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SV40 T antigen binds directly to the large subunit of purified DNA polymerase alpha

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Purified SV40 large T antigen and purified DNA polymerase α – primase form a complex detectable by ELISA and by a modified immunoblotting technique. The interaction is specific for the large catalytic subunit of polymerase α. The amino terminal 83 amino acids of T antigen are both necessary and sufficient for binding to the polymerase. However, antibody epitopes located in the carboxy terminal ATPase domain of T antigen are masked in the polymerase – T antigen complex, and complex formation is inhibited by an antibody directed against the carboxy terminus of T antigen, suggesting that this region of T antigen, though not required for binding, is in close proximity to the bound polymerase. The affinity of human DNA polymerase α for T antigen is ~10-fold greater than that of polymerase α from calf thymus, consistent with the interpretation that polymerase α is at least in part responsible for the primate-specific replication of SV40 DNA *in vivo* and *in vitro*. The results suggest that specific protein – protein interaction between DNA polymerase α and T antigen plays an important role in viral DNA replication.

Key words: DNA polymerase α/initiation of DNA replication/SV40 T antigen

Introduction

The replication of the simian virus 40 (SV40) minichromosome in infected monkey cells has been long regarded as a useful model system for the study of eukaryotic DNA replication mechanisms and their control. One important asset is that SV40 replication is accomplished by the host cell replication machinery with the assistance of only one viral protein, T antigen. Moreover, a cell-free SV40 replication system that reflects many features of replication *in vivo* has been developed (Li and Kelly, 1984). This assay, combined with extensive fractionation of cellular extracts in several laboratories, has facilitated the elucidation of the components of the replication machinery and their functions (reviewed by Stillman, 1989; Challberg and Kelly, 1989). Although the current picture of SV40 DNA replication is far from complete, its main outlines and the primary replication proteins are known.

The initiation of SV40 replication is governed by T antigen, a regulatory protein of remarkable complexity. Genetic and biochemical data demonstrate that specific binding of T antigen to a palindromic sequence in the minimal origin of DNA replication is an essential step in initiation (reviewed by Hay and Russell, 1989; Stillman, 1989; Borowiec *et al.*, 1990). A second biochemical activity of T antigen, DNA helicase (Stahl *et al.*, 1986), is required in concert with cellular replication proteins, in particular a single-strand DNA binding protein (RF-A/RP-A), to unwind the double-strand DNA at the origin, forming a pre-initiation complex (reviewed by Hay and Russell, 1989; Stillman, 1989; Borowiec *et al.*, 1990).

The actual initiation of replication is probably catalysed by the cellular DNA polymerase α – primase complex, but how this enzyme and other replication proteins are directed to the origin region, a key step in coupling formation of the pre-initiation complex with DNA synthesis, is not yet clear (Lee *et al.*, 1989; Tsurimoto and Stillman, 1989; Weinberg and Kelly, 1989). One attractive possibility is that protein – protein interactions between T antigen and cellular replication proteins may be involved. Indeed, an association between T antigen and DNA polymerase α activity was detected in crude cell extracts (Smale and Tjian, 1986; Gough *et al.*, 1988; Gannon and Lane, 1987, 1990). However, the nature of the reagents and the detection systems used precluded a detailed analysis of the components involved and the molecular specificity of the interaction.

We have now used highly purified DNA polymerase α-primase and T antigen to explore these questions further. We report here that T antigen binds directly and specifically to one subunit of DNA polymerase α, that the amino terminal 83 amino acids of T antigen are both necessary and sufficient for association with purified polymerase, and that polymerase isolated from human tissue, in keeping with the permissivity of primate cells for SV40 replication, has a higher affinity for T antigen than that prepared from calf thymus.

Results

Purification of DNA polymerase α and primase

DNA polymerase α was purified from calf thymus by immunoaffinity chromatography to near homogeneity. The modifications of previous procedures increased the yield several-fold while maintaining a high specific activity (Table I). The isolated polymerase α (Figure 1, lane POL) was composed of four types of polypeptides: a family of high molecular weight polypeptides that represent the catalytic subunit, called p180 in this report, a p72 subunit of unknown function, and a p58 and a p48 subunit, which together make up the DNA primase (reviewed by Lehman and Kaguni, 1989; Wang *et al.*, 1989b). DNA primase was isolated from the polymerase α – primase complex with only trace contamination by the p180 subunit (Figure 1, lane PRI). The isolated primase retained enzymatic activity in a primase assay (O.Zacharova, personal communication). DNA polymerase α was also isolated from human placenta by immunoaffinity chromatography (Table I). The yields were considerably lower than from calf thymus, though the specific activity and subunit composition were similar (Table I; data not shown).

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Table I. Immunopurification of DNA polymerase–primase complex

Step	Total protein (mg)	Total act. (units)	Sp. act. (units/mg)	Yield (%)	Enrichment
Crude extract[a]	294 000	380 000	1	100	1
phosphocellulose	11 310	262 112	23	69	23
heparin–Sepharose	686	208 000	303	55	303
antibody column	2.5	47 300	18 920	1.2	18 920
Crude extract[b]	180 000	n.d.[c]	n.d.[c]		
phosphocellulose	480	824 000	1 716	100	
heparin–Sepharose	76	204 000	2 684	25	
antibody column	0.14	3 000	21 430	0.4	

[a]Crude extract was prepared from 1 kg of calf thymus.
[b]Crude extract was prepared from 1.5 kg of human placenta.
[c]n.d., not determined.

