RESEARCH ARTICLE

A Mammalian Host-Vector System That Regulates Expression and Amplification of Transfected Genes by Temperature Induction

Donald C. Rio, Scott G. Clark, Robert Tjian

Abstract. *SV40-transformed simian cells that permit temperature-dependent regulation of vector DNA replication were isolated and characterized. These cell lines (ts COS cells) produce high levels of thermolabile large T antigen under the transcriptional control of the Rous sarcoma virus long terminal repeat. The ts COS cell lines can complement SV40 A gene mutants and support replication of SV40-origin containing vectors at 33°C but not at 40°C. It should now be possible to regulate the copy number of transfected plasmid DNA's and also maintain selectable vector sequences either as integrated DNA or as autonomously replicating episomes by modulating T antigen activity in ts COS cells.*

The transfer of cloned genes back into host cells has been a powerful tool for studying eukaryotic gene expression (*1*). Many eukaryotic host-vector systems have been developed to allow either transient expression or stable integration of cloned genes (*2*). A well-known vector system is based on the use of recombinant bacterial plasmids that can be propagated and expressed in mammalian cells because they carry the origin of DNA replication and transcriptional regulatory elements derived from the genome of a DNA tumor virus, simian virus 40 (SV40) (*3*). These vectors also include the SV40 large T antigen gene, expression of which is required to activate vector replication of the SV40 origin in monkey cells (*3*). The utility of these SV40-derived vectors was greatly improved when permissive cells carrying integrated copies of the viral A (T antigen) gene were isolated (*4*). These so-called COS (*C*V1-*o*rigin-*S*V40) cells express the early viral gene product, large T antigen, and are thus able to support replication of any DNA that contains an intact SV40 origin sequence. The combined use of COS cells and SV40-based vectors has proved valuable for studying different aspects of eukaryotic gene regulation. For instance, this vector system has been used to identify transcriptional regulatory elements in eukaryotic genes (*5*). In addition, the use of COS cells simplified the investigation of T antigen, a multifunctional regulatory protein, known to be involved in viral and cellular replication, transcription, and neoplastic transformation (*6, 7*). Finally, COS cells have permitted the propagation of pure SV40 virus stocks that are defective in early viral functions (*8*).

Despite the convenience and widespread use of COS cells, it was apparent that the flexibility and diversity of the COS cell system could be increased by creating a new COS cell line that could regulate expression of a functional T antigen in an inducible manner, unlike wild-type COS cells, that constitutively express active T antigen. For example, it would be advantageous to have permissive simian cells transformed by SV40 tsA mutants such that temperature shift could control T antigen activity. COS cells expressing a temperature-sensitive T antigen would permit modulation of the degree of replication, transcription, integration, and expression of SV40-based vectors merely by shifting temperature. Such host cells would also facilitate investigation of the regulatory functions of T antigen because experiments could be performed in an isogenic host and allow measurement of the effects of T antigen function by temperature inactivation.

We now report the isolation and characterization of SV40-transformed permissive CV1 cell lines that express a T antigen whose activity can be modulated by temperature shift. Previous attempts to obtain ts COS cells by conventional methods were unsuccessful. We therefore incorporated modifications that allowed us to isolate ts COS cells. First, a temperature-sensitive T antigen expressed by the virus tsA1609 (*9*) that encodes a relatively stable T antigen polypeptide was used (*8*). Second, the tsA1609 T antigen coding region was placed under the transcriptional control of a strong RNA polymerase II promoter that is not subject to repression by T antigen. The ts COS cells that we have isolated should allow development of a mammalian host-vector system that can regulate the copy number of free episomal plasmids simply by temperature shift. In addition, it should also be feasible to mediate the excision of integrated origin-containing plasmid vectors from the host cell genome in a temperature-dependent manner.

Isolation of simian cells expressing temperature-sensitive T antigen. We constructed a plasmid, pRSV-1609 that places the SV40 T antigen (A) gene from mutant tsA1609 (9) under transcriptional control of the promoter in the Rous sarcoma virus (RSV) long terminal repeat (LTR) (*10*). This promoter was chosen because previous attempts to obtain SV40 tsA58 or 209 (*11*) transformed simian cells by means of the SV40 early region promoter yielded cell lines that expressed such low levels of functional T antigen that they were unable to support the replication of SV40 A gene mutants or SV40 origin-containing plasmids (*12*). In order to increase the level of functional T antigen, we needed to increase both its expression and stability. We chose the RSV promoter because it is known to be a more efficient RNA polymerase II promoter in simian cells than the SV40 early promoter (*10*) and it is not subject to transcriptional repression by T antigen. The SV40 mutant tsA1609 (*9*) was chosen rather than the more common tsA58 or 209 mutants (*11*) because this allele encodes a T antigen protein that is functionally very sensitive to temperature variation but is not as rapidly proteolyzed as the tsA58 or 209 T antigens (*8*).

Permissive CV1 monkey cells were transfected with pRSV-1609 by the standard calcium phosphate coprecipitation procedure (*13*) and incubated at the semipermissive temperature of 37°C.

