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Structure and function of SV40 large-T antigen

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The small eukaryotic DNA tumour virus, SV40, has long provided a very useful model for the study of eukaryotic DNA replication and cellular transformation. The viral gene product, large-tumour (large-T) antigen, is essential for the initiation of viral DNA replication and the initiation and maintenance of SV40-virus-mediated cellular transformation. The large-T antigen is a complex multifunctional protein, and to delineate its activity more precisely in viral DNA replication and cellular transformation, small functional domains of the protein have been expressed in *Escherichia coli* and analysed by using a very extensive library of anti-T monoclonal antibodies.

Introduction

Purified T antigen protein displays a number of discrete activities *in vitro*. These are summarized in table 1. This extensive list is complemented by those activities summarized in table 2, which are the *in vivo* biological properties ascribed to the protein.

To place the biochemical and biological properties of T antigen in context it is helpful to attempt to integrate these functions within the virus lytic cycle and within its action as an oncogenic virus.

The role of T antigen in the lytic cycle

T antigen is the first viral protein expressed after viral infection of permissive cells (human or monkey). It is reviewed in Tooze (1981) and Rigby & Lane (1983). The protein is found predominantly in the nucleus in a range of oligomeric forms, some of which are associated with the cellular p53 protein at later times in infection. One of the primary functions of T antigen is to activate the cell for DNA synthesis. Thus the protein efficiently induces the host cell into S-phase, extending the host range of the virus to quiescent as well as growing cells. The biochemical basis of this induction is not understood as yet. Once the cellular pools of the enzymes and metabolites required for DNA synthesis have reached the required level, viral DNA synthesis can start. T antigen is essential for the initiation of viral DNA synthesis and with the development of the *in vitro* replication system (Li & Kelly 1984) the role of T in the process has been clarified.

T antigen binds to specific sequences within the viral origin and then probably initiates, in concert with as yet unidentified cellular proteins, a local unwinding of the DNA through its ATP-dependent DNA helicase activity (Stahl *et al.* 1986; Dean *et al.* 1987). The underwound origin then represents the site for entry of the replication machinery. Here, T may play a further role because T can bind polymerase α *in vitro* (Smale & Tjian 1986) and, as shown below, is complexed to polymerase α *in vivo*. The further role of T antigen in the replication cycle is less clear but the T antigen helicase may actually act at the replication fork for the

Table 1. Activities of large T *in vitro*

1. sequence specific DNA binding
2. ATPase
3. helicase
4. p53 binding
5. DNA polymerase α binding
6. initiation of SV40 DNA synthesis
7. repression of transcription

Table 2. Functions of large T *in vivo*

1. initiation and maintenance of transformation
2. immortalization
3. viral DNA replication
4. host DNA replication
5. viral transcriptional regulation
6. host transcriptional regulation
7. binding and modification of p53
8. adenovirus helper function
9. host range function
10. tumour transplantation antigen

entire round of synthesis (Stahl *et al.* 1985), presumably substituting for the normal cellular replicative helicase.

In addition to this direct role in the replicative cycle, other activities of T antigen are also expressed during the lytic cycle (Tooze 1981; Rigby & Lane 1983). The protein acts to regulate viral transcription, by autoregulating transcription of its own RNA and stimulating transcription of the late viral mRNAs. In certain host cells the protein also acts to facilitate the efficient translation of the late mRNAs.

The role of T antigen in transformation

The action of T antigen in cellular transformation is less clear. The direct replicative functions of the protein do not seem to be involved because mutants lacking both ATPase or DNA binding activities transform with wild-type efficiency (Manos & Gluzman 1984, 1985). The weight of current evidence would imply some role for the N-terminus of the molecule in the mitogenic activity of the protein (Pipas *et al.* 1983). The binding of the cellular proto-oncogene p53 by T antigen, an activity localized to a more central region of the molecule (Lane & Gannon 1986), may also be important for complete cellular transformation. However, the biochemical reactions mediated by these activities are still unclear.

The aim of our studies is to obtain a precise understanding of the action of large T in both transformation and replication.

Materials and methods

Construction of β-galactosidase–large-T fusion proteins

The techniques used are described in Mole & Lane (1985). SV40 fragments were cloned into unique restriction sites of pUR290, pUR291 and pUR292 (Rüther & Müller-Hill 1983). Fusion proteins were induced for expression in *E. coli* strain BMH 71-18, which has the lacZ phenotype, by using isopropyl thiogalactoside (IPTG).

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Construction of CAT–large-T fusion proteins

The 'EcoRI cassette' derived from a pUR plasmid containing an SV40 fragment was cloned into the unique *Eco*RI site of pSEMCat$_R$1 (Mole & Lane 1987). The bacterial host used for CAT–large-T fusion protein expression was *Escherichia coli* strain HB101.

Monoclonal antibodies

Antibodies belonging to the PAb400 series were given by Dr E. Harlow (Harlow *et al.* 1981); those of the PAb600 series were given by Dr L. Gooding (Gooding *et al.* 1984) and those of the PAb200 series were produced in this laboratory. PAb203, PAb204 and PAb205 were described by Clark *et al.* (1981). PAb250 and PAb251 are two new antibodies raised against a β-galactosidase–large-T fusion protein expressing the SV40 *Hind*III D fragment (Mole & Lane 1985) and PAb281, which was raised against SV40 small-t antigen (Montano & Lane 1984) binds to the common N-terminus of large-T and small-t antigen. The T6, T11 and T12 series (part of the PAb200 series) of anti-large-T monoclonals were raised by Dr J. W. Yewdell (J. W. Yewdell & D. P. Lane, unpublished data) and were obtained from Dr D. P. Lane. The anti-p53 antibody, RA3-2C2 (Rotter *et al.* 1981), was obtained from Dr V. Rotter. The anti-DNA polymerase α antibody, SJK 287-38 (Tanaka *et al.* 1982) was obtained from the American National Type Culture Collection.

