Volume 10 Number 22 1982 Nucleic Acids Research

Isolation and characterization of rabbit anti-$m_3^{2,2,7}G$ antibodies

Reinhard Luhrmann, Bernd Appel, Peter Bringmann, Jutta Rinke, Rolf Reuter and Susanne Rothe
Max-Planck-Institut für Molekulare Genetik, 1000 Berlin 33 (Dahlem), FRG, and

Rolf Bald
Institut für Biochemie (Fachbereich Chemie), Freie Universität Berlin, 1000 Berlin 33 (Dahlem), FRG

Received 28 September 1982; Accepted 18 October 1982

ABSTRACT

Antibodies specific for intact 2,2,7-trimethylguanosine ($m_3^{2,2,7}G$) were induced by immunization of rabbits with a nucleoside-human serum albumen (HSA) conjugate. Competition radioimmunoassay showed that the antibody distinguishes well between intact $m_3^{2,2,7}G$ and its alkali-hydrolysed form ($m_3^{2,2,7}G^*$). Antibody specificity is largely dependent on the presence of all three methyl groups in $m_3^{2,2,7}G$: none of the less extensively methylated nucleosides m^7G, m^2G and $m_2^{2,2}G$ is able to compete efficiently with the homologous hapten. Little or no competition was observed with m^1G, m^1A, m^6A, m^5U and each of the four unmodified ribonucleosides. Binding studies with nucleoplasmic RNAs from Ehrlich ascites cells suggest that the antibody reacts specifically with the $m_3^{2,2,7}G$-containing cap structure of the small nuclear U-RNAs (U-snRNAs). Thus the antibody should be a valuable tool for studying the role of the 5'-terminal regions of the U-snRNAs of eucaryotic cells.

INTRODUCTION

The highly modified nucleoside 2,2,7-trimethylguanosine ($m_3^{2,2,7}G$) is part of the cap structure present at the 5'-termini of the most abundant species of a group of metabolically stable small nuclear RNAs (U-snRNAs) of eucaryotic cells (1). Except for the snRNA-species U3, which is located in the nucleoli, the other snRNAs (U1,U2 and U4 to U6) are found predominantly in the nucleoplasm (2). The function of these snRNAs and thus the role of their unusual caps are not yet understood. However, the discovery that the nucleoplasmic snRNAs exist in discrete ribonucleoprotein complexes (snRNPs), and their coexistence with heterogenous nuclear RNPs (hnRNPs) suggests a role of snRNPs in processing of hnRNA (3,4). It has been proposed that the small RNAs might ensure the proper alignment of premessenger RNA sequences for splicing (5). The presence of the highly

0305-1048/82/1022-7103$2.00/0

methylated $m_3^{2,2,7}G$ in the snRNA caps provides a basis for the investigation of the functional importance of the 5' terminal regions of snRNAs by the use of $m_3^{2,2,7}G$-specific antibodies.

In this paper we describe the preparation of rabbit antibodies with high specificity for 2,2,7-trimethylguanosine. The antibody distinguishes well between the homologous hapten and its alkali-hydrolysed form. Little or no cross-reactivity was found with $m_2^{2,2}G$, m^1G, m^7G, m^6A and the four unmodified ribonucleosides. Binding experiments using the membrane filter binding technique suggest strongly that the antibody reacts specifically with snRNAs via the $m_3^{2,2,7}G$-moiety of their 5' terminal caps.

MATERIALS AND METHODS

Nucleosides and nucleotides.

2-Methylguanosine (m^2G) and 2,2-dimethylguanosine ($m_2^{2,2}G$) were prepared from 6-chloro-2-amino-9-β-D-ribofuranosylpurine via the 6-O-benzyloxy intermediate as described by Gerster and Robins (6). 2,2,7-Trimethylguanosine ($m_3^{2,2,7}G$) was synthesized by methylation of $m_2^{2,2}G$ with dimethyl sulphate (7). $[^{14}C]m_3^{2,2,7}G$ (specific activity: 58 Ci/mol) was prepared from $m_2^{2,2}G$ and ^{14}C-labeled dimethyl sulphate.

