PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

In vitro Replication of DNA Containing Either the SV40 or the Polyoma Origin

C. R. Wobbe, F. B. Dean, Y. Murakami, J. A. Borowiec, P. Bullock and J. Hurwitz

Phil. Trans. R. Soc. Lond. B 1987 **317**, 439-453
doi: 10.1098/rstb.1987.0071

Phil. Trans. R. Soc. Lond. B **317**, 439–453 (1987)
Printed in Great Britain

In vitro replication of DNA containing either the SV40 or the polyoma origin

By C. R. Wobbe, F. B. Dean, Y. Murakami, J. A. Borowiec, P. Bullock and J. Hurwitz

Memorial Sloan Kettering Cancer Center, Graduate Program in Molecular Biology, 1275 York Avenue/Box 97, New York, New York 10021, U.S.A.

The replication of DNA containing either the polyoma or SV40 origin has been done *in vitro*. Each system requires its cognate large-tumour antigen (T antigen) and extracts from cells that support its replication *in vivo*. The host-cell source of DNA polymerase α – primase complex plays an important role in discriminating between polyoma T antigen and SV40 T antigen-dependent replication of their homologous DNA. The SV40 origin- and T antigen-dependent DNA replication has been reconstituted *in vitro* with purified protein components isolated from HeLa cells. In addition to SV40 T antigen, HeLa DNA polymerase α – primase complex, eukaryotic topoisomerase I and a single-strand DNA binding protein from HeLa cells are required. The latter activity, isolated solely by its ability to support SV40 DNA replication, sediments and copurifies with two major protein species of 72 and 76 kDa. Although crude fractions yielded closed circular monomer products, the purified system does not. However, the addition of crude fractions to the purified system resulted in the formation of replicative form I (RFI) products. We have separated the replication reaction with purified components into multiple steps. In an early step, T antigen in conjunction with a eukaryotic topoisomerase (or DNA gyrase) and a DNA binding protein, catalyses the conversion of a circular duplex DNA molecule containing the SV40 origin to a highly underwound covalently closed circle. This reaction requires the action of a helicase activity and the SV40 T antigen preparation contains such an activity. The T antigen associated ability to unwind DNA copurified with other activities intrinsic to T antigen (ability to support replication of SV40 DNA containing the SV40 origin, poly dT-stimulated ATPase activity and DNA helicase).

Introduction

In vitro DNA replication systems for SV40 and polyoma have recently been described (Ariga & Sugano 1983; Li & Kelly 1984; Stillman & Gluzman 1985; Wobbe *et al.* 1985; Murakami *et al.* 1986*a*, *b*). These systems demonstrate most of the protein, DNA sequence and host range requirements observed for these viruses *in vivo* and should therefore be useful for studying the mechanism of viral and, to a certain extent, cellular DNA replication. Some progress has been made in identifying and isolating viral and host factors required for papovavirus DNA replication. For example, the large-T antigen, which appears to be the only virus protein required for DNA replication *in vivo* (De Pamphilis & Wassarman 1982), can be readily purified and has been studied extensively. This protein has been implicated in both the initiation (see Tooze (1980)) and elongation (Stahl *et al.* 1985, 1986) phases of DNA synthesis. The host DNA polymerase α – primase complex also appears to be required *in vivo* (Tooze 1980) and *in vitro* (Li & Kelly 1984; Stillman & Gluzman 1985; Wobbe *et al.* 1985; Murakami

et al. 1986*a*, *b*) and may play some role in determining cell permissiveness for papovavirus DNA replication (Murakami *et al.* 1986*a*, *b*). It also seems likely, based on prokaryotic replication systems (Marians 1984), that single-stranded DNA (ssDNA) binding, topoisomerase, RNase H and DNA ligase activities are required for complete replication. Recent results (Stahl *et al.* 1986) suggest that another activity, that of a DNA helicase, is intrinsically part of T antigen. With these observations and predictions in mind, we have sought to isolate host factors required for papovavirus DNA replication *in vitro* and reconstitute this process with purified components. This report describes the partial reconstitution of SV40 DNA replication with apparently purified components of known function, one of which is a previously uncharacterized single-stranded DNA binding activity. In addition, an origin-dependent DNA melting reaction done by T antigen is described that partly clarifies the complex role of this multifunctional protein in papovavirus DNA replication.

Materials and methods

Materials

SV40 ori$^+$ DNA (pSV01 EP), T antigen and crude extract of HeLa cells were prepared as described previously (Wobbe *et al.* 1985). Construction of plasmid pSVLD6-1, containing an inactivating deletion within the SV40 origin of plasmid pSVLD, has been published (Dean *et al.* 1987). HeLa DNA polymerase α – primase was purified to apparent homogeneity by immunoaffinity chromatography (Y. Murakami, unpublished data) and HeLa topoisomerase I was purified to apparent homogeneity by the procedure of Liu & Miller (1981). *E. coli* single-stranded DNA binding protein (SSB) and S1 nuclease were purchased from P–L biochemicals. *E. coli* topoisomerase I and DNA gyrase were kindly provided by Dr K. Marians of the Memorial Sloan Kettering Cancer Center.

Assays

DNA replication assays and product analyses were done as described previously (De Pamphilis *et al.* 1982). Single-stranded and double-stranded DNA binding assays, similar to those used for nuclear factor I (Nagata *et al.* 1983), are described elsewhere (Wobbe *et al.* 1987). The procedures for the DNA unwinding, DNA helicase and ATPase assays have been described (Dean *et al.* 1987). The substrate for the DNA unwinding reaction was prepared by relaxation of pSVLD or pSVLD6-1 with topoisomerase I.

Fractionation of HeLa extracts

By using ammonium sulphate precipitation, HeLa crude extract was resolved into two fractions, AS30 and AS65, both of which are required to reconstitute replication activity (Wobbe *et al.* 1987). Activity present in the AS30 fraction was further purified by Biorex-70 and DNA cellulose column chromatography as described (Wobbe *et al.* 1987), eluting as a single component in each case.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Results and discussion

Replication of SV40 and polyoma origin-containing DNAs in crude extract

The requirements for the replication of SV40 origin-containing DNA in crude extract of HeLa cells and of polyoma origin-containing DNA in crude extract of mouse FM3A cells are summarized in tables 1 and 2, respectively. Both systems are dependent on the cognate origin and T antigen, cell extract, dNTPs and an ATP regenerating system. Inhibition by aphidicolin

Table 1. Requirements for SV40 DNA replication *in vitro*

(Replication reaction mixtures containing crude extract of HeLa cells were prepared as described previously (Wobbe *et al.* 1985).)

component omitted (−) or added (+)	dNMP incorporated pmol per 2 h
complete	620
−SV40 T antigen	10
−SV40 DNA	<4
−SV40 DNA+pBR322 DNA	8
−SV40 DNA+topoisomerase I treated SV40 DNA	516
−SV40 DNA+*Pst* I digested SV40 DNA	16
−ATP	214
−creatine phosphate and creatine phosphokinase	56
−dATP, dGTP, dTTP	19
−CTP, GTP, UTP	496
+aphidicolin (100 μM)	9
+camptothecin (500 μM)	260

Table 2. Requirements for polyoma DNA replication *in vitro*

(Replication reaction mixtures containing crude extracts of FM3A cells were prepared as described previously (Murakami *et al.* 1986).)

component omitted (−) or added (+)	dTMP incorporated pmol
complete	65.7
−extract	<1
−polyoma DNA	<1
$-Mg^{2+}$	<1
−polyoma DNA+pBR322 DNA	<1
−polyoma T antigen	<1
−ATP	17.0
−creatine phosphate	<1
−CTP, GTP, UTP	36.3
−dATP, dCTP, dGTP	6.9
+aphidicolin (200 μM)	<1

suggests that DNA polymerase α is required for nucleotide polymerization. The extract requirement is further constrained by the fact that it must be from a source permissive for the replication of the given virus *in vivo* (table 3). SV40 DNA is not replicated in (non-permissive) mouse cell extracts and polyoma DNA is not replicated in (non-permissive) HeLa cell extracts.

The *in vitro* host-range specificity may be at least partly explained by the requirement for a

TABLE 3. EXTRACT, ORIGIN AND T ANTIGEN SOURCE REQUIREMENTS FOR PAPOVAVIRUS DNA REPLICATION *IN VITRO*

(Reaction mixtures were prepared as described previously (Murakami *et al.* 1986).)

source of:			dTMP incorporated
origin DNA	extract	T antigen	pmol
polyoma	FM3A	polyoma	76
polyoma	FM3A	SV40	<1
polyoma	HeLa	polyoma	5
polyoma	HeLa	SV40	3
SV40	HeLa	SV40	93
SV40	HeLa	polyoma	<1
SV40	FM3A	SV40	3
SV40	FM3A	polyoma	<1
pBR322	FM3A	polyoma	<1
pBR322	HeLa	SV40	<1

DNA polymerase α – primase complex from a permissive cell source. HeLa extracts depleted of polymerase α – primase by immunoaffinity chromatography were inactive for SV40 replication. These extracts were reactivated by the addition of polymerase α – primase from permissive cells (monkey or human) but not from non-permissive cells (Murakami *et al.* 1986*b*). Non-permissive mouse cell extracts were activated for SV40 replication by supplementation with HeLa polymerase α – primase complex (Murakami *et al.* 1986*b*). Similarly, polyoma replication occurred only in the presence of mouse polymerase α – primase complex (Murakami *et al.* 1986*a*). Together, these results suggest that DNA polymerase α – primase plays a major role in determining permissiveness for papovavirus replication and that all other factors can be supplied by permissive or non-permissive cells.

Fractionation of HeLa extracts

We have previously shown that crude cytosolic extracts could be fractionated by ammonium sulphate precipitation (Wobbe *et al.* 1986). This procedure has been modified to give two fractions, a 0–30% fraction (AS30) and a 35–65% fraction (AS65). Both fractions are absolutely necessary for replication activity (figure 1). The activity in the AS30 was further purified by using complementation with the AS65 fraction as an assay for SV40 DNA replication activity. Complementing activity in the AS30 eluted as a single fraction between 0.05 and 0.1 M NaCl from Biorex-70. This activity was further purified by native DNA cellulose chromatography, where it eluted in a single step between 0.25 and 1.0 M NaCl (Wobbe *et al.* 1986). Titration of the native DNA cellulose eluate in the presence or absence of saturating levels of the AS65 fraction is shown in figure 1. Both fractions are absolutely required for replication activity as are SV40 T antigen and the SV40 origin of replication.

The AS65 fraction contains DNA polymerase α, DNA primase, topoisomerase I and II, RNase H and DNA ligase activities, whereas the native DNA cellulose eluate did not have detectable levels of these activities. Experiments using various combinations of the enzymes listed above indicated that a combination of HeLa polymerase α – primase complex and topoisomerase I efficiently substituted for the AS65 fraction in stimulating nucleotide incorporation with the native DNA cellulose fraction (figure 1 and table 4). Replication in this purified system was also dependent on SV40 T antigen, a DNA containing the SV40 origin and

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

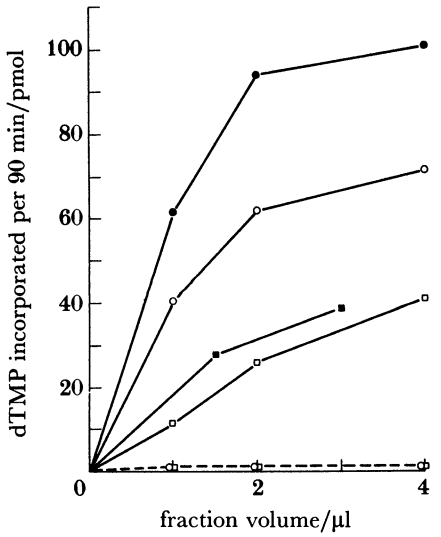


FIGURE 1. Titration of fractions required for SV40 DNA replication *in vitro*. The AS30 (12.4 mg protein per millilitre) (□, ■), or double-stranded DNA (ds DNA) cellulose (0.1 mg protein per millilitre) (○ and ●) fractions were added to reactions containing either 85 μg AS65 fraction protein (closed symbols) or HeLa DNA polymerase α – primase and topoisomerase I as indicated in Materials and methods (open symbols) in the presence (solid lines) or absence (dashed line) of SV40 T antigen. After incubation at 37 °C for 90 min, acid-insoluble radioactivity was determined.

dNTPs. Like the crude system, replication was observed in the presence of permissive cell (HeLa) polymerase α – primase but not the presence of the enzyme from non-permissive (mouse or calf thymus) cells.

