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Renaturation and DNA looping promoted by the SV40 large tumour antigen

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Simian virus 40 large tumour antigen (T antigen) is shown to catalyse the formation of duplex DNA from complementary strands in specific conditions. The activity is dependent on an excess of unspecific double-stranded DNA and seems not to function by T antigen mediated destabilization of secondary structure. Rather, protein-protein contacts between T antigen molecules appear to be involved. Protein-protein interactions between T antigen molecules bound to physically separated DNA sites are also demonstrated by the formation of specific DNA loops and by cyclization of DNA molecules with 3'-extended single-stranded ends where T antigen specifically binds to the single-stranded/double-stranded junctions. The relevance of these properties for T antigen functions in DNA replication, transcription and(or) recombination is discussed.
Key words: DNA loops/DNA reassociation/DNA recombination/protein-protein interactions/T antigen

Introduction

The simian virus 40 (SV40) large tumour antigen (T antigen) is a DNA binding protein that plays diverse roles in the life cycle of the virus (for reviews see Tooze, 1981; Stahl and Knippers, 1987). During lytic infection, T antigen functions in the control of viral and cellular gene expression (Hansen *et al.*, 1981; Keller and Alwine, 1984) and in viral DNA replication (for review see DePamphilis and Bradly, 1986). The transcriptional regulation activities of T antigen are not yet understood. For viral DNA replication, the intrinsic DNA helicase activity of T antigen (Stahl *et al.*, 1986) is essential to the melting of the double helix at the viral replication origin (Tegtmeyer *et al.*, 1983; Jones and Tjian, 1984; Wold *et al.*, 1987; Scheffner *et al.*, 1989a,b; Borowiec *et al.*, 1990) and to the separation of parental DNA strands during the subsequent elongation phase (Stahl *et al.*, 1985; Wiekowski *et al.*, 1987).

SV40, as well as polyomavirus, can also lead to the transformation of susceptible cell lines (for reviews see Tooze, 1981; Rigby and Lane, 1983) by integration of the viral genome into the host DNA, most probably promoted by T antigen (Chia and Rigby, 1981; Della Valle *et al.*, 1981; Hacker and Fluck, 1989). T antigen is also involved in the reverse reaction: the excision of integrated viral DNA from host chromosomes (Miller *et al.*, 1984). Here it is known that T antigen acts at some point prior to or during the homologous recombination process that yields excised circular viral DNA. Several other investigations of SV40 and polyomavirus infected or transformed cells also suggest that T antigen functions in DNA recombination (Michel *et al.*, 1967; Nichols *et al.*, 1978; Brown and Basilico, 1982; Jasin *et al.*, 1985; Norkin *et al.*, 1985; Rubnitz and Subramani, 1985; Piche and Bourgaux, 1987; Heinzel *et al.*, 1988; Gurney and Gurney, 1989; Strauss *et al.*, 1989) most probably in conjunction with its DNA replication function (Stary *et al.*, 1989). In addition to these possible *in vivo* roles in some recombination processes, T antigen shows amino acid sequence similarity to the *Escherichia coli* RecA protein, the key enzyme of recombination in the bacterial cell (for review see Cox and Lehman, 1987; West, 1988; Smith, 1989), and antibodies raised against T antigen cross-react with RecA (Seif, 1982). T antigen and the RecA protein also share some biochemical properties such as double-stranded (ds) and single-stranded (ss) DNA binding activity, a DNA dependent ATPase activity and the DNA unwinding activity (Ogawa *et al.*, 1978; Roberts *et al.*, 1978; Giacherio and Hager, 1979; Spillman *et al.*, 1979; Stasiak *et al.*, 1982; Kowalczykowski *et al.*, 1987).

Recently, we have begun to analyse homogeneously purified T antigen for biochemical activities in order to explain its *in vivo* functions (Stahl *et al.*, 1986; Scheffner *et al.*, 1989a-c). Here we demonstrate that purified T antigen is able to promote the renaturation of complementary ss DNA in the presence of a large excess of ds DNA, most probably by protein-protein interactions. Moreover, we also show defined protein-protein interactions between T antigen molecules bound to physically separated ds DNA sites leading to DNA loops and joining of different DNA molecules via T antigen bridges. We demonstrate that T antigen, specifically bound to the ss/ds junction of partially ds DNA (Wiekowski *et al.*, 1988), can combine DNA sites of this common general structure via protein bridges separated by a distance of several kilobase pairs. The structure of ds/ss junctions of partially ds DNA also occurs in the forks of replicating DNA molecules, suggesting that T antigen combines different replication forks, particularly those of replicating SV40 minichromosomes where T antigen has been proposed to be functionally bound (Stahl *et al.*, 1985). As one interpretation of our observations therefore, we propose that T antigen favours finding and pairing of small homologous ss DNA regions like those in the vicinity of replication forks to create hybrid structures that can subsequently be processed to recombinant DNA molecules by suitable enzymes.

