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Ambiguity and Transcriptional Errors as a Result of Methylation of N-1 of Purines and N-3 of Pyrimidines†

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ABSTRACT: Poly(A), poly(C), and poly(U) containing about 10% modified nucleoside were used as templates in transcription experiments using DNA-dependent RNA polymerase in the presence of Mn^{2+}. The presence of 3-methylcytidine, 3-methyluridine, 1-methyladenosine, and N^6-methyladenosine did not prevent transcription but decreased the rate. Polymers containing xanthosine were transcribed with the same rate as homopolymers, but those with 11% 1-methylguanosine were virtually inactive as templates. Nearest-neighbor analysis of products of transcription of various copolymers showed that, in the presence of all four nucleoside triphosphates, 3-methylcytidine was able to direct AMP, CMP, and UMP equally well into the complementary strand. 1-Methyladenosine also directed incorporation of AMP, GMP, CMP, and UMP but with a preference for AMP and UMP. 3-Methyluridine directed AMP and UMP incorporation. 3-Methyluridine-directed CMP incorporation was low but could be verified when ATP and UTP were absent. Polymers containing N^6-methyladenosine, xanthosine, or 5-fluorouridine directed incorporation of the expected complementary nucleotide only. The data presented indicate that, under competitive conditions (all four NTPs present), nucleosides modified on the N-3 of Urd or Cyd and the N-1 of Ado have little or no specificity in transcription, thus behaving ambiguously. Although incorporation of GMP could only be found for 1-methyladenosine due to technical problems, it is likely that the N-3 methylated pyrimidines also direct GMP. It is postulated that no specific hydrogen bonds are formed between the modified bases in the template and the nucleoside triphosphate, but instead other factors such as stacking forces and the RNA polymerase itself direct incorporation of two, three, or four nucleotides. In templates, 3-methylcytidine, 3-methyluridine, and 1-methyladenosine are by this criterion mutagenic.

The general understanding regarding the mechanism of point mutation is that a base is modified in such a way that it behaves in transcription or translation as if it were another base. This assumes that the proper number and location of potential hydrogen bonds are required for a base to become incorporated. However, in vitro testing of the template activity of modified bases in polynucleotides has led to results which cannot be interpreted as resulting from normal hydrogen bonding (Topal & Fresco, 1976; Singer et al., 1978). Certain alkylated bases have already been studied in terms of their base-pairing abilities, but there is no uniform theory to fit the reported properties: 3-MeC is reported to be a mutagenic base, acting like A, U, or G in transcription (Ludlum, 1970a; Singer & Fraenkel-Conrat, 1970). Three *O*-alkyl bases have been studied in the same way more recently. O^6-MeG acts like A or U (Gerchman & Ludlum, 1973; Mehta & Ludlum, 1978), while O^2- and O^4-alkyl U act like C or G in transcription (Singer et al., 1978) but like U in tRNA binding (Singer et al., 1979). The presence of 3-MeU was considered to be a lethal event (Szer & Shugar, 1961; Grunberger et al., 1968), but in doublets (pGp3MeU) it was found that 3-MeU could function as C in tRNA binding (Singer et al., 1979).

Thrierr & Leng (1971) found that 15% 1-MeA in poly(A) appears to allow helix formation with poly(U), but the 1-MeA prevented binding of DNA-dependent RNA polymerase to such polymers. However, these polymers were prepared by methylation of poly(A) and not by de novo polymerization. Pochon & Michelson (1967) reported 1-MeG as being incapable of forming a base pair, both on experimental and theoretical grounds.

Methylation of the N-1 of purines and the N-3 of pyrimidines should have the same effect in preventing transcription if only base pairing were involved. The fact that 3-MeC is accepted as being mutagenic while 3-MeU is generally

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considered to be lethal has resulted from the use of different methods to test for possible base pairing. In addition, the secondary structure of the polymer may influence recognition of a methylated base in transcription or translation (Stahl & Chamberlin, 1978).

The present series of experiments was designed to investigate, under identical conditions, whether the presence of a methyl group at a position considered essential for base pairing would allow transcription if the amount of modified base were low enough to prevent gross changes in the polymer's secondary structure (Kröger & Singer, 1979). The modifications chosen were on the N-3 of cytidine and uridine, and on the corresponding N-1 of adenosine and guanosine.

For the purpose of comparison, we chose to study three additional modified nucleosides which can base-pair in a predictable manner. These are N^6-methyladenosine (N^6-MeA), xanthosine (X), and 5-fluorouridine (FU). N^6-MeA can replace A in both polymers and codons (Griffin et al., 1964; Michelson & Pochon, 1966; Kohlschein et al., 1974; Engel & von Hippel, 1978), X can base-pair with A or U (Michelson & Monny, 1966) and FU behaves like U in codons and polymers (Grunberg-Manago & Michelson, 1964; Massoulié et al., 1966).

Nearest-neighbor analysis of transcription products from polyribonucleotides is a sensitive and quantitative test for presumed base pairing, and this method was applied to transcripts directed by poly(C) and poly(U) containing 10–15% of modified base. Complete or partial ambiguity without evidence for specific hydrogen bonding was found for the behavior of 1-MeA, 3-MeC, and 3-MeU.

Materials and Methods

Chemicals. [^{3}H]ATP (26 Ci/mmol), [^{3}H]CTP (21 Ci/mmol), [^{3}H]GTP (15 Ci/mmol), and [^{3}H]UTP (18 Ci/mmol) are products of Schwarz/Mann; [α^{32}P]ATP, [α-^{32}P]CTP, [α-^{32}P]GTP, and [α-^{32}P]UTP are from New England Nuclear at highest specific activity available. 1-Methyladenosine, 1-methylguanosine, 3-methylcytidine methosulfate, and 3-methyluridine were from Sigma. Xanthosine 5′-diphosphate was obtained from P-L Biochemicals. All other chemicals used were of highest purity from commercial sources. Poly(U,C) 24:1 was orginally prepared for a previous study (Singer et al., 1978), and poly(U,FU) and poly(C,3-MeU) 13:1 are part of a generous gift from Dr. A. M. Michelson. We are also indebted to Dr. J. T. Kuśmierek for a preparation of 3-methyl-UDP.

