Methylated, Blocked 5' Termini of Yeast mRNA*

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mRNAs of the yeast, Saccharomyces cerevisiae, have modified 5' termini similar but not identical with those present in the higher eukaryotes. Poly(A)-containing RNA isolated from spheroplasts or from polyribosomes of cells labeled with [methyl-3H]methionine or [3H]p, contain methylated, blocked 5'-terminal structures of composition: m'G(5')pppXp Ap and m'G(5')pppGp, occurring in a relative distribution of 75% and 25%, respectively. Furthermore there is no N*-methyladenosine nor any other methylated nucleoside in the entire molecule of poly(A)-containing RNA. Experiments with a mutant, temperature-sensitive for the transport of mRNA to cytoplasm, suggest that the methylation and addition of the blocking group take place in the nucleus and that the genetic lesion in the mutant does not affect these mechanisms.

The addition of blocking groups at the 5' end of mRNA, and their methylation, have recently been recognized as a general feature in the formation of mRNA in mammalian cells and their viruses (1-12). In mammalian cells these modified 5'-terminal structures are of the type: m'G(5')pppXp Ap in which X and often Y are 2'-O-methylribonucleosides containing any of the four bases (8-11). The 5' terminus of the mRNA of animal viruses is m'G(5')pppXppXp where X is limited to A or G (3-6, 12), while the two plant viruses, TMV (13) and BMV (14) contain a similar structure in which X is unmethylated guanosine. A common feature in all cases is the presence of m'G(5')p attached by a pyrophosphate linkage through a middle phosphate residue to the 5'-terminal nucleotide of the mRNA chain. These dinucleotide structures, by virtue of the pyrophosphate linkage between the two 5'-nucleotides, are resistant to the action of nucleases and alkaline phosphatase, and thus can be readily isolated and analyzed by paper electrophoresis.

In addition to these methylated modified structures, cellular mRNA (7, 9-11, 15) and the mRNA of a DNA virus which multiplies in the nucleus (9) contain at least 1 residue of N*-methyladenosine per molecule, located near the 5' end. While the terminal methylation in the 5' structure has been shown to be essential for ribosome binding of mRNA (16, 17), the other methylations are thought to be important in the processing of potential mRNA molecules (2, 15).

In hopes of using the genetically tractable yeast, Saccharomyces cerevisiae, to study mRNA synthesis, we have looked for modified 5' termini on its mRNA. As will be described in this report, yeast mRNA molecules do contain modified 5' termini, which are similar but not identical with those found in the higher eukaryotes. This structure therefore is likely to be a common feature of all eukaryotic mRNA.

EXPERIMENTAL PROCEDURES

Strains—The parent strain of Saccharomyces cerevisiae, A364A, gal-1 ade-1 ade-2 ade-1 his-7 lys-2 try-1 (ATCC 22244) was obtained from Dr. L. H. Hartwell, University of Washington, Seattle. The isolation and preliminary characterization of the temperature-sensitive mutants ts 136 and ts 386 have been described (18, 19).

Growth Conditions and Radioactive Labeling—For labeling with [methyl-3H]methionine, cells were grown for several generations to a concentration of 1 × 10^7/ml in synthetic medium (20). The cells were then converted to spheroplasts with snail enzyme (Glusulase, Endo Laboratory, New York) as previously described (21). The suspension was centrifuged for 6 min at 2,500 × g at 23° and the spheroplasts were resuspended at a concentration of 1 × 10^7/ml in synthetic medium supplemented with 1 M sorbitol to provide osmotic support. These spheroplasts were incubated for 5 min at 37° with [methyl-3H]methionine (specific activity 6.7 Ci/mmol; New England Nuclear) and washed twice in cold 1 M sorbitol.

For labeling with [3H]p, cells were grown at 23° in broth free of inorganic phosphate (22) to a concentration of 1 to 2 × 10^7/ml.

