*TEMPLATE FUNCTIONS IN THE ENZYMIC FORMATION OF POLYRIBONUCLEOTIDES, I. INTEGRITY OF THE DNA TEMPLATE**

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Despite much excellent work, one of the most fundamental problems in biology, namely, the mechanism through which the cell produces and reproduces sequentially specific heteropolymers (proteins, nucleic acids, polysaccharides, etc.), still remains far from being well understood. The nearest we have come to an understanding is through the postulation of template mechanisms in which a preformed polynucleotide prescribes, either directly by base pairing or through an intricate "coding" procedure, the order in which the precursors of the newly forming polymer, simultaneously or subsequently linked by enzyme action, are aligned.

It is, perhaps, not surprising that the conventional concepts of enzymology are not entirely adapted to dealing with enzyme complexes whose action depends on, or is directed by, high-molecular templates that surpass the enzyme protein itself in size and often in complexity and sensitivity. We may describe the dilemma as follows. An enzyme can be defined by its substrate or by its product. If we define RNA polymerase, the enzyme studied here, by its substrate, the situation is clear: the ribonucleoside triphosphates are the only substrate and the enzyme is a ribonucleotidyl transferase. But in trying to define the enzyme by its product, we encounter a predicament of ever-shifting variables: there are as many products, and consequently as many enzymes, as there are templates.

The central function of DNA in specifying the enzymic formation of polyribonucleotides of specific composition and, presumably, sequence, prompted us to investigate the problem of a template in some detail—a study that will be continued in subsequent communications.

RNA polymerase (EC 2.7.7.6) has formed the subject of several recent reviews.[1-3] The abolition or the alteration of template activity has been observed in several instances, e.g., on storage,[4] irradiation with ultraviolet light[5, 6] or X rays,[7] and also by chemical modification.[8, 9] In the present study, we have used the enzyme preparation from *E. coli* and the DNA of calf thymus; we have investigated the changes in template activity exhibited by the latter when subjected to ultrasonic oscillations, changes in pH, and the partial removal of the purine constituents. The effects of temperature will be discussed later, as will be the action as templates of such extreme degradation products of DNA as apurinic and apyrimidinic acids. As we were naturally interested in both the quantity and the quality of the products of the enzyme action, we also examined the composition of the polyribonucleotides synthesized in the presence of the treated templates.

Materials and Methods.—RNA polymerase: The enzyme was isolated from frozen *E. coli* W cells (Grain Processing Co., Muscatine, Iowa) essentially according to a published procedure.[10] In the last step of purification, elution from the column of DEAE-cellulose was performed, instead of stepwise, by a KCl gradient (0.16–0.64 *M*). The fractions containing the enzyme were pooled, brought to a 50% glycerol concentration, and stored at −20°.

DNA: Three preparations from calf thymus were used. Preparation 1 was isolated by extraction with strong salt solution and deproteinization with chloroform (p. 324 of ref. 11); for

preparations 2 and 3, the Duponol technique[12] was employed. The composition of these specimens is included below in Table 2.

Ribonucleoside triphosphates: Unlabeled and C^{14}-labeled preparations (in position 2 of the pyrimidine and 8 of the purine rings) were supplied by Schwarz BioResearch, Inc. When the base composition of the enzymic products was to be determined, the specific activities of the precursors were accurately measured immediately before their use. They were first diluted with appropriate amounts of the corresponding unlabeled nucleoside triphosphates, separated by paper chromatography (buffered isobutyrate) from the small amounts of mono- and diphosphates usually present, and the ultraviolet absorption and radioactivity of the eluates of the separated triphosphate areas were determined. At least four individual determinations of the specific radioactivity of each nucleotide were performed and the averages used for the conversion of the data on C^{14}-incorporation into mμmoles.

When no complete analysis of the polyribonucleotide product was required, the radioactivity data supplied with the preparations were used; in this case, the tables and figures list counts incorporated per minute rather than mμmoles, and the specific activities are designated as approximate.

