Comparison of DNA Polymerases α, δ, and ε of Mouse Cell Line FM3A and Its Temperature-Sensitive Mutant tsFT20*

HIRONOBU IKEHATA†

Cellular Physiology Laboratory, The Institute of Physical and Chemical Research (RIKEN), Wako, Saitama 351-01

IKEHATA, H. Comparison of DNA Polymerases α, δ, and ε of Mouse Cell Line FM3A and Its Temperature-Sensitive Mutant tsFT20. Tohoku J. Exp. Med., 1994, 172 (1), 65-81 DNA polymerises (pol) α, δ and ε of a mouse cell line FM3A and its temperature-sensitive derivative tsFT20, which is defective in DNA replication at a non-permissive temperature, were purified by chromatographic procedures monitored by a set of relatively specific assays for the respective DNA polymerase activities. The pol ε activity was separated into two fractions with similar enzymatic properties except for their optimal KCl concentrations and processivities. The fractions of pol δ and ε were not homogeneous, but their identities were confirmed by their sensitivities to DNA polymerase inhibitors, their associated 3'→5' exonuclease activities, optimal concentrations of salts, dependencies on the proliferating cell nuclear antigen and processivities in polymerization, which also excluded significant contamination with other DNA polymerases. Of the DNA polymerases prepared from tsFT20 cells, only pol α showed greatly decreased activity and remarkable sensitivity to the non-permissive temperature, demonstrating that pol δ and ε, the other polymerases supposed to be involved in nuclear DNA replication, are unequivocally different entities from pol α. The level of pol ε activity tsFT20 was also significantly lower than in the parental cells, suggesting cooperation and/or interaction between pol α and ε, and some relevance of pol ε to DNA replication. ——— DNA polymerase; DNA replication; temperature-sensitive mutant; mammalian cells; protein purification

Of the five species of mammalian DNA polymerases (pol) so far known, at least three, pol α, δ and ε are thought to be involved in nuclear DNA replication (Wang 1991; Araki et al. 1992). The role of pol α in DNA replication is indicated in cytological studies with monoclonal antibodies against pol α (Miller et al. 1985; Kaczmerek et al. 1986), and with a mouse temperature-sensitive cell line tsFT20 (Murakami et al. 1985), which has heat-labile pol α (Takada-Takayama et al. 1991), and in a biochemical study with an in vitro model system for eukaryotic

Received October 1, 1993; revision accepted for publication December 22, 1993.
*This work was supported by grants from the Ministry of Education, Science and Culture of Japan and by a Special Researchers' Basic Science Program Grant from RIKEN and the Ministry of Science and Technology of Japan.
†Present address: Department of Radiation Research, Tohoku University School of Medicine 2–1 Seiryo-machi, Aoba-ku, Sendai 980, Japan.
DNA replication with simian virus 40 (SV40) origin and its large T antigen (Murakami et al. 1986a). Since pol α has tightly bound primase subunits (Hirose et al. 1988) and tsFT20 cells are deficient in initiation of replication (Eki et al. 1986), it has been proposed that pol α works as a lagging strand DNA polymerase at a replication fork as well as an initiating enzyme of DNA replication at the origin. This idea is supported by findings in the in vitro SV40 DNA replication system (Prelich and Stillman 1988). The contribution of pol δ to DNA replication has been suggested by the dependence of its DNA synthesis activity and processivity on the proliferating cell nuclear antigen (PCNA) (Tan et al. 1986; Prelich et al. 1987a), which is expressed at an increased level in cells stimulated to proliferate (Celis et al. 1987). In addition, pol δ was found to be necessary as a leading strand DNA polymerase in the in vitro SV40 DNA replication system (Prelich and Stillman 1988; Lee et al. 1989; Weinberg and Kelly 1989), where PCNA is also required as one of the accessory proteins of pol δ (Prelich et al. 1987b). The much higher processivity of pol δ in the presence of PCNA than that of pol α supports the idea that pol δ synthesizes the leading strand at the replication fork. Another highly processive enzyme of mammalian origin has been identified as pol ε (Burgers et al. 1990), which shows little or no dependence on PCNA (Syväsuoja and Linn 1989). Although there is as yet no direct evidence that pol ε is relevant to DNA replication in mammals, in Saccharomyces cerevisiae, DNA polymerase II, which is the yeast homolog of pol ε, was shown to be essential for cell growth and chromosomal DNA replication (Morrison et al. 1990; Araki et al. 1992). Recently, in human, the genetic independence of these three DNA polymerases that are likely to be involved in nuclear DNA replication has been established by base sequencing of genes for the catalytic subunits of human DNA polymerase α, δ and ε (Wong et al. 1988; Chung et al. 1991; Kesti et al. 1993).

