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Comparative transcription of right- and left-handed poly[d(G-C)] by wheat germ RNA polymerase II

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The template properties of left-handed synthetic polymers, the Z* form of poly[d(G-C)] and the Z form of poly[d(G-m^{5}C)], have been investigated using an eucaryotic RNA polymerase, the class II enzyme from wheat germ. Results from a comparative kinetic study of transcription using the polynucleotide substrates in the B and Z conformations are reported. Optimal conditions for enzyme activity compatible with the preservation of the desired template conformation were determined. On the basis of several criteria, both physical (c.d. spectra of the polymers, sedimentability of the Z* form) and biochemical, it was demonstrated that the left-handed conformations of poly[d(G-C)] and poly[d(G-m^{5}C)] serve as templates for wheat germ RNA polymerase II. The level of incorporation was less than that exhibited by the B form of poly[d(G-C)], the relative activity being a function of the precise experimental conditions. Activity ratios (Z*/B or Z/B) ranged from 0.1 to 0.5. The effect of various incubation parameters, including pH, salt concentration, temperature, and the presence of dinucleoside monophosphate primers were investigated. The *Km* values for nucleoside triphosphate substrates were slightly smaller for the Z* form of poly[d(G-C)] than for the B conformation. Titration of DNA (Z* or B) with enzyme and reciprocal experiments suggested that the reduced activity of left-handed templates might derive from the availability of fewer and/or lower affinity sites for initiation and/or translocation on these templates. Specific antibodies raised against left-handed DNA strongly inhibited the observed transcription of Z* and Z DNAs by wheat germ RNA polymerase II.

Key words: poly[d(G-C)]/transcription/RNA polymerase II/Z DNA

Introduction

Poly[d(G-C)] undergoes a structural transition in concentrated salt (Pohl and Jovin, 1972) or ethanol (Pohl, 1976) solutions which is accompanied by large changes in c.d., optical absorbance and in other physical properties. The high salt conformation of poly[d(G-C)] is a member of the 'Z' family of left-handed helices (Wang *et al.*, 1981), while the low salt structure corresponds to the right-handed B form. There is immunochemical evidence for the existence of Z DNA structures in natural sequences (Nordheim *et al.*, 1981; Jovin *et al.*, 1982; Pohl *et al.*, 1982; Arndt-Jovin *et al.*, 1983; Zarling *et al.*, 1983). Furthermore, the stability of alternating purine-pyrimidine sequences having the Z conformation and adjoining natural right-handed B DNA has been demonstrated using recombinant negatively supercoiled plasmids (Klysik *et al.*, 1981; Singleton *et al.*, 1982; Zacharias *et al.*, 1982; Nordheim *et al.*, 1982). The possible implications of these observations for the regulation of gene activity are still largely speculative. For example, the left-handed configuration may serve as a structural control element or, alternatively, it may support biological functions directly. The latter hypothesis has stimulated the search for mild conditions under which Z DNA can be established *in vitro* and examined biochemically. It has been recently demonstrated that a left-handed form of poly[d(G-C)] denoted 'Z* DNA' can be produced at low ionic strength by the combined effects of Mg^{2+} and ethanol (van de Sande and Jovin, 1982) or of Mn^{2+} or other transition metals and a brief transient exposure to elevated temperatures (van de Sande *et al.*, 1982). Certain results support the contention that Z(Z*) DNA under such conditions may be biochemically active. For example, it can be used as a template by *Escherichia coli* RNA polymerase (van de Sande and Jovin, 1982), it can be hydrolyzed by various nucleases (Behe *et al.*, 1981; Singleton *et al.*, 1982; Nickol *et al.*, 1982; Jovin *et al.*, 1983), and it can interact with histones (Miller *et al.*, 1983). The left-handed conformation can also be stabilized at low ionic strength by base modification (methylation: Behe and Felsenfeld, 1981; bromination: Lafer *et al.*, 1981; Malfoy *et al.*, 1982; iodination: McIntosh *et al.*, in preparation) and phosphate modification (Jovin *et al.*, 1983).

The control of gene expression at the level of transcription may be exerted, at least in part, through alterations in DNA conformation. Thus, a consideration of the possible roles attributable to Z DNA in eucaryotic nuclei requires the evaluation of its effect on the transcription process. We report here a kinetic study of the transcription of of poly[d(G-C)] in the right- and left-handed conformations by wheat germ RNA polymerase II.

Results

The B-Z equilibrium in RNA polymerase reaction mixtures containing $MnCl_2$*

The left-handed configuration of poly[d(G-C)] was generated in solutions containing 4 mM Mn^{2+} by heating at 60°C for 5-7 min, and then cooling to room temperature. This treatment was accompanied by optical spectral changes identical to those described by van de Sande *et al.* (1982) and the poly[d(G-C)] was completely sedimentable at low *g* forces. The latter property is characteristic of the associated state of this conformation (designed Z* DNA) established in the presence of divalent cations, e.g., Mg^{2+} or the transition metals (van de Sande and Jovin, 1982; van de Sande *et al.*, 1982). In unheated Mn^{2+} solutions the B form of poly[d(G-C)] is maintained.

