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Transcription of Left-Handed Z-DNA Templates: Increased Rate of Single-Step Addition Reactions Catalyzed by Wheat Germ RNA Polymerase II†

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ABSTRACT: Wheat germ RNA polymerase II is able to transcribe polynucleotide templates in the poly[d(G-C)] family, adopting either the right-handed B or left-handed Z conformations depending on the ionic environment and temperature. Thus, with poly[d(G-C)] either the B state (in $MgCl_2$) or the associated Z* state (in $MnCl_2$) can be established. Poly[d(G-m^{5}C)] adopts the Z form readily in $MgCl_2$, and poly[d(G-br^{5}C)] can be regarded as being "constitutively" in the Z state. In transcription studies with CpG as a primer and templates in the left-handed conformation, it is found that the rate of productive elongation, i.e., the synthesis of poly[r(G-C)], is depressed, in accordance with the results of previous studies. However, with a single triphosphate substrate, CTP, the rate of formation of the first phosphodiester bond, i.e., the synthesis of CpGpC, is about 4-fold greater with both the Z and Z* templates than with B-DNA. This transcriptional activity is also catalytic in the sense that product concentrations exceed that of the enzyme. The synthesis of CpGpC is reduced in the presence of GTP. However, the apparent K_m value for GTP utilization is lower for the trinucleotide synthesis (0.1 μM) than that obtained for productive elongation (0.8 μM), a result that also holds for B-DNA templates. All transcription reactions are specifically inhibited by the fungal toxin α-amanitin, and, in the case of the left-handed templates, by monoclonal anti-Z-DNA antibodies. The relative probabilities of single-step addition and productive elongation imply that the major distinction between transcription of templates in the B and Z conformations involves a step following the synthesis of the first phosphodiester bond. As a result, fully competent elongation complexes do not form on the left-handed DNA.

The control of gene expression at the level of transcription may be exerted, at least in part, through alterations in DNA helical conformation or topology. As an example, in vitro transcription experiments conducted with procaryotic or eucaryotic RNA polymerases have shown that the transition between B-DNA and Z-DNA is associated with a decrease in the rate of RNA synthesis on the left-handed template (van de Sande & Jovin, 1982; Durand et al., 1983; Butzow et al., 1984; Santoro et al., 1984; Peck & Wang, 1985).

In one experimental design, alternating d(C-G)$_n$ sequences were inserted downstream to promoter sites in a circular plasmid (Peck & Wang, 1985). The results indicated that the conversion of the inserts to the left-handed Z-helical form induced by negative supercoiling introduces a strong transcriptional stop signal. The polymerase, together with its nascent transcript, stops at or near the B–Z junction. That is, a few nucleotides (about four) of the alternating d(C-G)$_n$ stretch are transcribed. Transcription resumes when the insert resumes the B conformation upon relaxation of the plasmid. Such a behavior was only encountered with alternating d(C-

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G)$_n$ sequences. In contrast, when alternating d[(A-C)·(G·T)]$_n$ sequences were placed downstream to the *lac* UV5 and *tac* promoters, the transcription complex proceeded through the inserts, even at superhelix densities stabilizing the Z conformation. However, the elongation of RNA transcripts was somewhat impeded, as evidenced by pausing near the d[(A-C)·(G·T)]$_n$ insert. From these experiments, it was deduced that left-handed DNA reduces transcription because the RNA polymerase cannot translocate efficiently into a DNA segment in the Z conformation, particularly in the case of the d(C-G)$_n$ sequence for which the B-to-Z transition is energetically more favorable compared to d[(A-C)·(G·T)]$_n$ [reviewed in Soumpasis and Jovin (1987)].

The effect of the B-DNA to Z-DNA transition on transcription has also been investigated on linear templates such as poly[d(G-C)]. The left-handed configuration of this polymer can be obtained under mild experimental conditions [reviewed in Jovin et al. (1987)] compatible with enzymatic polymerization in the presence of RNA polymerase. Thus, it was shown that a left-handed form of poly[d(G-C)], denoted Z*-DNA, can be produced at low ionic strength by the effects of divalent metals such as Mn^{2+} or Mg^{2+} (which are also cofactors of the RNA polymerases) combined with organic cosolvents or by using a brief transient exposure to elevated temperatures (van de Sande & Jovin, 1982; van de Sande et al., 1982). Another useful template in transcription studies has been poly[d(G-m^{5}C)], since methylation of the dC residue provides substantial stabilization of the left-handed structure (Behe & Felsenfeld, 1981). The main results previously reported with the linear templates are the following: (i) Both Z*-poly[d(G-C)] and Z-poly[d(G-m^{5}C)] can serve as templates for *Escherichia coli* RNA polymerase (van de Sande & Jovin, 1982; Butzow et al., 1984) or wheat germ RNA polymerase II (Durand et al., 1983). (ii) The level of nucleotide incorporation into RNA is less than that exhibited by the B form of poly[d(G-C)], such that the template activity ratios (defined as the relative incorporation with the two templates Z*/B or Z/B) have ranged from 0.1 to 0.5. In the case of wheat germ RNA polymerase II, we suggested on the basis of titration experiments that the reduced activity encountered with the left-handed configuration might derive from the availability of fewer and/or lower affinity sites for initiation or translocation on these templates (Durand et al., 1983).

The DNA-dependent single-step addition reactions described by Oen and Wu (1978) [reviewed in McClure (1985) and von Hippel et al. (1984)] have led to a precise description of the mechanism of formation of the first phosphodiester bond in RNA synthesis catalyzed by the *E. coli* RNA polymerase. The enzyme catalyzes the addition of a single nucleotide to a short primer such as a dinucleoside monophosphate or a ribonucleoside 5′-monophosphate. In the presence of a single nucleoside triphosphate substrate, this reaction necessarily constitutes an "abortive" event, thus leading to the use of the terminology "abortive synthesis", as opposed to "productive elongation", which refers to the formation of polymeric product in the presence of a full set of substrates. It is now well established that purified eucaryotic RNA polymerases II from yeast (Lescure et al., 1981), calf thymus (Vaisius & Wieland, 1982), and wheat germ (Dietrich et al., 1985) can also catalyze the DNA-dependent reaction of condensation of ribonucleotides to dinucleotide primers, leading to trinucleotide product formation. In these reactions, the extent of abortive synthesis as well as the nonprocessive behavior exhibited by the wheat germ enzyme (Durand et al., 1982; Dietrich et al., 1985) is dependent, at least in part, on the stability of transcription complexes (Dietrich et al., 1986; Job et al., 1987, 1988).