Direct interaction between purified DNA polymerase α and SV40 T antigen

DNA polymerase α in crude extracts of human and mouse cells was shown to bind to SV40 T antigen *in vitro* in an apparently specific fashion (Smale and Tjian, 1986; Gough *et al.*, 1988; Gannon and Lane, 1987, 1990). Since these results did not allow a definitive conclusion whether polymerase α–primase itself or another polymerase-associated protein was responsible for T antigen binding, purified calf thymus DNA polymerase α was used as the solid phase in an enzyme-linked immunosorbent assay (ELISA) to measure complex formation with purified large T antigen (Figure 2A). Purified monoclonal antibody Pab419, directed against the amino terminus of large T (Figure 2C), and peroxidase-conjugated second antibody were used to detect bound T antigen. The amount of complex formation depended on the T antigen concentration added, showing that T antigen can bind directly to purified DNA polymerase α. Similar results were obtained when Pab101, which binds a carboxy terminal epitope, was substituted for Pab419 in the detection system (Figure 2C; data not shown). On the other hand, some anti-T monoclonal antibodies, such as Pab204, detected an amount of T antigen that was scarcely above the background level determined in the control assays lacking polymerase α, T antigen or anti-T antibody (Figure 2A). This result suggests that the Pab204 epitope was partially masked in the T antigen–polymerase α complex.

The specificity of the T antigen–polymerase interaction was further tested with another anti-T monoclonal antibody, Pab414 (Figure 2C), that was reported to inhibit both SV40 DNA replication *in vitro* and T antigen binding to polymerase α, but not origin DNA binding, ATPase, or helicase activity (Smale and Tjian, 1986; Wiekowski *et al.*, 1987; Gough *et al.*, 1988). Purified T antigen was pre-incubated with increasing amounts of purified Pab414 before addition to the DNA polymerase solid phase of the ELISA. Detection of bound T antigen by Pab419 showed that complex formation was efficiently inhibited by 10 μg of Pab414 (Figure 2B). Our results with purified proteins thus completely confirm earlier reports of specific T antigen–polymerase α interaction in crude cell extracts (Smale and Tjian, 1986; Gannon and Lane, 1987, 1990; Gough *et al.*, 1988) and provide new evidence that this interaction involves only these two components.

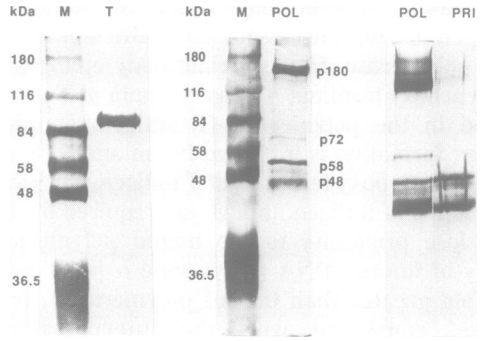

Fig. 1. Protein purification. Purified baculovirus expressed T antigen (T), purified calf thymus DNA polymerase α (POL) and purified calf thymus primase (PRI) were analysed by denaturing PAGE (Laemmli, 1970) and silver staining (Heukeshoven and Dernick, 1988). M, prestained marker proteins (Sigma, Munich).

Species specificity of T antigen binding to DNA polymerase α

The association of T antigen with DNA polymerase was postulated to play an essential role in the initiation of SV40 DNA replication and possibly in elongation as well (Smale and Tjian, 1986; Braithwaite *et al.*, 1987; Gannon and Lane, 1987, 1990; Gough *et al.*, 1988; Wiekowski *et al.*, 1987, 1988; Hay and Russell, 1989; reviewed by Stillman, 1989). Indeed, primate polymerase α or polymerase-associated factors rendered mouse cell extracts permissive for SV40 DNA replication *in vitro*, suggesting that they may represent the key factor in the host cell specificity of SV40 DNA replication (Murakami *et al.*, 1986). Thus, it was of interest to compare the interaction of T antigen with DNA polymerase α from permissive and non-permissive host cells. DNA polymerase α purified from human placenta or from calf thymus was added to a monoclonal anti-polymerase antibody SJK 132-20 ELISA solid phase (Figure 3). This antibody bound equally well to polymerase α of many different mammalian species (Miller *et al.*, 1988). Increasing amounts of T antigen were added and bound T antigen was detected directly with peroxidase-conjugated Pab419 anti-T antibody, which gave little or no background in control assays. Human DNA polymerase α bound significantly more

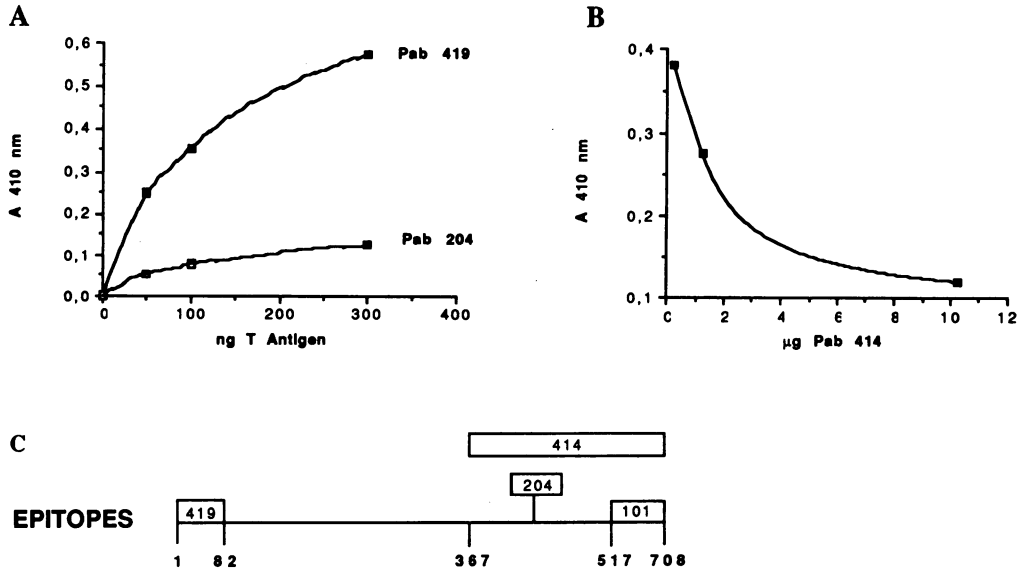

Fig. 2. Binding of T antigen to purified calf thymus DNA polymerase α. (**A**) Purified DNA polymerase immobilized on ELISA plates was incubated with the indicated amounts of purified T antigen for 1 h. After washing, bound T antigen was detected by incubation with 1 µg purified anti-T monoclonal antibody, rabbit anti-mouse immunoglobulin and a chromogenic substrate. Bound T antigen was quantitated spectrophotometrically. Background values of control wells without DNA polymerase, without T antigen, without the first or second antibodies were negligible (0.1 or less). (**B**) 300 ng T antigen were pre-incubated with the indicated amount of purified Pab414 for 1 h before adding it to DNA polymerase solid phase. After washing, bound T antigen was quantitated by incubation with 1 µg Pab419, followed by second antibody and enzymatic assay as in A. Control assays without DNA polymerase, without T, without Pab419 or second antibody gave negligible background values (0.1 or less). (**C**) The localization of T antigen epitopes recognized by selected monoclonal antibodies is shown schematically. The amino acid residue numbers of T antigen are indicated at the bottom.