To determine the relative kinetic parameters for the transcription of poly[d(G-C)] in the right- and left-handed conformations, it was necessary to establish the optimal conditions for enzyme activity compatible with the preservation of the desired template conformation. However, the enzyme requires the protective effect of sulfhydryl reagents [usually

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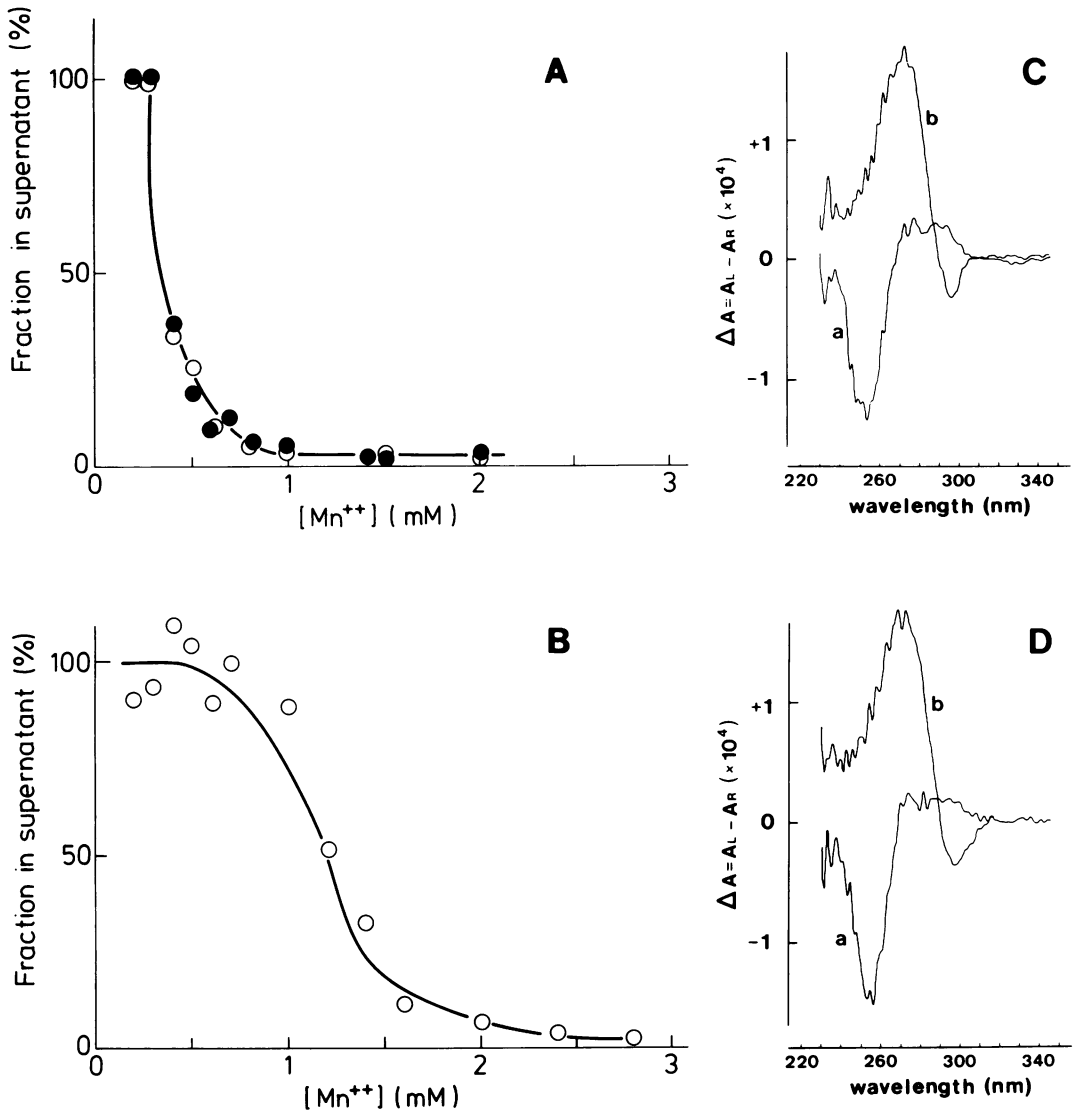

Fig. 1. Titration of [^{3}H]poly[d(G-C)] with $MnCl_2$. **(A)** Reverse Z*→B transition at 35°C in the absence of DTT. Samples for measurements were made by diluting to appropriate $MnCl_2$ concentrations. Each sample was centrifuged for 5 min. The radioactivity in the supernatant was used to calculate the fraction of rapidly sedimentable DNA. The samples contained 25 μM [^{3}H]Z*-poly[d(G-C)] in buffer C, 10 μM of both GTP and CTP, in the presence of (●) or in the absence (○) of 15 nM RNA polymerase. **(B)** Reverse Z*→B transition at 35°C in buffer C augmented with 1.1 mM DTT, 1.5 mM NaF, and an additional 39 mM Tris-HCl. Conditions otherwise as described in **(A)**. Identical results were obtained for the B→Z* transition in the presence or absence of 15 nM RNA polymerase. **(C,D)** c.d. spectra of poly[d(G-C)] under the conditions specified, respectively, in **(A)**, 1 mM $MnCl_2$; and **(B)**, 2.5 mM $MnCl_2$. The poly[d(G-C)] concentration was 0.25 mM and the optical pathlength 1 mm. C.d. spectra before **(a)** and after **(b)** 7 min heat step at 60–65°C. Similar spectra as in **(b)** were recorded after addition of 18 nM RNA polymerase to both the B and Z* forms of poly[d(G-C)]. The c.d. and u.v. absorption (not shown) spectra in **(a)** and **(b)** are very similar to those reported for the right-handed and left-handed forms, respectively, of poly[d(G-C)] in the presence of Mn^{2+} (van de Sande *et al.*, 1982).

the combination of 2-mercaptoethanol, dithiothreitol (DTT), and α-thioglycerol] which can potentially react with the Mn^{2+} cation essential for the generation of the Z* conformation. Figure 1A,B demonstrates the combined effects of solvent and solute additions on the B-Z* equilibrium. In the absence of DTT but in the presence of all other factors required for RNA synthesis, a transition curve was obtained (Figure 1A) similar to that previously reported (van de Sande *et al.*, 1982). However, the inclusion of DTT together with the greater concentrations of Tris-HCl shifted the transition to higher total Mn^{2+} concentrations and reduced the apparent cooperativity (Figure 1B). The effects probably were largely due to the increase in ionic strength (van de Sande *et al.*, 1982).

