INTERACTION OF POLY-L-LYSINE WITH CHROMATIN

Inhibition of In Situ Priming for
Escherichia coli DNA Polymerase

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ABSTRACT

The priming capacity of chromatin of fixed nuclei and chromosomes for exogenous DNA polymerase can be evaluated radioautographically by the incorporation of labeled nucleotides. It had previously been reported that acid fixation or acid treatment of alcohol-fixed tissues led to increased priming when calf thymus DNA polymerases, specific for single-stranded DNA, were used. We employed Escherichia coli DNA polymerase and sequential treatments of the fixed tissue with acid and poly-L-lysine in order to elucidate the mechanism through which the acid effect is produced. Acid treatment enhanced chromatin priming for the E. coli DNA polymerase, and saturation of the chromatin with poly-L-lysine strongly inhibited the reaction. This inhibition was reversible through subsequent treatment with acid. Wide differences in priming were observed between cell types of alcohol-fixed chicken blood smears: thrombocyte and lymphocyte nuclei exhibited strong priming ability whereas erythrocyte nuclei failed to support any detectable priming. We conclude that the acid effect is readily interpretable in terms of acid-mediated changes in the association between DNA and protein in the chromatin complex.

INTRODUCTION

The enzymes DNA polymerase and terminal transferase, as derived from calf thymus tissue, have been used in several studies on fixed eukaryote chromosomes aimed at an elucidation of the distribution of ends or replicative initiation points of DNA molecules (Bollum, 1963, Modak and Bollum, 1970; Modak et al., 1969, Price et al., 1971; Von Borstel et al., 1969, Von Borstel et al., 1966). Either acid fixation, or acid treatment after alcohol fixation of nuclei and chromosomes, was found to increase the incorporation of labeled nucleotides mediated by these enzymes. In view of the fact that both of the aforementioned enzymes require, as primer, single-stranded DNA with free 3'-hydroxyl ends (Yoneda and Bollum, 1965), these results were interpreted as resulting from the increased availability of single-stranded ends of DNA through denaturation by acid treatment (Bollum, 1963; Modak and Bollum, 1970; Modak et al., 1969; Price et al., 1971; Von Borstel et al., 1969, Von Borstel et al., 1966). It occurred to us that there might be an alternative interpretation, that the enhancement of enzyme-catalyzed nucleotide incorporation into acid-fixed chromosomal DNA could be brought about by the removal of basic protein from DNA by acid treatment (Dick and Johns, 1968), particularly since histones have been demonstrated to inhibit priming of DNA for calf thymus DNA polymerase (Bazill and Philpot, 1963, Bollum, 1963). Such an interpretation would imply that the previous data are not directly indicative of the state of the DNA
alone. In order to test this possibility, we have carried out in situ nucleotide incorporation studies with the DNA polymerase of *Escherichia coli* which can use, as primer, native DNA, denatured DNA (Harrington and Ricanati, 1966), partially single-stranded DNA (Richardson et al., 1963), and viral single-stranded DNA (Mitra et al., 1967). At low temperature (15°C) this enzyme catalyzes nucleotide incorporation with a predominantly single-stranded DNA primer, whereas, at the more usual 37°C it utilizes native as well as denatured or single-stranded DNA (Deutscher and Kornberg, 1969). Moreover, both Billen and Hnilica (1963, 1964) and Kornberg (quoted by Lehman, 1964) have reported that both histones and polypeptide, when bound to the DNA primer, are strong inhibitors of the *E. coli* polymerase. Our results, while not excluding the possibility of an acid effect by denaturation, suggest that increased availability of DNA as primer through removal of basic protein is a more direct and reasonable interpretation for the effect.

**Materials and Methods**

**Preparation of Acid-Fixed Salivary Glands**

Squashes of salivary gland chromosomes of *Drosophila melanogaster* were prepared as described by Plaut et al. (1966). Glands were dissected from third instar larvae in Ephrussi-Beadle medium. The glands were fixed and squashed on gelatinized slides in 45% acetic acid, frozen in liquid nitrogen for coverslip removal, post-fixed in acetic acid alcohol (1:3), transferred to 75% ethanol, and allowed to air-dry. The slides were washed three times, 10 min per wash, in deionized water, air-dried, and glass rings were glued on.

