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DETECTION OF A DNA-BINDING FACTOR ASSOCIATED WITH MAMMALIAN DNA POLYMERASE-α

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Summary : A DNA-binding factor able to bind to a double-stranded DNA containing no free ends (SV40 DNA I) has been reproducibly detected in highly purified (to 30,000 units/mg) regenerating rat liver DNA polymerase-α. This factor could not be separated from the catalytic unit by any of the separation procedures so far used (ion-exchange chromatographies, gel filtration, sucrose gradients and DNA affinity chromatographies). Dissociation of the DNA binding unit from the catalytic unit may be observed after formation of a nucleoprotein complex between DNA polymerase-α and SV40 DNA I.

INTRODUCTION

Circumstantial evidence for the involvement of DNA polymerase-α activity in the DNA replication process has been found in a variety of experimental systems (1,2,3,4,5). In a previous work, we have described the purification and detailed catalytic properties of the DNA polymerase-α from regenerating rat liver (6,7). DNA polymerase-α was now prepared from regenerating rat liver using some modifications of the procedure described previously (6,7) which allowed us to obtain a purified enzyme fraction having a specific activity of 20,000 to 30,000 units/mg (manuscript in preparation). In order to dissociate the catalytic and the binding events associated with the synthesis of DNA, the binding of DNA polymerase-α to DNA was determined using the covalently closed SV40 DNA I as a substrate for a number of reasons. From a biological point of view, this viral double-stranded DNA replicates in the nuclei of mammalian cells (CV_1 monkey cells) and the majority of reactions needed to replicate the SV40 genome are catalyzed by cellular enzymes (8 and 9 for a review). Moreover, the DNA polymerase activity that was the most enhanced early in infection of growing cells by SV40 was that of DNA polymerase-α (5). Secondly, this supercoiled molecule contains no free ends, so the binding experiments were made in absence of 3'OH termini which initiate the catalytic process of replication (10) . We would like to report here that highly purified DNA polymerase-α binds to SV40 DNA I and that the catalytic activity can be dissociated from the binding activity.

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MATERIAL AND METHODS

1 - Purification of DNA polymerase-α

The six-step purification scheme used was the following : Step I through III were exactly as described previously (6). Briefly, a post microsomal supernatant from liver cell homogenates (Step I) was fractionated by ammonium sulfate precipitation (30 to 40 % saturation) to obtain the Step II preparation. It was then further fractionated by DEAE cellulose chromatography (Step III).

The enzyme preparation was then chromatographied on hydroxylapatite (6) under high ionic strength conditions (0.5 M NaCl) in order to dissociate aggregated forms of the enzyme (10,11). Polymerase activity was eluted in a single peak at 0.12 M KPO_4 (Fraction IV) with a linear gradient from 0.050 to 0.30 M KPO_4. Fraction IV was dialyzed against 50 mM Tris-HCl pH 7.5, 6 mM KCl, 1 mM $MgCl_2$, 1 mM β-mercaptoethanol, 30 % glycerol (buffer A) and applied onto a phosphocellulose column equilibrated with the same buffer. The column was washed with buffer A and proteins eluted with buffer A containing 0.15 M NaCl and then with a linear gradient from 0.2 M to 0.6 M NaCl in buffer A. Polymerase activity, eluted at 0.3 M NaCl (Fraction V), was loaded onto a denatured DNA-cellulose column prepared according to Alberts and Herrick (12) and equilibrated with buffer A containing 2 mM EDTA. The column was washed with the same buffer and the polymerase activity was eluted with 0.15 M NaCl. Fraction VI was dialyzed against buffer A and stored at -80°C. When analyzed by native gel electrophoresis in different conditions a single protein band was obtained, whereas by sodium dodecyl sulfate gel analysis two polypeptides of 160,000 and 60-70,000 daltons were detected (Méchali, M., Abadiedebat, J. and de Recondo, A.M., unpublished datas). We could not exclude that the 160,000 daltons band represented an aggregated form of the 60-70,000 daltons band. Similar results have been described recently by Fisher and Korn (13).