Colony blotting

Colonies to be screened were gridded out on nitrocellulose filters laid on L-agar plates supplemented with the appropriate antibiotic and IPTG if inducing the expression of β-galactosidase fusion proteins, grown overnight, lysed in chloroform saturated vapour and incubated at 4 °C for 3 h in blocking buffer (bovine serum albumin (BSA) (3% by mass)) in phosphate-buffered saline (PBS; 150 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4 at pH 7.2). After rinsing in PBS, incubation in the monoclonal antibody was for 3–18 h, followed by rinsing in PBS, then washing twice for 15 min in PBS containing Nonidet P-40 (NP-40) (1% by volume) and twice for 15 min in PBS. Incubation in preadsorbed rabbit anti-mouse immunoglobulins coupled to horseradish peroxidase was for 3–18 h, followed by rinsing in PBS, then washing twice for 15 min in PBS containing NP-40 (1% by volume) and twice for 15 min in PBS.

The substrate used was 50 mg of 4-chloro-1-naphthol in 1 ml ethanol; this preparation was freshly diluted 1:100 in PBS and filtered, and hydrogen peroxide (30% by volume) added at a dilution of 1:5000.

McKay immunoprecipitation assay for DNA binding

The procedure used was based on that described by McKay (1981). Overnight bacterial cell extracts were subcultured at a 1:5 dilution and grown for 1 h at 37 °C. The cells were washed in 1 × McKay dilution buffer (10 mM Hepes at pH 7.5, 0.5 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF and glycerol (2.5% by volume)), resuspended in 0.5 × dilution buffer and sonicated on ice for 30 s. The cell debris was removed by centrifugation and the supernatant used in the McKay assay.

The McKay assay reaction mix (total volume 200 μl) consisted of 300 ng of end-labelled *Eco*RII digested SV40 DNA, bacterial cell extract prepared as described or purified SV40 large-T antigen (Simanis & Lane 1985) in 1 × dilution buffer with 75 mM KCl. The assay was

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

done on ice. This was incubated for 15 min, then 100 µl of monoclonal antibody supernatant added, and incubated for 1 h.

25 µl of a 10% (by mass) suspension of fixed *Staphylococcus aureus* Cowan 1 (washed five times in NET buffer (150 mM NaCl, 5 mM EDTA pH 8.0, 50 mM Tris–HCl pH 7.5) containing NP-40 (0.5% by volume)) was added, incubated with occasional mixing for 30 min and the immune complexes pelleted. After washing four times at room temperature with NET buffer containing NP-40 (0.05% by volume), the DNA was released from the immune complex by incubation of the final pellet in 20 µl of a solution containing SDS (1% by mass), Ficoll (8% by mass), 25 mM EDTA at pH 8.0, and bromophenol blue (0.5 g l^{-1}), at 65 °C for 30 min. After centrifugation the supernatant was analysed by electrophoresis on an agarose gel (2% by mass).

The gel was fixed in acetic acid (10% by volume) followed by ethanol, dried and autoradiographed.

In vitro *assay of p53–SV40 large-T association*

(*a*) *Preparation of cell extracts*

After harvesting the cells were centrifuged and snap-frozen in a bath of ethanol and solid CO_2. The pellets were stored at −70 °C.

Extracts were prepared by thawing the cell pellets rapidly and resuspending them in extraction buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris–HCl at pH 8.0, 2 mM PMSF, NP-40 (1% by volume)) at a ratio of 1 ml of buffer per 4×10^7 cells. The extracts were kept on ice for 30 min and then clarified by centrifugation at 10000 *g* for 30 min. The supernatants were used immediately. For titration, the cell extracts were diluted in extraction buffer.

(*b*) *Preparation of antibody-coated plates*

The wells of a microtitre dish (Falcon) were coated with antibody by incubating overnight with the antibody at a concentration of 30 µg per ml in 10 mM phosphate buffer, pH 7.5, in a humidified chamber. The plate was rinsed in PBS and blocked by using BSA (3% by mass) in PBS for 3 h. After a final rinse in PBS, the plates were used immediately or stored at −20 °C.

(*c*) *Assay*

This assay is based on the solid-phase radioimmunoassay (RIA) of Benchimol *et al.* (1982).

Clarified cell-lysate (50 µl) from T3T3 cells and 50 µl of a given dilution of a clarified lysate from Ad5-SVR111-infected 293 cells were incubated together for 3 h. The mixture was transferred to either an RA3-2C2 antibody-coated well in a plastic microtitre plate or to a PAb205 antibody-coated well, allowed to incubate overnight, and then rinsed in PBS. 50 µl of ^{125}I-labelled PAb419, PAb414 or PAb250 were added to the wells, incubated for 3 h, rinsed in PBS and the individual wells cut out and counted in an LKB Wallac 80000 gamma counter.

In vitro *assay of polymerase α-SV40 large-T association*

(*a*) *Preparation of cell extracts*

Ad5-SVR111 infected 293 cells were harvested, centrifuged and the pellet frozen in a bath of ethanol and solid CO_2. Extracts were prepared by adding the extraction buffer, HMK

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

(10 mM HEPES, 1.5 mM $MgCl_2$, 5 mM KCl, pH 7.4), at a ratio of 1 ml of buffer per 10^8 cells. The cells were homogenized by using a dounce homogenizer (20 strokes) and incubated on ice for 30 min. The abstract was clarified by centrifugation at 50000 rev. min^{-1} for 10 min at 4 °C in the Beckman TL100 ultra-centrifuge, and the supernatants used immediately.

