of recent investigations indicating the effectiveness of anti-m^{7}G antibodies to isolate m^{7}G-containing oligonucleotide sequences (Munns et al., 1979a; Malek et al., 1981), to inhibit the in vitro translation of mRNAs (Munns et al., 1979b; Dante & Niveleau, 1980), to distinguish 5′ from 3′ termini via immunoelectron microscopy techniques (Castleman et al., 1980), and to interact with a variety of mRNA populations (Munns & Liszewski, 1980; Nakazato, 1980).

However, while the above investigations imply feasibility, they do not provide rigorous documentation as such for isolating intact, translationally active mRNA. Further, no attention has been focused upon establishing conditions (pH, ionic strength, temperature) which are optimal and/or acceptable for mRNA binding to anti-m^{7}G antibodies. The data presented herein provide this information by demonstrating that anti-m^{7}G antibodies immobilized to Sepharose (i) retain their high affinities and specificities for m^{7}G-containing mono- and oligonucleotides and mRNA over broad ranges of temperature, pH, and ionic strength and (ii) select for mRNAs by virtue of their 5′-terminal, m^{7}G-containing cap in an efficient and specific manner without apparent degradation to the molecule. Moreover, antibody-selected mRNAs are readily recovered and translated in vitro with efficiencies comparable to or greater than those of their poly(A)-selected counterparts.

Materials and Methods

Materials. Commerical preparations of rabbit globin mRNA were obtained from Miles Laboratory and Bethesda Research. As evaluated by sucrose gradient sedimentation, these preparations were contaminated with 18S rRNA to an extent of 20–40%. Additional globin mRNA devoid of rRNA contamination was prepared in our laboratory by the method described by Chu & Rhoads (1980). Aliquots of the latter preparation were periodate oxidized and subsequently labeled at the 5′-terminal m^{7}G and 3′-terminal A with NaB^{3}H$_4$ and recovered by oligo(dT)–cellulose chromatography (specific activity 6×10^4 cpm/μg). These procedures have been described in detail by Breter et al. (1979) and Malek et al. (1979).

NaB^{3}H$_4$ (8.7 Ci/mmol, Amersham/Searle) and NaBH$_4$ (Sigma) were also used in preparation of the tritiated and unlabeled trialcohol derivative of m^{7}G according to the procedure of Randerath & Randerath (1973), the trialcohol derivative of m^{7}G being designated as m^{7}G′ throughout the text. Characterization of the resulting m^{7}G′ and [^{3}H]m^{7}G′ products via thin-layer chromatography in two solvent systems revealed a single radioactive component migrating identically with the m^{7}G standard. The specific activity of [^{3}H]m^{7}G′ was 2.0 Ci/mmol or 3×10^6 cpm/μg, based upon the extinction coefficient of m^{7}G. The identical procedure was used to synthesize [^{3}H]m$_2^2$G hapten with a resulting specific activity of 2.5 Ci/mmol.

Immunochemical Reagents and Procedures. The production and purification of anti-m^{7}G antibodies and their subsequent immobilization to Sepharose have been described (Munns & Liszewski, 1980). A second immunoadsorbent, anti-N^2,N^2-dimethylguanosine antibody coupled Sepharose (anti-m$_2^2$G/Sepharose), was prepared in an identical manner by utilizing m$_2^2$G-coupled bovine serum albumin (m$_2^2$G-BSA) as the immunogen and m$_2^2$G-BSA/Sepharose as the matrix for antibody purification. Approximately 10 mg of affinity-purified antibody (both anti-m^{7}G and -m$_2^2$G antibody) was reacted with each gram (dry weight) of CNBr-activated Sepharose 4B (Pharmacia). Each microliter of adsorbent (swelled bed volume) contained approximately 2.3 μg of immobilized antibody, based upon a coupling efficiency of 80% (A_{280} measurements before and after coupling).

Binding of [^{3}H]m^{7}G′ and mRNA to Anti-m^{7}G/Sepharose. Binding assays with [^{3}H]m^{7}G′ and globin mRNA were conducted in a volume of 0.3 mL by using capped, conical, polypropylene tubes of 1.4-mL capacity. The immunoadsorbent was initially washed several times by pelleting at 8000g in a Beckman microfuge for 15–30 s and resuspending in the buffer to be used for binding (i.e., prewashed adsorbent). Typical incubation conditions were 30–60 min at room temperature in Hepes- or phosphate-buffered saline (Hepes/NaCl or PO$_4$/NaCl; 150 mM NaCl and 10 mM Hepes or PO$_4$, pH 7.0). Tubes were rotated at 30–60 rpm to suspend the immunoadsorbent during the binding reaction. The immunoadsorbent was then washed 4 times with 1-mL aliquots of the original buffer. Bound material was eluted with two aliquots (0.2–1.0 mL) of 1% NaDodSO$_4$ at 24 °C for 5 min. Parallel experiments with anti-m$_2^2$G/Sepharose were conducted to estimate nonspecific binding of [^{3}H]m^{7}G′. This was found to vary from 0.1 to 0.5% of input radioactivity, depending on the relative amounts of [^{3}H]m^{7}G′ and immunoadsorbent, and was subtracted from immunospecifically adsorbed radioactivity.