2 - Purification of DNA from Simian Virus 40

SV40 [^{3}H]DNA I was prepared as previously described (6).

3 - Binding assay

We used the capacity of nitrocellulose filters to retain a nucleoprotein complex while they cannot retain free native DNA (14) to study the binding of DNA polymerase to DNA. Binding reactions were carried out in 100 µl of 40 mM Tris-HCl pH 7.5, 10 mM KCl, 0.2 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 0.002 mM EDTA, 15 % glycerol (buffer B) containing 400 µg/ml bovine serum albumin (BSA, nuclease free), 0.02 to 1 µg SV40 [^{3}H]DNA I (34,000 cpm/µg) and enzyme. After 30 min at 37°C, the reaction media were diluted to 1 ml with buffer B and filtered immediately through 25 - mm Schleicher and Schull BA 85 nitrocellulose filters. The filters were washed with 0.6 ml buffer B, dried, and the radioactivity determined. The amount of DNA retained in the absence of enzyme ranged from 1 to 3 %. DNA polymerase-α activity was retained on the filter as checked by the lack of activity in the filtrate (not shown). The integrity of SV40 DNA I in the reaction medium was checked according to the procedures previously described involving alcaline sucrose gradients and filter disc assay (7). In all the experiments reported here, the amount of covalently closed DNA I was more than 95 % at the end of the incubation period.

4 - DNA polymerase assay

DNA polymerase-α activity was assayed using 50 µM poly(dC). $(dG)_{12-18}$ (base ratio 4 : 1) in 50 mM Tris-HCl pH 7.6, 1.5 mM $MgCl_2$,

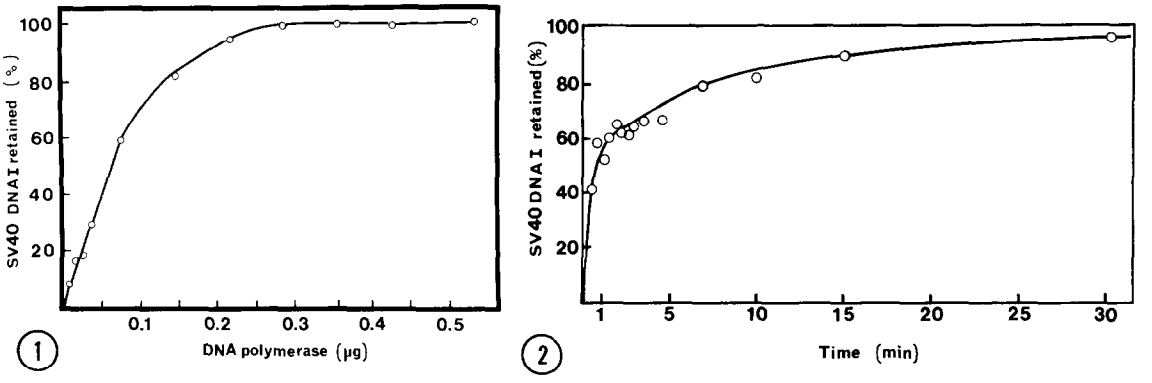

Figure 1 : Binding of DNA polymerase-α to SV40 [^{3}H] DNA I.

The binding activity was determined using the nitrocellulose filter assay described in Material and Methods. Reaction media contained 0.139 µg SV40 [^{3}H] DNA I (38,000 cpm/µg) and increased quantities of enzyme. The amount of DNA retained in the absence of enzyme was 1.4 %.

Figure 2 : Kinetic of formation of the nucleoprotein complex.

The reaction media contained 0.745 µg [^{3}H] SV40 DNA I (34,000 cpm/µg) and 1.14 µg DNA polymerase-α in 2.4 ml buffer A (see Material and Methods) containing 250 µg/ml bovine serum albumin. The solution was preincubated for 10 min at 37°C without the enzyme and the reaction was initiated by the addition of DNA polymerase-α. After the indicated incubation periods, 0.1 - ml samples were removed, diluted in 1 ml of buffer A and filtered immediately. In this experiment , the time required between dilution and complete filtration was about 10 to 15 sec.