It was also possible to reconstitute nucleotide incorporating activity by using the polyoma system supplemented with the HeLa native DNA cellulose fraction. In this case replication was dependent on polyoma T antigen and mouse DNA polymerase α – primase complex, and HeLa polymerase α – primase being inactive (table 4). These results are consistent with the proposal that factors other than DNA polymerase α – primase can be supplied by permissive or non-permissive cells (see above).

Products synthesized by the purified system

Labelled products synthesized in reactions containing the purified factors described above were examined by agarose gel electrophoresis in the presence of ethidium bromide (figure 2). In contrast to the products of reactions containing the ammonium sulphate fractions (lane 1), those from reactions containing the purified fractions contained no detectable covalently closed circles (lane 2). This is not surprising because these reactions do not contain activities required for completion of covalently closed circular DNA synthesis, RNase H and DNA ligase. Attempts to stimulate such synthesis in these reactions by adding RNase H and DNA ligase were unsuccessful. Further supplementation with topoisomerase II yielded small amounts of RFI (data not shown). Covalently closed circles were synthesized in reactions containing the purified components supplemented with the AS65 fraction, but not the AS30 fraction (lanes 4, 5, 6). These results suggest that factors in addition to RNase H, ligase and topoisomerase II are required to form covalently closed products and these factors are present in the AS65 fraction.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

TABLE 4. REQUIREMENTS FOR REPLICATION WITH THE PURIFIED SYSTEM

component omitted (−) or added (+)	dTMP incorporated pmol per 2 h
(*a*) complete	108
−polymerase α − primase, topoisomerase I, +AS65	112
−T antigen, or ds DNA cellulose fraction or HeLa polymerase α − primase	<1
−HeLa polymerase α − primase, +mouse polymerase α − primase	4
−HeLa polymerase α − primase, +calf thymus polymerase α − primase	<1
−HeLa topoisomerase I	51
−dATP, dCTP, dGTP	<1
−CTP, GTP, UTP	61
−ATP	23
−DNA	<1
−ori$^+$ DNA, +pBR322 EP	3
−ori$^+$ RFI DNA, +*Pst* I linearized ori$^+$ DNA	78
(*b*) FM3A crude extract+T antigen	57
−Py T antigen	2
FM3A polymerase α − primase+HeLa ds DNA cellulose fraction+HeLa topoisomerase I+Py T antigen	32
−Py T antigen, or FM3A polymerase α − primase or ds DNA cellulose fraction	<1
−FM3A polymerase α − primase, +HeLa polymerase α − primase	<1
−HeLa topoisomerase I	27

(*a*) The complete system contained HeLa DNA polymerase α − primase (0.32, 0.5 unit, respectively), HeLa topoisomerase I (1000 units), ori$^+$ DNA (180 ng) and 0.25 μg protein of the ds DNA cellulose fraction.

(*b*) 300 μg protein of FM3A (mouse) cell cytosolic extract and 0.6 μg protein polyoma (Py) T antigen, both prepared as described (Murakami *et al.* 1986) were added to reactions previously described. Cytosolic extract was replaced by 0.4 unit FM3A DNA polymerase α − primase and HeLa topoisomerase I and ds DNA cellulose fraction in amounts indicated above.

Properties of the activity in the native DNA cellulose eluate

As mentioned above, the native DNA cellulose fraction contained no detectable polymerase α − primase, topoisomerase I or II, RNase H and DNA ligase activities. Nitrocellulose filter binding assays indicated, however, that this fraction does contain a DNA binding activity preferential for single-stranded DNA (figure 3). The binding to duplex DNA saturates at a low level of DNA retention (approximately 10%) whereas single-stranded DNA was quantitatively retained.

When the native DNA cellulose fraction was applied to a denatured DNA cellulose column and eluted with a linear 0.5–2.0 M NaCl gradient, a single peak of replication activity was detected eluting at approximately 1.1 M NaCl (figure 4). Coeluting with this activity were a pair of proteins of 72 and 76 kDa visualized by SDS–polyacrylamide gel electrophoresis (figure 4, bottom). The high affinity for single-stranded DNA cellulose suggests that the replication activity in this fraction is a single-stranded DNA binding protein.

The above data were further supported by glycerol gradient sedimentation analysis of the native DNA cellulose fraction. As shown in figure 5, the replication activity, DNA binding activity and proteins of 72 and 76 kDa precisely cosedimented at 5.1 *S*. The replication activity behaved as a single component and was not stimulated by the addition of other glycerol gradient fractions.

Table 5 summarizes the purification of the replication activity from the AS30 fraction through the denatured DNA cellulose step. Taken together, these data suggest that one of the primary replication activities of the AS30 fraction is a single component, a 72–76 kDa

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

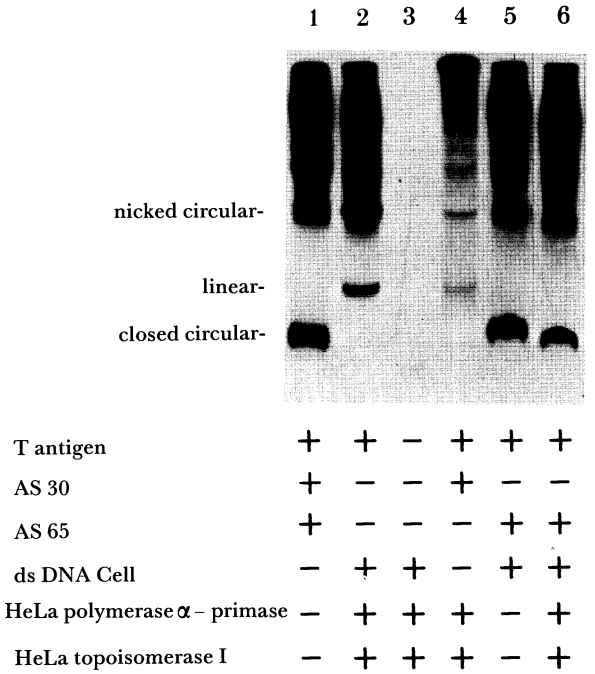


FIGURE 2. Comparison of products synthesized by crude and purified fractions. [α-^{32}P]dCTP labelled products from 60 min reactions containing the fractions indicated were isolated and electrophoresed as described previously (Wobbe *et al.* 1986). Amounts of fraction added per reaction (in micrograms of protein) were: 37 μg AS30, 85 μg AS65, 0.2 μg ds DNA cellulose fraction and HeLa DNA polymerase α – primase, topoisomerase I and SV40 T antigen as indicated in table 4.

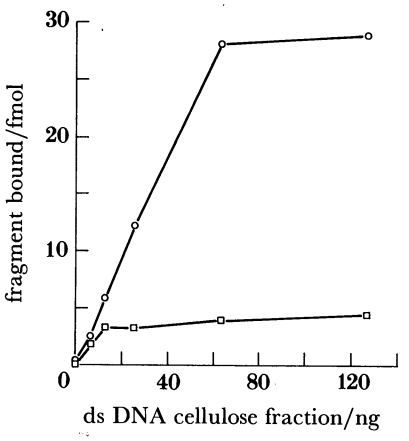


FIGURE 3. Binding of native (□) or heat-denatured (○) DNA by the ds DNA cellulose fraction. Assays were done as described previously (Nagata *et al.* 1983).

polypeptide having a single-stranded DNA binding activity. Moreover, the single-stranded DNA binding activity and the replication activity are sensitive to heat ($t_{\frac{1}{2}}$ equals 45 s at 55 °C) and resistant to *N*-ethylmaleimide (5 mM, 10 min, 0 °C). This would constitute the first isolation of a mammalian single-stranded DNA binding protein demonstrably required for DNA replication.

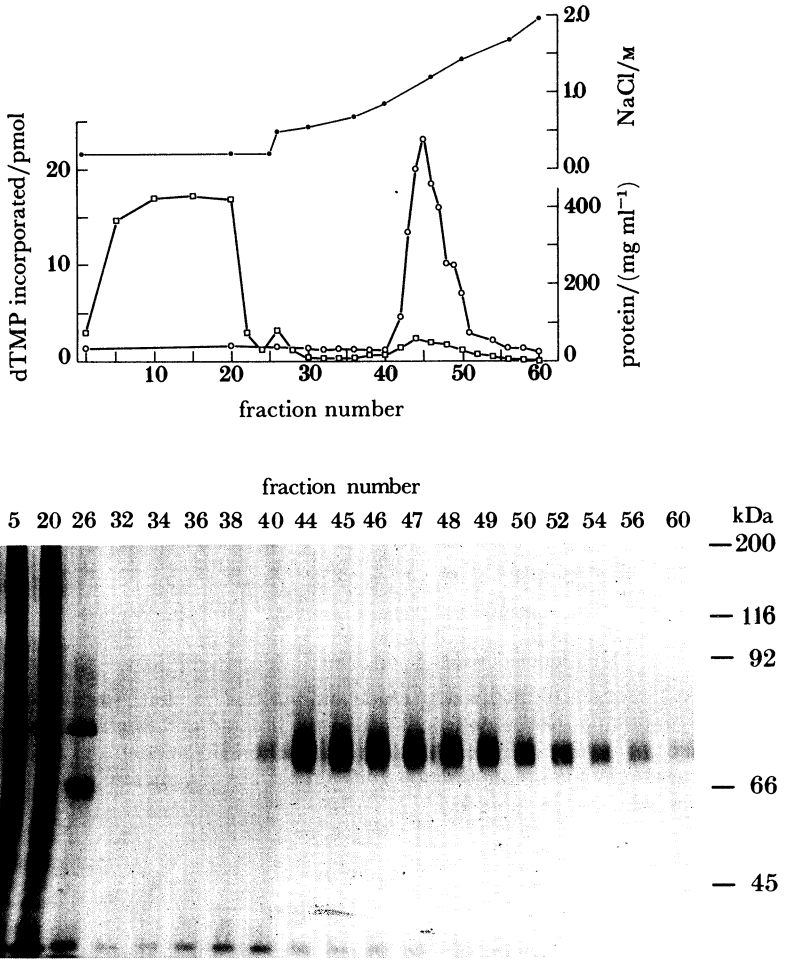


FIGURE 4. Single-stranded DNA cellulose chromatography of the ds DNA cellulose fraction. The ds DNA cellulose fraction was applied to a denatured DNA cellulose column as described previously (Wobbe *et al.* 1986), which was then eluted with a linear 0.5–2.0 M NaCl gradient (filled circles) in 20 mM HEPES–NaOH (pH 7.0), 1 mM DTT, 0.1 mM EDTA, 10% (by volume) glycerol. Fractions were assayed for protein (□) and replication activity complementing the AS65 fraction (○). (Bottom) 10 µl portions of the indicated fractions were electrophoresed on an SDS–polyacrylamide gel and stained as described previously (Wobbe *et al.* 1986). Molecular mass standard mobilities are given in kilodaltons (kDa).

Origin-dependent DNA unwinding catalysed by T antigen

The apparently small number of protein factors necessary for nucleotide incorporation in the system described above suggests that at least some of these factors perform multiple functions. Indeed, T antigen has recently been shown to possess an intrinsic DNA helicase activity probably linked to its ATPase activity (Stahl *et al.* 1986). Thus, in addition to its role in origin-specific DNA binding and initiation, this protein may also play a role in elongation.

