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POLYRIBONUCLEOTIDES AS TEMPLATES FOR POLYDEOXYRIBONUCLEOTIDES

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The mechanism by which DNA transfers its information for the synthesis of both DNA and RNA has not been established. Base analysis and nearest-neighbor frequencies imply that the DNA acts as a template *in vitro*. In the case of the RNA-polymerase reaction *in vitro*, the experimental evidence indicates overwhelmingly that the DNA template remains intact and helical.[1, 2] On the other hand, in DNA synthesis the identity of the template and the product make a similar conclusion less certain. In this paper we show that the helical complex poly (A + U), which is a two-stranded polyribonucleotide, serves as a template for the synthesis of a polydeoxyribonucleotide, poly (dA + T), using DNA polymerase *in vitro*. The poly (dA + T) product has been shown to be an ordered macromole-

cule (probably helical) in which each molecular chain contains only one type of base, like the template poly (A + U). The evidence presented here lends support to the suggestion made some time ago that the parental DNA remains intact during DNA synthesis.[3]

Materials and Methods.—Poly A and poly U were purchased from the Miles Laboratories, New Jersey. Poly (A + U) primer was prepared by mixing equimolar amounts of poly A and poly U in the presence of 10^{-4} *M* Mg^{++}.[4] The sedimentation constants of poly A, poly U, and poly (A+U) in 0.2 *M* sodium chloride were 21.8, 14.1, and 30 Svedbergs, respectively.

C^{14}-labeled deoxynucleoside-5′-triphosphates were obtained from Schwarz BioResearch, Inc. P^{32}-labeled deoxynucleoside-5′-triphosphates were kindly supplied by Dr. F. J. Bollum. Unlabeled deoxynucleoside-5′-triphosphates were purchased from Pabst Laboratories. Spleen phosphodiesterase was purchased from Worthington Biochemical Corporation. Micrococcal DNase was a gift from Dr. Charles Dekker.

DNA polymerase was prepared from *E. coli B*. The bacteria were grown with vigorous aeration at 37°C. The medium contained, per liter, 7 gm of K_2HPO_4, 3 gm of KH_2PO_4, 0.05 gm of Na-citrate·$2H_2O$, 0.1 gm of $MgSO_4$ (anhyd.), 1 gm of $(NH_4)_2SO_4$, 6 gm of yeast extract, and 10 gm of dextrose. The culture was chilled and harvested at the end of log phase. The cells were collected and washed by suspension in 0.5% NaCl–0.5% KCl, centrifuged, and then stored at −20°C.

DNA polymerase was prepared by the method of Furth, Hurwitz, and Anders.[5] The purification results are shown in Table 1.

TABLE 1

PURIFICATION OF DNA POLYMERASE

Step	Total units	Protein, mg/ml*	Specific activity†
Crude	21,000	56.44	1.07
DEAE cellulose‡	5,000	0.205	2,500

* Protein was determined by the procedure of Lowry *et al.*[6] Before the determination, the enzyme solution was dialyzed against 0.005 *M* Tris, pH 8.4.

† Enzyme activity was measured by following the incorporation of C^{14}-labeled deoxynucleoside-5′-triphosphates into acid-insoluble product. Enzyme activity is expressed as units per milligram of protein. A unit of enzyme is defined as that amount of enzyme which catalyzes the incorporation of 1 mμmole of labeled substrates into an acid-insoluble product in 20 min at 37°C.

‡ Before column chromatography, the DNA polymerase was separated from RNA polymerase by fractional precipitation with $(NH_4)_2SO_4$. No RNA polymerase activity was demonstrable in the purified DNA polymerase fraction when the ribonucleoside-5′-triphosphates were used as substrates with a DNA primer.

