BINDING OF RNA POLYMERASE TO T7 DNA: EVIDENCE FOR MINIMAL NUMBER OF POLYMERASE MOLECULES REQUIRED TO CAUSE RETENTION OF POLYMERASE - T7 DNA COMPLEX ON MEMBRANE FILTERS*

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The binding of RNA polymerase to DNA can be measured by retention of the complex on a membrane filter (Jones and Berg, 1966). Initially, it appeared that a linear relation existed between amount of DNA retained on the filter and amount of enzyme present. This suggests that a single enzyme molecule bound to a DNA molecule is sufficient to cause retention of DNA on the filter and that polymerase, at limiting concentrations, will not bind to a DNA molecule which already has enzyme bound to it. An alternative hypothesis is that most binding sites on a single DNA molecule have equal affinity for RNA polymerase (Richardson, 1966a; Stead and Jones, 1967a) and therefore enzyme molecules will distribute themselves statistically among the DNA molecules. As will be shown below, this type of distribution is best described by a sigmoid-shaped curve for binding. Moreover, a minimum number of 5 enzyme molecules must bind to each molecule of T7 DNA before the DNA is retained on the filter.

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MATERIALS AND METHODS

E. coli RNA polymerase was prepared through fraction IV according to the method of Chamberlin and Berg (1962). This enzyme fraction had a specific activity of 3000 when assayed according to Chamberlin and Berg using salmon sperm DNA as template. One fraction of RNA polymerase was used for all experiments. This fraction was subjected to zone sedimentation in sucrose (Stead and Jones, 1967b). Representative fractions of enzyme protein were recovered from the distribution of RNA polymerase in the gradient. Using equivalent amounts of protein, each fraction was assayed separately for RNA synthesis and binding to T7 DNA by the membrane filter technique (Jones and Berg, 1966). From this we estimate that the enzyme used for the experiments described below is at least 85 percent homogeneous.

Tritiated DNA was prepared from bacteriophage T7 by extraction in buffer-saturated phenol and dialysis in sterile 0.01M Tris-HCl pH 7.9. The absorbancy ratio at 260mu/280mu was 1.91. DNA concentrations were determined by the method of Dische (1955).

Each binding assay was performed in a volume of 0.25 ml containing 10 umoles Tris-HCl pH 7.9, 1.0 umole $MgCl_2$, and 3.0 umoles mercaptoethanol, 3.6 micrograms T7 [^{3}H] DNA and varying amounts of RNA polymerase. The reaction was started with the addition of enzyme and incubated five minutes at 37° C. Five ml. of chilled 0.01M Tris buffer pH 7.9 containing 0.05M NaCl was added and samples were kept chilled until filtered through a Millipore Filter (Type HA) at 2-6 centimeters of mercury and washed with approximately 20 ml of the Tris-NaCl solution. Allowing the diluted samples to stand for as long as 60 minutes did not alter DNA retention. The filters were dried and counted in a Nuclear Chicago Liquid Scintillation counter.

RESULTS AND DISCUSSION

The effect of adding increasing amounts of RNA polymerase to T7 DNA is shown in figure 1. For each enzyme concentration, from five to

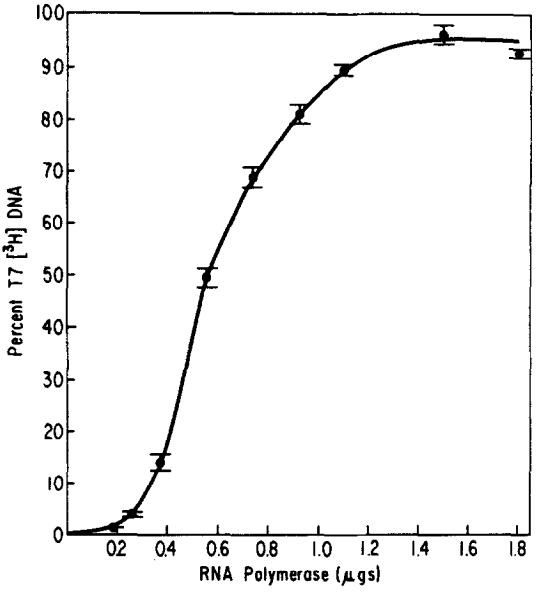

Figure 1: Retention of T7 [^{3}H] DNA on membrane filters. Each point represents an average of 5-8 binding assays performed as described in Methods and plotted as percent retained on the filter. The standard error of the mean for each point was calculated from $\frac{\sigma}{\bar{x}} = \frac{\sigma}{\bar{N}}$. 13,640 cpm as T7 [^{3}H] DNA is equivalent to 100 percent retention. In the absence of RNA polymerase less than 100 cpm are retained on the filter.

