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Production of simian virus 40 large tumor antigen in bacteria: Altered DNA-binding specificity and DNA-replication activity of underphosphorylated large tumor antigen

(papovavirus tumor antigen/DNA helicase/origin-specific unwinding/posttranslational modification)

IAN J. MOHR*, YAKOV GLUZMAN†, MICAELA P. FAIRMAN‡, MICHAEL STRAUSS§, DUNCAN MCVEY, BRUCE STILLMAN, AND ROBERT D. GERARD¶

Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724

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ABSTRACT **A bacterial expression system was used to produce simian virus 40 large tumor antigen (T antigen) in the absence of the extensive posttranslational modifications that occur in mammalian cells. Wild-type T antigen produced in bacteria retained a specific subset of the biochemical activities displayed by its mammalian counterpart. *Escherichia coli* T antigen functioned as a helicase and bound to DNA fragments containing either site I or the wild-type origin of replication in a manner identical to mammalian T antigen. However, T antigen purified from *E. coli* did not efficiently bind to site II, an essential cis element within the simian virus 40 origin of replication. It therefore could not unwind origin-containing plasmids or efficiently replicate simian virus 40 DNA *in vitro*. The ability of protein phosphorylation to modulate the intrinsic preference of full-length T antigen for either site I or site II is discussed.**

Protein phosphorylation has been demonstrated to be an effective means of regulating both the catalytic activity of numerous enzymes and the assembly of macromolecular structures (1–7). Subtle alterations in the sites that are phosphorylated may result in conformational changes that could, in turn, affect protein function. It is conceivable that, in some instances, basal levels of phosphorylation may be required for proper folding of a polypeptide and full activity, whereas subsequent levels of modification may have a more pronounced regulatory effect. The wealth of genetic and biochemical information concerning the large tumor antigen (T antigen) encoded by simian virus 40 (SV40) (for review, see ref. 8) makes this phosphoprotein an excellent model with which to examine the consequences of multiple phosphorylation events.

T antigen is phosphorylated at two clusters of serine and threonine residues (9–11). Biochemical studies have indicated that 80% of the phosphate moieties on the molecule can be removed by treatment with calf intestinal alkaline phosphatase (CIAP), while the remaining 20% are refractory to enzymatic dephosphorylation (12–15). Furthermore, analysis of CIAP-treated protein revealed an increase in its ability to direct origin-specific DNA synthesis *in vitro* (13, 14). Although the ATPase activity of the CIAP-treated protein was not altered (13–15), its ability to bind specific DNA sequences in the SV40 origin of replication increased several-fold (13, 15, 16). The SV40 origin of replication contains two T antigen binding sites (17, 18). While each of these sites contains multiple copies of the 5′ GAGGC 3′ sequence recognized by T antigen, the arrangement of the pentanucleotide motifs differs markedly, and T antigen binding to either site has different biological consequences (18–20). Site I contains a high-affinity T antigen binding site, participates in the autoregulation of early transcription, and is an auxiliary component of the origin of replication (19–21). Site II also functions in transcriptional control but is primarily an essential cis element within the minimal SV40 origin of replication (20, 22). CIAP-treated T antigen displays a 4-fold increase in binding to site II and a 2-fold increase in binding to site I (13, 15).

Phospho amino acid analysis performed in several laboratories has confirmed that the phosphates present on serine residues are removed by CIAP treatment, while those covalently attached to the two threonine residues (124 and 701) are unaffected (12, 14, 15). Furthermore, genetic studies have indicated that, while Thr-701 is dispensible for T antigen function and virus viability (23, 24), Thr-124 is absolutely essential (24, 25). To examine the activity of T antigen that lacks the posttranslational modifications provided by a mammalian cell, the protein was expressed in bacteria, and several biologically relevant biochemical parameters were evaluated *in vitro*.

