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Distinction between mouse DNA polymerases α and β by tryptic peptide mapping

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ABSTRACT

Results presented here and in a previous paper (Tanabe et al. (1979) Biochemistry 18, 3401-3406) indicate that mouse β-polymerase is a single polypeptide with an apparent molecular weight of 40,000. This polypeptide has now been analyzed by tryptic peptide mapping. Comparison of the results with identical analysis of mouse α-polymerase reveals that the tryptic peptides derived from the two enzymes are different. These results indicate that β-polymerase is neither a subunit of α-polymerase nor a proteolytic degradation product of α-polymerase.

INTRODUCTION

Two of the well-known mammalian cellular DNA polymerases, α-polymerase and β-polymerase, can be readily distinguished due to a number of clear-cut differences in both catalytic and physical properties. These enzymes also have been distinguished immunologically by Mizutani and Temin[1], Smith et al.[2], Spadari et al.[3], and Brun et al.[4]. The various distinctions do not mean, however, that α-polymerase and β-polymerase share no common subunit or regions of primary structure homology. Indeed, there are several experiments pointing to the possibility that the two enzymes might be so related. For example, there are several reports that α-polymerase and β-polymerase can be recognized by the same antiserum: (1) Chang and Bollum[5] showed that an antiserum prepared against calf thymus α-polymerase was capable of recognizing β-polymerase from calf thymus and several other mammalian species; (2) Mizutani and Temin[6] found that an antiserum prepared against chicken embryo β-polymerase was capable of recognizing α-polymerase from pheasant liver and duck liver; and (3) Smith et al.[2] found that an antiserum prepared against human lymphoid cell α-polymerase was capable of recognizing human β-polymerase. The possibility of a common subunit in the two enzymes was consistent with the work of Yoshida et al.[7] and Holmes et al.[8] who found that urea treatment of calf thymus α-polymerase

changed the native molecular weight of the enzyme from about 180,000 to about 50,000. Thus, it appeared that calf thymus α-polymerase contained an enzymatically active polypeptide with an apparent molecular weight approximately the same as that of calf thymus β-polymerase[9] (i.e., 40,000-50,000). Using a similar approach, Bandyopadhya[10] found that mouse α-polymerase could be dissociated into an active species with a molecular weight of approximately 50,000-60,000, and Chen et al.[11] recently found that mouse α-polymerase (native Mr = 190,000) is composed of two nonidentical subunits with apparent molecular weights of 54,000 and 47,000. The size of the smaller mouse α-polymerase subunit is near the 40,000-Mr value obtained for the subunit Mr of mouse β-polymerase by Tanabe et al.[12].

In the present study we have analyzed the subunit of mouse β-polymerase by tryptic peptide mapping, and together with similar analysis of mouse α-polymerase, the results indicate that tryptic peptides derived from these two classes of mouse DNA polymerase are different.

MATERIALS AND METHODS

Preparation of DNA Polymerase Beta. Three different purification methods were compared. The "standard method" was exactly as described by Tanabe et al.[12], and it included the following sequence of steps: extraction and high speed centrifugation to obtain a soluble protein fraction; chromatography on DEAE-cellulose and phosphocellulose columns coupled in sequence; ammonium sulfate fractionation to obtain the proteins precipitated between 50 and 80% saturation; gel filtration on Sephacryl S-200; chromatography on a column of ssDNA-cellulose; rechromatography on ssDNA-cellulose. Two variations of the standard method are referred to as modifications 1 and 2, respectively. In summary, these modifications differed from the standard method as follows: Modification 2, the ammonium sulfate fractionation was performed before, instead of after, the DEAE-cellulose:phosphocellulose chromatography, and the gel filtration step and the rechromatography on ssDNA-cellulose step was omitted; modification 1, a detail of ammonium sulfate fractionation was changed (i.e., the adjustment to 80% saturation was by dialysis rather than addition of a saturated solution of ammonium sulfate). This change allowed a much longer period for proteins to precipitate.