To obtain 2,2,7-trimethylguanosine-5'-phosphate, 2,2-dimethyl-guanosine-5'-phosphate ($pm_2^{2,2}G$) was methylated with dimethyl sulphate. $pm_2^{2,2}G$ was synthesized by phosphorylation of 2',3'-O-ethoxymethylen-2,2-dimethylguanosine at the 5' position by the β-cyanoethylphosphate procedure (8).

7-Methylguanosine was purchased from P-L Biochemicals, Inc. 6-Chloro-2-amino-9-β-D-ribofuranosylpurine was bought from Pharma Waldhof GmBH, Düsseldorf. Other nucleosides were from Sigma Chemical Co. Di[^{14}C]methyl sulphate (specific activity: 58 Ci/mol) was purchased from Amersham/Buchler, Braunschweig.

Preparation of $m_3^{2,2,7}G$-HSA conjugate

2,2,7-trimethylguanosine was conjugated to human serum albumen (HSA) via the periodate-oxidized nucleoside (9,10). To prevent alkali-catalysed fission of the imidazole ring, reduction of intermediate Schiff bases was performed with t-butylamine borane at pH 8.9 in the cold (11). The product was isolated by gel

filtration on Sephadex G25.

Immunization

Two randomly-bred rabbits were immunized at multiple intradermal sites with 4 mg of $m_3^{2,2,7}$-G-HSA in complete Freund's adjuvant on days 0, 21, 35 and 42. Six days after the final injection the rabbits were bled; thereafter they were bled 6 days after each monthly boost. The response was followed by radioimmunoassay.

Immunoglobulins (IgG) were purified from the sera by chromatography on Sephadex G150.

Radioimmunoassays

To measure binding of ^{14}C-labeled 2,2,7-trimethylguanosine, reaction mixtures containing 0.5 mg of a globulin fraction of antibody and 0.3 nmol of [^{14}C]$m_3^{2,2,7}$G (specific activity: 58 Ci/mol) in a total volume of 200 µl PBS (0.01 M KH_2PO_4, pH 7.5, containing 0.15 M NaCl) were incubated for 1 h at 0°C. Then 150 µl of an $(NH_4)_2SO_4$ solution (saturated in distilled water and adjusted to pH 7.5 at 4°C) were added, the mixture was vigorously mixed and allowed to stand at 4°C for 1 h. The suspensions were centrifuged for 15 min at 4°C and 10,000 xg and the supernatant was removed by aspiration. The pellet was resuspended in 200 µl of ice-cold $(NH_4)_2SO_4$ solution, mixed, recentrifuged, and drained as before. The washed pellets were dissolved in 250 µl concentrated formic acid and the mixture was diluted by addition of 2 ml distilled water and added to 10 ml of Instagel scintillation fluid. Radioactivity was measured in a liquid scintillation spectrophotometer.

Competitive radioimmunoassay was performed as described above except that antibody and inhibitor were preincubated for 30 min at 0°C before adding [^{14}C]$m_3^{2,2,7}$G.

Cell growth and labeling conditions

Ehrlich ascites cells were grown in suspension culture at 37°C in a medium consisting of Eagle's MEM (Flow Laboratory) supplemented with 5 % newborn calf serum, 50 µg/ml penicillin and 100 µg/ml streptomycin. Cell density was maintained at approximately 8 x 10^5 cells/ml by daily feeding. For preparation of [^{3}H]uridine-labeled RNA, cells were labeled with 10 µCi of [^{3}H]uridine/ml for 14 h.