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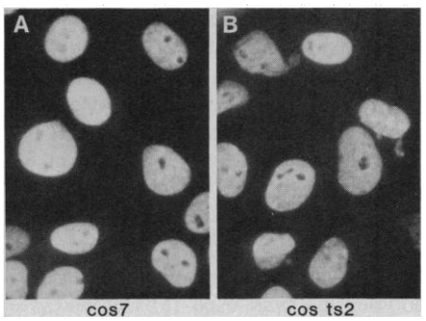

Fig. 1. Comparison of T antigen immunofluorescence in COS ts1 and COS7 cells. T antigen in COS7 cells (A) and in COS ts2 cells (B) was detected by indirect immunofluorescence. Nuclei are seen as bright areas with visible dark nucleoli. Plates of confluent cells were washed with phosphate-buffered saline (PBS), cooled to 0°C, and treated for 3 minutes with a cold (−20°C) mixture of methanol and acetone (1:1) and air-dried. A 50-μl sample of hamster antiserum to SV40-induced tumors (Flow Labs), diluted 1:50 in PBS, was spread onto the cells and incubated at room temperature (22°C) for 15 minutes. The cells were washed with PBS and then similarly treated with a 1:100 dilution of rhodamine-conjugated rabbit antibody to hamster immunoglobulin G (Cappel Labs), washed with PBS, and air-dried. The cells were viewed in a photomicroscope (Nikon UV).

The ability of SV40 T antigen to induce neoplastic transformation and colony formation was exploited to identify cells that express T antigen. Colonies were isolated, propagated individually, and assayed for T antigen expression by indirect immunofluorescence. One population, although heterogeneous, contained a fraction of cells that were strongly positive for T antigen. Our initial attempts to clone the T antigen–positive cells by limiting dilution were unsuccessful. Instead, we used a procedure in which micromanipulation of single colonies was used to obtain homogeneous T antigen–positive cell lines, designated COS ts1, COS ts2, and so on; these were all derived from the same initial isolate. A comparison of the COS ts2 cell line with COS7 cells by indirect immunofluorescence indicated that the nuclei of COS ts2 and COS7 cells contained comparable amounts of T antigen (Fig. 1, A and B) and that they were essentially homogeneous for the expression of T antigen.

Rates of synthesis and steady-state levels of T antigen in ts COS lines. Extracts prepared from COS ts1, COS ts2, and COS7 cells were subjected to immunoprecipitation with a T antigen–specific monoclonal antibody, PAB905 (*8*, *14*). The T antigen–antibody complexes were adsorbed to formalin-fixed *Staphylococcus aureus* cells (*15*), analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and visualized by staining with Coomassie blue (Fig. 2A). Both COS ts2 and COS7 cells express comparable steady-state levels of T antigen when maintained at 33° or 37°C (Fig. 2A; compare lanes 2 and 8 and lanes 3 and 9). However, at 40°C, a lower level of T antigen was observed in COS ts2 cells, whereas the level of T antigen was unchanged in COS7 cells. By contrast, COS ts1 cells show somewhat lower levels of T antigen at all three temperatures (Fig. 2A, lanes 5 to 7). These results are consistent with previous findings that temperature-sensitive T antigens are more susceptible to proteolysis than the wild-type counterpart at nonpermissive temperatures (*8*). More important, at the permissive temperature (33°C), COS ts2 and COS7 cells contain similar amounts of T antigen.

The rate of T antigen synthesis was measured by pulse-labeling cells with [^{35}S]methionine at either 33°, 37°, or 40°C. The cells were then lysed, and equal amounts of radioactive extract were subjected to immunoprecipitation (*8*, *14*). The T antigen–antibody complexes were analyzed by SDS-PAGE and autoradiography. The autoradiogram shows that the amount of labeled methionine incorporated into T antigen during a short pulse is comparable at all three temperatures in COS ts1, COS ts2, and COS7 cells (Fig. 2B, lanes 1 to 3, 4 to 6, and 7 to 9). These findings indicate that the rate of T antigen synthesis is approximately equivalent in these three cell lines. Consequently, the lower steady-state level of T antigen in COS ts2 cells at 40°C and in COS ts1 cells at all three temperatures probably reflects a higher turnover rate (lower stability) for the thermolabile protein.

Structure of the integrated RSV-tsA1609 gene. The organization of the pRSV-1609 DNA incorporated into the genomes of the transformed CV1 cells was deduced by standard genomic DNA blot hybridization analysis (*16*). High molecular weight DNA isolated from COS ts1, COS ts2, COS7, and CV1 cells was cleaved with various restriction endonucleases, subjected to agarose gel electrophoresis, and analyzed by blot hybridization with a labeled DNA fragment isolated from the early region of SV40. COS ts1 and COS ts2 DNA digested with either BGl II or Sac I produced a single DNA fragment that hybridized to SV40 DNA (Fig. 3A). In contrast, wild-type COS7 DNA that was treated with the same enzymes yielded multiple fragments. Because neither of these enzymes cleave within pRSV-1609 or SV40 sequences, this evidence suggests that the COS ts cell lines have SV40 DNA