Results

T antigen associated renaturation activity

Promotion of renaturation of complementary single strands under physiological conditions most likely plays a direct role

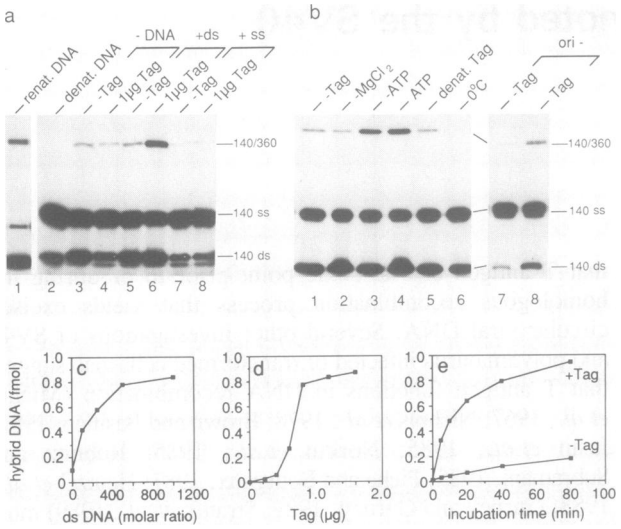

Fig. 1. T antigen associated DNA renaturation activity. (**a**) Formation of hybrid DNA depends on unspecific ds DNA. After renaturation reactions in the absence (−Tag; lanes 3, 5 and 7) or presence (1 µg Tag; lanes 4, 6 and 8) of T antigen and in the presence of unspecific ds (+ds; lanes 5 and 6) or ss (+ss; lanes 7 and 8) salmon sperm DNA or in the absence of competitor DNA (−DNA; lanes 3 and 4), mixtures were analysed for hybrid DNA, consisting of one small (140 base) and one larger (360 base) strand (see Materials and methods), by SDS−PAGE and autoradiography. Substrate DNA before the assay is shown in lane 2 (denat. DNA). As a positive control, 3 ng of the mixture of denatured DNA fragments were incubated in the presence of 400 mM NaCl at 90°C and slowly cooled down to room temperature (renat. DNA, lane 1). Running positions of the denatured (140 ss) and ds (140 ds) forms of the 140 bp DNA fragment as well as of the 140 base/360 base hybrid DNA (140/360) are indicated. (**b**) The renaturation reaction requires native T antigen and Mg^{2+}, but not ATP or the SV40 origin sequence (lanes 1 and 7, reaction in the absence of T antigen; lanes 2−6 and lane 8, reaction with 1 µg T antigen; lanes 2 and 3, $MgCl_2$ or ATP were omitted; lane 4, reaction in the presence of 3 mM ATP; lane 5, before use T antigen was denatured at 90°C; lane 6, same as in 4, but reaction was carried out at 0°C; lanes 7 and 8, fragments of identical length but without the SV40 origin sequence (*ori*$^-$) were used as substrate DNA). (**c**−**e**) Reassociation activity determined by quantification of the radioactivity in the hybrid DNA band and plotted as a function of unspecific ds DNA (c), T antigen concentration (d) and incubation time (e), respectively.

in genetic recombination and also is one of the *in vitro* activities of the RecA protein (Weinstock *et al.*, 1979). Because of the similarities of both proteins, we tested purified T antigen in a DNA renaturation assay.

As substrate DNA we used a mixture of two denatured DNA fragments of different size, a ^{32}P-labelled small (140 bp) fragment complementary to a second (unlabelled) 360 bp DNA fragment (Figure 1). We observed a relatively high rate of spontaneous rehybridization of the denatured fragments, even if they were immediately cooled in ice water after heat denaturation (Figure 1a, lane 2, see below). Therefore, we scored for hybrid DNA, consisting of one small (140 base) and one larger (360 base) strand. This hybrid DNA was formed (though at a low yield) under DNA hybridization conditions (Figure 1a, lane 1) but its spontaneous formation was much reduced under the experimental conditions used in the renaturation assay (Figure 1a, lane 3). Gel electrophoretic analysis under denaturing conditions of the 140/360 hybrid DNA after digestion with P1 nuclease showed an intact 140 base strand, confirming that it was completely base paired over the homologous region. Moreover, a hybrid DNA with the same electrophoretic mobility was also obtained when the 360 bp fragment was radioactively labelled instead of the smaller 140 bp fragment (data not shown).