Comparative studies on transcription in vitro with wheat germ RNA polymerase II and *E. coli* RNA polymerase have indicated that the purified plant enzyme exhibits catalytic properties characteristic of the core form of its bacterial counterpart (Chandler & Gralla, 1980, 1981; Lewis & Burgess, 1980). However, recent phenomenological studies of the first steps of transcription by RNA polymerase II in vitro using HeLa cellular extracts, adenovirus DNA, and dinucleotide primers (Luse et al., 1987; Luse & Jacob, 1987; Rappaport & Weinmann, 1987) have indicated many similarities between the mechanisms of commitment to elongation of transcription complexes formed with the mammalian RNA polymerase II in the extract or with the purified plant enzyme (Dietrich et al., 1985; Job et al., 1987, 1988). This prompted us to investigate whether the B-DNA to Z-DNA transition could affect the rate of formation of the first phosphodiester bond in reactions catalyzed by wheat germ RNA polymerase II.

The general finding is that with left-handed templates the abortive pathways are enhanced, whereas the productive elongation reactions are diminished. In the present study, reactions were conducted in the presence of poly[d(G-C)], poly[d(G-m^{5}C)], and poly[d(G-br^{5}C)] as templates in order to clearly separate the effects of DNA conformation, base substitution, and aggregation on the enzymatic activity. The conformational transitions of the polymers were induced in the presence of Mn^{2+} or Mg^{2+}.

Materials and Methods

Reagents. Ribonucleoside triphosphates and the dinucleoside monophosphates were purchased from Sigma and Boehringer Mannheim. Nucleotide concentrations were calculated from absorbance measurements. [α-^{32}P]CTP (410 Ci mmol^{-1}) was from Amersham. Poly[d(G-C)] and poly[d(G-m^{5}C)] were from P-L Biochemicals; they were dissolved in 10 mM Tris-HCl[1] pH 7.8 buffer containing 2 mM EDTA. All buffer components were of reagent grade. [^{32}P]Poly[d(G-m^{5}C)] and poly[d(G-br^{5}C)] were synthesized with *Micrococcus luteus* DNA polymerase I (Pharmacia) according to adaptations (McIntosh and Jovin, unpublished results) of published procedures (Gill et al., 1974).

Wheat germ RNA polymerase II (essentially in the IIA form) was purified by the method of Jendrisak and Burgess (1975), as previously described (Job et al., 1984). The specific activity was 1080 units mg^{-1} for 10-min incubation. RNase H (800 units mL^{-1}) was from Pharmacia. Monoclonal anti-Z-DNA antibody 23B6 was generated by D. J. Arndt-Jovin using chemically brominated poly[d(G-C)] as the immunogen (Arndt-Jovin and Jovin, unpublished results). It was used as a purified IgG fraction.

Reaction Assays. Unless otherwise noted in the figure legends, the reaction mixtures contained 20 nM enzyme, 18 μM DNA (in nucleotide units), 1.7 mM dinucleotide CpG primer, 1 μM [α-^{32}P]CTP (10–20 Ci mmol^{-1}), and appropriate amounts of unlabeled nucleoside triphosphate substrates and metal salts ($MnCl_2$ or $MgCl_2$). In all assays, other components were 64 mM Tris-HCl, pH 7.8 buffer, 12.5% (v/v) glycerol, 12.5 mM 2-mercaptoethanol, 5 mM α-thioglycerol, 1.1 mM dithiothreitol, 0.05 mM EDTA, 0.05% Triton X-100, and 1.5 mM NaF [conditions for transcription assays used in Durand

[1] Abbreviations: TLC, thin-layer chromatography; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane. Dinucleoside monophosphate primers and trinucleoside diphosphate products are referred to as dinucleotides and trinucleotides, respectively.

Table I: Absorbance Ratios and Conformations of Template Polymers

polymer	condition	A_{280}/A_{260}	A_{295}/A_{260}	conformation
poly[d(G-C)]	no metal	0.503	0.13	B
	2.5 mM $MnCl_2$	0.56	0.17	B
	2.5 mM $MnCl_2$[a]	0.71	0.35	Z*
poly[d(G-m^{5}C)]	no metal	0.592	0.256	B
	0.3 mM $MgCl_2$	0.657	0.295	B
	5 mM $MgCl_2$	0.759	0.416	Z
	2.5 m$MnCl_2$	0.8	0.471	Z
poly[d(G-br^{5}C)]	no metal	0.794	0.567	Z
	2.5 m$MnCl_2$	0.857	0.616	Z

[a] Heated 10 min at 60 °C.

et al. (1983)]. Final volumes were 10 μL, and assays were usually incubated for 45 min at 35 °C. Reactions were stopped by mixing the 10-μL reaction mixtures with 20 μL of stop solution containing 1 mM EDTA, 80% formamide, and 0.1% xylene cyanol (XC). Control experiments lacking the DNA template were performed routinely, and all the rate measurements shown in the figures were corrected by such blanks.

Conformational Transitions and Spectra. The conformational states of poly[d(G-C)] and poly[d(G-m^{5}C)] were analyzed quantitatively by (i) the absorbance ratio A_{295}/A_{260} determined in an Uvikon 810 spectrophotometer thermostated at 35 °C (Table I) and (ii) sedimentation analysis (7000 rpm, 10000*g*, for 15 min) in a Jouan centrifuge according to the method of van de Sande and Jovin (1982). The Z* state of poly[d(G-C)] was generated by heating the DNA solution in transcription buffer containing 2.5 mM $MnCl_2$ at 60 °C for 10 min. Under these conditions, the Z*-DNA was easily sedimentable, as reported previously (Durand et al., 1983). The Z state of poly[d(G-m^{5}C)] was generated by adding $MnCl_2$ (1 or 2.5 mM) or $MgCl_2$ (5 or 10 mM) to DNA solutions in transcription buffer. Under these conditions, the Z-DNA was not sedimentable. The B state of the methylated polymer was obtained in the presence of $MgCl_2$ (<0.5 mM) in buffers used for transcription assays. With poly[d(G-br^{5}C)] as the template in the transcription buffer, the A_{295}/A_{260} ratios were similar in the absence and presence of 2.5 mM $MnCl_2$ (Table I), testifying to the constitutive (salt-independent) Z-state of this DNA (Malfoy et al., 1982).

