Mammalian DNA Polymerase: Separation of Binding from Incorporation of Deoxyribonucleoside Triphosphates

Abstract. *An assay for the binding of deoxyribonucleoside triphosphate with an enzyme-DNA complex has been developed. This binding requires active enzyme and magnesium ion, takes place equally with native or denatured DNA, and may proceed in the absence of demonstrable DNA synthesis. The binding reaction appears to be specific for deoxyribonucleoside triphosphates, and studies on competition indicate that one active site accommodates the four common triphosphates.*

The DNA polymerase activity is generally assayed with a partially purified enzyme preparation by the incorporation of deoxyribonucleoside triphosphates into DNA. It is assumed either that the binding of nucleotides to an enzyme-DNA complex is not rate-limiting for the reaction or that it is at least an integral part of polymerase function. Our study shows that the binding process can be dissociated from that of polymerization. This binding requires primer DNA, Mg^{++}, and an active enzyme fraction.

The DNA polymerase activity in the assay (*1*) is terminated by placing the incubation tubes into ice. Two or more portions (0.05 ml each) of reaction mixture are applied to two different sheets of diethylaminoethyl (DEAE) impregnated paper (Whatman D 81). To determine total binding, one of the papers is treated (chromatographically) with 0.75*M* NH_4HCO_3 in a descending system. When the solvent front reaches 15 cm from the origin, the paper is removed and dried in a stream of warm air; the spots at the origin are identified with a mineral light, cut out, and the radioactivity is counted in Bray's solution (*2*). The radioactivity (count/min) remaining in these spots at the origin represent the sum of ^{3}H-triphosphate nucleotides incorporated into DNA and those bound to the DNA polymerase complex. To determine incorporation the second paper is eluted with 0.05*M* potassium phosphate buffer, *p*H 5.2; the conditions otherwise are identical to those above. Binding is defined as the difference between the total remaining at the origin after elution with 0.75*M* NH_4HCO_3 and that remaining after acid phosphate elution. An alternative method to determine incorporation of triphosphate nucleoside is as follows. Duplicate origin spots are removed after chromatography in 0.75*M* NH_4HCO_3. One of the spots is dried and placed directly into a counting vial; the other is washed in trichloroacetic acid, then in Hokin's reagent (*3*), and then in ether. The radioactivity is then determined. Both systems, chromatography in *p*H 5.2 buffer and the trichloroacetic acid wash, yielded identical results. Furthermore, results obtained with both methods agree with those obtained when acid precipitable radioactivity (count/min) is determined by our usual assay (*4*). Binding and incorporation data were obtained with enzyme derived from a crude *p*H 5.0 fraction of hepatoma 7777 and with partially purified Sephadex G-200 fractions I and II prepared from the *p*H 5.0 fraction (*5*).

Binding is independent of the state of the primer (Table 1). Equal binding is obtained whether native or denatured DNA is used as primer, whereas incorporation does depend on the primer. In studies of incorporation, the *p*H 5.0 fraction of hepatoma 7777 and fraction I show a marked preference for denatured DNA primer. Fraction II, on the other hand, has its preference for native primer (*5*). Both binding and incorporation depend on an active enzyme fraction, primer DNA, and $MgCl_2$. In the absence of primer DNA or magnesium ion, less than 0.01 nmole of ^{3}H-nucleotide was bound per milligram of enzyme protein. Heat-inactivated *p*H 5.0 protein fraction (0.2 mg heated at 56°C for 30 seconds) bound only 0.01 and 0.03 nmole per milligram of protein with native and denatured primer, respectively. Rat serum (0.2 mg) used as a source of protein fraction gave values of 0.01 nmole with both native and denatured primer. Binding appears to be specific for the deoxyribonucleotide triphosphates. Uridine triphosphate, thymidine monophosphate, and thymidine do not bind to any appreciable extent. Deoxyadenosine triphosphate and deoxycytosine triphosphate, however, give binding and incorporation values similar to those of thymidine triphosphate.