With each purification method, β-polymerase was extracted from a crude nuclear fraction (wet weight, 85 g) obtained from approximately 200 g of

mouse myeloma, MOPC 104E. Three extracts were processed through the high speed centrifugation step as described[12] for the standard method. For modification 2, solid ammonium sulfate was added to the "S-100 fraction" to give 50% saturation and the proteins that precipitated were discarded. The supernatant fraction was adjusted to 80% saturation in ammonium sulfate by addition of saturated ammonium sulfate at pH 7.0. Proteins that precipitated were chromatographed on coupled DEAE-cellulose:phosphocellulose columns as described for the standard method. The pooled fractions (24 ml) corresponding to the sharp peak of enzyme activity were then chromatographed on a single-stranded DNA-cellulose column. The pooled fractions (14 ml) corresponding to the peak of DNA polymerase activity were dialyzed against 50% glycerol, 20 mM Tris·HCl, pH 8.0, 200 mM NaCl, 1 mM dithiothreitol, 0.1 mM EDTA for 7 hrs. The dialyzed solution (7.4 ml) was stored at -20° and was the final fraction. For modification 1, the extract was processed through the DEAE-cellulose:phosphocellulose chromatography step as described for the standard method. The enzyme solution (206 ml) was adjusted to 50% saturation with solid ammonium sulfate, and the proteins that precipitated were discarded. The supernatant fraction was dialyzed against 90% saturated ammonium sulfate, pH 7.0, for 16h at 0°, and the proteins that precipitated were dissolved in the Sephacryl S-200 column starting buffer. The chromatography on the S-200 column and subsequent steps were as described for the standard method, except the elution of the 2nd ssDNA-cellulose column was with NaCl instead of dextran sulfate; this change in elution had no effect on the polypeptide content of the final fraction.

Sephacryl S-200 Gel Filtration. The solution of fraction III β-polymerase obtained by the standard purification method[12] contained 20 mM Tris·HCl, pH 8.0, 0.5 M NaCl, 10% glycerol, 1 mM dithiothreitol, 0.1 mM EDTA. This solution was loaded onto a 0.9 x 90 cm column of Sephacryl S-200, which was connected in tandem to an identical column. The columns had been equilibrated with the solution described and were run at a flow rate of 10 ml/h. Recovery of enzyme activity applied to the column was 75%. Molecular weight calculations were according to the method described by Andrews[13]; marker proteins were BSA (68,000), ovalbumin (45,000) and chymotrypsinogen (25,100).

Tryptic Peptide Mapping. The method was a modification[11] of the method described by Elder et al.[14]. All conditions for analysis of each polypeptide were identical. The following stained protein bands were sliced from the gels of β-polymerase preparations, some of which are shown in Figure 1:

standard method, 39,000-, 40,000-, and 45,000-Mr; modification 1, 40,000- and 45,000-Mr; modification 2, 39,000-,and 40,000-Mr. The gel slices were washed and dried, and proteins were radioiodinated with [^{125}I] as described by Elder et al.[14]. After washing with 10% methanol to remove free [^{125}I], each slice was transferred to a siliconized 12 x 75 mm glass tube, and 475 µl 50 mM NH_4HCO_3 (pH 8.4) and 25 µl 1 mg/ml trypsin (Worthington Biochem. Corp.) in 0.001 N HCl were added to each tube. After incubation for 18 h at 37°, the liquid in each tube was placed in a siliconized 12 x 75 mm glass tube and lyophilized to dryness. The residue was dissolved 0.5 ml H_2O, and the lyophilization to dryness was repeated. The residue was then dissolved in 100 µl H_2O and the lyophilization was again repeated. The final residue was dissolved in 20 µl of acetic acid:formic acid:H_2O (15:5:80), and the solution was stored at -20°C. A 2-4 µl portion of this solution was spotted on a silica gel thin layer plate and peptides were resolved using electrophoresis in the first dimension and ascending chromatography in the second dimension. Electrophoresis was at 2° and 1000 volts for 80 min in a buffer of acetic acid:formic acid:H_2O (15:5:80). The plate was dried in air at 25° for 6-8 h. The plate was then suspended in a thin layer chromatography chamber equipped with a hanging vertical plate holder, and 250 ml of butanol: pyridine:acetic acid:H_2O (32.5:25:5:20) was added to the bottom of the chamber. After equilibration for 12 h at 25°, the plate was lowered into the liquid and allowed to develop for 5 h until the front reached the top. The plate was dried and peptides were located by autoradiography using Kodak X-Omat R film.

