On the base-stacking in the 5'-terminal cap structure of mRNA: a fluorescence study

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ABSTRACT

The fluorescence at 370 nm of the 7-methylguanosine residue (m^7G) is found to be quenched when the base residue is involved in a stacking interaction with the adenosine residue in the cap structure $m^7G^{5'}pppA$ of an eukaryotic mRNA. On the basis of the observed degree of quenching, the amounts of the stacked and unstacked forms in the cap structure have been determined at various temperatures and pH's. It has been found that at pH 6.2 effective enthalpy and entropy in the unstacked → stacked change are $\Delta H° = -4.4 \pm 0.1$ kcal/mole and $\Delta S° = -14.3 \pm 0.2$ e.u., respectively. The pk_a value for the m^7G residue is found to be 7.7 at 10°C and 7.3 at 30°C. The stacked structure seems to be less favourable in the deprotonated form that occurs in the higher pH solution. A similar analysis of some other cap structures indicates that the stacked form in $m^7G^{5'}pppN$ structure is favourable if N is a purine nucleoside or a 2'-O-methylpyrimidine nucleoside but not for an unmethylated pyrimidine nucleoside.

INTRODUCTION

The 5'-terminus of mRNAs of eukaryotic cells and viruses have an unusual structure(cap); the terminal phosphate of RNA chain is blocked by 7-methylguanosine 5'-diphosphate[1-4]. The structure is expressed as $m^7G^{5'}pppN(m)pN_p\cdots$. Such a structure is found to stabilize the mRNA for nucleolytic attack and to play a certain positive role in the translation[5, 6].

In the present work, we have examined, by the use of a synthetic model compound, $m^7G^{5'}pppA$ and a few other $m^7G^{5'}pppN$'s, how the base-base stacking interaction takes place in the terminal structure. It has been shown that the stacking-destacking equilibrium is finely controlled by changing pK_a of the m^7G moiety (i.e., by changing the

electronic structure of 7-methylguanine) and/or by changing pH and temperature of the medium.

MATERIALS AND METHODS

P^1-7-methylguanosine-5'P^3-adenosine-5'triphosphate (abbreviated as m^7G^5'pppA) has been prepared as described in the previous paper[7]. The product was confirmed by degradation with snake venom phosphodiesterase (from Boerlinger Mannheim); here 7-methylguanosine 5'-phosphoric acid (m^7GMP) and adenosine-5'-phosphoric acid(AMP) were obtained as expected. m^7GMP was prepared by a modification of the method of Yoshikawa et al[8]. P^1-7-Methylguanosine-5' P^3-guanosine-5' triphosphate(m^7G^5'pppG), P^1-7-methylguanosine-5'P^3-2'-O-methyluridine-5' triphosphate (m^7G^5'pppUm) and P^1-7-methylguanosine-5'P^2-adenosine-5' diphosphate (m^7G^5'ppA) have been prepared by an exactly the same method as described in the previous paper[7].

Fluorescence spectra were measured by the use of a Hitachi MPF4 fluorospectrometer. The sample was kept in a constant-temperature cell holder and its temperature was controlled by the use of a Coolnics. Absorption spectrum was examined with a Hitachi 124 spectrophotometer.

RESULTS AND INTERPRETATION

Quenching of the Fluorescence of the 7-Methylguanosine Residue through a Base Stacking Interaction.