An early step in the replication of circular duplex DNA must involve unwinding of the duplex, exposing templates for the synthetic machinery. In the *Escherichia coli oriC* system, for example, this process is done by a complex of proteins in separable steps: the Dna A protein binds to the origin followed by the formation of a multiprotein complex at the origin, then the combined action of Dna B, Dna C, HU protein, SSB and gyrase melts the duplex and reduces

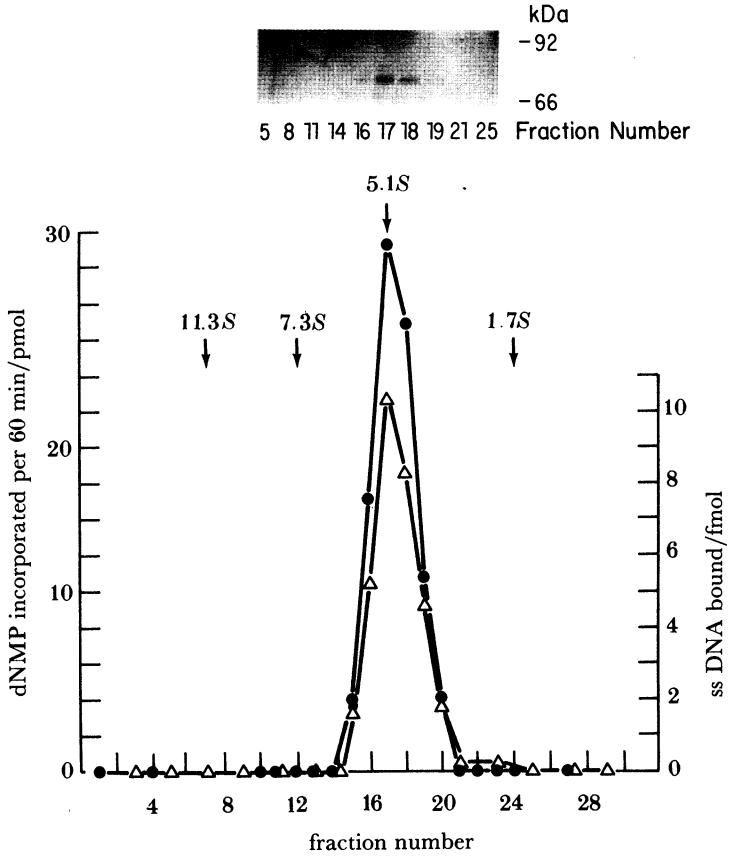


FIGURE 5. Glycerol gradient sedimentation of the ds DNA cellulose fraction. The ds DNA cellulose fraction (50 µl) was sedimented through a linear, 15–30 % (by volume) glycerol gradient as described (Wobbe *et al.* 1986). Fractions (175 µl) were collected from the bottom. 3 µl portions were assayed for replication activity complementing the AS65 fraction (●) and 2 µl portions were assayed for ss DNA binding activity (△). Arrows marked 11.3 *S*, 7.3 *S* and 1.7 *S* indicate positions of markers (catalase, aldolase and cytochrome *c*, respectively) and 5.1 *S* indicates the sedimentation coefficient corresponding to the peak of replication and binding activities. Top, silver-stained SDS–polyacrylamide gel of indicated fractions.

TABLE 5. PURIFICATION OF HeLa 72/76 kDa DNA BINDING ACTIVITY

fraction	protein/mg	activity/units	specific activity / units mg^{-1}
crude	1055	110	0.1
AS30 pellet	174	152	0.9
Biorex-70 eluate	9.1	194	21.3
dsDNA cellulose eluate	0.23	53	230
ssDNA cellulose eluate	0.03	8.1	270

Starting material was crude extract from 10 l of late log-phase culture of HeLa cells prepared and fractionated as described previously (Wobbe *et al.* 1987). One unit of replication activity stimulates incorporation of one nmol dTMP into acid insoluble material in 60 min at 37 °C in reactions containing 0.6 µg SV40 T antigen, 0.32 unit DNA polymerase α, 0.5 unit DNA primase and 1000 units HeLa topoisomerase I (see Wobbe *et al.* (1987) for complete description of all units).

the topological linking number of the DNA (Baker *et al.* 1986). The unwinding reaction itself is presumably mediated directly by the Dna B helicase activity (LeBowitz & McMacken 1986).

We have assayed for a similar DNA unwinding reaction dependent upon the SV40 origin, reasoning that T antigen, with its origin-specific DNA binding and helicase activities, may be involved. Relaxed circular DNA (containing the SV40 origin) was incubated with T antigen, a topoisomerase and an SSB in the presence of ATP. The products were then examined for the formation of negatively supercoiled DNA by agarose gel electrophoresis. Relaxed DNA substrates allowed easier visualization of the products, but RFI could also be used as a substrate (data not shown).

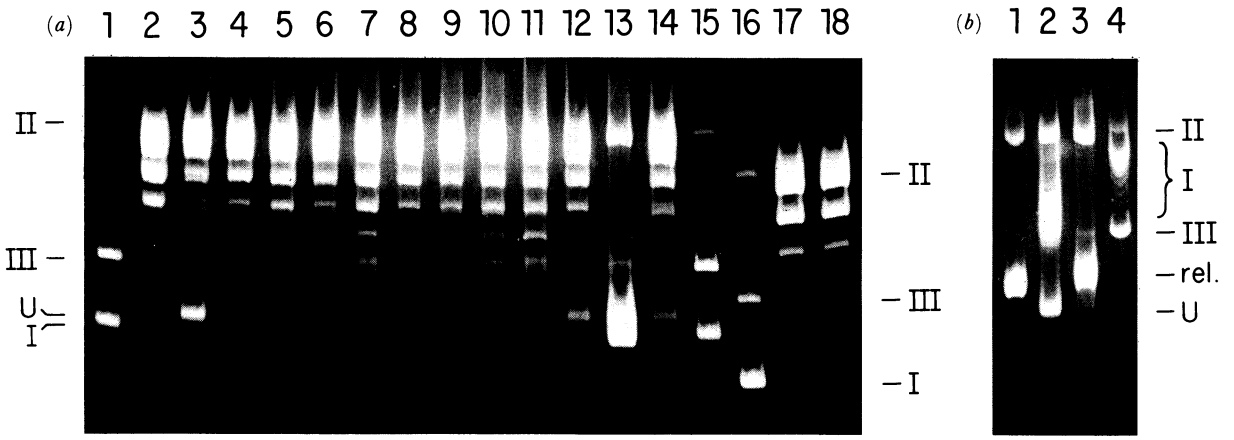


FIGURE 6. Detection and requirements for form U formation. (*a*) Lanes: 1–15, pSV01 EP (ori$^+$) DNA; 16–18, pBR322 EP (ori$^-$) DNA; 1, 15, and 16 contained nicked, linear, and supercoiled plasmid DNA markers; 2 and 17 contained relaxed substrate plasmid DNA; 3 and 18 contained complete reaction; 4, T antigen omitted; 5, HeLa topoisomerase I omitted; 6, *E. coli* SSB omitted; 7, ATP omitted; 8, $MgCl_2$ omitted; 9, creatine phosphate and creatine kinase omitted; 10, incubation was 25 °C instead of 37 °C; 11, topoisomerase I omitted and 20 units of *E. coli* topoisomerase I (ω protein) added; 12, topoisomerase I omitted and 30 units of HeLa topoisomerase II added; 13, topoisomerase I omitted and 16 units *E. coli* DNA gyrase added; 14, *E. coli* SSB omitted and 0.4 μg of adenovirus DNA binding protein added. Markers for lanes 15–18 are at right. (*b*) Chloroquine gel. Electrophoresis was done with chloroquine phosphate added to a concentration of 15 μg ml^{-1}. Reactions contained (ori$^+$) DNA. Lanes: 1, relaxed substrate plasmid DNA, 2, topoisomerase I omitted and 16 units *E. coli* DNA gyrase added; 3, complete reaction (containing HeLa topoisomerase I); 4, nicked, linear, and supercoiled plasmid DNA markers. I, II, and III, RFI RFII, and RFIII, respectively; U, form U; rel., relaxed DNA.

As shown in figure 6, incubation of ori$^+$ DNA with SV40 T antigen, HeLa topoisomerase I and *E. coli* SSB resulted in the formation of a DNA product with an electrophoretic mobility slightly slower than RFI. Formation of this product, designated as form U, was dependent on the proteins listed above in addition to ATP, Mg^{2+} and an ATP regenerating system. It did not occur if the incubation temperature (normally 37 °C) was lowered to 25 °C (figure 6, lanes 4–10). *E. coli* topoisomerase I, which cannot relax positive supercoils, did not substitute for HeLa topoisomerase I, which can relax positive supercoils. HeLa topoisomerase II and *E. coli* DNA gyrase, both of which can relax positive supercoils, efficiently replace HeLa topoisomerase I (lanes 11–13), suggesting that, as expected, the unwinding reaction generates positive superhelicity. The SSB requirement could also be met by either the adenovirus DNA binding

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

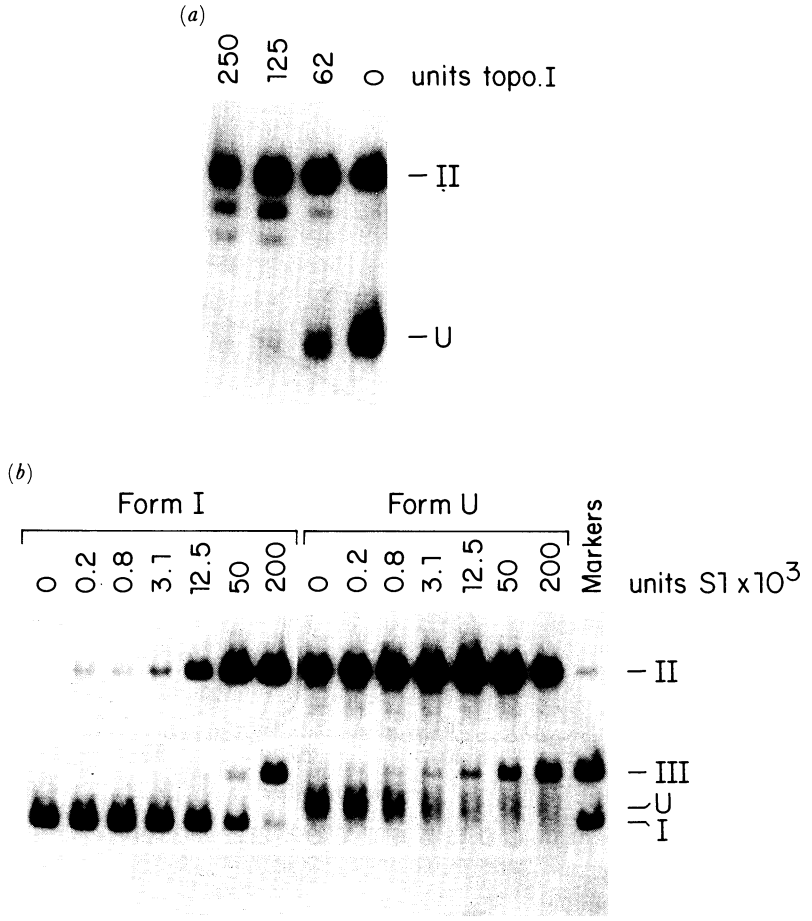


FIGURE 7. Structure analysis of form U. (*a*) Treatment with HeLa topoisomerase I. Form U was isolated from an agarose gel as described (Dean *et al.* 1987). Form U (2 ng) was incubated with the indicated units of HeLa topoisomerase I in 20 mM Tris–HCl at pH 7.5, 5 mM EDTA and 0.2 mM KCl containing 50 μg of bovine serum albumin per millilitre for 30 min at 37 °C and electrophoresed. (*b*) Treatment with nuclease S1. Supercoiled DNA (2 ng, form I) or the isolated form U (2 ng) was incubated with the indicated amount of nuclease S1 (in units S1 × 10^3) in 30 mM sodium acetate at pH 4.6, 50 mM NaCl, 1 mM $ZnSO_4$ and 5% (by volume) glycerol for 30 min at 30 °C. Reactions were terminated and electrophoresed as described (Dean *et al.* 1987). Southern blots of the gels were probed with labelled pBR322 sequences, and autoradiograms are shown.