RESULTS

Polypeptides in β-Polymerase Preparations. During evaluation of our standard method for purification of β-polymerase, we found that only minor modifications yielded final enzyme preparations containing different proportions of three polypeptides with apparent molecular weights of 39,000, 40,000 and 45,000. This is illustrated by the dodecyl sulfate-polyacrylamide gel electrophoretic analysis shown in Figure 1. The three β-polymerase preparations analyzed did not contain relatively abundant polypeptides with apparent M_r lower than 39,000 or higher than 45,000. Since the native M_r of mouse β-polymerase is about 40,000 (Figure 2), any or all of these three main polypeptides in the 39,000 - 45,000 range might be a β-polymerase subunit. All three enzyme preparations shown in Figure 1 were abundant in the 40,000-Mr protein, whereas the 45,000-Mr protein was abundant only with mod-

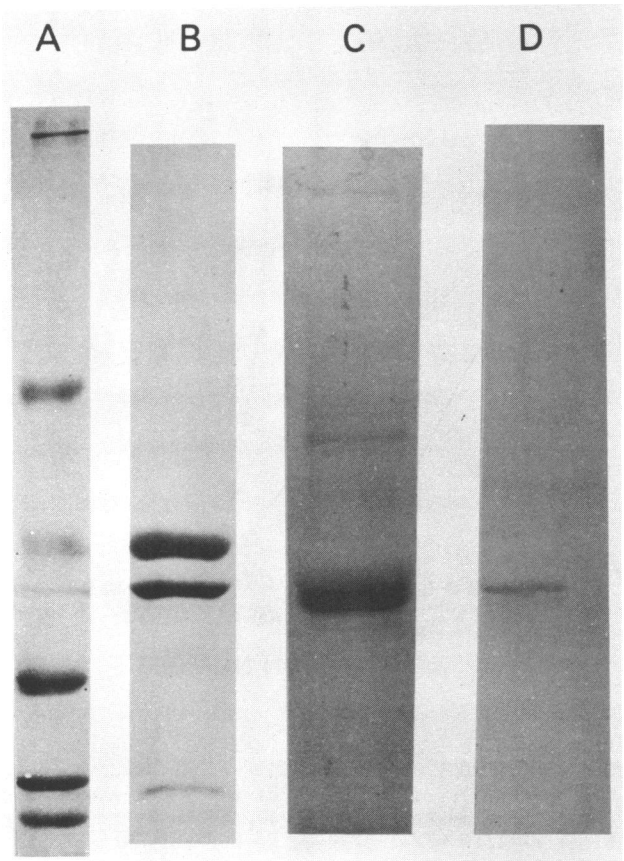

Figure 1: Sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis of different preparations of mouse β-polymerase. The final fraction obtained by each of the three purification methods described in the text was analyzed on a 10% polyacrylamide gel according to the method described by Laemmli[15]. Lane A; protein standards BSA (68,000), ovalbumin (45,000), chrymotrypsinogen (25,100), myoglobulin (17,200), lysozyme (14,300). Lane B; modification 1. Lane C; modification 2. Lane D; standard method. The main band in lane D, the upper band of the "doublet" in Lane C, and the lower of the two abundant bands in lane B corresponded to an apparent molecular weight of 40,000.

ification 1 and the 39,000-Mr protein was abundant only with modification 2. Comparison of the specific activities of the three preparations revealed that the enzyme activity did not correlate with the 45,000-Mr protein (Table 1). For example, the enzyme preparations obtained by modification 2 and the standard method contained only small amounts of 45,000-Mr protein; yet, their specific activities were higher than that of the preparation obtained by mod-