**Preparation of Chicken Blood Smears**

Fresh blood from adult chicken was smeared on gelatinized slides, fixed for 1 hr in 70% ice-cold ethanol, transferred to 80% ethanol, and air-dried. Acid treatment consisted of a 10 min exposure of the tissue to 0.1 N HCl at room temperature, followed by washing, and air-drying.

**Treatment with Poly-L-Lysine**

Poly-l-lysine type I (mol wt 130,000) and poly-l-lysine type II (mol wt 2600), purchased from Sigma Chemical Co. (St. Louis, Mo), were used in aqueous solution (400 μg/ml) to treat the slides. A drop (0.020 ml) of poly-l-lysine solution was placed on the tissue and covered with a 22 mm round coverslip to ensure uniform distribution and to minimize evaporation. After 10 min at room temperature the coverslip was removed with a stream of water. The slides were washed three times, 10 min per wash, in deionized water, air-dried, and glass rings were glued on.

Several slides were treated with acetic acid and ethanol after the treatment with poly-l-lysine. These slides were placed sequentially for 10 min in each of the following: 45% acetic acid, acetic acid ethanol (1:3), 100%, 95%, 75%, and 30% ethanol, water, and were finally air-dried.

**DNA Synthesis In Situ**

Unlabeled deoxyadenosine triphosphate (dATP), deoxyguanosine triphosphate (dGTP), deoxycytidine triphosphate (dTTP), and deoxythymidine triphosphate (dCTP) were purchased from Sigma, and dTTP-3H (sp. act 17.4 Ci/mmole) from Schwarz Bio Research Inc., Orangeburg, N. Y. DNA polymerase from *E. coli* was prepared as described by Jovin et al. (1969). Both fraction six and fraction seven were used in the in situ studies, with similar results.

The incubation mixture (Richardson et al., 1964 b) (0.5 ml/well) contained 33.3 μmole of glycine buffer, pH 9.2, 3.3 μmole of MgCl2, 0.5 μmole of 2-mercaptoethanol, 12 nmole each of dATP, dCTP, dGTP, and dTTP, 0.5 nmole of dTTP-3H (sp. act 17.4 Ci/mmole), and 5 units (Exo II assay, Lehman and Richardson, 1964) of DNA polymerase. In control incubations the enzyme was omitted from the incubation mixture. Slides were incubated at 37°C for 30 min. The enzymatic reaction was stopped by replacing the reaction mixture in the well with ice-cold 5% trichloroacetic acid (TCA). After 10 min the TCA was removed from the well and the glass ring was separated from the slide. The slides were washed three times in cold TCA followed by ethanol-ether (1:1) and ether, allowing 10 min for each wash. The slides were then air-dried. Experiments carried out with potassium phosphate buffer (pH 7.4) (Mitra et al., 1967) yielded the same results.

**Treatments with DNase I and RNase**

Bovine pancreatic RNase and DNase I were purchased from Worthington Biochemical Corp., Freehold, N. J. The RNase was heated in aqueous solution at 100°C for 15 min to inactivate contaminating DNase. The incubation mixture for DNase I (0.5 ml/well) contained 0.12 μmole Tris-HCl buffer, pH 8.0, 1.2 μmole MgCl2, and 30 μg DNase I. The incubation mixture for RNase I (0.5 ml/well) contained 0.12 μmole Tris-HCl buffer, pH 8.0, and 60 μg RNase. The control incubation mixture (0.5 ml/well) contained 0.12 μmole Tris-HCl buffer, pH 8.0, 1.2
mole MgCl₂, and no enzymes. All incubations were carried out at 37°C for 2 hr, and stopped as in the case of the DNA polymerase incubations.

**Preparation of Poly-L-Lysine-¹⁴C**

Formaldehyde-¹⁴C (sp. act. 8.1 μCi/mmol), purchased from New England Nuclear, Boston, Mass., was used to prepare labeled poly-L-lysine according to the method of Rice and Means (1971). After reducing with sodium borohydride, the protein solution (2 ml) was dialyzed for 24 hr at 4°C against 450 ml of deionized water. The water was changed twice during the dialysis period. At the end of dialysis the polylysine solutions were centrifuged for 30 min at 10,000 g in order to remove any precipitate. The supernatant was analyzed for polylysine content by Lowry’s technique (Lowry et al., 1951) and ¹⁴C was counted on a Packard Tricarb liquid scintillation spectrometer (Packard Instrument Co., Downers Grove, Ill.).