10 mM KCl, 5 mM 2-mercaptoethanol, 15 % glycerol, 600 µg/ml bovine serum albumin and 0.1 mM [^{3}H] or [^{32}P] dGTP (200 cpm/pmol). After incubation at 37°C, the reaction mixtures were transferred to Whatman GF/C glass fiber filter discs processed for acid-insoluble material as previously described (6), and the radioactivity was determined by liquid scintillation counting.

RESULTS

Detection of the binding activity

When DNA polymerase-α (Step VI) was preincubated with a DNA containing no free ends, SV40 [^{3}H] DNA I, a nucleoprotein complex was formed, as indicated by the retention of labelled DNA on the nitrocellulose filter (Fig. 1). The binding is not cooperative since the amount of SV40 [^{3}H] DNA I retained by the filter was linearly dependent on the amount of DNA polymerase-α added to the reaction medium in the range from 0 to 60-95 % of the added DNA with the different enzyme preparations tested. The non-linearity of the upper part of

the DNA binding curve did not vary with DNA concentration, and hence was not caused by an equilibrium dissociation of the nucleoprotein complex but could correspond to the existence of more than one binding site on the DNA molecule. As the ratio protein/DNA increases, an increased amount of protein molecules will bind to SV40 DNA molecules which can even be retained to the filter. The linear part of this binding curve indicates that 1 µg of DNA can be retained to the filter by 0.73 µg of protein.

The corresponding enzyme/DNA molar ratio was 13, assuming a molecular weight of 180,000 for DNA polymerase-α (from sedimentation experiments not shown) and 3.3×10^6 for SV40 DNA I (15). Yet, this value should be taken with caution for many reasons. First, the enzyme may aggregate under the low ionic strength conditions used in the binding assay (10,11). Secondly only a fraction of the purified enzyme could be active with respect to binding to DNA. Moreover the efficiency of retention of the nucleoprotein complex on the filter may be less than unity (16) and vary with the amount of enzyme molecules bound per DNA molecule. Taken together these uncertainties do not allow us either to interprete these filter binding datas in terms of a Scatchard plot, to quantify an association constant.

Properties of the binding activity

The kinetic of formation of the complex was relatively rapid with a half-time of reaction inferior to 50 sec (Fig. 2). Moreover the form of the curve suggested at least two kinds of binding sites on SV40 DNA I. The binding activity was inhibited by an increase of ionic strength (Table I) as DNA polymerase-α activity (6,10) and did not necessitate $MgCl_2$ or bivalent cation since DNA polymerase-α activity also bound to DNA at 2 mM EDTA (7). Moreover at $MgCl_2$ concentration above 1 mM the formation of the nucleoprotein complex was reduced. Dilutions at various salt concentrations immediately before filtration did not affect the efficiency with which the complex was retained by the filter (not shown), and hence the variations observed under different cation conditions reflected the properties of the formation of the nucleoprotein complex. The mucopolysaccharide heparin prevented the formation of the complex, and this was probably due to the polyanionic state of this reagent. ATP (in the range from 0.2 to 0.6 mM) and mixtures of deoxynucleotides triphosphates (0.2 to 0.6 mM) had no effect on the amount of nucleoprotein complex retained on the filter.

Dissociation of the binding activity from the catalytic activity after binding to SV40 DNA I

The presence of this binding activity in highly purified DNA polymerase-α led us to try to dissociate the catalytic and the binding activity

Table I : Requirements for the binding activity

Incubation conditions	SV40 DNA I retained (%)
Standard reaction medium	100
plus EDTA 2 mM	100
plus $MgCl_2$ 1 mM	81
plus $MgCl_2$ 3 mM	42
plus $MgCl_2$ 6 mM	20
plus NaCl 50 mM	12
plus KCl 50 mM	31
plus SDS 0.1 %	0.0
plus Heparin 2.5×10^{-9} M	1.8
plus ATP 0.6 mM	98
Incubation at 20°C	40

