Analysis of a Large-T-Antigen Variant Expressed in Simian Virus 40-Transformed Mouse Cell Line mKS-A

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Earlier reports had suggested that the large T antigen expressed in simian virus 40 (SV40)-transformed mKS-A cells may be replication defective. Our experiments support these earlier observations showing that the mKS-A T antigen has a reduced DNA-unwinding activity *in vitro*. To investigate the molecular basis for this defect, we have isolated from an mKS-A genomic library an EMBL-3 bacteriophage clone carrying in its insert a full-length SV40 DNA element that most likely encodes the expressed T-antigen variant. DNA sequencing revealed only one nonconservative amino acid exchange, Asp to Asn at residue 636. Surprisingly, when a plasmid clone carrying the mKS-A T-antigen-coding sequence was transfected into monkey cells, we found that it replicated quite efficiently, probably suggesting that a high nuclear concentration of the variant T-antigen form compensates for the partial biochemical defect. However, a high nuclear concentration of T antigen was also found in mKS-A T-antigen-transformed mouse cells, yet a fusion of these cells to permissive monkey cells failed to induce *in situ* replication and excision of integrated SV40 DNA. We discuss possible reasons for the different behavior of T antigen in monkey cells and in mouse cells and suggest that one possibility for the replication-negative phenotype in transformed cells may be related to the fact that T antigen forms a tight complex with the cellular p53 protein in mouse cells but not in monkey cells.

Large T antigen, a phosphoprotein of 708 amino acids, performs several essential functions in simian virus 40 (SV40)-infected permissive monkey cells. A reasonably well understood biochemical function is associated with the role of T antigen as an initiator of viral DNA replication (reviewed in references 33, 44, 54, and 59). T antigen binds specifically to the SV40 origin of replication, where it locally unwinds duplex DNA at the expense of ATP. This is the first step establishing replication forks (8, 55, 61).

T antigen is also necessary for the establishment and, at least in some cases, for the maintenance of the transformed phenotype of SV40-infected nonpermissive rodent cells (59). The replication functions are probably not required for the (unknown) role that T antigen plays in transformed cells. In fact, T-antigen mutants have been described that are able to transform established cells to tumorigenicity and yet possess either impaired specific DNA-binding or impaired ATPase activity (6, 7, 19, 21, 35, 43, 44). (The ATPase activity is most likely a part of the active center of the T-antigen DNA-unwinding domain.) Moreover, replication-defective mutants of T antigen frequently arise spontaneously in SV40-transformed cell lines (for reviews, see references 36 and 44), either because replication-deficient T antigens are selected for during the establishment of the transformed phenotype or because replication functions are not required for the maintenance of the transformed state and mutations in the replication domains of T antigen accumulate in the absence of a selection pressure.

An example to illustrate this point seemed to be the stably SV40-transformed mouse cell line mKS-A. Published biochemical experiments suggested that the T antigen expressed in mKS-A cells seemed to possess reduced ATPase activity (27) and reduced DNA-unwinding activity (52). A defect of the replicative activity of mKS-A T antigen was also proposed because a fusion of mKS-A cells to permissive

TC7 monkey cells does not induce *in situ* replication and mobilization of integrated SV40 DNA, whereas a fusion of mKS-A cells to monkey COS-1 cells (which express a wild-type T antigen) (20) leads to the excision and extrachromosomal replication of integrated SV40 DNA elements (27; S. Dora, C. Schwarz, and R. Knippers, *J. Mol. Biol.*, in press).

Since we are studying the biochemistry of the T-antigen-catalyzed DNA-unwinding reaction, we found it interesting to investigate the genetic basis of this putative T-antigen mutant. For this purpose, we have started an investigation of the SV40 elements integrated in the genome of mKS-A cells (Dora et al., in press). We also assayed some relevant biochemical and biological properties of the T antigen expressed in these cells.

MATERIALS AND METHODS

Cells. The SV40-transformed cell line mKS-A, originally isolated and described by Dubbs et al. (11), was obtained through W. Deppert. We have been cultivating this cell line in our laboratory since 1982 and could not detect changes in its phenotype, the amount of T antigen expressed, and the pattern of integrated SV40 DNA as determined by Southern blotting (unpublished observations; Dora et al., in press). COS-1 cells are from T. Gluzman, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (20). These cell lines and the permissive African green monkey kidney cell line TC7 (a subline of CV1 cells) were cultivated as described previously (53). Cell fusion was performed essentially as described by Botchan et al. (2). Excised and mobilized SV40 DNA sequences were determined in Hirt supernatants (26) by Southern blotting (51) and hybridization to nick-translated SV40 DNA (45). For transfection assays, we used the DEAE-dextran method as described elsewhere (37).