(*b*) *Assay*

Dilutions of cell extracts prepared as described above were made up by using the HMK extraction buffer with BSA (1 % by mass) included as a carrier protein. Portions (50 µl) of these prepared extracts were applied to microtitre wells (Falcon), which had been previously coated with the anti-polymerase α antibody, SJK 287-38. The plates were incubated overnight and washed in PBS containing NP-40 (0.1 % by volume). 50 µl of ^{125}I-labelled PAb419 (labelled to a specific activity of 10 µCi μg^{-1}) was added at a concentration of 10 ng per well in PBS containing BSA (1 % by mass) and NP-40 (0.1 % by volume). After a 3 h incubation, the plates were washed in PBS containing NP-40 (0.1 % by volume), dried, and counted in a Packard Multiprias 2 gamma counter.

ELISA assay with synthetic peptides

The synthetic peptides were kindly supplied by Dr J. Rothbard (Imperial Cancer Research Fund). They had been conjugated to thyroglobulin through a carboxy terminal cysteine residue. The peptide protein conjugates were adjusted to a concentration of 0.1 mg ml^{-1} by dilution in PBS. The wells of a plastic 96 well microtitre dish (Falcon 3912) were then coated with peptide by incubating them for 12 h with 50 µl of the appropriate peptide conjugate. The unbound peptide conjugate was washed away and 100 µl of a blocking solution (5 % by mass non-fat dried milk in PBS) added to each well. After incubation with the blocking solution for 1 h, the wells were again washed out and the appropriate test monoclonal antibodies added to the wells. (All antibodies were diluted in PBS containing foetal calf serum (10 % by mass) PBS–FCS.) After 1 h the antibody was washed out using PBS containing NP-40 (0.1 % by volume), and replaced with a detecting antibody (peroxidase conjugated rabbit antibodies to mouse immunoglobulins (DAKO Laboratories) diluted 1:1000 in PBS–FCS). After a 2 h incubation, this reagent was washed out and a chromogenic peroxidase substrate added (3′3′5′5′ tetramethyl benzidene dissolved in DMSO at a concentration of 1 mg ml^{-1} and diluted 1:100 in PBS containing H_2O_2 (0.01 % by volume)). After a 15 min period for colour development a stop solution (1 M H_2SO_4) was added to the wells and the absorbance of each well at 450 nm measured by using an ELISA plate reader (Flow Multiscan).

Results

The β-galactosidase fusion proteins

A total of nine different fusion proteins between β-galactosidase and SV40 large-T antigen have been constructed by using the pUR series of vectors. The region of T that these new proteins contain is illustrated in figure 1. The ability of a large number of different anti-T monoclonal antibodies to bind to these fragments of T expressed in *E. coli* was tested with the colony blot procedure. The results are listed in table 3 and presented in diagrammatic form in figure 2. A number of interesting observations were made. Firstly, all of the antibodies were able to bind to at least one of the fusion proteins. Thus none of them recognize an epitope that cannot, at least in part, be represented on a fragment of T produced in a prokaryotic cell.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

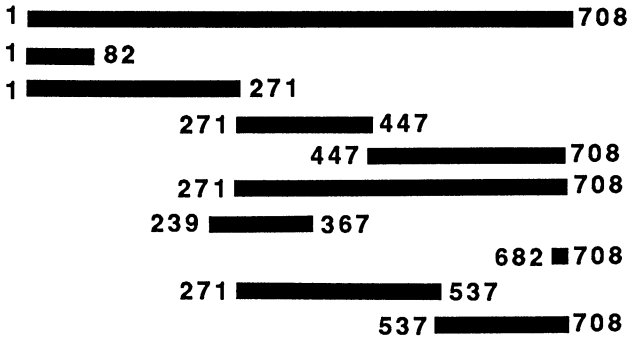

FIGURE 1. SV40 large T consists of 708 amino acid residues, represented by the uppermost solid black line. The regions of T expressed as fusion proteins are illustrated underneath, together with the amino acid residues encoded by each fragment.

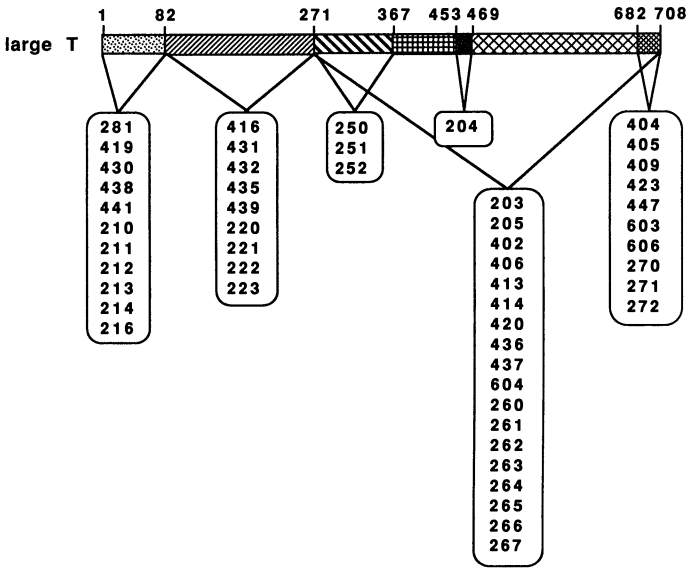

FIGURE 2. The mapping of epitopes recognized by a variety of anti-large-T monoclonal antibodies is illustrated. Each region of T is defined by the amino acid residues.

Secondly, the binding sites for the antibodies are not evenly distributed. A large number of antibodies are directed to sites at the extreme N- or C-terminus of the protein. Most of these antibodies in fact recognize epitopes that are resistant to denaturation, as the epitopes are not irreversibly lost on treating either T or the appropriate fusion protein with SDS (2% by mass). Thirdly, a large group of antibodies that recognize denaturation sensitive sites fail to interact with small fragments of T because they only show binding to fusion proteins that contain large segments of the protein.