MATERIALS AND METHODS

Expression of T Antigen in *Escherichia coli*. The coding sequences for SV40 T antigen were cloned into a T7 expression plasmid, pRK170 (obtained from R. Kostriken, Cold Spring Harbor Laboratory). pRK170 was derived from pAR3038 (26) by deletion of the sequences between *Eco*RI and *Eco*RV downstream of the T7 transcription termination signal. The *Nde* I restriction site of the vector contains the ATG start codon for translation of the cloned sequence. The sequence surrounding the ATG of T antigen was converted to an *Nde* I site by site-directed mutagenesis (27) using a cDNA copy of the SV40 *Hin*dIII fragment (nucleotides 4002–5171) in pUC119. Appropriate clones were identified first by positive hybridization to a ^{32}P-labeled oligonucleotide and subsequently by their sensitivity to *Nde* I. The *Nde* I–*Hin*dIII fragment from this plasmid was ligated together with the remainder of T antigen cDNA coding sequences (purified *Hin*dIII–*Bam*HI fragment, nucleotides 2533–4002) into *Nde* I–*Bam*HI-digested pRK170. The resulting pT7Tag plasmid

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Abbreviations: T antigen, large tumor antigen; CIAP, calf intestinal alkaline phosphatase; SV40, simian virus 40.

*Present address: Department of Molecular Biology, University of California, Berkeley, CA 94720.
†Present address: Lederle Laboratories, American Cyanamid Co., Pearl River, NY 10965.
‡Present address: Department of Zoology, University of Cambridge, Downing Street, Cambridge CB2 3EJ, UK.
§Present address: Zentralinstitut fur Molekularbiologie, 1115 Berlin-Buch, G.D.R.
¶Present address: Department of Biochemistry, University of Texas Health Science Center, 5323 Harry Hines Boulevard, Dallas, TX 75235.

was used to transform BL21(DE3) bacteria (26) to ampicillin resistance. Isolated colonies demonstrated less than 1% viability when plated on agar containing isopropyl β-D-thiogalactoside and ampicillin. To create an expression plasmid that conferred resistance to kanamycin upon the host bacteria, the *Bgl* II–*Eco*RI fragment from pAR3038 was cloned into *Bam*HI–*Eco*RI-digested pMK16#1 (22). The resulting plasmid was digested with *Bam*HI and *Xba* I and ligated to the *Xba* I–*Bam*HI fragment from pT7Tag. This final construct was termed pABT7T.

Proteins. T antigen from a mammalian source was produced in HeLa cell suspension cultures (5×10^5 cells per ml) infected with a mixture of wild-type adenovirus 5 (as a helper) and a defective adenovirus carrying the coding sequence of SV40 T antigen in early region 1 at multiplicities of infection of 10 and 100, respectively. Cells were harvested 42–44 hr postinfection, and extracts were prepared as described (28).

Full-length T antigen from *E. coli* was prepared in the following manner. Cultures of BL21(DE3)Lys^s (ref. 19) carrying the pABT7T plasmid were grown at 37°C in LB medium containing kanamycin and chloramphenicol. When the OD_{600} reached 0.5, isopropyl β-D-thiogalactoside was added to a final concentration of 0.4 mM and the flasks were kept in a shaking incubator at 37°C for an additional 2 hr. Bacteria were harvested by centrifugation and suspended in a small volume of buffer C [50 mM Tris·HCl, pH 7.4/500 mM LiCl/1 mM EDTA/10% (vol/vol) glycerol]. Phenylmethylsulfonyl fluoride was added to a final concentration of 1 mM, and the suspension was lysed under 1500 psi (1 psi = 6.89 kPa) of nitrogen in a french press. After centrifugation at 76,800 × *g* (r_{avg}), the clarified extract was loaded onto a 3.5-cm × 47.5-cm Sephadex G-100 column equilibrated in buffer C, and the column was eluted at a flow rate of 12 ml/hr. This step allowed some separation of the full-length protein from shorter contaminants. Fractions containing full-length T antigen were pooled and loaded sequentially onto a protein A-Sepharose column followed by a protein A-Sepharose column onto which the monoclonal antibody Pab419 (29) had been coupled (28). The column was then washed and eluted as described (28).

DNA Replication Reactions. Cytoplasmic extracts were prepared from human 293 cells grown in suspension (30). After adjusting the final concentration of NaCl in the supernatant to 0.1 M, the extract was clarified by centrifugation at 100,000 × *g*, and the resulting supernatant fraction (S100) was divided into aliquots and stored at −70°C. This fraction was used for all DNA replication reactions. DNA replication *in vitro* was measured by the incorporation of [α-^{32}P]dATP into trichloroacetic acid-insoluble material (30).

