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Effect of Tautomeric Shift on Mutation: N^4-Methoxycytidine Forms Hydrogen Bonds with Adenosine in Polymers†

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ABSTRACT: N^4-Methoxycytidine (mo^4C), previously found to act only as uridine (U) in transcription [Singer, B., & Spengler, S. (1981) *Biochemistry 20*, 1127], was tested for its ability to base pair as U in copolymers of (U,mo^4C) annealed with poly(A) or transcribed with ATP and DNA-dependent RNA polymerase. Mixing curves have now indicated that the derivative is retained in a poly(U,39% mo^4C)·poly(A) helix, unlike unmodified C in poly(U,35% C). The presence of 13–39% mo^4C in U polymers lowered the melting temperature, T_m, observed in annealed complexes both with poly(A) and after transcription with ATP. However, complexes isolated after transcription had a large hyperchromicity and melted cooperatively, which indicated that they are hydrogen bonded. The decreased T_m for poly(U,mo^4C)·poly(A) compared to that for poly(U)·poly(A) can be attributed to stacking changes and adjacent base-pair disruption by mo^4C. The greater cooperative melting of transcribed poly(U,39% mo^4C) as compared to the annealed complex may indicate that the methoxy substituent is normally a mixture of rotamers and that the syn rotamer is required for transcription. The interference of the methoxy substituent was also shown by the loss of helix formation by poly(C,mo^4C) in acid solution. mo^4C decreased the T_m much more than A, which stacks well in acid. U, which neither stacks nor participates in an acid structure, caused more distortion than either of the other bases. It is inferred that mo^4C has the base-pairing ability of U but that the planarity of the substituent is lost.

Previous studies in this laboratory on the effects of modified nucleotides on transcription of polymers by DNA-dependent RNA polymerase [reviewed by Singer (1981)] have shown that modifications which block essential hydrogen-bonding sites produce high levels of ambiguity (Kröger & Singer, 1979a). Modifications on exocyclic groups not necessarily blocking these sites cause a variety of effects (Singer & Spengler, 1981). N^4-Methoxycytidine (mo^4C)[1] appears to be only in the imino

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[1] Abbreviations used: mo^4C, N^4-methoxycytidine; ho^4C, N^4-hydroxycytidine; m^6A, N^6-methyladenosine; i^6A, N^6-isopentenyladenosine; m^4C, N^4-methylcytidine; ϵA, 1,N^6-ethenoadenosine; ϵC, 1,N^4-ethenocytidine; SSC, 0.15 M NaCl–0.015 M sodium citrate; N indicates any nucleotide in a polynucleotide while the single letters A, U, and C are used for the base moieties in a polynucleotide; HPLC, high-pressure liquid chromatography.

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form, since it acted exclusively like uridine (U) in directing the incorporation of one specific nucleotide, adenosine (A). The analogous hydroxylamine derivative, N^4-hydroxy-C, exhibited mainly U and C behavior.

The complete or extensive ambiguity seen with modifications which block the Watson–Crick sites but do not terminate transcription probably is not the result of base pairing but of nonspecific incorporation. In contrast, it has been assumed that the ability of a modified nucleotide to direct the specific incorporation of another nucleotide into a transcript reflects the ability of the two bases to form a hydrogen-bonded base pair which is acceptable to the polymerase (Topal et al., 1980). The polymerase itself might be able to participate in forming such a pair, for example, by rotating the exocyclic substituent which could potentially block the base-pairing sites (Engel & von Hippel, 1978; Singer & Spengler, 1981).

In the present experiments, we examined how modified bases affected the acid-helical structure of poly(C) and how one of these modifications, mo^{4}C, in U copolymers affected the ability of poly(U) to anneal with poly(A) or to form a double strand after transcription of U copolymers with ATP and DNA-dependent RNA polymerase. We find that mo^{4}C again acts like U, forming a base pair with A. It does not act like C, either in U polymers at pH 7.0 or C polymers at acidic pHs. We propose that steric interference with the adjacent base pair accounts for most of the observed destabilization.