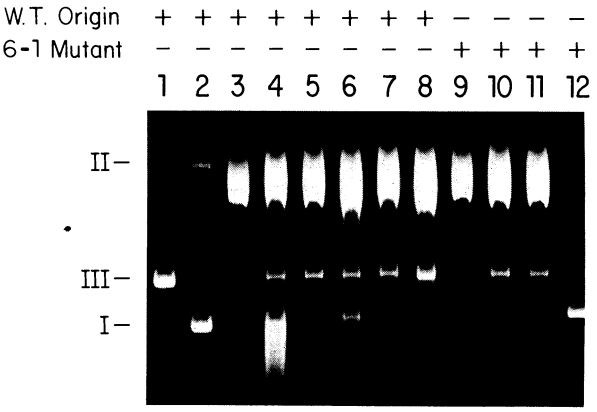


FIGURE 8. Comparison of unwinding of intact SV40 origin with mutant SV40 origin. Lanes: 1–8, pSVLD (wild-type) plasmid; 9–12, pSVLD6-1 (mutant) plasmid. Lanes: 1, linear plasmid; 2 and 12, supercoiled and nicked plasmid circles; 3 and 9, relaxed plasmid substrate; 4 and 10, complete reaction; 5 and 11, T antigen omitted; 6, topoisomerase I omitted; 7, *E. coli* SSB omitted; and 8, ATP omitted. Formation of form U in lane 6 was due to detectable topoisomerase I activity in this T antigen preparation.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

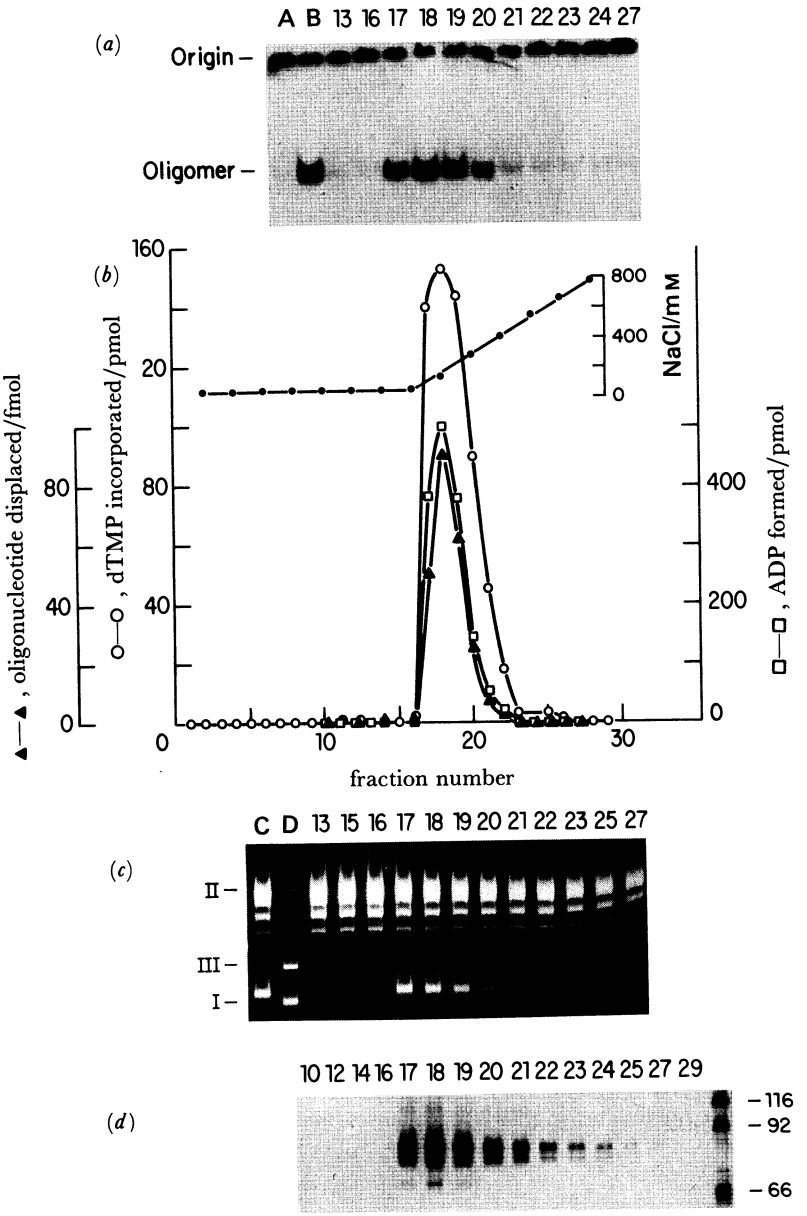


FIGURE 9. Chromatography of SV40 T antigen on DEAE–Sepharose and the coelution of various enzymic activities. Immunoaffinity purified T antigen (60 µg of protein) was loaded onto a 100 µl DEAE–Sepharose CL-6B column (Pharmacia), which had been equilibrated with 20 mM HEPES–NaOH at pH 7.5, 1 mM EDTA, 50 mM NaCl, 1 mM dithiothreitol and 40% (by volume) ethylene glycol. The column was washed with 300 µl of this buffer, and then T antigen was eluted with a linear NaCl gradient (50 mM–1 M NaCl, 600 µl total). Each fraction contained 50 µl of eluate. (*a*) Helicase assays (Dean *et al.* 1987) were done with 3 µl samples as indicated. At the peak fraction, 65% of the input substrate was used. The values presented in the graph were obtained by cutting out the oligonucleotide area of the gel and counting by liquid scintillation. Lane A contained SV40-oligonucleotide (130 fmol) hydrogen bonded to M13mp9 DNA and no enzyme. Under the conditions used, 3% of the input ^{32}P-labelled DNA migrated as free oligonucleotide. Lane B included 250 ng of T antigen. (*b*) The assays for ATPase and DNA replication were done with 2 µl and 3 µl, respectively, of each sample. (*c*) Unwinding assay was done with 3 µl of each fraction indicated. Lane C, control reaction using 0.75 µg of T antigen; lane D, nicked (II), linear (III), and supercoiled-plasmid markers. (*d*) SDS–polyacrylamide gel electrophoresis was done on 3 µl of the indicated fractions, and the T antigen was visualized by silver staining.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

protein (lane 14) or the HeLa DNA binding activity described above (data not shown). DNA lacking the 311 base pair (b.p.) cloned origin-containing fragment was inactive in this assay (lanes 16–18).

To characterize the structure of form U DNA, the product was electroeluted from the gel, purified and subjected to enzymic analysis. Form U was converted to relaxed circles by both HeLa and *E. coli* topoisomerase I (figure 7*a* and data not shown) suggesting that it was circular and negatively supercoiled. When form U was treated with dilutions of nuclease S1, it was found that approximately 16-fold less of this enzyme was required to give 50% nicking of form U than RFI (figure 7*b*). This suggests that form U has more single-stranded character and is therefore more highly negatively supercoiled than RFI.

To determine whether the unwinding reaction specifically required the origin of replication or whether other T antigen binding sites would suffice, a substrate with a 6 b.p. deletion

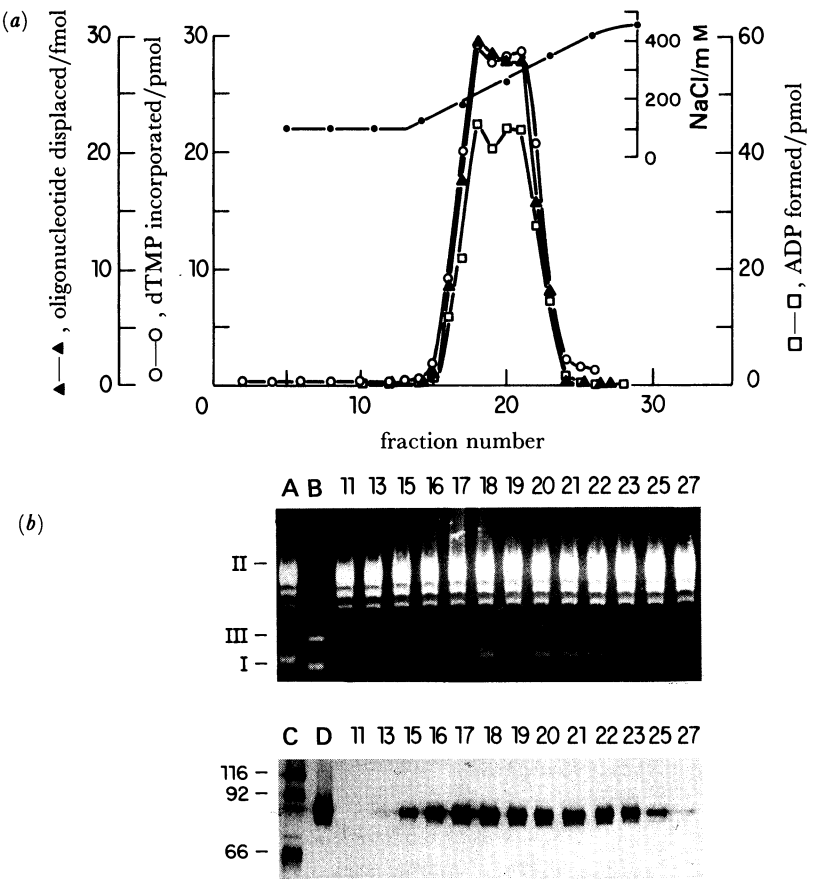


FIGURE 10. Chromatography of SV40 T-antigen on phosphocellulose. Immunoaffinity purified T antigen (50 μg of protein) was loaded onto a 100 μl phosphocellulose column (Whatman P-11) that had been equilibrated with 20 mM HEPES–NaOH at pH 7.5, 1 mM EDTA, 50 mM NaCl, 1 mM dithiothreitol and 40% (by volume) ethylene glycol. The column was washed with 300 μl of this buffer and then T antigen was eluted with a linear NaCl gradient (50 mM–1 M NaCl, 500 μl total). Each fraction contained about 50 μl of eluate. (*a*) Helicase, ATPase, and DNA replication assays were done by using 3 μl and 2 μl of the indicated fractions. (*b*) Unwinding assays were done by using 4 μl of each fraction as indicated. Lane A, control reaction with 1 μg of T antigen; lane B, nicked, linear, and supercoiled plasmid markers. (*c*) SDS–polyacrylamide gel electrophoresis with 3 μl of the indicated fractions, and the proteins were visualized by silver staining. Lane C, relative molecular mass size markers ($M_r \times 10^{-3}$); lane D, immunoaffinity purified T antigen.

(pSVLD6-1), disrupting T antigen binding site II (the core origin) but leaving sites I and III intact, was assayed. This DNA was inactive in the unwinding reaction, whereas a comparable plasmid (pSVLD), which contains an intact origin, was active (figure 8). This suggests that an intact, fully functional origin, T antigen binding site II, is required for the unwinding reaction.

Multiple activities copurify with T antigen

The results discussed above suggest that T antigen is a multifunctional replication protein, possessing sequence specific DNA binding, ATPase, helicase and origin specific DNA melting activities. To investigate further this possibility, immunoaffinity purified T antigen was further chromatographed on DEAE–sepharose (figure 9) or phosphocellulose (figure 10). In both cases, replication, ATPase, helicase and origin-dependent unwinding activities coeluted with the protein peak containing T antigen visualized by SDS–polyacrylamide gel electrophoresis. Thus we conclude that all of these activities are intrinsic to the T antigen polypeptide. Whether different modified forms of this protein, not yet resolved, contain varying levels of each of these activities remains to be determined.

In summary, this report suggests that the protein requirements for nucleotide incorporation during SV40 DNA replication *in vitro* are surprisingly simple. This may be due in large part to the multiplicity of functions performed by T antigen. In addition, a possible interaction between T antigen and DNA polymerase α – primase complex (Smale & Tjian 1986) may explain the species specificity of the polymerase α – primase requirement in SV40 and polyoma replication. Moreover, the combination of activities present in the partly purified SV40 replication system is similar to that found necessary for *E. coli* chromosomal replication *in vitro* (Baker *et al.* 1986). Other activities that stimulate nucleotide incorporation (both rate and length of products) and promote the formation of RFI products remain to be found. Some candidates for such factors, including RNase H, DNA ligase and topoisomerase II, are easily purified. Isolation and characterization of these activities should shed light on the mechanism of papovavirus and, to some extent, mammalian cellular DNA replication.

We thank Ms C. Turck and Ms N. Belgado for their assistance. This work was supported by National Institutes of Health Grant 5R01-GM34559; F. B. D. is supported by a fellowship from the National Institutes of Health; P. B. is supported by a fellowship from the American Cancer Society; C. R. W. is supported by a Damon Runyon – Walter Winchell Cancer Fund Fellowship.

References

Ariga, H. & Sugano, S. 1983 Initiation of simian virus 40 DNA replication *in vitro*. *J. Virol.* **48**, 481–491.

Baker, T. A., Sekimizu, K., Funnell, B. E. & Kornberg, A. 1986 Extensive unwinding of the plasmid template during staged enzymatic initiation of DNA replication from the origin of the *Escherichia coli* chromosome. *Cell* **45**, 53–64.

Dean, F. B., Bullock, P., Murakami, Y., Wobbe, C. R., Weissbach, L. & Hurwitz, J. 1987 Simian virus 40 (SV40) DNA replication: SV40 large T antigen unwinds DNA containing the SV40 origin of replication. *Proc. natn. Acad. Sci. U.S.A.* **84**, 16–20.

De Pamphilis, M. L. & Wassarman, P. M. 1982 Organization and replication of papovavirus DNA. In *Organization and replication of viral DNA* (ed. A. S. Kaplan), pp. 37–114. Boca Raton, Florida: CRC Press.