7-Methylguanosine has its absorption peak at 257 nm and a shoulder at 280 nm. On the deprotonation in a higher pH region, the 257 nm peak is lowered and a peak at 282 nm appears. In a strong alkaline solution, the imidazole ring is broken, and a strong peak comes up at 265 nm. When excited at 280 nm, 7-methylguanosine shows a fluorescence with a peak at 370 nm (λ_{max}). On deprotonation this becomes weaker and a weak fluorescence appears at about 400 nm. If the imidazole ring is broken, no fluorescence is observed. The absorption and fluorescence spectra (as well as the effects of pH elevation on them) of m^7GMP (see Figure 1) and of m^7GTP are all quite similar to those of 7-methylguanosine so far

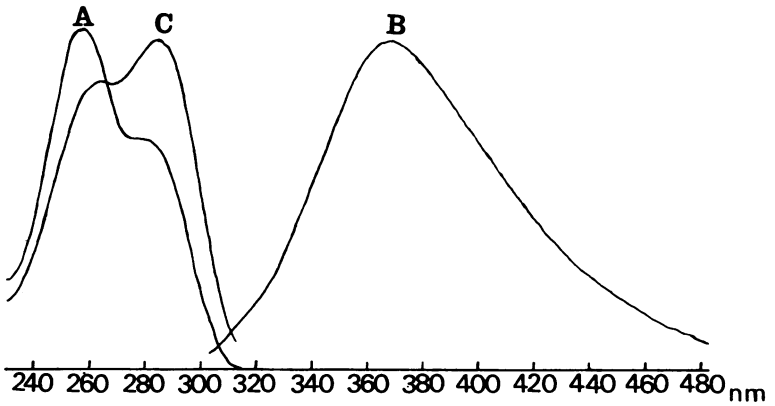

Figure 1. Absorption spectrum (A), fluorescence spectrum (B) (excited at 280 nm), and fluorescence excitation spectrum (C) (emission was observed at 370 nm) of m^7GMP in 0.01 M phosphate buffer, pH 4.8.

described.

$m^7G5'pppA$ shows a similar fluorescence spectrum to that of m^7GMP but its fluorescence peak (at 370 nm, see Figure 2) is lower than that of m^7GMP. This fact may be taken as indicating that the fluorescence of the 7-methylguanosine residue of $m^7G5'pppA$ is partially quenched by an intramolecular interaction with the adenosine residue. Thus, on incubating $m^7G5'pppA$ with snake venom phosphodiesterase, the fluorescence peak at 370 nm becomes higher as shown (B') in Figure 2. This inter-base interaction is probably a base-base stacking interaction.

Unconcern of the Excited State pK_a in the Fluorescence of the 7-Methylguanosine Residue. As is shown in Figure 3, the fluorescence intensity (at 370 nm) of 7-methylguanosine residue changes with pH in an exactly the same manner with that of the absorption intensity at 257 nm. This fact indicates that the fluorescence of 7-methylguanosine residue reflects its ground electronic state so that its change with pH gives the pK_a of the ground state 7-methylguanosine, and it requires no need to take the excited state pK_a into account.

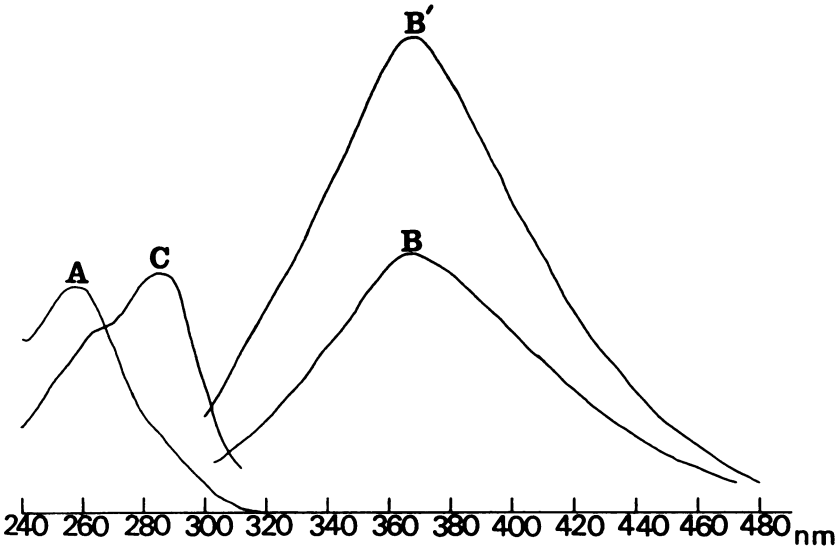

Figure 2. Absorption spectrum (A), fluorescence spectrum (B) (excited at 285 nm), and fluorescence excitation spectrum (C) (emission was observed at 370 nm) of $m^7G^{5'}pppA$ in 0.01 M citrate buffer with 0.1 M NaCl, pH 6.2. (B') Fluorescence spectrum after a phosphodiesterase treatment. Temperature at 25°C.