In this study, I isolated pol α, δ and ε separately from mouse tsFT20 cells and from the parental FM3A clone 28, examined their biochemical properties, and compared the temperature sensitivities of each polymerase from the two cell lines. Results obtained here provided a biochemical support of the genetic independence of pol α from pol δ and ε in mouse, which was consistent with the genetic studies of the human enzymes. The results also suggested cooperation or interaction between pol α and ε.

**Materials and Methods**

**Materials**

[^3H]dTTP was purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO, USA). [32P]dTTP was from Amersham (Little Chalfont, UK); poly(dA), oligo(dT), and poly(dA-dT) were from Pharmacia LKB (Uppsala, Sweden) except for poly(dA)4000, which were obtained from Life Sciences Inc. (St. Peterburg, FL, USA). Calf thymus DNA and deoxyribo-nuclease I were from Sigma (St. Louis, MO, USA). Anti-DNA polymerase α monoclonal antibodies SJK132-20 and SJK287-38 were prepared as described previously (Takada-Takayama et al. 1990). Mouse PCNA was kindly supplied by Dr. Seki (University
Mouse DNA Polymerases α, δ and ε in ts Mutant

of Tokyo, Tokyo). Rat DNA polymerase β (pol β) expressed in Escherichia coli was a gift from Dr. Matsukage (Aichi Cancer Center Research Institute, Nagoya).

Cells

Large scale suspension culture of tsFT20 (Murakami et al. 1985) and its parental cell line FM3A clone 28 (JCRB0701; Japanese Cancer Research Resource Bank), originally established from a spontaneous mammary carcinoma in a C3H/He mouse (Nakano 1966), were incubated at 33°C in spinner flasks (Bellco Biochemistry, Vineland, NJ, USA) containing RPMI1640 (Nissui, Tokyo) supplemented with 5% bovine calf serum (HyClone Laboratories, Logan, UT, USA).

DNA polymerase and 3'-5' exonuclease assays

DNA polymerase activities were assayed in a total volume of 30 μl containing 2 mM 2-mercaptoethanol, 2% (v/v) glycerol, 200 μg/ml bovine serum albumin (crystallized, Sekagaku Kogyo, Tokyo), and 20 μM [3H]dTTP (0.5 Ci/mmol). In addition, the mixture for assay of pol α activity included 20 mM Tris-HCl, pH 8.0, 5 mM MgCl₂, 500 μg/ml activated calf thymus DNA (Aposhian and Kornberg 1962), and 100 μM each of dATP, dGTP and dCTP, but without glycerol; the pol δ assay mixture contained 50 mM Hepes-KOH, pH 6.5, 3 mM MgCl₂, 15 μg/ml activated poly(dA-dT) prepared as described by Goulian et al. (1990), and 100 μM dATP; and the pol ε assay mixture contained 50 mM Hepes-KOH, pH 7.0, 20 mM MgCl₂ and 15 μg/ml poly(dA) • oligo(dT)₁₀ (20:1, w/w ratio). The mixture for assay of PCNA-dependent DNA polymerase activity was the same as that for assay of pol ε except that pH of the buffer was 6.5 and 2 mM ATP and 3.6 μg/ml mouse PCNA were added. Materials labeled with [³²H]dTTP during 30-min incubation at 33°C were trapped on DE81 paper (Whatman, Springfield Mill, UK) and the incorporated radioactivity was measured as described previously (Hanaoka et al. 1981). One unit of activity was defined as the amount that catalyzed the incorporation of 1 nmol of total deoxynucleotides in 1 hr at 33°C.

The assay of 3'→5' exonuclease activity was carried out at 33°C for 30 min in 20 μl of solution containing 50 mM Hepes-KOH, pH 7.0, 5 mM MgCl₂ and 1 μg/ml activated (3'-³²H)dT)-poly(dA-dT) (total: 30,000 cpm). Remaining radioactivity in the substrate was determined as described above. Activated (3'-³²H)dT)poly(dA-dT) was prepared as described by Goulian et al. (1990).

Thermalolability of DNA polymerases

DNA polymerase fractions were incubated at 39°C in the respective assay mixtures for the polymerases but without template DNA and deoxyribonucleoside triphosphates. Roughly equal amounts of polymerase activity of the fractions of each DNA polymerase from FM3A and tsFT20 cells were added to the incubation mixtures. After incubation for various times, the polymerization reaction was started at 33°C by addition of the substrates. The conditions of the reaction and the measurement of activity were as described above.