Processing of Globin mRNA. Globin mRNA, initially retained by anti-m^{7}G/Sepharose and subsequently eluted in 1% NaDodSO$_4$, was recovered by ethanol precipitation. After a single ethanol wash, the mRNA was dissolved in 5 mM Hepes buffers and (i) applied to the top of 15–30% sucrose gradients (Morrow et al., 1981) or (ii) added to a wheat germ translational system. Details regarding the in vitro translational system have been described in detail by Gordon et al. (1977). Briefly, rate-limiting quantities of RNA were translated in a methionine-dependent wheat germ system that was supplemented with 30 μCi of [^{35}S]methionine (1200 Ci/mmol, Amersham/Searle). At selected time intervals, 5-μL aliquots were withdrawn and processed for determination of the trichloroacetic acid precipitable radioactivity and/or electrophoretic characterization via NaDodSO$_4$–acrylamide gels (Gordon et al., 1977). Additional details regarding gel electrophoresis and autoradiography appear in the legends of appropriate figures. Unfractionated globin mRNA as well as that RNA not retained by anti-m^{7}G/Sepharose was processed in similar fashion, i.e., sucrose gradient sedimentation and in vitro translation.

Analysis of [^{3}H]pm^{7}G′ and [^{3}H]pA′. Immunospecifically retained and nonretained mRNA fractions (see previous sections) were chromatographed in a 0.5×30 cm column of Sephadex G-50 (fine) equilibrated in 5 mM EDTA, pH 7.0. The material eluting with the void volume was precipitated with ethanol in the presence of carrier RNA and digested with P$_1$ nuclease and tobacco acid pyrophosphatase as previously described (Breter et al., 1979). Quantities of radiochemically labeled [^{3}H]pm^{7}G′ and [^{3}H]pA′ were determined by isocratic high-pressure liquid anion exchange chromatography in the presence of unlabeled standards on a Whatman Partisil PXS 1025 SAX column equilibrated with 30 mM potassium

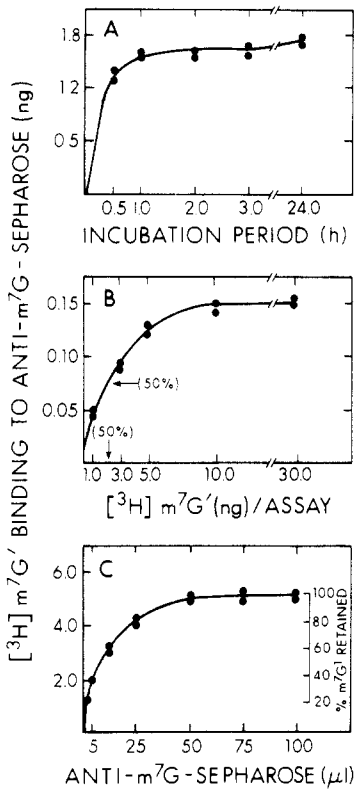

FIGURE 1: Assessment of binding of [^{3}H]m^{7}G′ to anti-m^{7}G/Sepharose as a function of time (A), hapten concentration (B), and adsorbent (C). All incubations were conducted at 24 °C for 1 h in PO_4/NaCl (final volume 0.3 mL) and contained 5 ng of [^{3}H]m^{7}G′ (15 000 cpm) and 5 μL of adsorbent unless indicated otherwise. In panel B, 0.25 μL of adsorbent was used in evaluating ^{3}H-labeled hapten saturation of antibody combining sites. The arrows in panel B denote the concentration of unbound hapten when 50% of the antibody combining sites are occupied, i.e., 1.82 ng or 0.2 × 10^{-7} M. The reciprocol of the unbound hapten concentration at 50% saturation is 5 × 10^7 M^{-1}.

phosphate, pH 3.48 (Breter et al., 1979).