T antigen than an equal mass of the calf thymus enzyme (Figure 3). The amount of T bound by calf thymus polymerase at the highest T antigen concentration (300 ng) was less than that bound by human polymerase at the lowest T antigen concentration (50 ng), suggesting that the difference in binding affinity is about one order of magnitude. Identical results were obtained when equal enzyme activity units of human and calf thymus polymerase were used directly as the solid phase of the ELISA (data not shown). Pre-incubation of T antigen with a saturating amount of Pab414 prevented complex formation with both human and calf thymus enzymes (Figure 3). The results indicate that human DNA polymerase α has a greater affinity for T antigen than polymerase α from calf thymus, consistent with its proposed role as a permissive cell factor for SV40 replication.

Subunit specificity of DNA polymerase α binding to T antigen

Preliminary experiments to address the question whether T antigen binds only to the intact DNA polymerase α, to one particular subunit of the enzyme, or possibly to the heterodimeric DNA primase were carried out using the isolated polymerase α and primase complexes (Figure 1, lanes POL and PRI) in an ELISA. The results demonstrated that T antigen bound to the intact polymerase, but did not recognize the isolated primase with sufficient affinity to be detectable in this sensitive assay (data not shown). Thus, we concluded that T antigen may associate with one or both of the other subunits, p180 and p72, or only with the intact enzyme.

This question was further investigated using a modified immunoblotting method. Purified DNA polymerase α and purified DNA primase were denatured and the subunits were separated by denaturing polyacrylamide gel electrophoresis. The proteins were renatured in the gel, transferred to nitrocellulose by electroblotting and then incubated with or without purified T antigen. Bound T antigen was then detected by incubation with monoclonal anti-T antibody and alkaline phosphatase-conjugated second antibody (Figure 4A). Lane 4 shows that T antigen bound strongly and specifically to the p180 subunit bands, and weakly to faint bands of smaller molecular weight proteins that were also detected in the control (lane 2), and in the lanes loaded with isolated primase (lanes 1 and 3). The isolated primase (lane 5) and polymerase α (lane 6) immobilized on the blot were visualized by staining with polyclonal anti-polymerase antibody and alkaline phosphatase-conjugated second antibody.

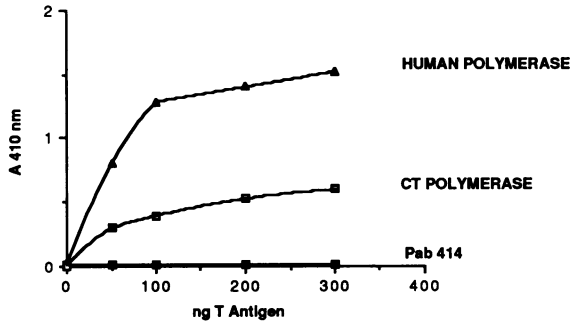

Fig. 3. Binding of T antigen to purified DNA polymerase α from human placenta. DNA polymerase purified from human placenta (▲) or calf thymus (□), 1 µg each, was bound to monoclonal IgG SJK 132-20 immobilized on ELISA plates. The indicated amounts of purified T antigen or T antigen pre-incubated with 10 µg Pab414 for 1 h were then added. Bound T antigen was detected by incubation overnight with horseradish peroxidase-conjugated Pab419 and then with a chromogenic substrate. Control reactions gave negligible background (A_{410} < 0.002). Pab414-treated T antigen binding to both polymerases was undetectable.

Parameters of the T antigen–p180 polymerase interaction

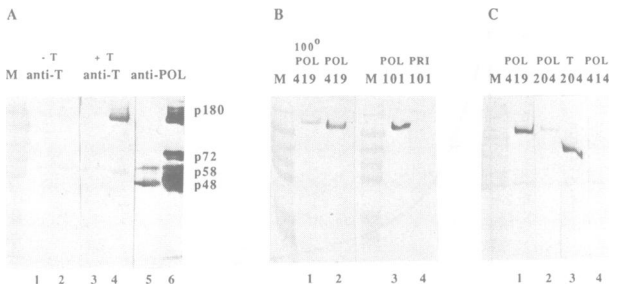

Fig. 4. Subunit specificity of DNA polymerase α binding to T antigen. Purified calf thymus DNA polymerase α or primase was denatured as described (Miskimins *et al.*, 1985) and electrophoresed in denaturing polyacrylamide mini-gels (Laemmli, 1970). The proteins in the gel were renatured and transferred to nitrocellulose as described (Dunn, 1986). (**A**) Lanes 1, 3, 5, primase; lanes 2, 4, 6, DNA polymerase α. Lanes 1, 2, 5 and 6 were incubated without T antigen, lanes 3 and 4 with T antigen, and after 1 h, lanes 1–4 were immunostained with Pab419 monoclonal anti-T antibody and a commercial detection system (Promega, Heidelberg). Lanes 5 and 6 were stained with rabbit polyclonal anti-polymerase α serum and detection system. (**B**) Lane 1 was loaded with polymerase α boiled in sample buffer; lanes 2 and 3 with polymerase denatured as in A; lane 4 with primase denatured as in A. All lanes were incubated with T antigen, followed by detection with Pab419 (lanes 1 and 2) or Pab101 (lanes 3 and 4). (**C**) Lanes 1, 2 and 4 were loaded with polymerase α, and lane 3, with T antigen. Lanes 1, 2 and 4 were incubated with T antigen and stained with Pab419 (lane 1), Pab 204 (lane 2) or Pab414 (lane 4). Lane 3 was stained directly with Pab204. M, prestained marker proteins (Sigma, Munich).