Materials and Methods

Chemicals and Polyribonucleotides. Hydroxylamine hydrochloride and methoxyamine hydrochloride, both Eastman chemicals, were used to prepare N^4-hydroxy-CDP (Janion & Shugar, 1968) and N^4-methoxy-CDP. 1,N^6-etheno-ADP, oligo(C_{10-20}) and unmodified nucleoside diphosphates were purchased from P-L Biochemicals. Polymers were prepared as described by Singer & Kröger (1979) and analyzed by HPLC on a Bio-Rad Aminex HP-C column eluted with 0.4 M ammonium formate, pH 7.0. Composition was determined by peak integration. [^{3}H]ATP (ICN) and Sephadex G-75 (Pharmacia) were used for transcript labeling and isolation, respectively.

Enzymes. Polymers were synthesized by using *Micrococcus luteus* polynucleotide phosphorylase from P-L Biochemicals. Snake venom phosphodiesterase, bacterial alkaline phosphatase, and acid phosphatase, used for polymer analysis, were all from Worthington. *Escherichia coli* DNA-dependent RNA polymerase was purchased from Miles.

Spectrophotometry. All absorption measurements were made with Teflon-stoppered cuvettes in a dual-beam Varian Cary 219 spectrophotometer equipped with jacketed sample and reference chambers. The temperature of the cell compartment was regulated by a Neslab Exa/Endo Cal programmed at a constant heating rate. Data from recordings were corrected for the thermal expansion of water and usually reduced to relative hyperchromicities [absorbance at temperature T/(absorbance at reference temperature−1)].

Mixing Curves. Mixing curves were performed by the method of continuous variation (Felsenfeld & Rich, 1957) as modified by Chamberlin & Patterson (1965), at 22–25 °C in 0.15 M NaCl–0.015 M sodium citrate (SSC). The absorbance at 260 and 280 nm, or spectra from 320 to 220 nm, was taken 30 min after each addition. In several cases, readings were also taken after 18 h. There were no significant changes in the readings.

Determination of Melting Temperature. The acid structures of poly(C) and poly(C,N) were determined at pH 4.5 and 5.4 in 0.05 M sodium acetate–0.1 M NaCl. Studies on poly(U)·poly(A) complexes were performed in SSC. Polymers in water were added to the appropriate dilution of buffer, mixed, and allowed to stand at 10–15 °C for 30 min before readings were taken. The temperature rise was programmed at 1 °C/2 min or 1 °C/3 min, as indicated in specific curves. Data points were taken every 25 s. Molar extinction coefficients at 260 nm were the following: C, 5.2×10^3; mo^{4}C, 7.98×10^3; U, 8.9×10^3; m^{3}C, 5.7×10^3; A, 9.6×10^3. The T_m was generally taken as the midpoint of the increase in absorption from 10% to 90%. In addition, the point of maximum inflection in the hyperchromicity curve, $\Delta H/\Delta T$, was determined in several cases. These were less than a 0.5 °C difference in the two types of determination.

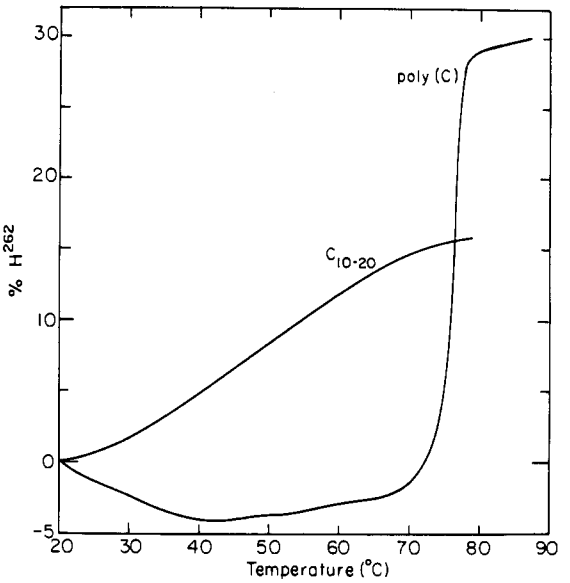

FIGURE 1: Thermal denaturation profiles of poly(C) and oligo(C_{10-20}) in 0.1 M NaCl–0.05 M sodium acetate, pH 4.5. Rate of heating, 1 °C/2 min.