Preparation of snRNAs

Nuclear and cytoplasmic fractions from Ehrlich ascites cells were obtained according to the procedure described by Zieve and Penman (2). To obtain nucleoplasmic RNAs intact nuclei were extracted with high-salt buffer (500 mM NaCl, 50 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4) at a density of 5 x 10^7 nuclei/ml by vigorous agitation on a Vortex mixer. Chromatin was separated from nucleoplasm by centrifugation at 12,000 rpm for 20 min in a Sorvall RC-3 with an HB4 rotor (12). The resulting supernatant is referred to in the Results section as "supernatant 1". The nucleoplasmic fraction was freed from high molecular weight RNAs and RNPs by centrifugation for 1 h at 45,000 rpm in a Ti60 rotor ("supernatant 2"). This supernatant was made 0.5 % in SDS and RNA was extracted with phenol and chloroform (12). The RNA-preparation predominantly contains the snRNA species U1, U2, U4 and U5 in addition to 5.8 S and 5S rRNAs as well as tRNAs (Fig.5, lane 2). This RNA preparation was used for binding studies with anti-$m_3^{2,2,7}G$ antibody (Figs. 6,7).

RESULTS

Preparation of $m_3^{2,2,7}G$-HSA conjugates

Since N-7 alkylated purine ribonucleosides are sensitive to alkali-catalysed fission of the imidazole ring (11,13) (Fig.1), special care was taken to prevent such degradation of $m_3^{2,2,7}G$ during preparation of the immunogen. For this purpose reaction of periodate-oxidized $m_3^{2,2,7}G$ with HSA and reduction of intermediate Schiff bases by t-butylamine borane (11) was performed at 4°C. Under these conditions hydrolytic ring fission of $m_3^{2,2,7}G$ is negligible (Fig.1). The difference spectrum of $m_3^{2,2,7}G$-HSA conjugate versus HSA (Fig.2) suggests that predominantly intact nucleoside is conjugated to the carrier protein (the spectrum of hydrolysed $m_3^{2,2,7}G$ differs significantly from that of the intact form). From the ratio of absorbance at 260 and 280 nm, respectively, the number of nucleoside residues bound per molecule of HSA was calculated to be eight (14).

Specificity for intact $m_3^{2,2,7}G$

Competitive radioimmunoassays showed that antibodies were able to distinguish well between intact and alkali-degraded

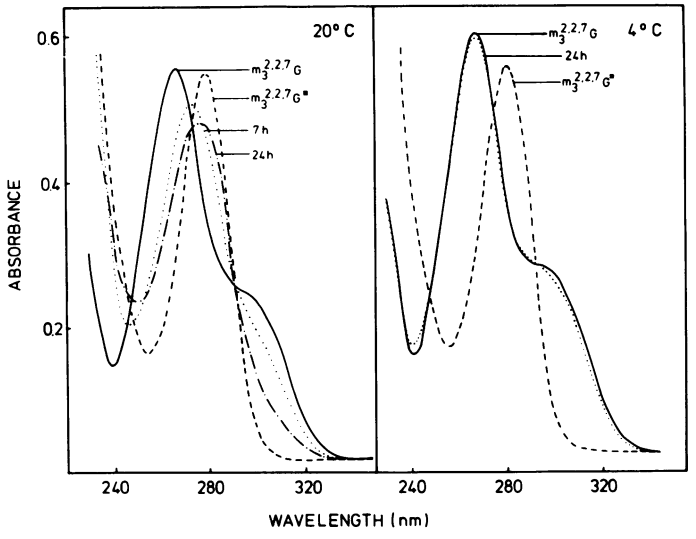

Fig. 1: Alkali sensitivity of $m_3^{2,2,7}G$ at pH 8.9 and 20°C and 4°C. A solution containing 0.2 mg $m_3^{2,2,7}G$ in 200 µl 0.3 M $NaHCO_3$, pH 8.9, was incubated at 20°C (left-hand panel) or 4°C (right-hand panel). At the indicated time intervals 20 µl aliquots were withdrawn and diluted 1:100 in 0.3 M sodium acetate buffer (pH5). A control sample of $m_3^{2,2,7}G$ was diluted directly into pH 5 buffer. To obtain the imidazole ring-opened form $m_3^{2,2,7}G^*$ an appropriate aliquot of $m_3^{2,2,7}G$ was hydrolysed for 24 h at room temperature with an equal volume of 2 N NaOH and then neutralized with 2N HCl. (Thin-layer chromatography on cellulose coated acetate TLC sheets revealed quantitative ring fission under these conditions). This solution was then diluted 1:100 in the pH 5 buffer.

