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DNA Binding Properties and Replication Activity of the T Antigen Related D2 Phosphoprotein†

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ABSTRACT: According to earlier genetic experiments, a region within the N-terminal 50-100 amino acids may be important for the replication function of T antigen, the initiator protein of simian virus 40 (SV40). We have investigated this possibility using the T antigen related D2 protein in several biochemical assay systems. D2 protein, a phosphoprotein coded for by the adeno-SV40 hybrid virus Ad2$^+$D2, shares its 594 C-terminal amino acids with authentic T antigen and its 104 N-terminal amino acids with an adenovirus structural protein. We confirmed earlier studies showing that D2 protein appeared to bind well to specific binding sites in the SV40 origin of replication. We found, however, that D2 protein was rather inefficient, inducing the unwinding of the double-stranded origin region, and was much less active than authentic T antigen as an initiator of in vitro SV40 DNA replication. We interpret these findings to indicate that D2 protein molecules associate with the origin to form an aberrant complex that is quite inefficient, inducing DNA unwinding and the establishment of replication forks. The possibility that the N-terminus may be required for an optimal arrangement of T antigen at the origin was supported by results of dephosphorylation studies. Dephosphorylation of N-terminal phosphoamino acids had significant effects on the stability of D2 protein-origin complexes.

Simian virus 40 (SV40) encodes its own replication initiator, commonly referred to as the large T antigen, a product of an "early" viral gene. T antigen, a nuclear phosphoprotein of 708 amino acids, has 2 functional domains known to be associated with its replication activity [for reviews, see Bradley and Livingston (1987) and Stahl and Knippers (1987)] (Figure 1).

One domain is responsible for the specific interaction of T antigen with the viral origin of replication. An analysis of nucleotide exchange and deletion mutants has shown that the DNA binding domain begins at amino acid residue 139 and extends at least to amino residue 259 (Paucha et al., 1986; Strauss et al., 1987; Arthur et al., 1988), but T antigen regions up to amino acid residue 371 are found in close contact with DNA in DNA-T antigen complexes (Simmons, 1988). This latter region includes a Zn-finger loop (Berg, 1986), a structural element known to be involved in DNA binding and, possibly, in protein-protein interactions (Klug & Rhodes, 1987; Evans & Hollenberg, 1988).

A second domain carries an ATPase/helicase activity which is required for the opening of duplex DNA at the origin (Dean et al., 1987a,b; Wold et al., 1987; Stahl et al., 1988) as well as for the continued unwinding of double-stranded DNA ahead of the replication forks (Stahl et al., 1986; Wiekowski et al., 1987). The boundaries of the ATPase/helicase domain are

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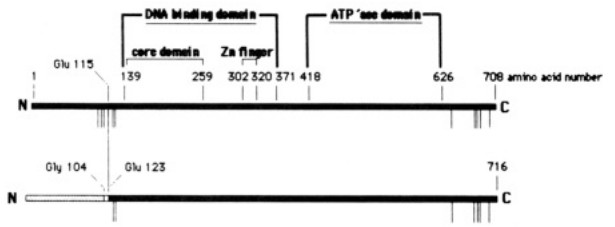

FIGURE 1: Authentic T antigen and D2-T antigen. Authentic T antigen consists of 708 amino acids and includes 2 relatively well-defined domains required for viral DNA replication. The "core" of the DNA binding domain is defined by genetic data (see text). Biochemical experiments suggest that regions of T antigen up to amino acid residue 371 form close contacts with DNA (Simmons, 1988). The vertical lines indicate the location of phosphoamino acids as described by Scheidtmann et al. (1982). D2 protein (lower line) consists of 716 amino acids and is composed of 3 parts: the 104 N-terminal amino acids up to Gly-104 are derived from the adenoviral 33K protein (stippled); this region is connected via a stretch of 18 amino acids of unknown origin (white) to the 594 C-terminal amino acids which are derived from SV40 large T antigen (black). The C-terminal part begins with a glutamic acid residue corresponding to Glu-115 in authentic T antigen (Baumann et al., 1985). The phosphoamino acids in the T antigen part of D2 are indicated as vertical lines.

less well-defined than those of the DNA binding domain. A series of C-terminal T antigen deletion mutants define the C-terminal boundary. The smallest of the truncated mutant T antigens with an active ATPase ends at amino acid residue 626 (Clark et al., 1983). The domain is also defined by a number of nucleotide exchange mutations that affect the ATPase but not the DNA binding activity of T antigen (Manos & Gluzman, 1985; Clark et al., 1983). Furthermore, a monoclonal antibody inhibiting the ATPase/helicase and not the DNA binding activity reacts with an epitope between amino acids 448 and 509 (Mole & Lane, 1985), and, ATP, covalently bound in vitro to T antigen, was recovered on a peptide ranging from amino acid 413 to amino acid 528 (Clertant et al., 1984).