Transcription of Polymers and Isolation of Products. A reaction mix of 0.5 mL for 0.5 absorbance unit of polymer (Singer & Spengler, 1981) contained 0.16 M total triphosphate as either [^{3}H]ATP or equimolar amounts of labeled ATP and cold GTP. The product was eluted in the void volume from Sephadex G-75 (1 × 15 cm in SSC, 23.5 mL cm^{-2} h^{-1}). The leading edge of the peak was used for determination of T_m because protein was located spectrophotometrically in the tail of the product peak. The product was well resolved from any lower molecular weight components.

Results

Acid Structure of Poly(C) and Poly(C,N). The acid structure of poly(C) formed at pH 4.5 (Figure 1), similar to that previously reported (Akinrimisi et al., 1963; Langridge & Rich, 1963; Guschlbauer, 1967; Chen & Charney, 1980), had a T_m of 78 °C. The hypochromicity observed before the temperature reached the T_m has been attributed to the removal of protons as the temperature rises. This hypochromic effect is generally lost as the percentage of C in the polymers decreases or the chain length is shortened to oligo(C) (Figure 1) (Brahms et al., 1967).

Increasing the pH from 4.5 to 5.4 did lower the T_m observed for poly(C) and the (C,U) and (C,mo^{4}C) copolymers (Figure 2). However, this change did not affect the relative influence of U and mo^{4}C on the helix structure. At least in this pH range, protonation of mo^{4}C was not a major factor. The pK_a of the mo^{4}C is $\simeq$ 2.5, similar to that of ho^{4}C (Brown et al., 1968; B. Singer, unpublished observation).

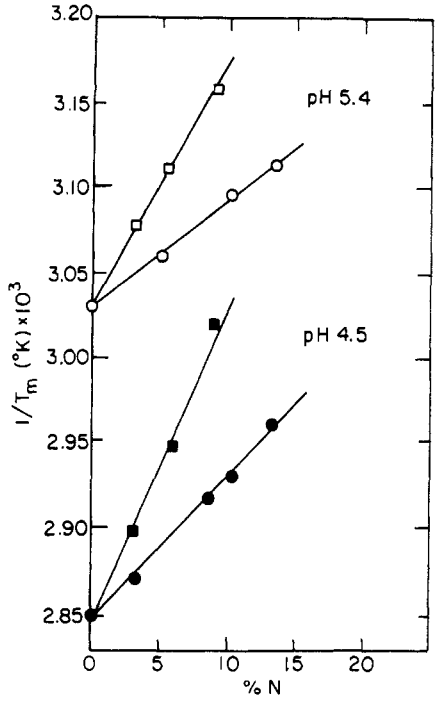

FIGURE 2: Effect of pH on the acid structure of C polymers: poly(C,U) (circles) and poly(C,mo^{4}C) (squares); pH 4.5 (●, ■) and 5.4 (○, □). T_m's were determined after melting of polymers at 1 °C/2 min in 0.1 M NaCl–0.05 M sodium acetate.

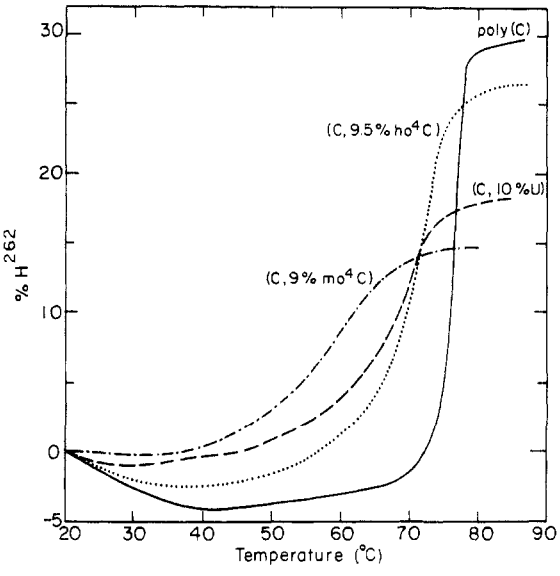

FIGURE 3: Thermal denaturation profiles of C copolymers. The effect of 10% replacement of C on the acid structure at pH 4.5 in 0.1 M NaCl–0.05 M sodium acetate. Rate of heating, 1 °C/3 min. Poly(C) (—); poly(C,9% mo^{4}C) (- - -); poly(C,10% U) (- - -); poly(C,9.5% ho^{4}C) (···).