Helicase Assay. mpSV2 contains the *Hin*dIII–*Hpa* II SV40 fragment (nucleotides 5171–346) cloned into *Sma* I/*Hin*dIII-digested M13mp9. A synthetic 42-base oligonucleotide (5′ GTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGAATT 3′) was synthesized on an Applied Biosystems 380-A DNA synthesizer and purified by electrophoresis on a denaturing polyacrylamide gel. Following adsorption onto a reverse-phase C_{18} SepPak cartridge (Waters), the oligonucleotide was eluted with 60% methanol, lyophillized to dryness, and resuspended in H_2O. Fifty nanograms of purified oligonucleotide was annealed to 1.5 μg of mpSV2 in 10 mM Tris·HCl, pH 8.5/1 mM $MgCl_2$ at 50°C for 1 hr. Following slow cooling to room temperature, the annealed template was labeled with the Klenow fragment of DNA polymerase I (in the presence of [α-^{32}P]dCTP and nonradioactive dGTP) and subsequently extracted by both phenol and chloroform. The labeled DNA was ethanol precipitated, resuspended, and loaded onto a 0.6-cm × 26-cm Sepharose CL-4B column. The column was equilibrated and developed in 10 mM Tris·HCl, pH 7.4/1 mM EDTA/100 mM NaCl, and peak fractions containing labeled DNA were collected and used directly. Helicase reaction mixtures contained ≈10 ng of labeled substrate in 30 mM Hepes-KOH, pH 8.0/4 mM ATP/7 mM $MgCl_2$/0.5 mM dithiothreitol and were incubated for 1 hr at 37°C. Reactions were terminated by adding SDS, EDTA, and glycerol to 0.6%, 30 mM, and 5%, respectively. Products were analyzed on 8% native polyacrylamide gels containing a gradient of 0.5× to 2.5× TBE (1× TBE = 90 mM Tris/64.6 mM boric acid/2.5 mM EDTA, pH 8.3). Gels were dried onto DE 81 paper (Whatman) and exposed to Kodak XAR film at room temperature.

DNA-Binding Reactions. Plasmid DNA templates containing site I (pOS-1; SV40 nucleotides 5171–5228), site II (pSVOdl3; SV40 nucleotides 5209–128), and the wild-type origin (pSVO+; SV40 nucleotides 5171–128) have been described (31, 32). An equimolar mixture of these plasmids was assembled and digested to completion with *Taq* I. This enzyme releases intact SV40 origin sequences and generates several plasmid-derived fragments. The mixture was labeled with the Klenow fragment of DNA polymerase I, extracted with phenol and chloroform, and precipitated with ethanol. Fifty nanograms of this mixture was incubated with various amounts of each T antigen in origin-binding buffer (10 mM Hepes, pH 7.4/100 mM KCl/1 mM $MgCl_2$/5% glycerol/bovine serum albumin at 50 μg/ml) for 1 hr at 0°C. Five micrograms of purified Pab419 (29) was added, and the incubation was continued for an additional 20 min. One hundred microliters of a 10% (vol/vol) protein A-Sepharose slurry in NET buffer (50 mM Tris·HCl, pH 7.4/150 mM NaCl/5 mM EDTA/0.05% Nonidet P-40) containing sonicated calf thymus DNA at 20 μg/ml was then added, and the reaction mixtures were incubated on a rocker for 50 min at 4°C. The beads were pelleted, washed three times with 1 ml of NET buffer, resuspended in 1% SDS/25 mM EDTA, incubated at 65°C for 15 min, and electrophoresed on a 6% native polyacrylamide gel in a TBE buffer system. Gels were dried onto DE 81 paper (Whatman) and exposed to Kodak XAR film at room temperature.

Origin-Specific Unwinding Assay. Various amounts of purified SV40 T antigen were added to reaction mixtures (50 μl) containing 40 mM Hepes-KOH (pH 7.5), 8 mM $MgCl_2$, 0.5 mM dithiothreitol, 4 mM ATP (from a 100 mM, pH 7.0 stock), 40 mM creatine phosphate, creatine phosphokinase at 1 μg/ml, 1 μg of bovine serum albumin, 100 ng of calf thymus topoisomerase I, 1.2 μg of RFA (a eukaryotic single-stranded DNA-binding protein) (33, 34), 20 μg of fraction SSI (33), and 100 ng of template DNA (pSV011; ref. 35). Reaction mixtures were incubated at 37°C for 15 min and terminated by the addition of SDS and EDTA to 0.4% and 100 mM, respectively. Protease XIII (50 μg/ml) and RNase A (10 μg/ml) were then added, and the samples were incubated for an additional 30 min at 37°C. The digested samples were then extracted once with an equal volume of phenol/chloroform, 1:1, and the DNA was precipitated with ethanol. The degree of unwinding was determined by electrophoresis through 1.3% agarose gels in TBE and visualized by staining with ethidium bromide and exposing the gel to x-ray film.