Transformation. The transformation capacity of cloned SV40 DNA was determined by using primary mouse fibroblasts and primary mouse kidney epithelial cells. The cells

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were grown on glass slides subdivided into small numbered squares. From 20 to 40 DNA molecules were microinjected per cell (22). T-antigen-positive cell clones were isolated and transferred to soft agar to determine their ability to form anchorage-independent colonies (23).

T antigen. Wild-type T antigen was prepared from SV40-infected TC7 monkey cells, using the immunoaffinity procedure described previously (50, 52). A similar method was used to isolate T antigen from mKS-A cells. It is known that nearly all of the soluble T antigen in mKS-A cells is tightly associated with the cellular p53 protein (4, 13, 27; A. Dorn, Ph.D. dissertation, University of Constance, Constance, Federal Republic of Germany). To isolate this complex, we used an immunoaffinity procedure coupling monoclonal antibody PAb 122 (which reacts with the p53 protein) (25) to tressyl-activated Sepharose 4B, following the instructions given by the manufacturer (Pharmacia). A lysate of proliferating mKS-A cells was prepared in buffer A (100 mM Tris hydrochloride [pH 9.2], 100 mM NaCl, 5 mM KCl, 1 mM CaCl_2 , 0.5 mM MgCl_2 , 0.5% Nonidet P-40, 50 mM leupeptin as a protease inhibitor). The extract from 20 9-cm plates was added to 0.5 ml of Sepharose 4B, carrying a total of 3 mg of bound antibody. The suspension was rolled for 2 h at 0 to 4°C, followed by five wash steps, each with 5 ml of NET buffer (100 mM NaCl, 10 mM Tris hydrochloride [pH 7.5], 1 mM EDTA, 0.05% Nonidet P-40), and three wash steps with 10 mM PIPES (piperazine-*N,N'*-bis(2-ethanesulfonic acid) buffer at pH 7.0. The p53-T-antigen complex was eluted with 2.5 M MgCl_2 in 10% glycerol and was immediately dialyzed against 50 mM Tris hydrochloride (pH 7.0).

Due to the relatively high pH value of the extraction buffer (a precaution against proteolytic degradation) (58), some p53-T-antigen complexes disintegrated during extraction (Dorn, Ph.D. dissertation). To isolate this p53-free fraction of T antigen, we added the PAb 122-treated cell lysate to Sepharose 4B, carrying monoclonal antibody PAb 101 (which reacts with a C-terminal epitope of T antigen) (10). The bound p53-free T antigen was eluted and processed exactly as described above.

All T-antigen preparations were stored in aliquots in 50% glycerol at -70°C.

For immunoprecipitation experiments, SV40-transformed cells were transferred to methionine-free medium. After 1 h, 0.1 mCi of [^{35}S]methionine was added per 10 ml of medium, and incubation was continued for 4 h. The cells were then lysed as described above to prepare a supernatant (10,000 $\times$ g) which was incubated with monoclonal antibody PAb 101 or PAb 122. The resulting immunocomplexes were precipitated by using inactivated *Staphylococcus aureus* as described previously (4, 13, 58). The precipitates were analyzed by polyacrylamide gel electrophoresis (30) and autoradiography.

DNA. SV40 DNA was prepared from lytically infected monkey TC7 cells by the procedure of Hirt (26) and was purified after pronase and RNase A treatment by two cycles of CsCl-ethidium bromide centrifugation. The 143-base-pair (bp) origin fragment included the SV40 DNA sequences between nucleotides 5171 (a *Hind*III restriction site) and 41 (an *Nco*I site) bracketed on both sides by plasmid sequences (1). The 1,118-bp origin fragment is the *Hind*III-C fragment from SV40 nucleotides 5171 to 1046. Both fragments were 3' end labeled with [α - ^{32}P]deoxynucleoside triphosphates, using the fill-in reaction of the Klenow DNA polymerase (34).