The presence of 7–16% N^4-hydroxy-C in C polymers lowered the T_m observed at either pH 4.5 or 5.4, but the decrease is less than that found when similar amounts of U are present in poly(C). N^4-Methoxy-C, however, caused a much larger destabilization of the acid structure of poly(C), as measured by the T_m. For example, at pH 4.5, poly(C,9% mo^{4}C) had a T_m of 58 °C, while the T_m for poly(C,9% ho^{4}C) is 71 °C and that for poly(C,10% U) 68 °C (Figure 3).

The presence of the etheno derivatives, 3N^4-etheno-C and 1,N^6-etheno-A, which have an extra ring on the Watson–Crick side, destabilized the acid helix to a level similar to that observed with N^4-methoxy-C. However, the equally large isopentenyl derivative of A, i^{6}A, did not cause a similar displacement of the melting curves. It has been reported that unless 12.5 mM Mg^{2+} is present the isopentenyl group lies perpendicular to the adenine (Thedford & Straus, 1974). When 12.5 mM Mg^{2+} is added to poly(C,i^{6}A) at pH 4.5, the melting curve shifted to a lower T_m, due to destabilization of the double-stranded form by magnesium (Akinrimisi et al., 1963; Shin, 1973).

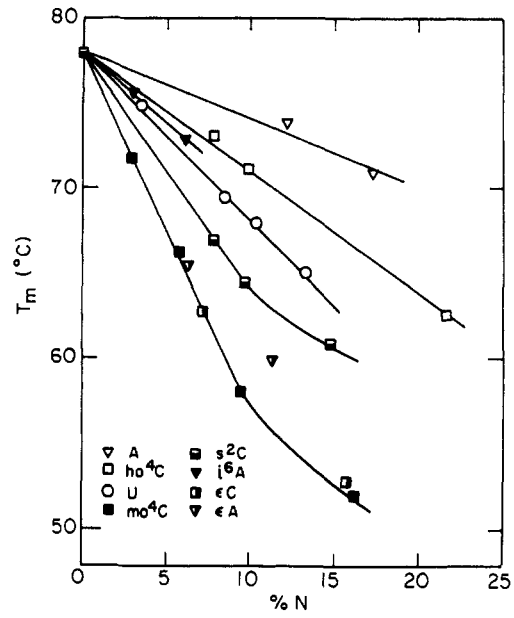

FIGURE 4: Thermal stability (T_m) of (C,N) copolymers as a function of the percent N. Polymer composition was determined by HPLC (see Materials and Methods). T_m's were determined at pH 4.5 in 0.1 M NaCl–0.05 M sodium acetate.

The minimal effect on T_m was observed in (C,A) copolymers, probably due to the effect both of good stacking interactions and of possible C·A pairing at acidic pH (Michelson et al., 1967). In contrast, 2-thiocytidine (s^{2}C), which has a high stacking energy (Kröger & Singer, 1979b), disrupts the C self-helix of (C,s^{2}C) copolymers, indicating perturbed base pairing is the cause of the T_m change. The changes in the T_m of the acid structure of poly(C) at pH 4.5 produced by copolymerization with these various nucleotides are summarized in Figure 4.

Stability of (U,C)·(A) and (U,mo^{4}C)·(A). Under the conditions used, i.e., equimolar amounts of A and U (plus any other nucleotide) with no Mg^{2+} present, the annealed polymers should be only double stranded. This is supported by the mixing curve (Figure 5A) for poly(U) with increasing poly(A) which has only one minimum, at 50% mole fraction A, at both 260 and 280 nm (Stevens & Felsenfeld, 1964). When this annealed polymer was melted, the T_m of 61 °C agreed with that reported by other investigators using the same conditions (Figure 6A).