Nucleotide exchange or deletion mutations mapping outside of the DNA binding or the ATPase/helicase domain affect other functions of T antigen in productively infected or transformed cells but usually do not interfere with the replicative activity of SV40 T antigen.

There are, however, some interesting exceptions. Gluzman and Ahrens (1982) isolated a T antigen mutant with amino acid exchanges at positions 30 (Met-Ile) and 51 (Lys-Asn) with a replication-negative phenotype even though its DNA binding and ATPase were intact. Likewise, Clark et al. (1983) discovered a replication-negative T antigen mutant with an in-phase deletion removing amino acids 17–27. Thus, the N-terminal one-tenth of T antigen appears to be somehow required for its replication function. This conclusion is in agreement with earlier observations (Wiekowski et al., 1987) showing that a T antigen specific monoclonal antibody reacting with the extreme N-terminus inhibited the replicative function of T antigen more drastically than an antibody reacting with the extreme C-terminus. Moreover, recent evidence suggests that the phosphorylation of certain N-terminal amino acids affects the specific DNA binding and replication function of T antigen even though these amino acids are clearly outside of the DNA binding domain (Figure 1) (Mohr et al., 1987; Grässer et al., 1988; Klausing et al., 1988). Taken together, these observations suggest that the N-terminus may play a role in the replicative function of T antigen. To investigate this possibility in more detail, we performed a biochemical study using the T antigen related D2 protein as a replicative initiator for SV40 DNA replication in vitro.

The D2 protein (or D2-T antigen), produced by the adeno-SV40 hybrid virus Ad2$^+$D2, is a chimeric protein composed of 3 sections: an N-terminal section of 104 amino acids derived from the N-terminus of an adenovirus structural protein, the adeno 33K protein; a linking region of 18 amino acids of unknown origin; and a 594 amino acid long C-terminal region corresponding to amino acids 114–708 of SV40 T antigen (Figure 1). Thus, D2 protein is composed of 716 amino acids and shares its C-terminal 594 amino acids, including the DNA binding and the ATPase/helicase domains, with SV40 T antigen (Baumann et al., 1985).

D2 protein, a nuclear phosphoprotein like SV40 T antigen, is produced in large quantities in Ad2$^+$D2-infected HeLa cells and has served as a substitute for authentic T antigen in earlier studies on the biochemistry of T antigen. These studies had shown that the D2 protein has the specific DNA binding properties and the ATPase activity of authentic T antigen (Tjian et al., 1979; Clark et al., 1981). Thus, D2 protein should be appropriate to address the problem concerning a possible function of the N-terminal section of T antigen.

We describe below our results using the D2 protein in DNA binding and DNA unwinding studies as well as an initiator for in vitro replication. In addition, we found it interesting to investigate the role that amino acid phosphorylation may play in D2 protein–DNA interaction and in DNA replication. Our results suggest that the D2 protein appears to bind as well to DNA as authentic T antigen but, clearly, has reduced in vitro DNA unwinding and DNA replication activity. We also find major differences between D2 and authentic T antigen as to the effects of dephosphorylation.

Materials and Methods

T Antigen and D2 Protein. Authentic T antigen was prepared from lytically infected monkey TC 7 cells by using an immunoaffinity procedure with monoclonal antibody PAb 101 as described before (Simanis & Lane 1985; Stahl et al., 1986) except that the column-bound T antigen was eluted with 20 mM triethanolamine, pH 10.8 (in 10% glycerol), instead of 3.5 M $MgCl_2$. D2 protein was prepared from AD2$^+$D2-infected HeLa cells. Infection of the HeLa cells and preparation of a crude protein extract were carried out as described by Baumann and Hand (1982). D2 protein was purified by the same immunoaffinity procedure used for the purification of authentic T antigen. The purified proteins were stored in aliquots at −70 °C in the presence of 50% glycerol.

^{32}P-Labeled D2 protein was extracted from Ad2$^+$D2-infected cells which were transferred 4 h before harvesting to 200 mL of phosphate-depleted medium containing 5 mCi of [^{32}P]phosphate (Amersham). The labeled protein was purified by the chromatographic procedure of Baumann (1985).

DNA. SV40 DNA was extracted from lytically infected monkey cells by using the procedure of Hirt (1967). The crude preparation was incubated with RNase and Pronase and purified by phenol–chloroform extraction followed by two cycles of ethidium bromide–CsCl centrifugation.