Isolation of DNA polymerases

All isolation steps were performed at 4°C. A frozen stock (−80°C) of 9.5×10⁹ cells of FM3A clone 28 or 9.7×10⁹ cells of tsFT20 was thawed, suspended in 20 mM potassium phosphate, pH 7.5, 2 mM MgCl₂, 2 mM 2-mercaptoethanol, 2 mM ethyleneglycol-bis(β-aminoethyl ether) N, N', N', N'-tetraacetic acid, 1% (v/v) ethanol, 25 mM potassium acetate, 10% (v/v) glycerol, 5 mM benzamidine-HCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 mM N-α-tosyl-L-lysine chloro-methyl ketone, 1 μg/ml leupeptin, 1 μg/ml pepstatin A and 20 μg/ml soybean trypsin inhibitor, and homogenized in a Kontes tissue grind tube. The homogenate was extracted for an hour after addition of KCl to a final concentration of 0.2 M, cleared by centrifugation at 150,000×g for 60 min, dialyzed against 50 mM Tris-HCl, pH 8.0 (4°C), 1 mM EDTA, 10% (v/v) glycerol, 2 mM 2-mercaptoethanol, 0.01% (v/v)
Nonidet P-40, 5 mM benzamidine-HCl and 1 mM PMSF (T buffer) supplemented with 20 mM KCl, and then centrifuged at 2,000 \( \times g \) for 10 min to clear the supernatant (the crude extract fraction).

The crude extract fraction was applied to a 20-ml bed column of DEAE-Toyopearl 650M (Tosoh, Tokyo) equilibrated with T buffer containing 20 mM KCl. The column was washed with the same buffer and developed with a gradually increasing concentration gradient of KCl (Fig. 1). The resulting fractions were assayed for pol \( \delta \) and \( \varepsilon \) activities and fractions included in the first peak (80 mM KCl) with pol \( \delta \) activity were pooled (the DEAE fraction of pol \( \delta \)). Fractions in the second peak with pol \( \delta \) activity which was also associated with pol \( \varepsilon \) activity (170 mM KCl; the pol \( \delta \) activity in this peak was derived from pol \( \alpha \)) were also pooled and dialyzed against a similar solution to T buffer except that the buffer used was 50 mM Heps-KOH, pH 8.0 at 4°C (H buffer), and was supplemented with 20 mM KCl (the DEAE fraction of pol \( \alpha \) and \( \varepsilon \)).

The DEAE fraction of pol \( \delta \) was loaded on a column of phosphocellulose P11 (Whatman) of 5 ml bed volume, which had been equilibrated with H buffer containing 100 mM KCl. The column was washed with the same buffer, and then the pol \( \delta \) activity was eluted with an increasing gradient of KCl concentration, pooled and dialyzed against T buffer with 20 mM KCl (the phosphocellulose fraction of pol \( \delta \)). The DEAE fraction of pol \( \alpha \) and \( \varepsilon \) was also applied to a phosphocellulose column (6-ml bed volume) equilibrated with buffer H plus 20 mM KCl and fractionated in the same way. The peak fractions with pol \( \alpha \) and \( \varepsilon \) activities were pooled (the phosphocellulose fraction of pol \( \alpha \) and \( \varepsilon \)).

Fig. 1. Profiles on DEAE-Toyopearl chromatography. Crude extracts from FM3A (A) or tsFT20 (B) cells were applied to a DEAE-Toyopearl column. DNA polymerase activities were determined by standard assays for pol \( \delta \) (closed circle) and pol \( \varepsilon \) (open circle) as described in "Materials and Methods". The peak activity around the fraction 110 detected by pol \( \delta \) assay was due to pol \( \alpha \).
Mouse DNA Polymerases α, δ and ε in ts Mutant

The phosphocellulose fraction with pol α and δ activities was passed slowly through a 1-ml bed column of SJK287-Sepharose, which had been equilibrated with H buffer supplemented with 300 mM KCl. The flow-through fractions were pooled and dialyzed against T buffer plus 20 mM KCl (the SJK287 fraction of pol ε). The column was washed with H buffer containing 1 M KCl and developed as described previously (Takada-Takayama et al. 1990). The peak fractions with pol α activity were pooled, dialyzed against T buffer supplemented with 100 mM KCl and 50% (v/v) glycerol, and stored at -20°C (FM3A) or at -80°C (tsFT20). SJK287-Sepharose was prepared as described previously (Takada-Takayama et al. 1990).

The phosphocellulose fraction of pol δ or the SJK287 fraction of pol ε was loaded on a FPLC column of Mono Q HR5/5 (Pharmacia LKB, Uppsala, Sweden) equilibrated with T buffer containing 20 mM KCl and fractionated with a KCl gradient up to 500 mM. The peak fractions with pol δ or ε activity obtained were pooled, dialyzed against T buffer containing 100 mM KCl and 50% glycerol and stored (-80°C for pol δ and -20°C for pol ε). In the case of pol ε, the activity was eluted as two peaks (with 180 and 270 mM KCl), which were collected separately and named pol ε-I and pol ε-II in order of their elution (Fig. 2). The purification of DNA polymerases from the two cell lines are summarized in Table 1.