Enzymes. Polynucleotide phosphorylase and pancreatic ribonuclease were products of P-L Biochemicals. RNA polymerase *E. coli* K12 was a product of Miles Laboratories, and bacterial alkaline phosphatase, acid phosphatase, and snake venom phosphodiesterase were obtained from Worthington.

Preparation of Nucleoside 5′-Phosphates. 5′-Phosphates of methylated nucleosides were obtained by enzymatic phosphorylation using crude wheat shoot extract as a source of phosphotransferase (Giziewicz & Shugar, 1975). The general conditions were as described recently by Singer et al. (1978). A 1.5 × 30 cm column packed with Dowex 1-X2 (HCO_3 form) was used with 2 × 750 mL gradient volumes of triethylammonium bicarbonate (TBK) buffer concentrations which varied depending on the properties of different nucleosides. Using the same molar amount of starting material (0.3 mmol), we obtained the following yields: 1-MeAMP 55% at 0.12–0.15 M TBK (0.1–0.3 M gradient); 3-MeCMP 55% at 0.127–0.147 M TBK (0.1–0.3 M gradient); 1-MeGMP 51% at 0.45–0.50 M TBK (0.1–0.5 M gradient with additional washing using 0.5 M TBK); and 3-MeUMP 56% at 0.28–0.33 M TBK (0.1–0.5 M gradient).

All nucleotides were easily separated from unphosphorylated nucleosides. 1-MeAdo and 3-MeCyd were not bound to the anion exchanger, 1-MeGuo was eluted at 0.12–0.18 M TBK, and 3-MeUrd was eluted at 0.12–0.14 M TBK. No contamination with inorganic phosphate was found in the nucleotide fractions which allowed us to use the nucleotides without further purification.

Preparation of Nucleoside 5′-Diphosphates. The general synthetic procedure was according to Hoard & Ott (1965) using tributylammonium phosphate instead of tributylammonium pyrophosphate. The same column as above was used for the separation procedure, except that the TBK gradient was 0.1–1.0 M. The following yields, based on the amount of original nucleoside, were obtained: 1-MeADP 9.5% at 0.44–0.55 TBK buffer; 3-MeCDP 24% at 0.46–0.61 M TBK; 1-MeGDP 43% at 0.88–0.94 M TBK; and 3-MeUDP 23% at 0.74–0.80 M TBK. The appropriate fractions containing the nucleoside 5′-diphosphate were collected and evaporated to dryness. Evaporation of the residue was repeated three times after adding aqueous ethanol. Without further treatment, an aqueous solution of the residue was used for the preparation of polyribonucleotides. The UV spectra of the nucleoside diphosphates were identical with those of the nucleosides (Singer, 1975).

N^6-Methyladenosine 5′-diphosphate (N^6-MeADP) was obtained earlier as an impurity after preparation of 1-MeADP (1.6% of the original 1-MeAdo) at 0.57–0.69 M TBK buffer or by alkaline rearrangement of 1-MeADP (Jones & Robins, 1963). Incubation of 55 A_{258} units of 1-MeADP in 1 mL of 0.1 M NaOH for 1 h at 100 °C yielded 95% N^6-MeADP as shown by LC analysis after treatment with alkaline phosphatase. This preparation of N^6-MeADP was, after neutralization, directly used for polymerization.

Preparation of Polyribonucleotides. The preparation and isolation of polyribonucleotides were as recently described (Singer & Kröger, 1978; Singer et al., 1978). Unmodified nucleoside 5′-diphosphate (1–4 mg) was incubated for 18 h with 0.2–0.3 mg of polynucleotide phosphorylase in the presence of various amounts of modified nucleoside 5′-diphosphates. By using a thin-layer isolation procedure (Singer & Kröger, 1978) a large number of polymers with varying content of modified bases were prepared. In general all five nucleoside diphosphates prepared in this laboratory, as well as xanthosine 5′-diphosphate, could be polymerized with ADP, CDP, and UDP. The specific polymerization conditions as well as the estimated yields are given in Table I.

Analysis of Polynucleotide Composition. About 0.5 absorbancy unit (at λ_{max}) of each polyribonucleotide dissolved in 100–500 μL of 50 mM Tris (pH 7.3) was incubated for 4 h, 37 °C, with 6 μg of ribonuclease A and 3 μg each of alkaline phosphatase, acid phosphatase, and snake venom phosphodiesterase. These digestion conditions yielded nucleosides, and no undigested polymer was detected. By using a 100-μL filler loop, 0.1–0.2 absorbancy unit of digested polymer was directly applied to a Bio-Rad LC cation exchange column Aminex HR-C (250 × 4 mm) by using a Model 1300 UV Monitor and Sensor (Bio-Rad, CA) and a Model 396 Milton Roy Instrument pump (Milton Roy, FL). These conditions are similar to those previously described or used (Khym, 1975; Kröger et al., 1976). The elution was performed by using carefully degassed pH 4.7 0.4 M ammonium formate at 55 °C with an 0.62 mL/min flow rate. The recorded elution profile was quantitated by a simple standardization procedure:

