Isolation and characterization of rabbit anti-m⁷G-5'-P antibodies of high apparent affinity

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ABSTRACT

Antibodies highly specific for intact pm⁷G (7-methylguanosine-5'-monophosphate) were induced by immunization of rabbits with a pm⁷G-BSA conjugate. Since the nucleotide is six-fold more stable than m⁷G (7-methylguanosine) to alkali-catalyzed fission of the imidazole ring, it is a more desirable antigen for obtaining antibodies capable of binding caps on eukaryotic mRNA. UV spectra demonstrated that the nucleotide in the conjugate was predominantly the intact form. Competition radioimmunoassay showed 1) high apparent affinities for pm⁷G, on the order of 10⁻⁸ M, 2) low competition by the ring-opened form of the homologous hapten (*pm⁷G), and by m⁷G, 3) little or no competition by AMP, GMP, CMP, UMP or m⁶A, and 4) high apparent affinities for m⁷GpppA^m, m⁷GpppN⁶mA^m, m⁷GpppG^m, m⁷GpppA.

INTRODUCTION

In this paper, we describe the preparation of antibodies of high affinity, with a specificity for 7-methylguanosine-5'-monophosphate (pm⁷G). These antibodies appear to be suitable for the study of cap structures (m⁷GpppN^(m)) found on the 5' termini of most eukaryotic mRNAs, and for the construction of a binding assay for the capped mRNAs.

Most of the previous attempts to obtain antibodies to 7-methylguanosine (m⁷G)^{1, 2, 3, 4} have not examined the possibility of ring fission (i.e. hydrolysis) of m⁷G to 2-amino-6-hydroxy-5-(N-methyl)formamido-4-ribosylaminopyrimidine (*m⁷G)^{5, 6}. This reaction can occur during conjugation of m⁷G to a protein or after immunization with the m⁷G conjugate, since the pH and temperature of blood are within the range of conditions for this reaction⁵⁻⁷. One immunological study in which hydrolysis was considered⁸ found equal inhibition by m⁷G and by *m⁷G in a quantitative precipitin procedure, but it was not clear whether the

antisera had cross-reacting antibodies or two populations, one with a specificity for m^7G and the other for $*m^7G$.

We have found that the instability of m^7G makes it an undesirable immunogen for the production of antibodies capable of binding the 5' end of capped mRNAs. On the other hand, 5' phosphorylation of m^7G confers a six-fold increase in stability (6, this paper). Moreover, the conformation of the ribose in pm^7G is more rigid than in m^7G ^{9,10}, suggesting that antibodies to pm^7G might have higher affinities to capped mRNA than antibodies to m^7G . In the light of these considerations, as well as the finding that the nucleotide rather than the nucleoside inhibits translation in vitro^{9,11,12}, antibodies to pm^7G were prepared. These antibodies were found to be highly specific for pm^7G , compared to $*pm^7G$, m^7G and $*m^7G$. A preliminary report of these studies has already appeared¹³.

While this paper was in preparation, Rainen and Stollar¹⁴ reported on anti- m^7G antibodies that could distinguish between the intact and the alkali-hydrolyzed form. However, the apparent affinity of their antisera for homologous hapten and for "cap structures" was about 1000 times less than the affinity of the antisera described in this paper, making it less desirable for purification or assay of capped mRNA.

MATERIALS AND METHODS

Chemicals - Caps and pm^7G were obtained from P-L Biochemicals. Inosine was purchased from Chemical Procurement Labs., College Point, N. Y.; D-2-deoxyribose from Mann Research. All other inhibitors in the radioimmunoassay were from Sigma Chemical Co. Macaloid was a kind gift of NL Industries.

Preparation of pm^7G -BSA and pm^7G -RSA - To 300 μ l of ice cold 0.1M $NaIO_4$ was added 14 mg of pm^7G (P-L Biochemicals) and the pH was titrated to 5.0 with 10% acetic acid. The mixture was covered with foil and stirred on ice for 20'. The periodate-oxidized pm^7G was divided equally and added to ice-cold solutions of BSA and RSA (Pentex Fraction V, 50 mg each dissolved in 2 ml saline, adjusted to pH 8.5 with 5% $NaHCO_3$). After addition, the mixtures were kept at pH 8.5 with 5% $NaHCO_3$, and stirred on ice for 20'. 200 μ l of a freshly prepared 0.25% (w/v in distilled H_2O) solution of $Na(H_3BCN)$ (Sigma) was added; the solution was titrated immediately to pH 6.5-7.0 with 10% HAc and allowed to stir on ice for 40'. Dialysis for 24 hours in the cold was against running tap water; the conjugates

were then lyophilized.