* The abbreviations used are: A, 2'-O-methyladenosine; G, 2'-O-methylguanosine; C, 2'-O-methylcytosine; U, 2'-O-methyluridine; m'G, 7-methylguanosine; N*-m, N'-methyladenosine; N, nucleoside; Pm, Penicillium nucleose; TMV, tobacco mosaic virus; BMV, brome mosaic virus; VSV, vesicular stomatitis virus; CPV, cytoplasmonic polyhedrosis virus; SV40, Simian virus 40; Pipes, pipazine N,N'-bis(2-ethanesulfonic acid); SDS, sodium dodecyl sulfate; hRNA, heterogeneous nuclear RNA.
Carrier-free \(^{3}H\)P, (New England Nuclear) was added to a final concentration of 200 \(\muCi/ml\). After 20 min, the culture was poured over crushed ice and the cells were collected by centrifugation. The cells were suspended in thioglycollate/Tris (0.5 sodium thioglycollate/0.1 M Tris, pH 8.8) and after 30 min at 2\(\(^{\circ}\)C, 5 volumes of cold \(H_{2}O\) was added to the suspension. Cells were collected by centrifugation, and washed once with cold \(H_{2}O\), suspended in cold 1 M sorbitol, and converted to spheroplasts at 4\(\(^{\circ}\)C by treatment with 2% glusulase for 45 min.

**Preparation of Polysomes**—All operations were carried out at 2-4\(\(^{\circ}\)C. Washed spheroplasts were resuspended at a concentration of 1.3 to 2 \(\times\) 10\(^{8}\) cells/ml in Buffer A (10 mM NaCl/5 mM MgCl\(_2)/10\) mM Pipes/1 mM EDTA/LiCl/0.2% SDS, containing 0.5% SDS, and reprecipitated with ethanol three times. The RNA and proteins were precipitated at -20\(\(^{\circ}\)C with 2.5 volumes of 95% ethanol. RNA was recovered by centrifuging the aqueous phase and RNA was precipitated at -20\(\(^{\circ}\)C with 2.5 volumes of 95% ethanol.

**Extraction of RNA**—Washed spheroplasts or the ethanol precipitated samples from the polysome gradients were dissolved in T&J (pH 5.0) was added and the RNA was precipitated with ethanol. The RNA and proteins were precipitated at -20\(\(^{\circ}\)C with 2.5 volumes of 95% ethanol.

**Fractionation of RNA**—The RNA was separated into poly(A)-containing and non-poly(A)-containing fractions by chromatography on oligo(dT)-cellulose (No. T-2, Collaborative Research, Waltham, Mass.). DNA samples were dissolved in a 0.5 M LiCl/0.2% SDS solution and then extracted three times with an equal volume of phenol/chloroform/isoamyl alcohol (96/4). One-tenth volume of 5 x~ LiCl was added to the aqueous phase and RNA was precipitated at -20\(\(^{\circ}\)C with 2.5 volumes of 95% ethanol. RNA was recovered by centrifuging the suspension at 20,000 \(x\) g, dissolved in sodium acetate 0.15 M (pH 5.0) containing 0.05% SDS, and reprecipitated with ethanol three times.

**Treatment of RNA with Enzymes**—For combined digestion with *Penicillium nigrum* (P, nuclease, Yamasa Shoyu Co.) and bacterial alkaline phosphatase (Worthington Biochemical Corp.). RNA was dissolved in 100 to 150 \(\mu\)l of 10 mM sodium acetate buffer (pH 6.0) and incubated with P, nuclease (1 mg/ml) for 60 min at 37\(\(^{\circ}\)C. The pH of the mixture was adjusted to 8.0 with 1 M Tris base and incubation was continued for 60 min with bacterial alkaline phosphatase. One unit of enzyme was added four times at 15-min intervals. For RNase T\(_{1}\), (Calbiochem) digestion, RNA was dissolved in 100 \(\mu\)l of 0.05 M sodium acetate buffer (pH 4.5) and incubated 15 hours at 37\(\(^{\circ}\)C with 5 units of the nuclease. Digestion with nucleotide pyrophosphatase (Sigma Chemical) was carried out at 37\(\(^{\circ}\)C in 50 mM Tris-Cl buffer (pH 7.5) containing 2 mM MgCl\(_2\) and 0.3 unit of the enzyme/ml.

