JOURNAL OF VIROLOGY, May 1988, p. 1598–1605
0022-538X/88/051598-08$02.00/0

Vol. 62, No. 5

Mutations in the Phosphorylation Sites of Simian Virus 40 (SV40) T Antigen Alter Its Origin DNA-Binding Specificity for Sites I or II and Affect SV40 DNA Replication Activity

JENS SCHNEIDER† AND ELLEN FANNING*

Institute for Biochemistry, Karlstrasse 23, 8000 Munich 2, Federal Republic of Germany

Received 27 August 1987/Accepted 2 February 1988

A series of mutants of simian virus 40 was constructed by oligonucleotide-directed mutagenesis to study the role of phosphorylation in the functions of large T antigen. Each of the previously mapped phosphorylated serine and threonine residues in large T antigen was replaced by an alanine or cysteine residue or, in one case, by glutamic acid. Mutant DNAs were assayed for plaque-forming activity, viral DNA replication, expression of T antigen, and morphological transformation of rat cells. Viable mutants were isolated, suggesting that modification of some residues is not essential for the biological functions of T antigen. Two of these mutants replicated more efficiently than did the wild type. Seven mutants were partially or completely deficient in viral DNA replication but retained cell transformation activity comparable with that of the wild-type protein. Biochemical analysis of the mutant T antigens demonstrated novel origin DNA-binding properties of several mutant proteins. The results are consistent with the idea that differential phosphorylation defines several functional subclasses of T-antigen molecules.

Protein phosphorylation is an important regulatory mechanism in eucaryotic cells. For example, it has been shown to modulate the activity of enzymes, such as those involved in glycogen metabolism, and proteins involved in the control of gene expression, such as hormone receptors (for a review, see reference 8). More recently, protein phosphorylation has been postulated to play a regulatory role in the reorganization of the nuclear lamins during mitosis (3, 40, 60). Finally, protein phosphorylation, particularly tyrosine phosphorylation catalyzed by oncogene and proto-oncogene products, has been associated with altered growth control (for a review, see reference 28).

Simian virus 40 (SV40) large tumor antigen (large T antigen), a multifunctional regulatory protein encoded by the viral genome, is a phosphoprotein (66). Multiple sites of phosphorylation, i.e., serine and threonine residues, have been mapped to two regions in the primary sequence of the protein (Fig. 1; 33, 56, 69). A subset of these sites can be phosphorylated by cytoplasmic protein kinases, whereas full phosphorylation also requires nuclear kinases (58). Furthermore, T antigen matures after synthesis in a time-dependent fashion, becoming phosphorylated in discrete stages and forming oligomers that differ in level of phosphorylation (16, 23, 57, 61). The phosphate groups associated with the protein were found to turn over faster than the protein itself (14, 70), raising the question of whether phosphorylation is involved in the regulation of T-antigen function.

An investigation of the role of phosphorylation in the biological functions of T antigen in virus replication and cell transformation would require a way to specifically inhibit modification of the protein in the cell. Oligonucleotide-directed mutagenesis of the DNA encoding T antigen offers a means to substitute the modified serines and threonines by residues that are similar in size and charge but that cannot be phosphorylated. A similar approach has been used successfully to investigate the role of protein phosphorylation in the biological activity of several viral oncogenes, proto-oncogenes, and growth factor receptors (4, 5, 19, 32, 47).

We might expect the biological properties of such mutant T antigens to fall into two classes. If the modification is dispensable for protein function, we would expect a wild-type phenotype. If either the modification or the residue itself is essential, a mutant phenotype should result. Biochemical characterization of such mutant proteins should then reveal what property of the protein is defective. Thus, we have used oligonucleotide-directed mutagenesis to create a set of mutations in the T-antigen-coding region, such that each phosphorylated serine and threonine residue is conservatively substituted by alanine or cysteine. In the present communication, we describe the construction and biological characterization of these mutants and present an initial characterization of the DNA-binding properties of T antigens encoded by the mutants.

(This research was conducted by J. Schneider in partial fulfillment of the requirements for the doctoral degree from the University of Munich.)

MATERIALS AND METHODS

Plasmids and strains. pSVwt is wild-type SV40 DNA (strain SV-S) cloned in the *Bam*HI site of pAT153 DNA (17). pSDL13 (1,890 base pairs) carries a suppressor (*supF*), ColEI and M13 origins of replication, and the polylinker from pUC13 (35). pSDL13-1.13 consists of the SV40 sequences from pSVwt cloned in the *Bam*HI site of pSDL13. *Escherichia coli* XS127 (35) carries the F′ from JM103 and the RP1 plasmid p3 [Kan^r *tet*(Am) *bla*(Am)]. pONwt carries a synthetic 19-base-pair sequence from T-antigen binding site I (51, 53, 54). p1097 carries a 31-base-pair deletion encompassing all of site I (12, 27).

Oligonucleotide-directed mutagenesis. A modified procedure based on that of Zoller and Smith (72) was developed for use with the pSDL13 vector. Single-stranded template DNA was isolated by M13 wild-type infection of *E. coli* XS127 carrying the wild-type SV40 parent DNA pSDL13-

* Corresponding author.
† Present address: Division of Biology, California Institute of Technology, Pasadena, CA 91125.

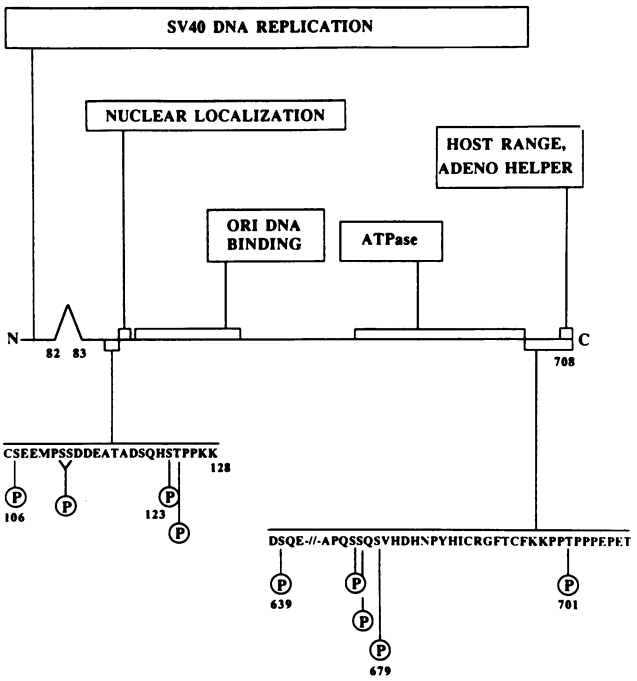

FIG. 1. Map of functional domains of T antigen and location of known phosphorylation sites. The sequences necessary and sufficient for each of the designated properties of T antigen were compiled from analyses of previously characterized SV40 mutants (7, 9, 31, 43, 48, 63; Arthur et al., in press). The phosphorylation sites shown Ⓟ are those mapped by Scheidtmann et al. (56).