The transcription product after a 15-min incubation of poly(U) with ATP (see Materials and Methods) had a T_m of 53 °C. This difference of 6–8 °C was seen for each transcript compared to the annealed polymers and may reflect the incomplete transcription of the polymer occurring during a short time. However, at longer times (60 min), it is clear that oligo(A) tails are synthesized (Figure 7A). These regions estimated to be approximately 12 base pairs long, form a short stacked structure which melted gradually at lower temperatures and made interpretation of the melting curves more difficult (Figure 7A, 60-min product). At very short times (5 min), the copolymers were transcribed to a very limited degree (less than 10% of the maximum). Therefore, as a compromise, 15-min transcripts of poly(U,35% C) and poly-

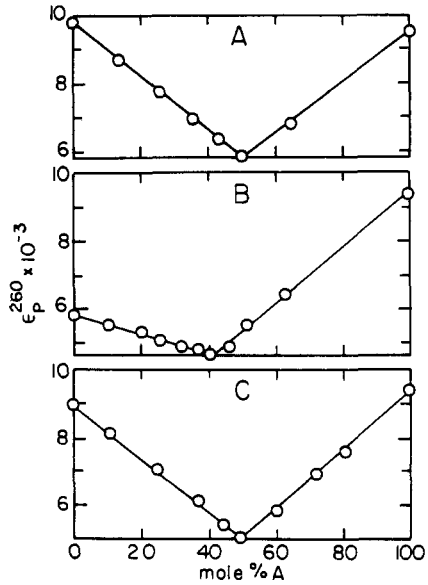

FIGURE 5: Mixing curves of poly(U,N) with poly(A) in SSC at 25 °C. (A) Poly(U) plus poly(A); (B) poly(U,35% C) plus poly(A); (C) poly(U,39% mo⁴C) plus poly(A).

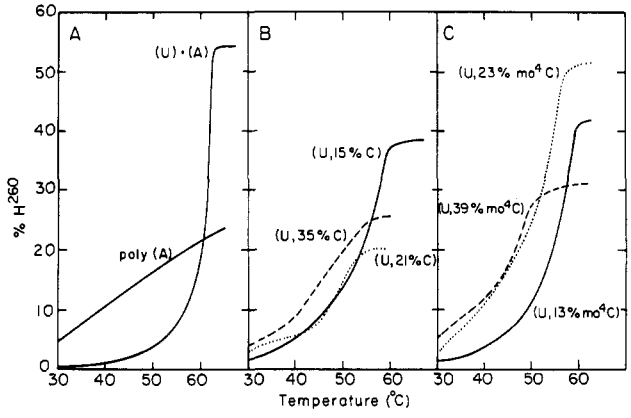

FIGURE 6: Thermal melting profiles of annealed poly(U)·poly(A) complexes in SSC. (A) Poly(U)·poly(A) and poly(A); (B) poly(U,x% C) annealed with poly(A); (C) poly(U,x% mo⁴C) annealed with poly(A). Temperature raised at 1 °C/2 min.

(U,39% mo⁴C) were used for the T_m determinations.

The annealed complex, poly(U,35% C)·poly(A), had a T_m of 43 °C. In contrast with poly(U)·poly(A), and as previously reported (Wang & Kallenbach, 1971), in this structure the C's were excluded from the helix as indicated by the mixing curve (Figure 5B). The 15-min transcript of the (U,C) copolymer with ATP melted with little cooperativity and an approximate T_m of 37 °C (Figure 7B). When both ATP and GTP were present in the reaction mix, transcripts contained G·C pairs which could be melted at about 94 °C (not shown). The existence of such regions indicates that accurate transcription occurred.