The course of polymer synthesis was followed both by incorporation of P^{32}-labeled deoxyribonucleoside-5′-triphosphates and, more frequently, by the hypochromic shift which accompanies the conversion of the substrates into an ordered polymer. The reaction was stopped by cooling and adding sodium chloride to a final concentration of 0.2 or 0.4 *M*. In some experiments, the enzymes also were inactivated by the addition of duponol to a final concentration of 1%. Helix-coil transitions and sedimentation velocity measurements were then carried out in its presence, after removal of the excess substrates by dialysis. In these cases the ratio of product to primer varied from 10 to 20. In other experiments, deproteinization was effected by shaking with chloroform-octanol in the cold. The solutions were then dialyzed in the cold against 0.2 *M* NaCl, H_2O, and finally against 0.02 *M* NaCl. The product was obtained by lyophilization or by precipitation with three volumes of cold ethanol.

The enzymatic hydrolysis of the product to 3′-deoxyribonucleotides by micrococcal DNase and spleen phosphodiesterase was carried out according to the method of Josse, Kaiser, and Kornberg.[7] The nucleotide analyses were carried out on Dowex-1 formate columns.[8] The results are given in Table 2. In each case the synthesis was carried by reacting 0.5 μmoles each of dATP and TTP in 1 ml of solution containing 0.46 optical density units of poly (A + U) and about 10 units of polymerase. The solution was 0.005 *M* in magnesium chloride, 0.05 *M* in Tris, pH 8.4, and 0.001 *M* in mercaptoethanol. The reaction was terminated after 20 min. The product was isolated as described by Hurwitz *et al.*[1] and hydrolyzed with micrococcal DNase followed by spleen phosphodiesterase.

Sedimentation velocity and CsCl density gradient measurements were made with a Spinco Model E ultracentrifuge equipped with ultraviolet absorption optics.

Light-scattering measurements were made in 0.2 *M* NaCl with a Brice-Phoenix universal light-scattering photometer. The poly (dA + T) solution was clarified by centrifuging for one-half hour at 30,000 × *g*, after which the top two thirds of the contents of the tube was removed and transferred to a dust-free conical cell. The solvent was cleaned by repeated filtration through a 0.45 μ Millipore filter. Turbidities were calculated for angles between 20° and 135°.

Helix-coil transitions were determined in a Beckman Model DU spectrophotometer equipped with a thermostatically controlled cell compartment.[9]

Results.—(1) *Synthesis of poly (dA + T) primed by poly (A + U):* Figure 1 shows the course of both an unprimed and a poly (A + U) primed reaction. It can be seen that there is a 7-hr lag period for the unprimed poly (dA + T) synthesis, while in the primed synthesis the induction period is markedly reduced and the maximum decrease in optical density at 260 mμ is found at 25 min.

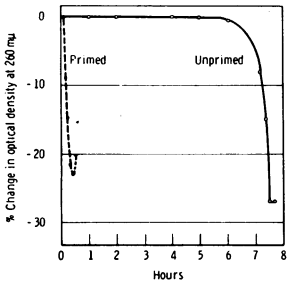

FIG. 1.—Comparison of poly (A + U) primed versus unprimed synthesis of poly dAT. The composition of the mixture was: 5×10^{-2} *M* Tris, pH 8.4; 5×10^{-3} *M* $MgCl_2$; 1×10^{-3} *M* mercaptoethanol. Each milliliter contained 0.5 μmoles each of dATP and TTP. The primed reaction contained 0.34 optical density units of poly (A + U).

(2) *Nearest-neighbor analysis of the product:* In order to determine that the poly (dA + T) was in fact homopolymeric in each chain just as the primer is, a nearest-neighbor analysis was carried out. Two reaction mixtures were studied: one contained dATP and $TP^{32}PP$ and the other, $dAP^{32}PP$ and TTP. The results are contained in Table 2, where it is shown that, within experimental error, each adenylate is followed by an adenylate in the product and each thymidylate, by a thymidylate.

