Immunological Comparison of Purified DNA Polymerase α from Embryos of *Drosophila melanogaster* With Forms of the Enzyme Present in Vivo*

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Specific antisera to purified DNA polymerase α from embryos of *Drosophila melanogaster* and to two of the four constituent subunits (α, β, γ, and δ) were prepared. These antibodies have revealed the following features of the enzyme. (i) The $M_r = 148,000$ α subunit is very likely derived by *in vitro* proteolysis from polypeptides with molecular weights of 185,000 and 160,000 that are present *in vivo*. (ii) The $M_r = 60,000$ β subunit occurs in rapidly replicating embryos as both an 85,000- and a 60,000-dalton form, but predominantly as a 60,000-dalton form in more slowly replicating cultured cells. (iii) There is no detectable immunologic cross-reactivity between the four subunits. (iv) There is an abundance of antigenic material in embryos that co-migrates with the δ subunit of the purified enzyme during polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

DNA polymerase α from *Drosophila melanogaster* embryos is a large, asymmetric 280,000-dalton protein composed of four nonidentical subunits with molecular weights of 148,000 (α), 58,000 (β), 46,000 (γ), and 43,000 (δ) (1). Of the four subunits, only the isolated α subunit retains catalytic activity (2). It is likely that one or more of the other subunits acts to modify or enhance the activity of the α subunit (3).

We have prepared specific antibodies against the intact α polymerase and against individual subunits in order to gain a better understanding of the enzyme and its constituent subunits. In addition to the functional studies that such antibodies permit, they provide a useful tool to examine the extent to which proteolysis may have occurred during purification of the enzyme. As demonstrated by Brakel and Blumenthal (4, 5), proteolysis can be a significant factor in the generation of multiple forms of the enzyme. It is this latter problem that is the principal subject of this paper. We have, in fact, found that significant proteolysis of the α and β and possibly the other subunits has indeed occurred during purification of the enzyme.

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**The abbreviation used is: SDS, sodium dodecyl sulfate.**

trophoresis in the presence of SDS. The resulting polyacrylamide slice was homogenized and mixed with Freund’s adjuvant. Five booster injections in Freund’s incomplete adjuvant of 70, 30, 30, 20, and 20 μg of β subunit and 100, 20, 30, 70, 50, and 50 μg of α subunit were given at 3-week intervals. Bleedings were performed 10 days after the last injections.

Purified IgG was prepared by ammonium sulfate precipitation and DEAE-cellulose chromatography (11).

**Enzyme Neutralization Reactions**—Equal volumes of phosphate-buffered saline and DNA polymerase α (Fraction VII, 260 units/ml) in Buffer N (30% glycerol, 2 mg/ml of bovine serum albumin, 40 μg/ml of leupeptin, 20 mM potassium phosphate (pH 7.1), and 2 mM β-mercaptoethanol) were mixed with a total volume of 50 μl, and preincubated for 2 h at 20 °C with the indicated amount of either preimmune or immune IgG. Aliquots of this preincubation mixture were then assayed as described (1). In the absence of added IgG, the DNA polymerase retained 100% of its initial activity under these conditions.

**Gel Electrophoresis and Protein Transfers**—Polyacrylamide gel electrophoresis in the presence of SDS was performed as described by Laemmli (12). *Drosophila* embryo extracts were prepared from dechorionated 0–16 h embryos which had been stored at −80 °C by homogenization in the presence of 5% SDS, 10% β-mercaptoethanol, 125 mM Tris-HCl (pH 6.8), 20% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 mM sodium metabisulfite, 10 μg/ml of leupeptin, 10 μg/ml of pepstatin, and 10 mM EDTA and then heated at 100% for 3 min. Cultured cell extracts were prepared, similarly, from Schneider’s cell line 2 (obtained from Dr. A. Sugino, National Institute of Environmental Health Sciences) which had been grown to 2–4 × 10^6 cells/ml in Schneider’s complete medium plus 10% fetal calf serum at 25 °C.