Assays. The specific DNA binding of T antigen was assayed by the DNase I footprinting procedure of Galas and Schmitz (16) as specified previously (1). ATPase activity was

FIG. 1. Isolated T antigen. Wild-type T antigen (lane 1, 0.5 μg), free mKS-A T antigen (lane 2, 0.2 μg), and the mKS-A T antigen-p53 complex (lane 3, 0.2 μg) were separated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (30) and were stained with Coomassie blue. The marker lane (M) shows the bands of proteins (from top to bottom) of 97,000, 67,000, 43,000, and 30,000 M_r , respectively.

tested as described elsewhere (5). The ATP-dependent DNA-unwinding activity of T antigen was assayed by using ^{32}P -end-labeled double-stranded SV40 origin DNA as a substrate, following the procedure of Stahl et al. (55). This assay measures the conversion of double-stranded to single-stranded DNA as catalyzed by T antigen in the presence of the *Escherichia coli* single-strand-specific DNA-binding protein (Pharmacia).

Cloning procedures. Isolated mKS-A genomic DNA was partially digested with *Sau*3A and was cloned in the EMBL-3 bacteriophage vector (15) according to established procedures (34). ^{32}P -labeled, nick-translated SV40 DNA was used to screen the phage library. A clone, L-SV2.1, carrying three contiguous copies of SV40 DNA (see below), was isolated. The central apparently intact copy was subcloned in the *Bam*HI site of plasmid pAT153 (34) for detailed restriction mapping. The SV40 early coding region was recovered from the plasmid clone and was digested by using various combinations of restriction endonucleases *Hind*III, *Kpn*I, *Bam*HI, *Taq*I, *Hinc*II, and *Pst*I. The individual restriction fragments were cloned in phage vectors M13mp18 and M13mp19 (39) and were sequenced by the chain termination method (47).

RESULTS

Biochemical properties of mKS-A T antigen. We compared wild-type T antigen, prepared from productively infected monkey cells, with two forms of mKS-A T antigen, a free form and a form complexed with the cellular p53 protein (Fig. 1). All three T antigens were prepared by the same procedure, namely, by binding to Sepharose-coupled monoclonal antibodies and elution with 2.5 M MgCl_2 (see Materials and Methods).

We first tested the DNA-binding activities of the three T-antigen forms by using a DNase I footprinting protocol (1, 16). Previous work, using the immunoprecipitation procedure of McKay (38), had shown that mKS-A T antigen bound specifically to a DNA fragment containing the genomic control region of SV40 DNA (27). It remained to be demonstrated, however, whether mKS-A T antigen binds specifically to binding site II in the origin of replication (9). We found that free mKS-A T antigen (results not shown) and the p53-T-antigen complex (Fig. 2) bound to the origin binding site as well as did wild-type T antigen (Fig. 2).

We also tested the ATPase activities of our T-antigen preparations and found no significant difference between wild-type T and mKS-A T, regardless of whether the latter

FIG. 2. Specific DNA binding as assayed by DNase I footprinting. A 1-ng sample of the ^{32}P -labeled 143-bp origin fragment (see Materials and Methods) and 10 ng of unlabeled salmon sperm carrier DNA were incubated with 0.05, 0.15, 0.45, and 0.9 μg of T antigen (lanes 1 to 4, respectively) in the presence of 6 μg of bovine serum albumin. After 30 min at room temperature, the resulting protein-DNA complexes were treated with DNase I as specified elsewhere (1). Protein-free DNA was treated under identical conditions in parallel assays (lanes 0). The resulting nuclease digestion products were separated on a sequencing gel and were visualized by autoradiography. The products of a Maxam-Gilbert A+G reaction (lanes A+G) served as markers. On the right we show the location of T-antigen-binding sites I and II as well as the AT-rich region (A/T) of the SV40 origin (9). We compared the DNA-binding properties of wild-type T antigen (from SV40-infected monkey cells [wt]) with those of the T-antigen-p53 complex (Fig. 1) from mKS-A cells.

was in the free or in the complexed form (Fig. 3). This result was in agreement with earlier observations showing that bound p53 protein does not block the ATPase function of T antigen (57). The discrepancy between our results and those reported by Huber et al. (27), who described a reduced ATPase activity of mKS-A T antigen, was probably due to technical problems, since Huber et al. used T antigen immunoprecipitated from crude extracts for their assays.

