MOLECULAR AND CELLULAR BIOLOGY, Nov. 1985, p. 3231–3240
0270-7306/85/113231-10$02.00/0

Vol. 5, No. 11

New Host Cell System for Regulated Simian Virus 40 DNA Replication

ROBERT D. GERARD* AND YAKOV GLUZMAN
Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724

Received 3 June 1985/Accepted 11 August 1985

Transformed monkey cell lines (CMT and BMT) that inducibly express simian virus 40 (SV40) T antigen from the metallothionein promoter have been isolated and characterized. Immunoprecipitation of pulse-labeled T antigen demonstrates a 5- to 12-fold increase in the rate of synthesis on addition of heavy-metal inducers to the culture medium. Radioimmunoassay of cell extracts indicates the accumulation of three- to fourfold more total T antigen after 2 days of induction by comparison with uninduced controls. A direct correlation was found between the level of T-antigen synthesis and the extent of SV40 DNA replication in inducible cells. Inducible BMT cells expressing a low basal level of T antigen were efficiently transformed by a vector carrying the neomycin resistance marker and an SV40 origin of replication. These vector sequences were maintained in an episomal form in most G418-resistant cell lines examined and persisted even in the absence of biochemical selection. Extensive rearrangements were observed only if the vector contained bacterial plasmid sequences. Expression of a protein product under the control of the SV40 late promoter in such vectors was increased after heavy-metal-dependent amplification of the template. These results demonstrate the ability of BMT cells to maintain a cloned eucaryotic gene in an amplifiable episomal state.

Simian virus 40 (SV40) vectors have been used to amplify eucaryotic genes in mammalian cells. Analysis of the mechanism of transcription (17, 28), studies on the posttranscriptional processing of RNA (16, 17, 18, 35), expression of exogenous genes at a high level (9, 34, 41), and study of the chromatin structure of the recombinant genome (6–8, 24, 25, 47) have all been possible by using SV40 vectors. The foreign DNA is usually inserted into either the early or late transcription unit in place of the viral gene. The late unit is suitable for high-level expression in permissive cells as a result of the amplification of the inserted gene by T-antigen-dependent DNA replication and transactivation of the late gene by T antigen (3, 26). Substitution of exogenous genes into the early region has been less widely used, because of lower levels of expression obtained from the early promoter (10).

The use of early-region replacement vectors has been facilitated by the development of COS cells (12). Because COS cells were derived by transformation of CV-1 cells with an origin-defective SV40 genome, they constitutively express wild-type T antigen and contain all necessary cellular factors required for SV40 replication. Transfection of COS cells with recombinant plasmids containing an SV40 *ori* element and an expression unit leads to efficient amplification of the genome and transient expression of the cloned DNA segment (33). If COS cells are infected with SV40 early-region replacement viral vectors, amplification results in viral capsid protein synthesis, virus production, and cell death. COS cells are therefore a host system for the propagation of pure stocks of early-region replacement viruses and for the transient replication of SV40 origin-containing vectors.

Inherent problems are associated with the use of COS cells as a host for the amplification of SV40-based vectors. One difficulty is that apparently not all cells within the COS population synthesize sufficiently high levels of T antigen to support replication (9, 43). T-antigen production in COS cells is heterogeneous, as determined by the intensity of immunofluorescent staining of T antigen. COS cells are known to synthesize about 5- to 10-fold less T antigen than do SV40-infected CV-1 cells (unpublished observations). These two observations suggest that the population of weakly stained cells may not contain sufficient T antigen to support DNA replication. A second disadvantage is that transfection of recombinant SV40 *ori* plasmids into COS cells does not lead to the establishment of permanent cell lines expressing the exogenous gene. Owing to the constitutive synthesis of T antigen in COS cells, replication proceeds unchecked until the cells die, presumably because they cannot tolerate the presence of high levels of extrachromosomally replicating DNA. An exception to this has been reported for the transformation of COS cells to mycophenolic acid resistance by using pSV2-*gpt* (44). These transformants were found to contain pSV2-*gpt* DNA episomally in the form of both monomers and high-molecular-weight concatemers which were rapidly lost on removal of selective pressure. Presumably in these cells a balance exists between DNA replication and the selective pressure to maintain the sequences conferring the drug resistance phenotype.