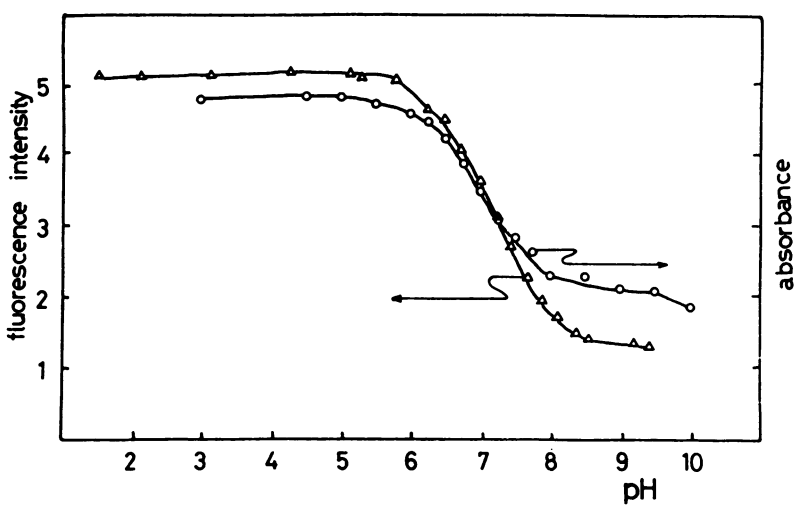

Figure 3. Fluorescence intensity at 370 nm (Δ), and absorption intensity at 257 nm (o) of 7-methylguanosine plotted against the pH of the solution, at 11°C.

This is understandable because the rate of the protonation and deprotonation in the excited state is considered to be much lower than the decay rate of the excited state itself. Thus, the fluorescence life time is estimated to be about 250 psec from the observed fluorescence quantum yield (0.015)[9] and the Strickler-Berg relation[10]. While, the protonation rate is diffusion (about 2 X 10^{11} M^{-1} sec^{-1}) limited, and it is much lower than 10^{10} sec^{-1} because H^+ concentration is 10^{-7}M at neutral pH.

Acidity Index pK_a of the 7-Methylguanosine Residue.

Table 1 gives the pK_a values determined of some 7-methylguanosine derivatives from the fluorescence intensity versus pH curves, such as that shown in Figure 3. The pK_a values are found to increase with the amount of the negative charge in the molecule:

$$\text{7-methylguanosine} < m^7GMP < m^7GTP \fallingdotseq m^7G^{5'}pppA.$$

The pK_a values of the first three (7-methylguanosine, m^7GMP, and m^7GTP) of these m^7G derivatives were once determined by absorption spectroscopy[11]. The results are in agreement with what we found by the fluorescence method. What is newly found in our present study is the temperature effect on pK_a. For each of these 7-methylguanosine derivatives, pK_a is lowered on raising the temperature of the solution. This fact is clearly demonstrated by an experiment in which 7-methylguanosine, for example, in pH 7 phosphate

Table 1. pK_a values determined by fluorescence measurements

Compounds	pK_a at 11°C	pK_a at 30°C
7-methylguanosine	7.1	6.8
m^7GMP	7.4	7.2
m^7GTP	7.6	7.3
$m^7G^{5'}pppA$	7.7	7.3

buffer is heated from 11°C to 60°C. At a lower temperature the solution shows the absorption spectrum of the protonated form whereas at a higher temperature it gives the spectrum showing deprotonated form. The change is so great that this is not attributable to a temperature effect on the pH of the phosphate buffer. The imidazole ring is not yet broken in such an experiment. It should also be mentioned here that no salt effect on pK_a has been found as long as the Na^+ concentration in the solvent was changed from 10 mM to 100 mM.