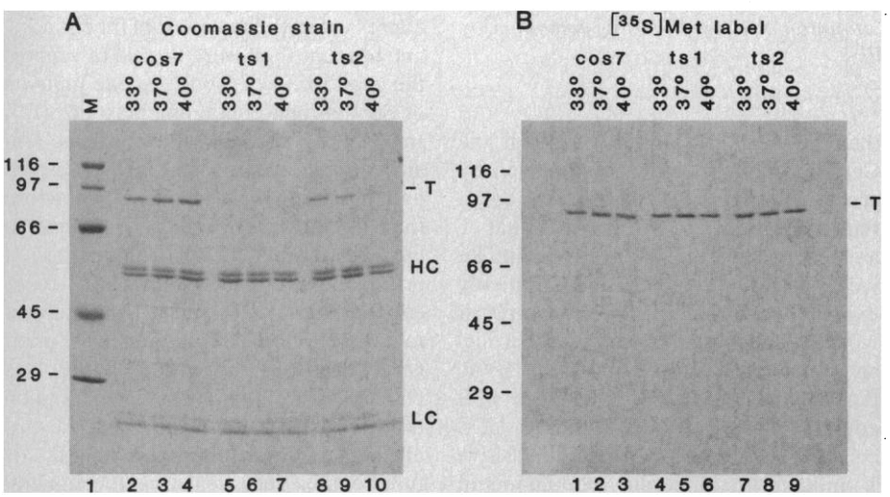

Fig. 2. Quantitation of T antigen in COS ts cells and COS7 cells. (A) T antigen immunoprecipitated from COS ts1, COS ts2 and COS7 cells. (Lane 1) Markers, size as indicated in kilodaltons; (lanes 2 to 4) T antigen immunoprecipitated from COS7 cells maintained at 33°, 37°, and 40°C, respectively; (lanes 5 to 7 and 8 to 10): T antigen immunoprecipitated from COS ts1 and COS ts2 cells maintained at 33°, 37° and 40°C, respectively. T, large T antigen; HC, heavy chain of antibody; LC, light chain of antibody. (B) Autoradiogram of polyacrylamide gel containing [^{35}S]methionine-labeled T antigen from COS ts1, COS ts2, and COS7 cells (3 hours, pulse label with no chase). (Lanes 1 to 3) Labeled T antigen immunoprecipitated from COS7 cells maintained at 33°, 37°, and 40°C, respectively; (lanes 4 to 6 and lanes 7 to 9) labeled T antigen immunoprecipitated from COS ts1 and COS ts2 cells maintained at 33°, 37°, and 40°C, respectively. Extracts (both radioactive and nonradioactive) were prepared and T antigen was immunoprecipitated with monoclonal antibody PAB905 (*14*) as described (*8*). Immunoprecipitates were analyzed by SDS-PAGE electrophoresis in a 7 to 15 percent gradient gel, and T antigen was visualized by Coomassie blue staining or autoradiography (*8*).

inserted into a single site, whereas COS7 cells have SV40 DNA integrated in many different locations.

Further analysis of genomic DNA with the endonuclease Bam HI or a combination of Bam HI and Eco RI is shown in Fig. 3B. (Hpa II was used instead of Eco RI for COS7 DNA because a different plasmid construction was used to transform these cells.) In order to quantify the A gene copy number in the transformed cells, a mixture containing CV1 genomic DNA and one copy per genome equivalent of pRSV-1609 plasmid DNA (that is, in a weight ratio of 1 to 1×10^6) was used as a standard. A single, intact copy of the pRSV-1609 Eco RI–Bam HI fragment, that includes the RSV promoter element and the complete A gene coding sequences, is present in both COS ts1 and COS ts2 genomic DNA (see below and Fig. 3). In contrast, there appear to be multiple copies of the Hpa II–Bam HI SV40 early region fragment integrated in COS7 cells in addition to many insertions of partial cryptic copies. These data, together with additional restriction endonuclease analysis (data not shown), reveal the structure of the pRSV-1609 DNA within the genome of ts COS cells (Fig. 3, bottom) and suggest that COS ts1 and COS ts2 cells have similar if not identical genome structures at this level of analysis. A truncated copy of the A gene is found adjacent to the complete Eco RI–Bam HI insertion in both COS ts1 and COS ts2. This type of cryptic insertion is commonly found in cells transformed by calcium phosphate–DNA precipitates and presumably arises by deletion and rearrangement during integration. Thus, apparently a single copy of the A gene is sufficient to express the high levels of T antigen found in COS ts2 cells. It is not clear why COS ts2 cells maintain a higher steady-state level of T antigen than COS ts1 cells given the similarity of the pRSV-1609 insertions. Perhaps subtle cellular variations, not detectable by blot hybridization, between these two cloned cell lines account for this disparity.