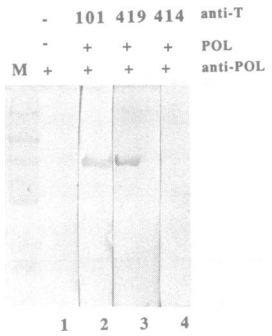

Fig. 5. Pab414 inhibits T antigen binding to polymerase. Purified T antigen was denatured (Miskimins *et al.*, 1985), separated by denaturing gel electrophoresis, renatured in the gel (Dunn, 1986) and transferred to nitrocellulose. Lane 2 was incubated with Pab101, lane 3 with Pab419 and lane 4 with Pab414. Lanes 2–4 were then incubated with purified DNA polymerase α and bound polymerase was detected with polyclonal rabbit antipolymerase serum. Lane 1 was treated directly with rabbit anti-polymerase serum as a control. M, prestained marker proteins.

in this assay were then analysed in more detail. The importance of polymerase renaturation for T antigen association was tested by boiling the polymerase in sample buffer before gel electrophoresis instead of denaturing it at room temperature (Figure 4B, compare lanes 1 and 2). Apparently, renaturation of the boiled sample was incomplete, leading to decreased but still detectable T antigen binding. Polymerase-bound T antigen was easily detectable with Pab419 and Pab101 (Figure 4B, lanes 2 and 3), but staining with Pab204 or Pab414 was weak or absent (Figure 4C, lanes 2 and 4), reflecting exactly the results obtained by ELISA (Figure 2A). Control experiments showed that Pab204 was able to detect immunoblotted T antigen (lane 3). These results demonstrate that the specific T antigen–polymerase α association detected by ELISA is due to a specific interaction between T antigen and the p180 subunit of the enzyme, and that the other subunits of the enzyme are not required for this interaction.

If the association between T antigen and p180 is a simple direct interaction, it should be possible to invert the binding reaction, allowing soluble polymerase α to bind to T antigen fixed on nitrocellulose, and to prevent this complex formation with Pab414 as shown in the ELISA (Figures 3 and 2B). Figure 5 shows the result of such an experiment. Immunoblotted T antigen was pre-incubated with Pab419, Pab101 or Pab414 and then with purified DNA polymerase α. Bound polymerase was then detected with polyclonal anti-polymerase serum and alkaline phosphatase-conjugated second antibody. Lane 1 shows a control incubated only with anti-polymerase and second antibody to rule out cross-reaction of the serum with T antigen. Polymerase α bound to the T antigen associated with Pab419 and Pab101, but not to that associated with Pab414 (lanes 2–4). Thus, not only was the Pab414 epitope masked in the T antigen–polymerase complexes (Figure 4C, lane 4), but Pab414 prevented complex formation (Figure 5, lane 4), in agreement with the results in Figure 3. These results provide additional evidence for a specific interaction between T antigen and purified DNA polymerase α.

Localization of the polymerase binding domain of T antigen

The ability of Pab414 to prevent formation of T antigen–polymerase complexes (Smale and Tjian, 1986; Gough *et al.*, 1988; Figures 3 and 5), the reported competition between polymerase α and p53 for T antigen binding (Braithwaite *et al.*, 1987; Gannon and Lane, 1987, 1990; Gough *et al.*, 1988; Sturzbecher *et al.*, 1988; Wang *et al.*, 1989a), and the loss of Pab204, Pab205 and Pab414 epitopes in polymerase-bound T antigen (Smale and Tjian, 1986; Gough *et al.*, 1988) have been interpreted as indirect evidence that the polymerase binding domain of T antigen may overlap with these epitopes and the p53 binding domain. Each of these epitopes lies within the region between amino acids 271 and 517 of T antigen, which was recently shown to suffice for p53 binding activity (Mole *et al.*, 1987; Schmieg and Simmons, 1989) (Figure 2C).

The modified immunoblot procedure described in Figure 4 was employed to localize the polymerase binding domain of T antigen more directly. DNA polymerase α immobilized on immunoblots was incubated with defined T antigen peptides expressed in bacteria or baculovirus (Figure 6A) and purified by immunoaffinity chromatography. Bound T antigen peptide was detected using an appropriate monoclonal antibody against T antigen. Binding of baculovirus wild-type T antigen was used as a positive control. The results are shown in Figure 6B. A peptide containing T antigen residues 272–708 failed to bind to polymerase α (lane 5), while unexpectedly, a peptide carrying T antigen residues 1–259 bound well (lane 2). Moreover, a small peptide containing T antigen residues 1–130 bound equally well (lane 3), as did a peptide containing only the first 83 amino acids of T antigen (lane 6). Conversely, peptides carrying residues 131–259 and 83–708 (lanes 4 and 7) were unable to bind to polymerase at levels above the background labelling observed upon antibody staining of blots incubated without any T antigen peptide (cf. lane 8). These data demonstrate that the polymerase binding domain of T antigen