Finally, we assayed for the DNA-unwinding function of T antigen by measuring the conversion of double-stranded to single-stranded DNA in the presence of ATP and the *E. coli* single-strand-specific DNA-binding protein (55). In Fig. 4 we show the results of experiments using the 143-bp origin fragment as a substrate (see Materials and Methods). We found that free mKS-A T antigen clearly had a reduced DNA-unwinding activity compared with that of wild-type T antigen. The amount of single-stranded DNA produced during 60 min of incubation was determined by densitometer scanning (data not shown) of autoradiograms like those shown in Fig. 4, demonstrating that free mKS-A T antigen was, on the average, about 1/10 as active as wild-type T in the assay system used. The p53-T-antigen complex was completely inactive in this assay system (Fig. 4).

FIG. 3. ATPase activity. The release of inorganic phosphate from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was measured as described previously (5). We show the results after 30 min at 37°C using the indicated amounts of wild-type T antigen (■), free mKS-A T antigen (□), and p53-T-antigen complexes (○).

Similar data were obtained by using a 1,118-bp origin fragment as a substrate for T-antigen-catalyzed unwinding (data not shown), suggesting again that free mKS-A antigen had a reduced DNA-unwinding activity and that p53-T-antigen complexes were essentially inactive.

We have also tested the T-antigen forms prepared from mKS-A cells in the *in vitro* replication system of Li and Kelly (32) and found that the free, uncomplexed mKS-A T antigen was approximately 5 to 10% as active as wild-type T antigen in inducing SV40 DNA replication in the presence of a HeLa cell extract, whereas p53-T-antigen complexes were completely inactive (results not shown). A similar effect of bound p53 on the *in vitro* replication efficiency of T antigen has been described previously (56).

To investigate the molecular basis for the defects of mKS-A T antigen, we constructed an mKS-A genomic library in the EMBL-3 phage vector and isolated a phage clone, termed L-SV2.1, carrying an integrated SV40 genome which most probably codes for the T antigen expressed in mKS-A cells.

An SV40 early coding region in the mKS-A genome. The insert of phage clone L-SV2.1 was characterized by restriction enzyme mapping and Southern blotting. The insert in clone L-SV2.1 was approximately 15 kbp long and contained

FIG. 4. DNA unwinding. The substrate used was 1 ng of the ^{32}P -labeled 143-bp origin fragment (see Materials and Methods) incubated for 30 min at 37°C under the conditions described by Stahl et al. (55) with wild-type T antigen (lane 3, 0.1 μg ; lane 4, 0.2 μg), with free mKS-A T antigen (lane 5, 0.1 μg ; lane 6, 0.2 μg), or with the mKS-A T-antigen-p53 complex (lane 7, 0.1 μg ; lane 8, 0.2 μg). The substrate incubated under standard conditions in the absence of T antigen (lane 2) and the heat-denatured substrate (lane 1) are shown. We present the results of polyacrylamide gel electrophoresis (55) followed by autoradiography. ss, Single-stranded DNA; ds, double-stranded DNA.

FIG. 5. A segment of the mKS-A genome carrying integrated SV40 DNA sequences. (Top) The insert in the EMBL-3 phage clone L-SV2.1 is approximately 15 kbp long and contains SV40 sequences (▨) flanked by cellular DNA sequences (□). *Hind*III restriction sites (|), *Bgl*II sites in the SV40 origin of replication (▲), and *Bam*HI sites (B) are shown. The central SV40 element (between two *Bam*HI sites) is 5.2 kbp long and contains all the restriction fragments expected for a full-length SV40 DNA. The left SV40 element includes early SV40 DNA sequences between the *Bam*HI site at nucleotide 2533 and a site around nucleotide 1 (the left border between cellular and viral DNA sequences has not been precisely determined). The right SV40 DNA element has been completely sequenced (Dora et al., in press). It contains a late deletion extending from nucleotides 114 to 2113 as indicated. On the right, SV40 DNA is linked to cellular DNA at nucleotide 3765. (Bottom) Plasmid pL-SV5. The L-SV2.1 DNA was restricted by *Bam*HI. The central SV40 element (▨) was cloned in the *Bam*HI site of plasmid pAT153. We show the *Bam*HI (B), *Eco*RI (E), and *Hind*III (single lines) sites in the resulting plasmid, pL-SV5. The position of the SV40 origin of replication (▲) is indicated. The early region is indicated by the internal segment (■, coding region; line, intron) (59).