$m_3^{2,2,7}G$. While 0.3 nmol $m_3^{2,2,7}G$ inhibited binding by 50 %, 200 nmol of ring-opened nucleosides was required to produce the same effect (Fig.3), the ratio being about three orders of magnitude. If the concentration of $m_3^{2,2,7}G^*$ was further increased, more than 90 % of the $m_3^{2,2,7}G$-binding IgG was capable of complex formation with the ring-opened form (Fig.3). This indicates true cross-reactivity of $m_3^{2,2,7}G$-specific antibody with the hydrolysed nucleoside. The high apparent affinity of the antibody for $m_3^{2,2,7}G$ is largely determined by the presence of the three methyl groups on the purine ring. Concentrations of the mono- or dimethylated nucleosides m^2G, m^7G and $m_2^{2,2}G$ between 10^4 and 10^5 times higher were necessary to produce the amount of inhibition produced by $m_3^{2,2,7}G$ (Fig.3).

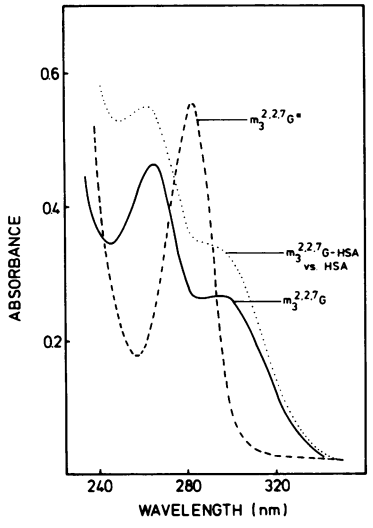

Fig. 2: UV spectrum of immunogen $m_3^{2,2,7}G$-HSA. $m_3^{2,2,7}G$-HSA and HSA were dissolved in PBS, pH 7.4, each to a concentration of 0.35 mg/ml. Appropriate dilutions of $m_3^{2,2,7}G$ as well as $m_3^{2,2,7}G^*$ (prepared as described in Fig.1) were made in the same buffer.

Little or no competition was observed by other methylated nucleosides (m^1G, m^1A, m^6A or m^5U) as well as by the unmodified ribonucleosides (Table 1), which underlines the high specificity of the antibody for $m_3^{2,2,7}G$.

Binding of antibody to $pm_3^{2,2,7}G$

One of the potential biological applications of an $m_3^{2,2,7}G$-specific antibody is to study capped snRNAs. For this reason it wasimportant to eliminate the possibility that binding by the antibody might be prevented simply by neighbouring negatively charged phosphate groups. Since preparative factors precluded the study of isolated snRNA caps, 2,2,7-trimethyl-guanosine-5'-

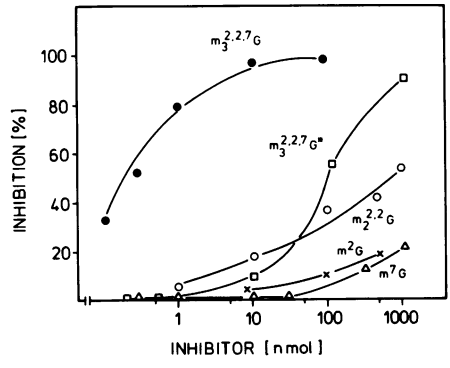

Fig. 3: Competitive radioimmunoassay with antiserum against $m_3^{2,2,7}G$-HSA. Assays were performed as described in Materials and Methods.

Table 1: Inhibition of binding of [^{14}C]$m_3^{2,2,7}$G to $m_3^{2,2,7}$G-specific antibodies by nucleosides.