Action of RNA polymerase: The incubation mixtures contained, in a final volume of 0.5 ml, 20 μmoles Tris-HCl buffer, pH 7.9, 2 μmoles $MgCl_2$, 0.5 μmole $MnCl_2$, 6 μmoles 2-mercaptoethanol, 50–200 μg DNA, 10–50 μg enzyme protein, and 100–200 mμmoles of each ribonucleoside triphosphate (ATP, GTP, CTP, UTP) of which one or more was labeled with C^{14}. The exact amounts of DNA, enzyme, and precursors, as well as the specific activities of the latter, will be specified below. Unless noted otherwise, incubation was carried out at 37° for 20 min, following which 5 ml of 10% trichloroacetic acid were added to each assay tube. After 30–100 min at 0°, the mixtures were filtered through membrane filters (Schleicher and Schuell, type B-6), and the precipitates washed 6 times with cold 5% TCA. The filters were introduced into scintillation vials, and 1 ml of 3% NH_4OH was added, followed 10 min later by 15 ml of scintillation mixture.[13] The radioactivity was counted in a Packard Tri-Carb scintillation spectrometer.

Results and Discussion.—Composition of enzymic product: The composition of the polyribonucleotides synthesized by the enzyme has often been investigated by a variety of techniques.[10, 14–18] Although a definite similarity between the base composition of the enzymic product and that of the particular DNA serving as the template was indicated, the analytical results reported were very often far from ideal. As it appeared desirable to recognize even small changes in product composition after various treatments of the template, we attempted to increase the accuracy of our measurements by (*a*) averaging four to six sets of complete determinations for each product of a given template, and (*b*) determining, in quadruplicate, the specific radioactivities of the diluted precursors directly before their use. The experimental arrangement is illustrated in Table 1; Table 2 presents the composition of three enzymically formed polyribonucleotides, in comparison with the

TABLE 1

EXPERIMENTAL ARRANGEMENT FOR ENZYMIC POLYRIBONUCLEOTIDE SYNTHESIS SHOWN IN TABLE 2

		Expt. 1	Expt. 2	Expt. 3
Incubation at 37°, min		20	30	30
Template DNA, μg		200	100	100
RNA polymerase, μg		21	17	25
Amount of each precursor, mμmoles		400	200	100
Specific radioactivity of precursors (cpm/mμmole)	A	220	1053	1073
	G	291	1444	1455
	C	310	1370	1155
	U	331	1454	1340

Four groups of 4–6 assay tubes contained all four precursors, with one triphosphate—either A, G, C, or U, respectively—in each group C^{14}-labeled. Division of the average counts, incorporated in each group, through the exact specific radioactivity of the respective precursor furnished the base proportions of the product. In each experiment a different DNA preparation and a different enzyme specimen were used.

TABLE 2

CALF THYMUS DNA AS TEMPLATE: COMPOSITION OF POLYRIBONUCLEOTIDE SYNTHESIZED BY RNA POLYMERASE*

Expt. 1					Expt. 2				
DNA, Prep. 1		RNA Product			DNA, Prep. 2		RNA Product		
Base	%	%	mμMoles	Base	Base	%	%	mμMoles	Base
A	28.8	28.5	4.13	U	A	27.9	27.2	1.214	U
G	20.7	21.8	3.16	C	G	21.7	20.9	0.929	C
C	21.7	21.1	3.05	G	C	22.1	22.2	0.989	G
T	28.8	28.6	4.15	A	T	28.4	29.7	1.322	A
			Molar Ratios						
$\frac{A + T(U)}{G + C}$	1.36	1.33				1.29	1.32		
Pu/Py	0.98	0.99				0.98	1.08		
$\frac{6\text{-Am}}{6\text{-K}}$	1.02	1.02				1.00	1.02		