Activity Measurements. The reaction mixtures were processed as follows (Job et al., 1984; Dietrich et al., 1985): (i) Total RNA synthesis was measured by trichloroacetic acid precipitation of 10 μL (reaction mixture + stop solution) spotted on Whatman GF/C filters; after eight washing steps in cold 5% trichloroacetic acid and 0.04 M sodium pyrophosphate and a washing step in absolute methanol, the filters were dried and counted for radioactivity in a liquid scintillation counter. (ii) Abortive synthesis of CpGpC was quantified after TLC on poly(ethylene imine)–cellulose sheets of 2 μL (reaction mixture + stop solution) (Randerath & Randerath, 1967) with 1 M HOAc as the solvent. (iii) The distribution of RNA chain lengths was analyzed by electrophoresis on 20% polyacrylamide gels (0.03 cm × 30 cm × 40 cm) with an 8 M urea, 50 mM Tris–borate, pH 8.0 buffer (Maniatis et al., 1982). Electrophoresis was conducted at a constant power of 90 W until the xylene cyanol had migrated 15 cm.

Nitrocellulose Filter Binding Assays. Filters (13-mm diameter, Millipore HAWP 0.45-μm pore size) were soaked in 0.3 M KOH for 20 min and rinsed with water and then with transcription buffer. Buffers were as described for transcription experiments but also contained 50 μg mL^{-1} BSA. Following filtration of 350 μL of transcription buffer, 450-μL reaction assays were filtered at a flow rate of 1 mL min^{-1} by using a Millipore vacuum pump. After drying, the filters were counted for radioactivity as for the Whatman GF/C filters. The assays contained 132 nM [^{32}P]poly[d(G-m^{5}C)]; 100% DNA retention corresponded to 3130 cpm. The DNA solutions in transcription buffer containing divalent metal salts as appropriate were incubated with wheat germ RNA polymerase or 23B6 antibodies for 30 min at 35 °C.

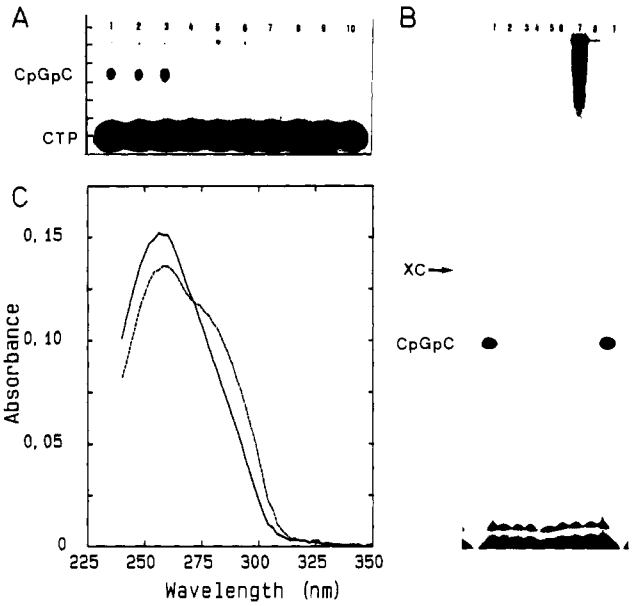

FIGURE 1: Synthesis of GpGpC with Z-poly[d(G-m^{5}C)] as template and CpG as primer. The divalent cation was 2.5 mM Mn^{2+}. Aliquots of 2 μL (A) and 7 μL (B) (reaction mixture + stop solution) were analyzed. (A) Radioautographic analysis by TLC: (lanes 1–3) complete reaction mixture; (lane 4) +16 μg mL^{-1} α-amanitin; (lane 5) minus enzyme; (lane 6) minus primer; (lane 7) minus $MnCl_2$; (lane 8) minus DNA template; (lane 9) +240 μM GTP; (lane 10) +240 μM GTP and 16 μg mL^{-1} α-amanitin. (B) Analysis by polyacrylamide–urea gel electrophoresis: (lane 1) complete reaction mixture; (lanes 2–8) see conditions for lanes 4–10 in (A). (C) UV absorption spectra of 0.21 μM poly[d(G-m^{5}C)] in transcription buffer: (—) no $MnCl_2$; (---) 2 mM $MnCl_2$. Optical path length, 1 mm; temperature, 35 °C. See Table I for summary.

Results

Formation of CpGpC Using CpG Primer. In a typical reaction assay, the synthesis of CpGpC occurred after incubation for 45 min of reaction mixtures containing wheat germ RNA polymerase II, CpG as primer, [α-^{32}P]CTP as substrate, and Z-poly[d(G-m^{5}C)] as template. In these experiments, the divalent cation was Mn^{2+} at 2.5 mM. When analyzed by TLC on poly(ethylene imine)–cellulose sheets, CpGpC migrated with a R_f value of 0.35–0.40, whereas unreacted CTP remained bound at the origin (Figure 1A). As shown in Figure 1A, the formation of this transcription product was absolutely dependent on the presence of enzyme, primer, DNA template, and divalent cation. The reaction was abolished upon addition of GTP, indicative of elongation to poly[r(G-C)] chains. The formation of CpGpC was strongly inhibited by α-amanitin, as expected for reactions catalyzed by class II RNA polymerases (Sentenac, 1985). However, as previously noted for UpApU formation on a poly[d(A-T)] template (Dietrich et al., 1985), the abortive reaction leading to CpGpC formation was not totally inhibited by the fungal toxin, which may be related to the fact that α-amanitin inhibits translocation but not the formation of phosphodiester bonds in reactions catalyzed by calf thymus RNA polymerase II (Vaisius & Wieland, 1982). Figure 1B shows the same reaction mixtures as in panel A but analyzed by high-resolution gel electrophoresis with

Table II: Steady-State Kinetic Parameters for CpGpC Synthesis with Wheat Germ RNA Polymerase II[a]

template	V_{max}[b,c] (pmol)	K_m values for CTP[c] (μM)	CpG[d] (mM)	enzyme[e] (nM)
B-poly[d(G-C)]	0.27	8.7	4.2	100
Z-poly[d(G-m^{5}C)]	0.9	9.5	5.3	120

[a] Reactions were conducted as described under Materials and Methods in a 10-μL volume in the presence of 18 μM (nucleotide units) DNA template and 2.5 mM $MnCl_2$. The numerical values were obtained by linear least-square analysis of Lineweaver–Burk plots. The mean correlation coefficient was 0.975. [b] CpGpC synthesis in 45 min at 35 °C. [c] [CpG] = 1.7 mM, 0.25 pmol of enzyme, [CTP] varied. [d] [CTP] = 1 μM, 0.2 pmol of enzyme, [CpG] varied. [e] [CTP] = 1 μM, [CpG] = 1.7 mM, enzyme varied.

results that are consistent with those obtained by TLC. The gels also resolved the α-amanitin-sensitive poly[r(G-C)] chains synthesized on Z-poly[d(G-m^{5}C)] (lanes 7 and 8). We conclude that wheat germ RNA polymerase II is able to catalyze the abortive reaction of single-step addition of a nucleotide to a dinucleotide primer on a left-handed DNA template.