Results

Binding of [^{3}H]m^{7}G′ to Anti-m^{7}G/Sepharose. Before the potential of anti-m^{7}G/Sepharose to select for mRNA on the basis of a 5′-terminal, m^{7}G-containing cap was examined, a series of binding studies with the [^{3}H]m^{7}G′ hapten were initiated to obtain information regarding the affinity and specificity of the immobilized antibody. As illustrated in Figure 1, investigations focused upon assessing the maximum degree of ^{3}H-labeled hapten binding to anti-m^{7}G/Sepharose as a function of time (panel A), hapten concentration (panel B), and immobilized antibody concentration (panel C). Kinetic analysis (Figure 1A) revealed that the maximum binding of ^{3}H-labeled hapten to immobilized antibody (immunoadsorbent) was attained within 1 h; i.e., the interaction between antibody and hapten was rapid and equilibrium achieved within this incubation period.

Information pertaining to (i) antibody affinity and (ii) active antibody concentration was obtained by incubating increasing quantities of [^{3}H]m^{7}G′ with a constant amount of immunoadsorbent until all available antibody combining sites were saturated with hapten. These data appear in Figure 1B and reveal that a maximum of 0.15 ng (0.5 pmol) of ^{3}H-labeled hapten could be bound to 0.25 μL of immunoadsorbent. The inability of additional hapten (10–30 ng) to increase the quantity of bound hapten indicated that all anti-m^{7}G antibody combining sites were saturated at these hapten concentrations. Such data provide the necessary information to approximate the affinity constant of the immobilized antibody (K_a = 5 × 10^7 M^{-1}), i.e., the reciprocal of the concentration of the unbound hapten at 50% saturation. They also indicate that the quantity of active antibody immobilized to each microliter of Sepharose was 0.15 μg or 1.0 pmol. The latter is based upon each antibody (molecular weight 150 000) possessing two combining sites and the finding that a maximum of 0.50 pmol of hapten can be bound per 0.25 μL of immunoadsorbent (Figure 1B). Thus, about 7% of the 2.3 μg of antibody originally coupled to 1.0 μL of Sepharose was active.

Table I: Inhibition of Binding of [^{3}H]m^{7}G′ to Anti-m^{7}G/Sepharose by Unlabeled Competing Haptens[a]

competing hapten	quantity of competing hapten required for 50% inhibition (pmol)	competitor: [^{3}H]m^{7}G′ (pmol/pmol)
m^{7}G′ (unlabeled)	13.7	0.83
m^{7}G	26.6	1.61
pm^{7}G	30.2	1.83
m^{7}GpppA^m	24.1	1.46
m^{7}GpppG^m	27.5	1.67
GpppG	ND[b] (at 30 nmol)	
pG	ND (at 27 nmol)	
pA	ND (at 29 nmol)	
pU	ND (at 31 nmol)	
pC	ND (at 31 nmol)	

[a] The specificity of immobilized anti-m^{7}G antibodies as evaluated by the ability of selected unlabeled haptens to inhibit the binding of [^{3}H]m^{7}G′ to anti-m^{7}G/Sepharose. Incubations (1 h) were the same as those described for Figure 1A except that various quantities of unlabeled competing haptens were included in the assay. Control assays without competing hapten revealed that 32% of the [^{3}H]m^{7}G′ (4800 cpm) was bound to the adsorbent. The amount of [^{3}H]m^{7}G′ present in each assay was 16.5 pmol. [b] ND is not detected.

For determination of the amount of adsorbent necessary to quantitatively bind a given amount of hapten, increasing quantities of immunoadsorbent were incubated with 5.0 ng of [^{3}H]m^{7}G′ (16.5 pmol), an amount equivalent to 5 μg of globin mRNA (16.5 pmol). These results are presented in Figure 1C and revealed that 50 μL of anti-m^{7}G/Sepharose was sufficient to bind greater than 95% of the ^{3}H-labeled hapten. Based upon antibody affinity and concentration (determined above), it was predicted that 6 μL of adsorbent would bind 50% of the ^{3}H-labeled hapten, a value in agreement with that experimentally obtained (ca. 10 μL; see Figure 1C).

Additional binding studies were conducted to assess the specificity of the immobilized antibody toward various nucleosides, nucleotides, and cap analogues. This was accomplished by measuring the degree to which various concentrations of these competing haptens (unlabeled) would inhibit the binding of [^{3}H]m^{7}G′ to anti-m^{7}G/Sepharose in standard incubation assays. The degree of inhibition observed with each competitor was graphed as a function of their concentration, i.e., percent inhibition vs. log of competitor concentration. In this manner, the picomoles of each competing constituent required to inhibit [^{3}H]m^{7}G′ binding by 50% were determined. These results are presented in Table I and indicated that unlabeled m^{7}G′ was the most effective competitor, requiring only 13.7 pmol to inhibit by 50% the binding of [^{3}H]m^{7}G′ (16.5 pmol). All other m^{7}G-containing compounds were approximately half as effective as m^{7}G′ with these variations being attributed to structural dissimilarities in their ribose moieties. Compounds ineffective as competitors included GpppG and the four common nucleoside 5′-phosphates, even when tested at 10^3–10^4-fold molar excess relative to [^{3}H]m^{7}G′