Levels of T antigen messenger RNA expressed in ts COS cells. Polyadenylated cytoplasmic messenger (mRNA) was isolated from COS ts2, COS7, and CV1 cells, treated with glyoxal, subjected to agarose gel electrophoresis, and analyzed by blot hybridization with ^{32}P-labeled SV40 DNA as a probe. An equivalent amount of large T antigen mRNA was found in both COS ts2 and COS7 cells (Fig. 4, lanes 1 and 2). No mRNA that hybridizes to SV40 DNA was detected in CV1 cells (Fig. 4, lane 3). These results indicate that a single copy of the A gene under the control of an RSV promoter element is sufficient to produce an amount of T antigen mRNA equivalent to that found in COS7 cells which contain multiple copies of the SV40 early promoter and T antigen coding sequences. This finding may partly explain the difficulty in obtaining functional ts COS cells with the conventional SV40 early promoter construction.

Temperature-sensitive complementation of SV40 A gene–defective viruses. In order to determine whether the tsA1609 T antigen expressed in ts COS cells is functional, we tested its ability to support SV40 replication. COS ts1, COS ts2, COS7, and CV1 cells were infected with an SV40 A gene deletion mutant, dl 1151 (*17*), and maintained at either the permissive temperature (33°C) or the restrictive temperature (40°C). The SV40 deletion mutant, dl 1151, is unable to carry out viral DNA replication because it encodes a defective T antigen (*17*). At various times after infection, low molecular weight DNA was isolated from the cells (*18*), subjected to agarose gel electrophoresis, ethidium bromide staining, and blot hybridization analysis (*16*). At the permissive temperature, both ts COS cell lines produced T antigen that was able to efficiently complement the

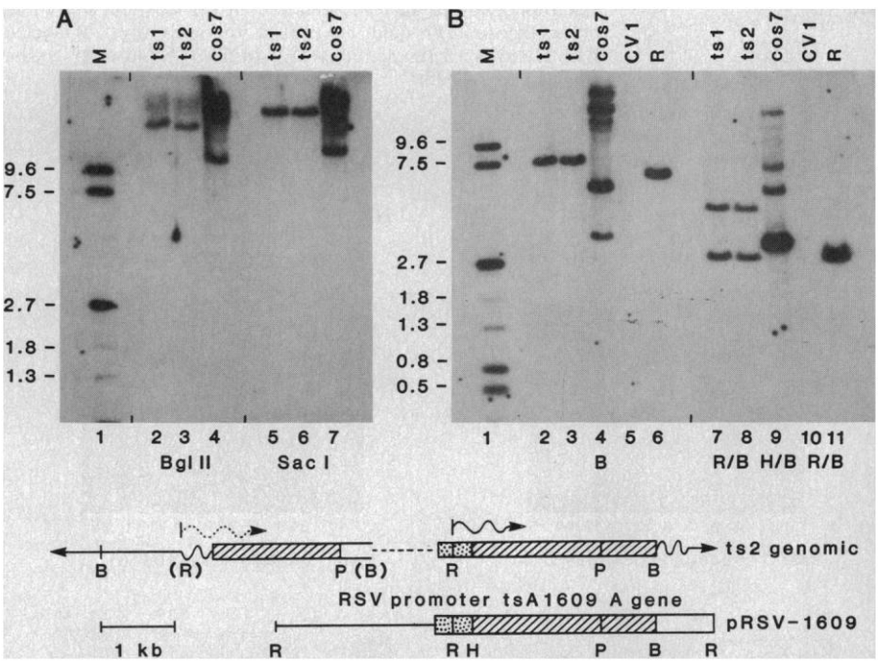

Fig. 3. (Top) Blot hybridization analysis of genomic DNA from ts COS cells. High molecular weight DNA isolated from ts1, ts2, COS7, and CV1 cells was cleaved with various restriction enzymes and analyzed by Southern blot hybridization (*14*). (A) (Lane 1) Markers are in kilobase pairs (kb); (lanes 2 to 4) ts1, ts2, and COS7 DNA, respectively, cleaved with Bgl II; (lanes 5 to 7) ts1, ts2, and COS7 DNA, respectively, digested with Sac I. (B) (lane 1) Markers; (lanes 2 to 6) ts1, ts2, COS7, CV1, and reconstituted mixture of CV1 DNA and pRSV-1609 DNA, respectively, cleaved with Bam HI; (lanes 7 and 8) ts1 and ts2 DNA, respectively, cleaved with Eco RI and Bam HI; (lane 9) COS7 DNA cleaved with Hpa II and Bam HI; (lanes 10 and 11) CV1 and reconstituted mixture cut with Eco RI and Bam HI; abbreviations are B, Bam HI; R, Eco RI; H, Hpa II; P, Pst. (Bottom) Genomic structure of pRSV-1609 and the integrated form of pRSV-1609 DNA found in CV1 genomic DNA sequences. The Eco RI and Bam HI sites in parentheses are missing in the integrated genomic pRSV-1609 DNA. The pRSV-1609 plasmid (approximately 5.5 kb) was constructed from pRSV-cat (*10*) which contains the Rous sarcoma long terminal repeat (RSV virus LTR) cloned in a derivative of pBR322. The pRSV-cat plasmid was cleaved with Hind III and Bam HI and the vector DNA fragment containing the bacterial origin and amp^R gene, as well as the RSV LTR, was purified by gel electrophoresis. Cloned SV40 tsA1609 DNA (*9*) was cleaved with Bam HI and partially with Hind III and the SV40 early region fragment (nucleotides 2516 to 5171) was purified by gel electrophoresis. This fragment was ligated to the DNA fragment that contained the pRSV-cat vector-RSV promoter so that the SV40 early region coding sequence was now downstream from the RSV LTR transcriptional initiation site (*10*). Trypsinized cells were scraped from plates, washed with PBS, and centrifuged. The pelleted cells were resuspended in 2.5 ml of cold lysis buffer (30 m*M* tris, *p*H 8.0, 100 m*M* EDTA, proteinase K at 150 μg/ml, and 1.0 percent sarcosyl) and then incubated at 37°C for 8 to 10 hours. The lysate was extracted with a mixture of TE (10 m*M* tris, *p*H 7.6, 1 m*M* EDTA) -saturated phenol and $CHCl_3$, SEVAG [chloroform and isoamyl alcohol (24:1)], and ether; then 1.25 g of CsCl per milliliter of solution was added. After 20 to 24 hours of centrifugation at 56,000 rev/min (Type 65 Beckman rotor), fractions containing DNA were pooled and dialyzed against TE. DNA was subjected to restriction enzyme cleavage and blot hybridization (*25*). The probe was an SV40 early region DNA fragment—Bam HI to Bgl I (nucleotides 2516 to 5243)—labeled in vitro by nick translation (*25*).