(3) *Physical properties of poly (dA + T):* (a) *Ultracentrifugation:* Figure 2 shows sedimentation velocity patterns of a typical preparation of poly (dA + T) which had been primed by poly (A + U). The sedimentation constant calculated from this run is 28 S, although different values were obtained for the various preparations. The range was 18–30 S. The variation is due principally to the size of the primer and the quantity of nucleases present in the polymerase used. The poly (dA + T) was found to be extremely sensitive to nuclease action, and in a number of instances the product was degraded to a very low molecular weight. Another cause for the variation in the molecular weight and sedimentation constant is the degree of aggregation. In one instance the molecular weight was decreased from 6×10^6 to 1.8×10^6 on further deproteinization by shaking with chloroform-octanol. The boundary shown in Figure 2 is quite sharp, indicating a narrow distribution of sedimenting species and implying a narrow molecular weight distribution.

The density determined in a cesium chloride density gradient is 1.674 ± 0.005.

TABLE 2

DETERMINATION OF NUCLEOTIDE SEQUENCE IN POLY (dA + T) PRIMED BY POLY (A + U)

		3′-deoxyadenylate		3′-thymidylate	
Experiment	Substrate	cpm	% P^{32} recovery	cpm	% P^{32} recovery
1	dAPPP + $TP^{32}PP$	—	—	20,000	100
2	$dAP^{32}PP$ + TPPP	19,500	100	—	—

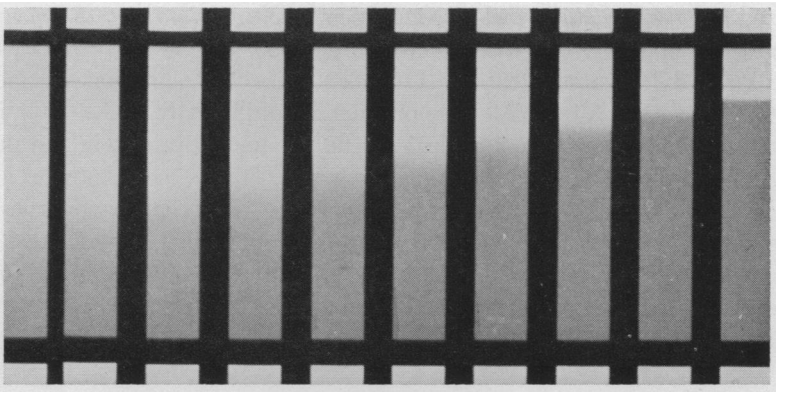

FIG. 2.—Ultracentrifuge pattern of poly (dA + T) primed with poly (A + U). Pictures were taken at 2-min intervals at a speed of 59,780 rpm. The dark region represents the ultraviolet light absorbing solution. The O.D.$_{260}$ of the poly (dA + T) was 0.4 in 0.2 *M* NaCl. The sedimentation constant is 28 S.

Equilibration was carried out at 8° but the value given is corrected to 25°. Equilibrations carried out at 25° showed considerable degradation of the poly (dA + T).

(b) *Light scattering:* Figure 3 shows the light scattering results for poly (dA + T). The weight-average molecular weight and radius of gyration are 1.8×10^6 and 2200 Å, respectively. The high radius of gyration is similar to that found for many natural native DNA's, indicating that the poly (dA + T) is a stiff chain. Its sedimentation constant is 21 S.

(c) *Helix-coil transitions:* Further evidence for the existence of an ordered structure in this polymer is given in Figure 4, where the change in absorption at 260 mμ is plotted as a function of temperature. The hyperchromic shift is quite sharp and occurs at 58° in 0.1 *M* NaCl and 41.2°C in 0.01 *M* NaCl. On fast cooling the optical density returns to its original value, indicating a reversible helix-coil transition. The values for the melting point at different ionic strengths are similar to those reported for the alternating poly dAT copolymer.[21] Figure 5 shows melting curves for two reaction mixtures in which there was approximately a one-fold synthesis. It is apparent that only two transitions are present: one for the product poly (dA + T) and one for the primer poly (A + U).[22]

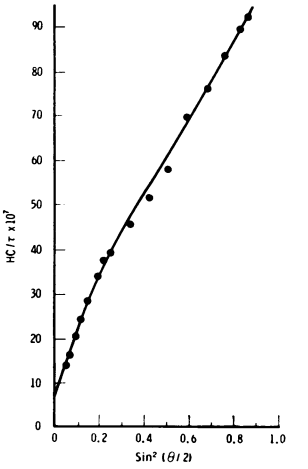

FIG. 3.—Light scattering results of poly (dA + T) primed with poly (A + U). The curve represents the zero concentration line of a Zimm plot. The ordinate intercept is the reciprocal of the weight-average molecular weight. The limiting low angle slope yields the radius of gyration. Molecular weight, 1.8×10^6; radius of gyration, 2200 Å.