Table II: Binding of [^{3}H]m^{7}G′ to Anti-m^{7}G/Sepharose. Effects of Temperature, Ionic Strength, and pH[a]

incubation conditions	[^{3}H]m^{7}G′ bound (cpm)	[^{3}H]m^{7}G′ bound relative to PBS (24 °C)
temp (°C)		
0	2520	0.66
24	3800	1.00
37	4670	1.23
56	5210	1.37
75	<100	<0.03
ionic strength (NaCl, mM)		
0	3410	0.90
150	3800	1.00
500	4260	1.12
1000	4420	1.16
pH		
5.0	3290	0.86
6.0	3720	0.98
7.0	3820	1.01
8.0	3970	1.04
9.0	4140	1.09

[a] All assays were conducted for 1 h at 24 °C (unless noted otherwise) in a final volume of 0.3 mL and containing 5.0 μL of adsorbent and 5 ng of [^{3}H]m^{7}G. PO_4/NaCl was employed in temperature and ionic strength studies, the latter containing 10 mM PO_4, pH 7.5, and NaCl as indicated (150 mM NaCl used in all temperature studies). Buffers for pH investigation were 10 mM sodium acetate (pH 5.0–6.0), 10 mM imidazole (pH 6.5–7.5), and 10 mM Tris (pH 8.0–9.0), all containing 150 mM NaCl.

(Table I). These latter results are considered mandatory if the immunoadsorbent is to be used to isolate mRNA or hnRNA populations, since such molecules contain 10^2–10^4 pG residues per m^{7}G cap residue.

Because the degree of secondary structure inherent in nucleic acids is dependent upon temperature, pH, and ionic strength, additional binding assays were performed to assess these parameters. These results are presented in Table II and revealed that ^{3}H-labeled hapten binding to anti-m^{7}G/Sepharose increased linearly with temperature to 56 °C, yet rapidly declined thereafter and was abolished at 75 °C. Binding was not significantly affected, however, by changes in ionic strength (0.01–1.0 M NaCl) or pH (5.0–9.0). These data suggest that the antibody combining site is hydrophobic in nature and are consistent with our unpublished observations (T. W. Munns and M. K. Liszewski, unpublished results) that ^{3}H-labeled hapten binding is reduced dramatically in aqueous buffers containing 50% ethanol or formamide.

Binding of Globin mRNA to Anti-m^{7}G/Sepharose. While the above investigations provide a comprehensive understanding as to the nature of the interaction between [^{3}H]m^{7}G′ and anti-m^{7}G/Sepharose, it cannot be assumed that complex molecules such as globin mRNA behave similarly. For the establishment of potential similarities, globin mRNA was incubated with anti-m^{7}G/Sepharose and its binding assessed by measuring the amount of A_{260} units retained by the immunoadsorbent after extensive washings. A typical elution profile for commercial preparations of globin mRNA is illustrated in Figure 2. Routinely, 20–30% of this RNA was eluted during the initial two wash fractions, while the remainder (70–80%) was removed only after $NaDodSO_4$ treatment of the immunoadsorbent (fractions 5 and 6, Figure 2), the former RNA being designated as nonretained (NR) and the latter as immunospecifically retained (IR).

As presented in Figure 3, sucrose gradient characterization of these RNA fractions together with unfractionated (UNFX in figure) globin mRNA revealed the following information.

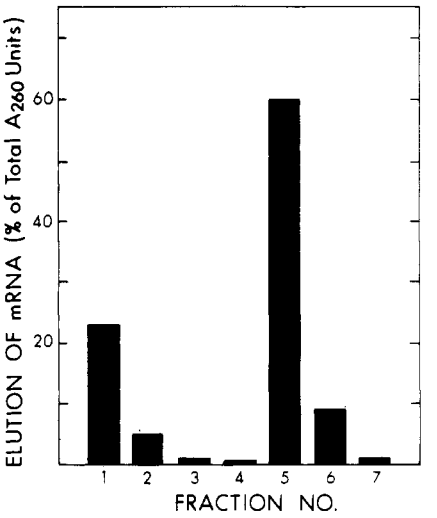

FIGURE 2: Elution of globin mRNA from anti-m^{7}G/Sepharose adsorbent. Globin mRNA (50 μg or 1.0 A_{260} unit) was incubated with 200 μL of anti-m^{7}G/Sepharose for 60 min at 24 °C in a final volume of 0.3 mL of PO_4/NaCl. Additional details regarding the elution of nonretained (NR, fractions 1 and 2) and immunospecifically retained (IR, fraction 5) RNA are described under Materials and Methods. The RNA in fractions 1 and 2 (NR) and in fraction 5 (IR) was recovered by ethanol precipitation and further characterized by gradient sedimentation (Figure 3) and by in vitro translation (Figures 5 and 6).