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eight binding assays were performed under identical conditions. Below 0.19 ug enzyme, less than 1.5% of the DNA is retained on the filter. The retention curve is linear between 0.26 and 0.92 ug enzyme, reaching maximum retention at a weight ratio of enzyme to DNA of 0.3-0.4, in agreement with previous observations (Jones and Berg, 1966; Stead and Jones, 1967a).

It can be seen that a plot of the percent of the DNA-RNA polymerase complex retained by the filter at each enzyme concentration gives a sigmoid-shaped curve. Because little DNA is retained below 0.19 ug polymerase it would seem that the first enzyme molecule bound to a DNA molecule is insufficient to cause that DNA molecule to be retained on the filter and that some minimum number of enzyme molecules might be required to cause

retention of the complex. Furthermore, the sigmoid-shaped curve suggests that at very low concentrations of polymerase, the DNA sites competing for the enzyme have equal polymerase binding affinity.

Assuming that an enzyme molecule will bind as readily to DNA already bound with, for example, 3 enzyme molecules as to DNA with none, and that a certain minimum number of enzyme molecules must bind to the DNA before it will be retained on the filter, one can use a statistical approach to determine the minimum number of enzyme molecules required for DNA retention. The molecular weight for E. coli RNA polymerase, under our experimental conditions is 8.8×10^5 (Richardson, 1966b) and for T7 DNA is 27×10^6 (Studier, 1967). We convert micrograms of enzyme added to the 3.6 micrograms of DNA in each assay into molecules of enzyme added per molecule of DNA. Since the largest amount of enzyme added is less than the maximum number of T7 DNA sites for polymerase, it can be assumed that all enzyme molecules in the reaction mixture are bound to DNA. The number of enzyme molecules added per molecule of DNA is therefore equivalent to the average number of enzyme molecules bound per molecule of DNA. Using the Poisson distribution

$$P(x) = \frac{e^{-u} u^{x}}{x!} \qquad (1)$$

where x is number of enzyme molecules bound, u is the average number of enzyme molecules bound per DNA molecule, and P(x) is the probability of having x enzyme molecules bound.

For an average of u polymerase molecules per DNA, one can calculate the probability of a DNA molecule binding $x = 0,1,2...n$ polymerase molecules. In a large enough sampling, this probability approximates the actual percentage of the total number of DNA molecules binding $0,1,2...n$ polymerase molecules. If n enzyme molecules must bind to a DNA molecule before it can be retained by the filter, the molecule will not be retained when $x = 0$ up to $x = n-1$ polymerase molecules have bound to it. If we state further, that S equals DNA which is not retained by the filter, then

$$S = e^{-u} \sum_{x=0}^{n-1} \frac{u^x}{x!} \qquad (2)$$

Using (2) the DNA in the reaction mixture may be subdivided into several classes, having <2, <3,...<n polymerase molecules per DNA. In Figure 2, the solid lines show this family of theoretical curves where the percent DNA in a particular class is plotted as a function of u. One can then compare the theoretical curves with the experimentally derived plot of percent DNA which is not retained by the filter. As shown by the broken line in figure 2,

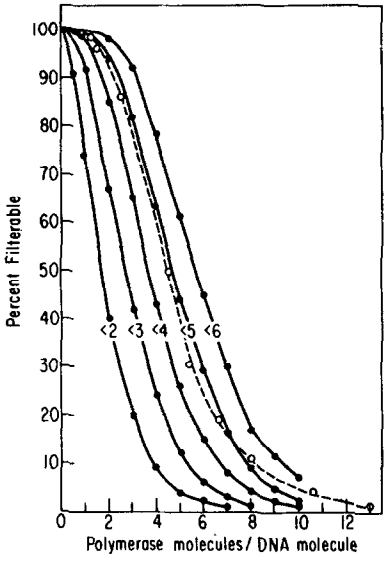

Figure 2: Theoretical plot for enzyme distribution among DNA molecules in the binding mixture compared with experimental curves for filterable T7 [^{3}H] DNA. The dashed line indicates percent filterable DNA at increasing concentration of enzyme molecules per DNA molecule, based on 85 homogeneity of the enzyme preparation. The solid lines show the percent DNA in the reaction mixture that should have less than 2, less than 3, less than 4, etc. enzyme molecules per DNA when an average 1,2,3,...n enzyme molecules/DNA have been added to the reaction mixture.