T antigen did not much increase the renaturation efficiency (Figure 1a, lane 4), except when the reaction mixture contained an excess of unspecific ds (salmon sperm) DNA (Figure 1a, lane 6; Figure 1c). Under these conditions the formation of hybrid DNA exceeded that obtained under optimal hybridization conditions (compare lanes 1 and 6 of Figure 1a). In contrast, the presence of unspecific ss DNA in the reaction mixture was inhibitory (Figure 1a, lane 8). We have tested several T antigen preparations purified by different methods and also control preparations from uninfected cells, and the results exclude the possibility that a cellular DNA renaturing activity could be strongly associated with the purified T antigen (data not shown, but for details see Scheffner *et al.*, 1989c). Moreover, preabsorption to T antigen specific monoclonal antibodies (that do not cross-react with cellular proteins: PAbs 108, 101, KT3; see Gurney *et al.*, 1980; MacArthur and Walter, 1984; Wiekowski *et al.*, 1987) coupled to protein A−sepharose removed the reassociation activity from T antigen preparations. However, the same antibodies did not inhibit the reaction when added directly to the reaction mixture (data not shown). An exception is monoclonal PAb 1604 which was partially inhibiting (~40%, data not shown) probably due to its interference with the T antigen DNA binding activity (Wiekowski *et al.*, 1987). The reassociation reaction was relatively insensitive to salt up to ~150 mM NaCl and was dependent on temperature, magnesium ions and incubation times, but ATP had no effect (Figure 1b, lanes 2, 4 and 6; Figure 1e).

The denatured DNA fragments used as a substrate in the renaturation reaction contained the SV40 origin sequence (*ori*$^+$ DNA, see Materials and methods). In contrast to its preferential binding to *ori*$^+$ ds DNA T antigen does not specifically bind to the same DNA sequence when denatured (Auborn *et al.*, 1988), and additional experiments using DNA strands of the same size but without the origin sequence (*ori*$^-$ DNA) clearly demonstrated that the T antigen renaturation activity was not sequence specific (Figure 1b, lanes 7 and 8). However, a quantitative evaluation showed that *ori*$^+$ DNA reassociated at twice the rate of *ori*$^-$ DNA (data not shown). But this was also found for the spontaneous renaturation reaction (compare lane 5 of Figure 1a and lane 7 of Figure 1b), and is probably a consequence of the different sequence or base composition of *ori*$^+$ and *ori*$^-$ DNA. We want to point out that the formation of the duplex 140 bp DNA was also stimulated by T antigen, independent of the presence of the origin sequence (see Figure 1b, lane 8; note that the spontaneous background reaction is high, and an effect of T antigen is hardly visible from the autoradiograph shown in Figure 1a for the *ori*$^+$ DNA). The T antigen renaturation activity decreased with increasing length of substrate DNA and could not be detected using DNA fragments longer than 500 bp (data not shown).

Native T antigen was essential for the reassociation reaction (Figure 1b, lane 5). Plotting the amount of hybrid DNA as a function of T antigen concentration gave a sigmoidal curve suggesting a cooperative interaction between T antigen molecules (Figure 1d).

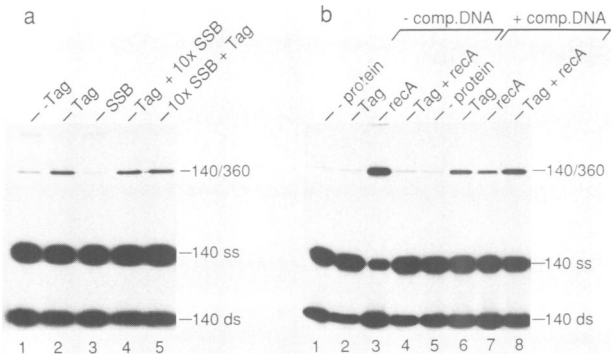

Fig. 2. Renaturation activity of T antigen in comparison with other recombination proteins. (**a**) Influence of the *E.coli* SSB. Reactions were carried out essentially as described in Figure 1 (lane 1, reaction in the absence of T antigen; lane 2, reaction with T antigen only; lane 3, reaction with SSB only (30 ng, 10-fold amount compared with 3 ng of substrate DNA); lane 4, T antigen was added first to the DNA, and after preincubation on ice (10 min) SSB was added; lane 5, same as in 4, but SSB was added first to the DNA and T antigen thereafter). (**b**) Comparison with the *E.coli* RecA protein. 800 ng of T antigen (Tag, lanes 2 and 6) or 400 ng of commercial RecA protein (RecA, lanes 3 and 7) were tested under our renaturation conditions (see Materials and methods) in separated reactions or in combination (Tag + RecA, lanes 4 and 8). As a control, both proteins were omitted (− protein, lane 1). Reactions were performed in the absence (−comp. DNA, lanes 1−4) or in the presence (+comp. DNA, lanes 5−8) of 1 μg ds salmon sperm DNA as a competitor.