Expt. 3				
DNA, Prep. 3			RNA Product	
Base	%	%	mμMoles	Base
A	28.8	29.6	1.84	U
C	21.3	20.6	1.28	C
C	20.8	20.3	1.26	G
T	29.2	29.6	1.84	A
	Molar Ratios			
$\frac{A + T(U)}{G + C}$	1.38	1.45		
Pu/Py	1.00	0.99		
$\frac{6\text{-Am}}{6\text{-K}}$	0.98	1.01		

* See Table 1 for experimental conditions.

base distribution recorded for the DNA specimens used as the respective templates.[19] These determinations form part of three separate experiments in which the template activity of an untreated intact DNA preparation is contrasted with that of the same DNA sample after some form of degradation to be discussed below. Although three different DNA specimens, three different enzyme preparations, and different conditions of assay were used, an excellent agreement in composition of the RNA products and the DNA templates will be seen. For instance, when the averages of the three experiments in Table 2 are compared, the dissymmetry ratio A + T/G + C of the template DNA is 1.34, the corresponding ratio A + U/G + C of the product is 1.36. (The most detailed examination of calf thymus DNA in the literature[20] yielded, in fact, a dissymmetry ratio of 1.36.)

Storage of DNA solutions: The loss of template activity on storage of a DNA solution has been noted briefly.[4] The observations summarized in Table 3 are, perhaps, not without interest. It will be seen that the storage of a DNA solution at 4° for lengthy periods entails the loss of 80 per cent or more of the initial activity. What is remarkable is that when the solutions under comparison, fresh and stored, were subjected to denaturation by heat and then cooled rapidly, a very similar level of activity with regard to the incorporation of precursors within 2 hr is encountered in all cases. Moreover, and very surprisingly, the activity of heated aged solutions eventually becomes higher than that of their unheated counterparts, although the initial rates (incorporation in 15 min) are always higher with the unheated templates. These observations may explain certain inconsistencies in the literature concerning the relative template activities of native and heated DNA.[6, 10, 16, 18, 21–23]

Ultrasonic vibrations: The drop of template activity, together with the con-

TABLE 3

TEMPLATE ACTIVITY OF UNHEATED AND HEAT-DENATURED CALF THYMUS DNA: EFFECT OF STORAGE IN SOLUTION

	Expt. 4		Expt. 5		Expt. 6	
Storage of solutions at 4° (weeks)	0		6		7	
Incubation time (min)	15	120	15	120	15	120
Unheated DNA	5740	16423	1310	3030	933	2327
Heated DNA	1234	3512	1136	3675	763	3250
Change of template activity on storage, %	—	—	−77	−82	−84	−86
Change of template activity on heat denaturation, %	−79	−79	−13	+21	−18	+40

The incubation mixtures (0.5 ml) contained, in addition to the standard supplements listed in the text, 100 mμmoles each of ATP, GTP, CTP, and UTP-C^{14} (approx. spec. activity 1000 cpm/mμmole), and 38 μg of always the same enzyme preparation. DNA prep. 2 served as the template, either as a freshly prepared solution (1 mg/ml of 0.001 *M* Tris buffer, pH 7.0) or after storage of the solutions at 4° for specified periods. Before use, the DNA solutions were diluted with distilled water to a concentration of 250 μg/ml, and portions were heated in a boiling water bath for 10 min and then cooled quickly to 0°. Portions of 0.22 ml of these DNA solutions, both heated and unheated, were added to each assay tube. The values reported (averages of two determinations) are the total incorporation as cpm.

comitant loss of viscosity, induced by the ultrasonic treatment of DNA solutions is shown in Figure 1. In all experiments, a characteristically rapid initial inactivation was followed by a plateau at about 30–40 per cent of the original activity. With DNA preparation 1 (Table 2) as the template, treated ultrasonically for 20 min, the composition of the enzymic product was, in mole per cent: A, 29.5; G, 21.4; C, 20.6; U, 28.4. The control for this experiment, carried out with undegraded template, is listed as experiment 1 in Tables 1 and 2. Though the total activity of the ultrasonically broken template declined to 32 per cent of the original, the base composition of the product will be seen as essentially unchanged.