Plasmid pSVm0-1 is a pUC8 derivative carrying a blunt-ended SV40 origin fragment (from *Hin*dIII at SV40 nucleotide 5171 to *Nco*I at nucleotide 41) in the *Sma*I cloning site (Baur & Knippers, 1988). The origin fragment was recovered from the plasmid using restriction nucleases *Eco*RI and *Hin*dIII. The 143 bp fragment was ^{32}P 3′ end labeled using the Klenow polymerase fill-in reaction (Maniatis et al., 1982).

The SV40 *Hin*dIII C fragment (SV40 nucleotides 5171–1046) was recovered from restricted SV40 DNA and cloned in the *Hin*dIII site of plasmid pAT 153 (Maniatis et al., 1982).

DNase I Footprinting. The radioactively labeled SV40 origin fragment was incubated with T antigen and D2 protein in the concentrations indicated in the legends to the respective figures. DNase I treatment and electrophoresis were carried out essentially as described by Galas and Schmitz (1978) with the modifications specified in Baur and Knippers (1988).

DNA Unwinding. As substrates we used 1–2 ng of either the 143 bp origin fragment or the 1169 bp *Hin*dIII C fragment in a reaction volume of 40 μL, containing 2 mM ATP, 7 mM $MgCl_2$, 20 mM Tris-HCl, pH 7.5, 10 mM creatine phosphate, 2 μg of creatine kinase, 300 ng of the *Escherichia coli* single-strand specific binding (SSB) protein, and 2 μg of bovine serum albumin. A 400-fold excess of linearized plasmid DNA was added when the origin-specific reaction was to be analyzed (Goetz et al., 1988; Stahl et al., 1988). After 90-min incubation at 37 °C, the reaction was stopped by addition of 20 mM EDTA and 0.5% sodium dodecyl sulfate (final) and electrophoresed on a 11% polyacrylamide gel.

DNA Replication in Vitro. The preparation of protein extracts from proliferating HeLa cells and the conditions for in vitro DNA replication were performed exactly as described by Li and Kelly (1985).

Dephosphorylation. Potato acid phosphatase (grade I, Boehringer-Mannheim) and bovine intestinal alkaline phosphatase (type VII S; Sigma Chemical Co.) were used exactly as described before (Klausing et al., 1988), incubating ^{32}P-labeled D2 protein for 60 min at room temperature.

Phosphopeptides. Purified ^{32}P-labeled D2 protein was precipitated by trichloroacetic acid, oxidized with performic acid, and digested with trypsin and Pronase E as described (Scheidtmann et al., 1982). The resulting phosphopeptides were separated by two-dimensional electrophoresis and chromatography and visualized by autoradiography exactly as before (Scheidtmann et al., 1982).

Results

DNA Binding. For DNA binding studies, we used an SV40 DNA fragment containing the two high-affinity T antigen binding sites. Binding site I consists of two tandem GAGGC pentanucleotides separated by an AT-rich spacer (Ryder et al., 1985), whereas binding site II consists of two GAGGC pairs forming a perfect palindrome as the central element of the viral origin of replication (Deb et al., 1986). The binding experiment was performed not only to confirm earlier reports on the specific DNA binding activity of D2 protein (Tjian et al., 1979) but also to compare the binding affinities of D2 protein and authentic T antigen. Using a DNase I protection assay, we found that similar quantities of both proteins were needed to protect comparable regions of the SV40 DNA fragments (Figure 2). Thus, it seemed that D2 protein bound almost as efficiently and specifically to DNA as authentic T antigen.

DNA Unwinding. It had been shown before that isolated T antigen in the presence of ATP unwinds any double-stranded DNA regardless of whether it is circularly closed or linear (Stahl et al., 1988; Goetz et al., 1988). However, linear DNA must be larger than 60 bp to allow for the formation of a functional T antigen–DNA complex (Scheffner et al., 1989).

At more stringent binding conditions such as high salt in the reaction mixture (Stahl et al., 1988) or at low protein:DNA ratios (see below), T antigen binds preferentially to the replication origin and, consequently, preferentially unwinds double-stranded DNA with an SV40 origin of replication (Stahl et al., 1988). Native and single-stranded DNAs migrate at different rates during polyacrylamide gel electrophoresis, allowing for a convenient determination of the fraction of