**Processivity of DNA polymerases**

The mixture for the polymerizing reaction was essentially the same as that for assay of a PCNA-dependent DNA polymerase, except that 3 mM MgCl₂, 100 μM [³²P]dTTP (1 Ci/mmole) and poly(dA)₁₄₀₀ • oligo(dT)₁₂₋₁₈ prepared with the ratio of one primer at about a 600-base interval on the template were used, and that for pol ε's, the pH was changed to 7.0. The amounts of DNA polymerases used for the reaction were 0.064, 0.017, 0.011 and 0.023 units for pol α, δ, ε-I and ε-II respectively, where the substrate DNA was present in excess with limited amount of enzymes. The reaction was carried out at 37°C for 5 min and then stopped quickly by cooling on ice and addition of EDTA. One-fifth of each reaction
mixtures were used for determination of dTTP incorporation, which was performed using a Whatman GF/C glass filter. The remaining materials were extracted with phenol/chloroform and concentrated with ethanol. The resulting precipitates were washed with 70% (v/v) ethanol, dried and dissolved in alkaline loading buffer consisting of 50 mM NaOH, 1 mM EDTA, 5% (v/v) glycerol and 0.025% (w/v) bromocresol green. The total volumes of each solution were subjected to electrophoresis in 1.5% alkaline agarose gel as described by Maniatis et al. (1982).

RESULTS

Isolation of DNA polymerases

The activities of pol α, δ and ε were isolated simultaneously from mouse FM3A clone 28 and tsFT20 cells as summarized in Table 1. Pol α was purified by SJK287-Sepharose affinity chromatography to almost homogeneity judging from its sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) profile, where four subunits of the tight complex of pol α and DNA primase were detected (data not shown). The total amount and specific activity of the purified pol α were about 4.5-fold and 4-fold less, respectively, in tsFT20 cells than in the parental cells (Table 1), indicating that both the intracellular amount and the specific enzyme activity of pol α are decreased in tsFT20 cells. On the other hand, only much smaller difference between the activities of pol δ in FM3A and tsFT20 cells was detected (Table 1), although this enzyme was not completely purified. Pol E was also not purified to homogeneity, but the activity eluted from a Mono Q column was separated in two peaks, which were provisionally named pol ε-I and pol ε-II, respectively, in order of their elution (Fig. 2). The total pol ε-I activity was about one-tenth of that of pol ε-II in both cell lines. The total and specific activities of the pol ε-I and II fractions from tsFT20 were less than half of those of these fractions from FM3A (Table 1), and this decrease in pol ε activities in tsFT20 cells was observed reproducibly. The reductions in the polymerase activities of pol α and ε in tsFT20 cells were evident from their profiles on DEAE-Toyopearl chromatography compared with those of the fractions from wild-type FM3A cells (Fig. 1).

Identification of DNA polymerases

I confirmed the identities of the isolated activities of DNA polymerases in several ways, such as by their PCNA dependencies (Table 2), associated 3′→5′ exonuclease activities (data not shown), processivities on primed homopolymer (Fig. 3) and optimal concentrations of salts (Fig. 4), using mainly the fractions from FM3A cells. The preparations from tsFT20 cells gave essentially the same results (data not shown). The identity of the pol α fraction, which was clearly demonstrated by the molecular weight and the composition of its subunits separated by SDS-PAGE (data not shown since identical results have been given in Takada-Takayama et al. 1990), was confirmed by its absence of 3′→5′ exonuclease activity (data not shown) and of PCNA dependence (Table 2 and Fig. 3A), and
Mouse DNA Polymerases α, δ and ε in ts Mutant

