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Identification of multiple cellular factors required for SV40 replication *in vitro*

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The replication of simian virus 40 has been studied by using cell-free extracts derived from human 293 cells. Fractionation of this extract has led to the identification of three fractions that are required for efficient DNA synthesis. Initial fractionation of the crude extract by phosphocellulose chromatography has produced two fractions, I and II, neither of which is able to support replication separately, but when they are combined, efficient synthesis is restored. Both fractions are required, with SV40 T antigen, for the formation of a presynthesis complex at the SV40 origin. The major replication enzymes, DNA polymerase, DNA primase and the topoisomerases I and II all reside in fraction II. Fraction I has been subdivided into two subfractions (A and B) by DEAE–cellulose chromatography. Fraction A is essential for replication and is required for presynthesis complex formation. Fraction B stimulates DNA replication and is only required at the elongation stage. This multicomponent system has provided the foundation for identification of individual components that are required for DNA replication *in vitro*.

Introduction

DNA viruses such as simian virus 40 (SV40) are valuable models for elucidating the mechanism of replication of eukaryotic chromosomes. The SV40 genome is a circular duplex molecule of about 5.2 kilobases, which exists in a chromatin structure similar to that of cellular chromosomes. Replication starts at a unique site (*ori*) and proceeds bidirectionally around each DNA molecule (see DePamphilis & Wassarman (1982) for review; Li & Kelly (1985)). DNA synthesis terminates when the replication forks meet at a point approximately 180° from *ori* on the circular DNA and the two daughter molecules segregate into two mini-chromosomes (Sundin & Varshavsky 1980, 1981; Weaver *et al.* 1985). Initiation of replication is dependent upon the presence of the virus-encoded large-tumour (T) antigen (Tegtmeyer *et al.* 1972) whereas the remaining factors required to replicate SV40 DNA are of cellular origin.

SV40 large-T antigen (TAg) is a multifunctional 90 kDa phosphoprotein which is involved in several cellular processes, including control of transcription (Tegtmeyer *et al.* 1975; Myers *et al.* 1981; Hansen *et al.* 1981), cellular transformation and DNA replication (Rigby & Lane 1983). TAg binds specifically to the origin of SV40 replication at three defined sites, one of which (binding site II) is essential for initiation of DNA replication as defined by mutant analysis of the SV40 template (Stillman *et al.* 1985; Li *et al.* 1986). TAg binding site I is not required for SV40 replication *in vitro*, but stimulates replication two fold.

In addition to binding to the SV40 *ori* region, TAg also exhibits several biochemical properties including an ATP-dependent DNA helicase function (Stahl *et al.* 1986). Mutant TAgs that are defective for either *ori* binding or ATPase activity have been shown to be also defective for replication (Clark *et al.* 1983; Kalderon & Smith 1984; Manos & Gluzman 1984,

1985; Stillman *et al.* 1985). However, certain mutant TAg molecules which do exhibit normal origin binding and ATPase activities do not efficiently replicate SV40 DNA (Manos & Gluzman 1984, 1985; Stillman *et al.* 1985), indicating possible additional roles for TAg in the initiation of DNA replication, possibly mediated by specific protein–protein interactions.

Biochemical studies on the mechanism of SV40 DNA replication have been greatly facilitated by the development of an efficient cell-free system that uses cytosol extracts derived from monkey cells (Li & Kelly 1984). Based on this, cytosol extracts capable of replicating SV40 DNA have been prepared from various human cell-lines which can be grown in suspension cultures (Li & Kelly 1985; Stillman & Gluzman 1985; Wobbe *et al.* 1985). The use of such an *in vitro* system has allowed the initial fractionation of a cytosol extract derived from human 293 cells into several components, which may ultimately lead to the purification of single replication factors. Initial fractionation of the cytosol extract by phosphocellulose chromatography has led to the identification of two fractions, I and II, which are individually inactive for SV40 replication, but when together, restore efficient replication. Subsequent DEAE–cellulose chromatography of fraction I has further subdivided the extract into two components, A and B, which have been partly characterized and should lead to the identification and eventual purification of essential replication activities.

Materials and methods

Preparation of cell extracts and T antigen

The cytosol extracts were prepared from suspension cultures of human 293 cells as previously described (Stillman & Gluzman 1985). The cytosol extract was adjusted to 0.1 M NaCl and then subjected to centrifugation for 1 h at 100000 *g*. The supernatant (S100 fraction) was stored in aliquots at −70 °C.