The kinetics of CpGpC synthesis were reasonably linear for 60 min when B-poly[d(G-C)], Z*-poly[d(G-C)], and Z-poly[d(G-m^{5}C)] was used as templates. However, the level of trinucleotide formation was higher with both Z- and Z*-DNA templates than with the B template. Thus, under the standard conditions given under Materials and Methods (2.5 mM $MnCl_2$), the concentration of substrate CTP converted into CpGpC was 6, 4, and 3 nM with Z-poly[d(G-m^{5}C)], Z*-poly[d(G-C)], and B-poly[d(G-C)] as the template, respectively. This increased activity encountered with the left-handed templates was not due to DNA aggregation, since higher rates of CpGpC synthesis were obtained with Z-poly[d(G-m^{5}C)] than with Z*-poly[d(G-C)]. Furthermore, centrifugation of the DNA-containing reaction solution prior to transcription produced little effect with the Z-methylated DNA template, as opposed to the very dramatic influence on the reaction with the Z*-poly[d(G-C)] template. In the latter case, and as described previously (van de Sande & Jovin, 1982; Durand et al., 1983), the supernatant was substantially depleted of template and therefore supported incorporation only to the relative extent of about 20%.

In order to clearly distinguish the effects of the divalent metal concentration on the enzyme activity from those caused by the use of a Z-DNA template, CpGpC synthesis was measured with B-poly[d(G-C)] or Z-poly[d(G-m^{5}C)] in either $MnCl_2$ (5, 3.3, 2.5, and 1 mM) or $MgCl_2$ (10, 6.7, and 4.5 mM). In all cases, the same pattern of behavior was observed; i.e., CpGpC synthesis on the left-handed template was enhanced relative to that on the B-poly[d(G-C)] template.

The influence of CpG primer and CTP substrate concentrations on CpGpC synthesis with B-poly[d(G-C)] and Z-poly[d(G-m^{5}C)] was assessed by determination of apparent K_m values. These were not significantly different for the two templates (Table II). However, the comparative V_m values (Table II) reflect the higher template efficiency of Z-poly[d(G-m^{5}C)]. Saturation with respect to enzyme could be achieved for both templates. With the data plotted in double-reciprocal form, 1/[observed CpGpC synthesis] versus 1/[enzyme], linear relationships are obtained, allowing an estimate of apparent K_m values for the enzyme that are nearly identical for the B and Z templates (Figure 2A, Table II). From these data, the calculated occupancy of the B or the Z template by the enzyme at the K_m was of the order of 75–90 base pairs. Since the size of the site occupied by wheat germ RNA polymerase II on SV40 DNA is 35–41 base pairs

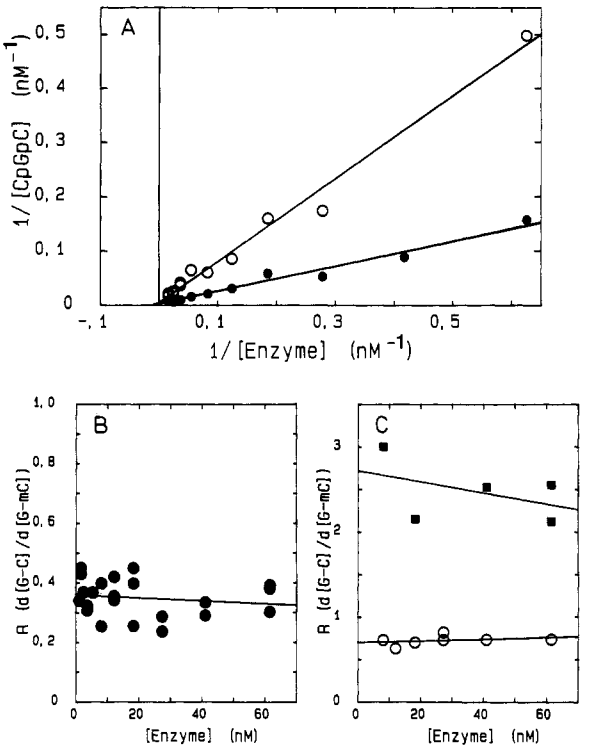

FIGURE 2: Saturation of B and Z templates with enzyme in CpGpC synthesis primed by CpG. (A) Templates: (○) B-poly[d(G-C)], K_m = 100 nM; (●) Z-poly[d(G-m^{5}C)], K_m = 120 nM. Divalent cation, 2.5 mM Mn^{2+}. (B) B/Z template activity ratio (R) for the data in (A). (C) B/Z template activity ratio (R) for experiments as in (B) except that the B and Z template conditions were established in (■) 0.5 mM and (○) 5 mM $MgCl_2$, respectively.

(Chandler & Gralla, 1980), the results in Figure 2 and Table II indicate that the saturation of the templates was complete and that the observed CpGpC synthesis was directed most presumably at double-stranded sequences rather than at any putative single-stranded nicks. The relative saturation of the B and Z templates by enzyme can be expressed as an activity ratio, B-poly[d(G-C)]/Z-poly[d(G-m^{5}C)]. Under experimental conditions stabilizing the Z form of the methylated polymer, this quantity did not depend on the enzyme concentration in the assay (Figure 2B,C), confirming the data of Figure 2A, a finding that also applied for poly[d(G-m^{5}C)] in the B state, i.e., in 0.5 mM $MgCl_2$ (Figure 2C). However, it can be seen that the conformational state of the template had a marked effect on the rate of CpGpC synthesis with wheat germ RNA polymerase II. Thus, the activity ratio was <1 (0.3–0.4 with $MnCl_2$ and 0.7 with $MgCl_2$) with the DNA template in the Z form but >1 (2.7) with the same template in the B conformation. The results presented in Figure 2 also demonstrate that the increased activity for CpGpC synthesis on Z-DNA (i) does not depend on the nature of the divalent cation used to induce the conformational transition of the DNA template and (ii) is greater than that inferred from a simple comparison of B-poly[d(G-C)] and Z-poly[d(G-m^{5}C)] as templates (Figure 2C). From the data obtained at 0.5 and 5 mM $MgCl_2$, we calculate that the B-to-Z transition leads to a ca. 4-fold increase in the rate of single-step addition reaction.