Li, J. J. & Kelly, T. J. 1984 Simian virus 40 DNA replication *in vitro*. *Proc. natn. Acad. Sci. U.S.A.* **81**, 6973–6977.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

LeBowitz, J. H. & McMacken, R. 1986 The *Escherichia coli* dnaB replication protein is a DNA helicase. *J. biol. Chem.* **261**, 4738–4748.

Liu, L. F. & Miller, K. G. 1981 Eukaryotic DNA topoisomerases: two forms of type I DNA topoisomerases from HeLa cell nuclei. *Proc. natn. Acad. Sci. U.S.A.* **78**, 3487–3491.

Marians, K. 1984 Enzymology of DNA replication in procaryotes. *Crit. Rev. Biochem.* **17**, 153–187.

Murakami, Y., Eki, T., Yamada, M.-A., Prives, C. & Hurwitz, J. 1986*a* Species specific *in vitro* synthesis of DNA containing the origin of polyoma virus. *Proc. natn. Acad. Sci. U.S.A.* **83**, 6347–6351.

Murakami, Y., Wobbe, C. R., Weissbach, L., Dean, F. B. & Hurwitz, J. 1986*b* The role of DNA polymerase α and DNA primase in simian virus 40 DNA replication *in vitro*. *Proc. natn. Acad. Sci. U.S.A.* **83**, 2869–2873.

Nagata, K., Guggenheimer, R. A. & Hurwitz, J. 1983 Specific binding of a cellular DNA replication protein to the origin of replication of adenovirus DNA. *Proc. natn. Acad. Sci. U.S.A.* **80**, 6177–6181.

Smale, S. T. & Tjian, R. 1986 T-Antigen – DNA polymerase α complex implicated in simian virus 40 DNA replication. *Molec. cell. Biol.* **6**, 4077–4087.

Stillman, B. W. & Gluzman, Y. 1985 Replication and supercoiling of simian virus 40 DNA in cell extracts from human cells. *Molec. cell. Biol.* **5**, 2051–2060.

Stahl, H., Droge, P. & Knippers, R. 1986 DNA helicase activity of SV40 large tumor antigen. *EMBO J.* **5**, 1939–1944.

Stahl, H., Droge, P., Zentgraf, H. & Knippers, R. 1985 A large-tumor-antigen-specific monoclonal antibody inhibits DNA replication of simian virus 40 minichromosomes in an *in vitro* elongation system. *J. Virol.* **4**, 473–482.

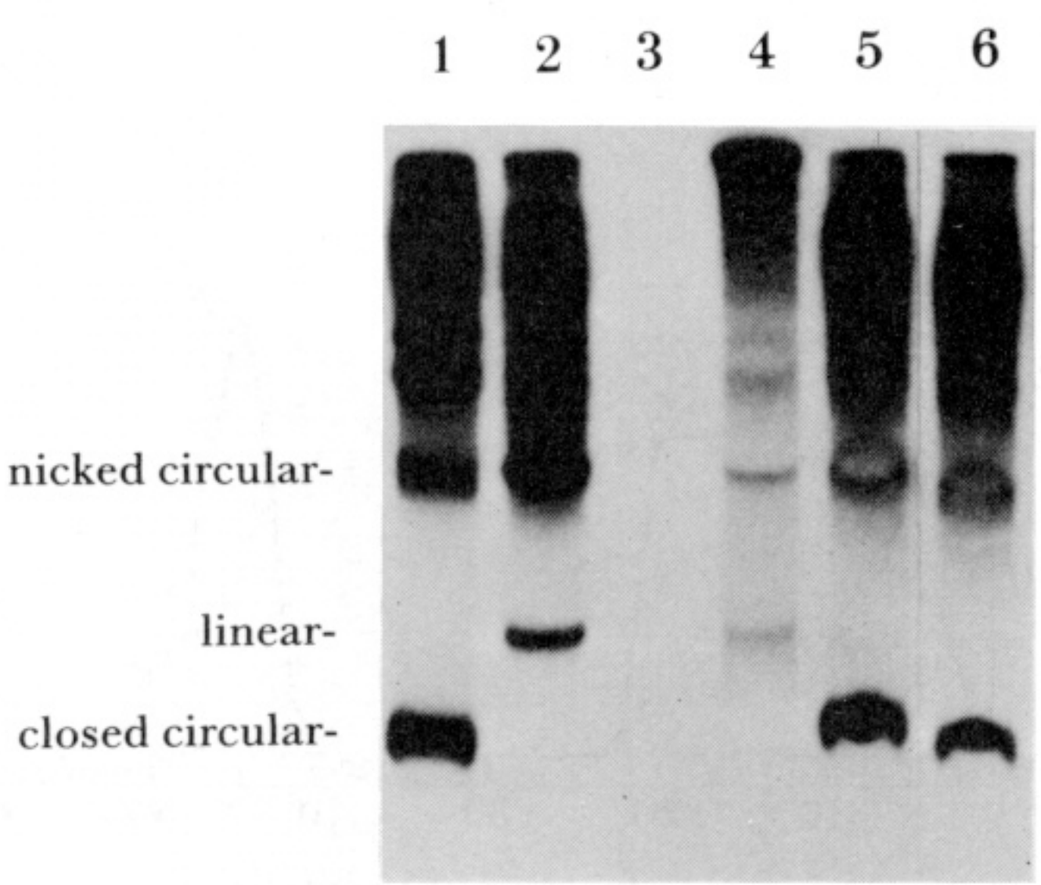
Tooze, J. (ed.) 1980 *Molecular and cellular biology of tumor viruses*, part 2. New York: Cold Spring Harbor Laboratory.

Wobbe, C. R., Dean, F. B., Murakami, Y., Weissbach, L. & Hurwitz, J. 1986 Simian virus 40 DNA replication *in vitro*: study of events preceding elongation of chains. *Proc. natn. Acad. Sci. U.S.A.* **83**, 4612–4616.

Wobbe, C. R., Dean, F. B., Weissbach, L. & Hurwitz, J. 1985 *In vitro* replication of duplex circular DNA containing the simian virus 40 DNA origin site. *Proc. natn. Acad. Sci. U.S.A.* **82**, 5710–5714.

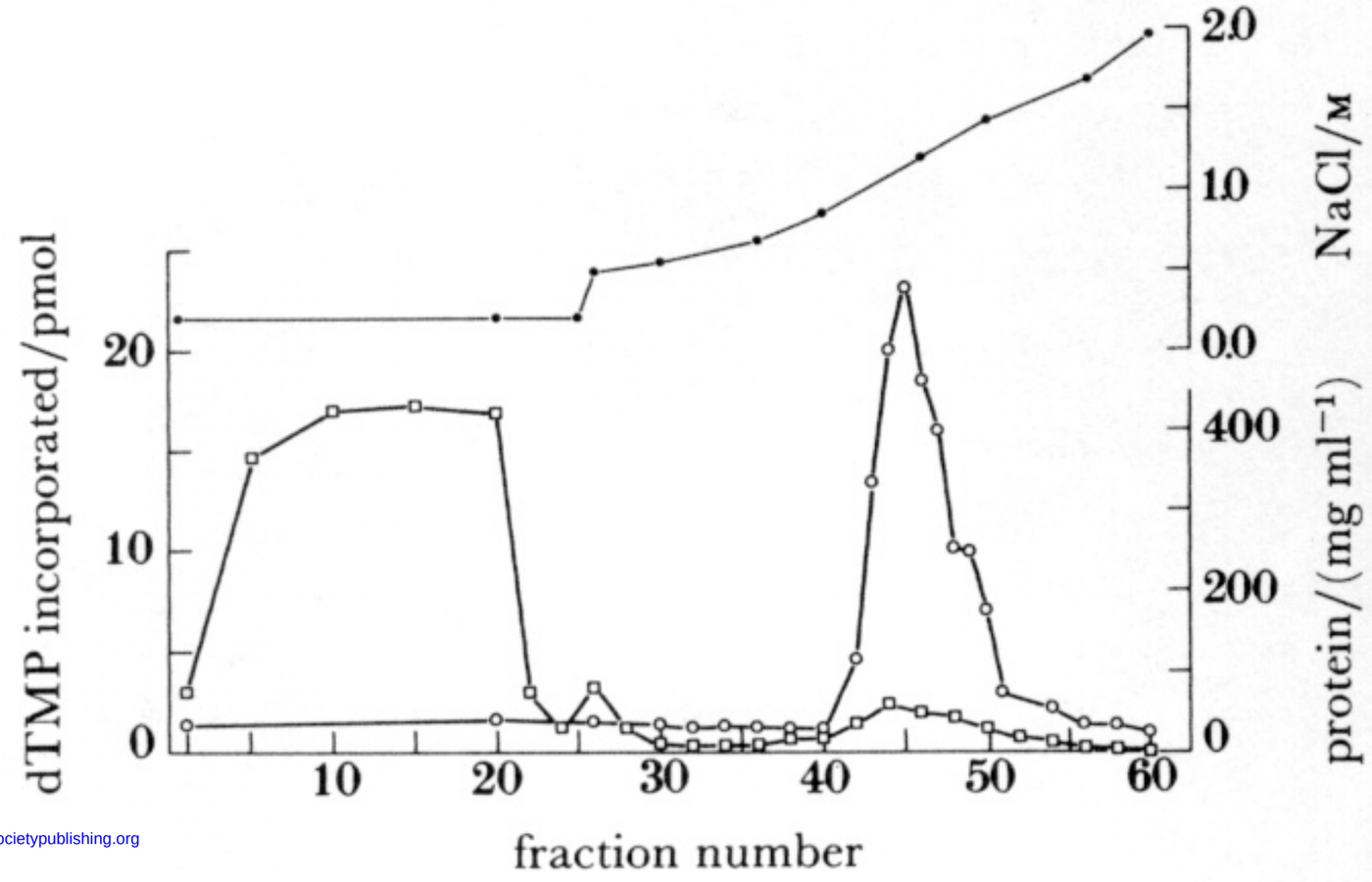
Wobbe, C. R., Weissbach, L., Boroweic, J. A., Dean, F. B., Murakami, Y., Bullock, P. & Hurwitz, J. 1987 Replication of SV40 origin-containing DNA *in vitro* with purified proteins. *Proc. natn. Acad. Sci. U.S.A.* **84**, 1834–1838.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES



T antigen	+	+	−	+	+	+
AS 30	+	−	−	+	−	−
AS 65	+	−	−	−	+	+
ds DNA Cell	−	+	+	−	+	+
HeLa polymerase α – primase	−	+	+	+	−	+
HeLa topoisomerase I	−	+	+	+	−	+

IGURE 2. Comparison of products synthesized by crude and purified fractions. [α-^{32}P]dCTP labelled products from 60 min reactions containing the fractions indicated were isolated and electrophoresed as described previously (Wobbe *et al.* 1986). Amounts of fraction added per reaction (in micrograms of protein) were: 37 μg AS30, 85 μg AS65, 0.2 μg ds DNA cellulose fraction and HeLa DNA polymerase α – primase, topoisomerase I and SV40 T antigen as indicated in table 4.