It would be desirable to develop a host cell in which the synthesis of T antigen required for SV40 *ori* replication could be expressed at a level higher than that present in COS cells and in a controlled manner. Recently, the isolation and characterization of *ts*COS cell lines that express a temperature-sensitive T antigen from a Rous sarcoma virus promoter have been reported (38). SV40 DNA replication in these cells is controlled by a temperature shift which inactivates the protein. Our approach to the development of an improved host cell was to modulate the level of T-antigen protein itself, rather than modulating the function of this protein. We have accomplished this by placing the structural gene for T antigen under control of the mouse metallothionein promoter and using this chimeric gene to transform permissive monkey cells. The isolation and characterization of these cell lines are the subjects of this report.

* Corresponding author.

MATERIALS AND METHODS

DNA constructions. Plasmid pK1 DNA containing the complete genome of SV40 cloned via the *Eco*RI site (13) was linearized with *Bgl*I and briefly treated with BAL 31 exonuclease to remove the SV40 *ori* and T-antigen binding site (2, 4). After treatment with the Klenow fragment of DNA polymerase I and all four deoxynucleoside triphosphates, *Bgl*II linkers were ligated, and the mixture was used to transform *Escherichia coli* DH-1 (20). Clones containing *Bgl*II linkers were identified, and a series of deletions in pK1 were sequenced by the dideoxy chain termination procedure (39) after cloning of the *Hin*dIII-*Kpn*I fragments into M13mp18. Such clones are identified as pK1*dl*1, pK1*dl*2, etc.

Construction of the metallothionein promoter-T-antigen structural gene was as follows. Plasmid DNA containing the desired deletion in pK1 was digested with *Bgl*II and *Kpn*I to remove the SV40 promoter and treated with alkaline phosphatase. Ligation of this DNA to *Bgl*II-*Kpn*I-digested pBRMT, a 3.8-kilobase genomic clone of the mouse metallothionein-I gene (19), resulted in directional insertion of the metallothionein promoter in place of the SV40 promoter.

Plasmid pS1 containing the *Hpa*II-*Bam*HI early-region fragment of SV40 cloned via the *Cla*I-*Bam*HI sites of pXf3 (20) was altered for use as a replication substrate. After digestion with *Nco*I to remove SV40 promoter and enhancer sequences, the DNA was blunt-ended with Klenow fragment, and *Bgl*II linkers were ligated. After digestion with *Bgl*II, the DNA was religated and used to transform *E. coli* DH-1. An appropriate clone was identified by restriction analysis of plasmid DNA with *Bgl*II and *Nco*I and was designated pS1*dl*2.

We constructed an SV40 *ori* recombinant vector (pSVHA$^-$*neo*) which contains the *neo* gene under control of the SV40 early promoter and a cDNA copy of a secreted form of the influenza virus hemagglutinin gene under control of the SV40 late promoter. The *Bgl*I-*Bam*HI fragment of pko-*neo* containing the neomycin resistance gene (45) and the *Bgl*I-*Bam*HI fragment of pSVEHA20A$^-$ containing the mutant hemagglutinin gene (10) were ligated in a trimolecular reaction into the vector pMK16-*Bgl*I^r (14) at the *Bam*HI site and transformed into *E. coli* DH-5. The structure of the recombinant was verified by extensive restriction analysis. Excision of SVHA$^-$*neo* DNA from the plasmid vector sequences was accomplished by *Bam*HI digestion and ligation of the DNA at a concentration of 2 μg/ml to promote recircularization.

Transfections. For stable transformation of monkey cells, DNAs were transfected overnight by the calcium phosphate precipitation method (46) with 20 μg of HeLa DNA as carrier and 1 μg of plasmid DNA per 100-mm-diameter dish. After transfection, cells were washed extensively and maintained in Dulbecco minimal essential medium (DME) containing 10% fetal bovine serum. Isolation of cell lines that inducibly express T antigen was accomplished by maintaining transfected cell cultures in the presence of heavy metals. DME containing 10% fetal bovine serum, 100 μM $ZnCl_2$, and 1 μM $CdSO_4$ was the standard inducer medium used throughout this study (5).