Unlike (U,C) copolymers, poly(U,39% mo⁴C) annealed with poly(A) on a 1:1 basis, indicating that the mo⁴C residues were held within the helix (Figure 5C). This retention of the derivative in the helix did not, however, prove that there was necessarily Watson–Crick base pairing between mo⁴C and A. The T_m for each annealed (U,mo⁴C)·(A) polymer was higher than that for the equivalent (U,C)·(A) complex but less than that observed for poly(U)·poly(A). The melting of the (U,39% mo⁴C)·(A) transcript was cooperative, similar to the (U)·(A) transcripts, but the product had a lower T_m, 43 °C vs. 53 °C (Figure 7B). This was the only polymer for which the profile of the transcript was more cooperative than the analogous

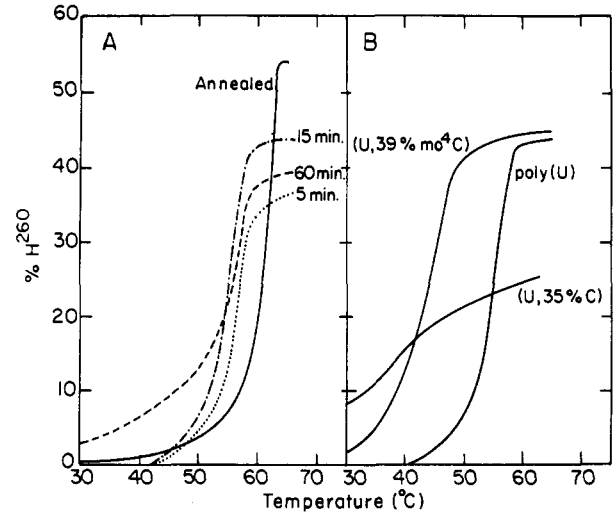

FIGURE 7: Thermal melting profiles in SSC of isolated products from polymer transcription with ATP and DNA-dependent RNA polymerase. (A) Variation in profiles with time of incubation (5, 15, and 60 min) compared to the annealed complex; (B) profiles of products from 15-min incubations of polymers as shown in the figure.

annealed complex. This may reflect more complete alignment which could be assessed by cooling followed by a second heating. Addition of GTP to the incubation mix did not change the melting profile of (U,mo⁴C) transcripts.

The T_m's observed for poly(U), poly(U,13–35% C), and poly(U,15–39% mo⁴C) annealed with poly(A) are linear with respect to the level of modification. This indicates that, even with clustering which may occur with high levels of another nucleotide, any destabilization caused by the C or mo⁴C does not extend beyond the nearest base pair.

Discussion

The presence of any nucleotide other than C disrupts the acid structure of poly(C) to an extent dependent on the specific nucleotide (Figure 4) and is proportional to the amount of modified base present, when they occur at low (less than 10%) levels. However, at higher levels, large deviations from linearity may occur. This may be the result of exclusion of these nucleotides from the helix, since such nonlinear behavior occurred particularly with some of the larger derivatives.

N^4-Methoxy-C acts like U in transcription experiments, but it has a much larger effect on the T_m of poly(C) than the presence of U (Figure 2). The magnitude of this disruption is similar for mo⁴C and two other derivatives, 3,N^4-etheno-C and 1,N^6-etheno-A (Figure 4). This large effect may be the result of the size of the substituent. All of these derivatives have large additional rings or groups. These may lie either out of the plane, such as mo⁴C (Birnbaum et al., 1979), or across the base-pairing plane and blocking the Watson–Crick sites, as with the etheno derivatives. Either of these behaviors would lead to the loss of both additional base-pairing and stacking interactions in excess of the single base pair lost by the replacement of C by a nucleotide which does not form a base pair at this pH.

The size of the substituent is not the only structural factor which affects stability. The orientation of the group also influences the helix distortion. The exocyclic isopentenyl derivative of A, which is transcribed only as A (Singer & Spengler, 1981), was much less destabilizing than the etheno derivatives, which are no larger. It is probable that, as during transcription, the isopentenyl moiety lies anti to the Watson–Crick sites, predominantly in the plane of the base.