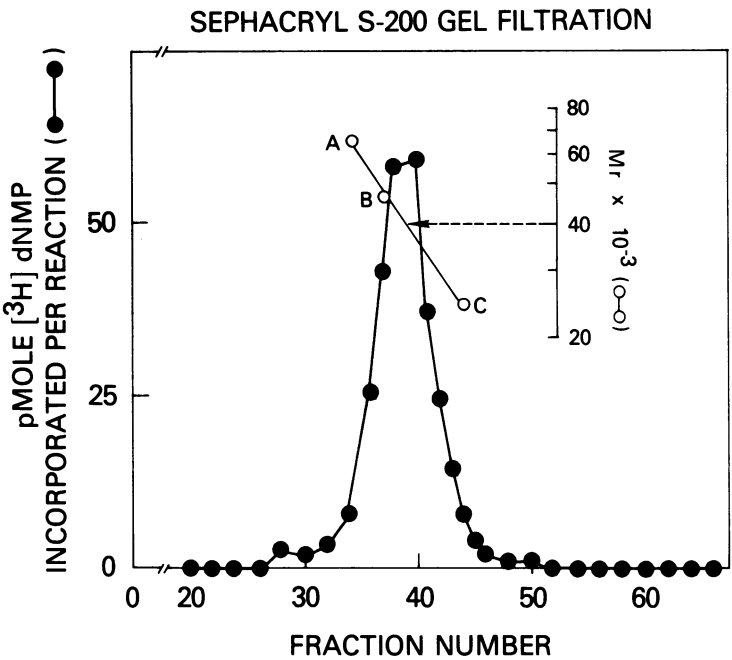

Figure 2: Profile of mouse β-polymerase after gel filtration column chromatography on Sepharyl S-200. Experiments were performed as described in the text with a sample of β-polymerase obtained by the standard purification method. Positions of the marker proteins are shown by the open circles. Virtually identical results were observed with β-polymerase obtained by modifications 1 and 2.

ification 1 which was rich in 45,000-Mr protein. These results are consistent with the earlier interpretation that the 40,000-Mr protein in the preparation obtained by the standard method is β-polymerase[12]. Further evidence in support of this interpretation is provided by the experiment shown in Figure 3. The enzyme preparation obtained by modification 2 was subjected to isoelectric focusing in a 5% polyacrylamide gel containing a narrow-range pH gradient. A portion of each gel slice was used for location of enzyme activity and another portion was used for polypeptide analysis by "direct-transfer" SDS-polyacrylamide gel electrophoresis. As can be seen in Figure 3, the slice from the isoelectric focusing gel corresponding to the peak of enzyme activity contained a single polypeptide with an apparent molecular weight of 40,000. A 45,000-Mr polypeptide was detected in gel slice 4, but this region did not contain enzyme activity.

TABLE I

Some Comparisons of Mouse β-Polymerase Purification Methods

Purification Methods	Recovery	Specific Activity of Final Fraction	Apparent Mr of Polypeptides in Final Fraction
	Units[a]/g tissue	Units[a]/mg protein	
Standard method	0.007	65	40,000
Modification 1	0.006	22	45,000 and 40,000
Modification 2	0.011	60	40,000 and 39,000

[a] 1 unit is equal to 1 μmol dNMP incorporated per 60 min; activated calf thymus DNA was the template-primer, and the reaction conditions were as described by Tanabe et al.[12].

Tryptic Peptide Mapping. Tryptic peptide mapping was performed with the 39,000, 40,000, and 45,000-Mr proteins obtained by each of the three purification methods. The maps of the three 40,000-Mr proteins contained many common signals, and indeed the maps of the proteins from modifications 1 and 2 were indistinguishable; examples of maps of the 40,000-Mr proteins from the standard method and from modification 1 are shown in the upper panels of Figure 4. These results indicate that the 40,000-Mr proteins obtained by the three purification methods shared extensive primary structure homology and were probably the same protein. Also shown in Figure 4 is that the 39,000-Mr protein obtained by modification 2 shared extensive homology with the 40,000-Mr proteins. In contrast, the map of the 45,000-Mr protein had a completely different pattern from that observed with the other proteins.

We conclude that the 39,000-Mr and 40,000-Mr proteins represent microheterogeneity of the same protein (i.e. β-polymerase) and that this protein and the 45,000-Mr protein are different. The possibility of microheterogeneity of the β-polymerase polypeptide had previously been suggested from gel electrophoresis results with enzyme preparations from another source[9].