Immunization - Three New Zealand White rabbits were immunized intramuscularly in each thigh with 0.4 mg of (pm^7G)-BSA (2 mg/ml in saline) in complete Freund's adjuvant for 3 weeks at weekly intervals. A 1:1 ratio (v/v) of complete Freund's adjuvant to antigen solution was used. For every injection, a fresh aliquot of pm^7G -BSA was employed; no more than 4 hours elapsed between the weighing of lyophilized, frozen pm^7G -BSA and the injection. Six to nine days after the final injection, the rabbits were bled; thereafter they were bled 6-9 days after each monthly boost. The response was followed by agglutination of derivatized acrylamide beads (see below). The results described in this paper are based on pools collected after the second and third boosts. One of the rabbits did not respond, even after repeated injections. Globulin fractions were prepared by four successive Na_2SO_4 precipitations at room temperature followed by dialysis against saline¹⁵.

Rate of ring fission - The procedure followed the method of Hendler *et al.*⁶ The change in OD_{273} with time was plotted as $\log (\text{OD}_0 - \text{OD}_t)$ vs. t . The data fell on a straight line from which half-lives could be calculated. Buffers used were 0.2M carbonate buffer, pH 9.0 in 0.5M NaCl and 0.1M phosphate, pH 8.0. To find OD_0 , a $\text{*pm}^7\text{G}$ stock solution was prepared by adding 40 μl of 1N NaOH to 1 ml of 3.64 mM pm^7G (in TBS, see below) incubating 26 hr at 23°C and then neutralizing with 40 μl 1N HCl. This stock solution was then diluted 1:50 in the appropriate buffer.

Agglutination with acrylamide beads - The procedure for derivatization of acrylamide beads was a modification of the methods of Robberson & Davidson¹⁶ and of Ohno & Stahmann¹⁷. To 500 μl of 0.05M NaIO_4 in 0.1M sodium acetate buffer, pH 5.0, was added equimolar quantities (by UV spectra) of nucleoside or nucleotide. The mixture was covered and stirred on ice for 20'. A volume of 2.5 ml of suspended, unpacked beads was added (Affigel 703 hydrazide derivative (Bio-Rad), capacity 20 $\mu\text{moles/ml}$, particle size 1-3 microns, washed once with 0.1M sodium acetate buffer, pH 5.0) and was stirred slowly for 2 hours. At the end of this incubation, the derivatized beads were washed twice with 1 volume of saline and stored at 10°C. To obtain $\text{*pm}^7\text{G}$ and $\text{*m}^7\text{G}$ derivatized beads, 1 ml of washed beads from each reaction mixture were re-suspended in 1 ml of 0.1M carbonate buffer, pH 9.0, and incubated for 74 hours

at 37°C.

Before agglutination, all beads were washed in TBS (0.01M Tris-HCl, pH 7.2, containing 0.15M NaCl) until the OD₂₆₀ of the supernatants was less than or equal to 0.02.

For the agglutination tests, serial two-fold dilutions of antibody were made in micro titer plates (rigid styrene microtitration plates with U walls, Cooke Laboratory Products). The diluent used was normal rabbit gamma globulin, 0.07 mg/ml, in a final volume of 25 µl. A 25 µl aliquot of a 1% suspension of derivatized beads was added to each well; the micro titer plates were shaken lightly and stored overnight in the cold. The end point was read as the reciprocal of the highest dilution of antibody showing agglutination.