We controlled the conformation of the template under all conditions used in this study by applying the sedimentation criterion for Z* DNA (see Materials and methods). In some cases, we also examined the u.v. and c.d. spectra of the DNA, before and after the initial heating step leading to the B→Z* transition, as well as after incubation with the RNA polymerase (Figure 1C,D).

RNA synthesis on Z-poly[d(G-C)] templates in $MnCl_2$*

Both the B and Z* conformations of poly[d(G-C)] support RNA synthesis (Figure 2). In the absence of DTT and at 1 mM $MnCl_2$ the relative incorporation (Z*/B) was 12% (Figure 2A). At higher concentrations of the divalent cation, both activities diminished. However, it was determined that

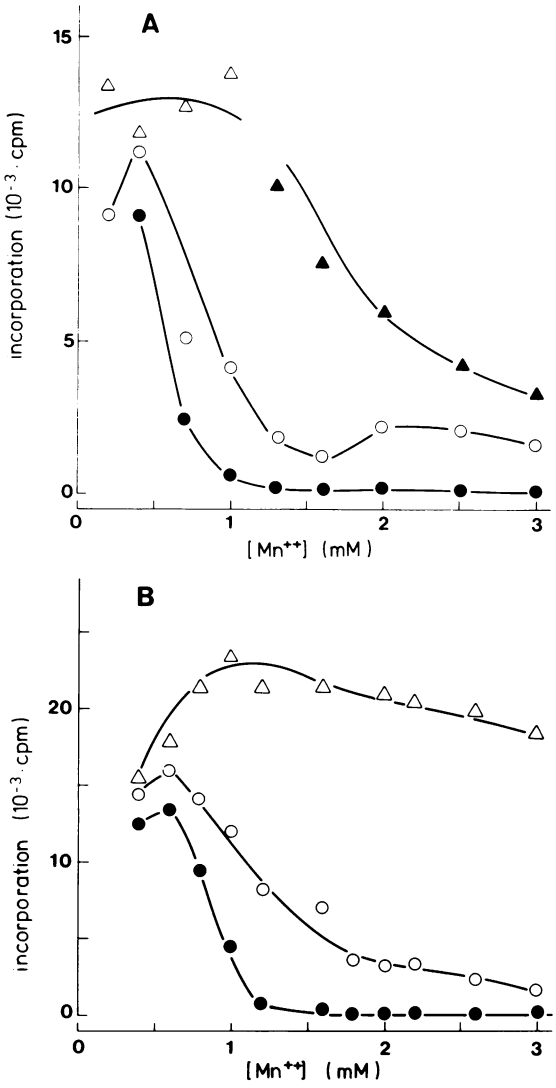

Fig. 2. Mn^{2+} dependence of the transcription of poly[d(G-C)] by wheat germ RNA polymerase II. Final concentrations were: enzyme 15 nM, DNA 25 μM, GTP = 10 μM, CTP = 10 μM. Labelled substrate was CTP with a final specific activity of 1.2 x 10^6 c.p.m./nmol. The pH was 7.8. **(A)** Reaction in the absence of DTT. Reactions were performed as described in Materials and methods. Incorporation of labelled substrate into RNA with Z*-poly[d(G-C)] as template was measured in both the unseparated reaction mixtures (○) and in their corresponding supernatants (●) after centrifugation. The data corresponding to the transcription of the B form of poly[d(G-C)] is also shown (△). The filled symbols (▲) represent the transition to a Z* form at 35°C (see text). **(B)** Reaction in the presence of 1.1 mM DTT (+NaF and additional Tris-HCl; see text). Conditions and symbols are otherwise as in **(A)**.

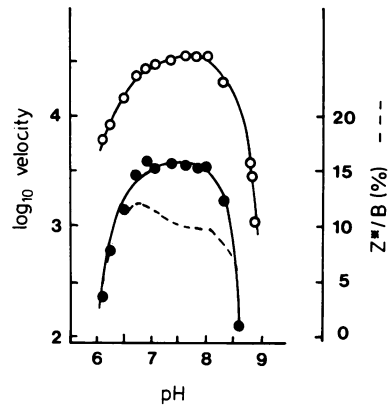

Fig. 3. pH dependence of poly[d(G-C)] transcription by wheat germ RNA polymerase II. Reactions were carried out as described in Materials and methods, in the presence of 2.5 mM $MnCl_2$ and 1.1 mM DTT. Final reactant concentrations were: enzyme 15 nM, DNA 25 μM, GTP = CTP = 5 μM. Labelled substrate was CTP with a final specific activity of 3.38 x 10^6 c.p.m./nmol. The pH was varied using 50 mM Tris-HCl buffers. The template was B (○) or Z* (●) poly[d(G-C)]. The activity ratio (Z*/B) is also shown (- -).