1.13. Oligonucleotides (Table 1) were synthesized by hand (13) or by using an automatic DNA synthesizer (model 380A; Applied Biosystems GmbH, Pfungstadt, Federal Republic of Germany), and they were purified by polyacrylamide gel electrophoresis. A 1-pmol amount of single-stranded template was annealed with 5 pmol of phosphorylated primer oligonucleotide (Table 1), filled in by Klenow DNA polymerase (New England BioLabs, Beverly, Mass.) and deoxynucleoside triphosphates, and ligated (72). Covalently closed circular DNA was transfected into competent *E. coli* XS127. Colonies resistant to ampicillin and tetracycline were hybridized with the ^{32}P-end-labeled mutant primer oligonucleotide as described previously (11, 20). Mutant colonies were then identified by autoradiography. The fraction of colonies positive by hybridization varied widely, from 1 to 45% depending on the mutant sequence, but was remarkably reproducible for a particular sequence (data not shown). The introduction of the mutation was confirmed by digestion of plasmid DNA with diagnostic restriction endonucleases (Table 1) and by supercoil DNA sequencing (6, 55). The carboxy-terminal mutations were further confirmed by recloning the mutant *Pst*I DNA fragment into the wild-type pSDL13-1.13 DNA and then sequencing the mutant fragments.

Cells and antibodies. TC7 monkey cells (50), COS-1 cells (21), and Rat2 cells (68) were grown in Dulbecco modified Eagle medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 8% newborn calf serum (Boehringer Mannheim Biochemicals, Indianapolis, Ind., or Biochrom, Berlin, Federal Republic of Germany) and antibiotics. Monoclonal immunoglobulin G antibodies Pab 416, Pab 419 (25), and Pab 108 (24) were purified as previously described (15).

DNA transfection. (i) DEAE-dextran method. One method of transfection used was that of McCutchan and Pagano (39). Mutant or wild-type plasmid DNA was excised from the pSDL13 vector by *Bam*HI digestion. Input DNA was visualized by ethidium bromide staining after agarose gel electrophoresis to ensure that equal amounts of each DNA were used. TC7 or COS-1 monkey cells (5×10^5 in 100-mm dishes) were washed with Tris-saline and exposed to 1 ml of DEAE-dextran (500 μg/ml) in Tris-saline containing the digested DNA (1 μg). After 1 h at 37°C, the dishes were washed thoroughly with Tris-saline, and fresh medium was added.

(ii) Calcium phosphate method. Another method of transfection used was that of Graham and van der Eb (22). Plasmid DNA was cleaved with *Eco*RI to release the SV40 early region from pSDL13. Rat2 cells (5×10^5 in 60-mm dishes) were fed 2 h before transfection. Medium was aspirated, and 0.5 ml of a calcium phosphate suspension containing 5 μg of plasmid DNA was added to the cells. After 30 min at 37°C, 5 ml of medium was added. After 4 h at 37°C, the cells were glycerol shocked, washed thoroughly with Tris-saline, and fed with fresh medium.

TABLE 1. Oligonucleotide-directed mutagenesis of the SV40 early region

Mutant[a]	Substitution (wild type:mutant)	New restriction site/nucleotide no.[b]	Mutant DNA sequences[c]
SV106	Ser:Ala	*Hha*I/4502	CTGTTTTGCgCAGAAGAA
SV111/112	Ser/Ser:Ala/Cys	*Alu*I/4486	AATGCCAgCTtGTGATGATG
SV120	Ser:Ala	*Hha*I/4460	CTGCTGACgCgCAACATTC
SV123-1	Ser:Ala		TCTCAACATgCTACTCCTCCA
SV123-2	Ser:Ala		TCAACATgCTACTCCTC
SV124	Thr:Ala		CTCAACATTCcgCTCCTCC
SV124E	Thr:Glu		CTCAACATTCggaaCCTCCAAAAAAG
SV639	Ser:Ala	*Hha*I/2903	GATGAAGACgcgCAGGAAAAT
SV676	Ser:Ala	*Alu*I/2791	CCCCTCAagCtTCACAGTCT
SV677	Ser:Ala	*Alu*I/2788	CTCAGTCagCtCAGTCTGTTC
SV679	Ser:Ala	*Alu*I/2782	CCTCACAagCTGTTCATGA
SV701	Thr:Ala	*Hha*I/2715	AACCTCCCgCgCCTCC

[a] The name of the mutant indicates the amino acid residue number of T antigen that has been mutated.

[b] In many constructions, silent mutations were created, in addition to the desired amino acid substitution, to facilitate confirmation of the mutation by restriction digestion.

[c] The sequence of the primer used for mutagenesis is shown 5′ to 3′ in the sense of the early mRNA (BBB numbering [67]). The mutant nucleotides are shown in lowercase letters.

Extraction of T antigen, quantitation of T antigen by immune blot, immunoprecipitation, and specific DNA-binding assays were carried out as described previously (16, 27, 42, 51, 71).

RESULTS

Oligonucleotide-directed mutagenesis. A modified mutagenesis procedure based on that of Zoller and Smith (72) was developed for use with the suppressor plasmid vector pSDL13 (35). This vector offers advantages for rapid production and identification of specific point mutations, i.e., its small size and stability, even with large inserts such as the SV40 genome, and the presence of both M13 and ColE1 replication origins. With few exceptions, the colonies identified by hybridization to the mutant primer carried the expected restriction site (Table 1 and Fig. 2) and had the expected sequence in the plasmid DNA (data not shown).

Biological activity of SV40 mutants. (i) Viability of virus. The ability of the SV40 mutants to produce infectious virions was tested by plaque assay after DNA transfection into TC7 monkey cells (Table 2). Of the 11 mutant DNAs tested, 7 produced detectable infectious virus, although SV111/112 and the carboxy-terminal mutants were slightly defective.