Effect of GTP Concentration on CpGpC Synthesis. Reaction mixtures were prepared in the presence of CpG as primer and [α-^{32}P]CTP as substrate, thereby leading to the formation of labeled CpGpC. The template was Z-poly[d(G-m^{5}C)] in 2.5 $MnCl_2$. Various amounts of unlabeled GTP were added prior to incubation for 45 min at 35 °C. Increasing the GTP concentration led to a pronounced decrease in the

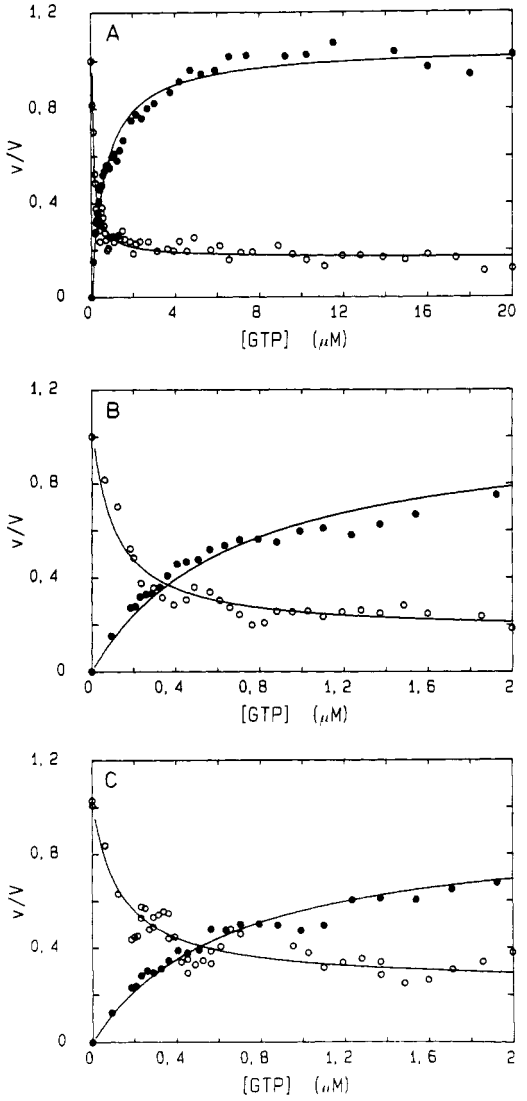

FIGURE 3: Effect of GTP concentration on CpGpC and poly[r(G-C)] synthesis. (A) Transcription with Z-poly[d(G-m^{5}C)] template and 2.5 $MnCl_2$ as in Figure 1. CMP incorporation after 45 min at 35 °C (○) into CpGpC and (●) into poly[r(G-C)]. The CpGpC and polymer synthesis were normalized to the values in the absence of GTP and to the V_{max}, respectively. The smooth lines are computer fits: CpGpC, $v/V = (1 + p_1[GTP])/(p_2 + p_3[GTP])$ with $p_1 = 1.5$, $p_2 = 1$, and $p_3 = 8.9$; poly[r(G-C)], $v/V = p_1[GTP]/(p_2 + [GTP])$ with $p_1 = 1$ and $p_2 = 0.7$. (B) As in (A) but for [GTP] ≤ 2 μM. (C) As in (A) but using a B-poly[d(G-C)] template. Computer fits: CpGpC, $p_1 = 1.8$, $p_2 = 1$, and $p_3 = 7.3$; poly[r(G-C)], $p_1 = 1$ and $p_2 = 0.8$.

rate of CpGpC synthesis and a concomitant increase in poly[r(G-C)] formation, as expected if CpGpC is elongated during the process of poly[r(G-C)] synthesis (Figure 3). It should be noted that the apparent K_m for GTP utilization is on the order of 0.1 μM for the CpGpC reaction, in contrast to a value of 0.8 μM for poly[r(G-C)] synthesis. Similar results were obtained with B-poly[d(G-C)] (Figure 3C).

Binding of RNA Polymerase to [^{32}P]Poly[d(G-m^{5}C)]. Ascribing a template activity to a given DNA molecule necessarily implies that the polymerase binds to the nucleic acid as the first step in transcription. That this is the case with wheat germ RNA polymerase II and the Z form of poly[d-(G-m^{5}C)] was established by direct binding assays using nitrocellulose filters and the ionic and temperature conditions of the incorporation reactions (2.5 mM $MnCl_2$, 35 °C). A maximal retention of about 70% of the radioactive probe (at 132 nM) was achieved with a half-saturation at about 1 nM

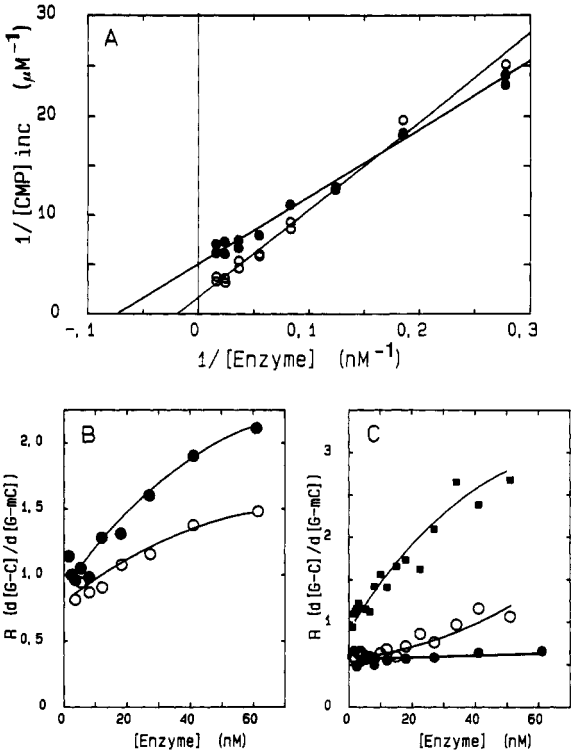

FIGURE 4: Saturation of template by enzyme during productive RNA synthesis. Divalent cation, 2.5 mM Mn^{2+}. (A) (●) Z-poly[d(G-m^{5}C)] template, apparent K_m = 14 nM; (○) B-poly[d(G-C)] template, apparent K_m = 60 nM. (B) B/Z template activity ratio (R) with (●) 2.5 mM $MnCl_2$ and (○) 1 mM $MnCl_2$. (C) B/Z template activity ratio (R) with (■) 5 mM $MgCl_2$, (○) 0.5 mM $MgCl_2$, and (●) 0.3 mM $MgCl_2$.

enzyme. Under identical experimental conditions, the same maximal retention of the radioactive DNA was obtained by using the 23B6 anti-Z-DNA antibodies, with a half-saturation at 1.25 nM (combining sites). There was no retention of the radioactive probe on the nitrocellulose filters upon incubation with the antibodies in the absence of $MnCl_2$, reflecting the specificity of the immunoglobulins for left-handed DNA (see Figure 5D below) and thereby confirming that the polymer in the polymerase binding experiment was in the Z conformation.