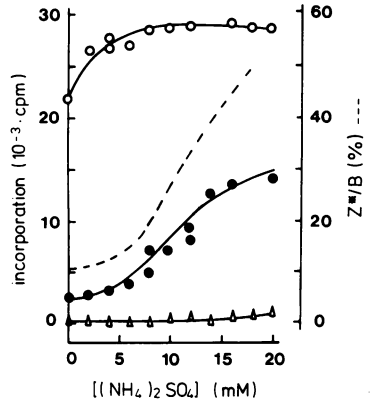

Fig. 4. Influence of ammonium sulfate concentration on the transcription of poly[d(G-C)] by wheat germ RNA polymerase II. Reactions were carried out as described in Materials and methods, in the presence of 2.5 mM $MnCl_2$ and 1.1 mM DTT. Final reactant concentrations were: enzyme 15 nM, DNA 25 μM, GTP 10 μM, CTP 10 μM. The labelled substrate was CTP with a final specific activity of 1.2 x 10^6 c.p.m./nmol. The pH was 7.8. The template form was B (○) or Z* (●) poly[d(G-C)]; (△) incorporation of labelled substrate in the supernatants after centrifugation of same reaction mixtures as (○). The activity ratio (Z*/B) is also shown (- -).

the B form was not stable under these conditions. For example, addition of 2.2 mM $MnCl_2$ at 35°C led rapidly ($t_{1/2}$ = 5 min) to the formation of a sedimentable Z* form. Apparently, the presence of 12.5% glycerol reduces the heating requirement for the B-Z transition, as reported previously with other mixed solvents (van de Sande *et al.*, 1982; Zacharias *et al.*, 1982). In 2.5 mM $MnCl_2$, 1.1 mM DTT (and the higher, 63 mM, Tris-HCl concentration), the Z*/B activity ratio was 12% (Figure 2B). Most of the additional experiments described below were carried out under this set of conditions.

The extent of incorporation relative to DNA phosphate did not exceed 1% at the levels of enzyme required for maintenance of steady state conditions. These low levels of synthesis are characteristic for the eucaryotic RNA polymerases (Job *et al.*, 1982). The RNA product synthesized from the Z* template was readily sedimentable, as reported previously in related studies with the *E. coli* RNA polymerase (van de Sande and Jovin, 1982). The base composition of the product was 52% and 48% (±2%) GMP and CMP, respectively, using the corresponding radioactive substrates. The reactions with both the B and Z* templates were inhibited to 90% by 1 μg/ml α-amanitin.

Effects of incubation parameters

The effects of numerous incubation parameters upon the extent of RNA synthesis were investigated. The pH profiles for poly[d(G-C)] transcription are shown in Figure 3. The pH optimum was 7–8 for both forms of the template and the ratio of activities was constant in this range. The effect of

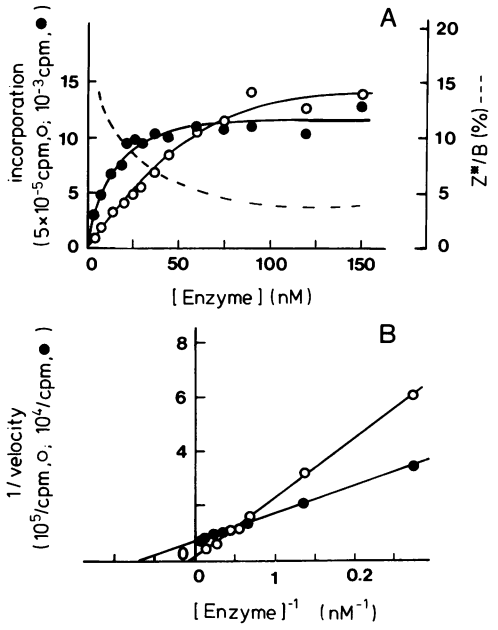

Fig. 5. Saturation of poly[d(G-C)] by wheat germ RNA polymerase II. Reactions were carried out as described in Materials and methods, in the presence of 2.5 mM $MnCl_2$ and 1.1 mM DTT. Final reactant concentrations were: DNA 25 μM, GTP 5 μM, CTP 0.74 μM. Labelled substrate was CTP with a final specific activity of 16 x 10^6 c.p.m./nmol (pH was 7.8). The data are represented as: **(A)** direct plot of incorporation of labelled substrate *versus* enzyme concentration; the templates were B (○) and Z*-poly[d(G-C)] (●), the activity ratio (Z*/B) is also shown (- -); **(B)** double reciprocal plots of the data in **(A)**, straight lines were calculated by linear least square analysis of the data; correlation coefficients were 0.999 for B (○) and 0.993 for Z* (●) forms of poly[d(G-C)].

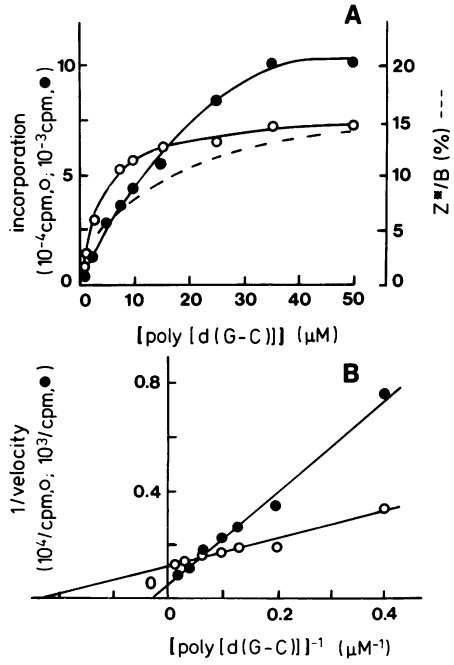

Fig. 6. Saturation of wheat germ RNA polymerase II by poly[d(G-C)]. The conditions were identical to those of Figure 5, except the enzyme concentration which was fixed at 15 nM. **(A)** direct plots of velocity *versus* DNA concentration with B (○) and Z* (●) poly[d(G-C)]. **(B)** double reciprocal plots of the data in **(A)**, straight lines were calculated by linear least square analysis of the data; correlation coefficients were 0.975 for B (○) and 0.995 for Z*-poly[d(G-C)] (●).