Depurination: The loss of template activity of DNA, incubated at pH 1.6 and 37°, is depicted in Figure 2. Again, as in Figure 1, two distinct rates of inactivation are observed: an initial very rapid one, followed by a much slower one, which latter is comparable in slope to the gradual loss of purines from the DNA.[24] It is possible that the removal of purines, which must set in at the very beginning, from particularly crucial positions in the DNA is responsible for the initial steep decline in activity. Also, in view of our findings on other means of inactivation, the latter is, more likely due to a change in the conformation of the template.

The effects on template activity of the treatment of DNA with HCl at pH 1.6 and with an acidic resin[25] are compared in Figure 3*A*. The incorporation of C^{14}-ATP and C^{14}-CTP was determined separately. The activity drop upon treatment with the resin is much slower than it is in the case of a milieu of pH 1.6, but still it is much faster than the rate of depurination induced by the resin.[25] Moreover, incorporation declines not only with CTP, but also with ATP, although the base complementary to the latter, thymine, is not being removed from the template.

The A/C ratios found in the enzymic products at different stages of this experiment, which are plotted in Figure 3*B*, permit conclusions on the nature of these products. These ratios change in all three instances—HCl at 37°, resin at 37° and 25°—but with different slopes. Again, there is no simple relation between this change and the change in the proportions of the complementary bases in the template: after the treatment of the DNA for 4 hr at pH 1.6 and 37°, when the T/G ratio can be estimated[24] to be near 1.5, the corresponding A/C ratio of the product has gone up to as high as 5.75.

Exposure of DNA to extreme pH values: Even the briefest contact with acidic or

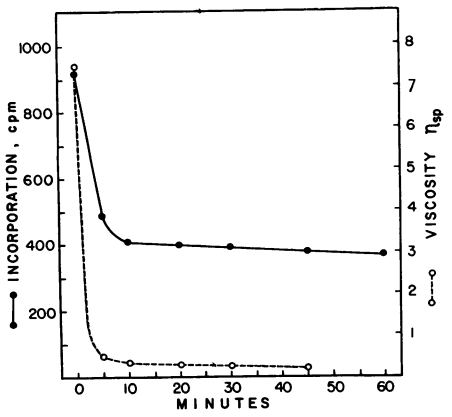

FIG. 1.—Effect of ultrasonic vibrations on template activity and viscosity of calf thymus DNA. A solution of prep. 2 (0.5 mg/ml of 0.001 *M* Tris buffer pH 7) was treated in an ultrasonic disintegrator (MSE model 300) at 19,000 cps, in an ice bath, for the periods stated on the abscissa. Template activity was tested in duplicate on 0.15-ml portions of the samples by the assay method described in the text, with 45 μg of enzyme protein and 100 mμmoles each of ATP, GTP-C^{14}, CTP-C^{14}, and UTP per assay tube. (The approximate specific activity of the labeled precursors was 1000 cpm/mμmole.) For the measurement of the loss of viscosity upon ultrasonic treatment, a solution of DNA prep. 2 (1 mg/ml of 0.001 *M* NaCl) was treated for the stated periods and measured in an Ostwald-Fenske viscosimeter at 25°.

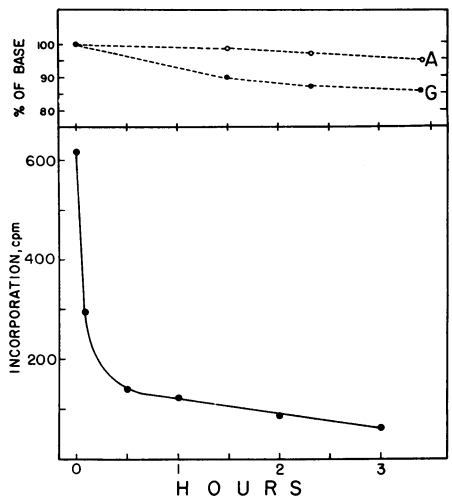

FIG. 2.—Effect of treatment with dilute acid on template activity. An aqueous solution of DNA prep. 1 (1 mg/ml) was brought to pH 1.6 with *N* HCl and kept at 37° for the stated periods. Duplicate samples of 0.1 ml were assayed with 28 μg of enzyme protein and 100 mμmoles each of ATP, GTP, CTP, UTP, all labeled with C^{14}, per assay tube. (Approximate specific activity of each precursor was 150 cpm/mμmole.) The rate of depurination, also shown, is based on ref. 24.