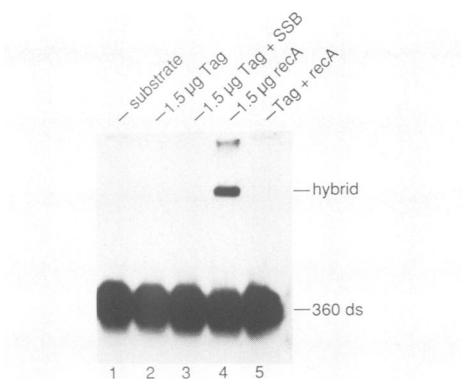

Fig. 3. T antigen has no strand exchange activity. Strand exchange between a ^{32}P-labelled 360 bp ds fragment and a ss M13 derivative encoding the sequence of the 360 bp fragment was tested in the presence of 1.5 μg T antigen (lane 2) or the same amount of RecA (lane 4) or both (lane 5). We show the gel electrophoretic and autoradiographic analysis of the experiment. Running positions of the 360 bp fragment (360 ds) and of the hybrid DNA (hybrid, arising from hybridization of the ss M13 hybridized to the complementary strand of the 360 bp fragment) are indicated. The activity of T antigen in the presence of SSB (40 ng, lane 3) was also tested.

The *E.coli* single-stranded binding protein (SSB) and the RecA protein have also been shown to promote the renaturation of homologous DNA single strands (Christiansen and Baldwin, 1977; Weinstock *et al.*, 1979). However, the SSB protein was completely inactive over a wide range of protein concentrations in our reannealing assay (Figure 2a, lane 3 and data not shown). In addition, SSB protein had no influence on the T antigen renaturation activity regardless of the order in which the proteins were added to the DNA (Figure 2a, lanes 4 and 5). We compared the T antigen renaturation activity with that of commercial RecA protein (Figure 2b). Under our assay conditions, similar amounts (in weight) of the RecA protein also showed a pronounced renaturation activity, which, however, in contrast to the T antigen activity, was strongly reduced by the addition of unspecific ds DNA. In the absence of competitor DNA, T antigen inhibited RecA, but in the presence of ds DNA the two activities were additive.

We also examined whether T antigen has a strand exchange activity−another *in vitro* activity of the RecA protein that plays a direct role in genetic recombination (Shibata *et al.*, 1979; Radding, 1982; Conley and West, 1989). However, under our very sensitive assay conditions (see Materials and methods and McCarthy *et al.*, 1988), we did not detect any evidence that our T antigen preparation promotes the formation of joint molecules from ss DNA plus duplex DNA (Figure 3). Instead, the RecA protein strand exchange activity could be completely inhibited by T antigen supporting recent data that DNA bound T antigen hinders homologous recombination enhancement conferred by an adjacent Z-DNA element (Wahls and Moore, 1990).

T antigen mediated DNA looping

A first step of a protein catalysed DNA pairing reaction might be an interaction between DNA bound proteins, bringing complementary DNA strands in juxtaposition for reassociation. Indeed, intermolecular interactions of T antigen in the presence of divalent cations have been described several years ago (Montenarh and Henning, 1983), and T antigen in form of multimeric complexes of different sizes can bind to DNA (Myers *et al.*, 1981; Dean *et al.*, 1987; Mastrangelo *et al.*, 1989). However, to our knowledge this analysis has not been extended to protein−protein interactions between T antigen bound to physically separated DNA sites.

Protein−protein interactions in combination with specific DNA binding can result in the formation of DNA loops as has been observed for other regulatory proteins (Griffith *et al.*, 1986; Krämer *et al.*, 1987; Hofmann *et al.*, 1989; for review see also Gralla, 1989). We therefore carried out a quantitative electron microscopic analysis of nucleoprotein complexes consisting of purified T antigen and a linear 11 kb DNA that contains twice the SV40 origin region, 0.95 and 2.75 kb away from the ends. In the absence of magnesium ions, T antigen was detected as a small protein knob, representing a T antigen monomer or dimer, specifically bound to the origin positions, as expected under the stringent binding conditions used (Figure 4a; see also Delucia *et al.*, 1983; Jones and Tjian, 1984). In the presence of magnesium, formation of regular DNA loops was observed at high frequencies, with T antigen always bound at the point where the DNA strands meet (Figure 4b and c). Since protein knobs now appeared substantially larger, T antigen seemed to be assembled into oligomers in the presence of the divalent cations (Montenarh and Henning, 1983; Mastrangelo *et al.*, 1989). We observed protein−DNA complexes consisting of several DNA molecules held together by T antigen, especially at increasing T antigen and DNA concentrations (Figure 4c). In the presence of EDTA instead of magnesium salt, DNA looping was never observed (Table I). However, when first bound to DNA in the presence of EDTA, T antigen could also be induced to form DNA loops by the subsequent addition of an excess of magnesium salt (data not shown). We measured the contour lengths, loop size and

