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Regulation of gene amplification and expression in cells that constitutively express a temperature sensitive SV40 T-antigen

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ABSTRACT

Simian cells have been transformed with SV40 origin-defective recombinant plasmids containing the tsA209 T-antigen gene. These plasmids contain deletions of either 5 or 52 nucleotides that include the BglI site at the SV40 ori, are defective for replication in COS-1 cells but retain a functional SV40 early promoter. Two cell lines transformed with these plasmids, U4 and S7, and their respective clonal derivatives E5 and F11, contain the tsA209 T-antigen gene integrated into the cell DNA and express T-antigen as detected by immunoprecipitation and immunofluorescence. These cells behave as ts-COS cells, since they complement in a temperature dependent manner the replication of an SV40 derived recombinant plasmid. When transfected with recombinant plasmids containing the chloramphenicol acetyl transferase (CAT) gene cloned into SV40 replicons, ts-COS cells were able to regulate the induction of the CAT activity by temperature. The ratios of CAT activity observed at permissive versus restrictive temperature were in the range of 20-400. Thus, these ts-COS cells are useful systems for the regulated expression of cloned genes in simian cells.

INTRODUCTION

Defective SV40 viruses and recombinant plasmids containing SV40 DNA segments, have been widely used as amplification and expression vectors in mammalian cells (for review see 1-3). Some SV40 derived expression vectors, such as pSV2 (1) or pBSV9 (4) lack the T-antigen gene and can only be amplified in simian cells that constitutively express SV40 T-antigen (5). The use of COS cells has allowed (i) the expression of a variety of genes in a functional form (6-9), (ii) the study of the mechanisms which control SV40 transcription and replication (10-14) and (iii) the cloning of SV40 early mutants (15).

The availability of COS cells which express temperature sensitive T-antigen, would be of help to further study the regulation of SV40 DNA transcription and replication and to design

mammalian systems able to regulate the amplification and expression of cloned genes. We have recently described a primer-vector system that regulates gene amplification and expression in mammalian cells, by including the tsA209 mutation (16) in the T-antigen gene (Portela et al., submitted for publication). Here we report the isolation and characterization of simian cells that constitutively express this ts T-antigen. They were derived by transformation with origin-defective SV40 recombinant plasmids containing the tsA209 T-antigen gene. These ts-COS cells are able to induce temperature-sensitive replication of SV40 replicons and to regulate the expression of the gene encoding chloramphenicol resistance, cloned in SV40 vectors. When this work was in progress, the construction of COS cells that contained the tsA1609 T-antigen gene was described (17).

MATERIALS AND METHODS

Plasmids and cells

The construction of plasmid pBSV9 has been described (4). Plasmid pSV2Neo (18) was a gift of P. Berg. Plasmid pSV2CAT (19) was obtained from W. Doerfler and plasmid pRGM18, containing tsA209 SV40 DNA cloned into pBR322, was provided by R.G. Martin. CV-1 and VERO cell lines were obtained from the American Type Culture Collection and the COS-1 cell line (5) was a gift of Y. Gluzman. The procedures for cell culture have been described (20). Molecular cloning was performed using *E. coli* MC1061 (21) or HB101 (22).

Enzymes and reagents

The origin of the enzymes and reagents used have been described (4). Hamster anti-T antigen serum was kindly provided by R. Carroll and K.H. Scheidtmann.

Construction and characterization of recombinants

The SV40 ori was mutagenized by treatment of 3 µg of pSV2Neo DNA, partially digested with BglI endonuclease, with 10 u of S1 nuclease for 30 min at 37ºC and filling in with *E. coli* DNA polymerase (Klenow fragment) as described (23). Plasmid DNA was then ligated and used to transform competent MC1061 *E. coli* cells. DNA sequencing was performed by the chemical degradation method (24).

Transfection and transformation

Transfection of monkey cells was performed by the DEAE-dextran procedure (25). After DNA adsorption, cell cultures were treated with 100 µM chloroquine in DMEM medium for 4 h at 37ºC (26), the drug was removed and the cultures were incubated at the indicated temperatures. The extraction of low molecular weight DNA from transfected cells was done as described (27). Cell transformation was performed using the calcium phosphate precipitation technique (28,29). Blot-hybridization of either genomic or episomal DNA was as described (30), using as probes DNA fragments labelled in vitro by elongation of random oligodeoxynucleotide primers (31).