N^4-Hydroxy-C, which is a classic example of ambiguity caused by a shift in the tautomeric equilibrium (Brown et al.,

1968), acts like both C and U under a variety of experimental conditions (Budowsky et al., 1972; Müller et al., 1978; Singer & Spengler, 1981). Copolymers of C containing a range of ho^{4}C all melted with T_m's higher than those of (C,U) copolymers of similar composition (Figure 4). In polymers, N^4-hydroxy-C shows very little stacking or other secondary structure (Janion & Shugar, 1968), like U (Richards et al., 1963), so that strong stacking is not the reason why the (C,ho^{4}C) polymers have higher T_m's than (C,U) polymers. This T_m effect probably results from the average presence of the amino tautomer of ho^{4}C, with the hydroxyl group anti to the Watson–Crick sites. Such an isomer is probably responsible for the inability of poly(ho^{4}C) to form any double-stranded structure with poly(A) (Janion & Shugar, 1968).

The ability of poly(U) and poly(A) to form a variety of multiple-stranded structures is well-known (Stevens & Felsenfeld, 1964; Felsenfeld & Rich, 1957; Blake & Fresco, 1966). Mixing curves performed under the conditions of annealing (Figure 4) showed only one minimum, whether at 260 nm, where the T_m was determined, or at 280 nm, where certain disproportionations would be observed (Stevens & Felsenfeld, 1964). Mixing curves for the copolymers of (U,35% C) and (U,39% mo^{4}C) showed that the minor base was excluded from the helix (Figure 5B), while the methoxy derivative was retained (Figure 5C). The indication of a slight curvature in the mixing curve of poly(U,35% C) with poly(A) (Figure 5B) may be the result of some additional loss of pairing residues when the C is looped out. This curvature is not observed in the mixing curve for poly(U,39% mo^{4}C), which further indicates that the mo^{4}C is likely to base pair like U (Cantor & Schimmel, 1980).

Particularly for copolymers, study of transcripts is important since a transcript may have more accurately aligned base pairs, zippered together by the polymerase. This appears to be the case with transcripts of (U,39% mo^{4}C) which are more cooperative, i.e., have a narrower transition width, than the annealed complex of this polymer with poly(A). Transcripts of poly(U,35% C) with ATP alone have very little cooperativity and a low T_m, but transcripts of this polymer with both ATP and GTP contain both A·U and G·C regions which melt independently. In contrast, poly(U,39% mo^{4}C) transcripts have only an A·U melting transition, even when transcribed with GTP and ATP. This further substantiates the nearest-neighbor transcription results previously reported (Singer & Spengler, 1981), in which mo^{4}C acts only like U.

Destabilization of helical structures can occur in at least three ways: bulges or looping out can occur in complexes; the stacking interactions can be changed; the stability of the Watson–Crick base pair may be changed. The change in the stability of the base pair even to its complete loss can be distinguished from the bulge or loop by mixing curves. However, even if a nucleotide is retained within the helix, and the destabilization energy is approximately equal, that need not imply a similar underlying mechanism. For example, m^{4}C (Brimacombe & Reese, 1966; Engel & von Hippel, 1974) and mo^{4}C (these experiments) have a similar destabilization energy (0.8 kcal/mol) as calculated from the change in T_m. The m^{4}C destabilization reflects the loss of the favored syn rotamer (Engel & von Hippel, 1974). This is not the case with mo^{4}C. Extension of crystal studies (Birnbaum et al., 1979) by computer models to the methoxy derivative indicates that the methyl hydrogens extend into the plane of the $n + 1$ base pair, regardless of whether the rotamer is syn or anti. Destabilization by this derivative thus appears to be caused by distortion of the adjacent base pair, rather than of the mo^{4}C·A pair, again indicating that the mo^{4}C·A pair has hydrogen bonds equivalent to those of a U·A pair. This unusual behavior by a modified derivative appears to be unique. It is attributed to the predominance of the imino tautomer in which the substituent lies anti to the ring N-3. In addition to being a mutagenic lesion, the presence of mo^{4}C in DNA would be expected to produce the same level of destabilization as m^{6}A (Engel & von Hippel, 1974). This could produce new restriction recognition sites, assuming that a bulge or level of destabilization, rather than a specific derivative, is recognized.

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