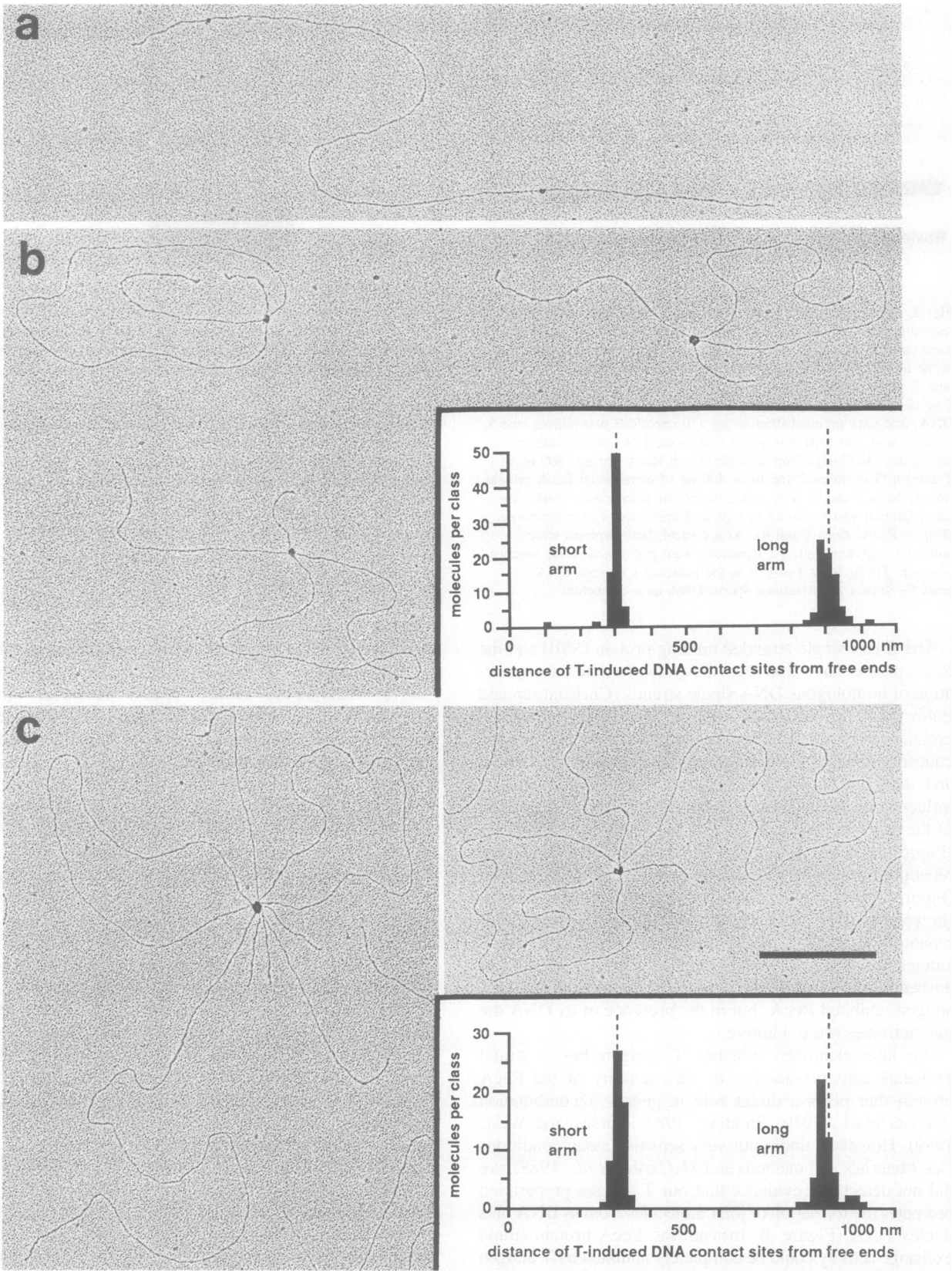

Fig. 4. T antigen induced sequence specific DNA looping, Electron micrographs of linear pATM13ori2× DNA (see below) with bound T antigen are shown. (**a**) DNA molecule with T antigen bound in the presence of EDTA (2 mM). Positions of bound T antigen correspond to the SV40 origin positions (50 molecules measured, data not shown). (**b** and **c**) DNA loops in the presence of Mg^{2+} (5 mM). The inserts show the distribution of DNA bound T antigen measured from free DNA ends of looped molecules. The dotted lines indicate the SV40 original positions calculated on the basis of the restriction map (size of classes is 20 nm). (**b**) Field view of DNA molecules showing intramolecular looping. (**c**) Higher order structures of DNA molecules forming intramolecular and intermolecular loops.