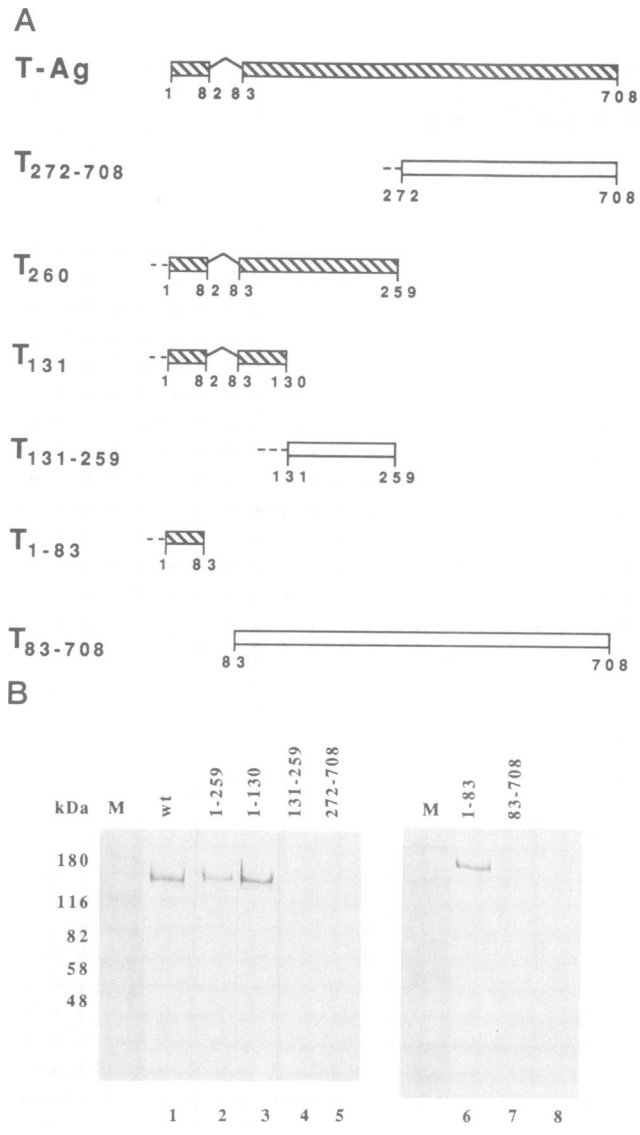

Fig. 6. Polymerase binding domain of SV40 T antigen. (**A**) The primary sequences included in each of the T antigen peptides used in (B) are diagrammed. Dashed lines indicate up to 10 amino acids from the *lacZ* polylinker used for bacterial expression (Arthur *et al.*, 1988). Shaded peptides bind to purified polymerase. (**B**) Purified calf thymus polymerase α immobilized on nitrocellulose as in Figure 4 was incubated with purified bacterial T antigen peptides (2 μg/ml) as indicated (lanes 2–6), baculovirus T containing only residues 83–708 (lane 7), or baculovirus wild-type T antigen as a control (lane 1). Lane 8 was incubated with buffer instead of T antigen as a control. Bound T antigen was detected using Pab419 (lanes 1–3 and 6), Pab220 (lane 4) or Pab101 (lanes 5, 7 and 8) and alkaline phosphatase-conjugated second antibody.

must be localized within the N-terminal 83 amino acids and that the C-terminal region of T antigen is not required for association with polymerase α. In addition, they imply that the inhibition of SV40 DNA replication caused by binding of monoclonal antibodies or p53 at the carboxy terminus of T antigen is probably due to indirect steric hindrance of polymerase binding rather than to a direct competition for the polymerase binding site on T antigen.

This interpretation predicts that complex formation between DNA polymerase α and the amino terminal fragments of T antigen should not be inhibited by Pab414, since this epitope is not present in these peptides. The prediction was tested in an ELISA using either baculovirus T antigen or the bacterial peptide T131 as the solid phase, and incubating it with increasing concentrations of Pab414,

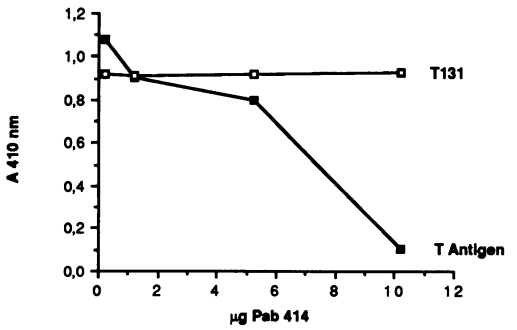

Fig. 7. Binding of an amino terminal truncated T antigen to DNA polymerase α is not inhibited by Pab414. Purified baculovirus T antigen or bacterial T131 (residues 1–130) immobilized on ELISA plates was incubated with the indicated amounts of Pab414 overnight. After washing, 1 μg of purified DNA polymerase α–primase in 50 μl of PBS was added to each well and incubated for 1 h. After washing, bound polymerase was detected by incubation overnight with peroxidase-conjugated monoclonal antibody against the p180 subunit of polymerase (2CT25, I.Dornreiter and E.Fanning, in preparation), followed by reaction with a chromogenic substrate.

followed by incubation with DNA polymerase α. Polymerase binding was then assayed with a peroxidase-coupled monoclonal antibody against DNA polymerase α (Figure 7). As expected Pab 414 interfered with polymerase binding to full-length T antigen, but binding to the amino terminal T131 was not affected.

Discussion

We have used ELISA and immunoblotting to study the interaction between DNA polymerase α and the SV40 replication initiator protein, T antigen. We demonstrate a specific, direct association between the two components, regardless of which was used for the solid phase. The amino terminal 83 residues of T antigen and the p180 subunit of polymerase α are both necessary and sufficient for complex formation. A monoclonal antibody against T antigen that inhibits cell-free SV40 DNA replication, but not ATPase, helicase or origin DNA binding activity of T antigen (Smale and Tjian, 1986; Wiekowski *et al.*, 1987), interferes with binding to polymerase α. Finally, the affinity of human DNA polymerase α for T antigen is ~10-fold greater than that of the calf thymus enzyme.

The results confirm and extend earlier observations made with crude extracts (Smale and Tjian, 1986; Gannon and Lane, 1987, 1990; Gough *et al.*, 1988) and suggest that T antigen–polymerase α complex formation plays an important functional role in initiation of SV40 DNA replication, and possibly elongation as well. The nature of this function has not yet been established. The most obvious possibility is that T antigen in the pre-initiation complex at the SV40 origin serves to bind polymerase α–primase in the appropriate orientation to begin primer synthesis (Hay and Russell, 1989), thereby coupling origin unwinding with DNA replication. Studies with mutant T antigens lend support to this interpretation. D2 T antigen, a chimeric protein composed of 104 amino acids derived from an adenovirus structural protein, followed by residues 115–708 of T antigen (Baumann *et al.*, 1985), has origin DNA binding and ATPase activity (Tjian, 1978; Tjian and Robbins, 1979). However, D2 T antigen replicated SV40 DNA very inefficiently in a cell-free assay (Klausing *et al.*, 1989).