Antibody inhibition of T-antigen function

A number of independent biochemical activities of the T protein can be selectively inhibited by certain anti-T antibodies. These inhibition reactions appear to be very selective and discrete, because in all cases the binding of a large range of other antibodies does not affect the measured function. Anti-T antibodies have been described that inhibit the ATPase activity (Clark *et al.* 1981), the helicase (Stahl *et al.* 1986), the binding to p53 (Lane & Gannon 1986)

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

TABLE 3. COLONY BLOTTING OF β-GALACTOSIDASE–SV40 FUSION PROTEINS

(Positive binding is indicated by +, negative binding by −.)

screening antibody†	region of T antigen encoded by fusion proteins (amino acid residues)						
	1–82	1–271	271–447	447–708	271–708	239–367	682–708
PAb204^{R}	−	−	−	+	+	−	−
PAb203^{S}	−	−	−	(+)	+	−	−
PAb205^{S}	−	−	−	−	+	−	−
PAb250^{R}	−	−	+	−	+	+	−
PAb251^{R}	−	−	+	−	+	+	−
PAb280^{R}	+	−	−	−	−	−	−
PAb281^{R}	+	+	−	−	−	−	−
PAb416^{R}	−	+	−	−	−	−	−
PAb419^{R}	+	+	−	−	−	−	−
PAb430^{R}	+	+	−	−	−	−	−
PAb431^{R}	−	+	−	−	−	−	−
PAb432^{S}	(+)	+	−	−	−	−	−
PAb435^{S}	−	+	−	−	−	−	−
PAb438^{S}	+	+	−	−	−	−	−
PAb439^{S}	−	+	−	−	−	−	−
PAb441^{R}	+	+	−	−	−	−	−
PAb402^{S}	−	−	−	−	+	−	−
PAb404^{R}	−	−	−	+	+	−	+
PAb406^{S}	−	−	−	−	+	−	−
PAb414^{S}	−	−	−	−	+	−	−
PAb420^{S}	−	−	−	−	+	−	−
PAb436^{S}	−	−	−	−	+	−	−
PAb437^{S}	−	−	−	−	+	−	−
PAb405^{R}	−	−	−	+	+	−	+
PAb409^{R}	−	−	−	+	+	−	+
PAb423^{R}	−	−	−	+	+	−	+
PAb447^{R}	−	−	−	+	+	−	+
PAb260^{S}	−	−	−	−	+	−	−
PAb261^{S}	−	−	−	−	+	−	−
PAb270^{R}	−	−	−	+	+	−	+
PAb271^{R}	−	−	−	+	+	−	+
PAb262^{S}	−	−	−	−	+	−	−
PAb252^{S}	−	−	+	−	+	+	−
PAb210^{R}	+	+	−	−	−	−	−
PAb267^{R}	−	−	−	−	+	−	−
PAb263^{S}	−	−	−	−	+	−	−
PAb264^{S}	−	−	−	−	+	−	−
PAb265^{S}	−	−	−	−	+	−	−
PAb272^{R}	−	−	−	+	+	−	−
PAb266^{S}	−	−	−	−	+	−	−
PAb216^{R}	+	+	−	−	−	−	−
PAb220^{S}	−	+	−	−	−	−	−
PAb221^{S}	−	+	−	−	−	−	−
PAb267^{S}	−	−	−	−	+	−	−
PAb211^{R}	+	+	−	−	−	−	−
PAb222^{S}	−	+	−	−	−	−	−
PAb212^{R}	+	+	−	−	−	−	−
PAb213^{R}	+	+	−	−	−	−	−
PAb214^{R}	+	+	−	−	−	−	−
PAb223^{R}	−	+	−	−	−	−	−
PAb603	−	−	−	+	+	−	+
PAb604	−	−	−	−	+	−	−
PAb606	−	−	−	+	+	−	+

† The superscript indicates whether the antibody recognizes denaturation resistant (R) or denaturation sensitive (S) epitopes.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

and the replicative functions (Smale & Tjian 1986) of T antigen *in vitro*. Some of these data are combined in table 4. This set of observations support and extend ideas about the functioning of T antigen *in vivo*. All antibodies that inhibit the ATPase activity of T antigen block helicase activity and the *in vitro* replication system. One antibody has been identified that blocks the helicase activity while leaving the ATPase activity undiminished. All the antibodies that block the binding to polymerase α also block the *in vitro* replication system. It is very provocative that these same antibodies also specifically block the binding of the cellular p53 protein to T. It is possible that either p53 and polymerase α bind to the same site of T or that polymerase α binds to T through p53. These studies need to be greatly extended and it is clear that more antibodies need to be produced to selected sites on T to obtain further antibodies that affect specific functions of large T. The binding sites of these, and existing inhibitory antibodies, should be mapped more closely.

TABLE 4. FUNCTIONS OF T ANTIGEN–ANTIBODY COMPLEXES

(The presence of a function of large T exhibited by the T-antibody complex is indicated by +, the abolition of a function of large T by the binding of the antibody is indicated by −. The table was compiled with data from Harlow *et al.* (1981), Lane & Gannon (1986), Stahl *et al.* (1986) and Smale & Tjian (1986). n.d., no data.)

complexed antibody	ATPase activity	helicase activity	p53 binding	DNA polymerase α binding	DNA replication	antibody recognition site on T antigen (amino acid residues)
PAb204	−	−	+	+	−	453–468
PAb205	+	+	(−)	−	−	*ca.* 362–708
PAb414	+	n.d.	−	−	−	*ca.* 362–699
PAb413	+	n.d.	−	−	−	*ca.* 362–699
PAb1630	+	−	n.d.	n.d.	n.d.	*ca.* 300–650

*Precise mapping of the epitope recognized by PAb*204

The only inhibitory antibody whose binding site has been precisely mapped is PAb204. This antibody was one of the first monoclonal anti-T antibodies to be characterized and was raised against the Adeno-T hybrid protein D2T (Lane & Hoeffler 1980). The antibody inhibits the ATPase (Clark *et al.* 1981), helicase (Stahl *et al.* 1986) and replicative functions of T antigen (Smale & Tjian 1986). Its binding site was mapped to within 63 amino acids with data obtained from its binding to the large-T fusion proteins and from data on the reactivity of the antibody with T deletion mutants (Mole & Lane 1985). PAb204 shows an exquisitely precise cross-reaction with a growth regulated evolutionarily conserved host protein, p68 (Lane & Hoeffler 1980). A series of cDNA clones for p68 have recently been isolated (M. J. Ford & D. P. Lane, unpublished observations) by screening a λgt11 library of human hepatoma cDNA (de Wet *et al.* 1984) with PAb204.