**Chromatography and Paper Electrophoresis**—DEAE-cellulose chromatography in 7 M urea was carried out on column (0.7 \(\times\) 22 cm) equilibrated with 50 mM Tris-Cl buffer (pH 7.6) containing 7 M urea and 5 mM MgCl\(_2\). Samples (0.5 to 1 ml) were applied together with tRNA hydrolysates and eluted with a linear gradient (100 ml) of NaCl (0.005 to 0.25 M in 50 mM Tris-Cl buffer (pH 7.6) containing 7 M urea). Absorbance at 260 nm was monitored and the radioactive content of each fraction was measured in Aquasol (New England Nuclear, Boston, Mass.).

The RNA and proteins were precipitated at -20\(\(^{\circ}\)C with 2.5 volumes of 95% ethanol.

**Results**

**Nucleoside- and Phosphatase-resistant 5'-Terminal Structures**—Radioactivity is present in poly(A)-containing RNA isolated from spheroplasts exposed to [methyl-\(^3\)H]-methionine for 10 min. (This strain is auxotrophic for purines and thus cannot incorporate methyl groups into the purine ring.) This [\(^3\)H]-methylated RNA was digested with nuclease P, from *Penicillium citrinum* (which cleaves phosphodiester linkages in polynucleotides, including those containing 2'-O-methylated residues, to yield 5'-nucleotides) (23) followed by bacterial alkaline phosphatase, and the product was analyzed by high voltage paper electrophoresis. As shown in Fig. 1a, about one-third of the radioactivity in the digest migrated towards the anode as two distinct peaks (I and II) intermediate between the AMP and GMP markers. The remaining radioactivity migrated towards the cathode in spots corresponding to all four ribonucleoside markers. From the specificity of P, and bacterial alkaline phosphatase, peaks I and II must be dinucleotides connected through a pyrophosphate linkage. By analogy with the 5'-terminal structures in mRNA of viruses and mammalian cells, peaks I and II probably arise from the 5' end of poly(A)-terminated RNA molecules. In order to identify their methylated nucleosides, peaks I and II were eluted, digested with nucleotide pyrophosphatase to liberate the mononucleotides, and again analyzed by paper electrophoresis. The radioactivity from peak I migrated as a spot corresponding to mGMP except for a trace of mG (Fig. 1b). The radioactivity from peak II also migrated as mGMP with a trace of mG (Fig. 1c).

The small amount of mG observed is likely to be due to the dephosphorylation of mGMP by traces of phosphomonoesterase activity present in the nucleotide pyrophosphatase preparations used. When the nucleotide pyrophosphatase digestion was followed by treatment with bacterial alkaline phosphatase, before electrophoresis, all the radioactivity from both peaks I and II migrated in a single spot identified as mG (Fig. 2). Thus, in both the presumptive 5'-terminal structures, methylated radioactivity is present exclusively in mGMP. Since peaks I and II have the same methylated compound, they must differ either in the number of phosphates in the pyrophosphate linkage or in other bases.