G418 selection experiments to isolate *neo* transformants were carried out with an active concentration of 300 μg/ml. Medium was changed twice weekly while G418-resistant cells were selected. Individual colonies were picked, and cultures were expanded for analysis. G418-resistant cell lines obtained by transformation with pON3 DNA were designated *neo*1, *neo*2, etc., to denote the individual clone number. Mass cultures obtained by expansion of a mixture of G418-resistant colonies were designated *neo*M. Similarly, SVHA$^-$*neo* transformants were designated *nha*1, *nha*2, etc. Cells were maintained continuously in selective medium unless otherwise indicated.

In DNA replication experiments, cells in 60-mm dishes were transfected with 100 ng of pS1*dl*2 DNA by using DEAE-dextran (250 μg/ml; M_r, 500,000) in DME buffered with 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.1) for 1 h at 37°C (32). Chloroquine (100 μM) in DME containing 5% fetal bovine serum was applied for 4 to 5 h to increase the frequency of transfected cells (30). Cells were then transferred to either inducer medium or control medium lacking heavy metals. Low-molecular-weight DNA was prepared by the Hirt procedure (23) and digested with *Bam*HI and *Dpn*I (37) before electrophoresis on an agarose gel, blotting to nitrocellulose (40), and hybridization to nick-translated pS1*dl*2 probe.

Labeling and immunoprecipitation of proteins. Cells propagated for 2 days in either control or inducer medium were starved for 1 h in either methionine-free DME or methionine-free DME plus inducer, respectively, before labeling with [^{35}S]methionine for 1 h. For analysis of T antigen, monolayers were washed twice with phosphate-buffered saline and extracted with 50 mM Tris hydrochloride (pH 8.0) containing 1% Nonidet P-40. Equivalent amounts of extract (based on cell number) were immunoprecipitated with PAb416 (21) and Protein A-Sepharose (Pharmacia Fine Chemicals) as previously described (22). Immunoprecipitates were washed twice with NET-gel (150 mM NaCl, 5 mM EDTA, 50 mM Tris hydrochloride [pH 7.4], 0.25% gelatin, 0.05% Nonidet P-40, 0.02% sodium azide). For analysis of hemagglutinin, labeled cells were chased for 1 h with DME containing 20 times the normal concentration of methionine. Cell culture supernatant was collected and immunoprecipitated with high-titer rabbit anti-hemagglutinin serum and Protein A-Sepharose. Immunoprecipitates were washed successively with NET-gel containing 0.5 M NaCl, NET-gel containing 0.1% sodium dodecyl sulfate and 1% Nonidet P-40, and 10 mM Tris hydrochloride (pH 7.5) containing 0.1% Nonidet P-40. Proteins were run on a 10% polyacrylamide gel, which was then dried and exposed directly to Kodak SB-5 film. Quantitation of labeled protein was performed by densitometric scanning of the autoradiogram.

RIA. Solid-phase radioimmunoassay (RIA) (1) was used to quantitate the relative amount of T-antigen protein in cell extracts. The assay involved the use of PAb419 (21) as the first antibody and ^{125}I-labeled PAb416 as the second antibody.

RESULTS

Construction of the metallothionein-T-antigen chimeric gene. Plasmid pK1 containing a complete copy of the SV40 genome was sequentially treated with *Bgl*I and BAL 31 to remove sequences at the SV40 origin of replication. This also resulted in the removal of T-antigen binding site II, which has been implicated in the autoregulation of T-antigen synthesis (36). The deletion in mutant *dl*26 was found to remove nucleotides 5212 through 9 (SV numbering system; 42) and insert three *Bgl*II linkers. This deletion retained the SV40 promoter and enhancer elements located upstream from nucleotide 10, but deleted the SV40 origin. pK1*dl*26 was therefore suitable as a positive control for transformation of CV-1 and BSC-1 monkey cells.