To ensure that the induced mutation—and not an inadvertently introduced second mutation elsewhere in the genome—was responsible for the phenotype, a second round of mutagenesis was performed using template DNA from the amino-terminal mutants and a wild-type oligonucleotide as the primer. Revertants were identified by colony hybridization to the wild-type primer. The revertants were then tested for plaque-forming activity and viral DNA replication. As expected, the defective amino-terminal mutants were restored to the wild-type phenotype by this procedure (Table 2, SV120MR, SV123MR, and SV124MR).

(ii) Cell transformation. The ability of the SV40 mutants to transform Rat2 fibroblasts was tested in a focus formation assay. All of the mutants formed foci, although slight differences in frequency of focus formation were observed relative to wild-type DNA in the same experiment. The amino-terminal mutants and SV639, SV676, and SV701 formed foci at about wild-type frequency (Table 2). The other carboxy-terminal mutants SV677 and SV679 transformed Rat2 cells at somewhat less than wild-type frequency (Table 2). The significance of these variations in the frequency of focus formation is not clear, since the activity of wild-type DNA in this assay varied between experiments, as also noted in other transformation studies (30). Nevertheless, all of the mutants tested here retained focus-forming activity in Rat2 cells, and foci obtained with each of the mutants also formed colonies in soft agar. For each mutant, single colonies were picked and expanded to cell lines.

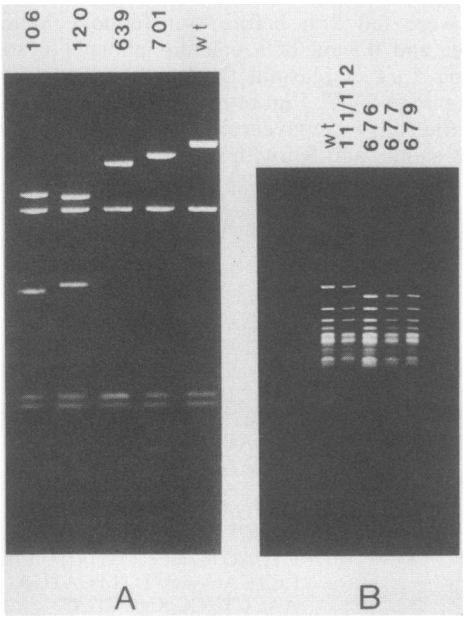

FIG. 2. Restriction endonuclease digestion of putative mutant DNAs identified by colony hybridization. DNA prepared from purified single colonies of the indicated mutants was digested with *Hha*I (A) or *Alu*I (B) (Table 1) under the conditions specified by the manufacturer and analyzed by agarose gel electrophoresis.

TABLE 2. Biological activities of mutant T antigens[a]

Mutant	% Viability[b]	% Transformation activity[c]
SV106	98	105
SV111/112	44	92
SV120	0	88
SV123	0	103
SV124	0	107
SV124E	NT[d]	100
SV120MR[e]	93	NT
SV123MR[e]	96	NT
SV124MR[e]	90	NT
SV639	59	209
SV676	48	177
SV677	67	7
SV679	70	36
SV701	78	94

[a] Viability and transformation activity values were 0% and <0.1%, respectively, in experiments performed with the vector alone and with no DNA.

[b] *Bam*HI-cleaved plasmid DNA (100 ng/60-mm dish) was transfected into TC7 cells in duplicate by using the DEAE-dextran method. Plaques were scored 10 to 12 days later as described previously (1). Results are expressed as a percentage of the wild-type value in the same experiment (53 and 57 PFU/100 ng of DNA).

[c] Transformation assays were performed as previously described (10). Briefly, *Eco*RI-cleaved plasmid DNA was transfected into Rat2 cells by the calcium phosphate technique. Cells were split 1:6 24 h later. Foci were stained with Giemsa and counted after 10, 15, and 20 days. The results are expressed as a percentage of the activity of wild-type DNA in the same experiment (930, 132, 180, and 252 foci per 5 μg of DNA in four different experiments).

[d] NT, Not tested.

[e] A wild-type oligonucleotide MR (5'-CTGCTGACTCTCAACATTCTAC TCCTCAA) was used as a primer to marker rescue the defective mutants SV120, SV123, and SV124 by repeating the oligonucleotide mutagenesis (see text).

(iii) SV40 DNA replication. The ability of the SV40 mutants to replicate their DNA was analyzed after transfection of plasmid DNA into monkey cells. To ensure that equal amounts of DNA were used for transfection, the restricted input DNA was visualized by agarose gel electrophoresis and ethidium bromide staining. Except for SV677, SV679, and SV701, all of the mutants defective in plaque formation were also defective in SV40 DNA replication (Fig. 3A). DNA replication of the marker rescue mutants SV120MR, SV123MR, and SV124MR was not detectably different from that of wild-type SV40 (results not shown). It is interesting that substitution of Thr-124 in T antigen by glutamic acid (SV124E) permitted a low level of replication, whereas substitution by alanine (SV124) eliminated replication activity (Fig. 3B). The mutant SV679 was unusual in its enhanced ability to replicate SV40 DNA (Fig. 3A and C). When parallel cultures of TC7 cells were infected with equal multiplicities of SV40 wild type and SV679, a similar over-