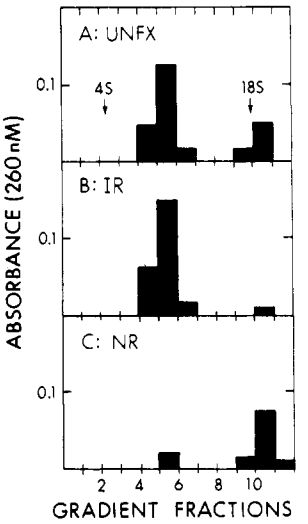

FIGURE 3: $NaDodSO_4$–sucrose gradient (15–30%) sedimentation of unfractionated globin mRNA (UNFX, panel A) and of immunospecifically retained (IR, panel B) and nonretained (NR, panel C) RNA eluted from anti-m^{7}G/Sepharose (see Figure 2). At the conclusion of centrifugation (SW41; 25 000 rpm; 24 h at 15 °C), each gradient was fractionated into 1.0-mL aliquots and the absorbance (260 nM) of each fraction determined (50 μg of RNA/mL is equivalent to 1.0 A_{260} unit). Sedimentation is from left to right.

First, approximately 75% and 25% of the unfractionated RNA (Figure 3A) sedimented as globin mRNA and 18S rRNA, respectively. Second, greater than 95% of the RNA retained by anti-m^{7}G/Sepharose was globin mRNA (Figure 3B) whereas the bulk of nonretained material sedimented as 18S rRNA (Figure 3C). Last, comparison of the sedimentation behavior of globin mRNA before (Figure 3A) and after retention to anti-m^{7}G/Sepharose (Figure 3B) implied that mRNA was not degraded during the adsorption and/or elution process.

For further substantiation of the above findings, additional experiments were conducted with ^{3}H-labeled globin mRNA in which only the 5′-m^{7}G and 3′-A terminal nucleotides were

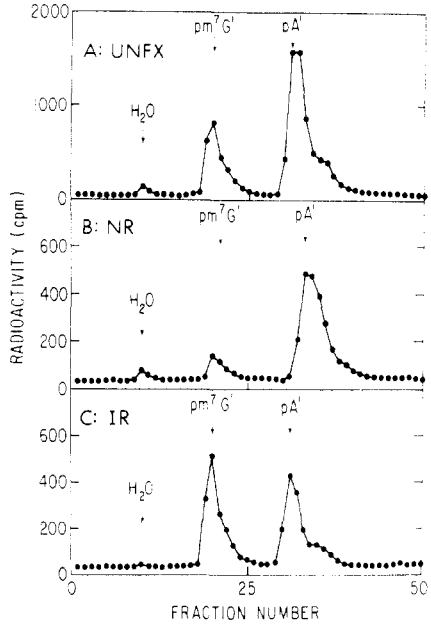

FIGURE 4: Identification of the ^{3}H-labeled nucleotides present in ^{3}H-labeled globin mRNA and representative of unfractionated (UNFX, panel A), nonretained (NR, panel B), and immunospecifically retained (IR, panel C) RNA. Each mRNA preparation was digested with P_1 nuclease and tobacco acid pyrophosphatase and analyzed by isocratic anion-exchange high-pressure liquid chromatography. Arrows indicate the positions of unlabeled standards included with each sample and detected by the absorbance at 260 nm. For additional details, see Materials and Methods.