(4) *Chemical and structural requirements of the primer:* Neither poly A nor poly U alone was able to act as a template using dATP and/or TTP as substrates. The three-stranded poly (A + 2U) also gave no product with these substrates. Apparently a two-stranded helical template structure is required. Using poly (A + U as primer with either dATP or TTP alone did not result in polymer synthesis; hence both strands must be

synthesized *simultaneously* in order to produce a polymer.

As might be expected, we have also found that poly (C + I) primes a reaction with dGTP and dCTP as substrates. Further studies on the product are under way. If poly (C + I) acts as a template, the product should be poly (dG + dC), in which each chain is a homopolymer, identical to the product obtained in the unprimed reaction.[10] Poly I does not serve as a primer with dCTP as the substrate. Neither poly (A + I) nor poly (A + 2I) serve as primers. For the former, the product would be poly (T + dG) if a helical structure were possible. The lack of product may therefore be a reflection of the fact that T and dG cannot couple in a helical structure. This would also suggest that the two strands are not synthesized independently.

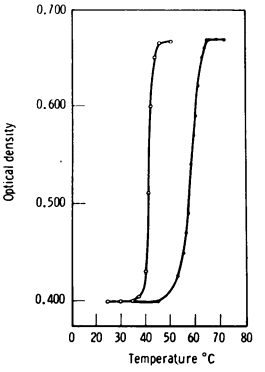

FIG. 4.—Optical density-temperature transition curves for poly (dA + T). Curve on left is in 0.01 *M* NaCl, melting point 41.2°; curve on right is in 0.1 *M* NaCl, melting point 58°. Measurements were made at 260 mμ.

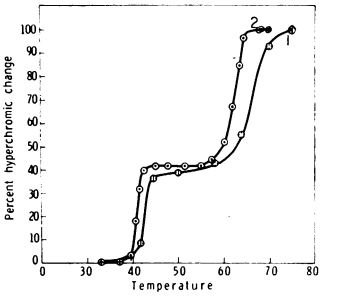

FIG. 5.—Hyperchromic change obtained on heating the reaction mixture after onefold, primed synthesis of poly (dA + T). The solvent is 0.02 *M* NaCl. One per cent duponol was also present, in curve 1. Measurements were made at 260 mμ.

Discussion and Conclusions.—It is evident that poly (A + U) serves as a primer in the DNA polymerase reaction, in that the lag period is greatly reduced. The fact that each chain of the product, poly (dA + T), is homopolymeric shows that poly (A + U) is the template. The poly (dA + T) product has a high molecular weight and an ordered structure. Since the melting curve of the reaction mixture of the onefold synthesis shows transitions only for the product and the template, it may be concluded that no hybrid intermediate is formed in measurable amount. Such a hybrid would presumably consist of a poly A strand complexed with the newly formed poly T strand; likewise, the poly U would be complexed with the poly dA. This would imply strand separation during synthesis. However, if the reaction were to continue, the hybrid molecules would then have to unwind so that the original poly A and poly U strands could reunite to form the helical poly (A + U); this would be a stringent requirement since we have shown that neither single-strand poly A nor poly U acts as primer in this reaction. The presence of most, if not all, of the original poly (A + U) after onefold synthesis is shown by its melting curve. Alternatively, one might postulate that only a

minute fraction is functional and that the hybrid it forms can itself act as template; but this appears unlikely since the system is always saturated with polymerase. It is thus unlikely that strand separation is involved in the function of poly (A + U) as a template.