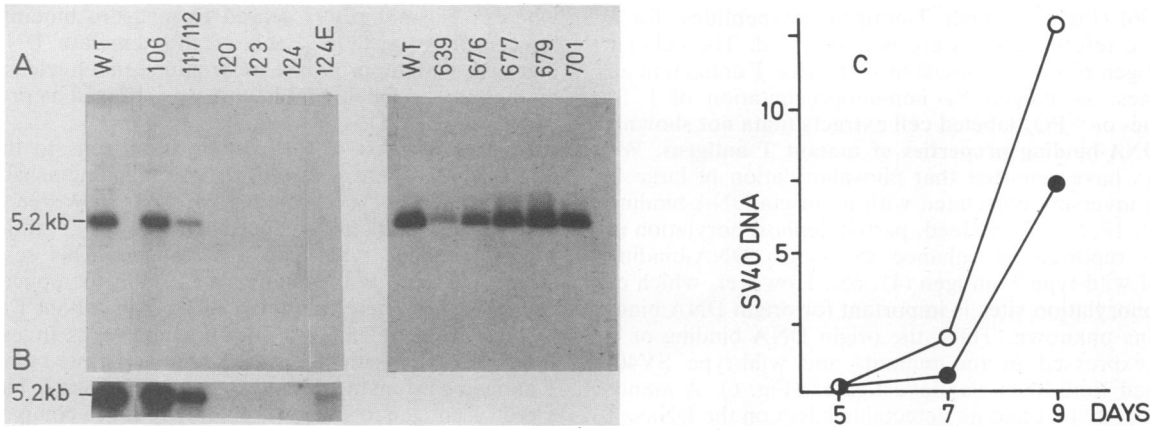

FIG. 3. Replication of mutant SV40 DNA in TC7 cells. *Bam*HI-digested plasmid DNA was transfected into TC7 cells by the DEAE-dextran method. Low-molecular-weight DNA (26) was isolated 9 days later ([A and B] or as indicated on the abcissa [C]), cleaved with *Bam*HI and *Dpn*I, and analyzed by agarose gel electrophoresis, ethidium bromide staining, and Southern blot hybridization to ^{32}P-nick-translated pSVwt DNA (49, 64). *Bam*HI-digested pSDL13-1.3 DNA served as a marker and was visualized by ethidium bromide staining. The position of the 5.2-kilobase SV40 band is indicated. (A) Autoradiography for 30 min. (B) Autoradiography for 5 h. (C) Bands of newly replicated SV40 DNA was quantitated by microdensitometry of the autoradiograms. ●, pSDL13-1.13 wild type; ○, SV679 DNA.

replication of SV679 DNA (two- to fivefold) was observed (B. Schnierle, A. Arthur, and E. Fanning, unpublished data). Marginally enhanced replication was also observed in some experiments with the mutant SV677 (results not shown).

COS-1 cells constitutively express a wild-type T antigen that should complement the mutant T antigens to allow replication of the mutant DNA (21). Thus, replication of mutant and wild-type DNA was tested after transfection into COS-1 cells (Fig. 4). In these cells, the mutant DNAs replicated to about the same level as did the wild-type DNA. The apparently weak replication of SV639 DNA was not observed in other experiments and can be explained by low recovery of the DNA in this experiment, as shown by the weak band of cellular DNA above the 5.2-kilobase SV40 band (Fig. 4).

(iv) T-antigen expression and subcellular localization. Expression of mutant T antigen after transfection of plasmid DNA in monkey cells and in the mutant-transformed rat cell lines was monitored by immunoperoxidase staining by using several different monoclonal antibodies as first antibody. Expression of the mutant T antigens in the nucleus resembled that of the wild-type protein (data not shown).

The T antigens expressed by the mutant SV40-transformed Rat2 lines were analyzed by immune blot (Fig. 5) and by immunoprecipitation of [^{35}S]methionine-labeled proteins (data not shown). Each of the lines expressed large T antigen of wild-type size and various amounts of the 100-kilodalton T

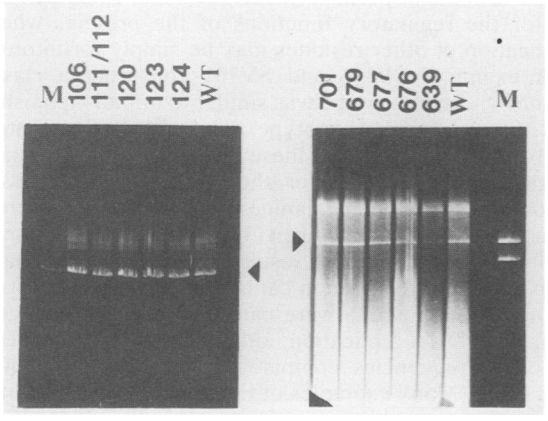

FIG. 4. Replication of mutant SV40 DNA in COS-1 cells. *Bam*HI-digested plasmid DNA was transfected into COS-1 cells by the DEAE-dextran method. Low-molecular-weight DNA (26) was isolated 6 days later, cleaved with *Bam*HI and *Dpn*I, separated by agarose gel electrophoresis, and stained with ethidium bromide. M, *Bam*HI-cleaved pSDL13-1.13 DNA; WT, wild type. Linear SV40 DNA is indicated by the arrowheads.

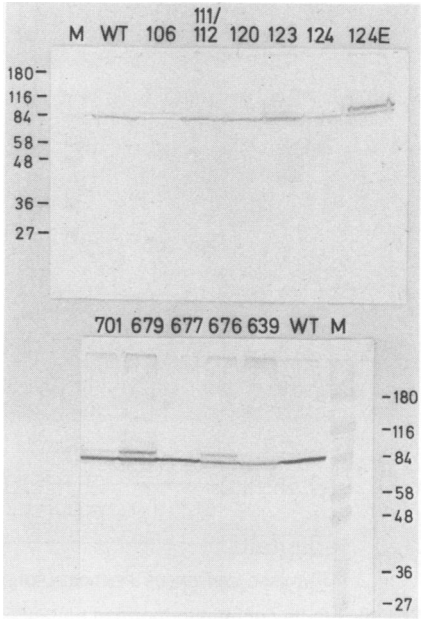

FIG. 5. Expression of T antigen in mutant and wild-type (WT) SV40-transformed Rat2 cells. Extract from about 3 × 10^5 cells of each line was analyzed by electrophoresis on 10% sodium dodecyl sulfate-polyacrylamide gels and immunoblotting (34, 71). T antigen was stained with Pab 416 immunoglobulin G and alkaline phosphatase-conjugated anti-mouse antibody (Promega Biotec, Heidelberg, Federal Republic of Germany). The migration of prestained molecular size markers (M), (Sigma Chemical Co., Munich, Federal Republic of Germany) is indicated. Molecular sizes are shown in kilodaltons.

antigen (36) (Fig. 5). Super T-antigen polypeptides (for a review, see reference 48) were not observed. The cellular tumor antigen p53 was associated with large T antigen in all of the lines, as judged by immunoprecipitation of [^{35}S] methionine- or $^{32}PO_4$-labeled cell extracts (data not shown).