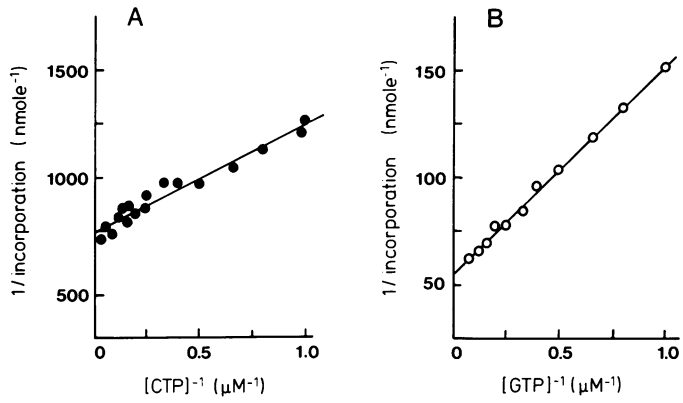

Fig. 7. Determination of the *Km* values for GTP of wheat germ RNA polymerase II with poly[d(G-C)] as template. Reactions were carried out as described in Materials and methods, in the presence of 2.5 mM $MnCl_2$ and 1.1 mM DTT. Final reactant concentrations were: enzyme 15 nM, DNA 25 μM, CTP 20 μM (pH was 7.8), *Km* values were determined by linear least square analysis of the data. **(A)** Z* form as template, correlation coefficient was 0.965; **(B)** B form as template, correlation coefficient was 0.998.

temperature on the absolute rates of incorporation was substantial; changing the incubation temperature from 15 to 37°C caused a 4-fold increase in the incorporation rates. However, the activity ratio (Z*/B; 2.5 mM Mn^{2+}, 1.1 mM DTT) was constant in the range 15° – 37°C. The influence of salt concentration is shown in Figure 4. Supplementation of the reaction by 0 – 20 mM ammonium sulfate resulted in a 1.3 and a 7-fold increase in incorporation for the B and Z* templates, respectively. Above 20 mM ammonium sulfate, a reversal of conformation from Z* to B occurred, as evidenced by an abrupt loss of sedimentability (data not shown). A similar competitive effect of monovalent cation (Na^+) on the Mn^{2+}-dependent B-Z* equilibrium has been reported previously (van de Sande *et al.*, 1982).

Km values

The influence of template, enzyme and ribonucleoside triphosphate substrate concentrations on transcription with poly[d(G-C)] were assessed by determinations of apparent *Km* values. Saturation with respect to enzyme at 25 μM DNA was achieved with both templates (Figure 5). However, the apparent *Km* with the Z* template was considerably lower than that of the B form (14 nM compared with 140 nM). The effect of 15 mM ammonium sulfate was to increase the enzyme *Km* value (with Z* template) ~3-fold (data not shown). At >0.2 μM enzyme, inhibitory effects were observed with both templates. The reciprocal titration with DNA at constant enzyme concentration (15 nM) also yielded saturation curves with *Km*s of 35 and 4.4 μM for the Z* and B templates, respectively (Figure 6). The *Km* values for each of the two nucleoside triphosphate substrates were determined in the presence of a saturating concentration (20 μM) of the other. Examples of double reciprocal plots of 1/velocity *versus* 1/[GTP] for the Z* and B templates are shown in Figure 7. Linear relationships were obtained in the substrate concentration range studied (1 – 20 μM). The *Km* values for GTP and CTP were in the micromolar range (0.7 – 5 μM) for both templates but were consistently about 50% lower in the case of Z* DNA (Table I).

Priming with dinucleoside monophosphates

The determination of initiation kinetics using ^{32}P-labelled substrates in the case of the eucaryotic RNA polymerases

Table I. *Km* values of wheat germ RNA polymerase II for nucleoside triphosphates

Template	Primer	*Km* for	
		CTP (μM)	GTP (μM)
B	–	5.0	1.8
Z*	–	2.3	0.7
B	GpC	0.2	4.1
Z*	GpC	0.2	1.7

The *Km* values were determined as shown in Figure 7.

poses severe experimental difficulties as compared with the experience with the procaryotic enzymes (Dynan and Burgess, 1979). However, dinucleoside monophosphates are effective primers with both the *E. coli* RNA polymerase (Oen and Wu, 1978) and the eucaryotic RNA polymerases (Lescure *et al.*, 1981), especially under conditions leading to initiation as the rate limiting process (low concentration of initiating purine triphosphate). We have determined that GpC, and to a lesser degree CpG, exert a strong stimulatory effect on the transcription of poly[d(G-C)] in either the B or Z* conformation and at low concentration of GTP.

Representative data for the Z* template are shown in Figure 8. The GpC primer leads to pronounced (10- to 25-fold) reductions in the *Km* for CTP and an ~ 2-fold increase in the *Km* for GTP (Table I).

Template properties of poly[d(G-m^{5}C)]

The associated nature of the Z* form of poly[d(G-C)] used as the left-handed template in these studies might be responsible in part for the decreased activity relative to the dispersed B form of the same polymer. We tested this point by employing poly[d(G-m^{5}C)] as an alternative template. The methyl substitution in the C-5 position of C in this polymer greatly potentiates the salt-induced B→Z transition (Behe and Felsenfeld, 1981). In particular, Mn^{2+} stabilizes the Z state at less than stoichiometric concentrations relative to DNA phosphate (van de Sande *et al.*, 1982). Under the standard conditions used in this study (1 mM $MnCl_2$, no DTT; 2.5 mM $MnCl_2$, 1.1 mM DTT), the Z state of poly[d(G-m^{5}C)] was established without aggregation to Z*. The B→Z transition in 1 mM $MnCl_2$ occurred with a $t_{1/2}$ of <1 min at 35°C. The template activity relative to the B form of poly[d(G-C)] was 27% (no DTT) and 19% (+DTT), that is, somewhat higher than the corresponding values for the Z* conformation.