Protein analysis

Cell cultures were labelled with ^{35}S-methionine in DMEM medium containing one tenth the normal methionine concentration and total cell extracts were prepared as described (32). T-antigen was immunoprecipitated with hamster anti-tumor serum using fixed S. aureus for immunoadsorption of antigen-antibody complexes(33). Analysis of immunoprecipitates by electrophoresis on SDS-polyacrylamide gels was as described (34). Indirect immunofluorescence was performed using fluorescein-labelled goat anti hamster serum as described previously (4).

Chloramphenicol acetyl transferase assay

To determine the level of chloramphenicol acetyl transferase (CAT) activity, 2-8 x 10^5 cells were transfected, washed with cold PBS, scraped off the plates and disrupted by sonication in 50 mM Tris.HCl pH 7.8. After removal of cell debris by centrifugation, CAT assay was performed in a reaction mixture containing 100 mM Tris.HCl pH 7.8, 150 µM ^{14}C-acetyl-CoA, 5-20 µg of protein and 200 µM chloramphenicol by incubation for 60 min at 37ºC (35). The appearance of ^{14}C acetyl-chloramphenicol was followed by extraction with toluene and liquid scintillation counting (36). All assays were performed under conditions for which the extent of the reaction was a linear function of protein concentration and time. Protein concentration was determined by the Bradford method (37).

RESULTS

Construction of plasmids containing defective SV40 origin of replication and temperature-sensitive T-antigen gene

The ts T-antigen gene contained in pRGM18 was used to construct plasmid pSEKts2 (Fig. 1). Upon transfection of monkey cells this plasmid is able to replicate at 33.5ºC but not at 40.5ºC (data not shown). The mutagenesis of the SV40 origin of replication was performed on pSV2Neo (18), by partial digestion with BglI endonuclease to generate linear DNA molecules and digestion with S1 nuclease. The plasmids lacking the BglI site located at the SV40 origin of replication were characterized by restriction analysis and by their ability to transform VERO cells to G418 resistance. Two of the clones obtained, clones 9 and 10, showed transformation efficiencies for G418 resistance comparable to that of the parental pSV2Neo plasmid but were unable to replicate when used to transfect COS-1 cells (data not shown). They were used to transfer their SV40 replication origin-promotor region

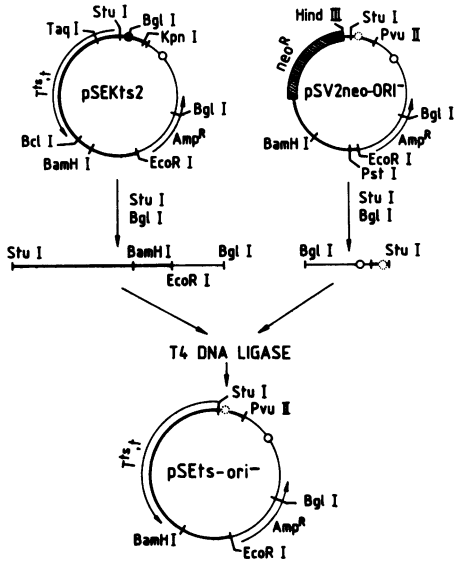

Fig. 1. Construction of plasmids pSEts ori⁻. Mutagenesis of the SV40 ori region of pSV2Neo was as described in the text. The indicated fragments of pSEKts2 and pSV2Neo ori⁻ (clones 9 or 10) were isolated and ligated to generate pSEts ori⁻, clones 9 or 10, as described under Materials and Methods.

to pSEKts2 as shown in Fig. 1, generating plasmids pSEts ori$^-$. The sequences around the SV40 origin of replication of plasmids pSEKts2, pSEts ori$^-$9 and pSEts ori$^-$10 were determined and are shown in Fig. 2. Origin-defective plasmids contain deletions of 5 or 52 nucleotides in length that affect the SV40 origin of replication. The precise location of the deletions is not known due to the redundancies of the SV40 DNA sequence in this region. The smaller deletion maps into the T-antigen binding site II and the larger one abolishes site II and partly site I.