Temperature Effect on the Stacking-Destacking Equilibrium of $m^7G^{5'}pppA$. When a $m^7G^{5'}pppA$ solution at pH 7.0 is heated from 20°C to 60°C, almost no change in the 257 nm absorption intensity is observed, whereas an appreciable increase is found when the solution is heated at pH 4.5. Our interpretation of this observation is as follows: at pH 7.0, the heating causes both the deprotonation and destacking on the 7-methylguanine residue of the $m^7G^{5'}pppA$ molecule, and the lowering in the absorption intensity at 257 nm due to the deprotonation is compensated with the elevation due to the destacking; whereas at pH 4.5, the heating causes only a destacking, and therefore only an intensity increase is observed.

With this interpretation in mind, we have avoided to examine the stacking-destacking equilibrium at the very neutral (pH=7.0) solution. Instead, we attempted to do it at pH 6.2 (see Figure 6 with later description on it). Thus, we next examined the temperature effects on the fluorescence intensities of $m^7G^{5'}pppA$ and m^7GMP at pH 6.2. The results are shown in Figure 4, where the intensity scale (ordinate) is common for these two compounds. The fluorescence intensity of m^7GMP has been found to decrease with elevation of the temperature. Such a decrease is observed with a nearly equal rate for the fluorescence of 7-methylguanosine, and therefore it cannot be ascribed to the phosphate group. The effect of the deprotonation (if any, even at pH 6.2) was estimated to be less than 10 % of the observed fluorescence intensity at 60°C, because the absorbance at 257 nm of m^7GMP is lowered only by

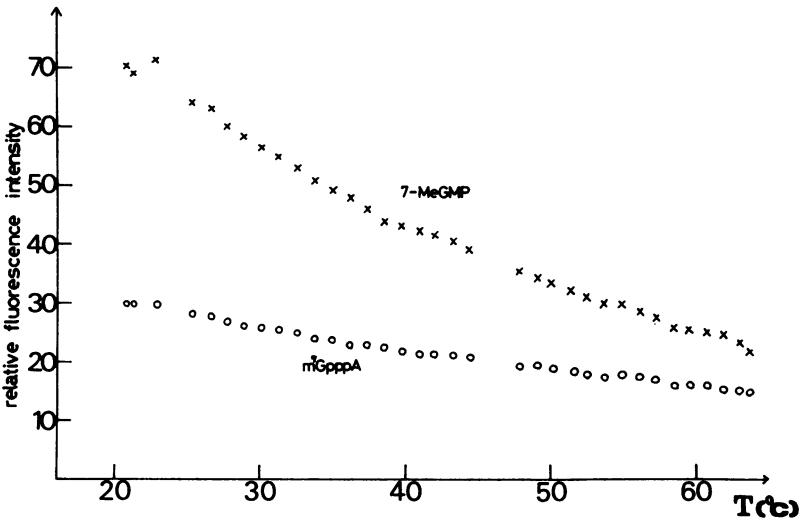

Figure 4. Temperature effects on the fluorescence intensity of m^7GMP (x) and $m^7G^{5'}pppA$ (o) at 370 nm (emission wavelength) excited at 285 nm.
Solvent : 0.01 M citrate buffer + 0.01 M NaCl, pH 6.20. The ordinate scale was normalized by assuming that the phosphodiesterase digestion of $m^7G^{5'}pppA$ converts it completely to m^7GMP.