dl 1151 mutant for viral replication (see Fig. 5, B and D, lanes 3 to 5 and 9 to 11). However, in ts COS cells propagated at the restrictive temperature, no viral DNA replication was observed, indicating that neither cellular- nor viral-derived T antigen molecules were active (Fig. 5, B and D, lanes 6 to 8 and 12 to 14). In contrast, the wild-type T antigen present in COS7 cells was able to complement the dl 1151 virus at both temperatures (Fig. 5, A and B, lanes 3 to 5 and 6 to 8). As expected, CV1 cells did not complement the dl 1151 virus at either temperature (Fig. 5, A and B, lanes 9 to 11 and 12 to 14). These results demonstrate that ts COS cells have a temperature-sensitive phenotype. The endogenous tsA1609 T antigen activates viral DNA replication only at the permissive temperature but not at the restrictive temperature.

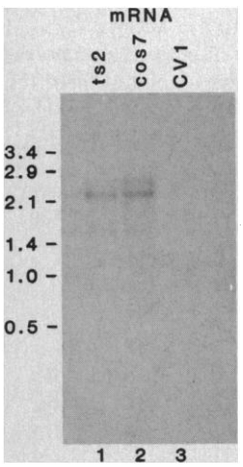

Fig. 4. Blot hybridization analysis of T antigen mRNA from ts COS cells. Northern blot hybridization of mRNA isolated from COS ts2, COS7, and CV1 cells. (Lane 1) Messenger RNA (mRNA) derived from COS ts2 cells; (lane 2) mRNA derived from COS7 cells; (lane 3) control CV1 mRNA; approximately 2 μg of polyadenylated RNA per lane. The larger RNA species corresponds to the small T antigen–specific mRNA, while the smaller RNA corresponds to the large T antigen–specific mRNA. Total cytoplasmic RNA was isolated (*26*). Polyadenylated RNA was isolated by oligo-dT (deoxythymidylate) cellulose chromatography (*27*). RNA was glyoxalated and then subjected to electrophoresis in agarose gels (*28*). RNA was transferred to nitrocellulose (*29*), and hybridized to uniformly ^{32}P-labeled Bam HI to Bgl I (nucleotides 2533 to 5243) SV40 early region DNA fragment (*25*).