Comparison of β-Polymerase and α-Polymerase Subunits by Tryptic Peptide Mapping. Earlier studies from this laboratory have shown that there are two

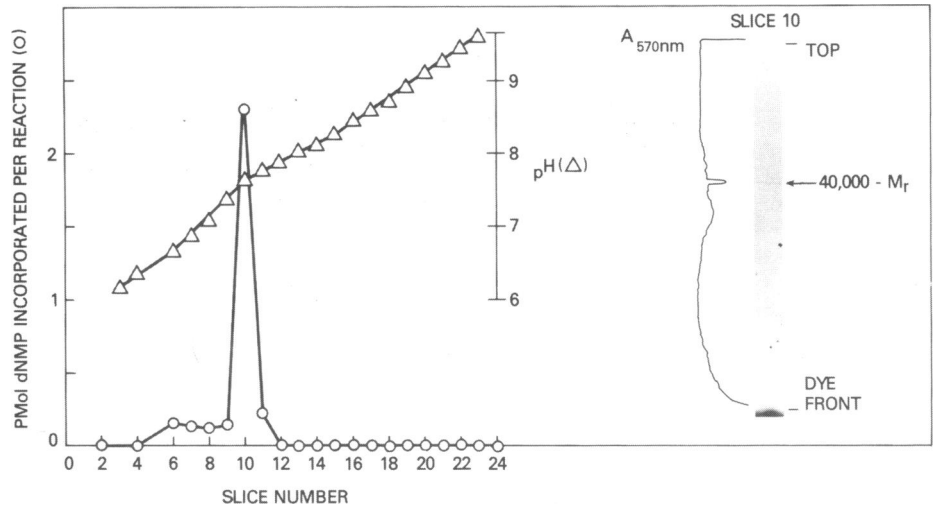

Figure 3: Isoelectric focusing of mouse β-polymerase and SDS-polyacrylamide gel electrophoresis of polypeptides in the gel slice containing the peak of enzyme activity. Isoelectric focusing was in a 5% polyacrylamide slab gel (24 x 1.5 x 0.3 cm) containing ampholines of pH range 6.5 to 9.5. After focusing for 5 hrs. at maximum settings of 10 mA and 2000 V, the gel was sliced into 24 pieces, 1 x 1.5 x 0.3 cm. About 15% of each gel slice was used for elution and assay of β-polymerase activity, and another portion of each slice, representing about 50%, was treated with 1% SDS and 40 mM dithiothreitol[16]. The gel piece was then transferred intact to the top of a 12% polyacrylamide gel containing 0.1% SDS, and electrophoretic analysis was as described previously[11]. Ovalbumin was isoelectric focused in parallel to the polymerase and also analyzed on the SDS-gel in parallel. Of the β-polymerase activity applied to the portion of the isoelectric focusing gel that was eluted, 42% was recovered in the eluate of slice 10 after soaking the slice for 120 hrs. at 4° in 150 µl 50 mM Tris·HCl, pH 8.4, 1 mM dithiothreitol, 0.1 mM EDTA, 20% glycerol, 1 mg/ml albumin. Each DNA polymerase assay contained 2 µl of eluate.

distinct α-polymerases in mouse myeloma. Each α-polymerase appeared to be composed of two dissimilar subunits with apparent molecular weights of about 54,000 and 47,000, and analysis of these subunits by tryptic peptide mapping has been presented by Chen et al.[11].

Comparison of tryptic peptide maps of the mouse α-polymerase subunits and the mouse β-polymerase subunit is shown in Figure 5. The pattern of spots in the map of each α-polymerase subunit was clearly different from the pattern of spots observed with the β-polymerase subunit, and composite tracings of the maps failed to reveal homologies of any of the major individual peptide spots of the α- and β-polymerase.