The binding activity was measured by the nitrocellulose filter assay described in Material and Methods. The results were expressed relative to that observed in the complete system (see Material and Methods). In all cases, between 45 and 70 % of the added SV40 [^{3}H] DNA I was retained by the filter under the standard assay conditions (linear part of the binding curve).

during the purification of the DNA polymerase-α. All the described chromatography procedures used to purify the enzyme (6, Material and Methods) failed to separate the two activities. Introduction of a Sephacryl S.200 gel filtration step during the purification of the enzyme did not separate the two activities which were excluded from such a column. Moreover, in the presence of 10 mM or 200 mM KCl, they co-sedimented in sucrose gradients and thus, they could not be separated on the criteria of size and shape of the molecules (datas not shown).

The last step of purification of DNA polymerase-α is an affinity chromatography on DNA-cellulose and therefore, it was interesting to analyse particularly this step. Fig. 3 shows that the two activities co-chromatographied on native DNA-cellulose. Identical results were obtained with denatured DNA- and native DNA-cellulose, using a salt gradient to elute the proteins adsorbed on the DNA-cellulose columns. In these cases, DNA polymerase

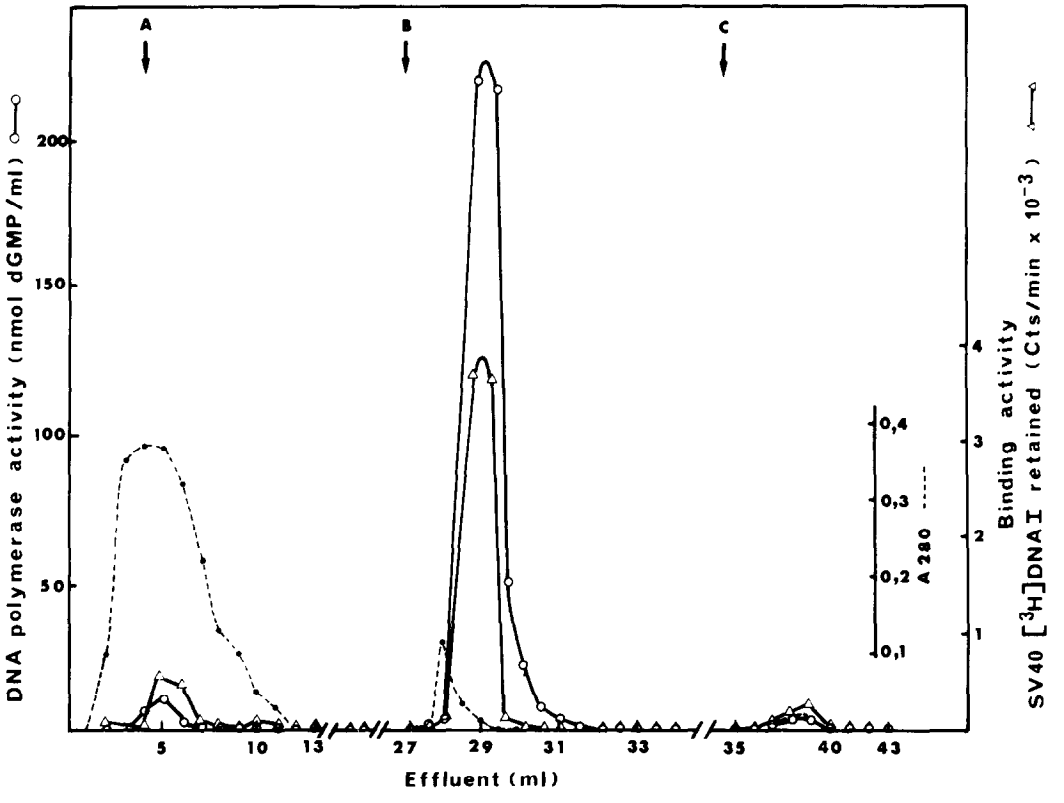

Figure 3 : Co-chromatography of DNA polymerase activity and DNA binding activity on native DNA-cellulose chromatography.