Productive Elongation. As in the case of Z*-poly[d(G-C)] (Durand et al., 1983), the apparent K_m value for the enzyme with the Z-poly[d(G-m^{5}C)] template was considerably lower than that of the B form of poly[d(G-C)] (14 nM compared to 60 nM, Figure 4A). If as described above (Figure 2) one defines the activity ratio R as the incorporation with the B-poly[d(G-C)] template divided by the corresponding value for the Z- or B-poly[d(G-m^{5}C)] template, one finds that this quantity varied with enzyme concentration, confirming the data in Figure 4A, as shown in Figure 4B for $MnCl_2$ (1 or 2.5 mM) and in Figure 4C for $MgCl_2$ (5 mM). However, the fact that R was >1 for higher enzyme concentrations is indicative of a reduction in the template activity of the Z form of poly[d(G-m^{5}C)]. Different results were obtained for the poly[d(G-m^{5}C)] template in the B conformation. Under these experimental conditions (0.3 mM $MgCl_2$), R was less than unity and did not depend on enzyme concentration (Figure 4C). These results indicate that B-poly[d(G-m^{5}C)] is a better template for productive RNA synthesis than B-poly[d(G-C)]. Thus, in this case as for the single-step addition reaction, the transition from B- to Z-DNA is associated with a larger effect than one might infer simply from a comparison of B-poly[d-(G-C)] and Z-poly[d(G-m^{5}C)].

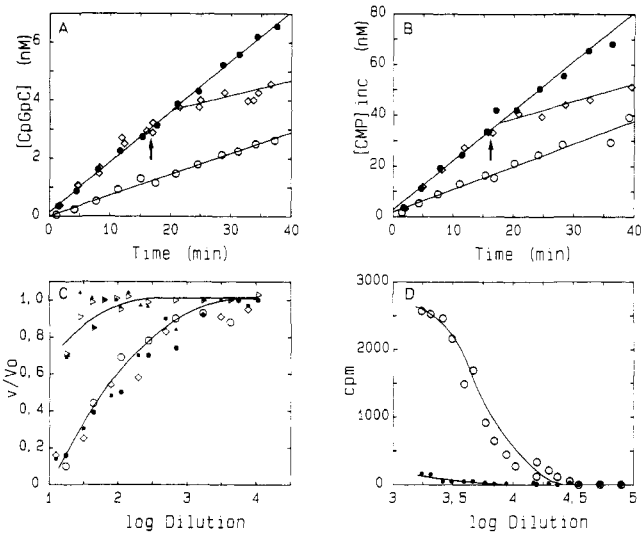

FIGURE 5: Inhibition of transcription by 23B6 anti-Z-DNA monoclonal antibody. (A) CpGpC synthesis with poly[d(G-m^{5}C)] template and 2.5 mM $MnCl_2$; (●) reaction in the absence of antibody; (○) reaction in the presence of 10 μg mL^{-1} antibody introduced 10 min before RNA polymerase; (◇) antibody addition 17 min (arrow) after initiation of transcription. (B) Poly[r(G-C)] synthesis: symbols as in (A). (C) Effect of antibodies on CpGpC synthesis and on productive elongation on B- and Z-DNA templates. Antibodies were added 10 min at 35 °C before the enzyme. Results are normalized to the rates obtained in the absence of antibodies. Symbols (filled, CpGpC synthesis; open, productive elongation): (triangles) poly[d(G-C)]; (circles) poly[d-(G-m^{5}C)]; (squares) poly[d(G-br^{5}C)]. The indicated dilution was in transcription buffer and of a stock 0.5 mg mL^{-1} antibody solution. (D) Formation of binary complexes between antibody and poly[d-(G-m^{5}C)]. Experiments were conducted in transcription buffer, as described under Materials and Methods. Incubations were for 30 min prior to filtration onto nitrocellulose filters. Divalent cation: (●) none; (○) 2.5 mM $MnCl_2$.

Transcription with Z-Poly[d(G-br^{5}C)] Template. In order to further establish the fact that the transcription in the above experiments was directed by the template in the left-handed conformation, similar assays were performed by using poly-[d(G-br^{5}C)] as the template. The same pattern of behavior was observed; i.e., CpGpC synthesis was enhanced relative to that achieved with the B-poly[d(G-C)] template, whereas in productive elongation, the Z-DNA template was relatively ineffective. Under the standard conditions described under Materials and Methods (2.5 mM $MnCl_2$), the template activity of Z-poly[d(G-br^{5}C)] relative to that of B-poly[d(G-C)] was 175% and 15% for the reactions of CpGpC and poly[r(G-C)] synthesis, respectively.

Inhibition of Transcription by Anti-Z-DNA Antibodies. Additional evidence that Z-poly[d(G-m^{5}C)] served as template in the reactions of single-step addition and of productive elongation was provided by the study of the effect of the 23B6 immunoglobulins specific for left-handed DNA. In both cases of abortive synthesis of CpGpC and of productive elongation, addition of 23B6 antibodies to the assay, either before the polymerase or during the course of the transcription, led to a marked inhibition (Figure 5). In contrast, the corresponding reactions carried out in the presence of B-DNA templates such as poly[d(G-C)] were insensitive to the presence of the anti-Z DNA antibodies (Figure 5).