Another deletion, *dl*11, was found to delete nucleotides 5238 to 47 and substitute two *Bgl*II linkers. As none of the

SV40 sequence encoding the 5′ untranslated region of T-antigen mRNA was removed, this deletion was chosen for fusion to the mouse metallothionein-I promoter. The SV40 promoter was removed by *Bgl*II-*Kpn*I digestion and replaced with approximately 600 base pairs of metallothionein gene containing the promoter fragment (11) to generate the plasmid pKMT11.

The chimeric T-antigen gene in pKMT11 should produce a transcript containing 5′ untranslated sequences derived from both the metallothionein gene and the SV40 early gene. It was therefore necessary to determine that T antigen would be produced by this construct. Transient expression in CV-1 cells verified that both pK1*dl*26 and pKMT11 synthesized sufficient T antigen to be detected by immunofluorescence in the presence of heavy metals, but that only pK1*dl*26 did so in the absence of heavy metals. The chimeric gene is therefore capable of efficient and inducible expression of T antigen in monkey cells and was subsequently used to generate monkey cell transformants.

Isolation of inducible cells. Plasmids pKMT11 and pK1*dl*26 were transfected into both CV-1 and BSC-1 monkey cells by the calcium phosphate technique to generate stably transformed cell lines. Transformed colonies (approximately 20 to 30 per μg of plasmid DNA) appearing 4 to 6 weeks after transfection were picked, grown up into a mass culture, and stained by indirect immunofluorescence for the presence of T antigen. Cultures containing T-antigen-positive cells were subcloned to isolate pure populations before further use. Cell lines derived by transformation of CV-1 and BSC-1 cells with pKMT11 were designated CMT and BMT lines, respectively, whereas BSC-1 cells transformed with pK1*dl*26 were designated BOS cells by analogy to COS. The different CMT clones (CMT1, CMT3, and CMT4) were subclones of the same original transformed cell, as determined by their pattern of integration of the pKMT11 DNA into the genome (data not shown), but expressed different levels of T antigen and were further characterized.

Inducible synthesis of T antigen. CMT and BMT10 cells were analyzed for their ability to synthesize SV40 T antigen in response to heavy-metal induction. Figure 1 shows the immunofluorescent staining pattern of CMT and BMT10 cells grown in the presence and absence of inducer. In the absence of inducer, CMT and BMT10 cells were faintly stained, which indicates a low level of T-antigen synthesis. After exposure of these cells to heavy metals for 2 days, the staining intensity was much brighter than that of control cells maintained in normal medium. In contrast, COS1 and BOS4 cells showed similar staining intensities in the presence and absence of heavy metals.

The rate of synthesis of T antigen by CMT and BMT10 cells was measured by pulse labeling cultures with [^{35}S]methionine and quantitating the amount of labeled T antigen after immunoprecipitation and gel electrophoresis (Fig. 2). Induced CMT and BMT10 cells synthesized T antigen at a 5- to 12-fold-higher rate than did uninduced controls (Table 1). By contrast, COS1 and BOS4 cells showed equivalent rates of synthesis in both the presence and absence of inducer. In addition, the rate of synthesis in COS1 cells was equivalent to that in BOS4 cells. All CMT cell lines synthesized T antigen at a greater rate than did COS1 cells, even under control conditions. BMT10 cells synthesized T antigen at a higher rate than either COS1 or BOS4 cells when induced, but at a lower rate under control conditions.