Transformation of monkey cells with pSEts ori$^-$ plasmids

Cultures of CV-1 cells were transformed with either pSEts ori$^-$9 or pSEts ori$^-$10 and incubated at 33.5 or 37ºC. After 2-3 weeks, foci of densely growing cells were apparent in the cultures incubated at both temperatures. Alternatively, VERO cell cultures were transformed with a mixture of pSEts ori$^-$9 and pSV2Neo ori$^-$9 or pSEts ori$^-$10 and pSV2Neo ori$^-$10 and G418 resistant colonies were selected at 40.5ºC to avoid any inhibition of neomycyn phosphotransferase expression by functional T-antigen. Individual foci or colonies were picked, grown into mass cultures and

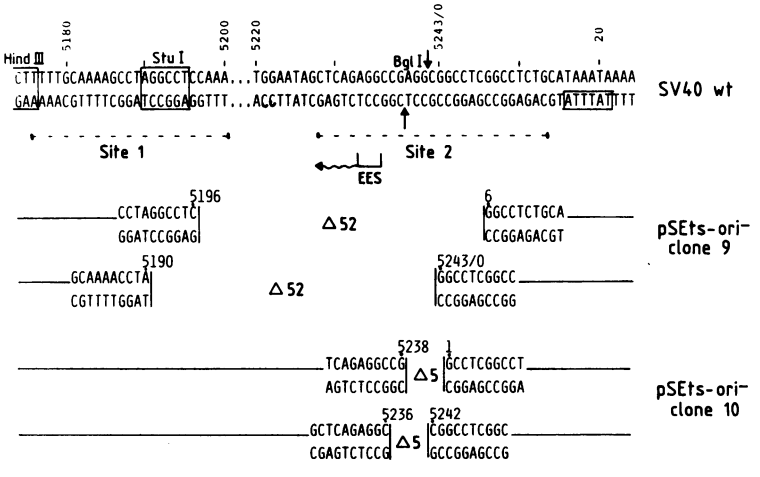

Fig. 2. Characterization of the deletions present in plasmids pSEts ori$^-$9 and 10. Plasmids pSEts ori$^-$9 and 10, as well as pBSV9 (4) were labelled by filling in with α^{32}P dATP the HindIII or NcoI sites near the SV40 origin of replication. Sequence determination was performed by the chemical degradation method (24).

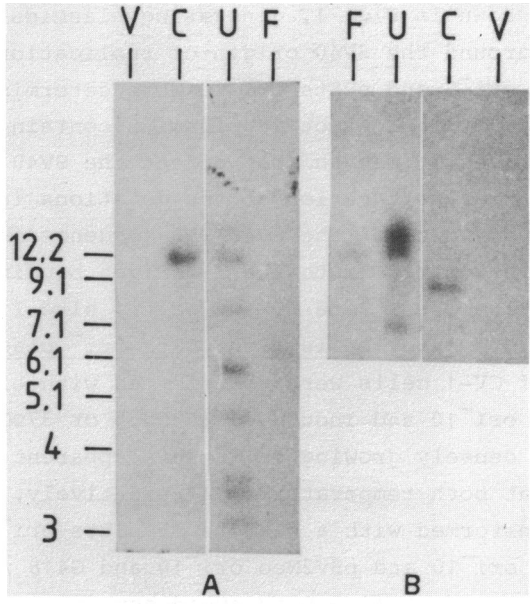

Fig. 3. Southern blot analysis of integrated pSEts ori$^-$ DNA in transformed cells. Total DNA from U4, F11, COS-1 and VERO cells was digested with EcoRI (A) or BglII (B) restriction endonucleases and separated by agarose gel electrophoresis. After DNA transfer to nitrocellulose sheets, hybridization was performed with a T-antigen specific probe. V, VERO cell DNA. C, COS-1 cell DNA. U, U4 cell DNA. F, F11 cell DNA.

tested for expression of T-antigen by immunofluorescence. Out of 22 foci and 16 G418 resistant colonies obtained, 14 foci and 3 colonies showed a strong T-antigen immunofluorescence signal, using COS-1 cells as control. Among them, U4 cells (derived by transformation of CV-1 cells by pSEts ori$^-$9), S7 cells obtained by transformation of VERO cells with pSEts ori$^-$10 and pSV2Neo ori$^-$10 and their respective clonal derivatives E5 and F11, have been characterized in detail. Since their isolation, these cells have been maintained in culture for 9 months without detectable decrease in the level of T-antigen expression.