Similarly, a truncated baculovirus T antigen containing only amino acids 83–708 was completely inactive in the cell-free replication assay (C.Schneider, I.Moarefi and E.Fanning, unpublished data). Our data indicate that both of these mutant T antigens lack the ability to bind specifically to polymerase α, suggesting that this defect is responsible for their poor replication activity. However, other biochemical defects in these truncated T antigens, such as inefficient origin unwinding activity, may also contribute to their replication defects (Klausing *et al.*, 1989; Fanning and Vogt, 1986).

Additional support for the role of T antigen–polymerase α binding in initiation of replication comes from studies with a new collection of monoclonal antibodies against DNA polymerase α (I.Dornreiter and E.Fanning, in preparation). An antibody that is unable to inhibit polymerase–primase activity, but interferes with T antigen binding to polymerase, efficiently inhibits initiation of SV40 replication *in vitro* (I.Dornreiter, C.Schneider and E.Fanning, in preparation). Moreover, the ability of Pab414 to inhibit SV40 replication and T antigen–polymerase binding but not origin DNA binding, ATPase or helicase activities of T antigen also argues for a role of complex formation in SV40 replication. In addition to a role in assembly of a replication initiation complex, the association of polymerase α with T antigen could influence the enzymatic properties of either protein in other steps of the replication process. Clearly, further studies with purified proteins in the cell-free replication assay will be required to determine the functional role of the polymerase α–T antigen interaction in SV40 replication.

The interaction between the human DNA polymerase α and T antigen is ~10-fold more efficient than with the calf thymus enzyme. This result is consistent with the proposed role of polymerase α in the species specificity of SV40 replication (Murakami *et al.*, 1986). However, the amount of SV40 DNA replication *in vitro* is at least 100-fold greater in primate cell extracts than in non-permissive cell extracts (Li and Kelly, 1985; C.Schneider and E.Fanning, unpublished data), raising the question of whether SV40 replication may require other, additional, primate-specific proteins (Brill and Stillman, 1989; Fanning *et al.*, 1988; Traut and Fanning, 1988). Experiments are in progress to address this question and identify such proteins.

The role of protein–protein interactions appears to be of general importance in initiation of DNA replication and has been most carefully studied in prokaryotic model systems. For example, the origin binding proteins required for initiation of *Escherichia coli oriC* and bacteriophage λ replication act to recruit other components of the replication machinery to the origin. They first associate with the helicase *dnaB* to generate a pre-initiation complex, to which the primase can then bind (Bramhill and Kornberg, 1988; Alfano and McMacken, 1989a,b; Dodson *et al.*, 1989). Primase and helicase remain associated with each other in a mobile primosome, which in turn associates with DNA polymerase III holoenzyme to carry out lagging strand replication. Protein–protein interactions between helicase, single-strand DNA binding protein, polymerase and primase are also an important feature of elongation reactions at the replication fork in other systems, such as bacteriophages T4 and T7 (Cha and Alberts, 1989, 1990; Bernstein and Richardson, 1988).

The protein–protein interactions involved in SV40 replication bear some similarity to the prokaryotic systems, but also show important differences. T antigen is of course a streamlined, multifunctional protein, combining both origin DNA binding and helicase activities in one molecule (reviewed by Borowiec *et al.*, 1990). In contrast to the prokaryotic systems, eukaryotic DNA primase is tightly associated with DNA polymerase α. Our data suggest that primase associates with the SV40 pre-initiation complex indirectly through the T antigen–polymerase α interaction, rather than binding to T antigen directly. Nevertheless, polymerase α–primase appears to serve as the lagging strand polymerase in analogy to the prokaryotic primosome–polymerase assembly (reviewed by So and Downey, 1988; Challberg and Kelly, 1989; Stillman, 1989). In analogy with the T4 replication fork components and their functions, one might expect to find additional interactions between DNA polymerase α–primase and the single-strand DNA binding protein RF-A/RP-A or other elongation factors (Brill and Stillman, 1989; Tsurimoto and Stillman, 1989; Kenny *et al.*, 1989, 1990). The methods used here to study T antigen–polymerase association may also be useful in identifying and analysing the molecular specificity of these interactions.

The DNA helicase activity of T antigen is clearly essential for initiation at the SV40 origin of replication and perhaps for elongation as well, but it is not certain whether T antigen is the only DNA helicase involved in SV40 DNA replication (Wiekowski *et al.*, 1987, 1988; Goetz *et al.*, 1988; Roberts and D'Urso, 1988). Clearly, eukaryotic cellular DNA helicases must participate in replication of chromosomal DNA, but they have proved difficult to identify and isolate. Based on the model character of the SV40 replicon, one may speculate that hypothetical cellular origin binding proteins and the DNA helicases involved in chromosomal replication may associate with DNA polymerase α–primase as reported here for T antigen. It will be of great interest to try to identify such proteins and compare their roles in replication of chromosomal DNA with that of T antigen in replication of the viral mini-chromosome.

Materials and methods

Antibodies

Hybridomas [Pab 419, 414, 101, 220, 204 against SV40 large T antigen (Gurney *et al.*, 1980; Lane and Hoeffler, 1980; Harlow *et al.*, 1981; Mole *et al.*, 1987) and SJK 132-20 and SJK 287-38 against DNA polymerase α (Tanaka *et al.*, 1982)] were cultured in Dulbecco's modified Eagle's medium with 10% fetal calf serum (GIBCO, Eggenstein). Immunoglobulins were purified by ammonium sulphate precipitation and affinity chromatography using a commercial kit (MAPS, BioRad, Munich). Pab419 was conjugated with horseradish peroxidase for use in ELISA assays using a commercial kit (Zymed Laboratories, So. San Francisco) as specified by the supplier. Rabbit antiserum against purified calf thymus DNA polymerase α was a generous gift from Frank Grosse and Heinz-Peter Nasheuer.