Table 1. Purification of mouse replicative DNA polymerases

<table>
<thead>
<tr>
<th>Cell</th>
<th>Polymerase</th>
<th>Fraction</th>
<th>Protein (mg)</th>
<th>Total activity (units)a</th>
<th>Specific activity (units/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FM3A</td>
<td>α</td>
<td>Crude extract</td>
<td>239</td>
<td>4,035</td>
<td>16.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DEAE</td>
<td>70.8</td>
<td>2,934</td>
<td>41.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Phosphocellulose</td>
<td>9.11</td>
<td>3,469</td>
<td>381.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SJK287</td>
<td>0.054</td>
<td>1,705</td>
<td>31,574</td>
</tr>
<tr>
<td></td>
<td>δ</td>
<td>Crude extract</td>
<td>239</td>
<td>269.4</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DEAE</td>
<td>29.9</td>
<td>188.8</td>
<td>6.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>phosphocellulose</td>
<td>3.74</td>
<td>409.9</td>
<td>109.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MonoQ</td>
<td>2.19</td>
<td>180.0</td>
<td>82.2</td>
</tr>
<tr>
<td></td>
<td>ε</td>
<td>Crude extract</td>
<td>239</td>
<td>90.0</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DEAE</td>
<td>70.8</td>
<td>142.6</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>phosphocellulose</td>
<td>9.11</td>
<td>401.2</td>
<td>44.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SJK287</td>
<td>8.66</td>
<td>436.2</td>
<td>50.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MonoQ</td>
<td>1.22</td>
<td>36.0</td>
<td>29.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>I</td>
<td>3.46</td>
<td>350.9</td>
<td>101.4</td>
</tr>
<tr>
<td>tsFT20</td>
<td>α</td>
<td>Crude extract</td>
<td>430</td>
<td>1,395</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DEAE</td>
<td>113</td>
<td>173.5</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Phosphocellulose</td>
<td>10.3</td>
<td>223.1</td>
<td>21.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SJK287</td>
<td>0.012</td>
<td>94.3</td>
<td>7,858</td>
</tr>
<tr>
<td></td>
<td>δ</td>
<td>Crude extract</td>
<td>430</td>
<td>121.7</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DEAE</td>
<td>51.1</td>
<td>136.2</td>
<td>2.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Phosphocellulose</td>
<td>4.29</td>
<td>456.1</td>
<td>106.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MonoQ</td>
<td>1.90</td>
<td>232.5</td>
<td>122.4</td>
</tr>
<tr>
<td></td>
<td>ε</td>
<td>Crude extract</td>
<td>430</td>
<td>15.7</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DEAE</td>
<td>113</td>
<td>22.2</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Phosphocellulose</td>
<td>10.3</td>
<td>169.1</td>
<td>16.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SJK287</td>
<td>9.30</td>
<td>115.1</td>
<td>12.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MonoQ</td>
<td>I</td>
<td>1.14</td>
<td>14.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>II</td>
<td>2.98</td>
<td>134.4</td>
<td>45.1</td>
</tr>
</tbody>
</table>

*aOne unit corresponds to the incorporation of 1 nmol dNTP per hour at 33°C.*

Interestingly, pol α of tsFT20 showed slightly lower activity for processive polymerization than pol α from the parental cells (Fig. 3B). The fraction of pol δ, which was isolated as activity on activated poly (dA-dT), was confirmed by demonstrating nearly five-fold stimulation of its polymerase activity (Table 2) and marked increase in its processivity (Fig. 3A) in the presence of PCNA. Release of more than 90% of the 3'-labeled nucleotide from substrate DNA by the pol δ fraction
was also observed in the 3′→5′ exonuclease assay (data not shown), although contamination of the preparation with unrelated nucleases could not be excluded. The two fractions isolated here by assay for pol ε activity were identified as those of pol ε by their highly processive polymerization activity without PCNA (Fig. 3A), but the definition of pol ε has not yet been established. PCNA rather inhibited the DNA polymerase activities of both pol ε fractions, as shown by assay of PCNA-dependent DNA polymerase activity (Table 2), but had little effect on their processivities (Fig. 3A). It should be noted that the processivity of the pol ε-II fraction (500–1,500 bases) was higher than that of the pol ε-I fraction (200–900 bases). Activity of 3′→5′ exonuclease was also detected (near-

TABLE 2. PCNA dependence of DNA polymerase activity

<table>
<thead>
<tr>
<th>Polymerase</th>
<th>PCNA</th>
<th>Polynucleotide activity (units)</th>
<th>Ratio of PCNA enhancement</th>
</tr>
</thead>
<tbody>
<tr>
<td>α</td>
<td>−</td>
<td>0.496</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+b</td>
<td>0.538</td>
<td>1.08</td>
</tr>
<tr>
<td>δ</td>
<td>+b</td>
<td>0.215</td>
<td>4.94</td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>0.164</td>
<td></td>
</tr>
<tr>
<td>ε-I</td>
<td>+b</td>
<td>0.084</td>
<td>0.51</td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>0.299</td>
<td></td>
</tr>
<tr>
<td>ε-II</td>
<td>+b</td>
<td>0.185</td>
<td>0.62</td>
</tr>
</tbody>
</table>

*aPreparations from FM3A clone 28 cells.

*bPCNA purified from FM3A clone 28 cells (3.6 μg/ml).