SV40 T antigen was obtained from HeLa cells coinfected with wild-type adenovirus and the recombinant adenovirus vector Ad5SVR112, and purified by immunoaffinity chromatography as described previously (Stillman & Gluzman 1985). The Ad5SVR112 vector was obtained from Y. Gluzman (Cold Spring Harbor Laboratory, New York, U.S.A.) and contains the SV40 T antigen gene expressed from the adenovirus major late promoter.

Fractionation of the 293 cell cytosol

The S100 cellular extract made from 8×10^9 human 293 cells was adjusted to 0.2 M NaCl and loaded onto a 50 ml phosphocellulose column that was previously equilibrated in Buffer A (25 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.01 % (by volume) Nonidet P-40, 10 % (by volume) glycerol, 1 mM dithiothreitol, 0.1 % phenyl methyl sulphonyl fluoride (by mass)) containing 0.2 M NaCl. The column was washed with two column volumes of buffer A containing 0.2 M NaCl and eluted with a further two column volumes of buffer A containing 1 M NaCl. The protein content of each fraction was determined by the method of Bradford (1976). The unbound peak (fraction I) and the bound peak (fraction II) were extensively dialysed against buffer A containing 25 mM NaCl plus sucrose (20 % by mass) at 4 °C.

Fraction I was then adjusted to 175 mM NaCl and applied to a 40 ml DEAE–cellulose (Whatman DE-52) column that had previously been equilibrated with buffer A containing 175 mM NaCl. The column was washed with two column volumes of buffer A plus 175 mM NaCl and the bound protein eluted with buffer A containing 1 M NaCl. The protein content

of each fraction was monitored as before and the pooled peaks designated A (unbound) and B (bound) were dialysed against buffer A containing 25 mM NaCl plus sucrose (20% by mass) at 4 °C, aliquoted and stored at −70 °C.

DNA replication reactions

Replication reactions contained (final concentrations): 40 mM Hepes-KOH pH 7.5, 8 mM $MgCl_2$, 0.5 mM dithiothreitol, 100 μM each of dCTP, dGTP and dTTP, 25 μM [α-^{32}P]dATP (1000 counts per minute pmol^{-1}), 4 mM ATP, 200 μM each of CTP, GTP and UTP, 40 mM creatine phosphate and 1 μg ml^{-1} creatine phosphokinase (rabbit muscle type 1, Sigma Chemical Co., St. Louis, U.S.A.). Each reaction contained either 300 ng of plasmid pSV010 (which contains the entire SV40 genome cloned into the BamH1 site of pUC18), pSV40 or its *ori*$^-$ derivative p8–4, (Stillman & Gluzman 1985), 1 μg of purified T Antigen and cellular extracts as indicated.

All reactions were prepared on ice and started by placing the tubes at 37 °C. Reactions were terminated by addition of EDTA to 10 mM and the radioactivity incorporated into acid insoluble material determined as previously described (Stillman & Gluzman 1985).

Analysis of replication products by gel electrophoresis

Replication reactions were terminated by the addition of 10 mM EDTA and 0.1% sodium dodecyl sulphate (by mass) and digested sequentially with 20 μg ml^{-1} pancreatic ribonuclease A and 1 mg ml^{-1} Protease XIII (Sigma Chemical Co.) for 15 min and 60 min at 37 °C respectively. The samples were extracted once with phenol and the labelled DNA products separated from unincorporated nucleotides by spin dialysis with Sephadex G-50 in 1 ml syringes. The DNA was then extracted once with phenol, once with phenol:$CHCl_3$:isoamyl alcohol (24:24:1) and once with $CHCl_3$:isoamyl alcohol (24:1) and it was then ethanol precipitated. High resolution agarose gel electrophoresis was carried out at 1 V cm^{-1} as described by Sundin & Varshavsky (1980).

Enzyme assays

DNA polymerase activity was assayed by using activated calf thymus DNA as template, and DNA primase by using a synthetic primer of poly(dA)–oligo (dT) as described by Gronostajski *et al.* (1984). Assays for topoisomerases I and II were as described by DiNardo *et al.* (1984) by using supercoiled plasmid DNA and *Crithidia* kinetoplast DNA as substrates respectively.

Results

Fractionation of the 293 cell S100 extract.