SV40 T antigen

T antigen was purified from Sf9 insect cells infected with a recombinant baculovirus (941T), generously provided by Robert Lanford. T83-708 baculovirus was provided by Peggy Bradley. Cells were infected with 10 p.f.u. per cell and T antigen was harvested after 36–40 h and purified by immunoaffinity chromatography essentially as described (Simanis and Lane, 1985; Lanford, 1988), except that the immunosorbent was Pab101–Sepharose prepared with Tresyl-activated Sepharose 4B (Pharmacia, Freiburg) (Höß *et al.*, 1990; M.Baack, personal communication). Protein concentration was estimated spectrophotometrically, assuming that one $A_{280\ nm}$ unit corresponds to 1 mg/ml of purified protein.

Bacterial T antigen peptides, expressed as pUC9 fusion proteins (Arthur *et al.*, 1988), were purified by immunoaffinity chromatography as described

(Arthur *et al.*, 1988; Höß *et al.*, 1988). Pab220-coupled Protein A–Sepharose was substituted for Pab419 immunosorbent in the purification of the peptide carrying T antigen amino acids 131–259. Pab101 immunosorbent was used to purify the peptide containing T antigen residues 272–708.

Immunoaffinity purification of DNA polymerase α

Calf thymus DNA polymerase α was purified from 1 kg of fresh calf thymus essentially as described (Nasheuer and Grosse, 1987), except that the ammonium sulphate precipitation step following elution from heparin–Sepharose was substituted by phosphocellulose chromatography, and the immunosorbent was SJK 132-20-Sepharose (Table I). Human DNA polymerase α was purified from fresh human placenta with a similar protocol using SJK 287-38-Sepharose (Table I). Calf thymus DNA primase was purified from the DNA polymerase α complex by eluting the SJK 132-20-Sepharose with 50 mM Tris–HCl pH 8.6, 2 M KCl instead of 0.1 M K-phosphate pH 12.5–13, 1 M KCl, 10% glycerol (Nasheuer, 1987). The eluate was dialysed against 10 mM K-phosphate buffer pH 7.8, adsorbed to a monoclonal anti-p58 primase (2CT6-6) Sepharose immunosorbent (I.Dornreiter and E.Fanning, in preparation), washed with 10 mM K-phosphate pH 7.8, then with 10 mM K-phosphate pH 7.8, 50 mM KCl, and eluted with 0.1 M K-phosphate pH 12.5–13, 10% glycerol. Eluates were neutralized immediately with 0.5 M KH_2PO_4, and dithiothreitol was added to a final concentration of 4 mM. Purified proteins were stored at −70°C in 50% glycerol. Concentrations of purified protein were estimated spectrophotometrically as for T antigen. One unit of DNA polymerase α activity was defined as the amount that incorporates 1 nmol of dNMP in 1 h at 37°C into acid-insoluble material using activated calf thymus DNA as a template–primer.

Enzyme-linked immunosorbent assay (ELISA)

Plastic 96 well microtitre trays (Immuno Maxi Sorb, Nunc, Wiesbaden) with 0.3 ml maximum volume per well were coated for 1 h with excess (1 μg) purified polymerase α. Unbound protein was washed away using phosphate buffered saline (PBS). Wells were blocked by incubating for 2 h with 0.3 ml 3% bovine serum albumin (BSA) (Nr. A-7030, Sigma, Munich) in PBS. After washing, various amounts of T antigen (in 50 μl) were added, incubated for 1 h at room temperature and then washed with PBS. Monoclonal anti-T antibody (1 μg in 50 μl) was added, incubated overnight at 4°C, washed and then visualized by incubation with anti-mouse immunoglobulin serum conjugated with horseradish peroxidase (Dakopatts, Hamburg) in the presence of 3% BSA for 1 h, followed by reaction with the chromogenic substrate 2, 2′-azino-bis-(3-ethyl-benzothiazoline-6-sulphonic acid) (ABTS) (Sigma, Munich). One ml of a stock solution of 40 mM ABTS was freshly mixed with 19 ml of 50 mM Na-phosphate pH 5.7 and 10 μl 30% H_2O_2 and 100 μl were added to each well. The reaction was allowed to proceed for 20 min in the dark and the resulting green colour was then quantitated spectrophotometrically at 410 nm. Alternatively, the solid phase was monoclonal anti-polymerase antibody SJK 132-20 (1 μg per well); complexes of purified polymerase α and T antigen were assembled as described in Figure 3 and detected with peroxidase-conjugated Pab419.

Immunoblot

Purified T antigen (1 μg), polymerase α (4 μg), or primase (1 μg) was denatured at room temperature as described (Miskimins *et al.*, 1985), electrophoresed in a 10% denaturing polyacrylamide minigel (Laemmli, 1970) and renatured in the gel before electrophoretic transfer to nitrocellulose as described (Dunn, 1986). Blots were incubated with 4 μg/ml of purified T antigen or 2 μg/ml of purified polymerase α in Tris-buffered saline (10 mM Tris–HCl pH 8.0, 150 mM NaCl) (TBS) for 1 h at room temperature as indicated in the figure legends. After four washes, detection of bound proteins was accomplished by incubation with 8 μg/ml of monoclonal anti-T antibody overnight at 4°C or rabbit anti-polymerase serum (1:500 in TBS) at room temperature for 2 h and then with commercial alkaline phosphatase-conjugated anti-mouse or anti-rabbit immunoglobulin antiserum and a chromogenic substrate as described by the supplier (Promega, Heidelberg).