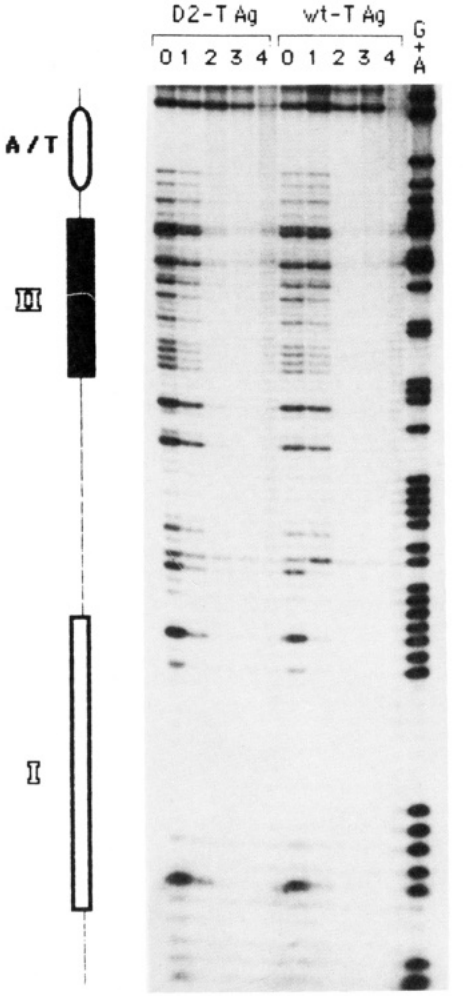

FIGURE 2: Binding of D2 protein (D2-T-Ag) and authentic T antigen (wt-T Ag) to the SV40 origin of replication. The vertical line at the left indicates the positions of T antigen binding site I, binding site II (in the replication origin), and the AT-rich element of the origin as identified by the G + A Maxam–Gilbert reaction (right electrophoresis lane). Aliquots of 2 ng of the [^{32}P]phosphate-labeled 143 bp origin fragment (see Materials and Methods) were incubated in the presence of 500 ng of unlabeled salmon sperm DNA each with 0 (lane 0), 50 (lane 1), 150 (lanes 2 and 4), and 450 (lane 3) ng of authentic T antigen or D2 protein. The binding reaction was done as described by Mc Kay (1981) (lanes 1, 2, and 3) or under replication conditions (lane 4). The protein–DNA complexes were treated with DNase I as outlined under Materials and Methods. We show the autoradiogram of a sequencing gel.

single-stranded DNA produced during the incubation with T antigen in the presence of ATP and SSB protein (see Materials and Methods).

Using this assay, we found that D2 was able to convert the 143 bp double-stranded origin fragment into single-stranded DNA, but with a 2–3 times lower efficiency than authentic T antigen. This was found for the "origin unspecific" as well as for the "origin specific" reaction (Figure 3).

We have performed experiments like that shown in Figure 3 using the SV40 *Hin*dIII C fragment as a double-stranded DNA substrate. The *Hin*dIII C fragment is about 1100 bp in size and includes the SV40 origin of replication. We obtained results (not shown) that were similar to those shown in Figure 3, demonstrating again that both authentic T antigen and D2 protein were able to unwind long stretches of double strandedness and that T antigen was 2–3 times more active than D2 protein in the unwinding assay.

DNA Replication in Vitro. Recently, in vitro replication systems have been described (Li & Kelly, 1984; Stillman &

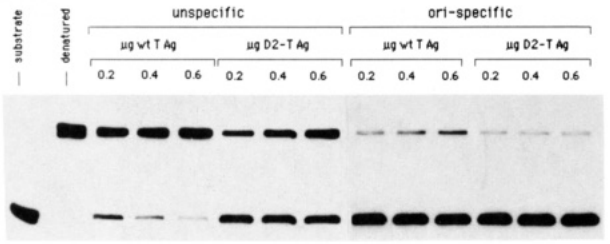

FIGURE 3: DNA unwinding. The [^{32}P]phosphate-labeled 143 bp origin fragment was used as the substrate in these experiments. The "unspecific" reactions were performed under standard conditions without competitor DNA. A 400-fold excess of competing linearized unlabeled plasmid DNA was present in the assay mixtures of the "ori-specific" reactions. Incubations were for 90 min at 37 °C in the presence of ATP, an ATP-regenerating system, *E. coli* SSB protein, and the indicated amounts of T antigen and D2 protein, respectively (see Materials and Methods). The reaction products were investigated by polyacrylamide gel electrophoresis. The left lanes show the substrate, incubated under standard conditions without T antigen, before (substrate) and after heat denaturation (denatured) to indicate the position of double-stranded and single-stranded DNA. For a quantitative evaluation, the bands containing single- or double-stranded DNA were cut from the gel to determine their radioactivity by scintillation counting.