Table I: Preparation of Poly(A), Poly(C), and Poly(U) Copolymers Containing Modified or Unmodified Nucleosides

polynucleotide	polymerization conditions[a]	input[b] (% NDP)	output[c] (% NMP)	yield[d] (%)
poly(A,1-MeA), 8:1	A	10	11[e]	13
poly(A,N^6-MeA), 8:1	A	15	11	52
poly(A,3-MeC), 7:1	A	59	13	35
poly(A,1-MeG), 8:1	C	9	11	19
poly(A,3-MeU), 13:1	A	64	7	76
poly(A,X), 5:1	A	5	15	32
poly(A,C), 9:1	A	9	10	53
poly(A,G), 5:1	C	6	18	6
poly(A,G), 5:2	B	5	28	13
poly(A,G,U,C), 5:3:1:1	D	G 8	27	23
		C 9	9	
		U 9	9	
poly(C,1-MeA), 16:1	A	8	6[e]	20
poly(C,1-MeA), 11:1	A	9	8	26
poly(C,1-MeA), 7:1	A	15	12	30
poly(C,N^6-MeA), 11:1	A	10	8	83
poly(C,3-MeC), 24:1	A	10	4	27
poly(C,3-MeC), 8:1	A	19	11	90
poly(C,3-MeC), 5:1	A	50	16	32
poly(C,1-MeG), 8:1	B	8	11	15
poly(C,3-MeU), 32:1	A	67	3	54
poly(C,3-MeU), 7:1[f]	A	20	12	97
poly(C,X), 7:1	A	5	13	60
poly(C,G), 7:1	C	5	12	13
poly(C,G,U,A), 12:6:2:1	D	G 7	30	28
		A 7	5	
		U 8	9	
poly(U,1-MeA), 10:1	A	9	9[e]	82
poly(U,N^6-MeA), 8:1	A	10	11	65
poly(U,3-MeC), 5:1	A	11	17	72
poly(U,3-MeU), 24:1	A	67	4	21
poly(U,X)[g]	A	5	nd	51

[a] Enzyme concentration and polymerization times are given under Materials and Methods. A is 5 mM $MgCl_2$ at 37 °C; B is 5 mM $MgCl_2$ and 5 mM $MnSO_4$ at 60 °C; C is 5 mM $MnSO_4$ at 60 °C; and D is 5 mM $MgCl_2$ and 5 mM $MnSO_4$ at 37 °C. [b] NDP stands for the modified nucleoside 5′-diphosphate or for the unmodified nucleoside 5′-diphosphate used as a minor component. [c] Data obtained after enzymatic digestion and analysis by using high pressure liquid chromatography. Values may have an absolute error of ±15%. [d] The percentage is an estimate using ϵ_{268nm} = 7100 for poly(C), ϵ_{260nm} = 9800 for poly(U), and ϵ_{256nm} = 13 700 for poly(A). No corrections are made for hypochromicity or the extinction coefficient of the modified base. [e] The amount of 1-MeA includes a small percent of N^6-MeAdo, which is present as a result of the Dimroth rearrangement (Griffin et al., 1964). [f] The sample of 3-MeUDP used was the sodium salt, obtained in crystalline form from Dr. J. T. Kusmierek, Academy of Sciences, Warsaw. The other polymers containing 3-MeU were made by using a solution of the triethylammonium salt of 3-MeUDP. [g] Composition not determined.

Under identical conditions with a known ratio of any two appropriate nucleosides, a second analysis was done. By using peak integration, the ratio of the polymer digestion could be calculated. The data are given in Table I. Under these conditions, all combinations of nucleosides except Urd and Xan, Guo and 1-MeGuo, and Urd and 3-MeUrd could be separated, but the last pair could be separated by using thin-layer chromatography. By using LC, the maximum elution volume (28 ± 1 mL) was that of 3-MeCyd.

Transcription of Polyribonucleotides. The incorporation of nucleoside triphosphates into polyribonucleotides using DNA-dependent RNA polymerase was performed according to Gleason & Fraenkel-Conrat (1976), but in the presence of inorganic phosphate to suppress any polynucleotide phosphorylase activity. The enzyme is capable of de novo synthesis (Chamberlin, 1976) but with a lag. The incubation time chosen minimizes this activity.

The 125-μL standard incubation mixture contained 0.03 absorbancy unit of polyribonucleotides and was 0.4 mM each in ATP, CTP, GTP, and UTP, 0.4 μM in one ^{3}H-labeled nucleoside 5′-triphosphate, 80 mM Tris (pH 7.8), 4 mM $MnSO_4$, 0.4 mM K_2HPO_4, 3 μg of RNA polymerase, and 40 mM β-mercaptoethanol. In some cases only one other nucleoside 5′-triphosphate was used in addition to the ^{3}H-labeled triphosphate. The concentration was then 0.8 mM for each triphosphate in order to maintain a constant 1.6 mM triphosphate concentration. After 30-min incubation at 37 °C, 100 μL was spotted on DEAE paper disks and washed seven times with 7% Na_2HPO_4 and briefly twice with water. When kinetic experiments were performed, only the reaction volume was increased and smaller aliquots were taken at various times and treated in the same manner.

Nearest-Neighbor Analysis of Transcription Products. DNA-dependent RNA polymerase transcription of polyribonucleotides was performed as described above but with a fivefold increase in scale. Most experiments were performed in the presence of all four triphosphates with the labeled nucleoside triphosphate in the α-^{32}P form. In other experiments, only two nucleoside triphosphates were used, in equal amounts, one being ^{32}P labeled. The overall nucleoside diphosphate concentration remained at the standard concentration of 1.6 mM. The following procedures of isolation, hydrolysis, and subsequent separation of the 2′(3′) nucleotides were similar to that used by Ludlum (1970b) and Singer et al. (1978).