One of the shortest clones that encoded the PAb204 epitope within p68 was sequenced. This sequence was then aligned with the sequence of 63 amino acids within T that encompassed the epitope, and a series of four synthetic peptides corresponding to this area were tested for their interaction with PAb204 in a solid phase ELISA assay. The results, shown in figure 3, localize the epitope to a region of 17 amino acids extending from residue 453 to 469 of large T. The

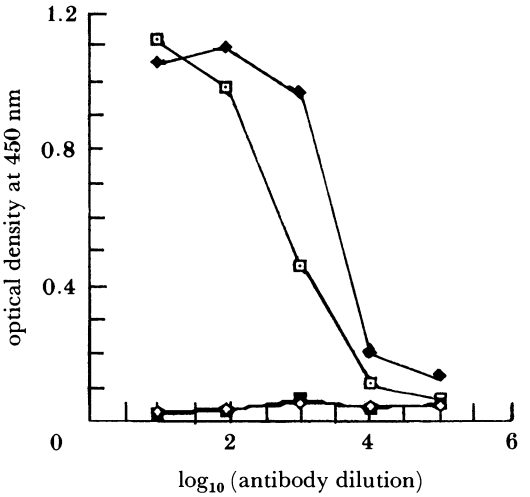

FIGURE 3. Localization of the epitope recognized by PAb204. The binding of a series of 10-fold dilutions of PAb204 to four synthetic peptides was measured in an indirect ELISA assay. The four peptides were: ⊡, NLPLDRLNFELGVAIDQFC; ◆, PLDRLNFELGVAIDQFC; ■, RLNFELGVAIDQFC; ◇, FELGV AIDQFC.

very close proximity of this site to the consensus nucleotide binding site previously noted in the sequence of T antigen (Seif 1984) is striking (see figure 4) and strongly supports the idea that the ATPase activity of T is intrinsic and that the nucleotide binding site has been correctly identified.

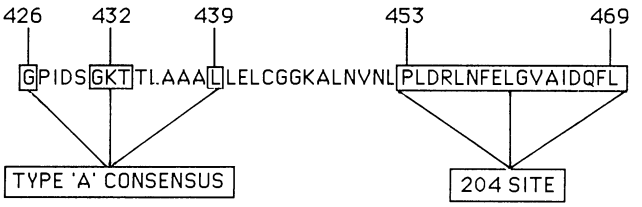

FIGURE 4. The type 'A' consensus sequence for nucleotide binding is located between amino acid residues 426 and 439 of SV40 large -T antigen. This is sited very close to the 17 amino acid sequence identified as containing the epitope recognized by PAb204, which inhibits ATPase and helicase activities of T antigen, both of which require nucleotide binding.

The CAT fusion proteins

The monoclonal mapping survey suggested that fragments of T produced in *E. coli* fold up correctly so as to assume aspects of their native structure in the complete molecule, because they were able to react with many different antibodies that recognize denaturation sensitive sites. It therefore seemed worthwhile to ask if the fusion proteins retained any of the biochemical activities of intact T antigen. Initial attempts at these experiments were frustrated by the insolubility of the majority of the β-galactosidase fusion proteins. To circumvent this problem, a new fusion protein expression vector, pSEMCat$_R$1 was developed (Mole & Lane 1987). This vector contains a plasmid unique *Eco*R1 site in the gene encoding bacterial chloramphenicol acetyltransferase (CAT) at the position corresponding to amino acid 72 of the protein. Any insert within a pUR vector can be excised as an '*Eco*R1 cassette' and transferred into this vector. The resulting recombinants can be identified by antibody screening and also by inactivation of the CAT gene.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

The vector, derived from pSV2-CAT (Gorman *et al.* 1982), permits the expression of the CAT fusion protein in both prokaryotic and eukaryotic cells. In several cases, the CAT fusion proteins have proved to be soluble while the corresponding β-galactosidase fusion proteins have been insoluble.

Sequence-specific DNA binding by a CAT–large-T fusion protein

Earlier studies with mutant T antigens had implied that the sequence-specific DNA binding activity of T antigen required the amino terminal half of the molecule (Paucha *et al.* 1986). Two of the CAT fusion proteins produced in *E. coli* were therefore chosen to test for sequence-specific binding in the DNA fragment immunoprecipitation assay of McKay (1981). Figure 5 shows that both the native large-T and the CAT fusion protein expressing the N-terminal third of T antigen (up to amino acid 271) are able to bind specifically to the origin-containing SV40 restriction fragment in this assay, while the CAT fusion protein expressing the C-terminal two-thirds of T is unable to do so. The CAT–large-T fusion protein is at least as efficient on a molar basis (data not shown) as wild-type T in DNA binding and appears to show less non-specific binding to non-origin containing DNA fragments than the native protein.