Inhibition of transcription of Z DNA templates by anti-Z DNA IgGs*

Additional evidence for the transcription of Z and Z* DNAs by wheat germ RNA polymerase II was supplied by the effects of immunoglobulins specific for left-handed DNA. Experiments were performed with three different anti-Z DNA IgGs: polyclonal rabbit anti-Br-poly[d(G-C)] (T4), rabbit anti-poly[d(G-br^{5}C)] (E5), and mouse monoclonal anti-Br-poly[d(G-C)] (D11, Pohl *et al.*, 1982). These IgGs bind the left-handed Z form but not the B form of poly[d(G-C)], (Zarling *et al.*, 1983). Addition of these anti-Z DNA IgGs to the reaction mixture containing a Z* template at zero time inhibited RNA synthesis by ~100% (T4), 95% (D11), or 80% (E5) at concentrations of 0.025, 0.072, and 0.59 mg/ml, respectively. Similarly, when these three different antibodies were added at 8 min after the start of the

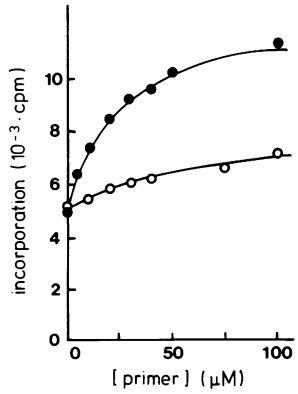

Fig. 8. Influence of dinucleoside monophosphates on the transcription of Z*-poly[d(G-C)]. Reactions were carried out as described in Materials and methods, in the presence of 2.5 mM $MnCl_2$ and 1.1 mM DTT. Final reactant concentrations were: enzyme 15 nM, DNA 25 μM, GTP 0.25 μM, CTP 0.4 μM. The labelled substrate was CTP with a final specific activity of 16 x 10^6 c.p.m./nmol. The pH was 7.8. Reactions in the presence of GpC (●) or in the presence of CpG (○).

polymerization reaction, further transcription was inhibited to the same extent. A control normal rabbit IgG had no effect on the transcription of Z* DNA. In addition, the transcription of the B form of poly[d(G-C)] was totally insensitive to the presence of the anti-Z DNA antibodies.

The requirement for a greater concentration of the E5 antibody was consistent with the known preference of this immunoglobulin for poly[d(G-C)] bearing a substitution in the C5 position of the pyrimidine, e.g., poly[d(G-m^{5}C)] (Zarling *et al.*, 1983).

Discussion

The results presented clearly demonstrate that under all experimental conditions examined, the left-handed conformations of poly[d(G-C)] (Z*) and of poly[d(G-m^{5}C)] (Z) serve as templates for wheat germ RNA polymerase II. Both biochemical and physical criteria were used to establish the integrity of the Z (Z*) form throughout the course of reaction. These included the sedimentability and c.d. spectrum of the Z* template. In addition, the RNA product was also sedimentable, suggesting the existence of active ternary complexes. These findings are in agreement with those presented previously for the *E. coli* RNA polymerase and support the proposal that left-handed DNA may directly support biological functions (van de Sande and Jovin, 1982). The level of incorporation was significantly less than that exhibited by the B form of poly[d(G-C)], the relative activity being a function of the precise experimental conditions. However, the general features of the reaction were common to both B and Z* templates: absolute requirement for both GTP and CTP substrates, α-amanitin sensitivity, pH activity profiles.

The highest absolute activity with the Z* template was obtained in the presence of added ammonium sulfate (20 mM, Figure 4). The assay used to assess synthesis was based on acid precipitation of the products and thus would not have scored smaller oligonucleotides. The latter have been shown to be produced in the case of the transcription of poly[d(A-T)] in the presence of Mn^{2+} (Durand *et al.*, 1982) but a similar product analysis with poly[d(G-C)] templates has not yet been completed. Preliminary results indicate that the ionic

strength influences the degree of processivity of RNA polymerase II using synthetic polymers as templates. The effects of salt combinations may be complex since the monovalent and divalent cations would be expected to exert different effects on the intra- and inter-molecular interactions determining the structure of the left-handed DNA and the binary complexes with the enzyme. For example, the binding of *E. coli* RNA polymerase to DNA has been shown to involve the release of counterions (Shaner *et al.*, 1983). In this connection, it is interesting that the increase in activity with the Z* template upon addition of ammonium sulfate was accompanied by a similar increase in the apparent *K*m for the enzyme to a value close to that obtained for the B DNA template. From the results presented, it appears that the main difference in the template efficiencies of B and Z* forms of poly[d(G-C)] derives from the relative accessibility of the template to the enzyme molecules. It should be noted that the results obtained with Z* template cannot be explained as being due to 'contamination' with small amounts of B DNA since any reasonable estimate (say 5% of the B form) would lead to a very low predicted level of activity from the B saturation curve and also since saturation should not have been achievable. Probably, the simplest explanation of the results in Figure 5 and 6, i.e., the fact that one needs more Z* DNA to saturate a given amount of enzyme, is that fewer sites and sites with lower affinity are available from titration with Z* DNA. Direct DNA-enzyme binding experiments designed to explore these questions in greater detail are in progress.

The basic features of the transcription reaction can be summarized as (reviewed by Kumar, 1981): (i) enzyme binding to DNA template and localized melting of the helix: (ii) initiation corresponding to the formation of the first phosphodiester bond: a purine nucleoside triphosphate at the 5′ end of the RNA synthesized is usually required; (iii) elongation involving the sequential incorporation of nucleoside monophosphates; and (iv) termination of the RNA chain. The differences observed during the transcription of B and Z* forms of poly[d(G-C)] by wheat germ RNA polymerase might therefore be reflected in one or several of these steps.