The structure of the T-antigen gene in these transformed cells has been studied by Southern blot analysis of their DNA. Digestion with EcoRI or BglII restriction endonucleases indicates that U4 cells contain several integration sites, whereas F11 cells show a single hybridization band (Fig. 3). The results ob-

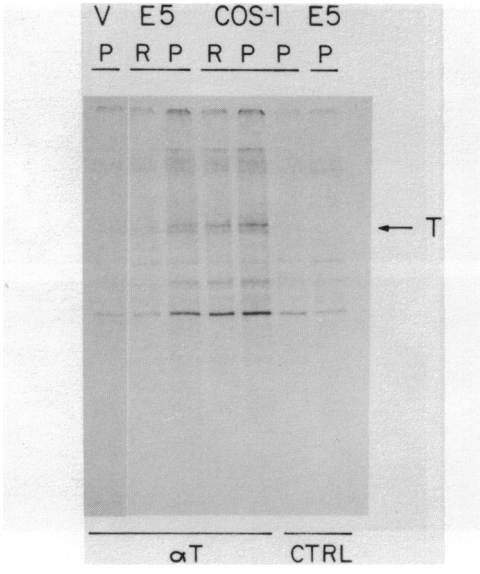

Fig. 4. Synthesis of T-antigen in transformed cells. Cultures of E5, F11, COS-1 and VERO cells were labelled with ^{35}S-methionine in Dulbecco's medium containing one tenth the normal methionine concentration. After labelling, cells were washed with cold PBS, and cell extracts were prepared. Aliquots of labelled extracts were used for immunoprecipitation with anti T-antigen serum and SDS gel electrophoresis as described under Materials and Methods.

tained with E5 cells were identical to those for U4 cells (data not shown).

Expression of SV40 T-antigen gene in transformed cells

The synthesis of T-antigen was studied by in vivo labelling with ^{35}S-methionine, immunoprecipitation with hamster anti-T serum and SDS-polyacrylamide gel electrophoresis. Figure 4 shows the presence of a 94 Kdalton band in extract of COS-1 cells labelled at 33.5 or 40.5ºC and a minor band of 84 Kdalton which probably represents a proteolytic degradation product of T-antigen (38). The results are similar in E5 cells except for an slight decrease of the T-antigen bands at 40.5ºC. To determine the location of T-antigen in the transformed cells, cultures incubated at either 33.5 or 40.5ºC were fixed and analyzed by indirect immunofluorescence using anti-T serum. The results are shown in Fig. 5 and indicate a similar level of accumulation of T-antigen

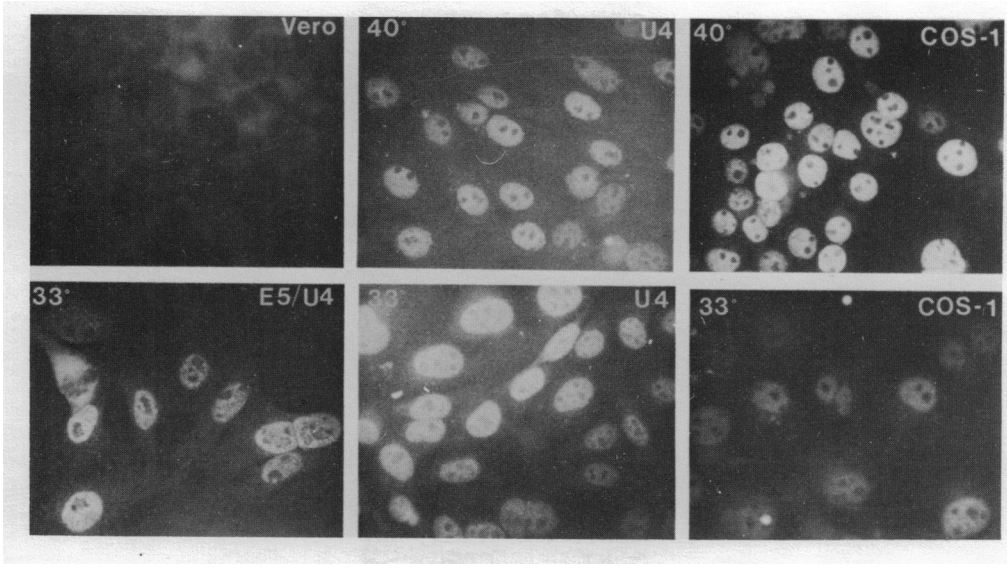

Fig. 5. Immunofluorescence of T-antigen in transformed cells. Cultures of U4, E5, COS-1 and VERO cells were incubated at either 33.5 or 40.5ºC and processed for immunofluorescence as described under Materials and Methods. Immunofluorescence was carried out in parallel and pictures were taken with the same exposure time.

in the nuclei of U4, E5 and COS-1 cells.