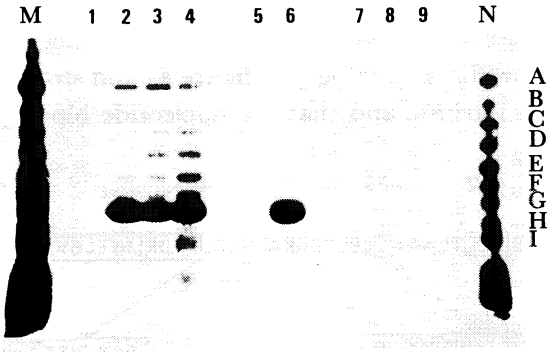

FIGURE 5. Immunoprecipitation of SV40 large-T antigen or CAT–large T fusion proteins to SV40 origin-containing DNA. Cat.T.HBsp encodes the N terminal 271 amino acids of large T and Cat.T.HDA encodes residues 271–708. Lane M: 15 ng of *Eco*RII-digested SV40 DNA (the origin is contained within the *Eco*RII G fragment). Lane N: 3 ng of *Eco*RII-digested SV40 DNA. Lane 1: 100 ng of SV40 large-T antigen incubated with PAb421. Lane 2: 100 ng of SV40 large-T antigen incubated with PAb416. Lane 3: 100 ng of SV40 large-T antigen incubated with PAb251. Lane 4: 100 ng of SV40 large-T antigen incubated with PAb204. Lane 5: Cat.T.HBsp extract incubated with PAb421. Lane 6: Cat.T.HBsp extract incubated with PAb416. Lane 7: Cat.T.HDA extract incubated with PAb421. Lane 8: Cat.T.HDA extract incubated with PAb251. Lane 9: Cat.T.HDA extract incubated with PAb204. The immunoprecipitates were separated out on an agarose gel (2% by mass).

p53 binding by a CAT–large-T fusion protein

The binding site for p53 has not been closely localized on T. The data from antibody inhibition studies imply that the interaction depends on sites in the central region of the molecule (Lane & Gannon 1986). Figure 6 shows that a CAT fusion protein produced in *E. coli* and encoding amino acids 271–708 of T antigen is able to associate with p53 *in vitro* as efficiently on a molar basis as wild-type T. In a further study we have recently been able to produce full length p53 in *E. coli*. This bacterially produced protein is also able to bind this CAT–large-T fusion protein, thus the entire interaction between large T and p53 can be modelled by using proteins isolated from prokaryotic sources (R. G. Greaves & D. P. Lane, unpublished observations).

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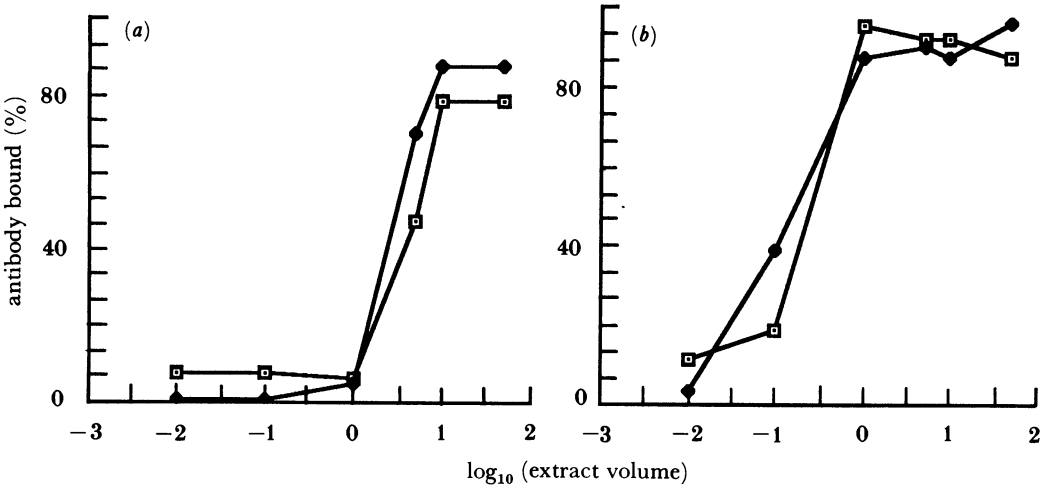

FIGURE 6. Cat.T.HDA, which encodes amino acid residues 271–708 of large T binds p53. (*a*) A crude soluble extract of bacteria expressing the Cat.T.HDA fusion protein was titrated in a sandwich radioimmunoassay with an anti-T monoclonal, PAb205, as the solid phase antibody and PAb414 as the iodinated antibody (◆). The same extract concentrations were also mixed with a murine p53 containing extract of transformed 3T3 cells and the resulting Cat.T.HDA–p53 complex measured with the same solid phase antibody (PAb205) but with the anti-p53 monoclonal 200.47 as the iodinated antibody (⊡). (*b*) The test was repeated substituting an extract of Ad5-SVR111 infected 293 cells which express large T for the Cat.T.HDA extract: ◆, PAb205 as the solid-phase antibody and PAb414 as the iodinated, detecting antibody; ⊡, PAb205 as the solid-phase antibody and 200.47 as the iodinated detecting antibody.

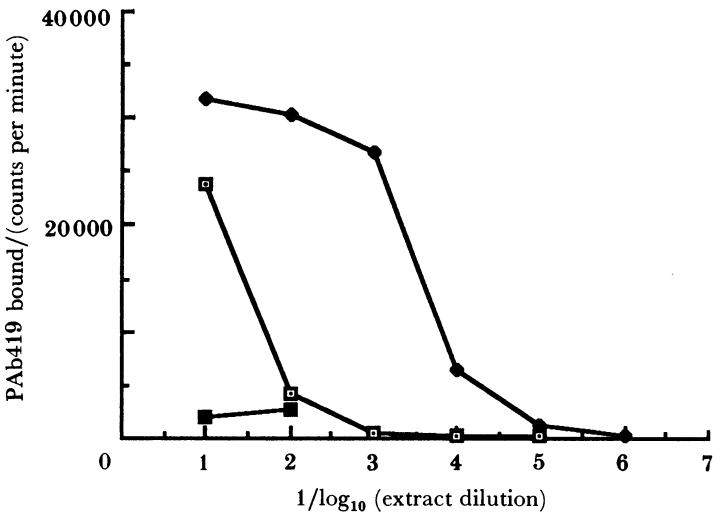

FIGURE 7. A series of 10-fold dilution of an extract of Ad5-SVR111 infected 293 cells were incubated with three different solid phase antibodies in plastic microtitre wells. The amount of T antigen retained by each solid phase was measured by determining the binding of iodinated PAb419 to the wells. The solid-phase antibodies were: ◆, PAb423, an anti-T monoclonal, to measure the total amount of T antigen in the extract; ⊡, 287.38, an anti-DNA polymerase α monoclonal, to measure the T antigen that binds polymerase α, ■, RA3-2C2, a control antibody that binds neither T antigen nor polymerase α.