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References

Alfano,C. and McMacken,R. (1989a) *J. Biol. Chem.*, **264**, 10699–10708.
Alfano,C. and McMacken,R. (1989b) *J. Biol. Chem.*, **264**, 10709–10718.
Arthur,A.K., Hoess,A. and Fanning,E. (1988) *J. Virol.*, **62**, 1999–2006.
Baumann,E.A., Bauer,C.P., Baack,M. and Beck,S. (1985) *J. Virol.*, **54**, 882–885.
Bernstein,J.A. and Richardson,C.C. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 396–400.
Borowiec,J.A., Dean,F.B., Bullock,P.A. and Hurwitz,J. (1990) *Cell*, **60**, 181–184.
Braithwaite,A.W., Sturzbecher,H.-W., Addison,C., Palmer,C., Rudge,K. and Jenkins,J.R. (1987) *Nature*, **329**, 458–460.
Bramhill,D. and Kornberg,A. (1988) *Cell*, **54**, 915–918.
Brill,S.J. and Stillman,B. (1989) *Nature*, **342**, 92–95.
Cha,T.-A. and Alberts,B.M. (1989) *J. Biol. Chem.*, **264**, 12220–12225.
Cha,T.-A. and Alberts,B.M. (1990) *Biochemistry*, **29**, 1791–1798.
Challberg,M. and Kelly,T.J. (1989) *Annu. Rev. Biochem.*, **58**, 671–717.
Dodson,M., McMacken,R. and Echols,H. (1989) *J. Biol. Chem.*, **264**, 10719–10725.
Dunn,S.D. (1986) *Anal. Biochem.*, **157**, 144–153.
Fanning,E. and Vogt,B. (1986) *Cancer Cells*, **4**, 471–475.
Fanning,E., Traut,W., Dornreiter,I., Dehde,S., Alliger,P. and Posch,B. (1988) *Cancer Cells*, **6**, 177–181.
Gannon,J.V. and Lane,D.P. (1987) *Nature*, **329**, 456–458.
Gannon,J.V. and Lane,D.P. (1990) *The New Biologist*, **2**, 84–92.
Goetz,G.S., Dean,F.B., Hurwitz,J. and Matson,S.W. (1988) *J. Biol. Chem.*, **263**, 383–392.
Gough,G., Gannon,J.V. and Lane,D.P. (1988) *Cancer Cells*, **6**, 153–158.
Gurney,E.G., Harrison,R.O. and Fenno,J. (1980) *J. Virol.*, **34**, 752–763.
Harlow,E., Crawford,L.V., Pim,D.C. and Williamson,N.M. (1981) *J. Virol.*, **39**, 861–869.
Hay,R.T. and Russell,W.C. (1989) *Biochem. J.*, **258**, 3–16.
Heukeshoven,J. and Dernick,R. (1988) *Electrophoresis*, **9**, 28–32.
Höß,A., Arthur,A.K., Wanner,G. and Fanning,E. (1988) *Bio/Technology*, **6**, 1214–1217.
Höß,A., Moarefi,I., Scheidtmann,K.-H., Cisek,L.J., Corden,J.L., Dornreiter,I., Arthur,A.K. and Fanning,E. (1990) *J. Virol.*, **64**, in press.
Kenny,M.K., Lee,S.-H. and Hurwitz,J. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 9757–9761.
Kenny,M.K., Schlegel,U., Furneaux,H. and Hurwitz,J. (1990) *J. Biol. Chem.*, **265**, 7693–7700.
Klausing,K., Scheffner,M., Scheidtmann,K.-H., Stahl,H. and Knippers,R. (1989) *Biochemistry*, **28**, 2238–2244.
Laemmli,U.K. (1970) *Nature*, **227**, 680–685.
Lane,D.P. and Hoeffler,W.K. (1980) *Nature*, **288**, 167–170.
Lanford,R.E. (1988) *Virology*, **167**, 72–81.
Lee,S.-H., Eki,T. and Hurwitz,J. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 7361–7365.
Lehman,I.R. and Kaguni,L.S. (1989) *J. Biol. Chem.*, **264**, 4265–4268.
Li,J.J. and Kelly,T.J. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 6973–6977.
Li,J.J. and Kelly,T.J. (1985) *Mol. Cell. Biol.*, **5**, 1238–1246.
Miller,M.A., Korn,D. and Wang,T.S.-F. (1988) *Nucleic Acids Res.*, **16**, 7961–7973.
Miskimins,W.K., Roberts,M.P., McClelland,A. and Ruddle,F.H. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 6741–6744.
Mole,S.E., Gannon,J.V., Ford,M.J. and Lane,D.P. (1987) *Phil. Trans. R. Soc. Lond. Ser. B*, **317**, 455–469.
Murakami,Y., Wobbe,C.R., Weissbach,L., Dean,F.B. and Hurwitz,J. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 2869–2873.
Nasheuer,H.-P. (1987) *Immunaufreinigung und Charakterisierung eines intakten DNA-Polymerase-α-Primase-Komplexes*. Dissertation. University of Hannover.
Nasheuer,H.-P. and Grosse,F. (1987) *Biochemistry*, **26**, 8458–8466.
Roberts,J.M. and D'Urso,G. (1988) *Science*, **241**, 1486–1489.
Schmieg,F.I. and Simmons,D.T. (1989) *Virology*, **164**, 132–140.
Simanis,V. and Lane,D.P. (1985) *Virology*, **144**, 88–100.
Smale,S.T. and Tjian,R. (1986) *Mol. Cell. Biol.*, **6**, 4077–4087.
So,A.G. and Downey,K.M. (1988) *Biochemistry*, **27**, 4591–4595.
Stahl,H., Dröge,P. and Knippers,R. (1986) *EMBO J.*, **5**, 1939–1944.
Stillman,B. (1989) *Annu. Rev. Cell Biol.*, **5**, 197–245.
Sturzbecher,H., Brain,R., Maimets,T., Addison,C., Rudge,K. and Jenkins,J.R. (1988) *Oncogene*, **3**, 405–413.

Tanaka,S., Hu,S.-Z., Wang,T.S.-F. and Korn,D. (1982) *J. Biol. Chem.*, **257**, 8386–8390.
Tjian,R. (1978) *Cell*, **13**, 165–179.
Tjian,R. and Robbins,A. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 610–614.
Traut,W. and Fanning,E. (1988) *Mol. Cell. Biol.*, **8**, 903–911.
Tsurimoto,T. and Stillman,B. (1989) *EMBO J.*, **8**, 3883–3889.
Wang,E.H., Friedman,P.N. and Prives,C. (1989a) *Cell*, **57**, 379–392.
Wang,T.S.-F., Wong,S. and Korn,D. (1989b) *FASEB J.*, **3**, 14–21.
Weinberg,D.H. and Kelly,T.J. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 9742–9746.
Wiekowski,M., Dröge,P. and Stahl,H. (1987) *J. Virol.*, **61**, 411–418.
Wiekowski,M., Schwarz,M.W. and Stahl,H. (1988) *J. Biol. Chem.*, **263**, 436–442.

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