Sensitivity of Transcription Products to RNase H. Dedrick and Chamberlin (1985) have shown that transcription in vitro is associated with different extents of RNA/DNA hybrid formation depending on whether the reactions are conducted with wheat germ or calf thymus RNA polymerase II or with *E. coli* RNA polymerase. Therefore, it was of interest to determine whether the reaction conditions used in this study could have altered the extent of hybrid formation on Z compared to B templates. We assessed the fractional distribution of products in the form of RNA–DNA hybrids by using RNase H. RNA was formed with B-poly[d(G-C)] or Z-poly[d(G-m^{5}C)] as templates in 10 mM $MgCl_2$, the optimal concentration for RNase H. The transcription was stopped by adding α-amanitin, followed by RNase H. Aliquots were removed at timed intervals and assayed for acid-precipitable radioactivity (residual polymeric product). The results presented in Figure 6 indicate that RNA–DNA hybrid formation normally accompanies in vitro RNA synthesis on alternating d(G-C) sequences. However, the proportion of hybrid in the product was approximately 20% higher with the B-poly[d(G-C)] template than with the Z-poly[d(G-m^{5}C)] template. The degradation of the hybrids by RNase H followed a simple exponential time course, with nearly identical values of the parameters for both B-poly[d(G-C)] and Z-poly[d(G-m^{5}C)]. Similar results were obtained with incubations in 5 mM $MgCl_2$.

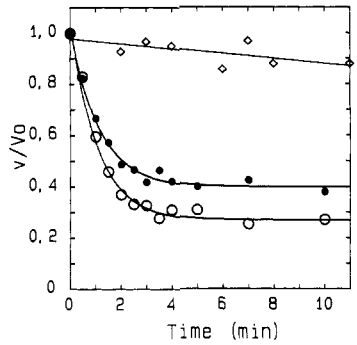

FIGURE 6: Sensitivity of transcription products to RNase H. The reactions (150 μL) leading to synthesis of labeled RNA were in 10 mM $MgCl_2$ and 18 μM DNA. After 45 min at 35 °C, 20 μg mL^{-1} α-amanitin was added, followed by 1.8 units mL^{-1} RNase H. Aliquots (10 μL) were analyzed with results normalized to the values at t = 0 (V_0). Conditions: (○) poly[r(G-C)] synthesis with a B-poly[d(G-C)] template; (●) poly[r(G-C)] synthesis with a Z-poly[d(G-m^{5}C)] template; (◇) control experiment with a Z-poly[d(G-m^{5}C)] template in the absence of RNase H. The smooth curves are computer fits to the equation $v/V_0 = p_1 \exp(-p_2 t) + p_3$: (●) $p_1 = 0.62$, $p_2 = 0.9$ min^{-1}, $p_3 = 0.39$; (○) $p_1 = 0.78$, $p_2 = 0.9$ min^{-1}, $p_3 = 0.20$.

DISCUSSION

The present study clearly demonstrates that the plant RNA polymerase II catalyzes the condensation of CTP to a CpG primer to form the trinucleoside diphosphate CpGpC in the presence of Z, Z*, or B alternating d(G-C) sequences as templates. The remarkable feature of the reaction catalyzed by wheat germ RNA polymerase II is the *enhanced* activity encountered with left-handed templates compared to that with those in the B conformation. However, the general properties of the reaction are common to both B and Z templates: (i) absolute requirement for enzyme, divalent cation, primer, and triphosphate substrate; (ii) α-amanitin sensitivity; and (iii) reduced rates under conditions leading to productive elongation. Anti-Z-DNA antibodies exert a strong inhibition on CpGpC synthesis but have no effect on the single-step addition reaction on B templates, findings which render unlikely that the observed reaction is simply due to a small amount of residual "contaminating" B-DNA in the left-handed templates. Even stronger evidence against the latter possibility is provided by the observations that (i) CpGpC synthesis is higher on Z as opposed to B templates, (ii) the Z template can be easily saturated with respect to enzyme, (iii) the activity persists at divalent cation concentrations for which the known thermodynamic B–Z equilibria correspond to vanishing fractions of

the right-handed conformation, e.g., as in the case of the Z-poly[d(G-br^{5}C)] template, and (iv) wheat germ RNA polymerase II binds readily to the left-handed DNA, Z-poly-[d(G-m^{5}C)]. The K_m values for CTP and CpG (Table II) indicate that the increased template efficiency is not due to a different substrate and/or primer utilization by the enzyme, since all corresponding parameters have very close numerical values. The saturation behavior of the templates with respect to enzyme was also investigated. The apparent K_ms for enzyme on the B and Z templates are very similar (Table II). It is noteworthy that the calculated occupancy of the poly[d(A-T)] template by wheat germ RNA polymerase II in the UpA primed synthesis of UpApU is on the order of 100 base pairs (Dietrich et al., 1986), which compares well with the determinations in Table II. The comparison indicates that the accessibility to the enzyme molecules and the number of available sites for trinucleotide synthesis do not differ appreciably with the sequence and/or conformation of the template. This interpretation is further supported by the observation that the template activity ratio (B/Z) does not depend on enzyme concentration (Figure 2). The values in Table II indicate that CpGpC synthesis can proceed catalytically, at least in the case of the Z-poly[d(G-m^{5}C)] template, such that the maximum amount of trinucleotide synthesized exceeds the enzyme concentration in the reaction. In reactions of single-step addition of a nucleotide to a dinucleotide primer catalyzed by *E. coli* RNA polymerase, the pyrimidine-terminated trinucleotides are formed catalytically and in larger amounts than the purine-terminated trinucleotides (Oen & Wu, 1978; Sylvester & Cashel, 1980). From Table II and Figure 2 we conclude that the most reasonable explanation for the greater rate of CpGpC synthesis with the left-handed templates is an enhanced rate of product release. Thus, the transition from B- to Z-DNA is associated with modifications of the stability of ternary transcription complexes involved in the catalysis of a single phosphodiester bond, for example, affecting the processivity of the reaction, the probability of enzyme translocation, and/or the relative stability of the various helical structures involved in the mechanisms (e.g., DNA duplexes upstream and downstream to the site of catalysis and product–DNA hybrids). The present study clearly establishes that the enhanced rate of trinucleotide formation is correlated primarily with the conformational transition in the template DNA and that neither aggregation nor methylation plays a significant role.

Under all experimental conditions examined, the left-handed conformations of poly[d(G-m^{5}C)] and of poly[d(G-br^{5}C)] also served as templates for productive RNA synthesis catalyzed by wheat germ RNA polymerase II, extending our previous results (Durand et al., 1983). The main characteristic feature of productive RNA chain elongation on Z-DNA (or Z*-DNA) during transcription by wheat germ RNA polymerase II is that the left-handed configuration has a reduced template efficiency, particularly at high enzyme concentrations. This effect seems to derive mainly from the relative accessibility of B and Z (or Z*) forms of the template to the enzyme molecules. All the results indicate that one needs less enzyme to obtain a functional saturation of a given amount of Z or Z* template as compared to that needed for a B template. Methylation of the DNA template cannot per se explain the reduced template activity of Z-poly[d(G-m^{5}C)], since the B form of this polymer is an even better template than B-poly[d(G-C)] (Figure 4).