4 % on heating the solution from 20°C to 60°C, but due allowance was made in interpreting the results below. One might further suspect that the fluorescence decrease with the temperature rise is due to a conformational change involving for example the syn-anti equilibrium. It is not probable, however, that this is the case. A similar fluorescence decrease takes place on heating 7-protonated guanosine and 7-protonated guanine solutions in a nearly equal rate. Thus, the fluorescence decrease of m^7GMP, now in question, must be due to the temperature effect on the internal conversion and/or on the intersystem crossing in the 7-methylguanine residue itself. The fluorescence intensity of $m^7G^{5'}pppA$ is always lower than that of m^7GMP, but the intensity ratio ($m^7G^{5'}pppA/m^7GMP$) becomes higher with the temperature. The lower fluorescence intensity of $m^7G^{5'}pppA$ is considered to be

caused by a intra-molecular base-base stacking interaction, and the increase of the relative intensity (in comparison with the fluorescence intensity of m^7GMP) with temperature is attributable to a thermal destacking. Let us assume here that the stacked form of $m^7G^5'pppA$ gives no fluorescence, and that the unstacked form gives a fluorescence of equal intensity to that of m^7GMP (discussions will be given below on this assumption). Then, from the fluorescence intensity ratio of $m^7G^5'pppA$ over m^7GMP observed at each temperature, the equilibrium constant K = (stacked form)/(unstacked form) is obtained. Here, the unstacked form* $\rightleftarrows$ stacked form* reaction in the excited electronic state has been neglected because this reaction rate is considered to be only 10^9 sec^{-1} in the order of magnitude and would not take place within the life time (about 10^{10} sec^{-1}) of the excited state. The unstacked form $\rightleftarrows$ stacked form equilibrium constant K for the ground state thus obtained is plotted in Figure 5 on a logarithmic scale against reciprocal absolute temperature (van't Hoff plot).

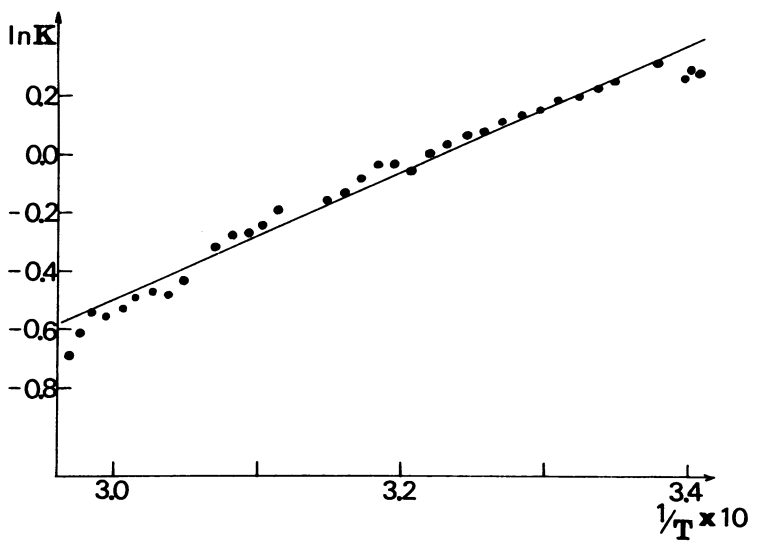

Figure 5. A van't Hoff plot. The equilibrium constant K for the unstacked $\rightleftarrows$ stacked forms of $m^7G^5'pppA$ (in 0.01 M citrate buffer + 0.1 M NaCl, pH 6.20) is plotted on a logarithmic scale against the reciprocal absolute temperature of the solution.