Gluzman, 1985; Wobbe et al., 1985), allowing the biochemical investigation of T antigen as an initiator for SV40 DNA replication. Replication in vitro depends on a DNA substrate, containing the intact SV40 origin of replication, on sufficient quantities of active T antigen, and on a protein extract from "permissive" monkey or human cells as a source for replication factors other than T antigen. It has been demonstrated that the interaction of T antigen with the origin sequence is a first and necessary step in the in vitro replication pathway (Wold et al., 1987; Dean et al., 1987a,b). This interaction leads to an opening of the duplex origin DNA and to the establishment of the replication apparatus at replication forks. As D2 and authentic T seemed to interact with similar efficiencies with the origin sequence (Figure 2), it was of interest to find out how these proteins compare in initiating the in vitro replication reaction. We have performed many experiments, using different T antigen and D2 protein preparations, and consistently found that D2 protein was only one-fifth to one-third as active as authentic T (Figure 4). It may be possible that D2 protein initiated the replication cycle but then was unable to catalyze a later step in the replication process. This possibility was investigated by analyzing the reaction products found at different times after the start of the reaction. The insert to Figure 4 shows an autoradiogram of electrophoretically separated DNA forms synthesized in vitro in the presence of T antigen or D2 protein. We did not find major qualitative differences between the reaction products (Figure 4). We have digested the in vitro replication products using *Bam*HI, a restriction endonuclease that cuts SV40 DNA at one single site, and obtained predominantly linear full-length radioactively labeled DNA from the T antigen as well as from the D2-initiated reactions (data not shown).

We note that a considerable fraction of the DNA, synthesized in our experiments (Figure 4) as well as in the experiments published by others, had a comparatively low electrophoretic mobility. The accumulation of these structures could indicate that in vitro replication did not always lead to mature viral DNA. We have labeled these structures in Figure 4 as high molecular weight DNA (HMW-DNA). A fraction of HMW-DNA may consist of replicative intermediates. However, it cannot be excluded that at least some of these structures were aberrant replicative forms as, for example, catenated DNA or rolling circle-type structures. In any case,

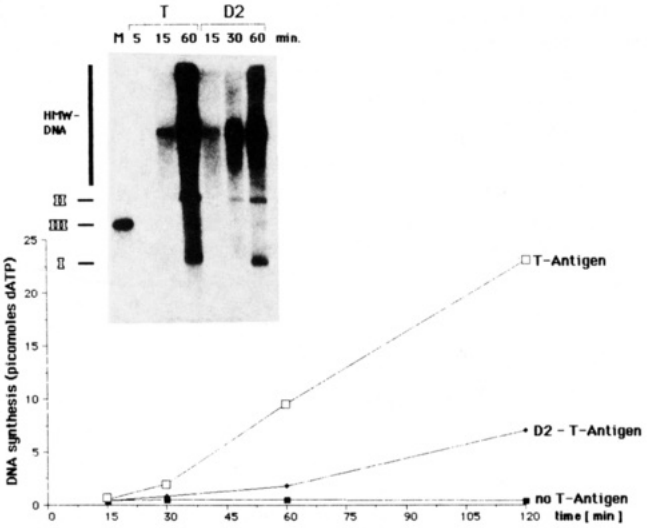

FIGURE 4: DNA replication in vitro. SV40 DNA (75 ng) was incubated under the in vitro replication conditions of Li and Kelly (1985) using [α-^{32}P]dATP as the radioactive tracer and 660 ng of T antigen or D2 protein per assay. Samples were removed at the indicated times to determine the incorporated radioactivity by precipitation with trichloroacetic acid. The insert shows the result of a parallel experiment using authentic T antigen (T) or D2 protein (D2). The DNA samples were extracted by phenol–chloroform from reaction mixtures, ethanol precipitated, and investigated by agarose gel electrophoresis and autoradiography (I, form I superhelical circularly closed DNA; II, form II relaxed circular DNA; III, form III linear unit-length DNA; HMW-DNA, high molecular weight DNA; see text).

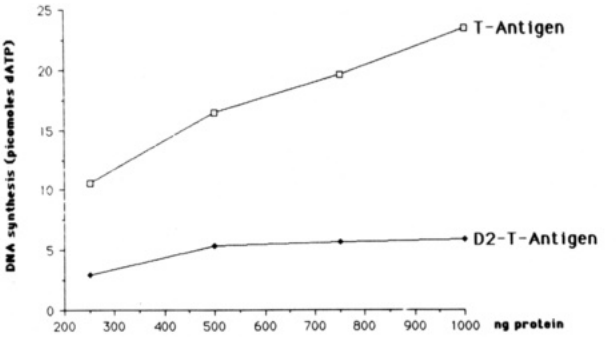

FIGURE 5: DNA replication in vitro as a function of T antigen or D2 protein concentration. The experiment was carried out as in Figure 4 except that increasing amounts of T antigen or D2 protein were used. The incubation time was 60 min at 37 °C. We show the incorporated radioactivity as determined by trichloroacetic acid precipitation.

for the present discussion, it is important to note that comparable structures were synthesized in the T antigen and in the D2-catalyzed reactions.