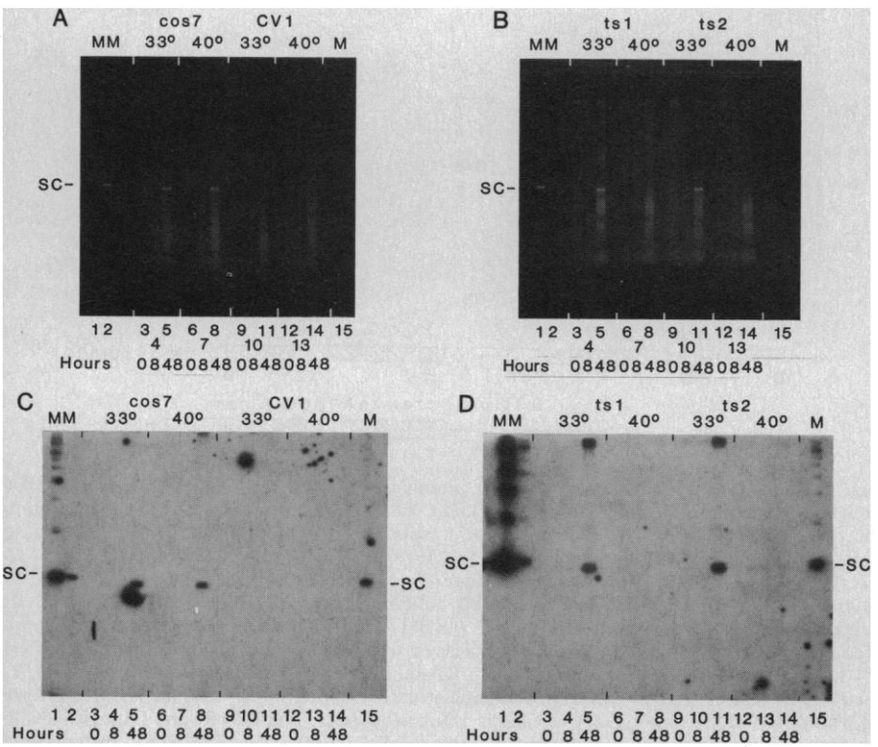

Fig. 5. Replication of SV40 A gene deletion mutants in COS ts1, COS ts2, and COS7 cells. (A) Ethidium bromide–stained agarose gel of low molecular weight DNA isolated from COS7 and CV1 cells at various times after infection with a dl 1151 SV40 (*17*) virus stock prepared by previous propagation on COS7 cells. (Lanes 1 and 2) Supercoiled SV40 DNA marker; (lanes 3 to 8) DNA isolated from COS7 cells maintained at either 33° (lanes 3 to 5) or 40°C (lanes 6 to 8); (lanes 9 to 14) DNA isolated from CV1 cells maintained at either 33° (lanes 9 to 11) or 40°C (lanes 12 to 14); (lane 15) supercoiled SV40 DNA marker. (B) Ethidium bromide–stained gel of low molecular weight DNA isolated from COS ts1 and COS ts2 cells at various times after dl 1151 virus infection. (Lanes 1 and 2) Supercoiled SV40 marker; (lanes 3 to 8) DNA isolated from ts1 cells maintained at 33° (lanes 3 to 5) or 40°C (lanes 6 to 8); (lanes 9 to 14) DNA isolated from ts2 cells maintained at 33° (lanes 9 to 11) or 40°C (lanes 12 to 14); (lane 15) supercoiled SV40 marker. (C) Blot hybridization of (A). (D) Blot hybridization of (B). Time point after infection is as indicated. Low molecular weight DNA was isolated (*18*), subjected to agarose gel electrophoresis, and blotted to nitrocellulose (*25*). The filters were first treated for 4 hours at 68°C in a buffer containing 5× SSC (saline sodium citrate), 5× Denhardt's solution, 0.5 percent sodium dodecyl sulfate, and 400 μg of denatured calf thymus DNA. The hybridization was continued for 20 to 24 hours at 68°C with the inclusion of a uniformly ^{32}P-labeled SV40 DNA probe (*26*).

Replication of SV40 origin-containing plasmids. The ability of ts COS cells to support replication of SV40-based plasmids was determined by transfecting cells (*19*) with the shuttle vector pSVT-2 (*17*) and maintaining them at either 33°, 37°, or 40°C. At 48 hours after transfection, low molecular weight DNA was isolated from the cells (*18*). After Mbo I cleavage, the plasmid DNA isolated from transfected cells was subjected to agarose gel electrophoresis and analyzed by blot hybridization using ^{32}P-labeled pBR322 DNA as a probe. To be sure that we were measuring de novo DNA synthesis in the transfection assay and not merely the persistence of input pSVT-2 DNA, low molecular DNA was treated with restriction enzyme Mbo I prior to gel electrophoresis. Mbo I cannot cleave the input plasmid DNA grown in *Escherichia coli* because the enzyme recognition sites are methylated by the adenine (*dam*) methylation system. However, the DNA synthesized in mammalian cells following transfection and replication is not methylated at these sites and should be quantitatively digested by the enzyme. A comparable level of pSVT-2 DNA replication was observed in COS ts1, COS ts2, and COS7 cells incubated at 33°C (Fig. 6, lanes 3, 6, and 9). This is apparent by comparing the two predominant bands indicated by arrows with those obtained after cleavage of pSVT-2 DNA with Sau 3A (Fig. 6, lane 2); Sau 3A is an isoschizimer of Mbo I that cleaves both methylated and unmethylated plasmid DNA. Replication of pSVT-2 was still evident in both COS ts1 and COS ts2 cells maintained at the semipermissive temperature of 37°C but not detectable in cells maintained at 40°C (Fig. 6, lanes 5 and 8). A somewhat higher level of replication was seen in COS7 cells grown at either 37° or 40°C probably because of increased metabolic rates at these higher temperatures (Fig. 6, lanes 10 and 11).