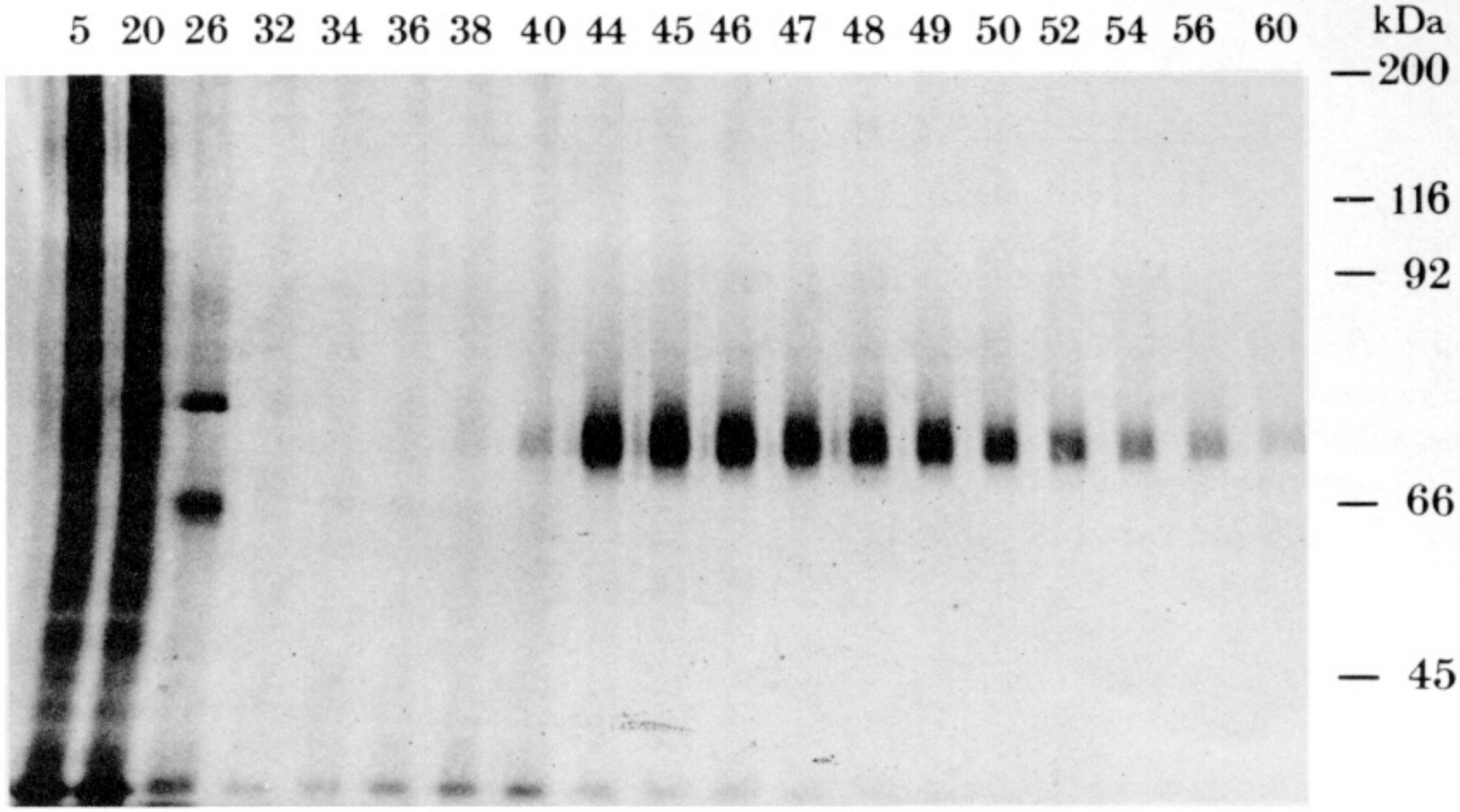
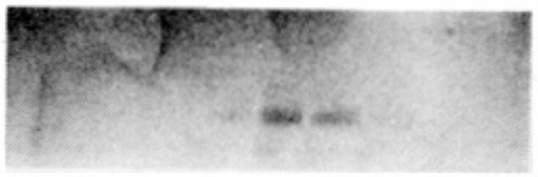
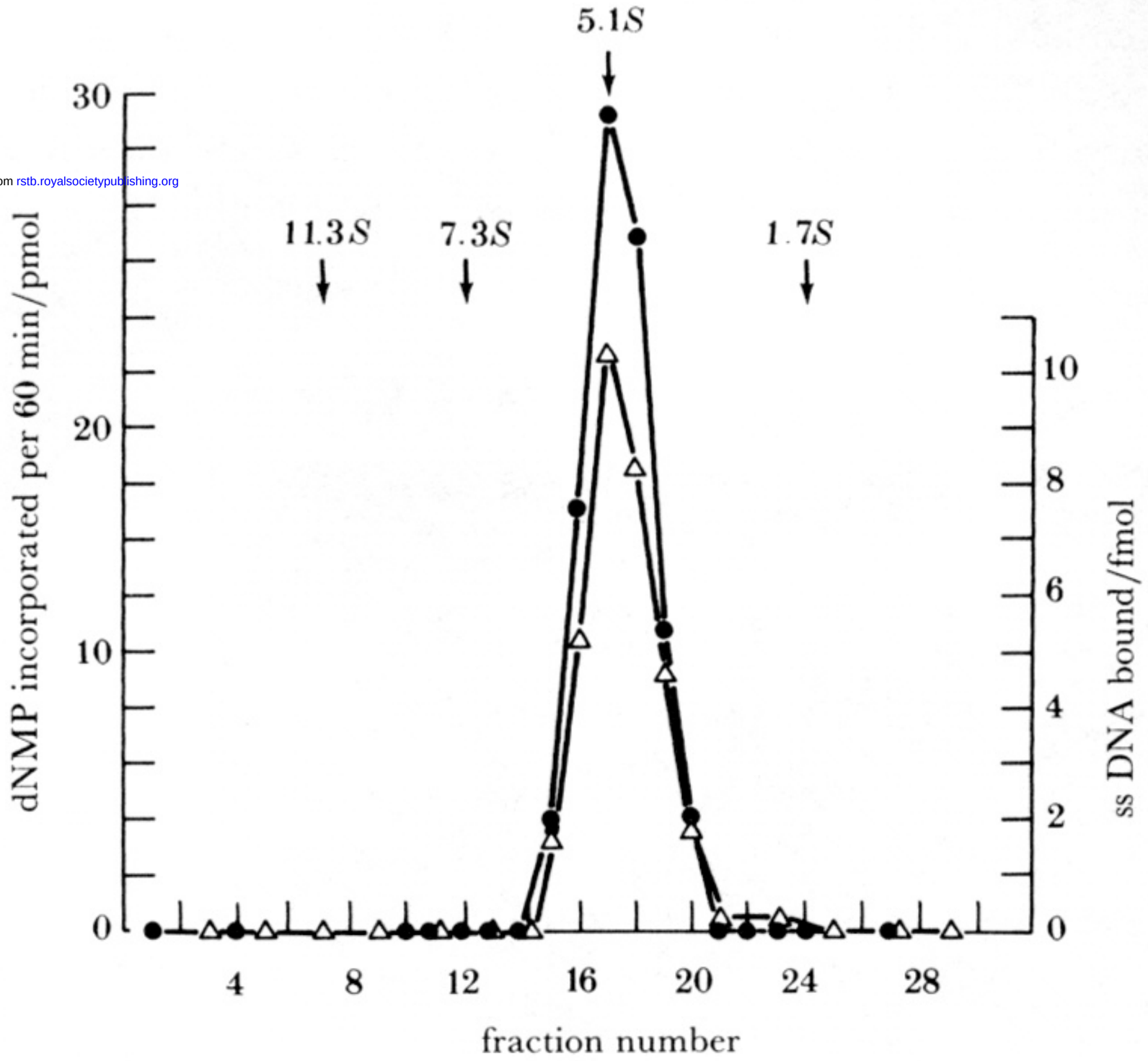


FIGURE 4. Single-stranded DNA cellulose chromatography of the ds DNA cellulose fraction. The ds DNA cellulose fraction was applied to a denatured DNA cellulose column as described previously (Wobbe *et al.* 1986), which was then eluted with a linear 0.5–2.0 M NaCl gradient (filled circles) in 20 mM HEPES–NaOH (pH 7.0), 1 mM DTT, 0.1 mM EDTA, 10% (by volume) glycerol. Fractions were assayed for protein (□) and replication activity complementing the AS65 fraction (○). (Bottom) 10 µl portions of the indicated fractions were electrophoresed on an SDS–polyacrylamide gel and stained as described previously (Wobbe *et al.* 1986). Molecular mass standard mobilities are given in kilodaltons (kDa).





GURE 5. Glycerol gradient sedimentation of the ds DNA cellulose fraction. The ds DNA cellulose fraction (50 µl) was sedimented through a linear, 15–30 % (by volume) glycerol gradient as described (Wobbe *et al.* 1986). Fractions (175 µl) were collected from the bottom. 3 µl portions were assayed for replication activity complementing the AS65 fraction (●) and 2 µl portions were assayed for ss DNA binding activity (△). Arrows marked 11.3 *S*, 7.3 *S* and 1.7 *S* indicate positions of markers (catalase, aldolase and cytochrome *c*, respectively) and 5.1 *S* indicates the sedimentation coefficient corresponding to the peak of replication and binding activities. Top, silver-stained SDS–polyacrylamide gel of indicated fractions.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