Table I. Dependence of *ori* specific DNA looping on magnesium ions

Conditions[a]	EDTA	Mg^{2+}
ori bound Tag[b]	90%	91%
ori specific looped structures	<1%	40%
ori specific higher order structures	none	23%

[a]Reaction conditions were as described in the legend to Figure 4; 170 DNA molecules with bound T antigen (Tag) were analysed in each case.
[b]T antigen was scored as *ori* bound, if the position of the SV40 origin sequence and the location of protein knobs could be superimposed.

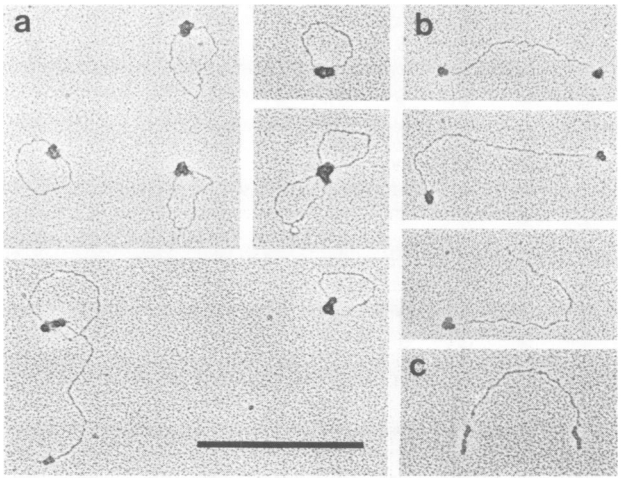

Fig. 5. T antigen dependent structure specific DNA looping. Electron micrographs of T antigen (a,b) or SSB (c) bound to single-stranded ends of linear pUC8 DNA in the presence of Mg^{2+} (see below) are shown. (**a**) Looped DNA molecules with bound T antigen. (**b**) Non-looped molecules with bound T antigen at the ends (note that the short single-stranded ends not complexed with protein were undetectable under the conditions used). Length measurements of the uncomplexed DNA confirmed that T antigen is bound to the single-stranded region of the molecules (data not shown), with highest affinity at the ss/ds junctions. (**c**) DNA molecule with bound SSB to demonstrate the single-stranded ends.

sites of protein–DNA interaction (Figure 4, inserts). The data clearly demonstrated that T antigen bound to widely separated origin regions undergoes defined protein–protein interactions, most probably in a cooperative manner, resulting in a looping out of intervening DNA regions. DNA loop formation was also observed using ds DNA not containing the SV40 origin sequence (data not shown). Since T antigen DNA binding was random in this case, the DNA loops were of different sizes but were also held together by a protein knob that was most probably representing oligomers of T antigen, the only protein present in these assays.

Joining of specific DNA structures by T antigen

A previous report from our laboratory showed that T antigen binds specifically to the ss/ds junction of partially ds DNA (Wiekowski *et al.*, 1988). We made use of this earlier observation to investigate whether T antigen can join DNA sites not only in a sequence specific but also in a structure specific manner (Figure 5). A linear plasmid DNA with ss regions of a few hundred bases at the 3′-ends (Figure 5c) was used in the loop formation assay and the resulting

Table II. T antigen induced structure specific DNA loops

Tag bound to ds DNA molecules with ss ends[a]	at the ends (ss/ds junctions)[b]	>99%
	within ds regions	<0.5%
Tag–Tag interactions	joining ss/ds junctions	43%
	combining internal DNA sites	<0.5%

[a]275 DNA molecules with ss ends (see Figure 5) occupied by T antigen were examined.
[b]27% showed bound T antigen at only one end and 72% at both ends.

protein–DNA complexes were analysed by electron microscopy as described above. In the absence (data not shown) and presence of magnesium ions, we found T antigen preferentially bound to the ss ends of the DNA molecules most probably at the ss/ds junctions in agreement with previously reported biochemical data (see above, Figure 5b). Interactions of DNA bound T antigen yielding circular DNA molecules held together at their ss ends were only observed in the presence of divalent cations (Figure 5a, Table II). As in the sequence specific DNA looping reaction, larger protein–DNA complexes with two or even more DNA molecules were occasionally observed. These larger complexes were always connected by T antigen bound to DNA ends.