After 30-min incubation at 37 °C a 75-μL aliquot is spotted on DEAE paper disks and washed with 7% Na_2HPO_4 and water, and the bound radioactivity determined in order to ascertain the total radioactivity in the reaction. To the remaining 550 μL, 1.6 mg of yeast RNA is added, and, immediately after mixing, a 30% solution of trichloroacetic acid is added so that the final concentration of Cl_3CCOOH is 5%. After cooling, the precipitate is centrifuged at 10 000 rpm for 10 min. The resulting pellet is washed four times with 0.5 mL of 5% Cl_3CCOOH and thereafter two times with ethanol at 0 °C. The washed pellet is hydrolyzed with 100 μL of 0.3 M KOH, 18 h at 37 °C. The entire sample together with 100 μg of each 2′(3′) nucleoside, as additional internal markers, is applied in a 1-cm streak to a Whatman 3MM 46 × 57 cm paper sheet for paper electrophoresis by using a Savant Model LT 48A chamber (Savant Instruments, Inc., Hicksville, NY) and the following buffer at pH 3.5: 5% (v/v) pyridine acetate, 5% (v/v) glacial acidic acid, 1 mM EDTA. The separation was complete for all four 2′(3′) nucleosides usually after 8 h at 1000 V. One sheet of paper could be used for up to 14 samples. The four UV absorbing areas were cut out, and the radioactivity was determined. In some experiments with very high radioactivity (>2 × 10^5 cpm), 1-cm strips were counted and it was found that there is a constant background of 0.2–1.0% of the total radioactivity, depending on the [α-^{32}P]triphosphate used. Nearest neighbor sequence data given as cpm are not corrected for this, while all data calculated in percentages include this correction.

Results

Preparation and Analysis of Polymers. By using polynucleotide phosphorylase and nucleoside diphosphates, a variety of polyribonucleotides could be prepared. However, there was considerable variation in the yields and efficiency of incorporation of modified nucleotide diphosphates (Table I). The incorporation of GDP or its derivatives was carried out in the presence of manganese (Pochon & Michelson, 1967) which decreases the total yield of polymer, but increases the

proportion of the guanosine derivative (Table I).

The only other nucleoside diphosphate incorporated into copolymers preferentially is xanthosine 5′-diphosphate, which has been reported as being easily polymerized (Michelson & Grunberg-Manago, 1964). N^6-MeADP was polymerized as well as unmodified diphosphates and high yields were obtained. We find pH 8.2 more favorable than pH 9.0 (Ludlum, 1970a) to prepare 3-MeCyd containing copolymers, but even at this pH the incorporation of 3-MeCDP is much lower than expected from the input ratio. The preparation of 1-MeAdo containing copolymers was also performed at pH 8.2 in order to minimize the formation of N^6-MeAdo. However, it was not possible to prevent some rearrangement occurring during polymerization. We find in polymers about 10% of the 1-MeA to be converted to N^6-MeA. This has also been reported by Griffin et al. (1964). 3-MeUDP was the only diphosphate which was difficult to polymerize, and it was necessary to use at least a tenfold higher input ratio (see footnote *f*, Table I). Szer & Shugar (1961) also used a high input ratio of 3-MeUDP in preparing copolymers of U and 3-MeU. The yields of polymer reported by Szer & Shugar and now in this study are similar.

Template Activity of Copolymers. The large number of polyribonucleotide preparations available allowed us to study the influence of modified and certain unmodified bases on the rate of transcription using DNA-dependent RNA polymerase. The same transcription conditions were used for all polymers, although it is recognized that polymers consisting primarily of A, U, or C differ in optimal conditions for transcription (Niyogi, 1972). For purposes of simplifying the results and discussion, we are introducing the term "carrier" to mean the base which predominates in a copolymer. Thus, in poly(A, 3-MeC), the "carrier" is A.

When a copolymer which contains about 15% of any other base is transcribed, the rate and extent of transcription of the carrier by the *E. coli* enzyme under our experimental conditions are generally lower than for the homopolymer (Figure 1). Even a normal base introduced into a copolymer decreases transcription of the carrier base. For example, poly(A) containing 12% C incorporates UTP with half of the rate of poly(A) (Figure 1A). The most dramatic effect is that of guanine, which only allows very slow transcription if it is present as 12% or 18% of the total polymer (Figure 1C,D), and inhibits transcription totally in copolymers containing more than 25% G. Xanthosine appears to have the least effect on transcription rate (Figure 1A,B).

The specific influence of different methylated bases on the rate of transcription of the carrier polynucleotide is also given in Figure 1. As already noted for unmodified bases, methylated bases also decrease the rate of transcription to varying degrees. Only 1-MeG decreases the rate of transcription of copolymers to as low an extent as unmodified G with the same carrier (Figure 1A,B). The effect of the modified base in transcription rates is not noticeably influenced by the carrier polynucleotide with one exception. This exception is the transcription of 3-MeU containing polymers.[1] While only 4% of poly(C, 3-MeU) 13:1 is transcribed after 30 min, 12% of poly(A, 3-MeU) 13:1 is transcribed in the same period of time (Figure 1A,B).

Figure 2 illustrates the apparent influence of secondary structure on template activity. If 3-MeC is present in poly(C) to only a limited amount (11% or less), the transcription rate

[1] Three separately prepared samples of 3-MeUDP were used for making polymers. One was prepared in this laboratory as described; the others were gifts from Drs. A. M. Michelson and J. T. Kušmierek.