The postribosomal supernatant (S100) derived from human 293 cells was initially divided by phosphocellulose chromatography into two components I and II, as described in Materials and methods. Neither fraction alone would support SV40 replication in a standard replication reaction containing TAg and *ori*-plasmid DNA, but when incubated together in optimum amounts, efficient replication was restored (figure 1). Routinely, greater than 80% of dAMP incorporation was achieved in these reactions when compared with the S100 extract. The reconstituted reaction was dependent on the presence of both TAg and a functional origin of SV40 replication (data not shown).

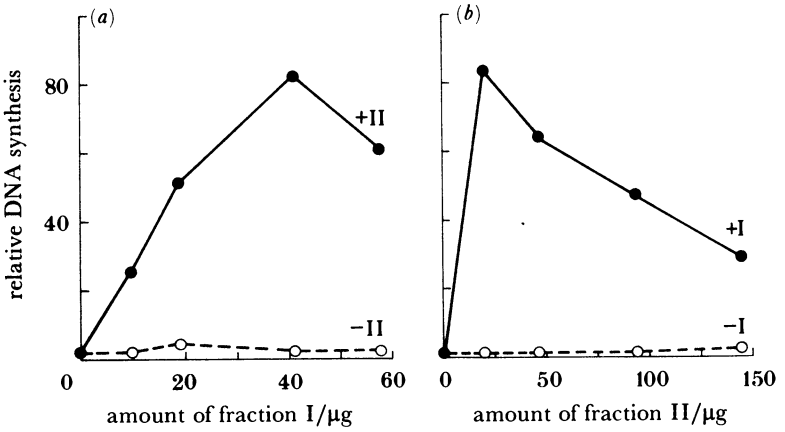

FIGURE 1. Fractionation of the 293 cytosol extract. The 293 S100 extract was divided into fractions I and II by chromatography on phosphocellulose as described in Materials and methods. (*a*) Reactions were done by using 300 ng of pSV40 plasmid and 1 μg of purified TAg in the absence (○) or presence (●) of fraction II (optimum amount 20 μg) with increasing amounts of fraction I as indicated. Reactions were terminated after 60 min at 37 °C. (*b*) Reactions were done as in (*a*). In this case reactions were either in the presence (●) or absence (○) of fraction I (optimum amount 42 μg) with increasing amounts of Fraction II. In both (*a*) and (*b*), DNA synthesis is shown as the percentage of dAMP incorporation obtained with an optimum amount (210 μg) of the unfractionated S100 extract assayed in the same experiment (36.4 pmol of dAMP incorporation in a 60 min incubation at 37 °C).

Analysis of both fractions has shown that greater than 98 % of the aphidicolin-sensitive DNA polymerase and primase activities as well as both topoisomerase I and II, are present in fraction II (table 1). These enzymes are assumed to be involved in replicative DNA synthesis.

Kinetics of the replication reaction

Previous experiments (Stillman & Gluzman 1985; Stillman *et al.* 1986; Wobbe *et al.* 1986) have demonstrated that during the time course of DNA synthesis with the unfractionated S100 extract, a 10–15 min lag occurred from the time the reactions were first transferred to 37 °C, before the onset of DNA synthesis. This lag in the replication reaction was overcome by a 15 min preincubation of the reaction mixture at 37 °C, but in the absence of precursor deoxynucleoside triphosphates (dNTPs). The preincubation reaction was shown to be dependent upon TAg, template DNA and cellular factors present in the S100 extract, as omission of any of these components resulted in the reappearance of the lag (Stillman *et al.* 1986;

TABLE 1. ENZYMIC ACTIVITIES IN FRACTIONS I AND II

	units per milligram	
	I	II
DNA polymerase†		
−aphidicolin	0.10	13.8
+aphidicolin	0.09	1.2
DNA primase†	1.8	76.9
topoisomerase I‡	<4	1739
topoisomerase II§	<4	57.9

† 1 unit = 1 nmol dAMP incorporated in 15 min at 37 °C.
‡ 1 unit = amount of enzyme required to relax 0.5 μg pBR322 in 30 min at 30 °C.
§ 1 unit = amount of enzyme required to decatenate 0.4 μg kDNA in 30 min at 30 °C.
All enzymes were assayed as described in Materials and methods.

Wobbe *et al.* 1986). These results suggest that a complex is formed between the SV40 *ori* DNA, TAg and cellular factors that promote immediate initiation of DNA replication upon addition of dNTPs. This observation therefore provides an assay for the identification of cellular proteins that are required for the presynthesis stage of the replication reaction.