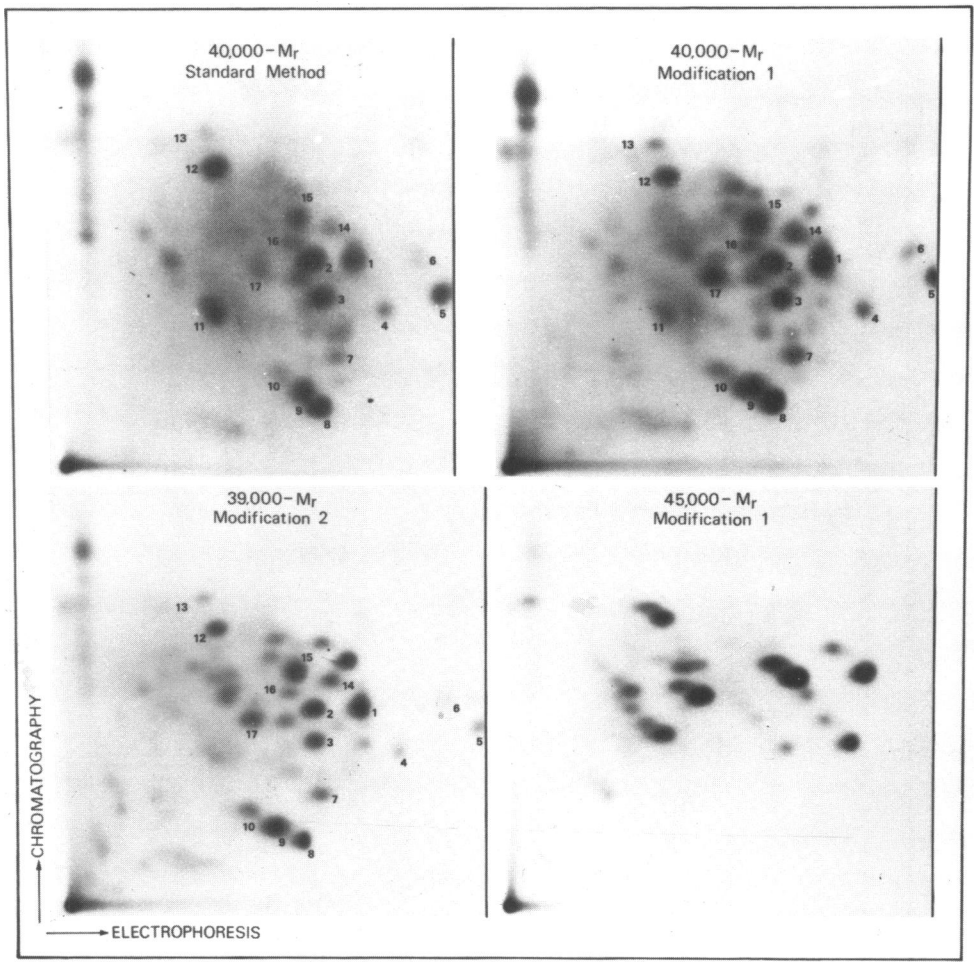

Figure 4: Tryptic peptide mapping of abundant polypeptides in three different preparations of mouse DNA polymerase β. Experiments were performed as described in the text. Polypeptides, as indicated above, were labeled with [^{125}I], and tryptic peptides were displayed on silica gel thin layer plates by electrophoresis in the first dimension and ascending chromatography in the second dimension. Autoradiographs are shown above. Signals that are numbered are clearly homologous in the maps of the 40,000-Mr and 39,000-Mr proteins.

DISCUSSION

The range in the apparent Mr values reported for β-polymerase polypeptides from various mammalian sources appears greater than expected from