The results obtained for poly[r(G-C)] synthesis with wheat germ RNA polymerase II are in good agreement with those reported from a similar study with procaryotic *E. coli* RNA polymerase (Butzow et al., 1984). For the latter enzyme, RNA synthesis on Z-poly[d(G-m^{5}C)] represented 40–50% of that measured on B-poly[d(G-C)]. Furthermore, much care was taken to avoid aggregation of left-handed DNA templates (Butzow et al., 1984). *E. coli* DNA polymerase I is almost totally inactive on Z-poly[d(G-m^{5}C)], although the efficacy of the B-poly[d(G-m^{5}C)] template is nearly identical with that of poly[d(G-C)] (Ramesh et al., 1986). From these comparisons, two points emerge: (i) The RNA polymerases seem to be much less inhibited by the B- to Z-DNA transition than the DNA polymerases. (ii) Methylation of B-DNA seems to exert a larger effect on the eucaryotic RNA polymerase than on the procaryotic RNA and DNA polymerases. This circumstance may have some physiological significance in view of the fact that DNA methylation plays an important role in the regulation of transcription in eucaryotic cells. We note that m^{5}C appears to be the only modified base in DNA of eucaryotes and that it occurs predominantly in the sequence CpG (Razin & Riggs, 1980; Doerfler, 1983).

With *E. coli* RNA polymerase, productive elongation is essentially an escape from the recycling phenomenon during which abortive products of short lengths are released (Carpousis & Gralla, 1980). If one compares the levels of products formed in the abortive and productive pathways under the various experimental conditions investigated in this study, it appears that a strong and inverse correlation is found between the rates for the two pathways, as described for bacterial RNA polymerase [reviewed in von Hippel et al. (1984)]. Conditions favoring formation of abortive products are associated with decreased rates of productive RNA synthesis (e.g., transcription of left-handed templates). This inverse relationship also holds in the opposite situation (reduced formation of abortive products and increased RNA synthesis), as in the transcription of methylated right-handed templates. The results indicate that on both B and Z templates purified wheat germ RNA polymerase II exhibits some of the characteristic features that have been well-documented for procaryotic transcription.

The commitment of transcription complexes to productive elongation was studied by measuring the effect of GTP concentration on the amount of CpGpC synthesis. Increasing the concentration of the elongating substrate caused a decrease in the formation of CpGpC and an increase in the rate of synthesis of poly[r(G-C)] chains (Figure 3). It is noteworthy that at saturating GTP concentrations (>5 μM) about 20% of CpGpC still remains synthesized and escapes elongation on both Z and B templates, suggesting that formation of abortive products normally accompanies productive RNA synthesis on the poly[d(G-C)] templates. Furthermore, the apparent K_m for GTP utilization is much smaller when calculated from the disappearance of CpGpC than the corresponding value for poly[r(G-C)] synthesis (Figure 3A,B). Similar results have been obtained for the B form of poly[d-(G-C)] (Figure 3C) and by studying the effect of ATP concentration on the rates of UpApU and poly[r(A-U)] synthesis with poly[d(A-T)] as template (Job et al., 1987). For the latter reaction it has been proposed that the very dramatic decrease in UpApU synthesis observed at low ATP concentrations is due to an ATP-induced stabilization of the ternary transcription complexes (Job et al., 1987). It is worth mentioning that this behavior has also been detected from phenomenological studies of the first steps of in vitro transcription using HeLa cellular extracts, adenovirus DNA, and dinucleotide primers (Luse et al., 1987; Luse & Jacob, 1987;

Rappaport & Weinmann, 1987). The results in Figure 3 may suggest that the mechanisms involved in the commitment of transcription complexes to elongation are similar on left- and right-handed DNA.

Since the results obtained for productive elongation indicate a reduced efficiency of the left-handed templates, we tested the possibility that the presence of nascent RNA chains somehow reduced the accessibility of left-handed templates to transcription complexes by RNA–DNA hybrid formation. An alternative possibility would be that the "renaturase activity" found in wheat germ RNA polymerase II and *E. coli* RNA polymerase (Dedrick & Chamberlin, 1985), i.e., the activity which displaces the RNA–DNA hybrids formed during transcription and therefore restores a free DNA template, may function on B but not on Z templates. A substantial proportion of poly[r(G-C)] chains was sensitive to the action of RNase H in the case of RNA synthesis carried out with both B-poly[d(G-C)] and Z-poly[d(G-m^5C)] as templates. However, the extent of hybrid formation was somewhat smaller with the left- than with the right-handed template. Furthermore, the rates of degradation by RNase H were similar with B- and Z-DNA, suggesting that in both cases the hybrids had same structure and similar accessibility to RNase H. We conclude that formation of nascent RNA molecules is not the case of reduced transcription of Z-DNA.

A tentative explanation for the different relative template efficiency of left-handed DNA in the single-step addition reaction and in productive RNA synthesis would be that the ternary transcription complexes involved in the first steps of RNA synthesis (those having formed a single phosphodiester bond) are not able to yield competent elongation complexes on Z-DNA, possibly due to a reduced competence for translocation along the left-handed helical structure because of inherent stereochemical factors or differential helical stability. DNA aggregation and/or C5 substitution (methylation–bromination) are probably not involved. Such a proposal would be in agreement with the observation that some, but not all, Z-DNA sequences placed downstream to promoter sites in suprcoiled plasmids hinder or block transcription (Peck & Wang, 1985). However, it should also be emphasized that in the cited experiments initiation of RNA synthesis was in a region of B-DNA and the transcription complex had to traverse a B–Z junction before reaching the left-handed insert. Clearly, linear DNA templates of homogeneous composition cannot reproduce this situation.

In conclusion, the present study provides evidence that wheat germ RNA polymerase II not only interacts with Z-DNA but is also able to bind the dinucleotide primer and to catalyze the formation of a phosphodiester bond. RNA synthesis is a complex process involving several substeps at which different control may be exerted. The present study illustrates that the transition from a B- to a Z-DNA template is associated with a variety of effects, from activation to inhibition in reactions catalyzed by wheat germ RNA polymerase II, a finding that emphasizes the central importance of DNA conformation upon gene expression.

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