The kinetics of DNA replication after a presynthesis reaction performed in the absence (none), or presence of fractions I, II or I plus II is shown in figure 2. When a component was

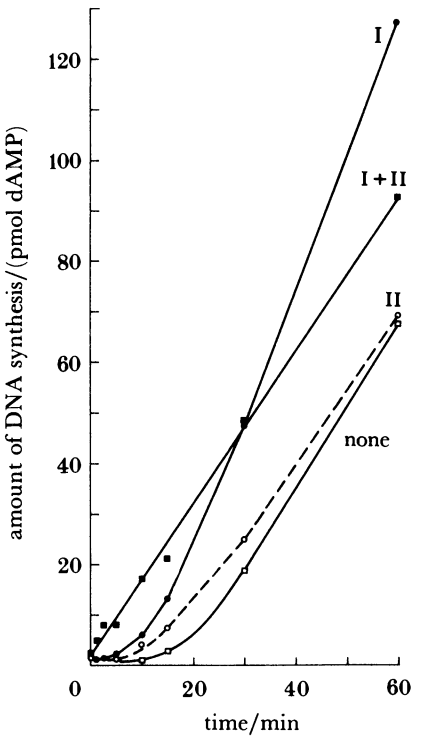

FIGURE 2. Kinetics of DNA replication in the presence or absence of fractions I and II. Time course of DNA synthesis after a 15 min preincubation at 37 °C in the absence of deoxynucleotide triphosphates (dNTPs). Preincubation reactions (100 µl) contained 600 ng pSV010, 2 µg TAg and optimum amounts of fraction I (430 µg; ●), fraction II (160 µg; ○) fractions I and II (■) or no cellular protein (□). At time zero, the omitted cellular fraction was added, together with the dNTPs to initiate DNA synthesis. Reactions were continued at 37 °C and samples were removed at the indicated time (minutes) and incorporation of dAMP into acid insoluble material was determined.

omitted during the presynthesis reaction, it was added at time zero with the precursor dNTPs to allow DNA synthesis. The absence of either fractions I or II during the presynthesis step resulted in a 10–15 min lag in dAMP incorporation, similar to that produced in the absence of both components or in the absence of unfractionated S100 extract (data not shown), whereas the presence of both components in the preincubation allowed immediate DNA synthesis. This indicated that cellular factors required for the formation of a presynthesis complex are present in both fractions I and II and could be reconstituted during the preincubation reaction.

Analysis of DNA replication products

The products synthesized by the S100 extract in the presence of TAg and template DNA are predominantly relaxed, covalently closed circular DNA molecules containing only a few supercoiled turns that migrate at the position of form II marker DNA (figure 3, lane 1).

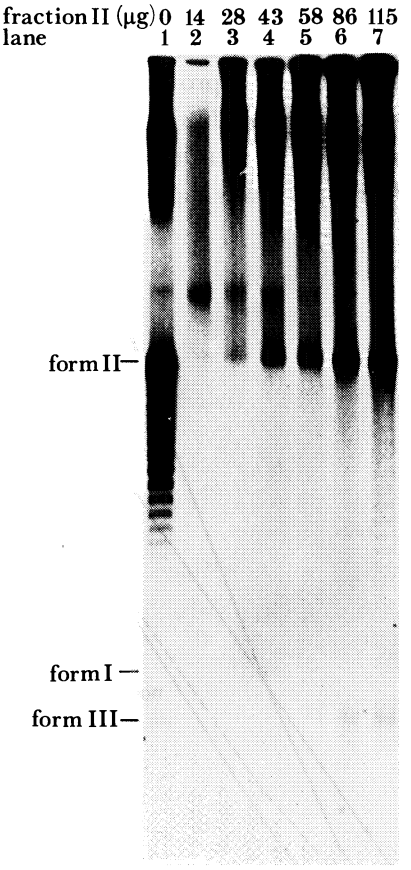

FIGURE 3. Agarose gel analysis of replicated DNA synthesized in the presence of increasing amounts of fraction II. DNA replication reactions containing 300 ng pSV40, 1 μg of purified TAg and either 300 μg of the 293 cell S100 extract (lane 1) or an optimum amount of fraction I (190 μg) in the presence of increasing amounts of fraction II (14–115 μg; lanes 2–7). After 2 h at 37 °C, the DNA was isolated as described in Materials and methods and subjected to electrophoresis in a 0.8% agarose gel at 1 V cm^{-1} after the method of Sundin & Varshavsky (1980). The gel was then dried and autoradiographed with an intensifying screen. The markers are forms I, II and III of pSV40 DNA.