Poly-L-lysine type I (mol. wt. 130,000) solution contained 105 μg/ml, 1.51 × 10⁴ cpm/mg, poly-L-lysine type II (mol. wt. 2860) solution contained 100 μg/ml, 1.0 × 10⁵ cpm/mg. Both solutions were used without further processing.

**Radioautography and Microscopy**

Slides were coated with K5 liquid emulsion (Ilford Ltd., Ilford, Essex, England), exposed for 2 wk at 4°C, and developed in D-19 (Eastman Kodak Co., Rochester, N. Y.). Microscopic observations were done with phase optics.

**RESULTS**

**Squashed Salivary Glands**

At 37°C, DNA polymerase produced extensive incorporation of thymidine-³H into chromosomal material (Fig. 1 d). The radioactivity was removed from the chromosomes by DNase treatment (Fig 2 f), but not by RNAse or buffer (Figs. 2 e, d), indicating that the product of the enzymatic reaction in situ is labeled DNA. The omission of DNA polymerase from the incubation mixture (Table I, group 1) eliminated detectable incorporation of thymidine-³H into chromosomes (Fig 1 a). In all cases, we could distinguish clearly between treatments which produced extensive priming (Fig. 1 d) and those that resulted in little, if any, DNA synthesis (Figs. 1 a–c, e, f). Carrying out the enzymatic reaction at 15°C also produced incorporation of thymidine-³H along the entire length of the chromosomes. Some quantitative variation was found in thymidine-³H incorporation into chromosomes of different nuclei on the same slide. This was more apparent in treatments with extensive DNA synthesis (Table I, groups 7–10). Quantitative variation in incorporation was also detected along the chromosomes, in that some chromosomal regions appeared less densely labeled (e.g., Fig. 2 e).

Treatment with poly-L-lysine before the DNA polymerase enzymatic reaction (Table I, groups 5, 6) resulted in strong inhibition of priming by chromosomal DNA (Figs. 1 e, f). When poly-L-lysine treatment was followed by acid treatment (45% acetic acid and ethanol) and incubation with DNA polymerase (Table I, groups 7, 8), the chromosomes’ priming ability was found to be restored (Figs. 2 a, b). We could not demonstrate any difference between the two types of poly-L-lysine used. No inhibition of DNA synthesis could be detected in experiments where treatments with the amino acid DL-lysine were substituted for poly-L-lysine.

Squashed preparations of salivary glands were incubated with polylysine-¹⁴C for 10 min at room temperature, followed by extensive rinsing in deionized water. After radioautography it was clear that polylysine indeed binds to chromosomal material (Fig. 2 e). No differences were found between type I and type II polylysine-¹⁴C.

**Chicken Blood Smear Preparations**

In alcohol-fixed blood smears, most of the cells (probably erythrocytes) did not exhibit any detectable priming for DNA polymerase (Figs 3 a, b) at either temperature. However, in the same slides some cell types showed moderate to heavy priming (Figs 5 a–d). The morphology of these priming cells suggested their identity as thrombocytes and lymphocytes (Lucas and Jamroz, 1961). Alcohol-fixed blood smears which were acid treated exhibited some priming at 15°C (Fig. 3 c) and extensive priming at 37°C (Fig 3 d). Poly-L-lysine treatment of alcohol-fixed and acid-treated blood smears led to inhibition of priming for DNA polymerase (Figs 4 a, b); this inhibition was reversed by a second acid treatment after the polylysine treatment (Fig 4 d). As with the salivary gland preparations, we found that poly-L-lysine-¹⁴C binds to nuclear material after acid treatment of the alcohol-fixed blood smears.
Figure 1  Inhibition of E. coli DNA polymerase by poly-L-lysine in situ, using D. melanogaster salivary gland chromosomal DNA as primer. All incubation mixtures contained dTTP-32P, dATP, dGTP, and dCTP (see text and Table I for details). (a–c) Control incubation omitting DNA polymerase, (a) no polylysine treatment, (b) pretreatment with poly-L-lysine type I, (c) pretreatment with poly-L-lysine type II. (d–f) Incubation mixture contains DNA polymerase, (d) no polylysine treatment, (e) pretreatment with poly-L-lysine type I, (f) pretreatment with poly-L-lysine type II. x 1000.
TABLE I
The Effect of Poly-L-Lysine and Acid Treatment on E. coli DNA Polymerase Activity in Vivo Using Drosophila melanogaster Salivary Gland Chromosomal DNA as Primer