To measure the relative amounts of T antigen which accumulate in the various transformed lines, the same cellular extracts from induced and control cultures used to quantitate the rate of T-antigen synthesis were assayed for total T antigen by RIA (Table 1). The results indicate that the quantity of T antigen which accumulates in a cell line corresponds to the rate of T-antigen synthesis in that cell line. After 2 days of induction, both CMT and BMT10 cells accumulated three- to fourfold more T antigen than did uninduced controls. By contrast, the amount of T antigen present in COS1 and BOS4 cells was essentially unchanged by treatment with heavy metals, although COS1 cells accumulated twice as much T antigen as did BOS4 cells. Clones CMT1 and CMT3 contained slightly more T antigen than did COS1 cells when uninduced, whereas CMT4 cells contained less T antigen than did COS1 cells. All three induced CMT cultures contained two- to fivefold-more total T antigen than did COS1 cells. Induced BMT10 cells accumulated as much T antigen as BOS4 cells, but only about half as much as COS1 cells did. The quantitative data for total T-antigen content obtained by RIA only roughly corresponded to the fluorescence intensities observed in Fig. 1. In particular, both COS1 and BOS4 cells were more brightly stained than would be expected on the basis of T-antigen content determined biochemically. The reason for this discrepancy is unknown, but may be related to the constitutive nature of T-antigen synthesis in COS1 and BOS4 cells. In any case, indirect immunofluorescence does not provide a quantitative measure of T-antigen content and is only shown for comparative purposes.

DNA replication of an SV40 origin-containing plasmid. The inducible cell lines were examined for their ability to support the replication of a recombinant plasmid containing an SV40 origin. Mutant pS1*dl*2 which contained no SV40 promoter or enhancer elements was isolated. As the plasmid was incapable of synthesizing T antigen, it was completely defective for replication in CV-1 or BSC-1 monkey cells lacking T antigen (data not shown). Transfection of pS1*dl*2 into permissive cells expressing endogenous SV40 T antigen demonstrated that this plasmid replicates efficiently. The gel shown in Fig. 3 demonstrates the accumulation of replicated pS1*dl*2 DNA after DEAE–dextran-mediated transfection. By comparison of the hybridization intensities of replicated DNA bands with the intensities of a standard curve of known DNA concentrations, a quantitative estimate of the number of copies per cell (obtained by counting the number of cells in a parallel culture at 2 days posttransfection) can be made (Table 2). At 2 days posttransfection, CMT1 and CMT3 cells were found to replicate SV40 DNA to levels comparable to those observed in COS1 cells (5,000 copies per cell), even in the absence of heavy-metal induction of T-antigen synthesis. These numbers are similar to previous estimates (29, 33) if differences in the method of calculating cell number are taken into account. However, in the presence of heavy metals, CMT1 and CMT3 cells accumulated even higher levels of replicated DNA (>30,000 copies per cell), and by 3 days after transfection they accumulated amounts of replicated DNA similar to those in SV40-infected cells. Uninduced CMT4 cells replicated SV40 *ori* DNA to a low level (750 copies per cell), but accumulated very high levels when induced with heavy metals (30,000 copies per cell). By comparison, BMT10 cells replicated DNA to a much lower level, with the level for induced BMT10 cells comparable to that for COS1 (5,000 copies per cell). Neither COS1 nor BOS4 cells showed any effect of heavy-metal induction on DNA replication.

Maintenance of SV40 origin-containing DNA as an episome. Previous work (44) has shown that COS1 cells are capable of