Elimination of RNase - The procedure used (J. Wollack, personal communication) is a modification of Inners and Felsenfeld¹⁸. Macaloid (NL Industries), a superior inhibitor of pancreatic RNase¹⁹, was washed with distilled water and autoclaved. A final concentration of 1 mg/ml in a globulin fraction was found to eliminate all detectable hydrolysis of the homoribopolymer most sensitive to pancreatic RNase¹⁹, poly C, if the incubation with Macaloid was for at least 48 hours in the cold. The hydrolysis of [³H]-poly C was assayed by incubating 10 µl of [³H]-poly C (Miles, 3000 cpm, 29.7 µCi/µmole phosphate) with 20 µl of antibody solution for 30' at 37°C. Then 25 µl of carrier RNA (10 mg/ml tRNA in TBS) and 200 µl of 5% TCA were added and the mixture allowed to stand on ice for 30 min. The suspension was centrifuged at 3000g for 5' in the cold, the pellet washed twice with 200 µl 5% TCA and all three supernatants were counted together. RNase was considered to be removed when counts in the supernatant were the same as a control without antibody. No changes in antibody specificity were found after incubation with macaloid over 48 hrs. All glassware was baked at 215°C overnight. Water was treated with diethylpyrocarbonate (Sigma, 4 drops/500 ml) before buffers were made up, and the buffers were then autoclaved. Antibody was routinely stored as a globulin fraction in the presence of 1 mg/ml of macaloid; before use, the macaloid was removed by centrifugation.

Radioimmunoassay - A globulin fraction of antibody (25 µl, 4 mg protein/ml, macaloid treated) was incubated with 15 µl of inhibitor and 15 µl of [³H]-pm⁷G

(NEN, 5000 cpm, spec. act. 13.2 Ci/mmol) for 24 hrs at 10°C in a 1.5 ml capacity polypropylene Eppendorf microcentrifuge tube (Brinkmann). Then 150 µl of normal rabbit gamma globulin (1.8 mg/ml) and 400 µl of an $(\text{NH}_4)_2\text{SO}_4$ solution (saturated in distilled H_2O at 4°C) were added, the mixture was vigorously mixed and allowed to stand at 4°C for 1 hour. All the above dilutions were made in TBS, pH 7.2. Each tube was then centrifuged at 4°C for 15 min at 10,000g, and 400 µl of supernatant was counted with 1250 µl of distilled H_2O in 10 ml of NEN formula 947 liquid scintillation cocktail. Each point was the average of duplicates or triplicates. Any inhibition curve of a heterologous hapten was always run with a standard inhibition curve by pm^7G . The concentrations of inhibitors were determined by OD (pm^7G , $^*\text{pm}^7\text{G}$, m^7G , $^*\text{m}^7\text{G}$, GMP, AMP, UMP, CMP), otherwise they were determined from weight measurements. The molar absorption coefficient for pm^7G was from Hendler *et al.*⁶

To prepare $^*\text{pm}^7\text{G}$, see above section on rate of ring fission. For $^*\text{m}^7\text{G}$ stock solution, 40 µl of 1N NaOH was added to 1 ml of 0.136 mM m^7G (in TBS) and incubated 26 hr at 23°C, then neutralized with 40 µl of 1N HCl.

Thin-layer chromatography of pm^7G and caps - A variety of caps were purchased from P-L Biochemicals. They were examined by analytical cellulose TLC plates (without fluorescence indicator, EM Laboratories, Inc.). R_f values were determined in two solvent systems (Table 1). All migrated as single fluorescent spots.

Table 1. Thin-layer chromatography of pm^7G and cap structures.

Nucleotides	R_f	
	Solvent I*	Solvent II
pm^7G	0.55	0.35
m^7G (5')ppp(5') A^m	0.62	0.10
$\text{m}^7\text{GpppN}^6\text{mA}^m$	0.66	0.20
m^7GpppG^m	0.46	0.08
m^7GpppA	0.47	0.05

*Solvent I: isobutyric acid/0.5M NH_3 (5:3v/v)

Solvent II: isopropanol/HCl/ H_2O (70:15:15) (ref. 20)

RESULTS

Immunization with m^7G -BSA - Initial attempts were made to prepare antibodies to m^7G by immunizing rabbits with m^7G -BSA, which was prepared by a modification of the method of D'Alisa and Erlanger²¹. Since the imidazole ring of m^7G can open to yield $*m^7G$ ^{5,6}, an effort was made to conjugate m^7G to protein carriers under conditions in which the imidazole ring remained intact. In the preparation of the nucleoside conjugate, $Na(H_3BCN)$ was used in the reduction step in place of $NaBH_4$. By UV spectrum, the product was predominantly intact m^7G conjugate. Preliminary data on sera obtained from rabbits injected with m^7G -BSA showed extensive cross-reaction with $*m^7G$, as shown both by agglutination of acrylamide beads linked to $*m^7G$ and by the decrease in titer of agglutination of m^7G -linked beads after absorption with $*m^7G$ -RSA (data not shown).