Proteins in polyacrylamide gels were transferred to activated amionophenylthioether paper by either blotting (13) or electrophoretic transfer with a Hoeffer Transphor apparatus (14) in 15 mM sodium fluoride, 10 mM potassium phosphate (pH 7.6), 20% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 mM sodium metabisulfite, 10 μg/ml of leupeptin, 10 μg/ml of pepstatin, and 10 mM EDTA and then heated at 100% for 3 min. Activated amionophenylthioether paper was used in place of nitrocellulose to circumvent the problem of nonspecific binding of IgG to some proteins, observed occasionally in nitrocellulose transfers. The transfer efficiency of both high and low molecular weight proteins was routinely monitored with the use of ^14^C-labeled marker proteins and autoradiography of the dried polyacrylamide gel after transfer by either blotting or electrophoresis. Transfer by electrophoresis was more efficient than by blotting, especially for large polypeptides. Under the conditions used for electrophoretic transfer, polypeptides of less than 130,000 daltons transferred at nearly 100% efficiency. Myosin transferred at about 30% efficiency. Transfers were probed with purified IgG at a concentration of 20 μg/ml as described (13). They were reprobed after removing bound IgG and protein A by treatment with 2 μg guanidine thiocyanate, 0.2 M acetic acid, and 10 mM dithiothreitol for 20 min at room temperature.

**Radioimmune Assay**—The radioimmune assay of Crawford and Lane (15) was used to determine the total amount of antigenic material detectable with anti-DNA polymerase α IgG. One unit of antigen was defined as that amount of antigenic material present in Fraction III of α polymerase that corresponds to one DNA polymerase unit (1).

**RESULTS**

**Inhibition of DNA Polymerase α by Specific Antibodies**—Antiserum to the *Drosophila* DNA polymerase inhibited the enzyme by 85% (Fig. 1); higher concentrations did not result in further inhibition. Under conditions where the antibody produced maximal inhibition of the *Drosophila* α polymerase, no inhibition of KB cell DNA polymerase α or β, or *E. coli* DNA polymerase I or III was observed.

Antibody directed specifically against the α subunit inhibited the *Drosophila* enzyme, although not completely, even at high concentrations of IgG. In contrast, the β subunit-specific antisera produced no inhibition of DNA polymerase activity (Fig. 1). These results are in accord with the previous finding that the α subunit is itself sufficient for catalytic activity and that the β, γ, and δ subunits most likely act to modify the activity of the α subunit, perhaps by increasing the processivity or stability of the enzyme (3).

**Specificity of Anti-DNA Polymerase Antibodies**—The protein transfer technique described by Renart et al. (13) was used to determine which subunits of the α polymerase were recognized by the various antisera. Purified DNA polymerase was subjected to electrophoresis on polyacrylamide gels containing SDS, transferred to diazotized paper, probed with the antibody to be examined, incubated with ^125^I-labeled *S. aureus* protein A, and then autoradiographed.

The results of such an experiment using DNA polymerase α antibody is shown in Fig. 2. The major antigenic determinant recognized is the α subunit; the γ and δ subunits reacted to lesser extents. Upon very long exposures a faint band was seen at the β position. In addition, a number of other bands were...
recognized by the antibody which are most likely minor contaminants present in the α polymerase preparation.

The specificity of the antisera prepared against the isolated α and β subunits is shown in Fig. 3. The α-specific antibody recognized only the α subunit (Fig. 3A). Similarly, the β-specific antibody recognized only the β subunit (Fig. 3B). These results indicate that the α and β subunits are distinct from one another and from the γ and δ subunits. They are also consistent with the observation that the polypeptide maps generated by protease digestion of the four subunits are dissimilar (2).