Structures with reduced electrophoretic mobility are also produced, which probably correspond to multiple catenanes and other replicative intermediate structures (figure 3, lane 1). The products produced by incubating increasing amounts of fraction II in the presence of a fixed amount of fraction I are shown in figure 3, lanes 2–7. The major products synthesized in the presence of low amounts of fraction II are species migrating slower than form II marker DNA, but these are progressively converted into monomer, relaxed form I replicated daughter molecules as the level of fraction II is increased. This apparent segregation of daughter molecules into relaxed circular structures may be caused by increasing levels of topoisomerase II as more of fraction II is added to the replication reaction. However, further analysis of these slower migrating forms is necessary to determine their structure.

Subfractionation of fraction I

Because fraction I contained factor(s) required for the formation of a presynthesis complex, but lacked the major known replication enzymes (figure 2 and table 1), it was necessary to

divide this fraction further as a prerequisite for their isolation. To this end, fraction I was subjected to DEAE–cellulose chromatography as described in Materials and methods, resulting in two fractions, A and B. As described above for fractions I and II, A and B were each tested for their ability to support SV40 replication when supplemented with an optimum amount of fraction II. Figure 4*a* shows a titration of increasing amounts of fraction A in the presence of optimum amounts of fraction II, with and without fraction B. In this case, fractions A and II alone supported a low level of SV40 replication (figure 4*a*) which was stimulated upon addition of optimal amounts of fraction B. Fraction B, however, is unable to support replication in combination with fraction II, but in the presence of optimal amounts of fractions II and A, fraction B stimulates DNA synthesis (figure 4*b*). Titration of fractions II, A and B resulted in a level of DNA replication that was comparable with levels obtained with the unfractionated S100 extract.

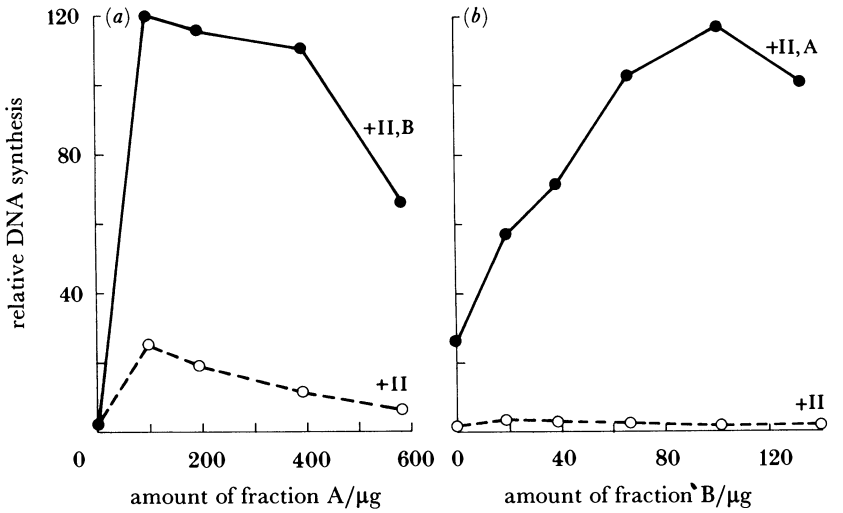

FIGURE 4. Subfractionation of the 293 cytosol. Fraction 1 derived from the 0.2 M wash from phosphocellulose chromatography of the S100 extract was further divided by DEAE–cellulose chromatography as described in Materials and methods. (*a*) Reactions were performed using 300 ng pSV010 and 1 μg purified TAg in the absence (○) or presence (●) of fraction B (optimum amount 108 μg) with increasing amounts of fraction A as indicated. All reactions contained an optimum amount of fraction II (20 μg). Reactions were terminated after 60 min at 37 °C and acid-insoluble radioactivity was measured. (*b*) Reactions were done as in (*a*). However, in this case, reactions were either in the absence (○) or presence (●) of fraction A (optimum amount 97 μg) with increasing amounts of fraction B as indicated. Again, all reactions contained an optimum amount of fraction II (20 μg). DNA synthesis is shown relative to the incorporation of dAMP with optimum amounts of fractions I and II (42 and 20 μg respectively) in the same experiment. (35.6 pmol of dAMP incorporated in a 60 min incubation at 37 °C is 100% synthesis.)