The cross-reaction between m^7G and $*m^7G$ appeared to be due to the ring fission of m^7G after injection of the conjugate, since m^7G -BSA has a short half-life (Table 2) at a pH near the pH of blood (7.3-7.5)⁷. However, the nucleotide, pm^7G , is about six-fold more stable than m^7G , suggesting that a pm^7G conjugate might elicit more specific antisera. The di- and tri-phosphate derivatives are only moderately more stable than pm^7G (Table 2). We chose, therefore, to immunize with pm^7G -BSA.

Preparation of pm^7G -protein conjugates - To synthesize pm^7G -protein conjugates, a modification of the periodate procedure²¹ was used. In the course of the reaction, the pH was kept as low as possible, but greater than 6.0; all

Table 2. Rate of ring fission of 7-methylguanosine derivatives at 37°C.

Derivative	pH	Half-life (hr)
m^7G (7-Methylguanosine)	9.0	5.7
m^7G	8.0	8.6
pm^7G (7-Methylguanosine 5'-mono-phosphate)	8.0	52
(m^7G)-BSA (m^7G conjugated to BSA)	9.0	9.5
pm^7G	8.9	40.6 [✓]
ppm^7G	8.9	46.0 [✓]
$pppm^7G$	8.9	53.5 [✓]

[✓] Data from ref. 6.

procedures were on ice, and the reducing agent was changed from NaBH_4 to $\text{Na}(\text{H}_2\text{BCN})^{22}$. Fig. 1 shows that, at pH 6.4, the difference spectrum of pm^7G -BSA (ca 3.8 moles of hapten per mole of BSA) versus BSA is similar to that of pm^7G . The hydrolyzed form, $^*\text{pm}^7\text{G}$, has a distinctly different absorbance spectrum with a peak at 274 nm. The spectrum of $^*\text{pm}^7\text{G}$ conjugate was similar to that of $^*\text{pm}^7\text{G}$ (data not shown).

Agglutination properties of anti- pm^7G - Globulin fractions of rabbit anti- pm^7G were sterile filtered and then treated for at least 48 hr with macaloid to remove RNase activity^{18,19}. Agglutination of derivatized acrylamide (Affigel) beads by the globulin fractions showed significant specificity for pm^7G (Table 3); there was some cross-reaction with m^7G but very little with $^*\text{pm}^7\text{G}$ or $^*\text{m}^7\text{G}$.

Absorption with $^*\text{pm}^7\text{G}$ -RSA reduced the titer to pm^7G , indicating, perhaps, the presence of some cross-reacting antibody, or, more likely, the presence

Fig. 1. UV spectrum of immunogen compared with pm^7G and $^*\text{pm}^7\text{G}$. BSA and pm^7G -BSA were dissolved in saline to a concentration of 1.33 mg/ml (pH 6.4). pm^7G was made up to 0.072 mM in 0.15M phosphate buffer, pH 6.4. To prepare $^*\text{pm}^7\text{G}$, 1 ml of a 3.6 mM solution of pm^7G in TBS was hydrolyzed by adding 40 μl of 1N NaOH, incubating at 23°C for 26 hr, neutralizing with 40 μl of 1N HCl, and diluting 1:50 in 0.15M phosphate buffer, pH 6.4.

Table 3 - Specificity for pm⁷G in pooled rabbit globulin. Titers are expressed as reciprocal of the highest dilution of antibody showing agglutination.