There is apparently one common binding site for all four triphosphates since the amount of labeled deoxyribonucleoside triphosphate bound is simi-

Table 1. Requirements for nucleotide binding with enzyme-DNA complex and for nucleotide incorporation into DNA. The complete system consists of 20 μmole of glycine-NaOH buffer (*p*H 8.0), 8 μmole of $MgCl_2$, 0.5 μmole of 2-mercaptoethanol, 65 μg of calf thymus DNA; 0.02 μmole each of the three nonlabeled deoxyribonucleoside triphosphates, and a labeled nucleotide or a nucleoside as indicated, in a total volume of 0.5 ml. Unless otherwise indicated the enzyme preparation used was 0.2 mg of a *p*H 5.0 protein fraction from hepatoma 7777. Incubation time was 60 minutes. Results are the averages from three different determinations with a variation of less than 10 percent; N indicates native DNA primer; D indicates denatured DNA primer.

Incubation conditions (*6*)	Binding plus incorporation (nmole ^{3}H-nucleotide mg^{-1} protein fraction)		Incorporation (nmole ^{3}H-nucleotide mg^{-1} protein fraction hour^{-1})		Binding (nmole ^{3}H-nucleotide mg^{-1} protein fraction)	
	N	D	N	D	N	D
Complete system						
^{3}H-TTP, 0.02 μmole	2.92	3.91	0.67	1.84	2.25	2.07
^{3}H-TTP, 0.02 μmole*	5.24	8.52	0.80	4.15	4.44	4.37
^{3}H-TTP, 0.02 μmole (minus dATP, dCTP, dGTP)*	15.81	17.31	0.09	0.47	15.72	16.84
^{3}H-TTP, 0.02 μmole†	6.94	6.19	1.56	0.63	5.38	5.56
Complete system						
^{3}H-UTP (minus other nucleotides)	0.09	0.07	0.03	0.04	0.06	0.03
^{3}H-TMP (minus other nucleotides)	0.23	0.24	0.14	0.18	0.09	0.06
^{3}H-TdR (minus nucleotides)	0.21	0.19	0.12	0.14	0.09	0.05
^{3}H-dATP	3.11	4.21	0.71	1.72	2.40	2.49
^{3}H-dCTP	2.85	3.95	0.59	1.57	2.26	2.38

* The enzyme source was 0.1 mg of Sephadex fraction I. † The enzyme source was 0.05 mg of Sephadex fraction II.

Table 2. Competition of deoxyribonucleoside triphosphates for the enzyme-DNA complex binding site. The experimental system is as given for Table 1 with nonlabeled triphosphates added as indicated. Sephadex G-200 fractions I and II provide the source of enzyme. Fraction I (0.1 mg) is assayed with denatured primer and fraction II (0.05 mg) is assayed with native primer.

Nonlabeled triphosphate added (6)	Amount (μmole)	Binding (nmole ^{3}H-nucleotide mg^{-1} protein fraction)	
		Fraction I	Fraction II
	^{3}H-TTP (0.02 μmole)		
		17.31	14.73
TTP	0.04	9.64	8.07
dATP	0.04	10.32	9.12
dCTP	0.04	11.70	8.17
dGTP	0.04	9.89	7.64
dATP, dCTP, dGTP each	0.02	8.52	6.94
	^{3}H-dCTP (0.02 μmole)		
		16.43	13.91
TTP	0.04	9.39	8.47
dATP	0.04	10.16	8.18
dCTP	0.04	8.68	6.84
dGTP	0.04	10.71	7.29
dTTP, dATP, dGTP each	0.02	7.94	5.82
	^{3}H-dATP (0.02 μmole)		
		18.45	15.75
TTP	0.04	11.78	8.48
dATP	0.04	10.12	7.27
dCTP	0.04	12.13	9.39
dGTP	0.04	10.56	7.89
TTP, dCTP, dGTP each	0.02	8.42	6.74

larly decreased by the addition of any of the four unlabeled triphosphates (Table 2). It appears from the competition experiments that binding requires between 0.03 and 0.04 μmole of triphosphate to saturate the amount of enzyme protein used in the assay. With 0.02 μmole of ^{3}H-TTP (6) and 0.01 μmole of nonlabeled TTP, the same number of counts is bound as with 0.02 μmole of ^{3}H-TTP alone. If 0.02 μmole of nonlabeled TTP is added, only slight inhibition of binding was found; it required 0.04 μmole of nonlabeled TTP to reduce binding by approximately one half. Similar amounts are required for competition with the other deoxyribonucleoside triphosphates.