0.21 mg of DNA polymerase-α, fraction V (see text), were applied to a DNA-cellulose column (0.5 x 7 cm) prepared according to Alberts and Herrick (12). The column was washed with buffer A (50 mM Tris-HCl 7.5, 6 mM KCl, 1 mM 2-mercaptoethanol, 3 mM EDTA and 30 % glycerol) then buffer A plus 0.15 M NaCl (buffer B), and finally with buffer A plus 2 M NaCl (buffer C). DNA polymerase activity was determined as indicated in Material and Methods with poly(dC).(dG)12-18 as template. DNA binding activity was measured as described in Material and Methods with 5-µl aliquots of enzyme fractions and 0.065 µg of SV40 [^{3}H]DNA I (34,000 cpm/µg). (○—○), DNA polymerase activity as nmol of [^{3}H]dGMP incorporated/ml/20 min ; (△—△), DNA binding activity as cts/min SV40 [^{3}H] DNA I retained on the filter by the binding assay ; (---------), absorbance at 280 nm. For a better analysis of the figure, the abscissa are different for the fractions between 0-27 ml, 27-34 ml and 35-43 ml effluent.

activity and binding activity were eluted together in a single peak at 90 mM NaCl.

Sucrose gradients containing covalently closed SV40 DNA I, or DNA polymerase-α , or nucleoprotein complexes formed at different protein/DNA ratios, were then made. Figure 4A shows the position of DNA I and DNA poly-

merase-α sedimented separately. In previously formed complexes, the higher the protein/DNA ratio was, the faster the DNA I sedimented in the gradient. Thus, at 38 % retention on the filter of the [^{3}H]DNA I involved in a nucleoprotein complex, the great majority of DNA I sedimented at 21-22 S, but about 27 % of SV40 DNA sedimented as a leading edge at 24-26 S (Fig. 4B). 62 % of DNA I was not complexed before the sedimentation and this result therefore agreed with the sedimentation curve obtained. At 72 % retention on the filter of DNA involved in a nucleoprotein complex, the [^{3}H]DNA I sedimented near the bottom of the gradient in a broad peak superior than 25 S (Fig. 4C). Only a minor part of SV40 DNA sedimented as free DNA and this result was consistent with the enzyme/DNA molar ratio used. In all cases, even when all the SV40 DNA I molecules were involved in a nucleoprotein complex (not shown), the DNA polymerase-α activity determined with poly(dC).$(dG)_{12-18}$ or activated DNA as template, did not co-sedimented with SV40 DNA I. However, a trailing edge of DNA polymerase-α activity towards the heavy regions of the gradient could always be observed when a nucleoprotein complex was formed.

The fractions containing DNA polymerase activity and SV40 DNA I were pooled separately (pool P and D of Fig. 4C). DNA polymerase contained in the pool P had lost its binding activity whereas SV40 DNA I contained in the pool D was retained on nitrocellulose filter (Table II). It was checked that SV40 DNA contained in the pool D (Fig. 4C) was form I DNA (as judged by electron microscopy and alkaline sucrose gradients). One would expect an alteration in the sedimentation behavior of α-polymerase activity if the binding factor was associated with DNA polymerase-α prior to binding to DNA. The centrifugation conditions of Fig. 4 did not however allow to observe such an alteration. It is also important to note that the recovery of α-polymerase activity was always lesser than in usual sedimentation experiments. Moreover, α-polymerase activity, when dissociated from the binding factor after binding to DNA, became very unstable and, so far, attempts to characterize the separated α-polymerase activity have thus failed.