Taken together, the results presented indicate the correct expression of tsA209 T-antigen gene integrated into the DNA of U4 and E5 transformed cells. The level of expression is similar to that in COS-1 cells, suggesting the usefulness of these transformed lines as ts-COS cells.

Temperature-sensitive replication of SV40 replicons in transformed cells

To test the functionality of the SV40 tsA209 T-antigen expressed in U4, E5 and F11 cells, they were transfected with pSV2CAT (19) at either 33.5 or 40.5ºC and low molecular weight DNA was isolated. The presence of plasmid DNA newly synthesized in the transfected cells was assayed by digestion with restriction endonuclease MboI which is able to cut DNA replicated in mammalian cells, and hence unmethylated, but not DNA synthesized in bacteria. As shown in Fig. 6, the parental plasmid DNA can be digested with Sau3AI restriction endonuclease, generating among

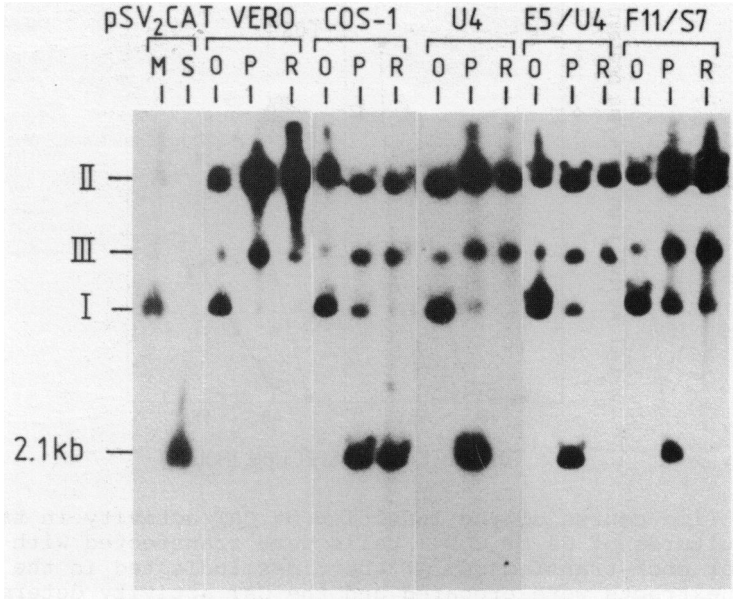

Fig. 6. Temperature dependent replication of pSV2CAT DNA in transformed cells. Cultures of U4, E5, F11, COS-1 and VERO cells were transfected with pSV2CAT DNA and low molecular weight DNA was isolated. DNAs were digested with MboI restriction endonuclea se, separated on an agarose gel and analyzed by blot hybridization using a CAT-specific probe. Purified pSV2CAT DNA was restricted with MboI (M) or Sau3AI (S) and run in parallel with the other experimental samples. O. Low molecular weight DNA isolated immediately after transfection. P. Low molecular weight DNA isolated after 72 h of incubation at 33.5ºC. R. Low molecular weight DNA obtained after 48 h of incubation at 40.5ºC.

others a 2.1 kbp DNA fragment, but is resistant to digestion with MboI restriction endonuclease. The low molecular weight DNA isolated from COS-1 cells incubated at either 33.5 or 40.5ºC is sensitive to MboI digestion, indicating the replication of pSV2CAT at both temperatures. However, the replication of pSV2CAT in U4, E5 or F11 cells took place at 33.5 but not at 40.5ºC, as indicated by the presence of the 2.1 kbp fragment only in the DNA of the cultures incubated at 33.5ºC. This result indicates that cell lines U4, E5 and F11 are able to complement an SV40 replicon in a temperature-dependent fashion and can therefore be considered as ts-COS cells.