Abundance of δ Subunit-associated Antigenic Material in Drosophila Embryos—Because high molecular weight proteins are transferred inefficiently to diazotized paper by the blotting technique of Renart et al. (13), the α polypeptide was initially indetectable in crude DNA polymerase fractions from Drosophila embryos using antibody directed against the intact polymerase (Fig. 4). However, a large quantity of an immunoreactive polypeptide appeared that had the same mobility on SDS-polyacrylamide gels as the δ subunit of the purified enzyme. After chromatography on DNA cellulose (1), the DNA polymerase fraction was free of excess δ subunit-associated antigen. No excess antigen was apparent in extracts of cultured Drosophila cells (data not shown).

As shown by the radioimmune assay, the excess of δ subunit-associated antigenic material is not retained on DNA cellulose (Table I) and was in this manner separated from the bulk of the DNA polymerase activity. There was no discrete protein band as judged by Coomassie blue staining that corresponded to the position of the δ subunit in this fraction (data not shown).

Detection of α and β Subunits in Drosophila Embryos and Cultured Cells in Vivo—Because the purified DNA polymerase α is the product of a substantial number of purification steps, it was important to determine whether modification of the enzyme occurred during this process. We, therefore, examined an extract prepared by directly homogenizing embryos in SDS. To ensure efficient transfer of high molecular weight polypeptides, the transfer was performed by electrophoresis of the proteins to diazotized paper. In addition, amounts of Fractions I through VII, equivalent to 20 units of DNA polymerase, were examined. As shown in Fig. 5, an extract prepared by homogenization of embryos in the presence of SDS (lane C) contained at least three high molecular weight polypeptides which reacted with antibody to the α subunit. The major species had an apparent molecular weight of 168,000, and the minor species had molecular weights of 185,000 and 158,000. Extracts of cultured Drosophila cells showed a similar pattern of polypeptides, although there was a greater abundance of the M, = 185,000 species (lanes A and B). Fraction I (lane D), a postmitochondrial supernatant obtained

![Fig. 3. Specificity of anti-α and β subunit sera. Eighty nanograms of Fraction VII were applied to an 8% polyacrylamide gel containing 0.1% SDS and 4 mM urea with diallyl tartarimide N,N'-diallyl tartarimide as the cross-linker, electrophoresed, treated with 2% periodic acid, and transferred electrophoretically to activated aminophenylthioether paper as described under "Methods." In this gel system the M, = 148,000 α polypeptide migrates anomalously at 135,000. The transfer was probed with either (A) anti-α subunit IgG, or (B) anti-β subunit IgG. MYO, myosin; ββ', subunits of E. coli RNA polymerase; βGAL, β-galactosidase; CAT, catalase; AP, E. coli alkaline phosphatase; LDH, lactate dehydrogenase.](image-url)

![Fig. 4. Separation of δ subunit-associated antigenic material during purification. Gel electrophoresis, protein transfer, and examination with the anti-DNA polymerase antibody probe were performed as described in Fig. 2. A, Fraction I (a postmitochondrial supernatant) from 0-16 h Drosophila embryos (36 units, 180 μg of protein); B, Fraction I after precipitation by and elution from Polymin P (62 units, 180 μg of protein); C, Fraction II, ammonium sulfate precipitate of the Polymin P eluate (146 units, 180 μg of protein); D, Fraction III, phosphocellulose (212 units, 80 μg of protein); E, Fraction IV, DNA cellulose (432 units, 20 μg of protein); F, Fraction V, hydroxylapatite (1260 units, 20 μg of protein); G, Fraction VI, glycerol gradient (3200 units, 20 μg of protein); H, Fraction VII, blue dextran-Sepharose (728 units, 4 μg of protein).](image-url)

| Table I | Separation of free δ subunit-associated antigenic material from DNA polymerase activity |
|---------|---------------------------------|
| Fraction or step | Volume | Protein | Antigen | DNA polymerase activity | Ratio of antigen to DNA polymerase activity |
| III. Phosphocellulose | 590 | 649 | 1770 | 1770 | 1.0 |
| IV. DNA-cellulose | 790 | 95 | 252 | 1600 | 0.16 |
| DNA-cellulose flow-through | 1180 | 189 | 1480 | <2.5 | >590 |

![Table I](image-url)
from embryo extracts, exhibited the same forms as seen in embryos homogenized in the presence of SDS. A number of additional bands of slightly lower molecular weight were also visible. As purification of the DNA polymerase proceeded, most of the higher molecular weight bands disappeared, and only a single major polypeptide remained in Fraction VII which corresponded to the α subunit.