Fig. 3. Processivities of mouse DNA polymerases. The conditions for assay of processivity were as described in "Materials and Methods" except for the modifications mentioned below. A, Effect of PCNA on the processivities of FM3A DNA polymerases. The amounts of nucleotides incorporated with pol α, δ, ε-I and ε-II, respectively, were 0.42, 0.21, 0.79 and 1.0 pmol in the absence of PCNA, and 0.67, 1.4, 0.92 and 1.0 pmol in the presence of PCNA. The absence and presence of PCNA in the reaction mixture are indicated by − and + above the lanes. B, Processivities of pol α from tsFT20 (lane T) and FM3A (lane F) cells. The reactions were carried out at 33°C without PCNA. The amounts of nucleotides incorporated by pol α's from tsFT20 and FM3A were 0.50 and 0.54 pmol, respectively. C, Effects of high salt concentrations on the processivities of pol ε-I and ε-II from FM3A cells. The reactions were performed without PCNA and with different concentrations of Mg2+ or K+. Mg and K above the lanes indicate reactions in mixture containing 20 mM MgCl2 and 83.4 mM KCl, respectively. Lane C shows results in standard conditions (3 mM MgCl2 and 16.7 mM KCl). The amounts of nucleotides incorporated in the reactions for lane C, Mg or K were 1.3, 0.45 or 1.3 pmol with pol ε-I, and 1.7, 2.0 or 1.7 pmol with pol ε-II, respectively. In each experiment, 5′-[32P] primer poly (dT)12-18 labeled with T4 polynucleotide kinase was also run in parallel (lane P). The sizes of 5′-[32P] labeled DNA markers are indicated in bases on the left of figures.
Mouse DNA Polymerases $\alpha$, $\delta$ and $\varepsilon$ in $ts$ Mutant

Fig. 3.
ly 80% release of 3’-base from the substrate, data not shown) in both pol ε fractions, although the possible contribution of contaminating nucleases could not be excluded. Another remarkable character common to the pol ε activities separated here was their high optimal concentration of magnesium ion of 20–25 mM (Fig. 4A), consistent with that of human pol ε (Syväsuoja and Linn 1989), although this concentration rather inhibited the processive polymerization (Fig. 3C). Moreover, an interesting difference between the two species of pol ε noted in this study was that the activity of pol ε-I was stimulated maximally by about 90 mM KCl, whereas pol ε-II activity was not affected by up to this concentration of KCl (Fig. 4B). Correspondingly, KCl was found to inhibit processive polymerization with pol ε-I at a concentration at which pol ε-II still maintained high processivity (Fig. 3C).

Inhibition experiments were carried out to exclude the possibilities of contamination of the preparations with other polymerizing activities such as those of pol β and γ, and cross-costaminations of pol α, δ and ε fractions. An anti-pol α monoclonal antibody SJK132-20 inhibited the mouse pol α purified here not
Mouse DNA Polymerases α, δ and ε in ts Mutant

Table 3. Effects of inhibitors on DNA polymerase activities

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Assay</th>
<th>Remaining polymerase activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>α</td>
</tr>
<tr>
<td>SJK132-20</td>
<td>56 μg/ml</td>
<td>α</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PCNA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ε</td>
</tr>
<tr>
<td>Aphidicolin</td>
<td>100 μM</td>
<td>α</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PCNA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ε</td>
</tr>
<tr>
<td>NEM</td>
<td>5 mM</td>
<td>α</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PCNA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ε</td>
</tr>
<tr>
<td>ddTTP</td>
<td>200 μM</td>
<td>other</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PCNA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ε</td>
</tr>
</tbody>
</table>

αRat DNA polymerase β provided by Dr. A. Matsukage (Aichi Cancer Center Research Institute).

Not tested.

Assay of PCNA-dependent DNA polymerase activity.

The dCTP concentration was 20 μM.

Reaction conditions were as for assay for DNA polymerase ε activity but with 5 mM MgCl₂.

only in the assay of pol α activity but also in those of pol ε and PCNA-dependent polymerase. On the other hand, the fractions of pol δ and ε were resistant to the antibody in the optimal conditions for their assays (Table 3), indicating little, if any, contamination of the pol δ or ε fractions with pol α. All the polymerase fractions isolated showed significant sensitivities to aphidicolin at a concentration that did not inhibit rat pol β (Table 3). This result confirmed reports of others (reviewed in Wang 1991) and excluded at least the possible misidentification of the activities with those of pol β and γ, which are insensitive to aphidicolin. N-ethylmaleimide (NEM) reduced the polymerase activities of the pol α and δ fractions as reported by others (Goulian et al. 1990; Wang 1991) and also strongly inhibited the activities of both pol ε-I and ε-II. Thus these polymerase preparations were devoid of pol β, which is known to be resistant to NEM (Wang 1991; Table 3). Moreover, none of my DNA polymerase fractions showed appreciable sensitivity to dideoxy TTP (ddTTP), whereas rat pol β was strongly inhibited by the same concentration of the drug (Table 3). This insensitivity not only confirmed negligible contamination by pol β, but also indicated little contamination with pol γ, which is very sensitive to ddTTP (Insdorf and Bogenhagen 1989; Gray and Wong 1992). Thus I concluded that at least the major parts of the polymerase activities detected in the fractions isolated here were due to the
respective DNA polymerases identified in these fractions.