RESULTS

Expression of T Antigen in *E. coli*. The cDNA encoding full-length SV40 T antigen was cloned in front of the promoter for T7 gene 10 (see *Materials and Methods*). This expression plasmid was transformed into a strain of *E. coli* harboring a λ prophage containing the gene for T7 RNA polymerase under the control of the lac UV5 promoter. Induction of cultures with isopropyl β-D-thiogalactoside results in the synthesis of large amounts of T7 RNA polymerase which, in turn, directs transcription from the T7 gene 10 promoter (26). T antigen was purified from induced cultures by modifications of existing immunoaffinity procedures (28). The yield of

T antigen purified by this method was approximately 50 μg of full-length protein from 2 liters of induced cultures. In addition to poor levels of expression, various amounts of immunologically related, truncated proteins copurified with the full-length polypeptide. The actual amounts of these proteins varied among preparations. The full-length polypeptide was the major protein species present in preparations used to perform the experiments that follow. Side-by-side analysis of proteins purified from bacterial and mammalian sources on SDS/PAGE reveals that *E. coli* T antigen migrates slightly slower than its mammalian counterpart (Fig. 1). Electrophoresis of both proteins in a single lane results in a doublet of bands corresponding to the individual components (data not shown). Although we do not precisely know the nature of this difference, one possibility is that the mobility difference may be attributed to differences in the phosphorylation of the proteins produced in *E. coli* vs. HeLa cells. ^{32}P labeling of induced cultures revealed that *E. coli* T antigen is phosphorylated several orders of magnitude less than the protein from HeLa cells (data not shown). Differences in both the phosphate pools and the efficiency of labeling these two very different cell types prohibit a more quantitative analysis. The overall amount of phospho amino acids in prokaryotes is known to be much lower than in eukaryotic organisms. Experiments performed with ^{32}P-labeled proteins from *E. coli* demonstrated that ≈96% of the acid-stable phospho amino acids are phosphoserine residues (see ref. 36 for review). It is worth noting, however, that phosphorylation can alter the migration pattern of proteins in one-dimensional SDS/polyacrylamide gels (for example, see ref. 37). This also holds true for T antigen, as mutation of the Thr-701 phosphorylation site to Ala (24) alters the protein's mobility on SDS/polyacrylamide gels (Fig. 1).

Biochemical Properties of *E. coli* T Antigen. The ability of *E. coli* T antigen or HeLa T antigen to direct the synthesis of SV40 DNA was evaluated by using an *in vitro* system (30, 38). Side-by-side analysis of equal microgram amounts of *E. coli* T antigen or HeLa T antigen (confirmed by SDS/PAGE) revealed that reactions programmed with *E. coli* T antigen replicated DNA to levels 10–15% of those directed by reactions programmed with its mammalian counterpart (Fig. 2). Furthermore, this reduced level of synthesis was origin specific, as it was completely abolished by the deletion of four base pairs within site II (22).

The specific DNA-binding property of these proteins was compared by incubating the purified proteins with a mixture of end-labeled DNA composed of three origin fragments present in equimolar amounts as well as several nonspecific

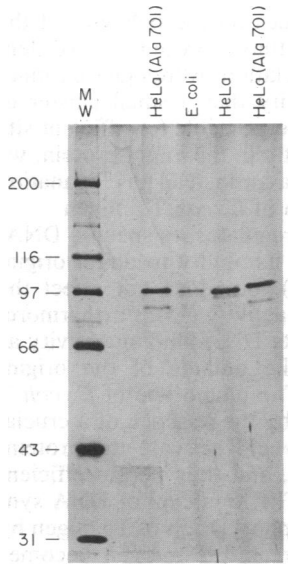

FIG. 1. Anamolous mobility of *E. coli* T antigen on SDS/polyacrylamide gels. Various amounts of immunoaffinity purified T antigen from HeLa cells or from *E. coli* were subjected to electrophoresis on a SDS/12.5% polyacrylamide gel. The gel was fixed and stained with silver. Molecular size markers (MW): 200 kDa, myosin; 116 kDa, β-galactosidase; 97 kDa, phosphorylase b; 66 kDa, bovine serum albumin; 43 kDa, ovalbumin; 30 kDa, carbonic anhydrase. The lane marked HeLa Ala-701 contains a mutant T antigen with a substitution of alanine for threonine at residue 701 (24).