Fig. 5II shows the proteins observed after probing with antibody to the β subunit. Embryos homogenized in SDS (lane C) and Fraction I (lane D) both showed two immunoreactive polypeptides: a major one at 85,000, another at about 60,000 daltons. The latter corresponds to the position of the β subunit in the purified enzyme. In addition, there was a faint band with a molecular weight of 105,000. Fraction II (lane E) showed considerably less of the M, = 85,000 species, although there was no loss of material at the 60,000-dalton position, and, indeed, there may have been some enhancement. The amount of β subunit-immunoreactive material associated with 20 units of enzyme appeared to remain constant throughout the purification, as would be expected for a subunit of the enzyme. In contrast to embryos, cultured Drosophila cells contained mainly the β subunit-immunoreactive polypeptide with a molecular weight of 60,000, although there was a small amount of the 85,000-dalton species.

It thus appears that the α and β subunits of purified DNA polymerase α derive from higher molecular weight forms that are present in vivo. To ensure that we were indeed examining forms found in vivo, extracts were also prepared by direct precipitation of cells with cold trichloroacetic acid followed by resuspension in SDS, and similar results were obtained (data not shown).

**DISCUSSION**

The use of antibodies specifically directed against the various subunits of the Drosophila DNA polymerase α demonstrates that the M, = 148,000 α subunit is the catalytic core of the enzyme, in accord with the previous observation that this polypeptide has catalytic activity after separation from the β, γ, and δ subunits (2). Furthermore, studies with these antibodies reaffirm the conclusion reached on the basis of peptide mapping of the isolated subunits that (i) the α and β subunits are unrelated to each other, and (ii) that the γ and δ subunits are most likely not derived from the α or β subunits by proteolytic cleavage (2).

We have also found that there is an abundance of antigenic material in Drosophila embryos that co-migrates with the δ subunit of the purified α polymerase on SDS-polyacrylamide gels. Although we have not yet demonstrated that this material is identical with the δ subunit, it is clear that it is not recognized by either α or β subunit-specific antibodies. The function of the δ subunit and the significance of its abundance in embryos remains to be determined.

Certainly the most striking result presented here is the observation that at least two of the four subunits of the purified DNA polymerase most likely derive from larger polypeptides present in vivo. In the case of the α subunit, it is clear that the 148,000-dalton polypeptide associated with the purified enzyme is generated from larger polypeptide(s) during the purification procedure. Most probably this modification is

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**Fig. 5. Fate of α and β subunits during purification.** Gel electrophoresis and electrophoretic protein transfer were performed as described in Fig. 3. The transfer was probed with either (I) anti-α subunit IgG or (II) with anti-β subunit IgG as described under “Methods.” A, 30 μg of SDS-homogenized cultured Drosophila cells (Schneider’s line 2); B, 150 μg of SDS-homogenized cultured Drosophila cells; C, 100 μg of SDS-homogenized 0-16 h Drosophila embryos; D, Fraction I (18 units, 100 μg of protein); E, Fraction II (18 units, 27 μg of protein); F, Fraction III (18 units, 9 μg of protein); G, Fraction IV (18 units, 1.8 μg of protein); H, Fraction V (24 units, 0.42 μg of protein); I, Fraction VI (21 units, 0.12 μg of protein); J, Fraction VII (19 units, 0.07 μg of protein). MYO, myosin; ββ', subunits of E. coli RNA polymerase; βGAL, β-galactosidase; AP, E. coli alkaline phosphatase.
the result of proteolysis \textit{in vivo}. However, we do not know whether the \( M_r = 166,000 \) or the \( M_r = 185,000 \) polypeptide, or both, are the functional moieties \textit{in vivo}. Although the 60,000-dalton \( \beta \) subunit of the purified enzyme most likely derives from an 85,000-dalton precursor, the possibility cannot be excluded that the latter species is an immunologically cross-reacting polypeptide unrelated to the \( \alpha \) polymerase. It is interesting to note, however, that it is much more abundant in rapidly dividing embryonic cells than in the more slowly replicating cultured cells.