Inhibitor	nmol inhibitor	% inhibition
$m_3^{2,2,7}G$	0.3	50
m^1A	1000	5
m^6A	950	0
m^1G	900	5
m^5U	1000	0
Uridine	1000	0
Cytidine	1100	10
Guanosine	1000	5
Adenosine	900	12

Competitive radioimmunoassay was performed as described in Materials and Methods.

phosphate had to suffice for this. Competitive radioimmunoassay showed that only 10-15 times more of $pm_3^{2,2,7}$ is necessary for 50% inhibition as compared with the nucleoside $m_3^{2,2,7}$G (Fig.4). Inhibition reached more than 90 % when $pm_3^{2,2,7}$G was present at higher concentrations. Thus 5'-adjacent phosphate groups do not abolish binding by the antibody, but do decrease to some extent the antibody's apparent affinity for the $m_3^{2,2,7}$G-moiety.

Reaction of antibody with snRNAs from Ehrlich ascites cells

Reactivity of antibodies towards snRNAs was studied with a nucleoplasmic RNA preparation from Ehrlich ascites cells which

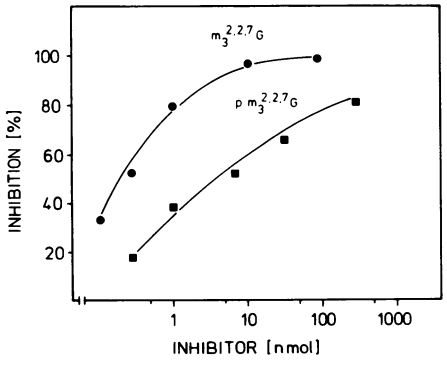

Fig. 4: Inhibition of binding of [^{14}C]$m_3^{2,2,7}$G to $m_3^{2,2,7}$G-specific antiserum by $pm_3^{2,2,7}$G. Competitive radioimmunoassay as described in Materials and Methods.

contained predominantly the snRNA species U1, U2, U4 and U5 in addition to the low molecular weight RNAs and some tRNAs (Fig.5, lane 2). The RNA preparation was essentially free of high molecular weight RNA (Fig.5). This was obtained by differential centrifugation of nucleoplasmic RNPs (see Materials and Methods). Complex formation between RNAs and antibodies was studied by the membrane binding technique (15). Anti-$m_3^{2,2,7}G$ antibody retained about 70 % of the radioactivity from [^{3}H]uridine-labeled RNAs on the filter (Fig.6). For comparison IgGs from pre-immune serum retained only 10-15 % of the radioactivity at all concentrations (Fig.6). Most importantly, complex formation between RNA and anti-$m_3^{2,2,7}G$ was effectively inhibited by $m_3^{2,2,7}G$ (Fig.7). As a control adenosine, which is not recognized by the antibody, did not inhibit at all (Fig.7). This shows that binding of most of the RNA occurs via an $m_3^{2,2,7}G$-moiety. The radioactivity from complexes insensitive even to high concentrations of $m_3^{2,2,7}G$ roughly equals the amount of radioactivity which was retained from the RNAs by IgGs from pre-immune serum (compare Figs. 6 and 7). Thus

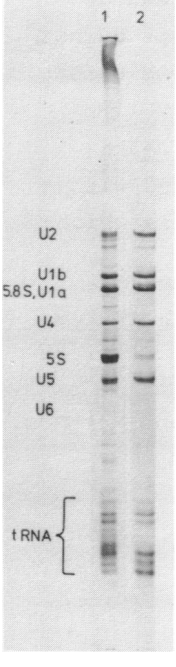

Fig. 5: Gel fractionation of snRNAs from Ehrlich ascites cells. Extraction, fractionation by differential centrifugation and deproteinization of nucleoplasmic RNPs from Ehrlich ascites cells was performed as described in Materials and Methods. RNAs were separated on 10 % polyacrylamide gels containing 7M urea, 1 mM EDTA and 90 mM Tris-borate, pH 8.3 (5). RNAs were visualized by staining with "stains all" (19). Lane 1 shows total RNAs from high salt buffer extracts of nuclei (RNAs from supernatant 1, Materials and Methods). RNAs in lane 2 were obtained from the high speed supernatant of HSB-extracted nucleoplasmic RNPs (RNAs from supernatant 2, Materials and Methods). The latter RNA preparation was used for binding studies with anti-$m_3^{2,2,7}G$ antibody.