Derivative on beads [†]	Agglutination Titer	
	Unabsorbed	Absorbed with *pm ⁷ G-RSA
pm ⁷ G	512	128
*pm ⁷ G	8	2
m ⁷ G	32	16
*m ⁷ G	2	2

[†]Affigel hydrazide acrylamide beads conjugated to these derivatives (treated with periodate) at pH 5.0, 4°C, incubation in the cold for 6 or more hours. All beads are equally substituted (by O. D.).

of antibody specific for the morpholino group^{21, 23}, the linkage group between the hapten and the lysine residues of the protein. The conjugation of Affigel beads to these derivatives was done under conditions that insured virtually no ring opening (pH 5.0, 4°C), and equal substitution. Storage of the beads at 4°C under saline (pH 6.5) for a period of 2 months did not result in a decrease in titer to pm⁷G or to m⁷G. Antisera collected 6-9 days after each of the first four boosts showed no detectable changes in agglutination titer to pm⁷G or to *pm⁷G.

Agglutination was used to follow immune responses and to roughly characterize the antibody. Radioimmunoassay was a more appropriate method of testing the utility of the antibody.

Competitive radioimmunoassay with unabsorbed globulin - Radioimmunoassays with [³H]-pm⁷G revealed a marked specificity for pm⁷G over *pm⁷G, m⁷G and *m⁷G (Fig. 2). About 4 pmoles of pm⁷G inhibited binding by 50%; 10⁴ pmoles of *pm⁷G or 2 x 10⁴ pmoles of m⁷G yielded equivalent inhibition *m⁷G was a less potent inhibitor than m⁷G by 2 orders of magnitude. When the reaction was run in PBS (0.01M phosphate, pH 7.2, containing 0.15M NaCl), instead of TBS, no difference in inhibition by pm⁷G was observed, indicating that phosphate ions did not compete with pm⁷G for antibody combining sites. Thus, the large preference for binding pm⁷G over m⁷G cannot be attributed to binding contacts with phosphate. These results are consistent with the suggestion put forward by Sarma^{9, 10} that the electrostatic interaction of the positive charge on N7 with the negative charge on the phosphate group

Fig. 2. Competitive radioimmunoassay with unabsorbed globulin. (●) pm^7G , (○) $^*\text{pm}^7\text{G}$, (■) m^7G , (□) $^*\text{m}^7\text{G}$. Serum dilution 1/5. Percentage of cpm bound without inhibitor = 46%.

confers a conformational rigidity upon the C-4'-C-5' bond. The greater inhibition by 7-methylguanine compared to 7-methylguanosine (Figs. 3 and 2) similarly suggests that in the nucleoside there is a predominant conformer(s) of the ribose not found in the nucleotide and, therefore, not recognized by the antibody.

Fig. 3. Competitive radioimmunoassay with unabsorbed globulin. (●) pm^7G , (▲) 7-methylguanine, (△) AMP, (○) GMP. Assay as described in legend of Fig. 2.

Of some other methylated nucleosides, only 7-methylinosine inhibited significantly but at a concentration 5 orders of magnitude greater than pm^7G (Table 4). Little or no inhibition was found by 6-methyladenosine, 7-methyl-xanthosine, inosine, D-ribose, D-2-deoxyribose, D-ribose-5'-phosphate at inhibitor concentrations greater than 10^5 picomoles. The four common ribonucleotides significantly inhibited binding of pm^7G at high concentrations only if they were purine nucleotides. Thus, GMP inhibited up to 50% at 5×10^5 picomoles, AMP up to 10% at 4×10^4 pmoles, but UMP and CMP showed little inhibition.

Binding of antibody to cap structure - NMR studies have shown that there are base stacking interactions between pm^7G and A^m in $\text{m}^7\text{G}(5')\text{ppp}(5')\text{A}^m$ ¹⁰. Of the four chromatographically pure caps tested, m^7GpppA^m , $\text{m}^7\text{GpppN}^6\text{mA}^m$, m^7GpppG^m , m^7GpppA , all required only about three to five times more for 50% inhibition than the homologous hapten (Table 5). Thus, base stacking slightly decreases binding by antibody, but does not abolish it. Inhibition reached 95-100% for all of the cap structures when in high concentration, indicating the absence of a detectable antibody population that bound pm^7G but not a given cap structure.

Methylation at the 2'-O or at N6 of A did not change the binding of anti- pm^7G antibody to cap: m^7GpppA^m and $\text{m}^7\text{GpppN}^6\text{mA}^m$ inhibited binding

Table 4. Inhibition of binding of [^3H]- pm^7G to anti- pm^7G by nucleosides, nucleotides, ribose substituents.