Comparison of temperature sensitivities of DNA polymerases in FM3A and tsFT20 cells

I examined the stabilities at 39°C of the DNA polymerase activities of each fraction isolated from FM3A and tsFT20 cells. As reported previously (Takada-Takayama et al. 1991), pol α from tsFT20 lost its polymerizing activity much more rapidly than pol α from the parental cells (Fig. 5A). On the other hand, no significant differences were detected in the heat stabilities of the pol activities of δ, ε-I and ε-II in the fractions from the two cell lines (Fig. 5B, C, D). Thus the temperature sensitivity of DNA replication in tsFT20 was not due to that of pol δ or pol ε. As the possible contributions of pol β and γ to the heat sensitivity have also been excluded (Murakami et al. 1986b) and pol α was the only polymerase so far identified in mammalian cells that showed heat-unstable activity in tsFT20, I concluded that the temperature-sensitive properties of this cell line could be attributed to putative mutation of its pol α to a heat-labile form. In other words, the genetic independence in mouse of pol δ and ε from pol α were demonstrated by the similar stabilities at 39°C of the pol δ, ε-I and ε-II activities in tsFT20 and FM3A cells. It is noteworthy that the pol δ activity was

Fig. 5. Temperature sensitivities of mouse DNA polymerases. Fractions with pol α (A), pol δ (B), pol ε-I (C) or pol ε-II (D) activity from FM3A (closed circles) and tsFT20 (open circles) cells were preincubated at 39°C for various times as indicated and then assayed for polymerase activity under standard conditions except that pol δ was examined in the conditions for PCNA-dependent DNA polymerase. Details of reaction conditions are described in "Materials and Methods." DNA polymerase activities are shown relative to the activity in each polymerase fraction with no preincubation at 39°C.
significantly more heat-stable than those of the other polymerases (Fig. 5).

**DISCUSSION**

Pol $\alpha$, $\delta$ and $\varepsilon$ were isolated simultaneously from mouse cells by a set of DNA polymerase assays that were relatively specific for each enzyme. However, since the pol $\delta$ and $\varepsilon$ fractions were not homogeneous, some contaminating polymerases may have contributed to their activities. The fraction isolated as pol $\delta$, monitored as polymerase activity on activated copolymer DNA, proved to contain a polymerase activity stimulated both in amount and in processivity by PCNA, specific characteristics of eukaryotic pol $\delta$ (Wang 1991). The pol $\varepsilon$ fractions, whose isolation was monitored as polymerizing activity in the presence of a high concentration of magnesium ion, were demonstrated to synthesize a very long stretch of poly-deoxynucleotides in a PCNA-independent manner even under highly diluted conditions, which is a characteristic distinguishing eukaryotic pol $\varepsilon$ from other DNA polymerases (Syväjoja et al. 1990). Moreover, 3$'$→5$'$ exonuclease activity, which is associated with authentic pol $\delta$ and $\varepsilon$ (Wang 1991), was also detected in both the pol $\delta$ and pol $\varepsilon$ fractions isolated here, although possible contamination with unrelated nucleases was not excluded. In addition, the profiles of inhibitor sensitivities of these fractions were completely consistent with observations by others (reviewed in Wang 1991). Pol $\gamma$ also has highly processive polymerase and 3$'$→5$'$ exonuclease activities (Insdorf and Bogenhagen 1989; Wang 1991) like pol $\varepsilon$, but the sensitivities of my fractions to aphidicolin and their resistance to ddTTP clearly excluded the possibility of their misidentification. The contaminations of the pol $\delta$ and pol $\varepsilon$ fractions with pol $\alpha$, $\beta$ or $\gamma$ were also shown to be insignificant by experiments with DNA polymerase inhibitors, but it is likely that some pol $\varepsilon$ was present in the pol $\delta$ fraction isolated here, and vice versa. Judging from the poor processive activity of the pol $\delta$ fraction without PCNA and from the similarly high processivities of the pol $\varepsilon$ fractions with and without PCNA, however, the influences of such contaminations, if any, were negligible. Another possible explanation for the pol $\varepsilon$ fractions is the coexistence of pol $\delta$ with PCNA. If this were so, a saturating amount of PCNA should be present, because the addition of PCNA to the pol $\varepsilon$ fractions did not stimulate their polymerizing activities. However, this was not the case because no PCNA was detected in Western blots of the pol $\varepsilon$ fractions probed with an anti-PCNA monoclonal antibody (unpublished observation).