We also like to emphasize that both the T antigen and the D2-initiated reaction depended on an intact origin. Circular plasmid DNA without a viral origin or with a mutationally altered cloned SV40 origin (Deb et al., 1986) was not accepted as a substrate by either T antigen or D2 protein (not shown).

We have tried to compensate for the poor initiating function of D2 protein by increasing the amount of D2 protein in the reaction mixture. However, in contrast to the results obtained with authentic T, an increase of the D2 concentrations in the in vitro replication assays had only minor effects on the DNA synthesizing capacity (Figure 5), suggesting that the DNA replication system was saturated with D2 protein. Taken together, the data of Figures 4 and 5 suggested that D2 protein served as an ineffective substitute for T antigen at the initiation of in vitro replication.

One possible reason for this could be the reduced capacity of D2 to unwind a double-stranded DNA substrate (Figure 3). This may be due to the fact that T antigen molecules must be arranged in a defined manner on the double-stranded DNA substrate to induce an effective unwinding (Scheffner et al., 1989).

The arrangement of T antigen on origin sequences is probably regulated by the phosphorylation of amino acids in the N-terminal cluster of phosphorylation sites (Figure 1) (Klausing et al., 1988).

To investigate this possibility, we performed studies with dephosphorylated D2 in analogy to previous work with authentic T antigen (Klausing et al., 1988).

Dephosphorylation of D2 Protein. Aliquots of [^{32}P]phosphate-labeled D2 protein were treated either with alkaline or with acid phosphatase (see Materials and Methods). Untreated as well as phosphatase-treated D2 protein was then digested using Pronase E and trypsin. The (partially overlapping) phosphopeptides were separated by two-dimensional electrophoresis–chromatography according to an established and well-calibrated procedure (Scheidtmann et al., 1982; Klausing et al., 1988). Untreated D2 protein yielded all the phosphopeptides described and identified before (Scheidtmann et al., 1982, 1984; Klausing et al., 1988) except those containing Ser-106, Ser-111, and Ser-112 which are absent in D2 (see the introduction) (Figure 6). We found that at least two phosphopeptides were recovered from D2 protein digests that were missing in authentic T antigen. To confirm that these phosphopeptides were derived from the adenoviral part of D2, we isolated the 33K adenoprotein from ^{32}P-labeled adenovirus-infected HeLa cells by immunoprecipitation as described by Baumann et al. (1985). The 33K adenoprotein was digested with trypsin and Pronase E and analyzed as above. We obtained phosphopeptides with electrophoretic and chromatographic properties like the additional phosphopeptides of D2 (data not shown; see below, Figure 6). Alkaline phosphatase dephosphorylated most of the phosphoserines (except for one phosphoserine in the C-terminal cluster of phosphorylation sites) but not the two phosphothreonines (Thr-124 and Thr-701). Acid phosphatase dephosphorylated essentially all phosphoamino acids with the exception of Thr-701 which was resistant against both phosphatases (Figure 6). (Both phosphatases dephosphorylated the phosphoamino acids in the adenoviral part of D2.) To find out how dephosphorylated D2 protein reacted with the SV40 origin of replication, we performed DNase I protection experiments. As shown in Figure 7, D2 protein, dephosphorylated by either phosphatase, bound to binding site I as efficiently as comparable amounts of untreated D2. However, the binding to site II in the origin of replication was strongly reduced after dephosphorylation. This effect was more pronounced after dephosphorylation with alkaline than after dephosphorylation with acid phosphatase (Figure 7). We have performed a number of additional DNA binding studies using dephosphorylated D2 protein and radioactively labeled origin DNA in an assay system which depends on the immunoprecipitation of the resulting protein–DNA complexes (McKay, 1981). The results of these studies (not shown) were consistent with the data of Figure 7 and showed once again that phosphatase treatment reduced the affinity of D2 to origin DNA [see also Baumann (1985)].

These results were surprising to us as they were significantly different from those obtained before with authentic T antigen. Authentic T antigen, dephosphorylated by alkaline phosphatase, had a higher affinity to binding site II in the origin of replication (Mohr et al., 1987; Klausing et al., 1988), whereas