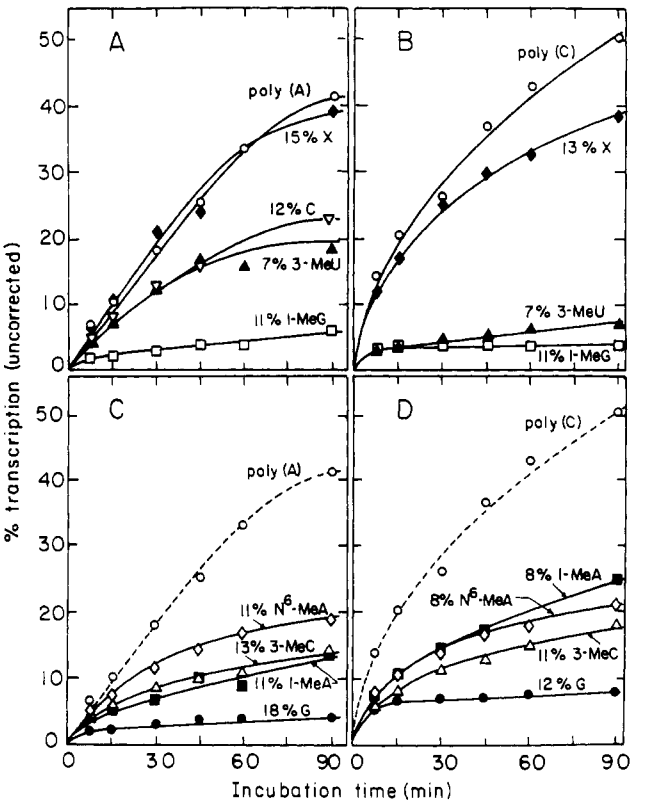

FIGURE 1: Rate of transcription of homoribopolynucleotides as compared with copolymers. The percent and type of nucleotide, other than the carrier, is shown in the figure. Percent transcription is the absolute amount of [^{3}H]UMP incorporated opposite poly(A) homo- or copolymers (panels A, C) and of [^{3}H]GMP opposite poly(C) homo- or copolymers (panels B, D). All four triphosphates were present. See Materials and Methods.

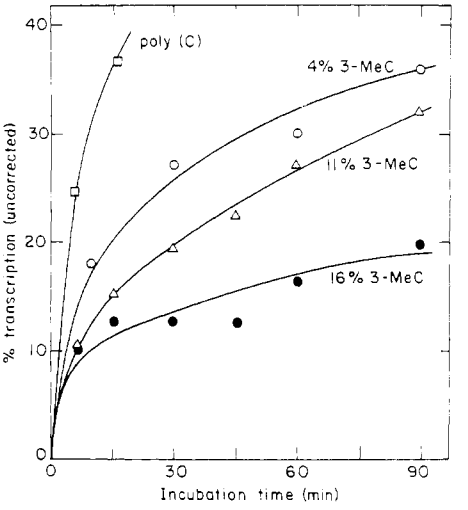

FIGURE 2: Effect of 3-methylcytidine on the rate of transcription of poly(C) by using [^{3}H]GTP and unlabeled ATP, UTP, and CTP. Percent transcription is the absolute amount of [^{3}H]GMP, uncorrected for the amount and transcriptional effect of 3-MeC.

is comparable to that of copolymers of unmodified bases. The presence of 16% 3-MeC, however, decreases the template activity to half, similar to the effect of 2-thiocytidine in cytidine copolymers (Kröger & Singer, 1979). For this reason, the poly(C, 11% 3-MeC) and polymers with about 10% of other modified bases are used exclusively for nearest neighbor analysis.

Product Identification by Nearest Neighbor Analysis. Generally two different approaches were used to identify the products of transcription: (i) by using an [α-^{32}P]NTP which

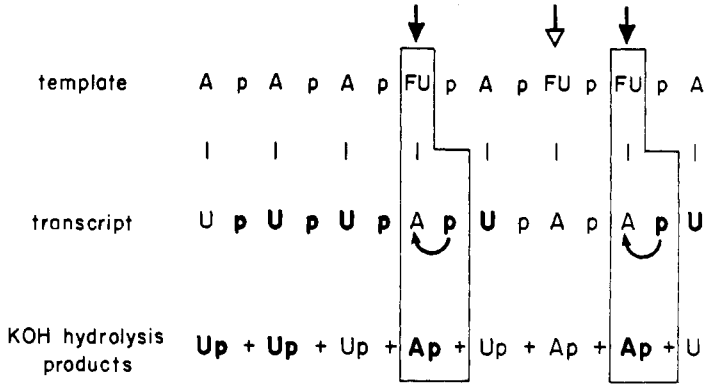

FIGURE 3: Schematic illustration of the use of nearest neighbor analyses. As an example, poly(A) containing 5-fluorouridine (FU) is shown as template and the transcript when transcribed with [α-^{32}P]UTP and unlabeled ATP. Solid arrows indicate the conditions under which the radioactivity is transferred to Ap, while the open arrow indicates no transfer occurs when two FUs are adjacent. Thus polynucleotides with clustered modified nucleotides will not give quantitative data by using nearest neighbor analysis.

is complementary to the carrier, e.g., poly(C) carriers are transcribed by [α-^{32}P]GTP, and (ii) by using a noncomplementary [α-^{32}P]NTP to determine the incorporation directed by the modified base. In the first case there is generally very high labeling of 2′(3′) nucleotides, while in the second case only low labeling can be expected. These two approaches are used for transcription under both competitive conditions with all four triphosphates and noncompetitive conditions, using the triphosphate complementary to the carrier and one other triphosphate.

Since the presence of a modified base in polymers did decrease overall transcription (Figure 1), and the absolute incorporation of noncomplementary triphosphates was low, it could not be excluded that the radioactivity found was due to terminal addition or self-polymerization. Nearest neighbor analysis supplied positive proof of internal incorporation. Figure 3 illustrates the use of the method. Data obtained with [α-^{32}P]ATP could not be interpreted since this triphosphate was the only one to exhibit a high degree of self-polymerization and led to predominant transfer of ^{32}P to A giving ApA sequences.