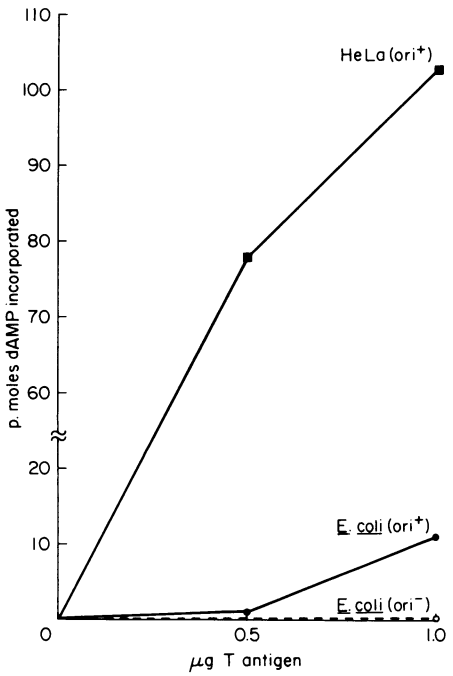

FIG. 2. Full-length T antigen from *E. coli* directs levels of *in vitro* DNA synthesis that are 10–15% of those directed by T antigen purified from a mammalian source. Various amounts of purified T antigen produced either in HeLa cells or in *E. coli* were used to program *in vitro* DNA synthesis reactions containing either wild-type origin (ori$^+$) or mutant origin (ori$^-$) templates. The amount of DNA synthesis is expressed as the number of picomoles of dAMP incorporated into acid-insoluble material.

DNA fragments. Protein–DNA complexes, once formed, are immunoprecipitated (39), and the DNA fragments bound are visualized on autoradiograms of polyacrylamide gels. Fig. 3 demonstrates that *E. coli* T antigen fails to form complexes with the site II fragment at concentrations where HeLa T antigen binds efficiently. *E. coli* T antigen also demonstrates greater binding to nonspecific DNA fragments than to the site II fragment. However, *E. coli* T antigen binds to fragments containing site I only or the wild-type origin with the same efficiency as HeLa T antigen. Binding of T antigen to the wild-type origin fragment primarily reflects binding to the high-affinity site I. DNase I footprinting of *E. coli* T antigen or HeLa T antigen bound to wild-type origin fragments has confirmed that both proteins completely protect site I, while only HeLa T antigen efficiently protects site II (data not shown).

T antigen from *E. coli* possesses an intrinsic ATPase activity, and this activity is inhibited by monoclonal antibodies (40) that inhibit ATP hydrolysis by HeLa T antigen (data not shown). The ATPase activity of the protein (41, 42) is required for it to function as a DNA helicase (43). This activity can be measured by monitoring the ability of the protein to displace a labeled 51-base oligonucleotide from a heteroduplex helicase substrate in the presence of ATP and Mg^{2+}. Fig. 4 demonstrates that *E. coli* T antigen and HeLa T antigen function identically in this assay. A second assay, which requires the helicase function of T antigen, measures the unwinding of a closed, circular DNA template containing the SV40 origin (44, 45) in the presence of several cellular proteins, including a eukaryotic single-stranded DNA-binding protein (RFA; refs. 33 and 34) and toposomerase I. Unwinding of the template is reflected by the appearance of faster migrating supercoiled forms of the labeled DNA evident in reactions containing HeLa T antigen (Fig. 5). The ability of *E. coli* T antigen to function in this assay parallels its ability to replicate SV40 DNA and its apparent inability to