I also found that the temperature sensitivity of tsFT20 cells was caused by heat lability of its pol $\alpha$, not by those of other DNA polymerases known to date. Pol $\alpha$ consists of four subunits, two of which are needed for the primase function, but the temperature sensitivity of pol $\alpha$ purified from tsFT20 cells has been shown to be due to one of the other two subunits with DNA polymerase activity, 180- and 68-kDa polypeptides (Takada-Takayama et al. 1991). Recently found in my laboratory was a missense mutation in a cDNA of the 180-kDa subunit isolated
from tsFT20 cells (manuscript in preparation), suggesting that the 180-kDa subunit, the catalytic subunit of pol α, causes the heat-instability of pol α in tsFT20 cells. The fact that the pol δ and ε activities in tsFT20 showed no temperature-sensitivity, indicates that their catalytic subunits are different from that of pol α. This is consistent with the fact that, in human, structures of the 125-kDa catalytic subunit of pol δ and the 258-kDa one of pol ε published recently (Chung et al. 1991; Kesti et al. 1993) differ from that of the 180-kDa polypeptide of pol α (Wong et al. 1988). These findings strongly suggest genetic independence of pol α and pol δ or ε in mouse, although some sharing of their non-catalytic subunits is conceivable.

The total polymerizing activities of both the pol ε-I and pol ε-II fractions from tsFT20 were found to be less than half those of the parental cells (see Table 1). This reduction of pol ε activities is highly probable if pol ε participates in chromosomal DNA replication in which pol α is known to play an important role. Since replication is thought to involve the cooperation of many kinds of cellular factors including DNA polymerases, decrease in pol α activity could impair or alter this cooperation, resulting in replicational deficiency, and then, to restore the replication activity, might lead to alteration in the equilibrium of replication factors, such as, for example, decrease in pol ε activity. Moreover, the reduced level of pol ε suggests an interaction of pol ε with pol α, pol ε possibly working as a leading strand polymerase in place of pol δ in the coordinated DNA replication model proposed by Tsurimoto and Stillman (1989). It is unclear whether the decrease of pol ε activities in tsFT20 cells was caused by repressive control of expression of pol ε activities or by genetic change produced by some selective pressure during establishment of the tsFT20 mutant cell line. On the other hand, the low activity of pol α in tsFT20 cells did not affect the cellular level of pol δ activity. This was unexpected considering its proposed function as a leading strand polymerase during in vitro SV40 DNA replication (Prelich and Stillman 1988; Lee et al. 1989; Weinberg and Kelly 1989). The intracellular activity of pol δ may be controlled indirectly by regulation of its accessory factors, PCNA and the replication factor-C (the activator I) (Tsurimoto and Stillman 1990; Lee et al. 1991), although this possibility has not been studied extensively.

The pol ε activities were stimulated by a high concentration of MgCl₂ and the activity of pol ε-I was also enhanced by 90 mM KCl. These high salt concentrations were found, however, to repress highly processive polymerization with the pol ε fractions (Fig. 3C). Thus high salt concentration may promote dissociation of the enzyme from substrate DNA and consequently enhance the turnover rates of the polymerases. Therefore, the stimulation of DNA polymerase activity by high salt concentrations may be useful as an indicator of highly processive DNA polymerases. In fact, on addition of MgCl₂, pol δ activity was increased in the presence of PCNA (data not shown), but inhibited in its absence (Fig. 4A). Interestingly, pol ε-II activity was not stimulated and was rather resistant to
inhibition by KCl (Fig. 4B) whereas only marginal inhibition of its processivity was observed (Fig. 3C). So the mechanisms of the stimulations by high concentration of MgCl₂ and KCl may be different, although no additive or synergistic effects of additions of the two salts on pol ε-I activity were detected (data not shown).

I isolated two fractions of pol ε with similar properties except for moderate differences in their degrees of processivity and different preferences to KCl concentration. These two species could be explained by structural variation of one enzyme; that is, differences in subunit composition, proteolytic fragmentation, or chemical modification such as phosphorylation. In fact, a proteolytically degraded form of pol ε was observed in S. cerevisiae (Hamatake et al. 1990) and in calf thymus (Siegal et al. 1992). The higher processivity of the pol ε-II fraction suggests that this fraction contains a more integrated form of pol ε than that in the pol ε-I fraction. An alternative explanation is the existence of genetically independent polymerases with highly similar features. Actually it was reported that antibodies against a 170-kDa form of pol ε prepared from human placenta (Lee and Toomey 1987; Lee et al. 1991) failed to react with a 215-kDa form from HeLa cells on immunoblotting (Syvööja and Linn 1989), suggesting the presence of two types of human ε-like polymerase. Further purifications of the fractions of pol ε are necessary for examination of this protein.

Acknowledgments

I thank Dr. A. Matsukage for supplying rat DNA polymerase β and Drs. M. Seki and T. Enomoto for providing mouse PCNA. I am grateful to Drs. F. Hanaoka and F. Yatagai, RIKEN Inst., for suggestions and to N. Kamiyama and S. Sato for assistance in preparing DNA polymerases.

References

sensitive mutant, tsFT20 strain, containing heat-labile DNA polymerase $\alpha$ activity.  


Mouse DNA Polymerases α, δ and ε in ts Mutant

142.