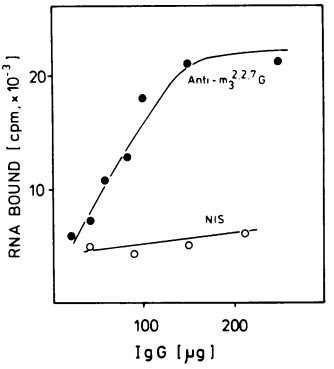

Fig. 6: Specific binding of low molecular weight nucleoplasmic RNAs by anti $m_3^{2,2,7}G$ antibody. Reaction mixtures (200 µl) containing 30.000 cpm of [^{3}H]uridine-labeled nucleoplasmic RNAs (prepared as described in Materials and Methods and Fig.5) and the indicated amounts of antibodies in buffer A (10 mM $MgCl_2$, 20 mM Tris-Cl, pH 7.5, 100 mM KCl) were incubated for 2 min at 37°C and put on ice. After 10 min the solutions were diluted with 2 ml of ice-cold buffer A and filtered through nitrocellulose membranes (0.45 µm). The filters were washed once with 1 ml of buffer A, dried and counted. ● - ●, IgGs from $m_3^{2,2,7}G$-specific serum; o - o, IgGs from preimmune serum.

this minor fraction of complexes may be considered to be caused by unspecific RNA-protein interactions.

Taking into account that snRNAs are the only molecules present in our RNA preparation containing $m_3^{2,2,7}G$ (16) it may be concluded from our data that the anti-$m_3^{2,2,7}G$ antibody reacts with the cap structures of intact snRNAs.

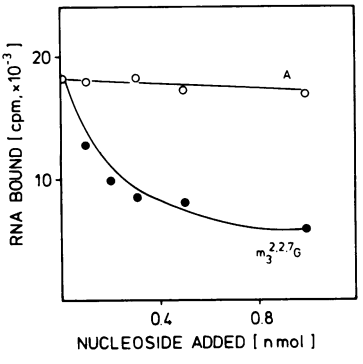

Fig. 7: Binding of RNAs by anti-$m_3^{2,2,7}G$ occurs via an $m_3^{2,2,7}G$-moiety. Competition assays were performed as described in Fig.6, except that the reaction mixtures contained 100 µg of anti-$m_3^{2,2,7}G$ and were preincubated with the indicated amount of nucleoside for 10 min at 37°C before adding the RNA. ● - ●, $m_3^{2,2,7}G$; o - o, adenosine.

DISCUSSION

The high specificity of the anti-$m_3^{2,2,7}G$ antibody described in this report is largely determined by the three methyl groups on the purine ring. The loss of only one or two of these methyl substituents reduce the apparent affinity of the antibody for the respective nucleosides ($m_2^{2,2}G$, m^7G, m^2G) by several orders of magnitude (Fig.3). The superior behaviour of $m_2^{2,2}G$ in competitive radioimmunoassays, as compared with m^7G, suggests that the dimethylated amino group contributes more to the strength of antibody-nucleoside complexes than does the N^7-methyl group. An intact purine ring is further required for high apparent affinity of the antibody for the homologous hapten as demonstrated by the poor recognition of the alkali-catalysed hydrolysis product of $m_3^{2,2,7}G$ (Fig.3).