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it would appear that the DNA not retained by the filter falls near that class of DNA in the reaction mixture having <5 polymerase molecules bound per DNA molecule, or conversely that DNA, to be retained by the filter, must bind at least 5 enzyme molecules per DNA molecule.

be defined as reversing this inhibition. Available data are consistent with the mechanism of energy-linked reduction of NAD^+ by succinate (Chance and Hollunger, 1960) blocking the pyruvate oxidation. Wenner and Cereijo-Santalo (1963) have shown that in Ehrlich-Lettré ascites tumor, mitochondrial oxidation of pyruvate is inhibited by the addition of α-glycerophosphate. Since mitochondrial α-glycerophosphate dehydrogenase is also a flavin-linked enzyme, the pyruvate oxidation in these tumors appears to be sensitive to the activity of flavin-linked dehydrogenases. A possibility exists that the pyruvate dehydrogenase of these tumors is inhibited by NADH, as is the α-ketoglutarate dehydrogenase of pig heart (Garland, 1964). Such characteristic behavior of the pyruvate oxidation may take part in the development of the Crabtree effect (Ibsen, 1961), since the Crabtree effect is known to involve a profound inhibition of pyruvate oxidation (Wenner and Paigen, 1961) and to be released by dinitrophenol (Racker, 1956). The present work suggests also that a concept analogous to "the concept of substrate competition in respiration" (Haslam and Krebs, 1963) may apply to certain inhibitors of the citric acid cycle, whose inhibitory action on one site of the cycle may induce a stimulation of other(s).

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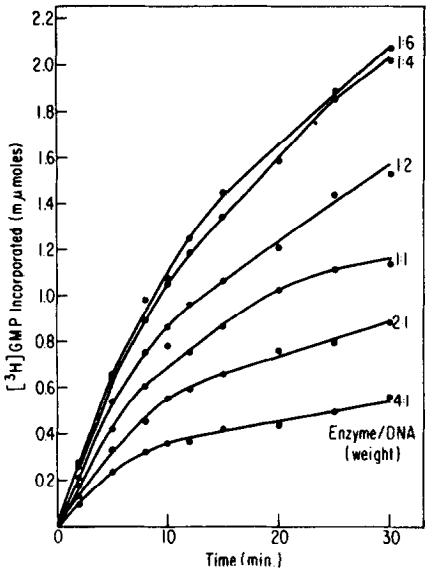

Figure 3: Rate of RNA synthesis in the presence of a constant amount of RNA polymerase and increasing concentrations of DNA. The assays measure incorporation of [^{3}H] GMP incorporated into acid precipitable RNA. Each reaction mixture (0.5 ml) contained 20 umoles Tris-HCl pH 7.9, 2.0 umole $MgCl_2$, 6.0 umoles 2-MSH, 200 mumoles [^{3}H] GTP (Specific activity = 2.5 x 10^3 cpm/mumole) and 200 mumoles each of unlabeled rUTP, rCTP, rATP. Each assay contained 8 ug RNA polymerase and increasing amounts of T7 DNA. Incubation was performed at 37°. The reaction was stopped by placing an aliquot of the reaction mixture on GF/C glass filters (Whatman) and washing the filter 3 successive times in 50 ml 5% TCA. The filters were washed in ether-ethanol 1:1, ether and then dried and counted in a liquid scintillation counter. The ratios shown in the figure are weight ratios of polymerase/DNA in each experiment.

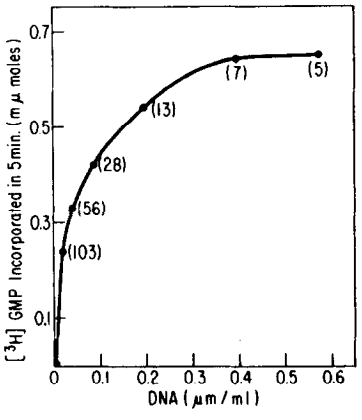

Figure 4: Initial rate of RNA synthesis as a function of increasing concentration of DNA. Each point is the amount of RNA synthesized at 5 minutes incubation (Fig. 3) when polymerase is constant and DNA increased. The numbers in parenthesis represent the number of enzyme molecules per DNA molecule at each point, assuming 85% homogeneity of this preparation of polymerase.

when there are 5-7 polymerase molecules per DNA molecule. Increasing DNA concentrations beyond this point does not increase RNA synthesis. This suggests that a small number of sites on the T7 chromosome are being transcribed preferentially by RNA polymerase. Whether these sites are related in any way to the minumum number for binding on membrane filters awaits further study.

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