three tandemly oriented SV40 DNA elements flanked by cellular DNA sequences. The SV40 element on the left (Fig. 5) contained an apparently full-length early coding sequence but lacked a large section of the viral genomic control region, including the promoter-enhancer sequences required for the expression of the T-antigen gene. The SV40 element on the right (Fig. 5) had a large deletion in the late genetic segment of the SV40 genome in addition to a truncated early coding region which was most probably not expressed in mKS-A cells, as no evidence for a truncated T antigen had been obtained in previous immunoprecipitation experiments performed with extracts from mKS-A cells (4, 13, 27; Dorn, Ph.D. dissertation). However, the centrally located SV40 element in the L-SV2.1 insert appeared to be essentially intact, since all the DNA fragments predicted from the SV40 nucleotide sequence (59) could be identified by restriction enzyme analysis.

Elsewhere, we have described in more detail the structure of the SV40 elements integrated in the mKS-A genome (Dora et al., in press). We have detected a minimum of six noncontiguous SV40 integration units separated by cellular DNA sequences of unknown lengths. However, most integrates are composed of truncated or rearranged SV40 DNA elements, and it is quite likely that the L-SV2.1 insert includes the only full-length SV40 DNA element in the mKS-A genome (Dora et al., in press). We assume, therefore, that the central SV40 DNA element in the L-SV2.1 insert encodes the T antigen expressed in mKS-A cells.

The central SV40 element was isolated by *Bam*HI restriction of L-SV2.1 DNA and was subcloned in plasmid vector pAT153. The resulting plasmid clone, pL-SV5, was used for additional restriction mapping which confirmed our conclusion that the central SV40 element in L-SV2.1 corresponded to an apparently full-length SV40 genome.

The SV40 nucleotide sequence in plasmid pL-SV5 be-

tween the *Kpn*I site at SV40 nucleotide 294 and the *Bam*HI site at nucleotide 2533 was determined. This sequence includes the entire early coding region plus relevant upstream elements needed for the expression of the T-antigen gene. The sequence of the upstream promoter-enhancer region differed from the published SV40 DNA sequence (59) at five sites, namely, by deletions of SV40 nucleotides 3, 8, and 82; by a C-T exchange at nucleotide 5209; and by a G-C exchange at nucleotide 44. These mutations appeared not to significantly affect the expression of the SV40 early gene (see below).

We detected a number of nucleotide exchange mutations in the T-antigen-coding region. But, as summarized in Fig. 6, most of these mutations were neutral, causing no amino acid exchanges, or conservative, causing an Ile-Val exchange (codon 623) and a Glu-Asp (codon 637) exchange in the predicted amino acid sequence. We detected only one non-conservative nucleotide exchange, namely, at codon 636, where an Asp codon was replaced by an Asn codon.

Previous studies on C-terminal deletion mutants of T antigen had shown that the shortest T-antigen variant with an active ATPase function ends at amino acid residue 626 (7). The mutations at codons 636 and 637 mapped outside of this region and were therefore not expected to affect the ATPase activity. However, the ATPase is probably only one part of the T-antigen domain needed for the unwinding of duplex DNA. Other parts may be required for the correct interaction of T antigen with the DNA substrate or, possibly, for the DNA-directed movement of T antigen during the helicase unwinding reaction (60).

Replication in vivo. The biochemical assays described above gave ambiguous results: free mKS-A T antigen was partially defective in DNA unwinding, an essential first step during the initiation of viral DNA replication, but p53-T-antigen complexes were found to be completely inactive.

FIG. 6. Early coding region of plasmid pL-SV5. The entire coding region for the SV40 large T antigen is represented by the horizontal parallel lines, with the codon corresponding to the N-terminal amino acid (N) on the left and the codon corresponding to the C-terminal amino acid (C) on the right. Numbers below the horizontal lines indicate the codons that deviate from the published wild-type sequence (59). The sections coding for two functional domains of T antigen (as discussed in reference 54) are indicated above the horizontal lines. aa, Amino acid.

To further investigate the replication potential of mKS-A T antigen, we transfected plasmid pL-SV5 (Fig. 5) into monkey TC7 cells and, as a control, into COS-1 cells. Plasmid pSV, carrying the entire wild-type SV40 genome, was used for comparison. In a transient replication assay, we tested whether *DpnI*-resistant SV40 sequences accumulated in the transfected cells as a function of time after transfection. (*DpnI* restriction is a convenient way to distinguish transfected, unreplicated plasmid DNA which is methylated at *DpnI* restriction sites from replicated DNA which is not methylated at these sites and which is therefore resistant to *DpnI* attack). We found that pL-SV5 DNA replicated almost as well in TC7 as in COS-1 cells and that there was no significant difference between the replication of pL-SV5 and that of the pSV control (Fig. 7).