It is clear from this and from previous studies (4, 5, 16) that DNA polymerase \( \alpha \) is extraordinarily susceptible to proteolysis and possibly other modifications \textit{in vitro} and that many of the proteolytic fragments retain catalytic activity, at least as judged by their ability to replicate activated DNA. It is highly likely, however, that such proteolysis influences the activity or function of the enzyme. In particular, association of the \( \alpha \) and \( \beta \) subunits with accessory proteins may be altered. As an example, the ability of the \textit{E. coli} dnaB protein to associate with dnaC protein is abolished by brief trypsin digestion of dnaB protein, although there is no effect on its ATPase activity.\(^2\) Similarly, trypsin treatment of phage T4 gene 32 protein abolishes cooperative binding of the protein to single-stranded DNA, although binding itself is unaffected (17). Purification and characterization of the form(s) of DNA polymerase \( \alpha \) that exists \textit{in vivo} are, therefore, essential prerequisites to the analysis of \textit{Drosophila} chromosomal DNA replication \textit{in vitro}.

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\textbf{REFERENCES}

1. Banks, G. R., Boezi, J. A., and Lehman, I. R. (1979) \textit{J. Biol. Chem.} 254, 9686-9692
2. Villani, G., Sauer, B., and Lehman, I. R. (1980) \textit{J. Biol. Chem.} 255, 9479-9483
3. Villani, G., Fay, P. J., Bambara, R. A., and Lehman, I. R. (1981) \textit{J. Biol. Chem.} 256, 8202-8207
4. Brakel, C. L., and Blumenthal, A. B. (1977) \textit{Biochemistry} 16, 3137-3143
5. Brakel, C. L., and Blumenthal, A. B. (1978) \textit{Eur. J. Biochem.} 88, 351-362
6. Burgess, R. R., and Jendrisak, J. J. (1975) \textit{Biochemistry} 14, 4634-4638
7. Aposhian, H. V., and Kornberg, A. (1962) \textit{J. Biol. Chem.} 237, 519-525
8. Seed, B. (1982) \textit{Nucleic Acids Res.} 10, 1799-1810
9. Fraker, P. J., and Speck, J. C., Jr. (1978) \textit{Biochem. Biophys. Res. Commun.} 86, 849-857
10. Markwell, M. A. K., and Fox, C. F. (1978) \textit{Biochemistry} 17, 4807-4917
11. Levy, H. B., and Sober, H. A. (1960) \textit{Proc. Soc. Exp. Biol. Med.} 103, 250-259
12. Laemmli, U. K. (1970) \textit{Nature (Lond.)} 227, 680-685
13. Renart, J., Reiser, J., and Stark, G. R. (1979) \textit{Proc. Natl. Acad. Sci. U. S. A.} 76, 3116-3120
14. Reiser, J., and Stark, G. R. (1982) \textit{Methods Enzymol.}, in press
15. Crawford, L. V., and Lane, D. P. (1977) \textit{Biochem. Biophys. Res. Commun.} 74, 323-329
16. Albert, W., Grummt, F., Hubscher, U., and Wilson, S. H. (1982) \textit{Nucleic Acids Res.} 10, 935-946
17. Lonberg, N., Kowalczykowski, S. C., Paul, L. S., and von Hippel, P. H. (1981) \textit{J. Mol. Biol.} 145, 123-138

\(^2\) K. Arai and A. Kornberg, personal communication.