The interaction of T antigen complexes bound to distantly located DNA sites was also observed in the additional presence of ATP (data not shown). Since these binding conditions are similar to those during SV40 DNA replication, where T antigen is found at replication forks (Stahl *et al.*, 1985; Wiekowski *et al.*, 1987), we tried to determine whether T antigen can induce the joining of replication forks in an *in vitro* SV40 DNA replication system (Li and Kelly, 1984; Stillman *et al.*, 1985). However, with the high T antigen concentrations necessary for efficient DNA replication, we obtained large protein–DNA networks that could not be analysed for details (data not shown) but were similar to those described before when we investigated T antigen during the unwinding of plasmid DNA (Scheffner *et al.*, 1989b).

Discussion

We have shown here that purified T antigen promotes the renaturation of homologous DNA strands, a characteristic function of proteins involved in DNA recombination (Weinstock *et al.*, 1979; Kmiec and Holloman, 1982; Bryant and Lehman, 1985; Hotta *et al.*, 1985; Ganea *et al.*, 1987). The relatively high protein concentration required probably indicates that only a subfraction of T antigen is active in DNA renaturation. T antigen is a phosphoprotein (Scheidtmann *et al.*, 1982), and its renaturation activity may be dependent on the state of post-translational phosphorylation as has been found for other T antigen functions (Mohr *et al.*, 1987; Klausing *et al.*, 1988; McVey *et al.*, 1989). This is confirmed by the preliminary result that dephosphorylation of T antigen reduces its renaturation activity suggesting that it is a highly phosphorylated subfraction which is most active in the reassociation reaction (unpublished observations). Moreover, the T antigen DNA renaturation activity functions optimally in the presence of a large excess of unspecific ds DNA, essential for the reaction *in vitro*. The ds DNA

probably eliminates this inactive subfraction of T antigen that may negatively interfere with the DNA reassociation reaction. Accordingly, we have found that T antigen, when bound to ss DNA in the presence of magnesium ions, forms coiled nucleoprotein aggregates that may be inhibitory for renaturation (R.Wessel, unpublished results). T antigen, therefore, may not function by extension of single strands and destabilization of secondary structure as has been suggested for the *E.coli* SSB and some other proteins (Arai *et al.*, 1981) consistent with the fact that the renaturation reaction is not influenced by the SSB and is specific for small ss DNA molecules. More likely, T antigen (or more precisely a specific subfraction of it) enhances the rate of DNA renaturation by protein–protein interactions leading to the formation of protein–DNA aggregates which increase the rate of strand collision.

Defined protein–protein interactions of T antigen bound to physically separated ds DNA sites are shown here by the induction of SV40 origin sequence specific DNA loops or by the formation of defined intermolecular bridges. However, DNA looping is not restricted to T antigen bound to specific DNA sequences. Rather, any DNA region can be attached to another DNA site via a T antigen bridge. Both the renaturation activity of T antigen and the DNA looping are dependent on magnesium ions, but looping is observed at a much lower protein/DNA ratio, implying that it may not be specific for a subfraction of T antigen. Attachment of physically separated DNA sites via T antigen bridges may play a role in transcription regulation, especially in the light of the results of Müller *et al.* (1989) who have shown that an enhancer can influence transcription *in trans* when attached to the promoter via a protein bridge.

Protein–protein interactions have also been demonstrated between T antigen molecules bound specifically to ss/ds junctions at the ends of a linear plasmid leading to circularization of the corresponding DNA molecules. These results suggest that T antigen may specifically join gapped DNA molecules *in vivo*. In combination with its reannealing activity this may eventually lead to recombination reactions like (sister) chromatid exchanges that have been shown to be induced in SV40 and polyomavirus transformed cells (Nichols *et al.*, 1978; Brown and Basilico, 1982; Cerni *et al.*, 1986).

Moreover, the structure of ss/ds junctions of partially ds DNA is reminiscent of the forks of replicating DNA molecules, where T antigen is bound during replication of SV40 minichromosomes (Stahl *et al.*, 1985; Wiekowski *et al.*, 1987). Protein–protein interactions between bound T antigen molecules should therefore cause an association of two replication forks. Since SV40 DNA replication is bidirectional, this should preferentially occur within the same replicating DNA molecule, as has been observed for the formation of intramolecular DNA loops. With its DNA renaturation activity, T antigen would then be able to create small hybrid DNA regions using ss complementary sequences within joined replication forks. Resolution of these DNA structures by appropriate enzymes could result in DNA excision or amplification reactions in which T antigen also seems to be involved (Matz *et al.*, 1985). This kind of (illegitimate) recombination does not necessarily depend on a strand exchange activity that could not be demonstrated in our T antigen preparations. Interestingly, an association of two replication forks has been proposed previously to be important for the process of DNA amplification in mammalian cells (Stark *et al.*, 1989). We want to add that this scenario of SV40 DNA replication with T antigen as the replicating helicase bridging two replication forks fits well to a previously presented model of bidirectional DNA replication with a pair of adjacent replisomes and the nascent DNA looped out between (Pardoll *et al.*, 1980).