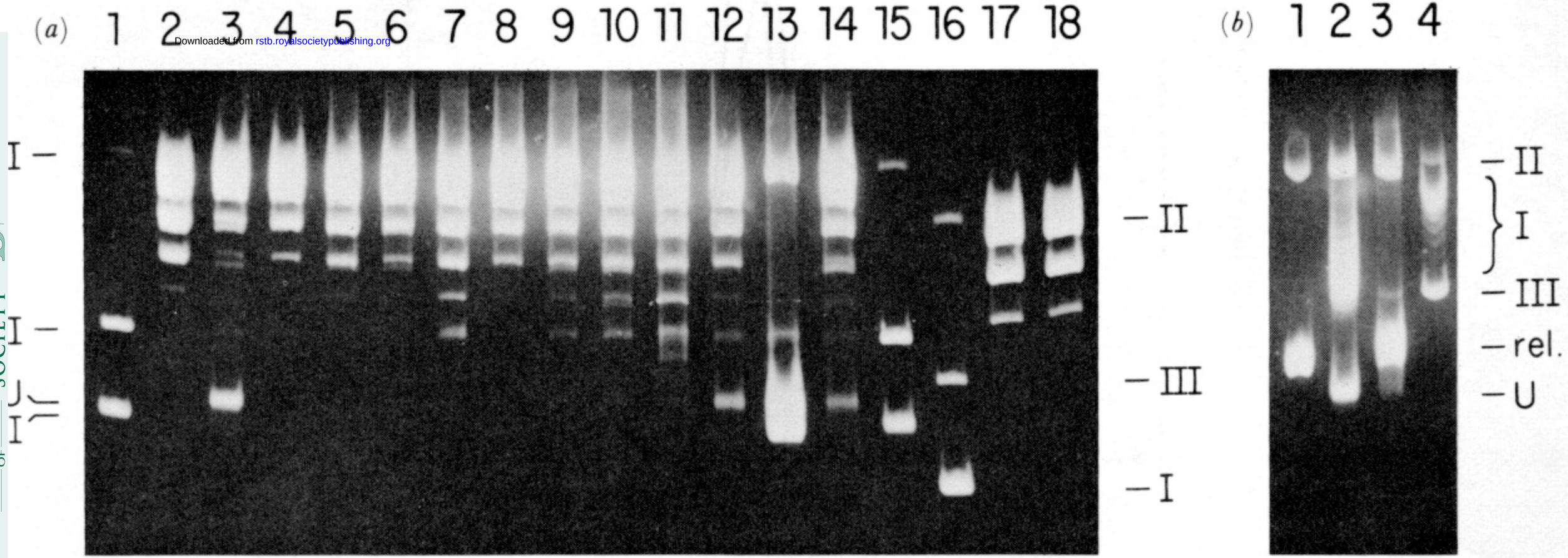
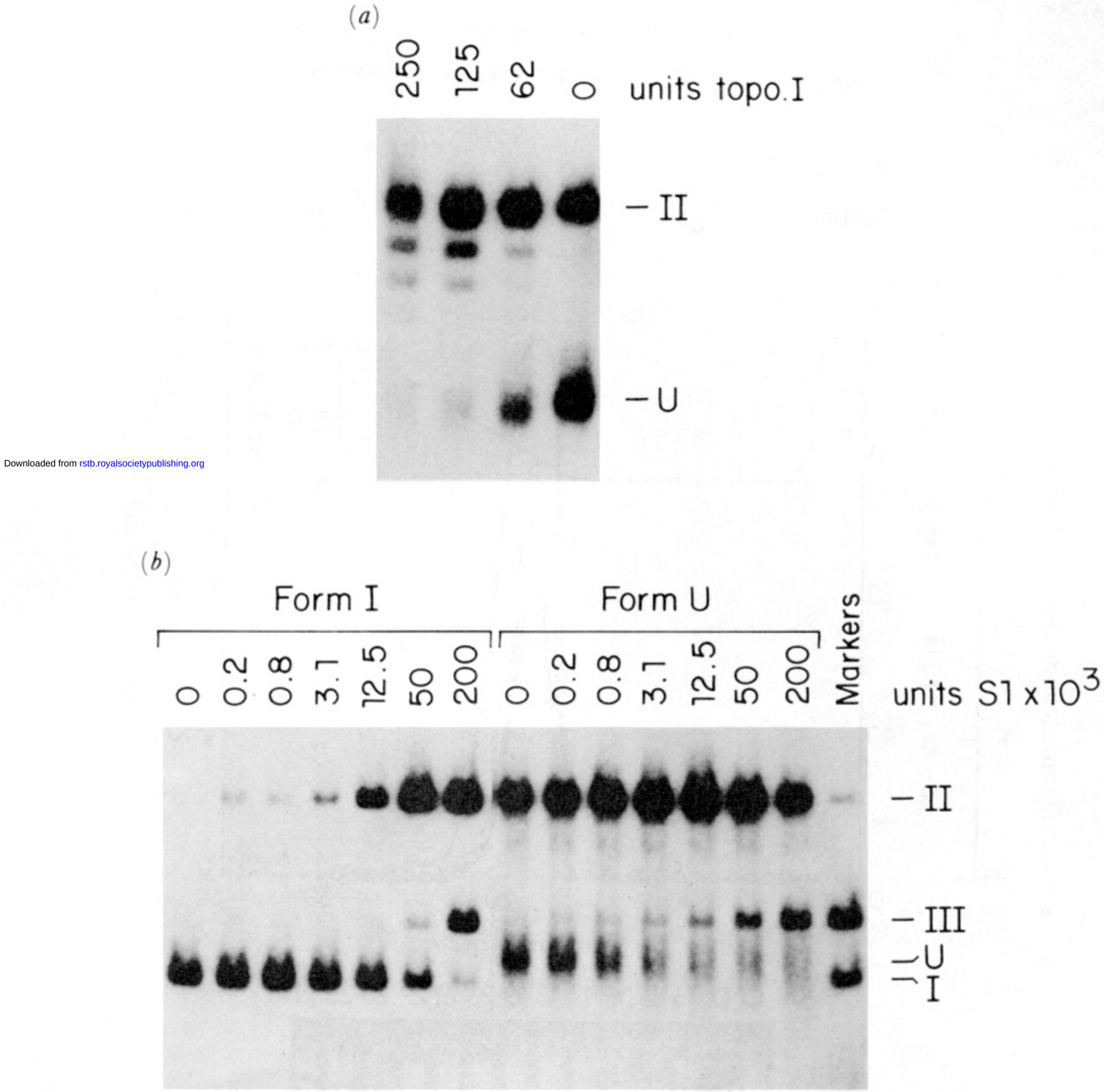
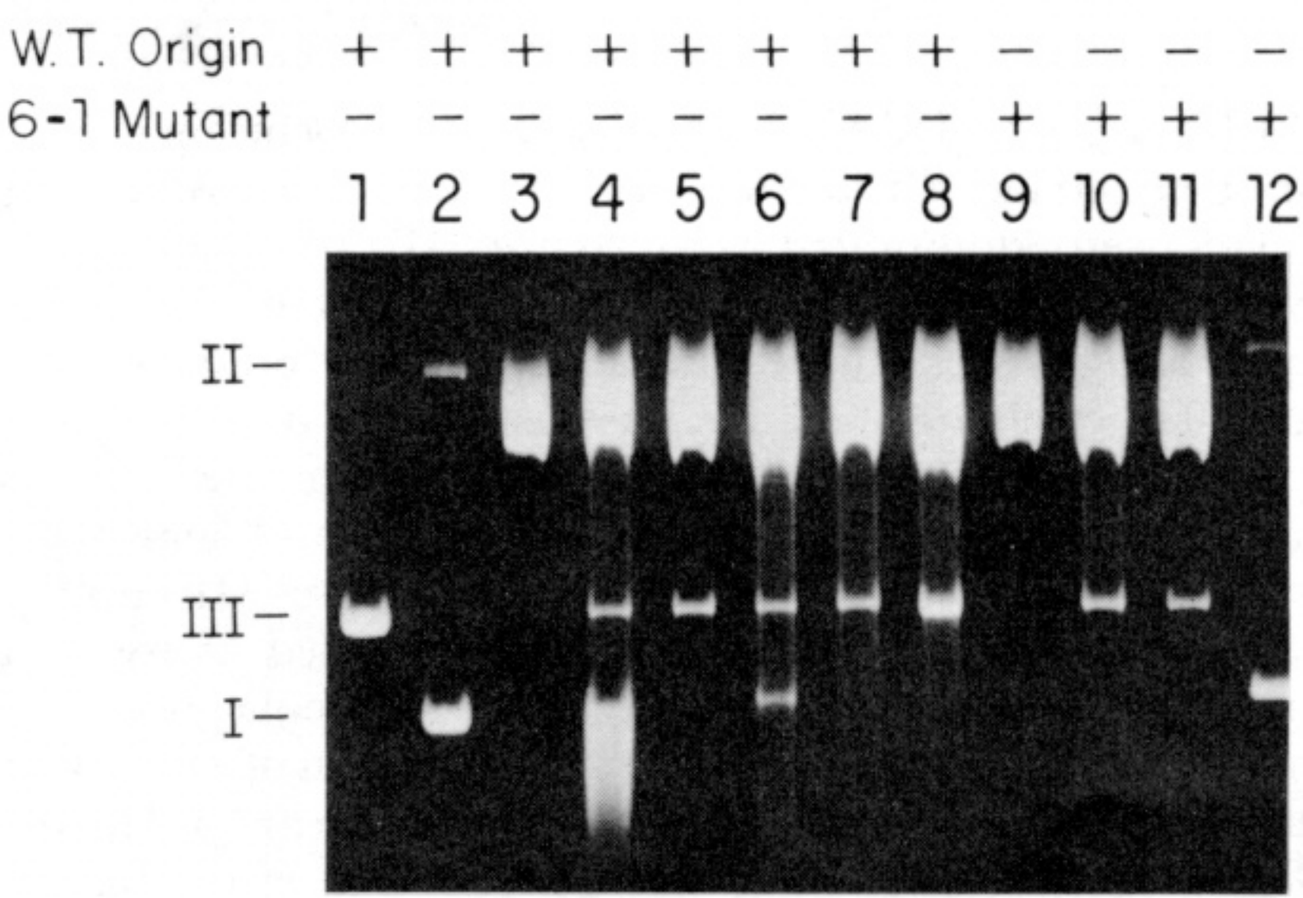


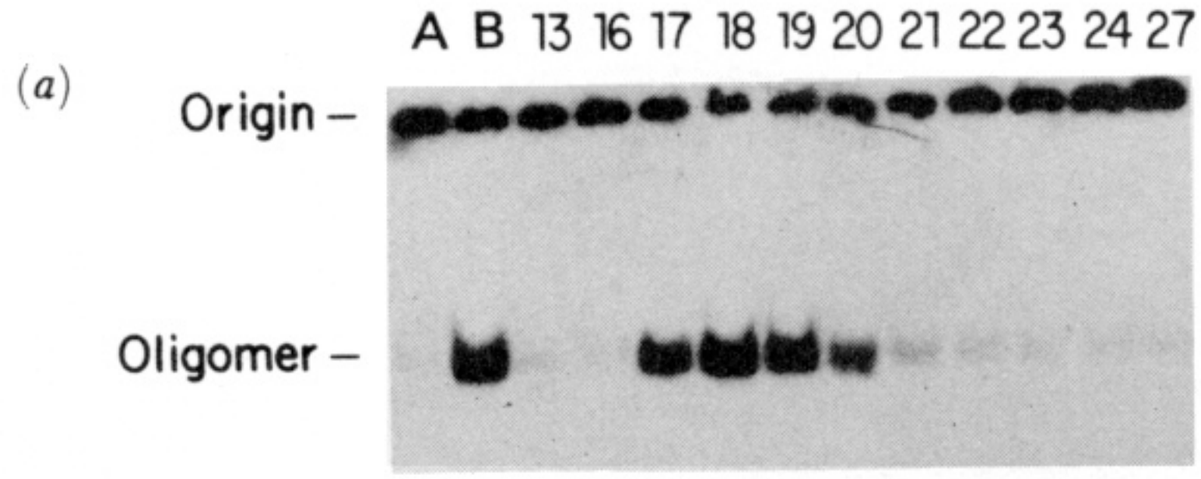
FIGURE 6. Detection and requirements for form U formation. (*a*) Lanes: 1–15, pSV01 EP (ori$^+$) DNA; 16–18, pBR322 EP (ori$^-$) DNA; 1, 15, and 16 contained nicked, linear, and supercoiled plasmid DNA markers; 2 and 17 contained relaxed substrate plasmid DNA; 3 and 18 contained complete reaction; 4, T antigen omitted; 5, HeLa topoisomerase I omitted; 6, *E. coli* SSB omitted; 7, ATP omitted; 8, $MgCl_2$ omitted; 9, creatine phosphate and creatine kinase omitted; 10, incubation was 25 °C instead of 37 °C; 11, topoisomerase I omitted and 20 units of *E. coli* topoisomerase I (ω protein) added; 12, topoisomerase I omitted and 30 units of HeLa topoisomerase II added; 13, topoisomerase I omitted and 16 units *E. coli* DNA gyrase added; 14, *E. coli* SSB omitted and 0.4 μg of adenovirus DNA binding protein added. Markers for lanes 15–18 are at right. (*b*) Chloroquine gel. Electrophoresis was done with chloroquine phosphate added to a concentration of 15 μg ml^{-1}. Reactions contained (ori$^+$) DNA. Lanes: 1, relaxed substrate plasmid DNA, 2, topoisomerase I omitted and 16 units *E. coli* DNA gyrase added; 3, complete reaction (containing HeLa topoisomerase I); 4, nicked, linear, and supercoiled plasmid DNA markers. I, II, and III, RFI RFII, and RFIII, respectively; U, form U; rel., relaxed DNA.