Dependence of replication with fractions A, B and II on a functional SV40 origin

The dependence of efficient DNA replication on a functional SV40 *ori* region with the S100 extract has previously been demonstrated (Stillman & Gluzman 1985). To ensure the separation of this extract into multiple components has not disrupted this requirement, replication was measured in reactions containing optimum amounts of fractions A, B and II and increasing amounts of either ori^+ or ori^- DNA templates. In this experiment, a pair of plasmids were utilized; pSV40, which contains the entire SV40 genome cloned into the $BgII^r$ pMK-16 vector (ori^+) and plasmid P8-4, which is an ori^- derivative of pSV40. This

second plasmid contains a 4 base pair (b.p.) deletion in TAg binding site II, which renders it non-functional for SV40 replication. The amount of dAMP incorporated after a 60 min incubation at 37 °C with increasing amounts of either template DNA is shown in figure 5. This demonstrates that, as with the unfractionated S100 extract (Stillman & Gluzman 1985), only plasmid pSV40 could support the efficient incorporation of dAMP, suggesting that the reaction is also still dependent upon TAg, as disruption of the essential binding site of this molecule abolishes replication.

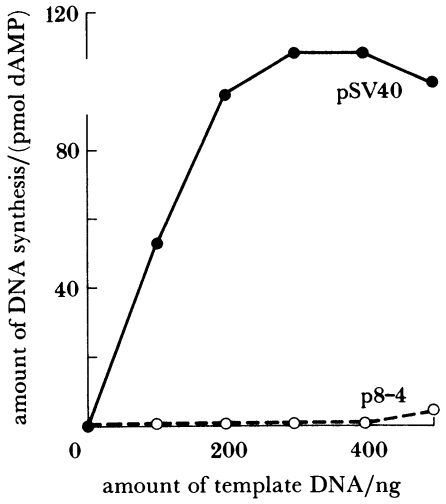

FIGURE 5. Replication with fractions II, A and B is dependent upon a functional *ori* sequence. Reactions were done with optimum amounts of fractions II (20 μg), A (97 μg) and B (108 μg) in the presence of increasing amounts of template DNA. Templates used were either pSV40, which contains a functional origin of replication, or p8–4 which contains a 4 base deletion thus inactivating the *ori* sequences. Reactions were terminated after 60 min at 37 °C and the amount of dAMP incorporated into acid insoluble material was determined.

Formation of the presynthesis complex requires fraction A

As described above, fraction I contains a component(s) that is(are) required for the efficient formation of a presynthesis complex. This has been demonstrated by the appearance of a lag in dAMP incorporation if fraction I is omitted from a 15 min preincubation period at 37 °C before DNA synthesis. To determine whether this component separated into fraction A, a similar experiment was done in which optimum amounts of fractions B and II were present in the presynthesis reaction, but in the presence or absence of an optimum amount of fraction A (figure 6*b*). A control showing the dependence of the presynthesis complex formation upon the S100 extracts is shown in figure 6*a*. Samples were taken at the time points indicated to a maximum of 45 min after the addition of dNTPs. Comparison of the curves produced in the absence or presence of fraction A in the presynthesis reaction indicates that the factor(s) required for the formation of a presynthesis complex reside in this fraction (figure 6*b*). A reciprocal experiment using fraction B as the omitted component in the presence of optimum amounts of A and II shows no effect on the rate of dAMP incorporation (Prelich *et al.* 1987*a*), indicating that the factor(s) required for complex formation has(have) segregated with fraction A. Subdivision of this fraction to identify and characterize further the functional component(s) is currently in progress.

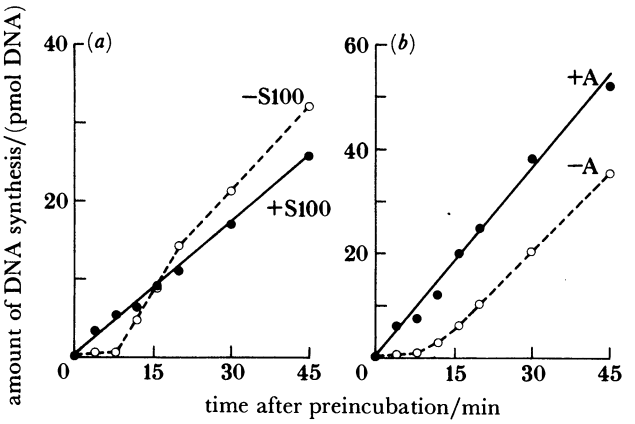

FIGURE 6. Kinetics of DNA replication after a preincubation in the presence or absence of either the S100 cytosol extract or fraction A. (*a*) Time course of DNA synthesis after a 15 min preincubation at 37 °C containing 300 ng pSV010, 1 μg TAg and the presence (●) or absence (○) of an optimum amount of the S100 cytosol extract (210 μg). In the latter case, the omitted component was added at time zero. All reactions were started by the addition of dNTPs and the reactions terminated at the times indicated after pre-incubation. (*b*) Reactions were set up as described in (*a*) with optimum amounts of fractions II (20 μg) and B (108 μg) in the presence (●) or absence (○) of an optimum amount of fraction A (97 μg). Again, the omitted component was added at time zero with the dNTPs and the reactions terminated at the times indicated after preincubation.