This result suggests that the T antigen coded for by the early region of the pL-SV5 viral DNA was expressed in sufficiently large amounts to initiate and maintain active viral DNA replication. The partial defect of mKS-A T antigen, as detected in DNA-unwinding assays (Fig. 4), may be compensated for in vivo by a high intranuclear concentration.

The results of the transient in vivo replication assay (Fig. 7) were surprising, since it had been shown before that mKS-A T antigen was unable to induce in situ replication and mobilization of integrated SV40 DNA elements after mKS-A cells were fused to permissive monkey cells. It was proposed that this may indicate a replication deficiency of mKS-A T antigen (see introduction).

We could reproduce these experimental data showing that integrated SV40 DNA was not mobilized in mKS-A-TC7 heterokaryons, whereas a fusion of mKS-A cells to COS-1 cells (expressing a replication-proficient T antigen) (20) resulted in fast and efficient in situ replication and extrachromosomal multiplication of the integrated SV40 DNA (Dora et al., in press).

The inability of mKS-A T antigen to support the mobilization of integrated SV40 DNA is not due to an insufficient amount of T antigen in mKS-A cells, since we were able to extract similar quantities of T antigen from productively infected monkey cells and from mKS-A cells (Fig. 1). However, we cannot exclude the possibility that some cell line-specific property of mKS-A cells prevented the induction of in situ replication. To investigate this point, we transformed primary mouse cells, using plasmid pL-SV5 (Fig. 5) in microinjection experiments.

Transformation. Between 20 and 40 molecules of pL-SV5 DNA were microinjected into the nuclei of primary fibroblasts (22). From 50 microinjected cells, 10 T-antigen-pos-

itive cell clones were isolated. These cell clones had the morphology of fully transformed fibroblasts, forming colonies in soft agar at high frequencies (50 to 70%) (Fig. 8). We also tested the ability of the pL-SV5 DNA to immortalize primary mouse kidney epithelial cells. For this purpose, primary kidney cells were cultivated until they reached confluency. Normally, these cells do not survive a second passage, but this block can be overcome by microinjection of SV40 wild-type DNA (data not shown; for details, see reference 23) as well as of pL-SV5 DNA. In both cases, about 20% of the microinjected cells were converted to transformed T-antigen-positive cells (Fig. 8) which survived many cell passages and efficiently formed colonies in soft agar (Fig. 8).

These experiments show that the mutated mKS-A T antigen transformed primary mouse cells as efficiently as did wild-type T antigen. Furthermore, the immunofluorescence data of Fig. 8 demonstrated that the T antigen encoded by pL-SV5 was actively expressed in transformed epithelial cells (EP-B-SV). This was independently determined by

FIG. 7. Replication in vivo. A 50-ng sample of pSV DNA (containing the wild-type SV40 genome cloned in the *Bam*HI site of plasmid pAT153) and a 50-ng sample of plasmid pL-SV5 (Fig. 5) were transfected into semiconfluent cells (COS-1 and TC7) grown on 9-cm plastic dishes. Hirt supernatants were prepared at 18, 44, and 68 h (lanes 1, 2, and 3, respectively) after transfection. The Hirt DNA was purified and restricted by using endonucleases *Bam*HI and *DpnI*. The digestion products were separated by agarose gel electrophoresis, were blotted onto nitrocellulose filters (51), and were hybridized with ³²P-labeled SV40 DNA.

FIG. 8. Transformation by pL-SV5. (1) Primary kidney cells. (2) Transformed mouse kidney epithelial cells (EP-B-SV cells). (3) Transformed mouse fibroblasts. (4) Immunofluorescence staining, using T-antigen-specific antibodies under the conditions of Graessmann et al. (23). (5) Single mouse fibroblast cells, 14 days after being plated into soft agar. (6) pL-SV5-transformed mouse kidney cells, 14 days after being plated into soft agar.

immunoprecipitation assays using extracts from [35 S]methionine-labeled cells. We found that more than 90% of the labeled T antigen could be precipitated with the p53-specific monoclonal antibody PAb 122 and was therefore present in a tight complex with p53 (Fig. 9).