Differences exist in the time course of the incorporation and binding reactions and in the relation to the amount of DNA primer. The incorporation reaction remains linear for at least 60 minutes; maximum binding is reached after 15 minutes of incubation and remains constant for up to 60 minutes. Binding increases linearly as the DNA in the system is increased to 150 μg with enzyme remaining constant at 0.2 mg of *p*H 5.0 protein fraction per 0.5 ml of reaction mixture. Incorporation, however, is linear only up to 50 μg of DNA and does not increase with increases in DNA. It is not possible at present to estimate the number of binding sites on an enzyme molecule since the enzyme preparations are not sufficiently pure.

The process of binding of deoxyribonucleoside triphosphates to a DNA–DNA polymerase complex can be dissociated from polymerization of nucleotides into DNA. The two reactions differ with respect to primer DNA preference and to time required for the completion of the reaction. Kornberg (7) postulated that *Escherichia coli* DNA polymerase molecules have five active sites, one of which binds triphosphates. When a correct base pair is within the active site, the enzyme can respond, presumably by a conformational change, so that the catalytic step may proceed. If an incorrect triphosphate were found, it would be rejected and no conformational change would occur. It is premature to suggest a similar postulate for mammalian DNA polymerase. It is still possible that two different protein molecules are involved in these two reactions, one acting as a nucleoside triphosphate acceptor and the other as a polymerase enzyme. In studying the triphosphate binding to DNA polymerase alone by equilibrium dialysis, Kornberg concluded that the four common nucleoside triphosphates compete for a single site (7). Our data with mammalian enzyme and DNA are in agreement with this finding.

PETER OVE*
JOHN LASZLO
Department of Clinical Pharmacology and *Medicine, Duke University Medical Center, Durham, North Carolina 27706*

References and Notes

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6. Abbreviations are: dATP, deoxyadenosine triphosphate; dCTP, deoxycytidine triphosphate; dGTP, deoxyguanosine triphosphate; TdR, thymidine; TMP, thymidine monophosphate; TTP, deoxythymidine triphosphate; and UTP, uridine triphosphate.
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* Present address: Department of Anatomy and Cell Biology, University of Pittsburgh Medical School, Pittsburgh, Pennsylvania 15213.

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Sex Attractant of Female Dermestid Beetle Trogoderma inclusum Le Conte

Abstract. *Two components of the sex attractant of a female dermestid beetle* Trogoderma inclusum *Le Conte were identified. They are (−)-14-methyl-*cis*-8-hexadecen-1-ol and (−)-methyl-14-methyl-*cis*-8-hexadecenoate. Other* Trogoderma *species also respond to the compounds.*

We report here the isolation, identification, and synthesis of two components of the sex attractant of a female dermestid beetle, *Trogoderma inclusum* Le Conte (1). This beetle is a common pest in dried-milk factories, and it consumes a wide variety of products such as grain, cereals, dried milk, spices, nuts, and other stored, dried foods. A closely related beetle *T. granarium* Everts (the khapra beetle) is a very serious pest of stored cereal products in hot, dry parts of the world. The response of the male *T. inclusum* beetle to the unmated female has been described by Burkholder and Dick (2) whose bioassay was used to monitor the isolation steps.

Each of the two identified compounds is active by itself. Responses of the male beetle to the synthesized attractants and to the isolated compounds are identical; the males respond to the compounds in a manner similar to that of males exposed to paper disks that have been in contact with females (2). Females do not respond in any of these tests, nor do they respond to paper disks that have been in contact with males.

In the laboratory bioassay, a response from the male *T. inclusum* was elicited by as little as 0.001 μg of synthesized compound 1 (3 males responded out of a total number of 40 tested) or 0.01 μg of synthesized compound 2. The effect of a mixture of the two synthesized compounds was additive; no synergistic or blocking effects were detected, but cross-attractance was general in the genus. The responses of males of several other *Trogoderma* species to 1 μg of each of the synthesized compounds are shown in Table 1. Forty males were used in each test, each male being placed in a 3.7-ml shell vial. An assay disk containing 1 μg of the attractant was suspended 1 cm above each male for 1 minute. The response was considered positive when the behavior reported earlier (2) was observed.

The interspecific responses for five