The *K*m values for nucleoside triphosphate substrates were determined. The results in Table I indicate that the difference in template efficiency was not due to substrate utilization by the enzyme since all corresponding parameters have very close numerical values. Furthermore, the values obtained with the Z* form of poly[d(G-C)] were slightly smaller than those measured using the B form. The low *K*ms for the nucleoside triphosphate substrates using the B form of poly[d(G-C)] are not a characteristic of this template, since measurements of corresponding values with other alternating purine-pyrimidine DNAs such as poly[d(A-T)] yield *K*m values for the substrates in the same concentration range (*K*m ATP = 3 μM, Job and Teissère, unpublished results). It should be noted that in the case of *E. coli* RNA polymerase (Anthony *et al.*, 1969, Downey and So, 1970), yeast RNA polymerase II (Dezélée and Sentenac, 1973) and wheat germ RNA polymerase I (Job *et al.*, 1982), the double reciprocal plots 1/velocity *versus* 1/[purine triphosphate] are non-linear for the substrate concentration range in which enzyme kinetics shift from an initiation rate-limiting to an elongation rate-limiting step. In contrast, linear plots of 1/velocity *versus* 1/[GTP] were obtained for both B and Z* poly[d(G-C)] templates (Figure 7). As previously stated (Job *et al.*, 1982), this may be either because the *K*m values for initiation and elongation steps are nearly identical, or because the GTP concentration necessary for the initiation step to occur is lower than the lowest substrate concentration studies (1 μM).

Dinucleoside monophosphates, complementary to the DNA sequence at the initiation site, can be used as primers for RNA synthesis by either *E. coli* RNA polymerase (Downey *et al.*, 1971, Oen *et al.*, 1979) or eucaryotic RNA polymerases (Lescure *et al.*, 1981). The effect of the dinucleoside monophosphate is particularly evident when the concentration of the purine initiating nucleoside triphosphate is very low, i.e., when the initiation step is rate-limiting. Therefore, increasing the dinucleoside monophosphate concentration results in a bypass of the initiation step and an enhancement of enzyme activity is observed (Anthony *et al.*, 1969, Downey and So, 1970). Such a result is also found for the transcription of Z*-poly[d(G-C)] in the presence of dinucleoside monophosphates (Figure 8). The enzyme discriminates markedly between the two possible dinucleotides (GpC and CpG) using either the B or Z* templates, a result reminiscent of those reported by Sylvester and Cashel (1980) for the abortive initiation reaction catalyzed by *E. coli* RNA polymerase on poly[d(A-T)] or poly[d(G-C)] in the presence of dinucleoside monophosphate primers. It is noteworthy that the GpC primer leads to a pronounced reduction in the *K*m for CTP in the case of both the B and Z* templates (Table I). It remains to be established whether this effect reflects primarily a process closely related to initiation (see, for instance, Shimamoto and Wu, 1980) or to the steady-state elongation phase.

The possibility that the aggregated state of the Z* form of poly[d(G-C)] was the cause of the reduced activity (compared with the B form) seen with this template was studied by employing poly[d(G-m^{5}C)] as an alternative template. We first checked that under the standard conditions used in this study (1 mM Mn^{2+}, no DTT; 2.5 mM Mn^{2+}, 1.1 mM DTT) the Z state of this template could be established without aggregation to the Z* state. The results indicated that only a slightly higher transcriptional activity (relative to the Z form of poly[d(G-C)]) is obtained with Z-poly[d(G-m^{5}C)], especially in the 2.5 mM Mn^{2+}, 1.1 mM DTT condition. DNA methylation may be an important factor in the regulation of gene expression (Razin and Riggs, 1980). In this connection, it is interesting to note the present demonstration that wheat germ RNA polymerase II is able to transcribe a highly methylated DNA such as poly[d(G-m^{5}C)]. We assume that methylated sequences in the B configuration are also templates for RNA polymerase II and conclude that the inhibition of genetic expression associated with methylation does not derive in a simple way from a block of the enzyme.

Independent evidence for the transcription of left-handed DNA by wheat germ RNA polymerase II was provided by the effect of antibodies specific for this conformational state of DNA. With all anti-Z DNA antibody preparations, a strong or complete inhibition of RNA synthesis was observed, whether the antibody was introduced at the outset of or later in the transcription assay. Since the anti-Z DNA antibodies had no effect on the transcription of the B form of poly[d(G-C)], these results rule out the possibility that the observed incorporation is simply due to a small amount of contaminating B DNA in the left-handed template.

In conclusion, the template efficiency of Z* (or Z) DNA in transcription assays containing an eucaryotic RNA polymerase has been established on the basis of different

physical (sedimentability, c.d. and absorption spectra) and biochemical (effect of antibodies) criteria. From the results described in this paper, we do not know how RNA polymerase binds to Z* poly[d(G-C)] and if during the binding process local transformation of Z* to B or other intermediate configurations occur (see also van de Sande and Jovin, 1982). The fact that under most experimental conditions studied: (a) Z* poly[d(G-C)] remained sedimentable; (b) the c.d. spectra of Z* DNA remained unchanged; and (c) the antibodies specific for left-handed DNA completely inhibited the transcription of Z*-poly[d(G-C)], argue against an appreciable conversion of the left-handed form back to the right-handed conformation. Other experiments, such as kinetic studies on the direct association between enzyme and template, or more detailed studies with the specific antibodies which bind to different sites on the Z-helix (Zarling *et al.*, 1983) would help in understanding the molecular basis of the recognition process.

Another relevant question concerns the mode of release of RNA products. Using poly[d(A-T)], it has been recently shown that the reaction is far more complicated than anticipated due to the high non-processive character of the wheat germ RNA polymerase (Durand *et al.*, 1982). Experiments to test the effects of the sequence and conformation state of the DNA template will be undertaken. The fidelity of transcription must also be evaluated.