One of our interests in the $m_3^{2,2,7}G$-specific antibody is to study the accessibility of the 5' termini of snRNAs either isolated or associated with hnRNPs. In this respect it is of special importance that the antibody shows only little or no cross-reactivity with m^7G and m^6A (Fig.3, Table 1), nucleosides which are present in the 5'-terminal caps of the hnRNAs or are found internally (17).

A further prerequisite for the usefulness of this antibody for such studies is the retention of high apparent affinity for $m_3^{2,2,7}G$ when the latter is a part of the cap in intact snRNA. A decrease in efficiency of binding to the m^7G-moiety of mRNA caps as compared with the free nucleoside was most probably responsible for failure to isolate mRNA by affinity chromatography with m^7G-specific antibodies (18). Fortunately such a drawback was not observed for the anti-$m_3^{2,2,7}G$ antibody. In competitive radioimmunoassays $pm_3^{2,2,7}G$ was only 10-15 times less efficient as an inhibitor as compared with the nucleoside $m_3^{2,2,7}G$ (Fig.4). Further, convincing evidence is provided by the membrane filter binding experiments: complex formation of the antibody with the $m_3^{2,2,7}G$-cap structure is strong enough to retain the snRNAs on the nitrocellulose filters (Figs. 6 and 7). Thus the antibody should be a valuable tool not only for studying the functional role of the 5'-terminal regions of snRNA but also for the isolation of snRNAs by immune affinity chromatography.

ACKNOWLEDGEMENT

This work was supported by grants from the Deutsche Forschungsgemeinschaft.

REFERENCES

1 Reddy, R. and Busch, H. (1978) In: The Cell Nucleus, Busch, H., Ed., Vol. VIII, pp. 261-305, Academic Press, New York.
2 Zieve, G. and Penman, S. (1976) Cell 8, 19-31.
3 Lerner, M.R. and Steitz, J.A. (1979) Proc.Natl.Acad.Sci.USA 76, 5495-5499.
4 Gallinaro, H. and Jacob, M. (1979) FEBS Lett. 104, 176-182.
5 Lerner, M.R., Boyle, J.A., Mount, S.M., Wolin, S.L. and Steitz, J.A. (1980) Nature 283, 220-224.
6 Gerster, J.F. and Robins, R.K. (1965) J.Am.Chem.Soc.87, 3752-3759.
7 Jones, J.W. and Robins, R.K. (1963) J.Am.Chem.Soc. 85,193-201.
8 Teneer, G.M. (1961) J.Am.Chem.Soc. 83, 159-168.
9 Erlanger, B.F. and Beiser, S.M. (1964) Proc.Natl.Acad.Sci. USA 52, 68-74.
10 D'Alisa, R.M. and Erlanger, B.F. (1974) Biochemistry 13, 3575-3579.
11 Rainen, L. and Stollar, B.D. (1978) Nucleic Acids Res. 5, 4877-4889.
12 Zieve, G. and Penman, S. (1981) J.Mol.Biol. 145, 501-523.
13 Townsend, L.B. and Robins, R.K. (1963) J.Am.Chem.Soc. 85, 242-243.
14 Inouye, H., Fuchs, S., Sela, M. and Littauer, U.Z. (1971) Biochim.Biophys.Acta 240, 594-603.
15 Van Vunakis, H. and Levine, L. (1977) in Immunoassays for Drugs Subject to Abuse, Mulé, S.J., Sunshine, I., Brande, M. and Willett, R.E., Eds., pp. 23-35 (CRC Press Cleveland, Ohio).
16 Sapanora, A.G. and Enger, M.D. (1969) Nature 223, 1365-1366.
17 Salditt-Georgieff, M., Jelinek, W., Darnell, J.E., Furnichi, Y., Morgan, M. and Shatkin, A. (1976) Cell 7, 227-237.
18 Munns, T.W., Oberst, R.J., Sims, H.F., and Liszweski, M.K. (1979) J.Biol.Chem. 254, 4327-4330.
19 Dahlberg, A.E., Dingman, C.W. and Peacock, A.C. (1969) J.Mol.Biol. 41, 139-147.