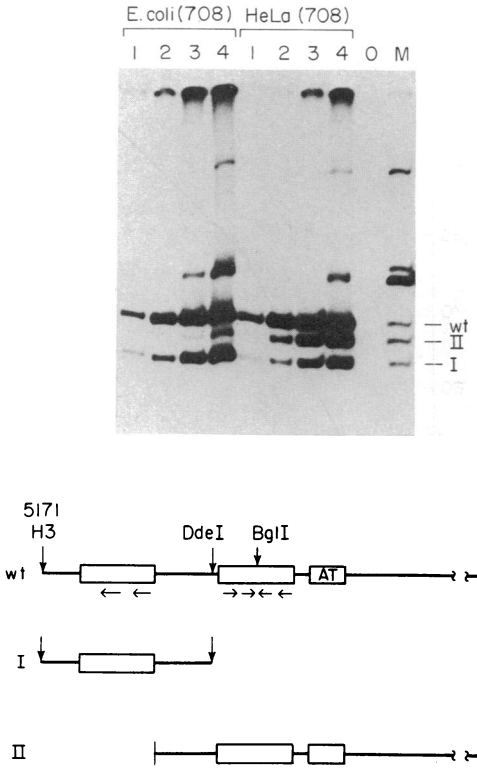

FIG. 3. Specific DNA-binding activity of *E. coli* T antigen versus HeLa T antigen. (*Upper*) Various amounts of immunoaffinity T antigen from *E. coli* or HeLa cells (lanes: 1, 50 ng; 2, 150 ng; 3, 450 ng; 4, 1000 ng) were incubated with a mixture of end-labeled DNA containing three origin fragments in equimolar amounts (lane M). After 1 hr at 0°C, T antigen was immunoprecipitated from the reaction mixture, and the DNA fragments bound were separated on native 6% polyacrylamide gels and visualized by autoradiography. (*Lower*) Schematic of the SV40 origin fragments used in the DNA-binding assay. wt, site I + site II; I, site I only; II, site II only.

form complexes with site II DNA fragments. This provides evidence that efficient site II binding, in addition to DNA helicase activity, is necessary for proper unwinding activity.

DISCUSSION

The biochemical activities of T antigen purified from prokaryotic or eukaryotic sources have been evaluated. In approximately half of these assays, the activity of *E. coli* T antigen was indistinguishable from that of HeLa T antigen. The reduced activity displayed by *E. coli* T antigen in the remaining assays can be explained in terms of its reduced

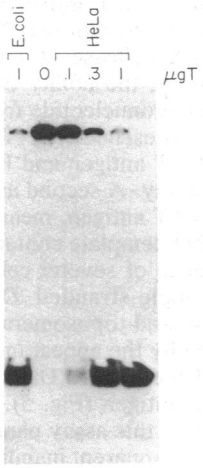

FIG. 4. *E. coli* T antigen possesses wild-type levels of helicase activity. Purified T antigen from *E. coli* or HeLa cells was incubated with the labeled helicase substrate in the presence of ATP for 1 hr at 37°C. The products of the reaction were separated on native 8% polyacrylamide/TBE buffer gradient gels and visualized by autoradiography.

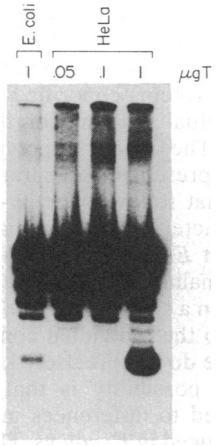

FIG. 5. The origin-specific unwinding activity of *E. coli* T antigen is dramatically reduced relative to HeLa T antigen. Purified T antigen from *E. coli* or HeLa cells was incubated with the purified, labeled DNA products of an *in vitro* DNA synthesis reaction in the presence of replication protein A, calf thymus topoisomerase I, fraction SSI, and ATP for 15 min at 37°C. The reaction products were analyzed on 1% agarose gels and visualized by autoradiography.

ability to bind site II. Although T antigen from *E. coli* binds to DNA fragments containing the wild-type origin and site I with the same efficiency as HeLa T antigen, it fails to complex with fragments containing only site II at concentrations where HeLa T antigen binds efficiently. The impaired ability of *E. coli* T antigen to complex with site II, an essential cis element within the SV40 origin of replication, directly affects its performance in replication and unwinding assays. *E. coli* T antigen directs origin-specific DNA synthesis at levels that are 10–15% of those directed by T antigen purified from HeLa cells infected with an adenovirus expression vector and functions at reduced levels in an origin-specific unwinding assay. Both *E. coli* T antigen and HeLa T antigen, however, exhibit similar levels of DNA helicase activity.