**Number of Blocked 5'-Terminal Phosphates**—To determine the number of phosphate residues present in the P, and bacterial alkaline phosphatase-resistant dinucleotides, material from peaks I and II (Fig. 1a) was eluted, mixed with oligonucleotide markers derived from RNase digestion of tRNA, and analyzed by DEAE-cellulose chromatography under conditions where nucleotides are eluted according to their negative charge. As shown in Fig. 3, radioactivity from both peak I and peak II eluted in the same position, between marker nucleotides of charge -2 and -3. P, and bacterial alkaline phosphatase-resistant dinucleotides having mG and a net charge of -2.5 must have 3 phosphate residues, each phosphate contributing 1 negative charge and the mG \(^{3}\) a positive charge. Hence, both dinucleotides have a structure, mG\(^{5}\)dGppN. Their difference can only be in the identity of the unmethylated nucleosides, N.
Second Nucleotide in 5'-Terminal Structures—In order to identify the nucleotides present in pyrophosphate linkage in the P-I and bacterial alkaline phosphatase-resistant 5' termini, 32P-labeled poly(A)-containing RNA was isolated from yeast cultures incubated with 32P for 20 min and digested with RNase T1. Resulting mononucleotides and oligonucleotides were separated by DEAE-cellulose chromatography at pH 7.6 in 7 M urea as shown in Fig. 4. Most of the radioactivity eluted as mononucleotides of charge -2 and a small fraction as a second prominent peak of charge about -4.5. Absence of 32P at positions corresponding to a charge of -5 and above indicates the absence of structures of the type m7G(5')pppXm'p and m7G(5')pppXm'pYm'p in the poly(A)-containing RNA of yeast. Fractions containing the second peak were pooled as indicated, desalted, and digested with nuclease P1 followed by bacterial alkaline phosphatase. The product was analyzed by paper electrophoresis (Fig. 5a). Negatively charged, nuclease- and bacterial alkaline phosphatase-resistant 32P-labeled dinucleotides migrated in two spots (peaks I and II) identical in position with those observed with enzyme-digested [3H]methyl-labeled RNA (Fig. 1a) between the markers, pA and pG. Material from peaks I and II was eluted, treated with nucleotide pyrophosphatase and analyzed further by paper electrophoresis. About 25% of the 32P from peak I migrated as m7GMP, 25% as AMP, and the rest as Pi (Fig. 5b). Analysis of peak II yielded 25% of the 32P as m7GMP and 25% as GMP, the rest being Pi (Fig. 5c). Thus, yeast poly(A)-containing RNA has blocked 5' termini of two different compositions: I, m7G(5')pppAp and II, m7G(5')pppGp. Since cells were labeled for only a few minutes in this experiment, the excess of Pi observed is at least partly due to the fact that the middle phosphate comes from the β-phosphate of a nucleoside triphosphate, which would have a higher specific activity than the phosphate residues in the α position.
Methylated Nucleoside Residues at other Positions In mRNA Molecule—Identification of other methylated nucleosides in the mRNA molecule requires isolation of poly(A)-containing RNA completely free from ribosomal RNA, which is highly methylated in yeast (26). However, poly(A)-containing RNA isolated by affinity chromatography from yeast always contains a trace of ribosomal RNA. In an attempt to overcome this problem, a mutant strain ts 368, was used. This mutant is temperature-sensitive for the formation of ribosomal RNA (18). At the nonpermissive temperature, 36°, mutant cells transcribe and methylate 35 S precursor RNA but fail to process it to 25 S and 18 S mature rRNA (27). In contrast, messenger RNA formation is unaffected.

Spheroplasts of ts 368, at 36°, were labeled for 10 min with [methyL3H]methionine. Spheroplasts of wild type cells were similarly labeled at 23°. From each culture, the cytoplasmic fraction was prepared, and polysomes isolated on a sucrose gradient were used for the preparation of poly(A)-containing RNA. Electrophoretic analysis of nuclease P1 and bacterial alkaline phosphatase digests of [3H]methyl-labeled poly(A)-containing RNA preparations are shown in Fig. 6. A majority of the radioactivity from the poly(A)-containing RNA digests from wild-type cells migrated in two spots (peaks I and II, Fig. 6a) corresponding to the 5' termini already characterized. The remaining radioactivity migrated as four positively charged spots corresponding to the four ribonucleoside markers. Three lines of evidence suggest that these methylated nucleosides arise not from mRNA but from contaminating rRNA. (a) There are no methylated nucleosides in the mRNA isolated from ts 368 cells labeled at the restrictive temperature (Fig. 6b), conditions in which no rRNA is formed. (b) The ratio of methylated adenosine to methylated cytosine seen in Fig. 6a is the same as that found in ribosomal RNA which is not bound to poly(dT). (c) The only methylated nucleoside found in the chain of mRNA molecule of mammalian cells is N6-mA (7, 9-11, 15). From the data of Fig. 6a it is clear that there is less than 1 methylated adenosine for every 3 m'G. Therefore, we conclude from Fig. 6 that the only methyl group in the mRNA of yeast is the m'G at the 5' terminus.

Ratio of m'G(5')pppA to m'G(5')pppG—We have now identified two modified 5' termini in mRNA molecules labeled either with "P or with [methyl-3H]methionine. Table I shows the relative amounts of m'G(5')pppA and m'G(5')pppG in RNA isolated after several labeling and cell fractionation protocols. It is clear that the ratio of m'G(5')pppA to m'G(5')pppG is about 3 in all situations studied, including the non-poly(A)-containing RNA isolated from polysomes. Thus, there are two classes of mRNA molecules in yeast, 75% terminated with m'G(5')pppAp, and 25% terminated with m'G(5')pppGp.