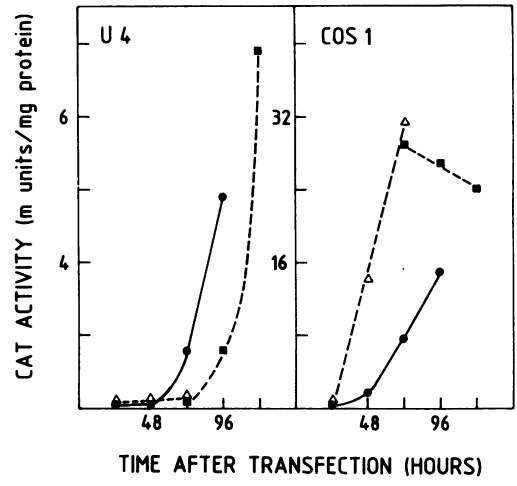

Fig. 7. Time course of the induction of CAT activity in ts-COS cells. Cultures of U4 or COS-1 cells were transfected with pECA plasmid or mock-transfected. At the times indicated in the Figure, cell extracts were prepared and the CAT activity determined as described in Materials and Methods and Table 1. ●——●, cultures incubated at 33.5ºC. Δ- - - -Δ , cultures incubated at 40.5ºC. ■- - - ■, cultures incubated for 48 h at 40.5ºC and then shifted down to 33.5ºC. Background values obtained with mock-transfected cells have been subtracted from all experimental values.

Use of ts-COS cells U4, E5 and F11 for the regulated expression of chloramphenicol acetyl transferase gene

To test the usefulness of ts-COS cells to regulate gene expression, U4, E5, F11, VERO and COS-1 cells were transfected with plasmid pSV2CAT or pECA, a plasmid containing the CAT gene under the HSV tk promoter in vector pBSV9 (4). The time course of the induction of CAT activity in transfected cells at 33.5 or 40.5ºC (Fig. 7) show that CAT activity was induced at either temperature in transfected COS-1 cells, with a faster kinetics at 40.5 than at 33.5ºC. In contrast, the induction of CAT activity was observed in U4 cells only when incubated at 33.5ºC. Furthermore, temperature shift-down of transfected U4 cells after 48 h of incubation at 40.5ºC resulted in the induction of the enzymatic activity with a kinetics similar to that found in cultures incubated at 33.5ºC (Fig. 7). Similar conclussions were reached in several transfection experiments using pSV2CAT or pECA (Table 1). It is noteworthy that the values for CAT activity found in trans-

Table 1. Regulation of chloramphenicol acetyl transferase expression in ts-COS cells.

Cell line	Plasmid							
	pSV2CAT				pECA			
	Experiment 1		Experiment 2		Experiment 3		Experiment 4	
	33.5	40.5	33.5	40.2	33.5	40.5	33.5	40.5
VERO[a]	<36[b]	<42	43	19	<39	<48	121	193
COS-1	2636	2834	6662	2610	15420	11665	14869	6631
U4	950	<36	2274	69	4226	<62	13761	33
E5	1150	<62	4983	128			14063	263
F11	246	52	1013	57	712	<63	3833	

[a]Cultures of the indicated cell lines were transfected with either pSV2CAT or pECA plasmids or mock-transfected, and incubated at either 33.5 or 40.5ºC. Extracts of the transfected cells were prepared at 48 hours post transfection (hpt) (cultures incubated at 40.5ºC), 72 hpt (cultures incubated at 33.5ºC, experiments 1 and 3) or 96 hpt (cultures incubated at 33.5ºC, experiments 2 and 4).

[b]The CAT activity is expressed as µunits per mg of protein (1 unit is defined as 1 µmol of acetyl-chloramphenicol formed per minute, under the conditions described under Materials and Methods). With the specific activity of the 14-C-acetyl-CoA used, one pmol is equivalent to 19 cpm (experiment 3) or 11.5 cpm (all other experiments). The background values obtained with extracts from mock-transfected VERO cells (632-1636 cpm) were subtracted in the corresponding experiments.

fected ts-COS cells incubated at 40.5ºC were in the range obtained in transfected VERO cells and often below the limit of detection. At 33.5ºC, however, the enzymatic activity was comparable to that obtained in COS-1 cells. The ratio of CAT activity at 33.5 versus 40.5ºC was about 20-30 for pSV2CAT and about 50-400 for pECA in the ts-COS cells used, whereas in COS-1 cells ranged between 0,9 and 3 (Table 1).