| Slide group | Pretreatment with poly-L-lysine | Treatment in 43% acetic acid | Incubation with dTTP-3H, dATP, dCTP, dGTP, and E. coli DNA-polymersase | Incubation in buffer, DNAse I, or RNase | Grain density in radioautographs |
|-------------|---------------------------------|-----------------------------|-----------------------------------------------------------------|----------------------------------------|---------------------------------|
| 1           | -                               | -                           | +Enzy †                                                          | -                                      | Background                      |
| 2           | Type I                          | -                           | -Enzy                                                           | Light                                  |                                 |
| 3           | Type II                         | -                           | -Enzy                                                           | Light                                  |                                 |
| 4           | -                               | -                           | +Enzy                                                           | Heavy                                  |                                 |
| 5           | Type I                          | -                           | +Enzy                                                           | Light                                  |                                 |
| 6           | Type II                         | -                           | +Enzy                                                           | Light                                  |                                 |
| 7           | Type I                          | +                           | +Enzy                                                           | Heavy                                  |                                 |
| 8           | Type II                         | +                           | +Enzy                                                           | Heavy                                  |                                 |
| 9           | -                               | -                           | +Enzy                                                           | Buffer                                  | Heavy                           |
| 10          | -                               | -                           | +Enzy                                                           | RNase                                   | Heavy                           |
| 11          | -                               | -                           | +Enzy                                                           | DNAse I                                 | Light                           |

† Followed by treatment in acetic acid-ethanol (1:3), 100%, 95%, 75%, 50% ethanols, and water, 10 min in each, and air-dried.

DISCUSSION

We have presented evidence showing that DNA polymerase derived from E. coli mediates incorporation of thymidine-3H into chromosomal material in fixed cytological preparations. Sensitivity to DNase digestion and resistance to RNase and buffer treatments permits the identification of the labeled chromosomal material as DNA. The activity of the polymerase in catalyzing such in vivo DNA synthesis was enhanced by prior treatment of the fixed tissue with acid. These results are in good agreement with those of previously reported studies on in situ calf thymus polymerase work had been presented (Bollum, 1963). The observations reported here on E. coli DNA polymerase-mediated in situ DNA synthesis bear out the validity of Hinilica and Billen's (1964) suggestion.

DNA polymerase from E. coli uses single-stranded DNA as primer at 15°C (Kornberg et al., 1964; Richardson et al., 1964); Mitra et al., 1967, Deutscher and Kornberg, 1969) but will act on native as well as denatured DNA with approximately equal efficiency when the reaction is carried out at 37°C (Harrington and Ricanati, 1966). This enzyme, like calf thymus DNA polymerase, is strongly inhibited by histones and polylysine (Hinilica and Billen, 1964; Lehman, 1964). As expected, our results at 15°C (Figs 3 a, c) were similar to previous observations with calf thymus enzymes (Modak et al., 1969), in that incorpora-
Acid treatment reverses polylysine inhibition of DNA polymerase; polylysine binds to chromosomes and dTTP-^H is incorporated into DNase-sensitive material. (a) Pretreatment with poly-L-lysine type I, followed by sequential treatments in acetic acid, acetic acid-ethanol (1:3), 100%, 95%, 75%, 50% ethanols, and water, after which slides were incubated with DNA polymerase. (b) Same as a, but using poly-L-lysine type II. (c) Slide was incubated for 10 min in poly-L-lysine-^14C type II, followed by extensive rinsing in water. (d-f) After incubation with DNA polymerase, the slides were incubated with buffer (d), RNase (e), and DNase (f). × 1000.
The effect of acid treatment on priming activity for *E. coli* DNA polymerase by chicken erythrocytes chromatin in situ. (a) alcohol-fixed blood smear incubated with DNA polymerase for 30 min at 15°C, (b) same as a, but incubated at 37°C, (c) alcohol fixed and acid treated, incubated with DNA polymerase for 30 min at 15°C, (d) same as c, but incubated at 37°C × 1000.