DISCUSSION

The S100 cytosol extract derived from human 293 cells has been divided into a number of fractions which, when reconstituted, restore efficient SV40 DNA replication. In the first fractionation step using phosphocellulose chromatography, fractions I and II have been identified, which are unable to support replication singly, but when they are combined 80% of the levels of dAMP incorporation into nascent DNA chains is achieved when compared with the unfractionated S100 extract. Analysis of both fractions has shown that the majority of polymerase, primase, topoisomerase I and II activities are present in fraction II, whereas both fractions contain components that are required for the formation of a presynthesis complex at the SV40 origin region. A role for both DNA polymerase α and DNA primase in the replication of SV40 *in vitro* has recently been described by Murakami *et al.* (1986). Subsequent fractionation of fraction I has yielded two essential fractions A and B. Fraction A is absolutely required for replication, whereas fraction B greatly stimulates the levels of dAMP incorporated. Factors required for the formation of a presynthesis complex, however, have been completely separated to fraction A, suggesting that factors in fraction B are only functioning at the elongation stage of replication.

Gel electrophoresis analysis of the products formed during replication reactions containing fractions I and II demonstrated that the distribution of the labelled products is dependent on the relative amounts of fractions I and II used, with limiting amounts of fraction II causing predominantly unsegregated, high molecular mass species. It appears that an increasing number of daughter molecules are segregated with increasing amounts of fraction II, which may correlate with the amount of topoisomerase II present in the reaction. DiNardo *et al.* (1984) have demonstrated that a mutant of *Saccharomyces cerevisiae*, defective in the production of topoisomerase II, produces similar unsegregated replication products.

Fractions II and A are absolutely required for the formation of the presynthesis complex, whereas the omission of fraction B in the preincubation reaction has no effect (data not shown). The formation of DNA–protein complexes at the origins of replication appears to be a common theme. The presence of preinitiation complexes formed before the replication of a number of prokaryotic DNAs has been reported for bateriophage ϕX174 (Wickner & Hurwitz 1975; Weiner *et al.* 1976), the *Escherichia coli* origin of replication *ori* C (van der Ende *et al.* 1985; Baker *et al.* 1986) and the origin of bacteriophage λ DNA (Dodson *et al.* 1985). Similarly, in eukaryotes, complexes are formed at the automonously replicating sequence (ARS) from the yeast *S. cerevisiae* (Jazwinski *et al.* 1983) and at the SV40 *ori* (Stillman *et al.* 1986; Wobbe *et al.* 1986). It is clear that formation of the presynthesis complex at the SV40 *ori* requires at least two cellular proteins, in addition to a functional *ori* sequence and TAg. Further characterization of these cellular proteins may identify those replication proteins that regulate initiation of DNA replication throughout the cell cycle.

Using fraction B as starting material, we have recently purified a protein that is an essential component for full length SV40 DNA replication *in vitro*. This 36 kDa protein was identified as the cell cycle regulated protein, alternatively called the proliferating cell nuclear antigen (PCNA) or cyclin (Prelich *et al.* 1987*a*). PCNA synthesis occurs just before DNA synthesis in each cycle of cell division, and it displays a specific nuclear immunofluorescent pattern only in S-phase nuclei (Tan 1982; Celis *et al.* 1986; Bravo 1986). Also Prelich *et al.* (1987*b*) demonstrated that PCNA was functionally equivalent to an auxiliary protein for the foetal calf thymus DNA polymerase δ, a novel DNA polymerase in mammalian cells (Lee *et al.* 1984; Tan *et al.* 1986; Crute *et al.* 1986). This result raises the possibility that polymerase δ is required for replication of SV40 DNA *in vitro*, and suggests that it is required for replication of cellular chromosomes. We expect that similar purification and characterization of other essential replication proteins will yield valuable insight into mammalian DNA replication.