Large-T antigen is bound to DNA polymerase α in infected cells

Smale & Tjian (1986) recently reported that large-T antigen could bind to DNA polymerase α in an *in vitro* reassociation experiment. To confirm and extend this result to the *in vivo* situation, a radioimmunoassay to measure large-T–polymerase α interaction was established.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

In this assay the wells of a plastic microtitre dish were first coated with a range of different monoclonal antibodies to DNA polymerase α (Tanaka *et al.* 1982). Extracts of cells that had been infected with the large T overproducing recombinant Adenovirus Ad5-SVR111 virus were incubated in these wells, which, after extensive washing, were tested for the retention of large-T antigen–polymerase α complex by incubating with a radiolabelled anti-T monoclonal antibody. The results of one such experiment are illustrated in figure 7. It is clear that a small fraction of the total T antigen present in the extract of SVR111 infected 293 cells is complexed to DNA polymerase α. The assay appears specific because control antibody coated plates bind only a tiny fraction of the large-T antigen retained by the anti-polymerase α antibody. Initial results suggest that a substantial fraction of the total polymerase α in the extract may be in the complex. The results need to be extended to wild-type SV40 infected and transformed cells.

Discussion

Using antibodies to probe T antigen function

To approach the analysis of the structure and function of complex protein molecules by using a large library of monoclonal antibodies is still relatively novel and these studies on T antigen to some extent represent a test case. The results obtained to date have been very encouraging and suggest that the approach may be generally applicable. However, a number of restraints on the method have become apparent and it is useful to discuss our results in this context.

Generating the library of antibodies

The majority of anti-T antibodies currently available have resulted from immunization with whole SV40 transformed cells rather than purified protein. This is due to historical reasons, as it is only with the advent of immunoaffinity chromatography procedures (Simanis & Lane 1985; Dixon & Nathans 1985) that the isolation of usable amounts of pure T antigen has become straightforward. The mapping studies described here suggest that the existing library of antibodies is incomplete and dominated by antibodies directed to certain epitopes at the N- and C-termini of the protein. Examination of the hybridomas resulting from the fusion of an individual spleen strongly suggest that there is individual variation in the dominant epitopes recognized. For instance, in our most recent series, mouse T12 gave rise to seven characterized antibodies, all of which recognized epitopes in the amino terminal third of T, whereas of the fourteen antibodies derived from mouse T6, only two were directed to epitopes in this region of the protein. The other point that emerges is the ease with which antibodies to novel epitopes can be generated by immunizing with altered forms of the antigen. The fusion that used mice immunized with the D2T hybrid protein generated the PAb204 antibody which is unique because it neutralizes some of the biochemical activities of the protein and yet recognizes a denaturation resistant epitope. Similarly, immunization with the β-galactosidase–large-T fusion proteins has generated new antibodies of novel specificity (Mole & Lane 1985; Lane & Gannon 1986). The next phase in expanding the library, designed to circumvent these problems, will involve further immunizations with pure and altered forms of the antigen. The resulting hybridomas will then be screened in very selective assays designed to identify antibodies directed to particular sites in the protein, or which inhibit particular functions.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Mapping antibody binding sites

Combined with the need to expand the library, as discussed above, is a requirement to map as accurately as possible the epitopes recognized by these antibodies. For antibodies that recognize epitopes primarily determined by a linear sequence of amino acids this is relatively straightforward. An optimal strategy will involve nested sets of small fusion proteins generated by random cloning of the entire T-coding region, followed by the use of short synthetic peptides to finally localize the site. This approach has already localized a major epitope at the C-terminus of T antigen (Gooding *et al.* 1984) and, as discussed here, has now also allowed the localization of the PAb204 epitope. The mapping of antibodies that recognize conformationally dependent epitopes is more difficult, although the finding that these epitopes can be expressed by large fusion proteins is encouraging. Again, a nested set of random fusion proteins will be required, but in this case the set will need to include large protein fragments with short overlaps and it can be anticipated that protein fragments containing random internal, in frame, deletions will be especially valuable. It is nevertheless desirable, in future fusions, to screen selectively for new antibodies that both inhibit function and recognize denaturation resistant epitopes.

Antibody inhibition of T antigen function

The selective inhibition of functions of large T by monoclonal antibodies has already yielded important results, and shows promise for the future. An important feature of the results, illustrated in table 4 and in figure 8, is the selectivity of individual inhibition reactions. For example, the binding of many antibodies that recognize C-terminal epitopes leaves all tested functions intact, whereas other antibodies selectively inhibit one activity such as the helicase while leaving another activity such as the ATPase intact. The most exciting new correlation that has emerged derives from a comparison of those antibodies that block p53 binding to T (Lane & Gannon 1986) and those that block DNA polymerase α binding to T (Smale & Tjian 1986). This comparison suggests, for the first time, a potential biochemical explanation for the role of p53 and its association with T antigen. Experiments are now in progress to determine whether the binding of polymerase α and p53 to T antigen are mutually exclusive or whether