... tion was observed only after acid treatment and in the absence of any logical means for dissociating basic protein removal from DNA denaturation as causative elements. The data from incubations at 37°C, however, do permit an approach to a causal analysis. Although the *E. coli* DNA polymerase should be able to use undenatured DNA as primer at this temperature, and in fact does so in thrombocyte and lymphocyte nuclei treated only with alcohol (Figs. 5 a–d), alcohol-fixed chicken erythrocyte nuclei show substantial incorporation only after acid treatment (Fig 3 d) and not without (Fig. 3 b). This latter difference would not be expected if the effect of acid were only on the strandedness of the DNA molecule, but can be readily understood in terms of the removal by acid of an inhibiting condition which prevents priming by double-stranded DNA.

We offer two lines of experimental evidence in support of the interpretation of the acid effect through removal of an inhibitor: basic chromosomal protein. The acid enhancement of priming capacity in our preparations at either of the two temperatures was observed only after acid treatment and in the absence of any logical means for dissociating basic protein removal from DNA denaturation as causative elements.

... It may be worth noting that we have also found *E. coli* DNA polymerase-mediated thymidylate-^3H^ incorporation at 15°C into acid-fixed *Drosophila* polytene chromosomes (unpublished observation), indicating the presence, at least after acid treatment, of single-stranded DNA primer in that material (cf. Von Borstel et al., 1969).
incubation temperatures can be suppressed by treatment of the tissue with poly-L-lysine (Figs 4 a, b), this secondary inhibition can be removed again by acid treatment (Figs 4 c, d). Moreover, we have demonstrated that labeled polylysine in fact binds to chromatin under the experimental conditions used (Fig. 2 c). (We are assuming that the labeled polylysine does not differ significantly from the unlabeled form with respect to DNA binding, and that polylysine is similar in its reaction with DNA to normal basic chromosomal protein.)

Our conclusion that the expressed priming capacity of chromosomal DNA in fixed cytological preparations is strongly influenced by the presence of basic chromosomal protein, as had been suggested by Hnilica and Billen (1964), does not imply that DNA denaturation plays no role in the enhancement of polymerase-mediated deoxyribonucleoside triphosphate incorporation by acid treatment. It does say that the effect of basic proteins must be controlled before the data obtained from such experiments can be used to evaluate the state of the DNA per se. We cannot exclude the possibility that some enhancement is brought about through an acid effect on the permeability of the cells and nuclei to the precursors and enzymes, nor do our data permit an unequivocal

**Figure 4** Inhibition of *E. coli* DNA polymerase by poly-L-lysine *in situ*, using chicken blood cells chromatin as primer. (Treatments are listed in sequential order of application). (a) Alcohol fixed, acid treated, poly-L-lysine (type II) treated, and incubated for 80 min at 15°C with DNA polymerase, (b) same as a, but incubated at 37°C, (c) alcohol fixed, acid treated, poly-L-lysine (type II) treated, acid treated again, and incubated with DNA polymerase for 80 min at 15°C, (d) same as c, but incubated at 37°C.
answer to questions on the mechanism by which basic proteins associated or associable with DNA in fixed preparations exert their inhibitory effect on the primer-polymerase interaction. It seems reasonable to visualize the mechanism in terms of the inaccessibility of DNA in a DNA-protein complex to the enzyme, it is not inconceivable, however, that inhibition may come about by more subtle interactions of histone or polypeptide with the polymerase.

It is tempting, finally, to speculate on the observation that the chromatin of some cell types (thrombocytes, lymphocytes) in alcohol-fixed chicken blood smears primes extensive incorporation while that of others does not (Figs. 5 a-d) in terms of possible differences between the cell types in the nature of the DNA-protein complex. Differences between tissues in in situ priming capacity have been reported in previous studies (Modak and Bollum, 1970; Price et al., 1971). Moreover, it was shown recently that only 50% of the DNA in chromatin is covered with proteins (Clark and Felsenfeld, 1971; Itzhaki, 1971) and protected from DNase digestion (Clark and Felsenfeld, 1971), and that polypeptide bound to DNA provides protection from nuclease (Clark and Felsenfeld, 1971). One might predict that 50% masking of chromatin DNA by basic proteins, or, more correctly, the titratability of 50% of the DNA binding sites in chromatin with polypeptide repre-
sents a gross tissue average, and that further studies may show deviations from this figure in more specifically selected cell types.

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