(a) [α-^{32}P]UTP. Table II presents data obtained by using all the types of polymers used in this investigation. The product of transcription using [α-^{32}P]UTP with unlabeled ATP, GTP, and CTP is shown in terms of its nearest neighbor sequence. If C is the carrier, then any radioactivity should be transferred to Gp. In the same way, if U is the carrier, radioactivity should be transferred to Ap. These transfers of label are in fact found for copolymers containing methylated bases except 1-MeG, indicating that the methylated derivatives used direct UTP incorporation into the complementary strand, thus simulating the presence of A.

The various polymers used as controls contain bases which have a predictable behavior. Every result obtained in this group can be explained as a consequence of normal base pairing. An example is poly(A, FU) which directs a transcript containing a high proportion of A^{32}pU sequences (Figure 3). On this basis it is also expected that polymers lacking A cannot transfer ^{32}pU to any nucleotide other than U. Data for U^{32}pU transfer are omitted since the small amount due to self-polymerization cannot be calculated. This level of self-polymerization (2000–5000 cpm in U^{32}pU) did not affect the data for other sequences. In the case of [^{32}P]ATP, the self-polymerization under the same conditions was 100 times higher.

(b) [α-^{32}P]CTP. If poly(C, 3-MeU) 13:1 is transcribed by DNA-dependent RNA polymerase using [α-^{32}P]CTP, completely different results are obtained depending on whether all four NTPs are present or only one other NTP, in addition to [α-^{32}P]CTP. 3-MeU directed G^{32}pC transfer can only be detected when GTP and [α-^{32}P]CTP are present and ATP and UTP are absent (data not shown). The fact that 3-MeU directs the incorporation of CTP poorly in the presence of all four triphosphates, while actually having the capability to incorporate CTP, is due to its relatively greater ability to direct incorporation of other triphosphates. Thus, CTP incorporation can only be detected under noncompetitive conditions. In the

Table II: Nearest-Neighbor Sequence Analyses Using [^{32}P]UTP in Transcription of Ribopolynucleotides Containing Modified Bases[a]

template[b]	nearest-neighbor sequence (cpm)[c] A^{32}pU	G^{32}pU	C^{32}pU
poly(C) containing[d]			
11% 3-MeC	194	*2842*[e]	183
7% 3-MeU	174	*4181*	126
11% 1-MeG	245	149	187
8% 1-MeA	218	*1165*	131
8% N^6-MeA	241	*3321*	136
13% X	270	*595*	162
poly(U) containing[f]			
4% 3-MeU	*427*	115	142
9% 1-MeA	*419*	101	132
11% N^6-MeA	*3522*	85	151
homo- or heteropolymer controls			
poly(C)	227	234	108
poly(C,G), 7:1	288	223	183
poly(A)	231	112	163
poly(A,G), 5:1	260	148	221
poly(A,FU)[g]	*11000*	197	352
poly(C,G,U,A), 12:6:2:1	295	*1322*	351

[a] See Materials and Methods. All four nucleoside triphosphates were present in these experiments. [b] All polymers were prepared and analyzed in this laboratory with the exception of poly(C, 7% 3-MeU) and poly(A, 33% FU) which were gifts from Dr. A. M. Michelson, Institut de Biologie, CNRS, Paris. [c] U^{32}pU sequences are not given in the table. This is due to two factors. There is some self-polymerization, or there is radioactivity due to undigested labeled UTP which electrophoreses partially in the same area as U^{32}p. All cpm are uncorrected. [d] By using copolymers with a C carrier, radioactivity due to incorporation resulting from the presence of bases other than C should be found in G^{32}pU sequences, if such bases simulate A. Figure 3 presents a schematic illustration of the principle of nearest neighbor analysis. [e] Significant numbers are italicized. [f] By using copolymers with a U carrier, radioactivity due to incorporation resulting from the presence of bases other than U should be found in A^{32}pU sequences, if such bases simulate A. [g] FU (5-fluorouracil) has the same base-pairing capacity as U (Grunberg-Manago & Michelson, 1964; Massoulié et al., 1966). In this experiment, the radioactivity in U^{32}pU was 28 500 cpm. See Figure 3.

Table III: Ambiguity of Modified Bases in Transcription Using [α-^{32}P]GTP. Nearest-Neighbor Sequence Analysis[a]

template[b]	nearest-neighbor sequence (cpm)			
	A^{32}pG	C^{32}pG	U^{32}pG	G^{32}pG[c]
expt 1				
poly(C,3-MeC), 8:1	*2120* (2.4)[d]	*2635* (3.0)	*2615* (3.0)	*80 800*
poly(C,3-MeU), 13:1	*1491* (3.3)	264 (0.6)	*3250* (7.2)	*45 000*
poly(C,1-MeA), 11:1	*2531* (2.5)	*948* (1.0)	*2170* (2.2)	*102 000*
poly(C,X), 7:1	1517 (1.6)	358 (0.4)	870 (0.9)	*92 000*
poly(C,G,U,A), 12:6:2:1	*2323* (7.0)	*2528* (7.7)	*1500* (4.5)	*33 000*
poly(C)	1214 (1.0)	261 (0.2)	984 (0.8)	*122 000*
expt 2				
poly(C,3-MeC), 8:1	*4047* (2.5)	*4540* (2.8)	*5632* (3.4)	*150 600*
poly(C,N^6-MeA), 11:1	1380 (1.1)	322 (0.26)	*6518* (5.3)	*114 200*
poly(C)	3073 (1.0)	448 (0.15)	2944 (0.95)	*304 800*
expt 3				
poly(C,N^6-MeA), 11:1	1020 (1.1)	220 (0.24)	*4660* (5.1)	*85 500*
poly(C,1-MeA), 7:1	*2016* (2.1)	*698* (0.73)	*4058* (4.3)	*88 500*
poly(C,3-MeU), 7:1	*3953* (2.4)	530 (0.32)	*7073* (4.3)	*150 000*
poly(C)	3264 (0.8)	441 (0.11)	1704 (0.4)	*411 500*
expt 4				
poly(U,1-MeA), 10:1	*1378*	145	727	494
poly(U,N^6-MeA), 8:1	442	136	988	750
poly(U,X)	503	145	914	722
poly(U,FU)	282	97	609	487
poly(U,C), 24:1	*3227*	136	535	569
poly(U)	520	77	390	1 184