Fig. 4. Column chromatography of RNase T1 digests of 32P-labeled poly(A)-containing RNA. Poly(A)-containing RNA isolated from cells incubated with [32P] for 20 min at 23° was digested with RNase T1 and analyzed by DEAE-cellulose chromatography as described under "Experimental Procedures." The positions of oligonucleotide markers of net charge -2 to -5 determined by absorbance at 260 nm are indicated by arrows. The region comprising fractions 54 to 66 was pooled as indicated for subsequent analysis described in Fig. 5.

Fig. 5. Electrophoretic analysis of products of digestion of 32P-labeled oligonucleotide from DEAE-cellulose column described in Fig. 4. Fractions 54 to 66, eluted from DEAE-cellulose column as described in Fig. 4, were pooled, desalted, digested with enzymes, and analyzed by paper electrophoresis. a, analysis of products of digestion with Penicillium nuclease and alkaline phosphatase. b, analysis of nucleotide pyrophosphatase digests of material eluted from peak I in panel a; c, analysis of peak II material as in b. The arrows indicate the position of radioactive markers. Marker m7GMP is a spot detected by its blue fluorescence under ultraviolet light.
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Fig. 6. Analysis of methylated constituents of poly(A)-containing RNA. Poly(A)-containing RNA was isolated from polysomes prepared from spheroplasts of wild-type cells labeled with [methyl-3H]methionine for 10 min at 20° (a) and of to 686 cells labeled for 10 min at 36° 60 min after being shifted to the nonpermissive temperature (b). The two samples were digested with Penicillium nuclease and alkaline phosphatase and analyzed by paper electrophoresis as described under "Experimental Procedures." The arrows indicate the positions of radioactive markers. Marker m'G is a spot detected by its blue fluorescence under ultraviolet light.

5' Termini in mRNA of ts 136—Hutchison et al. (10) isolated a temperature-sensitive mutant, ts 136, in which, at the nonpermissive temperature, poly(A)-containing RNA continues to be made but is not transported to the cytoplasm (28). In order to determine if the modified 5' termini could be involved in this mutant phenotype, 32P-labeled poly(A)-containing RNA was isolated from cells exposed to azP, for 20 min at the permissive and nonpermissive temperatures. The RNA was digested with RNase T, and mono- and oligonucleotides were separated by DEAE-cellulose chromatography. Oligonucleotide fractions (identical with Fractions 54 to 66 shown in Fig. 4) were desalted, digested with nuclease P, and bacterial alkaline phosphatase, and the 5' termini were analyzed. Table II shows that poly(A)-containing RNA made at both permissive and nonpermissive temperatures contain methylated, blocked 5' termini and that the ratio of °P in termini to °P in mononucleotides of the poly(A)-containing RNA formed at both temperatures is identical. Thus the genetic lesion in the mutant does not affect the mechanisms of methylation nor addition of m'G to mRNA. Furthermore, since the bulk of the poly(A)-containing RNA present in the mutant at nonpermissive temperature is nuclear RNA, we conclude that the 5'-5' addition of GTP to poly(A)-containing RNA, as well as its methylation, take place in the nucleus.

DISCUSSION

5' Termini of Yeast mRNA—The evidence presented above demonstrates that the 5' termini of yeast mRNAs contain m7G(5')pppA and m'G(5')pppG in a ratio of 3:1. No other methylated residues were observed in poly(A)-containing RNA under conditions where 1 methyl group per 40 molecules would have been detected. The methylated structures of yeast mRNA are compared with those of other organisms in Table III. There are several striking points.