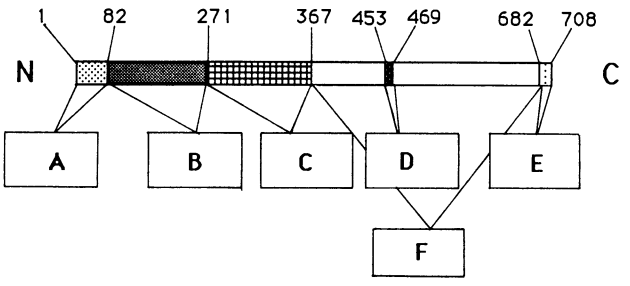

FIGURE 8. The binding of monoclonal antibodies to different regions of large T can affect its biochemical activities and functions: region A: the binding of PAb419 stimulates DNA replication (Smale & Tjian 1986); region B: no antibodies have been observed to affect functions of this region; region C: the binding site of PAb250 is lost on the formation of the T–p53 complex (Lane & Gannon 1986); region D: the binding of PAb204 inhibits ATPase, helicase and DNA replication activities of T antigen (Clark *et al.* 1981; Stahl *et al.* 1986; Smale & Tjian 1986); region E: no antibodies have been observed to affect functions of this region; region F: the binding of PAb203 inhibits ATPase activity (Clark *et al.* 1981); the binding of PAb1613 and PAb1630 inhibits helicase activity (Stahl *et al.* 1986); the binding of PAb205, PAb402, PAb413 and PAb414 inhibits DNA replication (Smale & Tjian 1986); the binding of PAb414 and PAb413 inhibits the formation of the T–p53 complex *in vitro* (Lane & Gannon 1986; J. V. Gannon & D. P. Lane, unpublished observations).

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

polymerase α binds to T via p53. Because some cell-lines have been identified that grow in the complete absence of p53 synthesis, we favour the hypothesis that p53 may represent an optional regulatory subunit of polymerase α that can be displaced by T antigen.

Functions expressed by fusion proteins

We show here that two biochemical properties of T antigen, sequence-specific DNA binding and p53 binding, are discrete functions that can be expressed on separate domains of large T synthesized as fusion proteins in *E. coli*. These observations provide definitive proof of the multi-domain nature of T antigen and also open up the possibility of a ready analysis of these separate biochemical activities. In particular, the amino terminal third large-T fragment can now be produced in milligram amounts in a pure and active form (J. V. Gannon, S. E. Mole & D. P. Lane, unpublished observations) and may well be suitable for structural characterization with X-ray crystallography. The efficient expression of a fragment of a eukaryotic protein in *E. coli* that binds specifically to DNA also has wider implications, and it may prove possible to devise a direct colony screening procedure to screen copy DNA (cDNA) expression libraries for sequence-specific DNA binding proteins by using these T-antigen fragments as a model.

Conclusions

The combination of protein expression, cloning of gene fragments and detailed immunochemical analysis with monoclonal antibodies is proving exciting and effective in the study of T antigen and its interaction with the host cell. The refinement of the approach seems technically straightforward and the time is now ripe to extend the strategy and apply it to the study of a complex protein machine such as that which mediates and regulates eukaryotic DNA replication.

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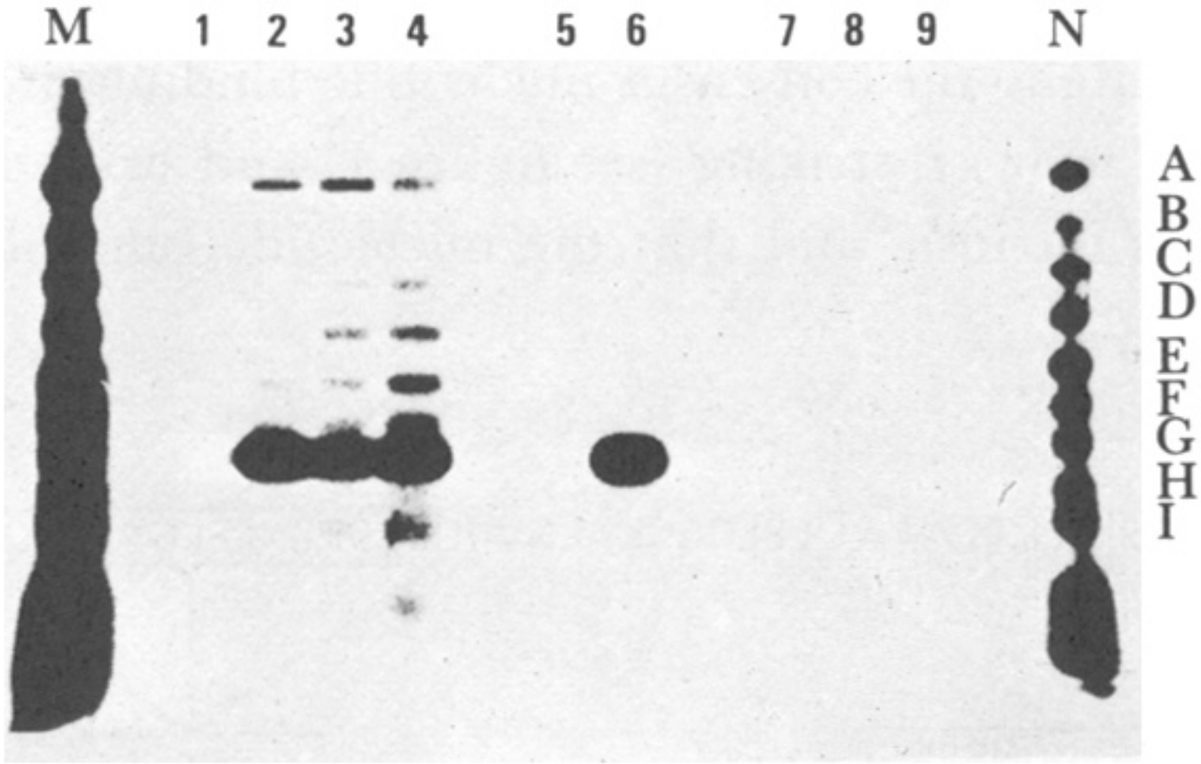

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