alkaline conditions affects the activity of the template, though not what could be considered its informational content. Table 4 shows that a very brief exposure of DNA to pH 2 or pH 12 decreases the template activity to 55 and 64 per cent, respectively, of the control value, without influencing the composition of the products formed on the treated templates. These observations also indicate that the initial rapid inactivation of the template, discussed above, is not due to the loss of purines.

Concluding Remarks.—We shall leave a detailed discussion of the functions of the DNA template in the action of RNA polymerase for a later communication. With regard to the directed synthesis of a sequentially specific nucleic acid by an enzyme, it is quite obvious that the participation of a second complex and sensitive macromolecular agent, the template, creates a new dimension not normally encountered in enzyme reactions. All the uncertainties besetting the attempt to define a DNA molecule will enter into consideration. The template is not a conventional cofactor; it determines not only the quantity, but also the nature of the product. DNA preparations that would be considered identical chemically show wide variations in their ability to function as templates.

It is for this reason that the definition of the activity of such conjugated enzymes is particularly troublesome, as we are dealing with two variables—the protein and the template—which, though capable of undergoing independent modifications,

can only be measured together. Although it is not easy to conceive of suitable reference standards, it may be hoped that the polymerase assay can be developed into one of the most sensitive tools for judging the quality of a DNA preparation.

When mild methods of inactivation are applied to a DNA template, interesting curves are obtained (cf. Figs. 1–3). The initial fast rate, changing abruptly to a much slower one, does not parallel the rate of chemical modification of the molecule and is even observed upon treatments, such as ultrasonic vibrations or storage, that do not result in detectable chemical changes. The type of structural alteration of the DNA that is responsible is not yet clear.

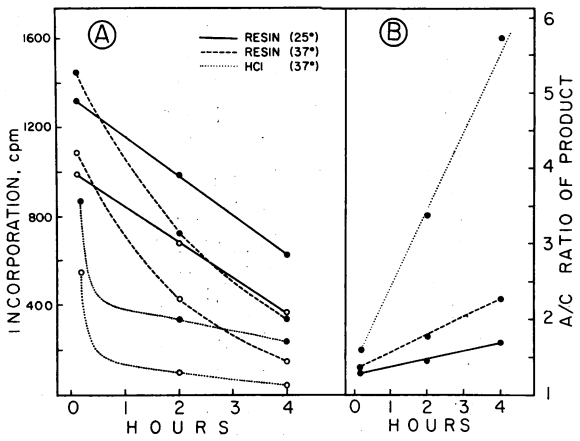

FIG. 3.—Effect of gradual depurination by acid or acidic resin on template activity and on composition of enzymic product. To each of two flasks containing 20 ml of an aqueous solution of DNA prep. 1 (1 mg/ml), 3 gm of dry Amberlite IR-120 (H^+) were added; one portion was shaken gently at 25°, the other at 37°. A third 20-ml portion of the same DNA solution was adjusted to pH 1.6 with HCl and also shaken at 37°. Duplicate samples of 0.1 ml were tested at intervals for template activity with 21 μg of enzyme protein per assay tube and 200 mμmoles of each precursor, ATP being C^{14}-labeled in one series and CTP in the other. (Approximate specific activites were 250 cpm/mμmole.) (*A*) Incorporation of ATP-C^{14} (*full circles*) and CTP-C^{14} (*open circles*). For the shape of the curves describing HCl treatment, account was also taken of the more detailed experiments in Fig. 2. (*B*) Ratios of A/C incorporated in the course of the experiment.