DISCUSSION

The results presented above indicate the presence of a DNA-binding activity associated with DNA polymerase-α in purified preparations. The ratio of protein/DNA obtained in the linear part of the binding curve was 1.2 + 0.6 (w/w) when tested with eleven different preparations. Association of the two activities was maintained throughout all our purification procedures and the separation has been obtained at this moment only by sucrose gradient sedimentation after formation of a nucleoprotein complex with a DNA devoid of free

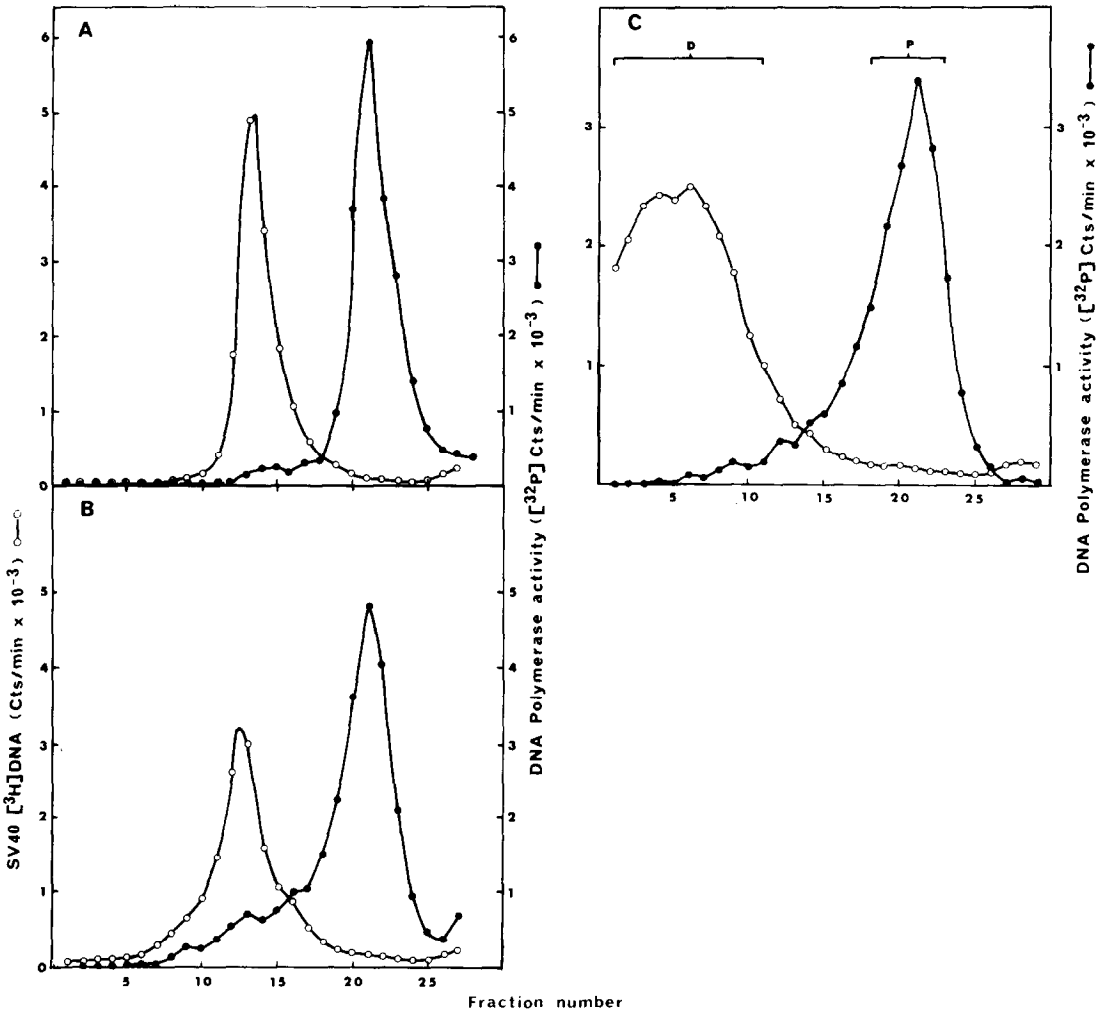

Figure 4 : Separation of the SV40 DNA I - DNA binding factor complex from the catalytic activity of DNA polymerase-α.