SV40 DNA-binding properties of mutant T antigens. We and others have reported that phosphorylation of large T antigen is inversely correlated with its origin DNA-binding activity (2, 17, 51, 57). Indeed, partial dephosphorylation in vitro was reported to enhance the origin DNA-binding activity of wild-type T antigen (41, 65). However, which of the phosphorylation sites is important for origin DNA binding remains unknown. Thus, the origin DNA binding of T antigens expressed in the mutant- and wild-type SV40-transformed Rat2 lines was investigated (Fig. 6). A monoclonal antibody that had no detectable effect on the DNase I footprint of purified T antigen (54; E. Vakalopoulou and E. Fanning, unpublished data) was used to immunoprecipitate T antigen from cell extracts. The purified immunocomplexed protein was then assayed for DNA binding at equilibrium by using excess SV40 plasmid DNA fragments in the absence of nonspecific competitor DNA. Three templates were used: pSVwt, pONwt, and p1097 DNA (Fig. 6C). Plasmid pONwt served to assay binding to site I independently of site II, whereas plasmid p1097 served to measure binding to the lower affinity site II. Each of the template DNAs was digested with appropriate restriction endonucleases, and binding to end-labeled fragments was assayed as previously described (51, 71).

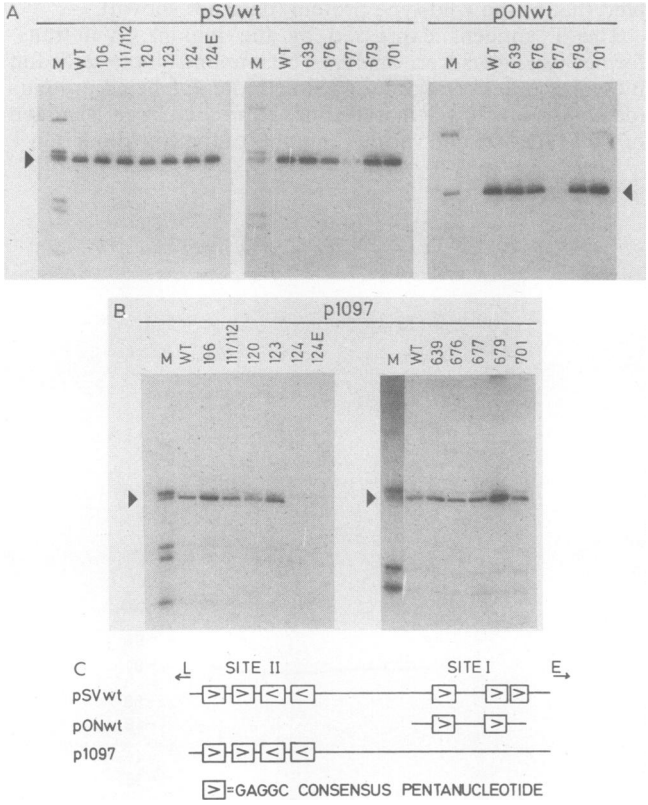

FIG. 6. Specific binding of immunopurified mutant and wild-type (WT) T antigens to SV40 DNA. Assays were performed exactly as described previously (51, 71). Briefly, equal amounts of T antigen (about 100 ng) were purified from extracts of SV40-transformed Rat2 cells by immunoprecipitation with Pab 108 immunoglobulin G and fixed *Staphylococcus aureus* and then assayed for specific binding to excess (250 ng) end-labeled DNA fragments. M, 5% of the input DNA. The origin-containing fragment is indicated by arrowheads. (A) *Hin*dIII-cleaved pSVwt and *Eco*RI-*Sal*I-cleaved pONwt. (B) *Hin*dIII-cleaved p1097 DNA. (C) Diagram of templates for SV40 origin DNA-binding assays.

Binding of most of the mutant T antigens to the SV40 origin DNA fragment of pSVwt was indistinguishable from that of the wild-type protein (Fig. 6A). However, specific binding was markedly reduced with SV677 T antigen and slightly reduced with SV676 T antigen. This result was surprising since both mutants were able to replicate viral DNA (Fig. 3). Therefore, the ability of the mutant T antigens to bind independently to sites I and II was investigated. Binding studies with pONwt DNA demonstrated that SV677 T antigen was unable to bind specifically to site I; binding of SV676 T antigen to site I was slightly reduced compared with binding of the wild-type protein and the other carboxy-terminal mutants (Fig. 6A). The other mutant T antigens bound to site I at wild-type levels (Fig. 6A; data not shown).

Analysis of site II DNA binding of the mutant proteins revealed more extensive heterogeneity (Fig. 6B). SV679 and, less strikingly, SV106 and SV123 T antigens reproducibly bound more site II DNA than did an equal amount of wild-type T antigen. Specific binding of SV124 and SV124E T antigens to site II was not detectable. SV677 T antigen bound specifically to site II DNA at a level comparable with that of wild-type T antigen, accounting for its ability to replicate SV40 DNA at wild-type levels. Binding of the other mutant proteins to site II DNA resembled that of the wild-type protein.

DISCUSSION

Several lines of evidence, reviewed above, suggest that phosphorylation of SV40 large T antigen modulates the multiple regulatory functions of this protein. Since the protein is heterogeneously phosphorylated, the question of the relationship between individual sites of phosphorylation and protein function, especially in vivo, is difficult to address. In this study, we used oligonucleotide-directed mutagenesis to specifically alter each of the previously mapped phosphorylated residues. The phenotypes of this series of mutants imply that the phosphorylation states of certain individual sites, or perhaps subsets of sites, could be important for the regulatory functions of the protein, whereas modification of other residues may be simply fortuitous.

For example, SV106 and SV701 constitute a class of mutants whose phenotype was similar to or indistinguishable from the wild-type phenotype. This result demonstrates clearly that modification of these residues is not essential for T-antigen function. However, the nonconservative substitution of Ser-106 by phenylalanine was reported to generate a replication-defective T antigen (30), suggesting that nonconservative substitutions that result in conformational changes in this region of the protein cannot be tolerated.

Many of the mutants were partially or completely defective in viral DNA replication, although they transformed rat cells with frequencies comparable with that of wild-type SV40 DNA. Many examples of this second class of mutants have been observed in earlier studies (18, 30, 38, 44, 46, 52). One of these mutants, SV120, carries the substitution at residue 120, which is not known to be phosphorylated in the wild-type protein (56). Nevertheless, this conservative substitution created a replication-defective protein, again suggesting that this region of the protein is particularly sensitive to conformation changes. Similarly, although changes in

phosphorylation of the mutant residues in the SV111/112, SV123, SV124, SV639, and SV676 T antigens may be involved in generating the mutant phenotypes, the present results do not rule out other explanations for the mutant phenotypes. Other amino acid substitutions at these sites could conceivably permit normal T-antigen function despite the inability of the amino acids to be phosphorylated. For example, substitution of glutamic acid for Thr-124 did not totally abolish replication activity, suggesting that in this case, a negative change may be more important than phosphorylation per se. Exhaustive mutagenesis of each site will be required to address this question.