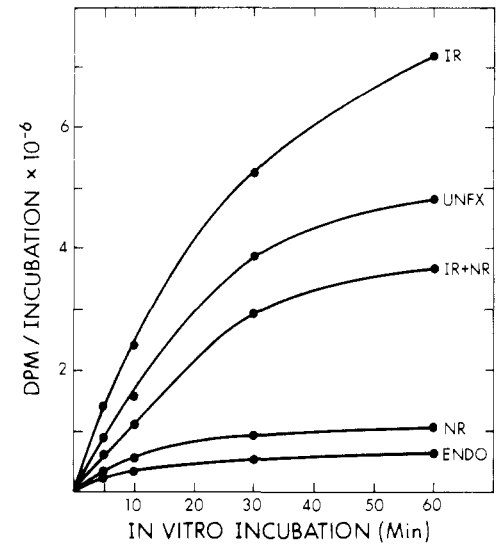

FIGURE 5: Kinetics of in vitro translation of unfractionated (UNFX), nonretained (NR), and immunospecifically retained (IR) globin mRNA. Aliquots of these individual RNA fractions (0.25 μg/assay) as well as equal quantities of the IR and NR fractions (0.125 μg of each) were translated in a wheat germ system (50 μL) in the presence of 10 μCi of [^{35}S]methionine (36 Ci/mmol). Aliquots (5 μL) were taken at the indicated times and processed for determination of trichloroacetic acid precipitable radioactivity (Munns et al., 1979b). All procedures pertaining to in vitro translation of mRNA are described under Materials and Methods. ENDO is the endogenous incorporation of [^{35}S]methionine into polypeptides, i.e., without exogenous mRNA added to the system.

labeled (see Materials and Methods for labeling protocol). Globin mRNA, labeled in this manner and recovered by oligo(dT)–cellulose chromatography, was digested with P_1 nuclease and tobacco acid pyrophosphatase, and the resulting ^{3}H-labeled nucleotides were identified by high-pressure liquid chromatography (Figure 4A). These results indicated that the [^{3}H]pm^{7}G′:[^{3}H]pA′ ratio present in ^{3}H-labeled globin mRNA was 0.41. This deviation from an anticipated ratio of 1.0 was attributed to the partial degradation of mRNA during radiochemical labeling and subsequent enrichment of 3′-terminal fragments as a result of the oligo(dT)–cellulose recovery step. This explanation is supported by the observation that [^{3}H]mRNA recovery via Sephadex G-50 filtration yields higher pm^{7}G′:pA′ ratios (R. Rhoads, unpublished results). To examine whether this explanation is correct, we incubated 1.5 μg of ^{3}H-labeled globin mRNA previously selected by oligo(dT)–cellulose (2 h, 24 °C) with 25 μL of anti-m^{7}G/Sepharose. Subsequent washing and elution steps revealed that 63% of the [^{3}H]mRNA was retained by the immunoadsorbent. Upon recovery, both nonretained (NR) and immunospecifically retained (IR) mRNAs were processed for determination of [^{3}H]pm^{7}G:[^{3}H]pA ratios (Figure 4). Whereas unfractionated [^{3}H]mRNA possessed a ratio of 0.41, those representative of nonretained and retained preparations were 0.15 and 0.95, respectively. These data indicate that anti-m^{7}G/Sepharose selects for mRNA solely on the basis of its 5′-terminal, m^{7}G-containing cap.

In Vitro Translation of Immunospecifically Retained mRNA. So that additional information regarding both the intactness and translatability of antibody-selected globin mRNA could be obtained, each of the three RNA preparations initially characterized in Figure 3 was evaluated for its ability to synthesize rabbit globin in a mRNA-dependent, in vitro translational system. The kinetics of [^{35}S]methionine incorporation into acid-precipitable proteins when equivalent and rate-limiting quantities (0.2 μg) of these RNA preparations were added to a wheat germ translational system are presented in Figure 5. These results indicated that the translation of antibody-selected RNA (IR) was 30–50% more efficient than unfractionated control preparations (UNFX), whereas the translation of nonselected RNA (NR) reflected less than 10% of the control value; efficiency of translation was defined as counts per minute of precipitable protein per microgram of exogenous mRNA per unit time. These data are in complete accord with those illustrated in Figure 3 which revealed that anti-m^{7}G/Sepharose selected intact globin mRNA while excluding the rRNA contaminant.

Electrophoretic analysis (Figure 6) of the resulting product(s) of translation demonstrated that both unfractionated and antibody-selected RNA synthesized a single protein with an approximate molecular weight of 15 000, i.e., a size comparable to rabbit globin. An identical product observed in those wheat germ extracts containing the nonretained RNA fraction was attributed to the small quantities of globin mRNA not selected by the immunoadsorbent. Additional studies were conducted in which equal quantities of various RNA fractions were mixed (i.e., UNFX + NR and IR + NR) prior to translation. These mixing experiments (shown in part; see Figures 5 and 6) confirmed that the quantities of RNA used during translation were rate limiting. They further implied the absence of any inhibitor of translation in the nonselected RNA preparations.