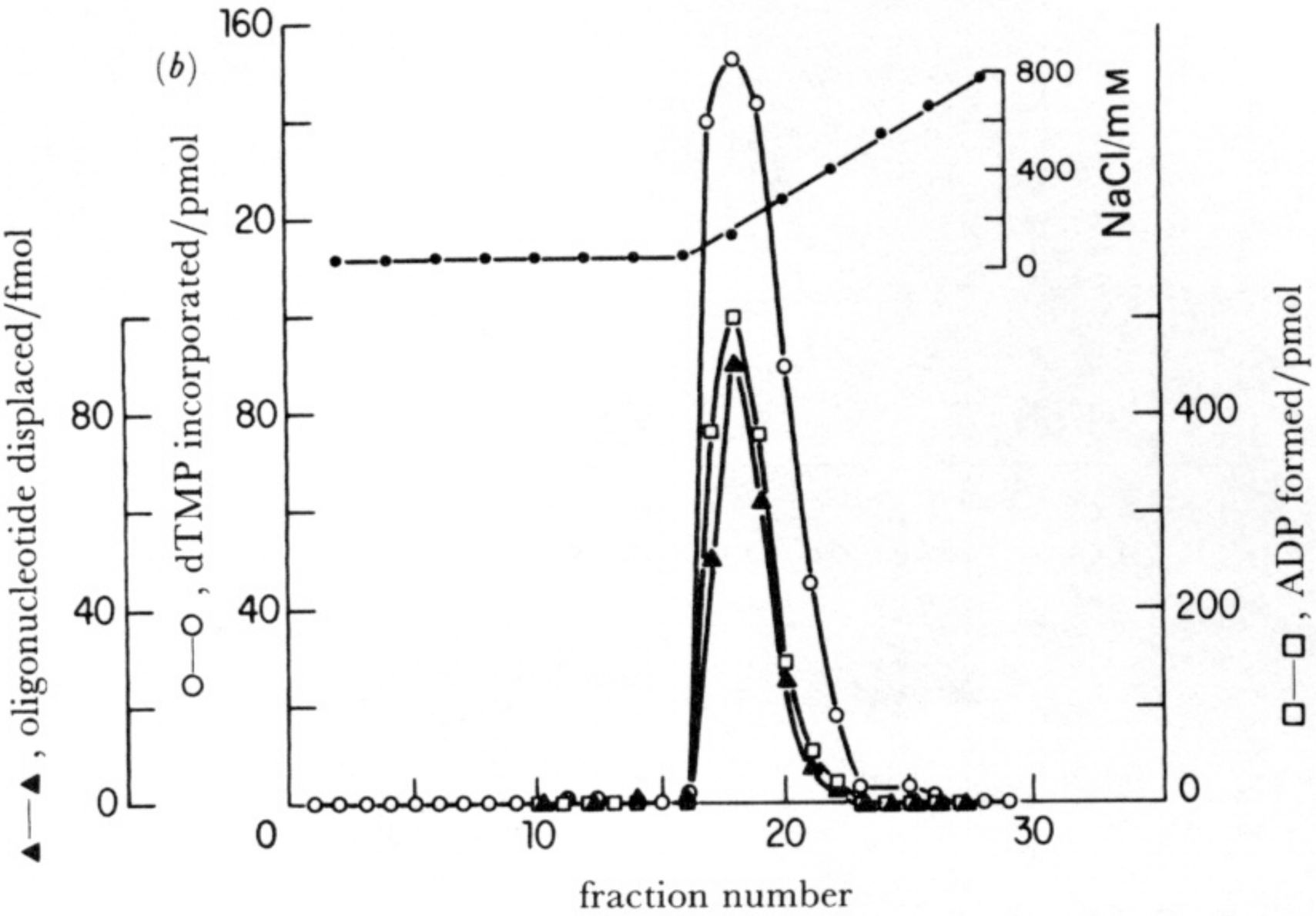


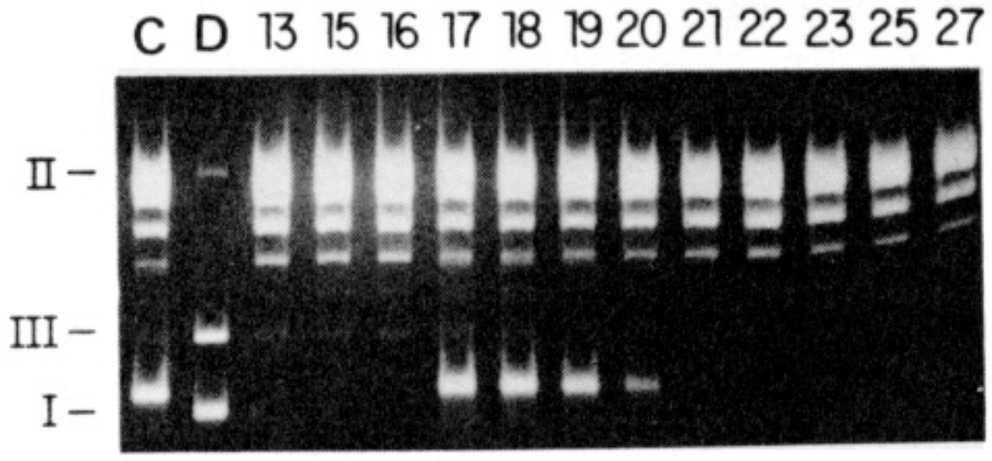
IGURE 7. Structure analysis of form U. (*a*) Treatment with HeLa topoisomerase I. Form U was isolated from an agarose gel as described (Dean *et al.* 1987). Form U (2 ng) was incubated with the indicated units of HeLa topoisomerase I in 20 mM Tris–HCl at pH 7.5, 5 mM EDTA and 0.2 mM KCl containing 50 μg of bovine serum albumin per millilitre for 30 min at 37 °C and electrophoresed. (*b*) Treatment with nuclease S1. Supercoiled DNA (2 ng, form I) or the isolated form U (2 ng) was incubated with the indicated amount of nuclease S1 (in units S1 $\times 10^3$) in 30 mM sodium acetate at pH 4.6, 50 mM NaCl, 1 mM $ZnSO_4$ and 5% (by volume) glycerol for 30 min at 30 °C. Reactions were terminated and electrophoresed as described (Dean *et al.* 1987). Southern blots of the gels were probed with labelled pBR322 sequences, and autoradiograms are shown.



GURE 8. Comparison of unwinding of intact SV40 origin with mutant SV40 origin. Lanes: 1–8, pSVLD (wild-type) plasmid; 9–12, pSVLD6-1 (mutant) plasmid. Lanes: 1, linear plasmid; 2 and 12, supercoiled and nicked plasmid circles; 3 and 9, relaxed plasmid substrate; 4 and 10, complete reaction; 5 and 11, T antigen omitted; 6, topoisomerase I omitted; 7, *E. coli* SSB omitted; and 8, ATP omitted. Formation of form U in lane 6 was due to detectable topoisomerase I activity in this T antigen preparation.







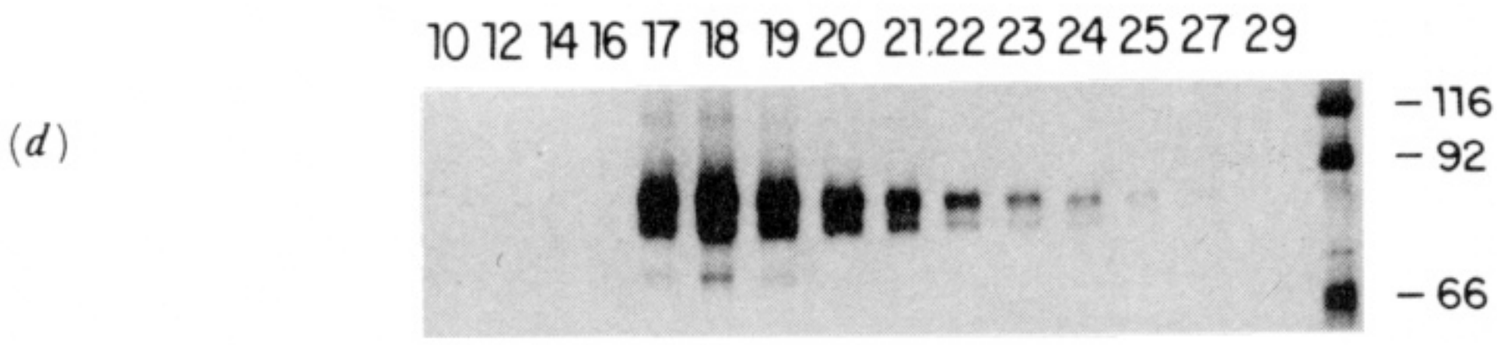


FIGURE 9. Chromatography of SV40 T antigen on DEAE–Sepharose and the coelution of various enzymic activities. Immunoaffinity purified T antigen (60 μg of protein) was loaded onto a 100 μl DEAE–Sepharose CL-6B column (Pharmacia), which had been equilibrated with 20 mM HEPES–NaOH at pH 7.5, 1 mM EDTA, 50 mM NaCl, 1 mM dithiothreitol and 40% (by volume) ethylene glycol. The column was washed with 300 μl of this buffer, and then T antigen was eluted with a linear NaCl gradient (50 mM–1 M NaCl, 600 μl total). Each fraction contained 50 μl of eluate. (*a*) Helicase assays (Dean *et al.* 1987) were done with 3 μl samples as indicated. At the peak fraction, 65% of the input substrate was used. The values presented in the graph were obtained by cutting out the oligonucleotide area of the gel and counting by liquid scintillation. Lane A contained SV40-oligonucleotide (130 fmol) hydrogen bonded to M13mp9 DNA and no enzyme. Under the conditions used, 3% of the input ^{32}P-labelled DNA migrated as free oligonucleotide. Lane B included 250 ng of T antigen. (*b*) The assays for ATPase and DNA replication were done with 2 μl and 3 μl, respectively, of each sample. (*c*) Unwinding assay was done with 3 μl of each fraction indicated. Lane C, control reaction using 0.75 μg of T antigen; lane D, nicked (II), linear (III), and supercoiled-plasmid markers. (*d*) SDS–polyacrylamide gel electrophoresis was done on 3 μl of the indicated fractions, and the T antigen was visualized by silver staining.

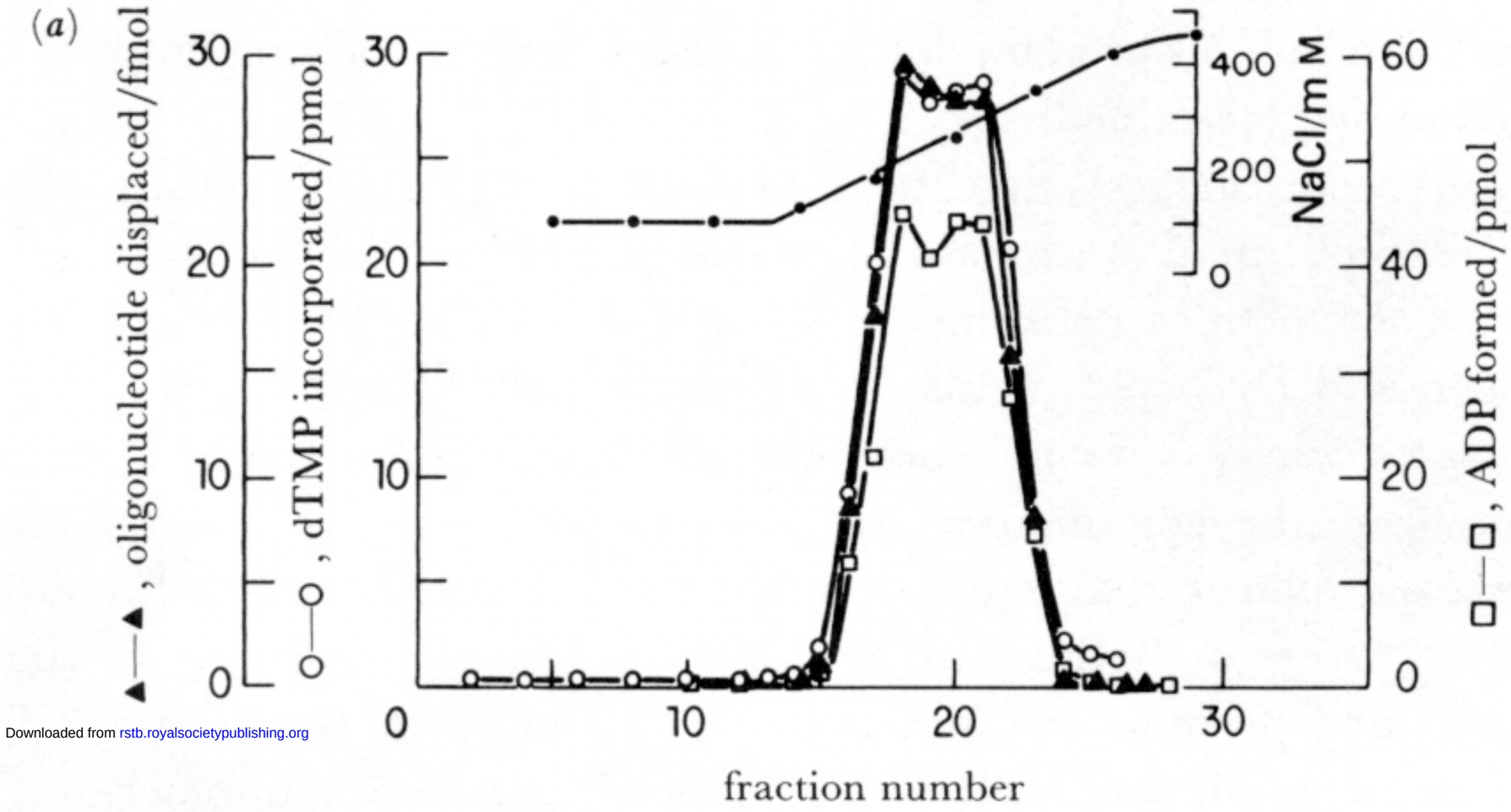


FIGURE 10. Chromatography of SV40 T-antigen on phosphocellulose. Immunoaffinity purified T antigen (50 μg of protein) was loaded onto a 100 μl phosphocellulose column (Whatman P-11) that had been equilibrated with 20 mM HEPES–NaOH at pH 7.5, 1 mM EDTA, 50 mM NaCl, 1 mM dithiothreitol and 40% (by volume) ethylene glycol. The column was washed with 300 μl of this buffer and then T antigen was eluted with a linear NaCl gradient (50 mM–1 M NaCl, 500 μl total). Each fraction contained about 50 μl of eluate. (*a*) Helicase, ATPase, and DNA replication assays were done by using 3 μl and 2 μl of the indicated fractions. (*b*) Unwinding assays were done by using 4 μl of each fraction as indicated. Lane A, control reaction with 1 μg of T antigen; lane B, nicked, linear, and supercoiled plasmid markers. (*c*) SDS–polyacrylamide gel electrophoresis with 3 μl of the indicated fractions, and the proteins were visualized by silver staining. Lane C, relative molecular mass size markers ($M_r \times 10^{-3}$); lane D, immunoaffinity purified T antigen.