Finally, we tested whether the SV40 DNA sequences in EP-B-SV cells could be mobilized after fusion to monkey cells. We found a fast and efficient excision and mobilization of SV40 DNA after the fusion of EP-B-SV cells to COS-1

cells but not after fusion to TC7 cells (Fig. 10). This is not due to some defect of the particular monkey cell line used, because the same cell line (TC7) had been used in the experiments of Huber et al. (27), who compared a large number of different SV40-transformed mouse and hamster cell lines. These workers identified a few rodent cell lines in which a mobilization of integrated viral DNA did occur after fusion to TC7 cells, and these transformed cell lines are believed to harbor a replication-proficient T antigen (27).

FIG. 9. T-antigen-p53 complexes in EP-B-SV cells. Equal samples of an extract from [35 S]methionine-labeled cells were kept for 90 min on ice with the T-antigen-specific monoclonal antibody PAb 101 (lane 1) or with the p53-specific antibody PAb 122 (lane 3). The immunocomplexes were precipitated by using inactivated *S. aureus*. The supernatant of the PAb 101 immunoprecipitate (lane 2) and the supernatant of the PAb 122 precipitate (lane 4) were again incubated with antibody PAb 101 to demonstrate the efficiency of the first immunoprecipitation reaction and to assay for the presence of p53-free T antigen. A control experiment using a third sample of the [35 S]methionine-labeled extract treated with mouse nonimmune serum (lane 5) is also shown. The immunoprecipitates were washed with NET buffer (13) and were analyzed by polyacrylamide gel electrophoresis and fluorography. Exposure time was 36 h.

In conclusion, plasmid pL-SV5 encodes a T-antigen variant that was able to support SV40 DNA replication in transfected monkey host cells. However, when the same permissive cells were fused to transformed mouse cells (either mKS-A or EP-B-SV cells), the T-antigen variant failed to induce *in situ* replication and mobilization of the integrated viral DNA.

DISCUSSION

We have confirmed an earlier report (27) showing that the mobilization of SV40 DNA sequences integrated in the

FIG. 10. Mobilization of SV40 DNA sequences in EP-B-SV cells. Hirt supernatants were prepared at 48 h after the fusion of EP-B-SV cells to EP-B-SV cells (control, lane 1), to TC7 monkey cells (lane 2), or to COS-1 cells (lane 3). The Hirt supernatant DNA was separated on 0.8% agarose gels, was transferred to nitrocellulose filters, and was hybridized to 32 P-labeled SV40 DNA. We show an autoradiogram after 5 h of exposure. An additional control includes the fusion of COS-1 cells to COS-1 cells. This control experiment was done many times; the results were always negative.

genome of transformed mKS-A cells requires not only permissive replication factors but also exogenous active T antigen. This conclusion was based on an observation showing that the fusion of mKS-A cells to COS-1 cells, but not to TC-7 cells, induced the excision and extrachromosomal replication of SV40 DNA sequences. It therefore appeared that the T antigen expressed in mKS-A cells was replication defective, like the T antigens of several other SV40-transformed cell lines (4, 19, 24, 27, 36, 44).

We found it interesting to investigate the genetic and biochemical bases for the defect of mKS-A T antigen and isolated from an mKS-A genomic library a phage clone carrying a full-length SV40 genome bracketed on both sides by truncated SV40 DNA copies linked to cellular DNA. We could show that the full-length integrated SV40 DNA element contained functional promoter-enhancer elements which direct the expression of the T-antigen-coding region.

The nucleotide sequence of the coding region revealed several deviations from the known wild-type SV40 DNA sequence. The most interesting difference was a replacement of Asp-Glu $\rightarrow$ Asn-Asp at amino acids 636 and 637. This mutation did not significantly affect the ATPase function of mKS-A T antigen as expected, since a T-antigen deletion mutant ending at amino acid 626 was found to be ATPase positive (7). The ATPase activity is an important part of the domain responsible for the DNA-unwinding function of T antigen. This can be concluded, for example, from the effects of a well-defined monoclonal antibody (PAb 204) (17) which inactivates the ATPase (5, 31) and, at the same time, the DNA-unwinding function (52, 55). Thus, the region of T antigen carrying the ATPase function is certainly a necessary part of the DNA-unwinding domain but probably not sufficient, since the mKS-A T antigen with a normal ATPase activity unwound double-stranded DNA less efficiently than did wild-type T. The DNA-unwinding domain may therefore be larger than the ATPase domain, but this point deserves a more detailed investigation using a set of defined SV40 nucleotide exchange and deletion mutants.