1. All mRNA molecules translated in eukaryotic cells seem to have 5' termini blocked with m7G(5')ppp. The addition of the primitive eukaryote, yeast, to this list substantiates the suggestion that methylated modifications of 5'-termini are required for the translation of mRNA on eukaryote ribosomes. The fact that m'G is the only methylated residue in the yeast

| RNA                        | Source | m7G(5')pppA | m7G(5')pppG | m7G(5')pppA/m7G(5')pppG |
|----------------------------|--------|-------------|-------------|--------------------------|
| [3P] Poly(A) RNA           | Whole cell | 27500     | 9000       | 3.1                      |
| [3H]methyl Poly(A) RNA      | Whole cell | 39800     | 13300      | 2.9                      |
| [3H]methyl Poly(A) RNA      | Polysomes (>90 S) | 1600     | 500        | 3.2                      |
|                            | Top of gradient (>90 S) | 405      | 130        | 3.1                      |
|                            | Polysomes (ts 368,36') (>90 S) | 3380     | 991        | 3.4                      |
|                            | Top of gradient (ts 368,36') | 744      | 248        | 3.0                      |
| [3H]methyl Poly(A) RNA      | Polysomes (ts 368,36') | 1110     | 335        | 3.1                      |

Table II

Ratio of °P in 5'-blocked termini to total mononucleotides of poly(A)-containing RNA

**P-labeled poly(A)-containing RNA was isolated from cells of strain ts 136 incubated with °P, for 20 min at 23° or for 20 min at 36° 10 min after being shifted to the nonpermissive temperature. The RNA samples were digested with RNase T, and the mono- and oligonucleotides were separated by DEAE-cellulose chromatography as described under "Experimental Procedures" and in Fig. 4. The 5' termini, m7G(5')pppA and m'G(5')pppG, corresponding to peaks I and II, were obtained from the oligonucleotide fraction as described in Fig. 5a.

| Poly(A)-containing RNA | Np | m7G(5')pppA | m7G(5')pppG | m7G(5')pppA/m7G(5')pppG |
|------------------------|----|-------------|-------------|--------------------------|
| Mutant ts 136 at 23°   | 11600 | 95        | 3.0         | 0.0082                   |
| Mutant ts 136 at 36°   | 1060 | 9.3       | 9.3         | 0.0088                   |

Table I

Ratio of m7G(5')pppA to m7G(5')pppG in yeast RNA

RNA was isolated from spheroplasts or from fractions of sucrose gradients, digested with Penicillium nuclease followed by alkaline phosphatase, and products were separated by paper electrophoresis as described under "Experimental Procedures." The 5' termini, m7G(5')pppA and m'G(5')pppG, were from peaks I and II, respectively, from experiments identical with those described in Figs. 1, 5, and 6.
mRNA, also supports the view that N₄-methylation of guanosine at the 5'-termini alone is sufficient to satisfy this requirement for translation (16).

2. The mRNA of all mammalian cells have ribose-methylated nucleosides in the first and often in the second position adjacent to m₇G, whereas animal viruses have such methylations only at the first position. Furthermore, unmethylated 5'-terminal structures of mRNA synthesized in vitro can be methylated by cell free extracts in the presence of S-adenosyl methionine. In this reaction, it is interesting to note that wheat germ extracts are capable of methylating only position 7 of the 5'-terminal guanosine, whereas extracts of mouse L-cells can methylate both guanosines and the 2'-OH of the second nucleotide in the structure (16). Thus, this extra methylation seems to be characteristic of only animal cells and their viruses. If this difference in methylation is true of plant mRNA in general, it would mark the first instance of a fundamental difference between protein synthesis in the animal and the plant kingdom.

3. The mRNA of animal cells and of those viruses which replicate in the nucleus have N₆-mA near the 5' end of the molecule. Yeast mRNA lacks this base.

4. The RNAs of animal cells can have either purines or pyrimidines at the internal position adjacent to m₇G(5')ppp. Yeast mRNAs have only purines.

It has been postulated that base and ribose methylations seen in mammalian mRNAs and those of viruses replicating in the nucleus serve as signals for the cleavage of hnRNA and the 5'-5' condensation reaction that occurs during or subsequent to this cleavage (2). In such a model for mRNA formation, these viruses. If this difference in methylation is true of plant mRNA in general, it would mark the first instance of a fundamental difference between protein synthesis in the animal and the plant kingdom.