Initial biochemical analysis of this second class of mutants to discover the nature of their replication defects revealed only that diminished binding of SV124 and SV124E T antigens to site II DNA may be responsible for their replication deficiency. Preliminary results indicate that all of the mutant T antigens have ATPase activity (A. Schmid, J. Schneider and E. Fanning, unpublished data); thus, further analysis of this class of mutant proteins will be required to identify their defects.

Mutants SV677 and SV679 provide more insight into the potential regulatory importance of T antigen phosphorylation. The mutations in SV677 and SV679 are localized in a region of the genome that is not essential for viral DNA replication (Fig. 1) (9, 45), but SV677 and SV679 replicated somewhat more efficiently than did wild-type SV40. A double mutant carrying both substitutions also replicated better than did the wild type (B. Schnierle, A. Arthur, E. Fanning, unpublished data). The origin DNA-binding domain of T antigen is localized between residues 131 and 259 and appears to be sufficient for site I as well as site II binding (Fig. 1) (43, 63; A. Arthur, A. Höss, and E. Fanning, J. Virol, in press; D. Lane, personal communication; Y. Gluzman, personal communication). This domain thus does not include the phosphorylated sites. Indeed, T antigen expressed in bacteria and lacking detectable phosphorylation binds to both sites (Arthur et al., in press). Yet mutation of the phosphorylation sites at residues 677 and, to some extent, 106, 123, and 124 had remarkably diverse effects on the origin DNA-binding properties of T antigen, altering its specificity for one site or the other.

These two binding sites play different roles in the control of the viral life cycle by T antigen. T-antigen-binding site II is essential for viral DNA replication, whereas site I is involved primarily in autoregulation of early transcription (12). Both sites harbor the same pentanucleotide binding signals, albeit in different arrangements (Fig. 6C). Each pentanucleotide directs the binding of a monomer mass of T antigen, and T antigen appears to interact with individual nucleotides in sites I and II in much the same fashion, so that it remains to be determined how the protein recognizes two different sites (29, 54). Since subunit interactions between protein molecules bound to the pentanucleotides appear to be essential for efficient binding (53, 54), we suggest that the phosphorylation status of T antigen determines a minimum of two basic types of protein-protein interactions between subunits on the DNA. These effects must be mediated indirectly through induction of subtle conformational changes in the origin DNA-binding domain of the protein. Indeed, heterogeneous phosphorylation of T antigen may define different functional subclasses of protein. For example, a T antigen unable to bind site I might be expected to be defective in autoregulation of early transcription (12); in fact, SV677 T antigen appears to be overproduced (B. Schnierle, A. Arthur, E. Fanning, unpublished data). Phosphorylation of residue 677 may thus define a subclass of T antigen active in autoregulation. Conversely, phosphorylation of Ser-679 and, to a lesser extent, Ser-106 and Ser-123 may downregulate site II DNA binding and, hence, viral DNA replication. A subclass of molecules active in replication may thus be distinguished by lack of modification at these sites, coupled with phosphorylation of Thr-124. Such a delicately balanced and rapidly adjustable control mechanism could explain how this single protein fulfills multiple regulatory functions. This interpretation is further supported by phosphopeptide mapping of wild-type T antigen of high and low specific activities of DNA binding (57) and by phosphopeptide mapping of our mutant T antigens (K.-H. Scheidtmann et al., manuscript in preparation).

Consistent with this proposal, alkaline phosphatase-treated T antigen is more active than untreated T antigen in binding to site II DNA and in replication of SV40 DNA in a cell-free system (41, 65). Although dephosphorylation of residues 106, 123, and 679 was not directly demonstrated, alkaline phosphatase has been shown previously to hydrolyze phosphoserine but not phosphothreonine residues of T antigen (59). It will be interesting to see whether SV679 T antigen also shows enhanced replication activity in vitro.

In summary, a series of mutants carrying conservative amino acid substitutions in the phosphorylated domains of SV40 T antigen displays a diverse set of properties ranging from wild-type to novel phenotypes not observed previously. The results suggest how differential phosphorylation of T antigen could modulate its biological and biochemical activities.

ACKNOWLEDGMENTS

We thank Ronald Mertz and Ernst Winnacker, Genzentrum, for oligonucleotides; Ed Harlow and Wolfgang Deppert for hybridoma cells; Martin Lipp for Rat2 cells; Brian Seed for making the pSDL13 system available to us; Cornelia Fischer for construction of pSDL13-1.13; Bärbel Schnierle and Avril Arthur for help with the reconstructions and criticism of the manuscript; Peter Heinrich for introducing us to supercoil DNA sequencing; Silke Dehde, Birgit Posch, and Andrea Schmid for excellent technical assistance; and Sabine Domrath and Maria Ihmsen for help with the manuscript.

The financial support for the Bundesministerium für Forschung und Technologie, Genzentrum, and Fonds der Chemischen Industrie is gratefully acknowledged.

LITERATURE CITED

1. **Baumgartner, I., C. Kuhn, and E. Fanning.** 1979. Identification and characterization of fast-sedimenting SV40 nucleoprotein complexes. Virology **96**:54–63.
2. **Burger, C., and E. Fanning.** 1983. Specific DNA binding activity of T antigen subclasses varies among different SV40-transformed cell lines. Virology **126**:19–31.
3. **Burke, B., and L. Gerace.** 1986. A cell free system to study reassembly of the nuclear envelope at the end of mitosis. Cell **44**:639–652.
4. **Carmichael, G., B. S. Schaffhausen, G. Mandel, T. J. Liang, and T. L. Benjamin.** 1984. Transformation by polyoma virus is drastically reduced by substitution of phenylalanine for tyrosine at residue 315 of middle-sized tumor antigen. Proc. Natl. Acad. Sci. USA **81**:679–683.
5. **Cartwright, C. A., W. Eckhardt, S. Simon, and P. Kaplan.** 1987. Cell transformation of pp60$^{c\text{-}src}$ mutated in the carboxy-terminal regulatory domain. Cell **49**:83–91.
6. **Chen, E. J., and P. H. Seeburg.** 1985. Supercoil sequencing: a fast and simple method for sequencing plasmid DNA. DNA **4**:165–170.
7. **Clark, R., K. Peden, J. M. Pipas, D. Nathans, and R. Tjian.** 1983. Biochemical activities of T-antigen proteins encoded by simian virus 40 *A* gene deletion mutants. Mol. Cell. Biol. **3**:

220–228.
8. **Cohen, P.** 1982. The role of protein phosphorylation in neural and hormonal control of cellular activity. Nature (London) **296:** 613–620.
9. **Cole, C. N., J. Tornow, R. Clark, and R. Tjian.** 1986. Properties of the simian virus 40 (SV40) large T antigens encoded by SV40 mutants with deletions in gene *A*. J. Virol. **57:**539–546.
10. **Crowe, R., H. Ozer, and D. Rifkin.** 1978. Experiments with normal and transformed cells: a laboratory manual for working with cells in culture. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
11. **Dalbadie-McFarland, G. L., L. W. Cohen, A. D. Riggs, C. Marin, K. Itakura, and J. H. Richards.** 1982. Oligonucleotide-directed mutagenesis as a general and powerful method for studies of protein function. Proc. Natl. Acad. Sci. USA **79:** 6409–6413.
12. **DiMaio, D., and D. Nathans.** 1982. Regulatory mutants of simian virus 40. Effect of mutations at a T antigen binding site on DNA replication and expression of viral genes. J. Mol. Biol. **156:**531–548.
13. **Dörper, T., and E. L. Winnacker.** 1983. Improvements in the phosphoamidite procedure for the synthesis of oligodeoxy ribonucleotides. Nucleic Acids Res. **11:**2575–2584.
14. **Edwards, C. A. F., G. Khoury, and R. G. Martin.** 1979. Phosphorylation of T-antigen and control of T-antigen expression in cells transformed by wild-type and *tsA* mutants of simian virus 40. J. Virol. **29:**753–762.
15. **Ey, P. L., S. J. Prowse, and C. R. Jenkin.** 1978. Isolation of pure IgG 1, IgG 2a, IgG 2b immunoglobulins from mouse serum using protein A-Sepharose. Immunochemistry **15:**429–436.
16. **Fanning, E., B. Nowak, and C. Burger.** 1981. Detection and characterization of multiple forms of simian virus 40 large T antigen. J. Virol. **37:**92–102.
17. **Fanning, E., K.-H. Westphal, D. Brauer, and D. Cörlin.** 1982. Subclasses of simian virus 40 large T antigen: differential binding of two subclasses of T antigen from productively infected cells to viral and cellular DNA. EMBO J. **1:**1023–1028.
18. **Farber, J. M., K. W. C. Peden, and D. Nathans.** 1987. *trans*-dominant defective mutants of simian virus 40 T antigen. J. Virol. **61:**436–445.
19. **Gallis, B., A. Lewis, J. Wignall, A. Alpert, D. Y. Mochizuki, D. Cosman, T. Hopp, and D. Urdal.** 1986. Phosphorylation of the human interleukin-2 receptor and a synthetic peptide identical to its C-terminal, cytoplasmic domain. J. Biol. Chem. **261:**5075–5080.
20. **Gergen, J. P., R. H. Stern, and P. C. Wensink.** 1979. Filter replicas and permanent collections of recombinant DNA plasmids. Nucleic Acids Res. **7:**2115–2136.
21. **Gluzman, Y.** 1981. SV40-transformed simian cells support the replication of early SV40 mutants. Cell **23:**175–182.
22. **Graham, F. L., and A. van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52:**456–467.
23. **Greenspan, D. S., and R. B. Carroll.** 1981. Complex of simian virus 40 large tumor antigen and 48,000-dalton host tumor antigen. Proc. Natl. Acad. Sci. USA **78:**105–109.
24. **Gurney, E. G., S. Tamowski, and W. Deppert.** 1986. Antigenic binding sites of monoclonal antibodies specific for simian virus 40 large T antigen. J. Virol. **57:**1168–1172.
25. **Harlow, E., L. V. Crawford, D. C. Pim, and N. M. Williamson.** 1981. Monoclonal antibodies specific for simian virus 40 tumor antigens. J. Virol. **39:**861–869.
26. **Hirt, B.** 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. **26:**365–369.
27. **Huber, B., E. Vakalopoulou, C. Burger, and E. Fanning.** 1985. Identification and biochemical analysis of DNA replication-defective large T antigens from SV40-transformed cells. Virology **146:**188–202.
28. **Hunter, T., and G. A. Cooper.** 1985. Protein-tyrosine kinases. Annu. Rev. Biochem. **54:**897–930.
29. **Jones, K., and R. Tjian.** 1984. Essential contact residues within SV40 large T antigen binding sites I and II identified by alkylation-interference. Cell **36:**155–162.
30. **Kalderon, D., and A. E. Smith.** 1984. In vitro mutagenesis of a putative DNA binding domain of SV40 large-T. Virology **139:** 109–137.
31. **Kalderon, D., W. D. Richardson, A. F. Markham, and A. E. Smith.** 1984. Sequence requirements for nuclear location of simian virus 40 large T antigen. Nature (London) **311:**33–38.
32. **Kmiecik, T. E., and D. Shalloway.** 1987. Activation and suppression of $pp60^{c\text{-}src}$ transforming ability by mutation of its primary sites of tyrosine phosphorylation. Cell **49:**65–73.
33. **Kress, M., M. Resche-Rigon, and J. Feunteun.** 1982. Phosphorylation pattern of large T antigens in mouse cells infected by simian virus 40 wild type of deletion mutants. J. Virol. **43:**761–771.
34. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
35. **Levinson, A., D. Silver, and B. Seed.** 1984. Minimal size plasmids containing an M13 origin for production of single-strand transducing particles. J. Mol. Appl. Genet. **2:**507–517.
36. **Levitt, A., S. Chen, G. Blanck, D. George, and R. E. Pollack.** 1985. Two integrated partial repeats of simian virus 40 together code for a super-T antigen. Mol. Cell Biol. **5:**742–750.
37. **Luthman, H., and G. Magnusson.** 1983. High efficiency polyoma DNA transfection of chloroquine-treated cells. Nucleic Acids Res. **11:**1295–1308.
38. **Manos, M. M., and Y. Gluzman.** 1985. Genetic and biochemical analysis of transformation-competent, replication-defective simian virus 40 large T antigen mutants. J. Virol. **53:**120–127.
39. **McCutchan, J. H., and J. S. Pagano.** 1968. Enhancement of the infectivity of simian virus 40 deoxyribonucleic acid with diethylaminoethyl-dextran. J. Natl. Cancer Inst. **41:**351–357.
40. **Miyake-Lye, R., and M. W. Kirschner.** 1985. Induction of early mitotic events in a cell-free system. Cell **41:**165–175.
41. **Mohr, I. J., B. Stillman, and Y. Gluzman.** 1987. Regulation of SV40 DNA replication by phosphorylation of T antigen. EMBO J. **6:**153–160.
42. **Montano, X., and D. P. Lane.** 1984. Monoclonal antibody to simian virus 40 small t. J. Virol. **51:**760–767.
43. **Paucha, E., D. Kalderon, R. Harvey, and A. E. Smith.** 1986. Simian virus 40 origin DNA-binding domain on large T antigen. J. Virol. **57:**50–64.
44. **Peden, K. W. C., and J. M. Pipas.** 1985. Site-directed mutagenesis of the SV40 large T antigen gene: replication-defective amino acid substitution mutants that retain the ability to induce morphological transformation. J. Virol. **55:**1–9.
45. **Pipas, J. M.** 1985. Mutations near the carboxyl terminus of the simian virus 40 large tumor antigen alter viral host range. J. Virol. **54:**569–575.
46. **Pipas, J. M., K. W. C. Peden, and D. Nathans.** 1983. Mutational analysis of simian virus 40 T antigen: isolation and characterization of mutants with deletions in the T-antigen gene. Mol. Cell. Biol. **3:**203–213.
47. **Piwnica-Worms, H., K. Saunders, T. M. Roberts, A. E. Smith, and S. H. Cheng.** 1987. Tyrosine phosphorylation regulates the biochemical properties of $pp60^{c\text{-}src}$. Cell **49:**75–82.
48. **Rigby, P., and D. Lane.** 1983. The structure and function of SV40 large T antigen. Adv. Viral Oncol. **3:**31–57.
49. **Rigby, P. W. J., M. Dieckmann, C. Rhodes, and P. Berg.** 1977. Labeling deoxyribonucleic acid to high specific activity in vitro by nick-translation with DNA polymerase I. J. Mol. Biol. **98:** 503–515.
50. **Robb, J., and K. Huebner.** 1973. Effect of cell chromosome number on simian virus 40 replication. Exp. Cell Res. **81:**120–126.
51. **Runzler, R., S. Thompson, and E. Fanning.** 1987. Oligomerization and origin DNA-binding activity of simian virus 40 large T antigen. J. Virol. **61:**2076–2083.
52. **Rutila, J. E., M. J. Imperiale, and W. W. Brockman.** 1986. Replication and transformation functions of in vitro-generated simian virus 40 large T antigen mutants. J. Virol. **58:**526–535.
53. **Ryder, K., S. Silver, A. DeLucia, E. Fanning, and P. Tegtmeyer.** 1986. An altered DNA conformation in origin region I is a determinant for the binding of SV40 large T antigen. Cell **44:**