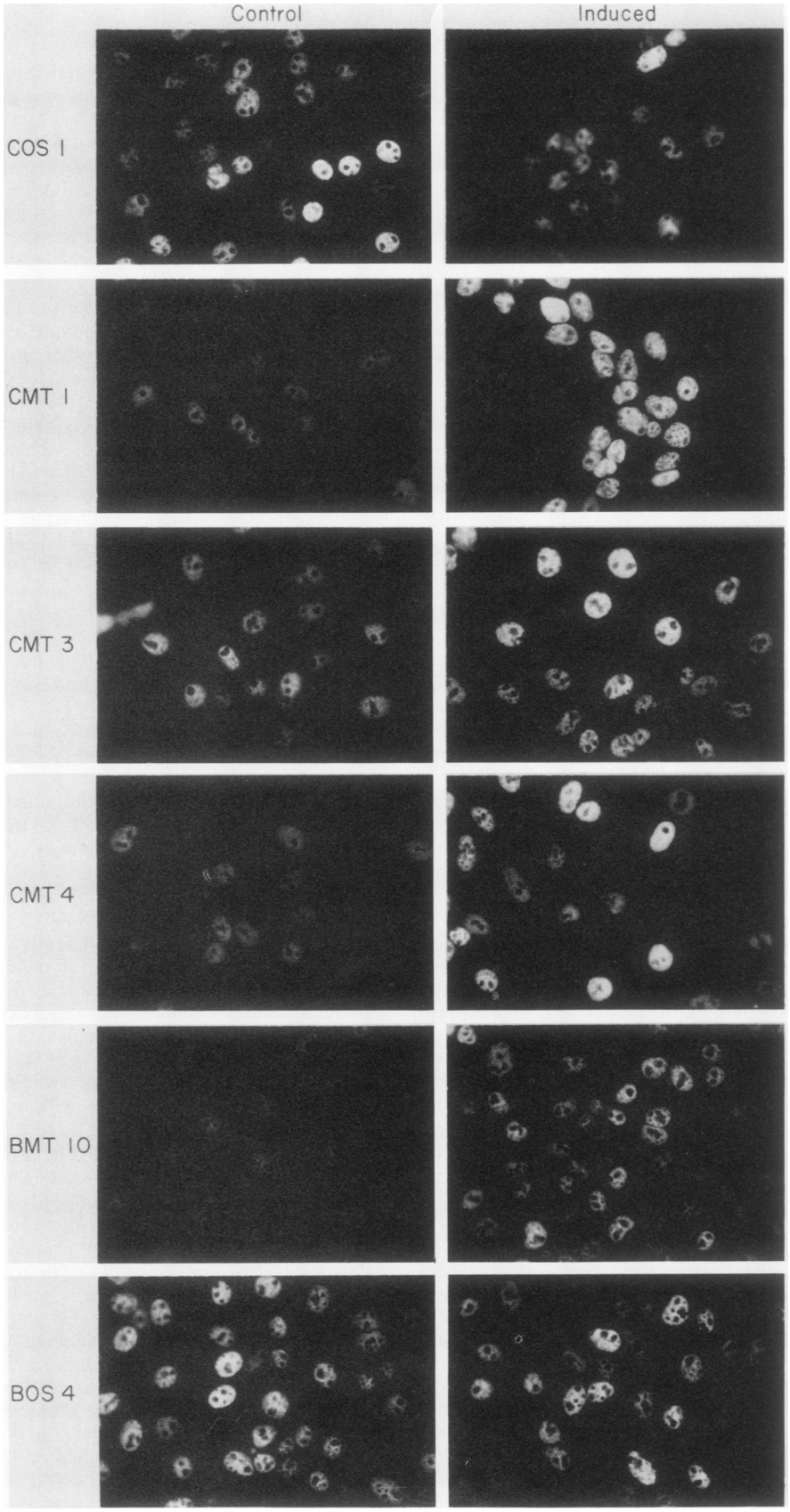

FIG. 1. Indirect immunofluorescence of T-antigen-producing monkey cell lines. Cells grown on glass cover slips were transferred to medium containing 100 μM $ZnCl_2$ and 1 μM $CdSO_4$ for 2 days (induced) or maintained in normal medium (control). Cells were fixed with methanol-acetone (1:1) and stained for indirect immunofluorescence of T antigen by using a 1:1 mixture of PAb 416 and PAb 419 and fluorescein isothiocyanate-goat anti-mouse immunoglobulin. Stained cells were photographed at equivalent exposures on Tri-X film.