The first event in the polymerase reaction, the specific interaction between two complex macromolecules, must be very sensitive even to minute conformational changes. Some of these changes may prevent the interaction altogether; in other cases protein and modified template may interact incorrectly. Numerous variations could result that would be unprofitable to discuss. Examples abound in the literature: e.g., the different effects of polyamines on intact and denatured DNA[5, 6, 26] and, most significantly, the finding that heat-denatured DNA, though usually a poorer template as judged by the rate of polymer formation, binds more

TABLE 4

CALF THYMUS DNA AS TEMPLATE: EFFECTS OF BRIEF EXPOSURE TO pH 2 AND pH 12

	Composition of RNA Products					
	Expt. 3 (Control)		Expt. 7 (DNA exposed to pH 2)		Expt. 8 (DNA exposed to pH 12)	
Base	mμmoles	%	mμmoles	%	mμmoles	%
A	1.84	29.6	0.989	29.0	1.18	29.7
G	1.26	20.3	0.698	20.5	0.791	19.9
C	1.28	20.6	0.743	21.8	0.858	21.6
U	1.84	29.6	0.980	28.7	1.14	28.8
Total	6.22		3.41		3.97	

An aqueous solution of DNA prep. 3 (1 mg/ml) was divided into three parts. One portion was brought rapidly to pH 2 with *N* HCl and immediately returned to pH 7 with *N* KOH. The second was brought to pH 12 and back to pH 7 with the same solutions in reverse order. To the third portion, an amount of KCl solution was added, calculated to match the salt concentrations produced by the neutralization of the other samples. The three solutions served as templates for the enzyme reaction in the arrangement described in Table 1 as expt. 3.

enzyme than does intact DNA.[16, 23, 27] This indicates that the conjugation of enzyme and template must follow certain rules.

Our observation that the composition of the polynucleotides produced was not affected by modifications of the template (ultrasonic treatment, pH extremes) that caused a reduction of its activity by 40–60 per cent could also be explained by the assumption that in these cases both "correct" and "incorrect" enzyme-template complexes were formed. As the tests of template activity were performed with constant amounts of enzyme protein, a change in the properties of the DNA that could permit some of the protein to be bound in an ineffective manner could actually reduce the concentration of active complex, thus decreasing the rate of formation of RNA product without affecting its composition. The modified template could then be compared to a noncompetitive inhibitor.

Another possible mechanism could postulate a change in the susceptibility of the complex between enzyme and modified template to product inhibition. In a series of as yet unpublished experiments in which ribonuclease was employed in the study of the reaction mechanism of RNA polymerase (cf. also ref. 28), observations were made pointing to differences in the degree of inhibition by RNA synthesized on intact and denatured templates. Whatever the final explanation will be, it is quite clear that the proper evaluation of experimental data on polymerase systems requires some indication of the state of the DNA template employed.

Summary.—As the first contribution to the study of the functions, and the concept, of the template in the enzymic synthesis of sequentially specific polynucleotides, the inactivation of calf thymus DNA as the template in the RNA polymerase system was examined. Treatments that avoid chemical alterations, such as ultrasonic vibrations, storage in solution, or very brief exposure to pH extremes, were applied as well as contact with acid conditions or with acid resins. In most cases, the composition of the polyribonucleotides formed on the treated templates was also determined. An initial rapid loss of template activity, presumably due to conformational changes, was followed by a much slower rate of decay; but the composition of the enzymic products remained unchanged. The studies are being continued.

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[19] Abbreviations used: A, G, C, T, U designate adenine, guanine, cytosine, thymine, uracil; ATP, GTP, CTP, UTP stand for the triphosphates of the corresponding ribonucleosides. Pu means purines; Py, pyrimidines; 6-Am, the sum of the 6-amino bases (A + C); 6-K, that of the 6-oxo bases (G + U or T).
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