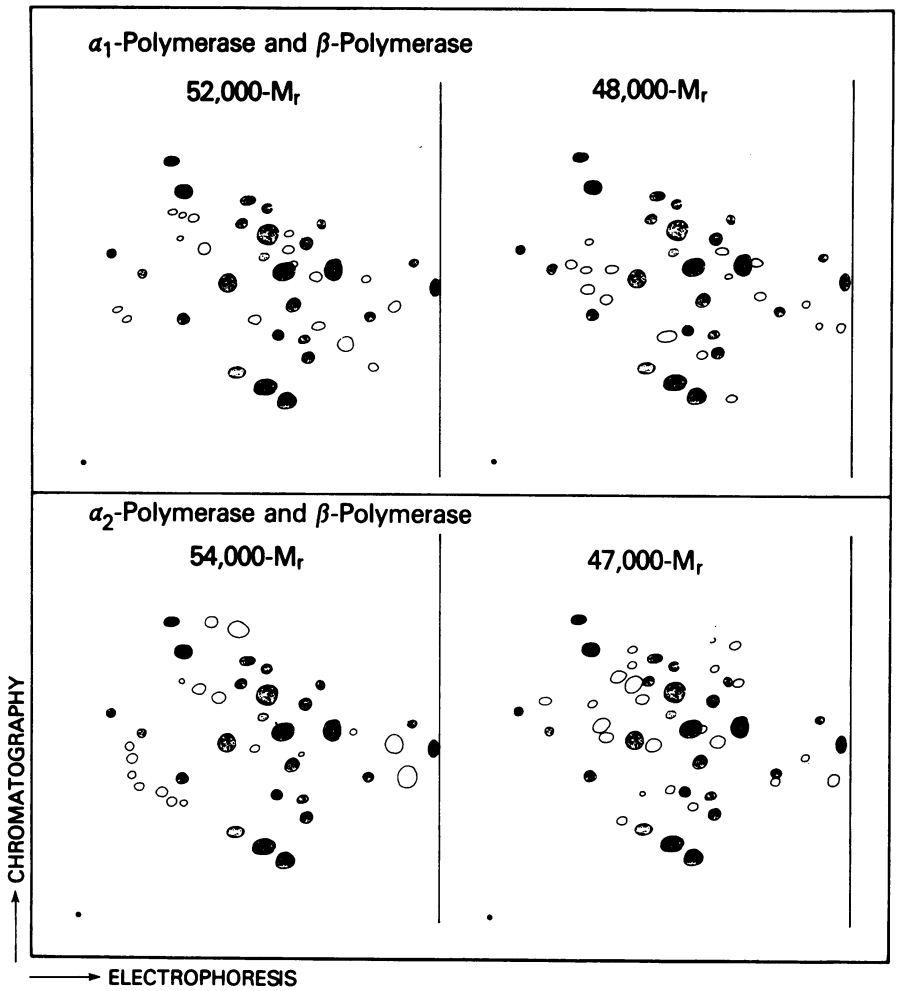

Figure 5: Composite tracings of tryptic peptide maps of α-polymerase subunits and the β-polymerase polypeptide. The open tracings represent the major peptide spots in the map of each of the subunits of α_1-polymerase and α_2-polymerase[11]. The peptide spots shown by stippling were traced from a map of β-polymerase (the 40,000-Mr polypeptide, modification 1), and the same tracing was superimposed on the map of each α-polymerase subunit.

experimental variation of the SDS-polyacrylamide gel analysis. Thus, Meyer and coworkers have demonstrated that β-polymerases purified from both rat Novikoff hepatoma[17] and guinea pig liver[19] were 32,000-Mr polypeptides, whereas earlier studies by Chang[9] and by Wang et al.[18] indicated that β-

polymerases purified from calf thymus and human KB cells, respectively, were 43,000- Mr and 44,000-Mr polypeptides. These two values are probably in agreement with the 40,000-Mr value recently attributed to β-polymerase polypeptides from human liver[20] and mouse myeloma[12]. Because of this range in reported Mr values for β-polymerase polypeptides and the presence of 39,000-, 40,000- and 45,000-Mr polypeptides in our various final fractions (Figure 1), we decided it was necessary to obtain additional evidence on coincidence of polypeptide(s) and the enzyme activity. Although it was already apparent that the amount of the 45,000-Mr polypeptide in the final fraction (Table I) did not correlate with enzyme specific activity, the results of the isoelectric focusing gel analysis clearly indicated that the 45,000-Mr polypeptide was a contaminant and that the 40,000-Mr polypeptide was β-polymerase. That the β-polymerase polypeptide can be degraded to a 39,000-Mr polypeptide peptide during its purification from mouse myeloma is suggested from our results with modification 2, where the ammonium sulfate precipitation step preceded the DEAE-cellulose:phosphocellulose chromatography.

The use of SDS-polyacrylamide gel electrophoresis for resolution of subunits and the tryptic peptide mapping procedure described by Elder et al.[14] permitted us to obtain an unambiguous answer to the question, is β-polymerase one of the α-polymerase subunits? Our results indicate that tryptic peptides derived from mouse β-polymerase and α-polymerase are different. It appears quite unlikely, therefore, that the two enzymes have a precursor:product relationship, and the results seem to clearly exclude the possibility that β-polymerase is a subunit in α-polymerase. It is interesting to consider that our failure to detect tryptic peptide homology between the two enzymes is not inconsistent with the reported immunological cross-reactivity (see above). In the immunological experiments, common antigenic determinants could have been conferred by regions of structural similarity, perhaps related to a common structure-function relationship among the two classes of DNA polymerase. Such regions of structural similarity may not be easily detected by tryptic peptide mapping, since this method tends to emphasize primary structure differences between proteins, rather than a certain degree of tertiary or primary structure similarity.

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