We found that the partially defective T antigen was quite active in initiating and maintaining SV40 DNA replication after the transfection of plasmid pL-SV5 into permissive TC7 cells. It is possible that the defect of the pL-SV5-encoded T antigen was compensated for by a high intranuclear concentration of the protein.

It was surprising to us that the same T antigen, when expressed in SV40-transformed mouse cells, failed to induce the *in situ* replication and mobilization of integrated viral DNA after the fusion of transformed cells with permissive monkey cells. This is not a general property of SV40-transformed mouse cells, since some SV40-transformed cell lines have been identified that mobilize the integrated SV40 DNA when fused to monkey cells (18, 27).

The observed differences in the transfection and cell fusion assays are not caused by different intracellular quantities of T antigen, as the amounts extracted from productively infected monkey cells and from transformed mouse cells were quite similar. Rather, it appears that the T antigen expressed in the transformed mouse cells is qualitatively different from that expressed in productively infected monkey cells.

What could this difference be? An obvious possibility is some kind of posttranslational modification such as phosphorylation, the formation of higher T-antigen aggregates, or the formation of complexes between T antigen and the cellular p53 protein.

It is known that the state of phosphorylation affects the

binding of T antigen to the viral origin of replication and thereby modulates the function of T antigen in initiating DNA replication (29, 40, 48). However, T-antigen preparations extracted from mKS-A cells and from monkey cells were found to bind to the SV40 origin with similar efficiencies and specificities (Fig. 2).

Higher T-antigen oligomers (the 14S to 16S forms) (4, 14) are usually more abundant in transformed cells than in productively infected cells, and higher T-antigen oligomers appear to bind to DNA less efficiently than do monomers or dimers (46 and references therein). We did not investigate the state of oligomerization of our T-antigen preparations, but it is possible that significant amounts of T antigen are present as high oligomers in mKS-A cells and, consequently, possess reduced DNA-binding activity.

However, we find it more likely that the replicative function of T antigen is inhibited in these cells by bound p53 protein. Almost all T antigen in mKS-A cells (4, 13; Dorn, Ph.D. dissertation) as well as in EP-B-SV cells (Fig. 9) is tightly associated with the cellular p53 protein. On the other hand, a very small percentage of T antigen occurs in p53 complexes in productively infected monkey cells (13; Dorn, Ph.D. dissertation). Also, according to our recent findings, bound murine p53 inhibits the replication activity of T antigen by occupying a site required for a functional interaction with a replicative DNA polymerase (3, 17). In addition, the biochemical experiments presented above suggest that mouse p53-T-antigen complexes were unable to efficiently unwind the SV40 origin of replication (see also reference 56). This is perhaps not surprising, since p53 covers the region of T antigen between amino acids 272 and 517 (49) overlapping the ATPase-helicase domain of the protein (Fig. 6). But p53 did not block the ATPase activity of T antigen (Fig. 3), and it must be assumed that p53 somehow interferes with functions of other parts of the helicase domain, for example, with parts involved in the proper alignment or orientation of T antigen on the DNA substrate.

Thus, there may be several reasons for the failure of mKS-A T antigen to induce the replication of integrated SV40 DNA in transformed mouse cell-monkey cell heterokaryons. These include the intrinsic mutational defect, a possible highly aggregated state, and the complex formation with p53 protein. The inhibition of the replication activity of T antigen may be an important function of the p53 protein in SV40-transformed mouse cells (3, 17), but it is most likely not its only function, since the p53 protein is probably an oncogene product in its own right (12, 28, 41, 42).

In this context, it would certainly be interesting to investigate those relatively rare SV40-transformed mouse cell lines that can be stimulated to mobilize SV40 DNA after being fused to monkey cells (18, 27). They most probably express a wild-type antigen, and it is also possible that its interaction with p53 is less tight than that between T antigen and p53 in mKS-A and EP-B-SV cells.

Finally, the enzymatic requirements for the induction of *in situ* DNA replication may be more stringent than those for ring-to-ring replication as in the transient replication assay, and the mKS-A mutant T antigen may be sufficiently active to perform the latter but not the former reaction.

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