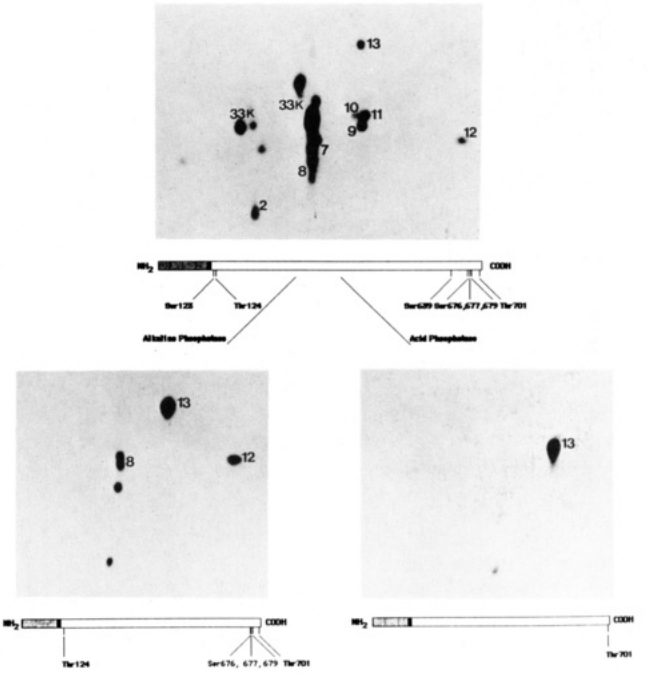

FIGURE 6: Dephosphorylation of D2 protein. D2 protein, [^{32}P]-phosphate labeled in vivo as described under Materials and Methods, was digested with trypsin and Pronase E. The resulting phosphopeptides were separated by two-dimensional electrophoresis–chromatography. Note that treatment of T antigen with trypsin/Pronase E results in partially overlapping peptides as described in detail by Scheidtmann et al. (1982) and by Klausing et al. (1988). (Upper part) Untreated D2 protein. To allow for a comparison of phosphopeptides with previously published data, we use the numbering system of Scheidtmann et al. (1982). Peptide 2 contains Ser-639; peptides 7 and 11, Ser-123 and Thr-124; peptides 8, 9, and 10, Ser-676, -677, and -679; peptide 12, Thr-124; peptide 13, Thr-701. The unnumbered phosphopeptides below and to the left of peptide 8 are from the adenoviral 33K protein part of D2 (see Materials and Methods and text). (Lower part) Dephosphorylation by alkaline or acid phosphatase removes the phosphate groups from most phosphoserines of the T antigen part as well as from the adenoviral part of D2. Both phosphothreonines are resistant against alkaline phosphatase, whereas acid phosphatase additionally removes the phosphate group of Thr-124.

T antigen, treated with acid phosphatase, bound to the origin with an efficiency comparable to that of untreated T (Klausing et al., 1988).

To find out whether phosphatase treatment affected the DNA replication activity of D2, we performed in vitro replication assays like those shown in Figure 4 and found that dephosphorylated D2 was quite inactive as an initiator of SV40 DNA replication (not shown). This was an expected result as dephosphorylated D2 protein bound poorly to the origin, the first and essential step in a replication cycle. However, again, this result was different from that obtained with authentic T antigen. In this case, dephosphorylation by alkaline phosphatase increased the replicative activity (Mohr et al., 1987; Grässer et al., 1988).

Discussion

D2 protein lacks the 114 N-terminal amino acids of authentic T antigen and has instead an N-terminus of 122 amino acids derived mainly from the adenovirus 33 K protein (Baumann et al., 1985). However, D2 protein does possess the two functional domains important for the replicative function of T antigen, namely, the DNA binding and the ATPase/helicase domain. In fact, we could confirm earlier work (Tjian et al., 1979) showing that D2 protein appears to bind specifically and efficiently to the two binding sites in the