A single domain encompassing residues 132–246 of T antigen contains all of the information necessary for the specific recognition of site I and site II (refs. 31, 46, and 47; D.M., M.S., and Y.G., unpublished observations). Although both sites contain multiple copies of the 5′ GAGGC 3′ sequence recognized by T antigen, the arrangement of the pentanucleotides is fundamentally different (18). Production of a truncated protein in *E. coli* composed of amino acids 132–246 has revealed that this isolated domain binds efficiently to both site I and site II (48). The ability of this region to function, in general, as a DNA-binding entity appears unaffected. Full-length *E. coli* T antigen, however, only binds efficiently to site I and wild-type origin fragments but fails to complex with site II. Packaging the DNA-binding domain within the superstructure of the protein has imposed constraints on the ability of the domain to recognize site II. The ability to modulate the conformation of the superstructure could have profound consequences on the behavior of the DNA-binding domain and would thus represent an excellent target for regulation by phosphorylation. Although we cannot completely rule out the possibility that a small cluster of amino acids within this domain responsible for efficient site II recognition cannot fold correctly in the intact protein, we believe this view is unlikely and favor instead an explanation centering on the phosphorylation of *E. coli* T antigen.

Phosphorylation of T antigen regulates its specific DNA-binding activity (13, 15, 16, 49), its ability to direct origin-specific DNA synthesis (13, 14), but does not affect the protein's intrinsic DNA helicase activity (15). Furthermore, this modification indirectly affects DNA-binding activity as both phosphorylation clusters lie outside of the origin-specific DNA-binding domain. The phenotype of *E. coli* T antigen could then be explained by the absence of a crucial phosphorylation event, which would activate the protein, allow efficient binding to site II, and thus permit efficient origin-specific DNA synthesis. The low level of DNA synthesis observed may reflect phosphorylation of T antigen by kinases in the S100, as CIAP-treated T antigen becomes

rephosphorylated under these conditions (14). Subsequent phosphorylation on serine residues would function to down-regulate the replication and DNA-binding activities of the protein. Consistent with this hypothesis, CIAP treatment of HeLa T antigen, which activates DNA replication and site II binding activity, cannot fully dephosphorylate the protein (12–15). Following CIAP treatment, phosphate is found predominately on Thr-124 and Thr-701 (14, 15). Although Thr-701 is dispensable for SV40 DNA replication, Thr-124 is absolutely essential (24). The final correlation is indeed striking, as full-length T antigen produced in *E. coli* is a partial phenocopy of a mutant at Thr-124. Mutants at this residue are totally defective for replicating SV40 DNA *in vivo* and fail to bind to DNA fragments containing only site II (24).

SV40 has successfully usurped the host cells' posttranslational modification machinery to further its own purposes. T antigen is a multifunctional protein and must be distributed to many different functions in a productive lytic infection. Phosphorylation is thus a mechanism in which extensive heterogeneity can be generated in an otherwise homogenous population of molecules. For example, molecules lacking phosphates on Thr-124 may be involved in autoregulation of T antigen synthesis (50–53), which requires binding to site I, while a hyperphosphorylated class may activate late transcription (54–57). Phosphorylation of Thr-124, however, may be obligatory on molecules participating in SV40 DNA replication. The creation and marking of T antigen subpopulations ensures that all molecules of T antigen will not be directed to replicate SV40 DNA and can thus be deployed to perform other functions crucial to the viral life cycle.

Protein phosphorylation can also account for heterogeneity among cellular proteins. Reversible covalent modification provides a means to modulate a variety of processes in a temporal fashion (for example, see ref. 58). Families of immunologically related proteins have been shown to be involved in regulation of cellular gene expression (59); furthermore, several of these families can function as nuclear oncoproteins (for review, see ref. 60). Multiple members of a given family are, in some instances, generated by differential splicing events (61) or are encoded by distinct genetic units (for example, see refs. 48 and 62). In several instances, however, extensive posttranslational modification accounts for the heterogeneity among members of a protein family (59, 63–66). Further study of these proteins will reveal any functional significance attached to this heterogeneity and will almost certainly unravel additional unexpected levels of complexity.

Note Added in Proof. The cdc2 protein kinase (purified from mammalian cells) phosphorylates T antigen on Thr-124. Phosphorylation of *E. coli* T antigen by the cdc2 protein kinase restores its ability to bind site II and to subsequently initiate SV40 DNA synthesis *in vitro* (D.M., L. Brizuela, I.J.M., D. Marshak, Y.G., and D. Beach, unpublished results).

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