Centrifugation of SV40 [^{3}H]DNA I, DNA polymerase-α, or mixtures, were performed at 4°C in 4.8 ml of 5 to 20 % sucrose gradients in 50 mM Tris-HCl pH 7.6, 10 mM KCl, 5 mM 2-mercaptoethanol, 0.2 mM $MgCl_2$, which have been layered on top of a 0.1-ml cushion of 50 % sucrose. After 225 min at 40,000 rpm in a Spinco SW50.1 rotor, 180 µl - fractions were collected from the bottom of the tubes. Panel A : 3 µg of DNA polymerase-α previously dialyzed against the above buffer, and 0.77 µg of SV40 DNA I were sedimented separately. Panel B : 3.5 µg of DNA polymerase-α previously dialyzed were preincubated at 37°C with 2.7 µg of SV40 DNA I. Filtration of an aliquote on nitrocellulose filter showed that 38 % of SV40 DNA I molecules were retained on the filter in these conditions. Panel C : 5 µg of DNA polymerase-α previously dialyzed were preincubated at 37°C with 2.3 µg of SV40 DNA I. Filtration of an aliquote on nitrocellulose filter showed that 68 % of SV40 DNA I molecules were retained on the filter in these conditions.

(●——●), DNA polymerase activity as cts/min [^{32}P]dGMP incorporated with poly(dC).(dG) 12-18 as template, as determined in Material and Methods (○——○), position of SV40 DNA I as [^{3}H] cts/min. Fractions containing SV40 [^{3}H] DNA I (1 to 11) and DNA polymerase activity (18 to 23) were pooled separately (pools D and P).

Table II : Separation of the SV40 DNA I - DNA binding factor complex from DNA polymerase-α activity

	Retention on nitrocellulose filter of pool D %	Binding capacity of pool P %	Control SV40 DNA I %
% SV40 DNA I retained on nitrocellulose filter	51	0.6	0.0

Pools D an P refer to Fig. 4C. 50-µl aliquots of pool D were filtered directly on nitrocellulose filters to determine the percentage of SV40 DNA I involved in a nucleoprotein complex. 68 % of SV40 [^{3}H]DNA I was involved in a nucleoprotein complex before sedimentation. The binding capacity of pool P was measured by addition of exogenous SV40 [^{3}H]DNA I (0.18, 0.71 or 1 µg, 34,000 cpm/µg) to 75-µl aliquots of the pool P. The SV40 [^{3}H]DNA I used as control was the free form I DNA taken from a sucrose gradient.

3'OH ends. These results may indicate an association between the DNA binding factor and the catalytic unit of DNA polymerase-α.

Competition experiments made in our laboratory, using α-polymerase with different synthetic templates indicated that the binding to template could be distinguished from the polymerization itself (Fichot, O., Pascal, M., Méchali, M., and de Recondo, A.M., preliminary results). Thus, the ongoing synthesis of the $(dA)_n.(dT)_{12-18}$ template can be stopped by the competing $(dC)_n.(dG)_{12-18}$ which was itself copied with a very bad efficiency, compared to the result obtained with $(dC)_n.(dG)_{12-18}$ alone. This final situation can be the result of the dissociation of the binding unit from the catalytic core DNA polymerase which cannot now assume a correct copy of the $(dC)_n.(dG)_{12-18}$ template. Our working hypothesis is that the same result is obtained by sedimenting the α-polymerase complexed with a DNA devoid of free 3'OH ends : centrifugation could dissociate the binding factor from the catalytic unit DNA polymerase-α which then becomes unstable. We do not exclude however that conditions permitting to obtain a stable nucleoprotein complex containing both activities could be achieved.

The possible presence of a "conversion factor" in DNA polymerase-α preparations has been pointed out by Probst *et al* (17) and Holmes

et al (18,19). Similar studies in progress in different laboratories lead to question the exact nature and role of the DNA polymerase-α subunits (13) and suggest that the polymerase-α holoenzyme could exist as more than one unique structural form possessing a common catalytic core. The answer should increase our understanding of the DNA-replication complex during the initiation and elongation of the new DNA strands.

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