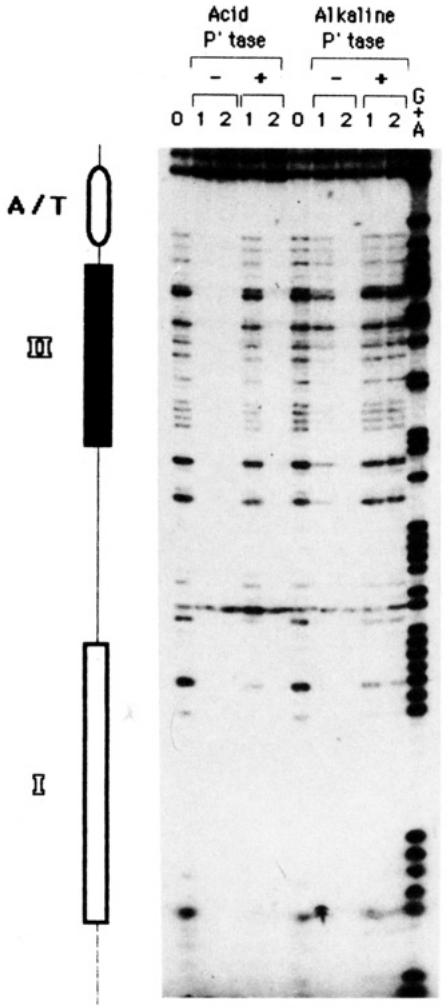

FIGURE 7: DNA binding of dephosphorylated D2 protein. D2 protein (150 ng, lane 1; 450 ng, lane 2) was incubated in parallel experiments in dephosphorylation buffers with (+) or without (−) acid or alkaline phosphatase (P'tase) and used for DNA binding to the 3'-^{32}P-labeled 143 bp origin DNA fragment in the presence of an excess of unlabeled salmon sperm DNA. DNase I digestion was performed as described under Materials and Methods. Lane 0 contains the digestion products of protein-free DNA. Lane G+A shows the results of a Maxam–Gilbert G + A reaction. On the left, we indicate relevant parts of the DNA fragment used, namely, the high-affinity binding sites I and II as well as the AT-rich element of the origin (which is only poorly attacked by DNase I).

genomic control region of SV40 DNA (Figure 2). However, D2 protein was clearly less active than authentic T in catalyzing the separation of duplex DNA strands and in inducing in vitro replication of SV40 DNA. This is most probably not due to an artifactual inactivation of the D2 preparations used in our experiments because (i) we obtained identical results with independently prepared batches of D2, (ii) the protein was prepared by exactly the same procedure used to purify authentic T (which was taken as a control in all of the experiments reported above), (iii) gel electrophoresis in the presence of sodium dodecyl sulfate did not reveal any structural changes of the D2 preparations used (data not shown), and(iv) D2 appeared to bind well to SV40 DNA. According to previous work, a negative charge on Thr-124 may be important for an effective association of authentic T antigen with the SV40 origin (Paucha et al., 1988; Arthur et al., 1988). Phosphate groups on adjacent serine residues may serve to down-regulate the origin binding activity of T antigen (Klausing et al., 1988). Consequently, authentic T antigen with dephosphorylated serines but intact phosphothreonines had an increased affinity to the origin (Mohr et al., 1987). In contrast, identically treated D2 protein had a drastically reduced affinity to the origin but not to binding site I which is located just outside of the minimal or core origin of replication. The arrangement of T antigen molecules, bound to site I, is most likely different from the arrangement of T antigen molecules bound to the origin. One T antigen molecule binds to each one of the recognition GAGGC pentanucleotides (Wright et al., 1984). Binding site I contains two tandemly oriented pentanucleotides separated by a 7 bp AT-rich spacer whereas the origin binding site II contains four closely spaced pentanucleotides, organized as two pairs in opposite orientations. Scanning transmission electron microscopy and other methods have shown that a tight cluster of up to four T antigen molecules (or multiples thereof) may bind to the origin (Mastrangelo et al., 1985).

The individual protein monomers at the origin are expected to be precisely arranged, forming a highly ordered complex that may be necessary for effective duplex DNA unwinding and, possibly, subsequent steps leading to the establishment of replication forks.

The organization of this complex may depend on specific protein–protein contacts. It is likely that these contacts are formed between regions in the N-terminal section of T antigen because phosphorylation and dephosphorylation of certain N-terminal amino acids have profound effects on the formation of a T antigen–origin complex.

A participation of N-terminal amino acids in complex formation would explain why D2 protein appeared to be unproductively bound to the origin. Untreated D2 protein molecules did bind to the GAGGC pentanucleotides of the origin site, but due to the absence of their cognate N-termini, their arrangement or orientation may be less than perfect. The negative effects of the "foreign" N-termini in origin complex formation were probably partially neutralized by the phosphate groups on N-terminal amino acids. This would explain why dephosphorylation converts an origin binding (though poorly functioning) D2 protein into a form that bound rather inefficiently, if at all, to the origin.

Nonoptimal binding to the origin may also be the reason why the in vitro replication activity of D2 protein did not increase even at D2 concentrations that were 2 times higher than those used under standard assay conditions (Figure 5). It is possible that D2 molecules saturated the available origin sequences but, in most cases, failed to induce DNA unwinding and initiation of DNA replication.

We do not suggest that the N-terminal region is the only part of T antigen responsible for protein–protein interaction. Arthur et al. (1988) interpret the results of their DNA binding studies to indicate that the region between amino acid residues 300 and 320 (including the Zn-finger loop) could participate in an interaction between individual T antigen molecules.

It is also possible that the N-terminal region of T antigen is necessary not only for the interaction between individual T antigen molecules at the replication origin but also for the interaction of T antigen with other proteins of replication apparatus. This may be the reason why the DNA unwinding activity of D2 protein was reduced to 30–40% whereas its replication activity was reduced to 20–30% compared to authentic T antigen. In any case, the organization of replication-competent T antigen molecules, bound to the SV40 origin, is certainly complex, and further studies, perhaps using a collection of specifically mutated T antigens, will be necessary to eludicate the structural requirements for the formation of functional T antigen–origin complexes.

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