Other enzymes such as *E. coli* RNA polymerase (van de Sande and Jovin, 1982), nucleases (Behe *et al.*, 1981, Singleton *et al.*, 1982; Nickol *et al.*, 1982), and proteins such as histones (Nickol *et al.*, 1982), polyarginine (Klevan and Schumaker, 1982), and cellular extracts (Nordheim *et al.*, 1982) have been shown to interact with left-handed DNA. In most cases, including this study, marked differences were observed when the left-handed DNA was used instead of the right-handed conformation, a finding which emphasizes the potential importance of DNA conformation upon gene regulation.

Materials and methods

Reagents

GTP, CTP and the dinucleoside monophosphates GpC and CpG were purchased from Sigma. [^{3}H]GTP and [^{3}H]CTP (11 and 27 Ci/mmol, respectively) were from Amersham. Poly[d(G-C)] was from P-L Biochemicals; it was dissolved in 10 mM Tris-HCl buffer pH 7.8 containing 10 μM EDTA and dialyzed against the same buffer (van de Sande *et al.*, 1982). Poly[d(G-m^{5}C)] was synthesized as previously described (van de Sande *et al.*, 1982). Uniformly labelled poly[d(G-C)] was prepared in the presence of [^{3}H]dGTP and [^{3}H]dCTP.

RNA polymerase

Wheat germ RNA polymerase II A was purified by the method of Jendrisak and Burgess (1975), as modified by Job *et al.* (1982). The specific activity of the enzyme was 300 units/mg, using denaturated calf thymus DNA as template. Protein determinations were effected according to Bradford (1976). Enzyme concentration was calculated assuming a mol. wt. of 650 000 (Bull and Garrido, 1982).

Antibodies

Polyclonal rabbit antibodies against poly[d(G-br^{5}C)] or Br-poly[d(G-C)] were raised in rabbits as described by Zarling *et al.* (1983). IgG fractions were purified by DEAE-A50 extraction and chromatography on protein A-Sepharose. The concentration of protein is given as the total IgG. The fraction of specific anti-Z DNA antibody in the polyclonal preparations is estimated at ~2% (Zarling *et al.*, 1983). The homogeneous monoclonal anti-Br-poly[d(G-C)] IgG, D11, was the kind gift of F.Pohl and R.Thomae.

Conformational transitions and spectra

The conformational states of poly[d(G-C)] and poly[d(G-m^{5}C)] were analyzed quantitatively by: (a) the absorption ratio A_{295}/A_{260} determined in an Uvikon 820 spectrophotometer; and (b) sedimentation analysis in an Eppendorf microcentrifuge (van de Sande and Jovin, 1982). Absorption spectra and transition kinetics (at 295 nm) were measured in the thermoregulated Uvikon 820 spectrophotometer with digital temperature readout. C.d. spectra were obtained with a computerized Jobin-Yvon Mark IV Dichrograph. Molar extinction coefficients used were: poly[d(G-C)], 7.1 x 10^3 (Pohl and Jovin, 1972) and poly[d(G-m^{5}C)], 7.0 x 10^3 M^{-1}, cm^{-1} (Gill *et al.*, 1974).

The Z* state of poly[d(G-C)] was generated by heating a 0.5 mM poly[d(G-C)] solution in 10 mM Tris-HCl, pH 7.8, 10 μM EDTA, and 4 mM $MnCl_2$. The heating step was at 60°C for 5–7 min. Ratios of absorbance at 295 and 260 nm were 0.16 and 0.35 for the B and Z* forms, respectively. Under these conditions the Z* DNA was sedimentable at 9000 *g*. The maintenance of the Z* state under the various experimental conditions examined in the transcription assays was also assessed by sedimentation and c.d. criteria. The Z state of poly[d(G-m^{5}C)] was obtained by adding $MnCl_2$ (1 mM or 2.5 mM) to 50 μM poly[d(G-m^{5}C)] in buffers used for the transcription assays (see below). Under these conditions, the Z form was not sedimentable.

Transcription assays

The reaction mixtures contained enzyme, DNA, GTP, CTP, dinucleoside monophosphates, $MnCl_2$ and ammonium sulfate as indicated in the legends of figures. They also contained a buffer C composed of: 25 mM Tris-HCl (final pH as indicated in the figure legends), 12.5% (v/v) glycerol, 12.5 mM 2-mercaptoethanol, 5 mM α-thioglycerol, 0.05 mM EDTA and 0.05% (v/v) Triton X-100. In some cases (see figure legends), the reaction mixtures also contained 1.1 mM DTT (together with an additional 39 mM Tris-HCl and 1.5 mM NaF).

In all experiments described, each reaction mixture (100 μl) containing all factors required for enzyme binding to DNA or for RNA synthesis, except the labelled substrate, was incubated for 10 min at 35°C, then chilled in an ice bath. Next, 50 μl were withdrawn and centrifuged for 5 min at 9000 *g*. The supernatant with withdrawn. The labelled substrate was then added to both the supernatant and to the unseparated reaction mixture (the remaining 50 μl from the initial reaction medium) and incubation was allowed to proceed for 40 min at 35°C (incorporation rates were linear for up to 60 min). This allowed the determination of the amount of DNA remaining in the Z* form under the various experimental conditions examined. Under conditions stabilizing the Z* state of the template, no DNA was left in the supernatant after the centrifugation step; therefore, no incorporation of radioactivity into RNA could occur following addition of the labelled substrate. This circumstance was observed in most assays, except those performed at low Mn^{2+} concentrations (Figure 2) or at high ammonium sulfate concentrations (Figure 6).

For activity measurements, reaction mixtures were absorbed on Whatman GF/C filters and washed, as previously described (Job *et al.*, 1982).

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