Inhibitor	Picomoles [†]	% Inhibition
pm^7G	4.0	50
m^7I	2.5×10^5	30
m^6A	1.0×10^5	2
m^7X	1.8×10^5	0
Inosine	1.8×10^5	0
UMP	1.0×10^5	5
CMP	1.0×10^6	1
D-ribose	5.6×10^5	0
D-2-deoxyribose	2×10^7	0
D-ribose-5'-phosphate	10^5	0

[†] Competitive radioimmunoassay as described in Fig. 2 and Materials and Methods.

Table 5. Inhibition of binding of [^3H]- pm^7G to anti- pm^7G by 5' terminal cap structures.[†]

Inhibitor	pmole for 50% inhibition
pm^7G	4.0
$\text{m}^7\text{GpppA}^{\text{m}}$	10
$\text{m}^7\text{GpppN}^6\text{mA}^{\text{m}}$	23
m^7GpppG	14
m^7GpppA	14

[†] Assay as described in Fig. 2 and Materials and Methods. The molar absorption coefficients employed for calculating concentration of caps are the following: $\text{m}^7\text{GpppA}^{\text{m}} = 2.18 \times 10^4$ liter/mole-cm at 257.5 nm, pH 7.5; $\text{m}^7\text{GpppN}^6\text{mA}^{\text{m}} = 1.97 \times 10^4$ at 260 nm, pH 7.5; $\text{m}^7\text{GpppG}^{\text{m}} = 1.93 \times 10^4$ at 253 nm, pH 7.5; $\text{m}^7\text{GpppA} = 2.09 \times 10^4$ at 260 nm, pH 7.0.

of antibody to pm^7G about as well as did m^7GpppA . The inhibition curves are indistinguishable (data not shown).

DISCUSSION

Previous attempts to isolate anti- m^7G antibody have either not examined the possibility of ring opening to $^*\text{m}^7\text{G}^{1,2,3}$ or have not fully characterized the extent of cross-reactivity of anti- m^7G for m^7G and $^*\text{m}^7\text{G}^8$. In principle, antibodies should easily distinguish m^7G from $^*\text{m}^7\text{G}$, but our initial results suggested that rabbit anti- m^7G antibody was largely cross-reactive. The 5'-phosphorylated nucleoside, pm^7G , was found to be more stable to ring opening (6, this paper) and bears a closer resemblance to the cap structure^{9,10}. Since our primary interest in these antibodies was to study capped mRNA, we chose pm^7G as a more suitable antigen. Care was taken to insure that ring fission did not occur during conjugation. Competitive radioimmunoassay of our rabbit anti- pm^7G antiserum showed high specificity for pm^7G ; the order of inhibition was $\text{pm}^7\text{G} \gg ^*\text{pm}^7\text{G} > \text{m}^7\text{G} > ^*\text{m}^7\text{G} \gg \text{GMP, AMP}$, with the difference between pm^7G and $^*\text{pm}^7\text{G}$ being four orders of magnitude. Rainen and Stollar¹⁴ have isolated rabbit and mouse anti- m^7G that are clearly specific for the intact nucleoside. However, competition radioimmunoassay of these anti- m^7G antisera showed 50% inhibition at concentrations of homologous inhibitor more than 1000-fold higher than found for our rabbit anti- pm^7G , indicating the need for correspondingly larger quantities of capped mRNAs

when used as an unlabeled competitor in a competitive radioimmunoassay with a radioactive probe.

Our anti-pm⁷G was inhibited by a variety of cap structures, m⁷GpppA, m⁷GpppA^m, m⁷GpppN⁶mA^m, m⁷GpppG^m, at concentrations close to that of pm⁷G. So little cross-reactivity occurred with AMP, GMP, UMP and CMP that an unlabeled, capped mRNA of sufficiently small length (ca 10,000 nucleotides/cap, or less) could be used to compete with [³H]-pm⁷G binding to anti-pm⁷G without cross-reaction by other nucleotides in the mRNA. Preliminary experiments with this procedure suggest that the antibody will allow interesting observations about accessibility of pm⁷G in capped mRNA, and the possible relationship of this accessibility to other segments of a given mRNA. Experiments with labeled whole cell mRNA are also in progress.

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