719–725.
54. **Ryder, K., E. Vakalopoulou, R. Mertz, I. Mastrangelo, P. Hough, P. Tegtmeyer, and E. Fanning.** 1985. Seventeen base pairs of region I encode a novel tripartite binding signal for SV40 T antigen. Cell **42**:539–548.
55. **Sanger, E., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
56. **Scheidtmann, K.-H., B. Echle, and G. Walter.** 1982. Simian virus 40 large T antigen is phosphorylated at multiple sites clustered in two separate regions. J. Virol. **44**:116–133.
57. **Scheidtmann, K. H., M. Hardung, B. Echle, and G. Walter.** 1984. DNA-binding activity of simian virus 40 large T antigen correlates with a distinct phosphorylation state. J. Virol. **50**: 1–12.
58. **Scheidtmann, K. H., J. Schickedanz, G. Walter, R. E. Lanford, and J. S. Butel.** 1984. Differential phosphorylation of cytoplasmic and nuclear variants of simian virus 40 large T antigen encoded by simian virus 40-adenovirus 7 hybrid viruses. J. Virol. **50**:636–640.
59. **Shaw, S. B., and P. Tegtmeyer.** 1981. Binding of dephosphorylated A protein to SV40 DNA. Virology **115**:88–96.
60. **Simanis, V., and P. Nurse.** 1986. The cell cycle control gene *cdc2*$^+$ of fission yeast encodes a protein kinase potentially regulated by phosphorylation. Cell **45**:261–268.
61. **Simmons, D.** 1984. Stepwise phosphorylation of the NH_2-terminal region of the simian virus 40 large T antigen. J. Biol. Chem. **259**:8633–8640.
62. **Simmons, D. T., W. Chou, and K. Rodgers.** 1986. Phosphorylation downregulates the DNA-binding activity of simian virus 40 T antigen. J. Virol. **60**:888–894.
63. **Simmons, D. T.** 1986. DNA-binding region of the simian virus 40 tumor antigen. J. Virol. **57**:776–785.
64. **Southern, E.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**: 503–515.
65. **Stillman, B., R. D. Gerard, R. Guggenheimer, and Y. Gluzman.** 1985. T antigen and template requirements for SV40 DNA replication *in vitro*. EMBO J. **4**:2933–2939.
66. **Tegtmeyer, P., K. Rundell, and J. K. Collins.** 1977. Modification of simian virus 40 protein A. J. Virol. **21**:647–657.
67. **Tooze, J.** 1981. Molecular biology of tumor virus, part 2. DNA tumor viruses, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
68. **Topp, W. C.** 1981. Normal rat cell lines deficient in nuclear thymidine kinase. Virology **113**:408–411.
69. **Van Roy, F., L. Fransen, and W. Fiers.** 1983. Improved localization of phosphorylation sites in simian virus 40 large T antigen. J. Virol. **45**:315–331.
70. **Van Roy, F., L. Fransen, and W. Fiers.** 1983. Metabolic turnover of phosphorylation sites in simian virus 40 large T antigen. J. Virol. **45**:442–446.
71. **Vogt, B., E. Vakalopoulou, and E. Fanning.** 1986. Allosteric control of simian virus 40 T-antigen binding to viral origin DNA. J. Virol. **58**:765–772.
72. **Zoller, M. J., and M. Smith.** 1982. Oligonucleotide-directed mutagenesis using M13-derived vectors: an efficient and general procedure for the production of point mutations in any fragment of DNA. Nucleic Acids Res. **10**:6487–6500.