The points fall on a straight line in the range of temperature between 20°C and 60°C, and from this straight line the enthalpy (ΔH) and entropy (ΔS) of stacking is determined by a least squares fit as $\Delta H = -4.4 \pm 0.1$ kcal/mole and $\Delta S = -14.3 \pm 0.2$ e.u.

pH Effect on the Stacking-Destacking Equilibrium of $m^7G^{5'}pppA$. A similar comparison of the fluorescence intensity (at 370 nm) of m^7GMP and $m^7G^{5'}pppA$ has been made at 11°C and at various pHs. The results are shown in Figure 6. On lowering pH from 4.5 to 3, the fluorescence intensity of $m^7G^{5'}pppA$ increases steeply. This is attributed to the protonation of the adenine moiety of $m^7G^{5'}pppA$. When the adenine resiude acquires positive charge, the base stacking interaciton with the 7-methylguanosine residue (which has a positive charge)

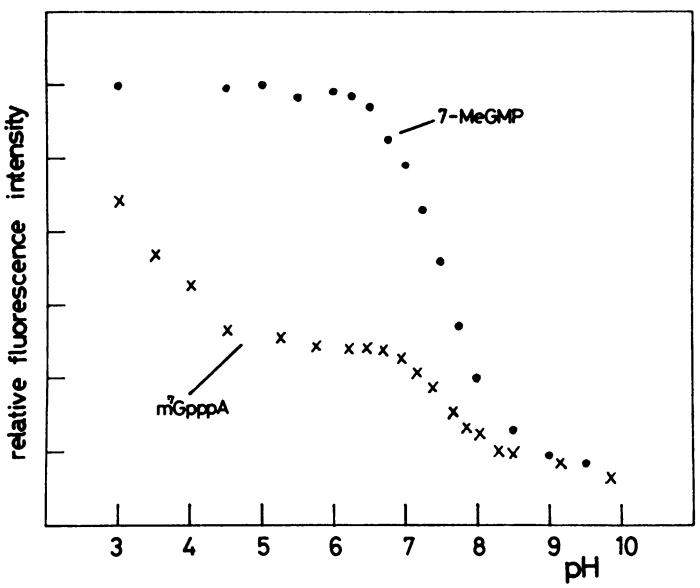

Figure 6. Fluorescence intensities of m^7GMP (●) and of $m^7G^{5'}pppA$ (x) plotted against pH of the solutions. Emission wavelength, 370 nm. Excitation wavelength, 285 nm. Temperature, 11°C. The ordinate scale was normalized by assuming that the phosphodiesterase digestion of $m^7G^{5'}pppA$ converts it completely to m^7GMP.

would be removed through an electrostatic repulsion, and this should cause a fluorescence increase. Any contribution from the adenosine residue does not need to be taken into account because its fluorescence intensity is known to be negligibly low at this conditions. In the pH = 4.5~7 region, about 70 % of $m^7G^{5'}pppA$ molecules are in the stacked form (and 30 % unstacked form) as estimated from the intensity ratio $m^7G^{5'}pppA$ over m^7GMP. On raising pH from 7 to 9, the fluorescence intensity ratio again increases, and this is apparently caused by a destacking due to the deprotonation (loss of its positive charge) of the 7-methylguanosine residue.

Base Stacking in Some Other Cap Structures and Their Analogues. Lastly the fluorescence intensities at 370 nm of $m^7G^{5'}ppA$, $m^7G^{5'}pppG$, and $m^7G^{5'}pppUm$ were examined and compared them with that of $m^7G^{5'}pppA$ as well as with that of m^7GMP. In Table 2, thermodynamic parameters in the unstacked → stacked changes and the equilibrium constants at pH 6.2 and at 25°C are shown.

As is seen in the first two columns of the table, the stacking seems to be more favourable in the system with 5', 5'-diphosphate than in the system with 5', 5'-triphosphate. This is found to be the case also for $m^7G^{5'}ppU$ and $m^7G^{5'}pppU$ pair. The equilibrium constant is found to be 0.65 at 25°C for $m^7G^{5'}ppU$ and 0.3 for $m^7G^{5'}pppU$[12].