The DNA and RNA polymerase seem to function in an almost identical manner; both utilize nucleoside triphosphates as substrates; both can bring about the incorporation of ribonucleoside triphosphates, in the presence of Mn^{++}.[11] Both reactions are primed by DNA and yield RNA or DNA products having the base composition[12-14] and nearest-neighbor pattern[8, 15] of the primer. The products, both RNA and DNA, are complementary and antiparallel to their templates, and both strands of the DNA can prime either product.[1, 16, 17] Since RNA polymerase utilizes helical DNA[2, 5] and poly (A + U)[18, 19] as templates, leaving them intact, and since we have shown the same is true of helical poly (A + U) in the DNA polymerase system, it is reasonable to conclude that with the DNA polymerase the conserved unit of DNA is the double helix.[3]

There is an interesting difference between the DNA and RNA polymerase systems when poly (A + U) is used as primer. With DNA polymerase our data show that dA and T are incorporated into the product to the same extent, whereas Nakamoto and Weiss[18] and Krakow and Ochoa[20] have shown that the amount of UTP incorporated exceeds that of ATP in the RNA polymerase system. It is possible that this type of difference provides a basis for the synthesis of noncomplementary RNA *in vivo*—there may be a preferential interaction of RNA polymerase with just one of the DNA chains at certain loci.

Evidence that certain other helical double-stranded, but not single- or triple-stranded, polyribonucleotides can prime the DNA polymerase reaction has also been presented.

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[22] The melting point of poly (A + U) complexed in the presence of Mg^{++} is considerably higher than that of the complex prepared in 0.02 *M* NaCl alone. The latter melts at 44°. Product and primer were precipitated with alcohol in the reaction represented in Figure 5. The Mg^{++} bound to the precipitate was therefore present in the mixture during the melting experiment. This accounts for the melting point between 60 and 64°. The melting point of pure poly (A + U) in 0.02 *M* NaCl and 10^{-4}*M* $MgCl_2$ was 64°. Exhaustive dialysis against 0.02 *M* NaCl reduced the value to 50°.

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*POLYPLOIDY IN THE MEXICAN AXOLOTL (AMBYSTOMA MEXICANUM) RESULTING FROM MULTINUCLEATE OVA**

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Spontaneous polyploidy in the Amphibia has been reported by several investigators.[1-4] Such polyploids among the progeny of diploid parents generally have been interpreted as resulting from one or more of the theoretically possible accidents which may occur in meiosis, fertilization, or the first cleavage division. This interpretation is much in accord with the fact that polyploidy may be induced by experimental procedures which suppress the second maturation division or disturb the normal progress of the first cleavage division, and it doubtless accounts for the great majority of spontaneous polyploids. Investigators rarely have offered the interpretation that polyploidy may result from a multinucleate condition in the egg. Humphries,[5] for example, hesitated to assume that diploidy in the egg of Triturus might be the result of a binucleate condition, in view of "the direct pathways actually seen to exist." More recently, however, Parmenter *et al.*[6] have reported the occurrence of binucleate and trinucleate young oöcytes in *Rana pipiens*. Their discovery of this condition suggested, as they point out, an important possible source of diploid parthenogenetic individuals. They point out, too, the possibility that the failure of one of the nuclei in a binucleate egg to undergo one or both meiotic divisions "could produce various chromosome numbers in mature eggs and in embryos resulting from their parthenogenetic stimulation or from fertilization."

Whether the multinucleate young oöcytes of *R. pipiens* observed by Parmenter *et al.* would have given rise to mature ova of unusually large size is uncertain. Briggs[7] states that he has observed eggs from females of this species with two or even three first polar bodies, but that they were of essentially the same size as mononucleate eggs, and could not be distinguished without the aid of a microscope. Multinucleate eggs of the axolotl, however, are readily distinguished by their larger size when eggs of a spawning including them are examined with the naked eye (Fig. 1). Their markedly large size, when they were first observed by the writer, caused them to be recorded as "giant" eggs.

The first oversized, or "giant," egg was found in 1953 in a spawning from a homozygous dark (D/D) female. No others were observed until 1956 when several appeared in two successive spawnings of a second female which was closely related to