Binding of Globin mRNA to Anti-m^{7}G/Sepharose. Effects of pH and Salt Concentration and the Types of Adsorbent and RNA Substrate. So that information as to the optimal and/or acceptable condition required for isolating mRNA could be provided, the binding of globin mRNA to anti-m^{7}G/Sepharose was examined by using a host of experimental conditions. Standard or control incubation conditions were selected to permit 60% of the total globin mRNA input to bind to the immunoadsorbent. The results of these investigations (not shown) indicated that variations in salt concentration

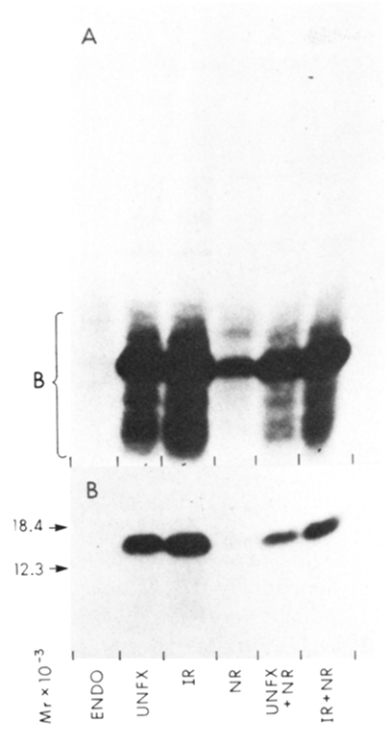

FIGURE 6: Autoradiographs of in vitro translational products as characterized by NaDodSO$_4$–acrylamide gel electrophoresis [20:0.6% acrylamide:bis(acrylamide)]. The quantity of radioactivity electrophoresed represents a 5-μL aliquot of wheat germ extract incubated in the presence of various RNAs for 60 min (see Figure 5). Prior to electrophoresis, each aliquot was heat denatured (100 °C for 3 min) in the presence of 0.1 M dithiothreitol and 2% NaDodSO$_4$. At the conclusion of electrophoresis, gels were fixed, dried, and exposed to XAR-5 Kodak film for 6 h (panel A, entire gel) and 30 min (panel B, bottom one-fourth of gel). The molecular weight (M_r) of the major ^{35}S-labeled polypeptide was approximated at 15 000 as evaluated by two ^{14}C-labeled protein standards; cytochrome *c* (12 300) and β-lactoglobulin (18 400).

(0.1–0.5 M NaCl) and pH (pH 5.5, 7.0, and 8.0) had minimal effect on globin mRNA binding. In contrast, both decapped globin mRNA and 18S rRNA (5–50 μg/assay) were not suitable substrates in such binding assays. Quite unexpected, however, was the finding that immobilization of a total IgG fraction comprising 7% anti-m^{7}G antibody and derived from anti-m^{7}G antiserum was not an effective immunoadsorbent for isolating globin mRNA. The identical adsorbent, however, was quite efficient in retaining [^{3}H]m^{7}G′ hapten (T. W. Munns and M. K. Liszewski, unpublished results). This observation stresses the importance of immobilizing an affinity-purified antibody population. Last, and as anticipated, anti-m$_2^2$G/Sepharose was not an effective immunoadsorbent for retaining globin mRNA.

While the above data imply that a variety of conditions are acceptable for immunochemically isolating capped mRNAs, we recommend 150 mM NaCl buffered with either 10 mM Hepes or PO$_4$, pH 7.4, the former buffer being used in conjunction with in vitro translational analysis (see Figures 5 and 6). As noted in Table II, other buffers appear to be quite acceptable as well.

Discussion and Conclusions

The uniqueness of the 3′-poly(A) terminus of most eukaryotic mRNAs permits these molecules to be isolated by affinity chromatographic techniques utilizing poly(U)- or oligo(dT)-coupled adsorbents (Edmonds et al., 1971; Aviv & Leder, 1972). In the present paper, we have evaluated the ability of anti-m^{7}G antibodies to interact with and subsequently retain mRNA on the basis of a second, unique structure, namely, the 5′-terminal, m^{7}G-containing cap. The data presented herein indicate that anti-m^{7}G antibodies, possessing both high specificity and affinity toward m^{7}G-containing mono-, oligo-, and polynucleotides (Figure 1 and Table I), are capable of immunospecifically retaining m^{7}G-capped globin mRNA in a rapid and efficient manner. Further, globin mRNA can easily be recovered and translated in vitro with efficiencies comparable to and in some instances greater than those of their poly(A)-selected counterparts (Figures 5 and 6).