These results indicate that shuttle vectors containing the SV40 origin can replicate in COS ts1 and ts2 cells at 33° and 37°C but not at the restrictive temperature of 40°C. This property of ts COS

cells should prove to be very useful in various experiments where modulating the level of vector replication and T antigen activity would be advantageous. First, it should be possible to modulate the copy number of transfected SV40-origin containing DNA's by maintaining the cells at different temperatures. Second, activities of SV40 T antigen, such as its repression and activation of transcription, should also be regulated by temperature shift. For example, ts COS cells could significantly facilitate the investigation of how large T antigen might be involved with the *trans*-activation of SV40 late transcription. Previous studies have suggested that T antigen in some way activates transcription of the SV40 late genes in the absence of DNA replication (*20, 21*), although the mechanism is not clear. In order to determine the effect of T antigen on SV40 late transcription, we have transfected vectors carrying wild-type and mutant SV40 replication origins into ts COS cells and measured RNA synthesis from the SV40 late promoter after a shift in temperature (*22*). Both "shift-up" and "shift-down" experiments suggest that in the absence of DNA replication and without specific T antigen binding sequences, T antigen has a stimulatory effect on one set of late transcripts that had previously been defined by in vitro transcription (*21*).

Transformation of COS ts2 cells with a dominant selectable marker. The temperature-dependent replication of origin-containing plasmid DNA's in COS ts2 cells suggests another use for this cell line. We reasoned that the induction of T antigen activity could be used to manipulate replication versus integration of vector DNA's in the host cells. For example, cells could be kept at the permissive temperature immediately after transfection with SV40 origin-containing plasmids which would allow transient replication so that more vector DNA is available for integration. After this initial burst of replication, cells could be shifted to the restrictive temperature to inhibit further replication and thereby favor conditions for plasmid integration. After integration and selection of transformants, a shift to the permissive temperature should restore functional T antigen allowing in situ replication and episomal excision to occur (*23*). This strategy will allow us to modulate the frequency of integration and could also be used to rescue cellular DNA sequences flanking the site or sites of plasmid insertion (*23*). A similar type of SV40 origin-dependent replication-excision event has been demonstrated by fusing nonpermissive cells carrying SV40 origin sequences with permissive cells that express T antigen (*23*). The advantage of using ts COS cells is that cell fusion is no longer necessary to induce origin-dependent replication and excision; rather, a shift in temperature should be sufficient to rescue the integrated sequences.

As a first step toward developing a mammalian host-vector system that would allow copy number regulation, we have transfected the plasmid pSV2-gpt (*24*) into COS ts2, COS7, and CV1 cells maintained at 33°C. This vector carries the SV40 replication origin and the SV40 early promoter directing transcription of the bacterial guanine phosphoribosyl transferase (*gpt*) gene, which can be used as a dominant selectable marker in mammalian cells (*24*). At 48 hours after transfection, the cultures were split at a ratio of 1 to 5 and incubated in medium containing mycophenolic acid to select for gpt expression (*24*) at either 33°, 37°, or 40°C. After 8 to 9 days of incubation in selective medium, the number of gpt$^+$ colonies was scored (Table 1). Our initial results indicate that there is a dramatic and reproducible increase in the number of gpt$^+$ colonies obtained from transfected COS ts2 cells at 37°C relative to either COS7 and CV1 cells at 37°C or COS ts2, CV1, and COS7 cells at either 33° or 40°C. This dramatic effect is not seen when the transfected COS ts2 cells are maintained at 40°C. Instead, all three cell types gave similar numbers of colonies at this temperature (Table 1). At 40°C, COS7 cells should still replicate pSV2-gpt, but neither COS ts2 nor CV1 cells allow vector replication. At 33°C, CV1 cells give a large number of gpt$^+$ colonies relative to COS7 or COS ts2, perhaps because high levels of pSV2-gpt replication in both COS7 and COS ts2 cells may preclude plasmid integration. The tenfold stimulation in gpt$^+$ colonies seen at 37°C in COS ts2 may be a consequence of the reduced level of functional T antigen and hence reduced vector replication at the semipermissive temperature (see Fig. 6). The mechanism that leads to the higher transformation frequencies of COS ts2 at 37°C is unclear but will be better understood when the state of the pSV2-gpt vector DNA in each type of gpt$^+$ colony is analyzed in more detail. We know, however, that there is a balance between a number of competing factors in COS ts2 cells at 37°C. The replication of pSV2-gpt DNA is reduced at this semipermissive temperature, as is the repression of gpt transcription from the SV40 early promoter by T antigen. These effects may result in an equilibrium between the episomal and integrated forms of pSV2-gpt as has been shown to occur in SV40-transformed human cells (*23*). Our initial findings are consistent with the idea that episomal maintenance of the gpt$^+$ marker in COS ts2 cells at 37°C may be

Table 1. Transformation frequency of COS ts2, COS7, and CV1 cells by pSV2-gpt DNA. Subconfluent COS ts2, COS 7, or CV1 cells (8×10^5 cells per 100-mm dish) were transfected with 10 μg of pSV2-gpt (*24*) DNA by the calcium phosphate coprecipitation method (*13*) and then treated with 15 percent glycerol in Hepes-buffered saline for 1 minute. The cells were grown for 40 hours at 33°C, and then replated at a lower density (6×10^4 cells per 100-mm dish) and incubated at 33°, 37°, or 40°C in gpt-selective medium containing mycophenolic acid (*24*). After 8 to 9 days of growth in selective medium, the colonies of transformed cells were counted after being treated with 0.6 ml of 25 percent aqueous glutaraldehyde and methylene blue staining fluid.