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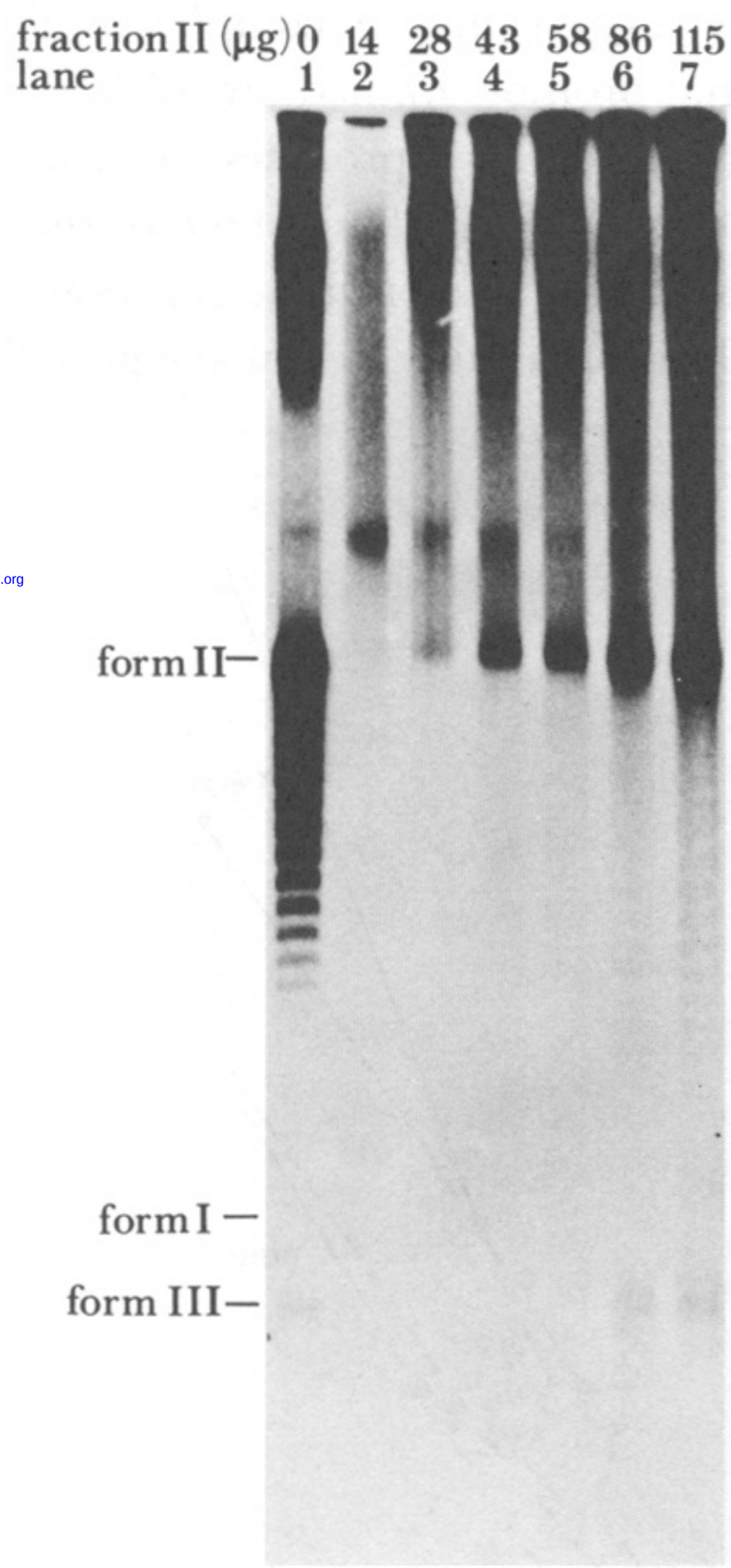

GURE 3. Agarose gel analysis of replicated DNA synthesized in the presence of increasing amounts of fraction II. DNA replication reactions containing 300 ng pSV40, 1 μg of purified TAg and either 300 μg of the 293 cell S100 extract (lane 1) or an optimum amount of fraction I (190 μg) in the presence of increasing amounts of fraction II (14–115 μg; lanes 2–7). After 2 h at 37 °C, the DNA was isolated as described in Materials and methods and subjected to electrophoresis in a 0.8% agarose gel at 1 V cm^{-1} after the method of Sundin & Varshavsky (1980). The gel was then dried and autoradiographed with an intensifying screen. The markers are forms I, II and III of pSV40 DNA.

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