Most important was the finding that anti-m^{7}G antibody selected mRNA is not degraded during its adsorption and subsequent elution. This was demonstrated by NaDodSO$_4$–sucrose gradient centrifugation (Figure 3), by characterization of the resulting in vitro translation product (Figure 6), and by analysis of the [^{3}H]pm^{7}G′:[^{3}H]pA′ ratio of ^{3}H-labeled globin mRNA specifically labeled at both the 3′ and 5′ termini (Figure 4). The latter results also revealed that only capped mRNA was retained by the immunoadsorbent, a finding confirmed with tobacco acid pyrophosphatase treated globin mRNA.

Little, if any, of the contaminating 18S rRNA in commercial globin mRNA preparations was retained by anti-m^{7}G/Sepharose (Figure 3). Further, by incubation of 10–50-μg quantities of this rRNA with immunoadsorbent, less than 1% appeared to be retained and reflected in all likelihood nonspecific binding. These results are somewhat surprising in view of the findings of Maden & Salim (1974) and Khan et al. (1978) which indicate that eukaryotic 18S rRNA contains a single m^{7}G residue. Our data, coupled with a similar observation by Nakazato (1980), suggest that the m^{7}G present in 18S RNA is "buried" within the three-dimensional confines of the molecule and thus not available for antibody binding. It appears that a similar situation exists for tRNAs since we have been unsuccessful in our attempts to select for m^{7}G-containing tRNAs [ca. 50% of the tRNAs in both pro- and eukaryotic systems possess m^{7}G; see Munns & Liszewski (1980)]. Together these findings support the concept that secondary and tertiary structures are important factors in antibody-dependent recognition of hapten-containing nucleic acids.

Equally important to the above thesis is the type of environment responsible for optimal and/or acceptable binding, particularly with regard to ionic strength. Thus, the abilities of anti-m^{7}G/Sepharose to bind ^{3}H-labeled hapten and particularly mRNA at intermediate and high ionic strength (e.g., 0.1–1.0 M NaCl; see Table II) are the same conditions that promote and stabilize secondary structures inherent in nucleic acids. Collectively, these results suggest that anti-m^{7}G antibody can selectively retain m^{7}G-capped-containing mRNA (and presumably low molecular weight nuclear RNAs; see below) in the presence of other m^{7}G-containing species, i.e., tRNA and 18S rRNA (28S rRNA does not contain m^{7}G; Khan et al., 1978). Further, by utilizing both m^{7}G- and poly(A)-selection techniques, it is possible to envision a protocol capable of isolating m^{7}G-capped yet nonpolyadenylated mRNAs (Milcarek et al., 1974; Nemer et al., 1976).

The use of anti-m$_2^2$G/Sepharose adsorbent is by no means restricted to monitoring nonspecific binding of [^{3}H]m^{7}G′. While it was used for this purpose in the present study, this particular adsorbent should have broad application in the isolation and characterization of low molecular weight nuclear RNAs and/or the corresponding ribonuclear protein particles [Lerner & Steitz, 1979; see review by Reddy & Busch (1981)].

Like mRNA, these nucleic acids possess a 5′-terminal cap structure of the type $m_3^{2,2,7}G(5')ppp(5')N$, in which the only significant difference is the presence of two additional methyl groups at the N-2 position of guanosine, i.e., m_2^2G. Recently, we have observed that the $m_3^{2,2,7}G$ nucleoside effectively competes with [^{3}H]m^7G' and with [^{3}H]m_2^2G' in binding assays with anti-m^7G antibody and anti-m_2^2G antibody, respectively (T. W. Munns and M. K. Liszewski, unpublished results).

Several obstacles remain to be resolved before a host of anti-nucleoside antibody-coupled adsorbents can be considered as univeral reagents for the isolation of hapten-containing nucleic acids. First, the coupling of antibody populations to adsorbents should be improved such that the bulk of antibody activity is not lost during conjugation (the adsorbent used in the present studies retained only 7% of the original antibody activity). Second, elution conditions have to be devised whereby the hapten-containing substrate can be dissociated from immobilized antibody without significant loss of antibody activity, i.e., a renewable adsorbent. (It should be noted that the $NaDodSO_4$ elution of RNA employed herein is accompanied by total loss of antibody activity.) Another problem inherent in such technology is the time-consuming documentation of antibody affinity and specificity toward hapten and hapten-containing nucleic acids. Such assays, however, are required for accurate definition of each antibody population since affinity and specificity parameters vary considerably among various antisera. In an effort to resolve some of these obstacles, we are in the process of selecting hybrid cells that secrete specific monoclonal antibody populations that recognize various methylated nucleosides (e.g., m^6A, m_2^2G, m^7G, m^5C, etc.). Those monoclonal antibodies possessing the necessary prerequisites of affinity and specificity will be employed for subsequent isolation and characterization of nucleic acids (Munns & Liszewski, 1980).

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