[a] All four triphosphates were present; the conditions of transcription are "competitive". See Materials and Methods. [b] The template compositions are in Table I, except poly(U,X) and poly(U,FU) which could not be analyzed by LC. It is likely that poly(U,X) contains 15% X on the basis that the input and output ratios of poly(C,X) are the same. Poly(U,FU) was a gift from Dr. A. M. Michelson and contains at least one-third FU. [c] The absolute incorporation of [^{32}P]GMP is dependent on the specific activity of [α-^{32}P]GTP in each experiment. [d] Significant numbers are italicized. The numbers in parentheses are the percent of total [^{32}P]GMP incorporation, uncorrected for the level of background radioactivity and nonspecific incorporation directed by poly(C). In experiment 4, the carrier is U but, since labeled ATP is not present, no percentages can be given.

case of 1-MeA, incorporation of all triphosphates is similar under competing and noncompeting conditions.

(*c*) *[α-^{32}P]GTP.* This triphosphate gives by far the most reliable data since almost no self-polymerization is observed in the standard 30-min incubation period. Therefore, [α-^{32}P]GTP was used for transcription experiments with two variations. Experiment 1–3 in Table III gives the results when a poly(C) carrier was used and all triphosphates were present. High amounts of radioactivity for G^{32}pG transfer were found as well as radioactivity in other sequences as a consequence of the extent and type of modification. In many cases significant amounts of radioactivity were found in more than one sequence other than G^{32}pG, indicating that 3-MeU, 1-MeA, and 3-MeC direct the incorporation of two or three unlabeled triphosphates.

Experiment 4 in Table III gives the results obtained by using a poly(U) carrier. Of the polymers tested, only poly(U) containing 11% 1-MeA or 4% C incorporated [^{32}P]GMP next to A. This confirms that, under competitive conditions, 1-MeA simulates the presence of C, while N^6-MeA, X, or FU does not.

The data in Table III (also shown as percent incorporation in Table IV, top section) can be interpreted as proof that 1-MeA and 3-MeC can simulate A, G, or U and 3-MeU can simulate A or U. This was further tested by a series of noncompetitive experiments in which each triphosphate was used, one at a time, with [α-^{32}P]GTP (Table IV). Incorporation of [^{32}P]GMP was compared by using as templates poly(C) containing 3-MeC, 3-MeU, 1-MeA, or N^6-MeA. The nonspecific incorporation of the poly(C) carrier (Table III) was subtracted. 3-MeC had no significant preference for incorporating [^{32}P]GMP in CpG, UpG, or ApG sequences, thus again generally acting like any of three bases. The total amount of misincorporation of C, U, and A directed by 11% 3-MeC in poly(C) is about 6.5% under both noncompetitive and competitive conditions (Table IV, last column). This value does not include incorporation of G (data not obtainable using C carriers). Poly(C, N^6-MeA) was used as a control for quantification and the expected sequence UpG was 4.4% while 8% N^6-MeA was present. Figure 3 illustrates the point that nearest neighbor analysis is not necessarily quantitative.

Both 3-MeU and 1-MeA showed increased incorporation of AMP, CMP, and UMP when only ATP or GTP or UTP singly were present in addition to GTP (Table V). Although N^6-MeA in competitive experiments only directed UMP incorporation, under noncompetitive conditions significant amounts of AMP or CMP can be incorporated.

(*d*) *[^{3}H]GTP.* The data in Tables II and III do not give information on whether 3-MeU or 3-MeC simulate C. The only approach which yielded information, although indirect, was to compare the absolute transcription of poly(C) containing these two bases, using [^{3}H]GTP with all four triphosphates present with data from [^{3}H]GTP alone. The results are that transcription with [^{3}H]GTP alone proceeds almost as well as with all triphosphates when 3-MeC or 3-MeU are in the poly(C). As earlier found, 1-MeA simulates C (Table III, experiment 4), and in this type of experiment the presence of 1-MeA does not prevent transcription of poly(C) by [^{3}H]GTP alone. Poly(C) containing N^6-MeA was not transcribed when only GTP was used.

Discussion

The principal purpose of this investigation was to study the effect on transcription of bases modified on positions essential for hydrogen bonding. We were able to prepare a number of different "carrier" polynucleotides containing methylated bases. The proportion of modified base was chosen to be high enough to obtain analytical data but not to markedly change

Table IV: Summary of Misincorporation of Nucleotides Directed by Poly(C) Containing 3-MeC, 3-MeU, 1-MeA, and N^6-MeA[a]

template	modification (%)	NTP in addition to [α-^{32}P]GTP[b]	^{32}P radioactivity (%) in nearest-neighbor sequence[c] ApG	CpG	UpG	total av
			competitive conditions			
poly(C,3-MeC)	11	all	1.5, 1.4	2.6, 2.8	2.4, 2.2	6.4
poly(C,3-MeU)	12	all	1.6	[~0.2]	3.8	5.6
poly(C,1-MeA)	12	all	1.3	0.6	3.8	5.7
poly(C,N^6-MeA)	8	all	–	–	4.3, 4.6, 4.7	4.5
			noncompetitive conditions			
poly(C,3-MeC)	11	ATP	4.9, 5.7	–	–	5.3
		CTP	–	6.6, 6.8	–	6.7
		UTP	–	–	6.4, 6.6	6.5
poly(C,3-MeU)	12	ATP	5.9	–	–	5.9
		CTP	–	2.7	1.0[d]	3.7
		UTP	–	–	6.6	6.6
poly(C,1-MeA)	12	ATP	3.7	–	0.6[e]	4.3
		CTP	–	2.6	1.9[d]	4.5
		UTP	–	–	6.6	6.6
poly(C,N^6-MeA)	8	ATP	2.2, 3.0	–	–	2.6
		CTP	–	0.8, 1.9	3.1, 1.1[d]	3.4
		UTP	–	–	6.7, 4.8	5.7