The ratio of m₇G(5')pppAp to m₇G(5')pppA (Table II) is the same in cells grown in broth (for ³²P labeling) or in spheroplasts labeled in minimal medium (for C¹¹H₄ labeling). The high glucose content of both media represses mitochondrial transcription. The ratio is the same in whole cells (including nuclei), in polysomes, and in the poly(A)-containing RNA not associated with polysomes. Between 10 and 20% of the mRNA in polysomes appears not to have poly(A), although fragmentation during isolation has not been rigorously excluded. Even this RNA has the same ratio of 5' termini. Does some special feature of the promoter sites in yeast lead to this ratio? Is it a result of specific processing enzymes? Does this ratio reflect the whole population of mRNA molecules, or only those present in very large amounts? Would it be possible to detect a different ratio under special conditions, such as during sporulation?

There Is Too Much m₇G—Table II indicates that the ³²P in 5' termini represents more than 0.8% of the total ³²P in poly(A) containing RNA after 20 min of labeling with ³²P. Since the average size of mRNA is 1300 nucleotides and of hnRNA is 2500 nucleotides, there appears to be a substantial excess of label in the terminal structures. Part of the excess is only apparent, since it reflects a number average rather than a weight average. Moreover, the specific activity of ³²P in all the constituent nucleotides of the mRNA molecule is not likely to be uniform under the conditions of the experiment. Another part of the excess may be due to the distribution of phosphates in the 5'-terminal structures. Fig. 5, b and c, shows that the middle phosphate has a higher specific activity than the two phosphates attached to the sugars. It almost certainly arises from a β-phosphate residue, and is expected to have a higher specific activity than the α-phosphates in the short labeling period. The fact that the two phosphates attached to the sugars have more or less the same specific activity suggests that they were both α-phosphates and that the γ-phosphate of both transcript and GTP are lost in the reaction leading to the presence at the 5' end of the transcribed molecule of only those nucleotides which initiate transcription, i.e. A or G, suggest the possibility that m₇G is added to an intact transcript, which has undergone no previous processing. On the other hand, there is evidence from this laboratory (28) and others (30) that in yeast the average size of poly(A)-containing hnRNA is twice that of mRNA. Only further experiments can determine whether yeast mRNA is derived from a larger precursor.

| Organism                        | m₇G(5')ppp  | Xp     | Yp     | N₄-mA | Reference     |
|---------------------------------|-------------|--------|--------|-------|---------------|
| Mouse myeloma                   | +           | A₅m, G₅m, or C₅m | U₅m   | +     | 11            |
| BSC-1 Monkey kidney cells       | +           | A₅m, G₅m, or U₅m  | U₅m   | +     | 9             |
| HeLa cells                      | +           | A₅m, G₅m, or C₅m | G₅m, C₅m or U₅m | + 8  | 10            |
| L-cells                         | +           | A₅m, G₅m, C₅m or U₅m | U₅m | +     | 29            |
| Simian virus 40                 | +           | A₅m or G₅m | Unmethylated | + 9  | 5,6           |
| Vaccinia virus                  | +           | A₅m or G₅m | Unmethylated | 0    | 12            |
| Vesicular stomatitis virus      | +           | A₅m | Unmethylated | 0    | 3             |
| Cytoplasmonic polyhedoresis virus | +     | A₅m | Unmethylated | 0    | 4             |
| Reovirus                        | +           | G₅m | Unmethylated | 0    | 13            |
| Tobacco mosaic virus            | +           | G | Unmethylated | 0    | 14            |
| Brome mosaic virus              | +           | A or G | Unmethylated | 0 | Present communication |
| Saccharomyces cerevisiae        | +           | A or G | Unmethylated | 0 | Present communication |
blocking of the termini. However, these experiments do not indicate whether the middle phosphate in the 5' structure arises from β-phosphate of GTP or of the RNA.

The Gene ma 1 Is Not Involved with 5' Termini—The strain carrying a mutation in the gene ma 1 is of interest because its single lesion blocks the transport of RNA from the nucleus to the cytoplasm at the restrictive temperature (19). Although we had hopes that this mutant might be defective in forming blocked 5' termini, such is not the case. Mutant cells form m7G(5')pppA and m7G(5')pppG to the same extent as do wild type cells.

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