Cell type	Colony number at*		
	33°	37°	40°
COS ts2	9	110	8
COS7	4	11	3
CV1L	27	13	5

*Given as number of colonies per 6×10^4 cells plated after 9 days in gpt-selective medium (*24*). The numbers are an average of several independent experiments.

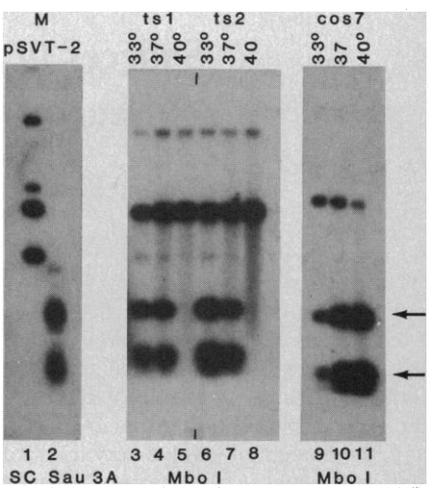

Fig. 6. Replication of the SV40 origin-containing plasmid pSVT-2 in COS ts1, COS ts2 and COS7 cells. Blot hybridization of low molecular weight DNA isolated 48 hours after transfection with pSVT-2 from COS ts1, COS ts2, or COS7 cells (legend to Fig. 3). (Lane 1) pSVT-2 supercoiled DNA; (lane 2) pSVT-2 digested with Sau 3A; (lanes 3 to 5) DNA cleaved with Mbo I from COS ts1 cells that were maintained at 33° (lane 3), 37° (lane 4), or 40°C (lane 5). (Lanes 6 to 8) DNA cleaved with Mbo I from COS ts2 cells that were maintained at 33° (lane 6), 37° (lane 7), or 40°C (lane 8); (lanes 9 to 11) DNA cleaved with Mbo I from COS7 cells that were maintained at 33° (lane 9), 37° (lane 10), or 40°C (lane 11). Arrows identify the fragments produced by Sau 3A cleavage of pSVT-2 (see lane 2).

favored by reduced replication at the semipermissive temperature and that there is more episomal DNA in pSV2-gpt transformed COS ts2 cells held at 37° than at 40°C. The potential to modulate copy number by temperature shift might be particularly useful for the isolation of transformed cells designed to overproduce toxic products. In principle, the copy number of the transfected gene could be kept low during maintenance and passage of these cells and then amplified thousands of times (*6*) by shifting to the permissive temperature to induce replication and overproduction.

References and Notes

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RESEARCH ARTICLE

Discovery of New Variable Radio Sources in the Nucleus of the Nearby Galaxy Messier 82

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There is growing evidence that short-lived periods of very large energy release in galaxies are due to intense bursts of star formation. Such bursts, whose intensity far exceeds that found in our own Galaxy, are thought to involve very massive stars which end as supernovae. Observations with the National Radio Astronomy Observatory's (NRAO's) Very Large Array (VLA) in New Mexico by Kronberg *et al.* (*1*) have revealed about 40 discrete radio sources in the inner, visually obscured nucleus of the enigmatic galaxy Messier 82 (M82). These radio sources, more luminous than any comparable objects in our Galaxy, are candidates for the supposed supernovae associated with the starburst source of energy.

A radio photograph of the 4.87-GHz map made in February 1981 with a resolution of 0.34 arc second is shown in Fig. 1. None of the myriad of radio sources has been optically identified because of the high visual extinction in M82's nucleus. However, the similarity of their radio luminosities to those of recently discovered extragalactic radio supernovae (*2*) strongly suggests that we are seeing an entire dynamic population of radio sources arising from supernovae associated with an intense burst of massive star formation in M82's nucleus.

Observations. It is well established that the brightest of the M82 nuclear sources, 41.9+58, has been declining in flux density since at least the early 1970's (*3, 4*). In doing so, it has maintained a nearly constant spectral index ($\simeq$0.9) in the optically thin part of its spectrum above 1 GHz.

Since the February 1981 observations, we have done repeated, multifrequency mapping of M82 with the VLA in order to search for time variability in this large, concentrated population of presumed radio supernovae and supernova remnants. Messier 82 was mapped at 4.87 GHz (wavelength λ = 6 cm) in April, May, and June 1982 and again in August and October 1983, thus covering a time span of 2.7 years at the same resolution and sensitivity. Measurements have also been made at 1.4, 15, and 23 GHz for some of the above epochs, but they are, so far, less suitable for the purpose of studying variability than the more complete 4.87-GHz measurements.

Calibration and errors. The flux density scale of all observations is relative to the strong radio source 3C286, whose brightness is accurately known. Its adopted integrated (zero-spacing) flux was 7.41 Jy at 4.87 GHz. Each VLA observing run between April 1982 and October 1983 was made within the same 8-hour local sidereal time range, thus giving similar interferometer baseline coverage. Local amplitude and phase calibration in February 1981 was performed via the nearby calibrator 0917+624 (*1*), whose flux density was tied to that of 3C286. In April 1982 and succeeding epochs, the local calibrator was 1044+719.

After applying the external calibrations, self-calibration in phase was used to improve the dynamic range of the

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