[a] Data from Table III and from other experiments not in the table. The nonspecific incorporation directed by the poly(C) carrier is subtracted. [b] In all experiments the total NTP concentration was equal. In competitive studies when all NTPs were present, ATP, CTP, and UTP were used at the same concentration. [c] Radioactivity in ApG sequences indicates that the modified nucleoside simulated the presence of U. Similarly CpG and UpG radioactivity indicate simulation of G or A, respectively. [d] The incorporation of radioactivity into UpG sequences is attributed to the high concentration of CTP which contains, as a contaminant, UTP. [e] Possible contamination.

Table V: Effect of Modified Nucleosides on Transcription Using DNA-Dependent RNA Polymerase[a]

modified nucleoside	simulates the presence of A	G	U	C
3-MeCyd	++	++	++	(+)[c]
3-MeUrd	++	(+)[b]	+	(+)[c]
1-MeGuo	nd[d]	nd	nd	
1-MeAdo	++	+	++	+
N^6-MeAdo	+++	(+)[b]	(+)[b]	(–)[b,c]
5-fluorouridine	–	–	++++	–
xanthosine	+	–	–	(–)[c]

[a] Data on which this summary is based are in Tables II–IV and other results in the text. Pluses are subjective. Minuses mean that there was no incorporation greater than double the background of incorporation directed by homopolymers. [b] Simulation only in noncompetitive experiments. [c] Conclusion derived from polymers with cytidine carrier and transcription with [^{3}H]-GTP alone. [d] nd means detection not possible due to poor transcription.

the character and properties of the polynucleotide carrier. That is, the number of modified residues should not contribute substantially to the secondary structure of the polyribonucleotide. The very sensitive method of high-pressure liquid chromatography was used for precise and rapid analysis of almost all polynucleotides prepared. The limits of modification chosen, 5–15%, provided information on transcription of methylated nucleosides, with one exception. 1-MeGuo showed a slightly stronger inhibiting behavior than guanosine itself and transcription dropped to such a low level that significant data could not be obtained.

The methylated bases studied did not change the rate of transcription to a significantly greater extent than did unmodified nucleotides. An artificial RNA made with 70% cytidine carrier was, for instance, a relatively poor template. It must be realized that the transcription conditions are unnatural and manganese forces DNA-dependent RNA polymerase to accept an RNA template. But this does not say that in vivo RNA is a poor template for any enzyme, and it does not say that manganese changes the base-pairing properties (Sirover et al., 1979; L. A. Leob, private communication), although, in the case of DNA transcription, the error frequency is enhanced by Mn^{2+}, but to such a low extent that it would not be detected by our method.

We therefore believe that we have a reliable method to study the effect of low levels of random methylation in a natural nucleic acid.

Applying nearest neighbor analysis to transcription products allows several conclusions to be reached. These are shown in summary form in Table IV. 3-MethylUrd, 3-methylCyd, and 1-methylAdo lost almost all specificity in transcription experiments. Alkylation on ring nitrogens involved in Watson–Crick base pairing removes all hydrogen acceptor–donor abilities. Studies on the influence of removal of hydrogen bonding capability clearly illustrated the importance of the hydrogen bond between the pyrimidine N-3 and the purine N-1 (Gassen et al., 1972; Hagenberg et al., 1973). In addition to the lack of a donor or acceptor function, the alkyl group prevents the correct positioning of a nucleoside triphosphate in transcription. Thus, it is not unexpected that all of these N-alkylated nucleosides show at least partial ambiguity and have lost all specificity for base pairing. Only 3-MeU may have the potential to interact with ATP stronger than with other bases. Unfortunately, 1-methylguanosine could not be classified in the same way since, even in copolymers, it strongly inhibits transcription.

When all four triphosphates are present as they are in cells, one cannot detect the capability of a modified base which should base-pair normally, such as N^6-MeA, to act ambiguously, but by using noncompetitive conditions this primarily unnatural behavior can be detected. It is not likely to happen in vivo but the ability to do so may occasionally lead to mutation.

From our data, a general mechanism can be postulated. The polymerase reads through along the template until it reaches a methylated base. Rather than releasing the template, the enzyme continues to search for a triphosphate to fit. Since there are only weak or nonspecific parameters for any triphosphate, we observe a random incorporation. Only if there is another determining force present, such as stacking interaction or stereochemical hindrance, one or the other tri-

phosphate may be incorporated to a higher or exclusive extent. We term this mechanism ambiguity. This interpretation is in line with the random misincorporations observed in transcription of depurinated poly[D(A)], poly[d(A·T)], and poly[d(G·C)] using the natural conditions for transcription of DNA (Shearman & Loeb, 1977).

In codon–anticodon ribosome binding studies which have different requirements for fit, Singer et al. (1979) observed ambiguity of a different type. 3-MeU did not replace U but acted like C, while 3-MeC did not replace C but acted like U. In the same series of experiments, O^2-alkyl-U and O^4-alkyl-U could act like either U or C. It is likely that all the alkyl bases with substitutions on positions involved in base-pairing will, under certain circumstances, exhibit ambiguity and thereby lead to mutation.

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