It is noticeable that 2'-O-methylation seems to cause a

Table 2. Thermodynamic parameters in the unstacked → stacked changes in cap structures

	Equilibrium const. K(at pH 6.2 and at 25°C)	Enthalpy diff. ΔH(Kcal/mol)	Entropy diff. ΔS(e.u.)
$m^7G^{5'}ppA$	1.9	-4.85 ± 0.06	-15.0 ± 0.3
$m^7G^{5'}pppA$	1.2	-4.41 ± 0.11	-14.3 ± 0.2
$m^7G^{5'}pppG$	1.7	-3.55 ± 0.03	-10.9 ± 0.3
$m^7G^{5'}pppUm$	1.3	——	——

better stacking. Thus, $m^7G^{5'}pppUm$ has a greater amount of the stacked form than $m^7G^{5'}pppU$ at 25°C. $m^7G^{5'}pppUm$ has nearly an equal amount of the stacked form to that in $m^7G^{5'}pppA$, whereas $m^7G^{5'}pppU$ has less (data are not shown here). From a circular dichroic study, Hattori et al.[13] showed that $m^7G^{5'}pppAm$ has a greater amount of stacked form than $m^7G^{5'}pppA$.

DISCUSSION

On the Estimation of the Amount of the Stacked Form from Fluorescence Intensity Measurement. We have shown in this work a new method of quantitative analysis of conformers for a nucleic acid with two base residues, one of which is fluorescent. The method is based upon an assumption that "base stacking" causes a complete quenching of the fluorescence and "destacking" removes the quenching completely. Such an assumption may be taken as equivalent to a difinition of the stacked and unstacked forms. In other words, we propose to describe a conformation of a dinucleoside by a "linear combination" of two representative conformers: one having two bases so well stacked, that the fluorescence is completely quenched, and the other having two bases far away from each other so that the fluorophore environment is equal to that of its monomer. We have determined, for $m^7G^{5'}pppA$, an effective enthalpy difference ΔH and an effective entropy difference ΔS of such representative conformers. It should be mentioned here that, for 7-methylguanylyl(3'→5')uridine(m^7GpU), our method gave an equal set of ΔH and ΔS values within an experimental error to that determined by hypochromic effect in the absorption spectrum[12]. It is quite possible, however, that our method gives different ΔH and ΔS values for a certian dinucleoside phosphate from those determined by another method, if this method regards, for example, a halfway stacked conformation as "stacked form" even when our fluorescence method regards it "unstacked" because it is still fluorescent[12].

A Flexibility of the Cap Structure and Its Possible Significance. It has been shown that the cap structure $m^7G^{5'}pppA$ has 50% stacked form and 50% unstacked at 37°C and at

pH 6.2. If the number of the phosphate groups in this structure is reduced from three to two (into $m^7G^{5'}ppA$), the structure becomes appreciably tight; it becomes to have a greater amount of the stacked form. If the adenosine residue is replaced by guanosine or by 2'-O-methyluridine, the stacked-form content also slightly increases. In the cap structure $m^7G^{5'}pppN$ so far known, N is always 2'-O-methylated if it is a pyrimidine nucleoside[14]. This fact may be related with the fact that the stacked form of $m^7G^{5'}pppN$ is appreciably less populated at 25°C when N is a pyrimidine nucleoside without 2'-O-methylation than that with methylation. Thus, it is speculated that the cap function requires the base-stacked conformation. In addition, it seems to require that the base-stacked conformaiton is readily formed and readily broken. The formation and breaking are found to be controlled by changing the pH of the environment in the pH = 7∼8 region. The amount of the stacked conformation may also be controlled by changing the effective pK_a value, through, for example, a specific interaciton with a protein. It may also be pointed out here that the base-stacking in the cap structure results in a prevention of the alkali decomposition of the 7-methyl-guanosine residue to some extent. The pK_a value of $m^7G^{5'}pppA$ is greater than that of m^7GMP. This is probably because the protonated form is stabilized by the stacking and the deprotonated form is not. Thus, the cap m^7G residue has a greater amount of protonated form than that in m^7GMP at pH 7.0, for example.

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