DISCUSSION

The expression of cloned genes in heterologous cell systems often requires the use of means to accomplish their regulation. In many cases, the overexpression of a extraneous protein from a multicopy plasmid or by the use of a strong promoter, affects the viability of the recipient cell. In mammalian cells, few systems are available for regulated gene expression. Some promoters, like metallothionein or MMTV LTR can be induced by heavy metals or glucocorticoids (39-43). Others, like those of αIFN (44) or βIFN genes (45,46) are inducible by virus infection. As an alternative we have prepared cells lines able to regulate gene amplification by temperature. The experimental approach was to transform simian cells with SV40 derived plasmids lacking the viral

replication origin and containing a temperature-sensitive T-antigen. Among the available ts markers in the T-antigen gene, we chose tsA209 (16) because its gene product appears to be reversible upon temperature shift-down (47) and has proven useful for the regulation of vector pSLts1 amplification (Portela et al., submitted for publication).

The *in vitro* mutagenesis of the SV40 ori was performed on pSV2Neo plasmid, to be able to select every plasmid lacking a *BglI* site by means of the kanamycin resistance afforded by the Neo marker. The level of G418 resistance of the ori$^-$ mutants was used to monitor the functionality of the early SV40 promoter. The size and location of the deletions present in pSV2Neo ori$^-$ clones 9 and 10 are strickingly similar to some reported previously (48) suggesting sites of preferential sensitivity to S1 nuclease within the SV40 ori or toxicity of other possible sequences to *E. coli*.

A large number of transformed foci and G418 resistant colonies was observed after transformation of CV-1 or VERO cells with either of the pSEts ori$^-$ plasmids. Only a fraction of the observed foci or G418-resistant colonies was actually isolated and, among those isolated, many were T-antigen positive. The cell lines derived from the T-antigen positive foci or colonies were difficult to clone, as described for wild type COS cells (5). Two of these cell lines, U4 and S7, have been cloned so far and show uniform staining with anti T-antigen serum by immunofluorescence (Fig. 5).

The expression of T-antigen gene in these transformed cells is comparable to that observed in COS-1 cells (Figs. 4 and 5). In contrast to the results reported earlier (17), the stability of the tsA209 T-antigen did not appear to be a problem for the use of E5 and F1 cells as ts-COS cells. Accumulation of ts-T antigen was similar to its wt counterpart as shown by labelling in a long pulse (Fig. 4) or by indirect immunofluorescence staining (Fig. 5). The reasons for the previous failures to construct ts-COS cells using tsA209 T-antigen gene (cited in 17), are not understood.

The temperature dependent replication of pSV2CAT DNA (Fig. 6) suggested the possible use of the ts-COS cells E5 and F11 as means to regulate the expression of genes cloned into SV40 repli-

cons. The results presented in Fig. 7 and Table 1 clearly indicate that regulation by temperature can be obtained during the transient expression of the CAT gene. Similar results have been obtained with the puromycin acetyl transferase gene and the influenza virus nucleoprotein gene present in plasmid pSVa963 (4) (data not shown). The ratio of enzymatic activity obtained at permissive versus restrictive temperatures reached values up to 400 when the CAT gene was under the tk promoter in plasmid pECA, lacking the pBR322 sequence toxic for DNA replication in mammalian cells (49). This is, to the best of our knowledge, the most efficient regulation system described for mammalian cells. Unfortunately, it is not possible to compare the ts-COS cells described here to those previously described (17). The transformation assay used to test temperature dependent gene expression in ts-COS cells (17) gave results difficult to interpret. Our results using pSV2Neo (data not shown) indicate that a transformation assay is not adequate to test regulation of expression, probably due to the low cloning efficiency of ts-COS cells.

On the other hand, temperature sensitive COS cells could be used to stably integrate at restrictive temperature genes cloned into SV40 replicons and induce their excission and amplification by activating T-antigen in a temperature shift down. Such a T-antigen dependent excission has been described both for polyoma (50) and SV40 virus (51). With this strategy, genes encoding proteins toxic for mammalian cells could be introduced into the chromosomes and their expression induced by a simple temperature shift down.

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