Materials and methods

Reagents and enzymes

Nucleotide, phosphocreatine, creatine phosphokinase, restriction enzymes and *E.coli* DNA polymerase Klenow fragment were purchased from Boehringer Mannheim, *E.coli* SSB and RecA protein from Pharmacia and λ exonuclease from BRL. T antigen was isolated by the immunoaffinity procedure as described previously (Stahl *et al.*, 1986; Scheffner *et al.*, 1989c).

DNA renaturation assay

As a substrate two DNA fragments were used: the ^{32}P-end-labelled SV40 MO-1 fragment of 140 bp length, containing the SV40 origin sequence (Scheffner *et al.*, 1989a), and an unlabelled 360 bp fragment excised from the same plasmid and containing the 140 bp fragment sequence. The homologous DNA fragments were each denatured by heat (10 min at 90°C) and subsequently chilled in ice water. The denatured fragments were mixed to equimolar concentrations and 3 ng of the DNA mixture (= 30 000 c.p.m.) were incubated for 40 min at 37°C in a 40 μl volume under the standard DNA renaturation conditions: 20 mM Tris–HCl pH 7.5, 4 mM $MgCl_2$, 1 mM DTT, 50 μg/ml BSA, 1 μg of unspecific salmon sperm ds DNA and 800 ng T antigen. Reactions were stopped by the addition of EDTA (20 mM) and SDS (0.2%) and analysed by SDS–PAGE (Laemmli, 1970) and autoradiography. In some assays we used as a substrate a pair of DNA fragments of identical size but without the SV40 origin sequence and containing the SV40 C4 fragment (Scheffner *et al.*, 1989a).

DNA strand exchange reaction

1 ng of the 360 bp ds DNA fragment with the SV40 origin (see above) and 20 ng ss M13 DNA (containing the sequence of the 360 bp fragment cloned within the *Eco*RI site) were incubated at 37°C for 60 min with indicated amounts of T antigen and RecA in the presence of 33 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$, 2 mM ATP, 1 mM DTT, 6 mM phosphocreatine, 50 μg/ml creatine phosphokinase and 50 μg/ml BSA. Reactions were stopped and analysed by gel electrophoresis as described above.

DNA looping assay

For sequence specific DNA looping, the MO-1 DNA fragment (see Figure 1) was subcloned into pAT and M13mp18 vectors at their *Hin*dIII–*Eco*RI sites. These constructs were ligated via their *Eco*RI sites to obtain the pATM13ori2× plasmid of 11.1 kb. pATM13ori2× DNA was cleaved with *Nru*I resulting in a linear molecule with SV40 origins 0.95 kb and 2.75 kb away from the ends. For structure specific looping 1.5 μg of *Sca*I cleaved pUC8 DNA were incubated for 20 s at 20°C in glycine–KOH, pH 9.4, 3 mM $MgCl_2$, 1 mM DTE, 30 mg/ml BSA with 30 U λ-exonuclease (BRL), to obtain 3′ single-stranded ends (Little, 1967). The reaction was stopped by the addition of EDTA (20 mM) and SDS (0.5%). After phenol extraction and alcohol precipitation the DNA was resuspended in 20 mM triethanolamine–HCl, pH 7.5 to a final concentration of 50 ng/ml. Quantitative electron microscopy revealed that >99% of the ends of the molecules were single-stranded, with a mean length of ~400 bp (data not shown). For DNA looping, reaction mixtures contained 100 ng DNA, 100–200 ng immunoaffinity purified T antigen, 20 mM triethanolamine–HCl, pH 7.5, 5 mM $MgCl_2$ and indicated amounts of salt. The mixtures were incubated for 15 min at 37°C, terminated by the addition of glutaraldehyde to 0.1% and incubated again.

Electron microscopy

Samples were spread by the BAC method (Vollenweider *et al.*, 1975). Electron microscopy was carried out as described previously (Wessel *et al.*, 1990). Micrographs were taken in a Hitachi H-7000 electron microscope. Lengths of molecules were measured on a CRP-digitizer (CRP = Cybernetical Research & Production, FRG) using magnified positives (30 000×). (bar = 500 nm).

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