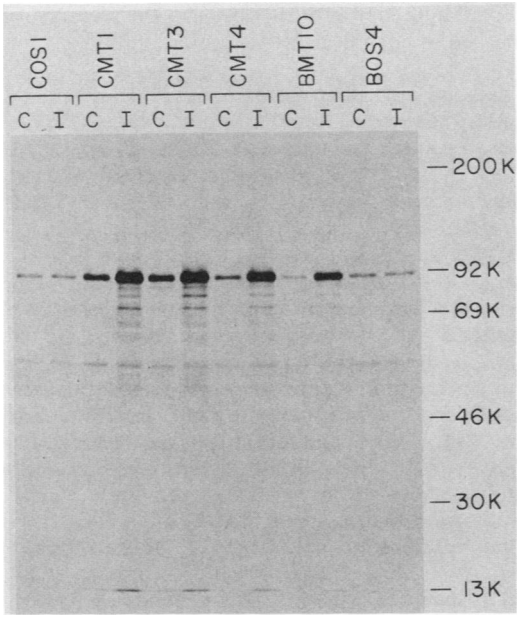

FIG. 2. Measurement of the rate of T-antigen synthesis in transformed monkey cell lines. Cells propagated in 60-mm dishes in either normal medium (C) or medium containing heavy-metal inducers (I) were starved for 1 h in methionine-free DME either without or with inducer as appropriate. Proteins were labeled with 125 μCi of [^{35}S]methionine per dish for 1 h. Cell counts were performed on parallel cultures, and the extract from 4×10^5 cells was immunoprecipitated and run on each lane of the gel. The gel was exposed to Kodak SB-5 film for 13 h.

maintaining an SV40 *ori* plasmid containing a dominant selectable marker as an episome. It was therefore of interest to determine whether CMT and BMT10 cells could support the episomal replication of an *ori*-containing plasmid containing the *neo* gene under control of the SV40 early promoter in pON3 (Fig. 4). In this plasmid, the SV40 early region has been replaced by the *Bgl*I-*Bam*HI fragment of pKO-*neo* encoding the neomycin resistance gene (45). Monkey cell lines were transfected with pON3 DNA by calcium phosphate coprecipitation and transferred to medium containing G418 but without added heavy metals to select for *neo* transformants.

TABLE 1. Synthesis of T antigen by transformed monkey cells

Cell line	Total amt of T antigen[a] from:			Amt of radiolabeled T antigen[b] from:		
	C[c]	I[c]	I/C	C	I	I/C
COS1	7.3	6.7	0.9	1.7	1.6	0.94
CMT1	9.0	39	4.3	5.6	47	8.4
CMT3	10	34	3.4	7.8	40	5.1
CMT4	4.0	17	4.4	2.4	30	12.5
BMT10	1.0	3.0	3.0	1.0	8.6	8.6
BOS4	3.4	3.4	1.0	1.6	1.2	0.75

[a] Data were obtained by solid-phase radioimmunoassay, as described in Materials and Methods, on the same extracts used to quantitate the rate of T-antigen synthesis. Values are normalized to control BMT10 cells.

[b] Data correspond to the experiment shown in Fig. 2. Values are expressed as the relative amount of T antigen determined by scanning densitometry and are normalized to that obtained for uninduced BMT10 cells.

[c] C, Cells grown under control conditions; I, cells grown in the presence of heavy-metal inducer.

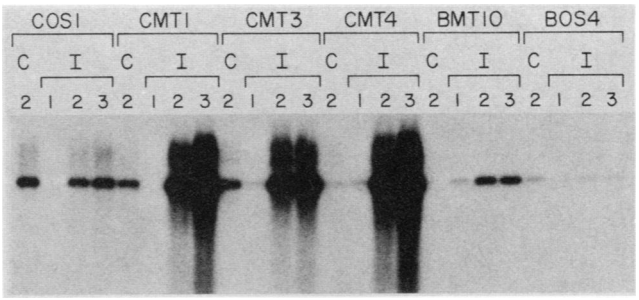

FIG. 3. Replication of a transfected SV40 origin-containing plasmid in T-antigen-producing monkey cells. Plasmid pS1*dl*2 DNA (100 ng) was transfected into cells in 60-mm dishes by using DEAE-dextran and a chloroquine boost as described in the text. Duplicate mock transfections served as parallel cultures on which cell counts were determined 2 days posttransfection. After chloroquine treatment, the cells were maintained in either normal medium (C) or medium containing heavy metals (I) until Hirt supernatant DNA was harvested at the indicated time in days. An amount of DNA equivalent to that from 6×10^4 cells was digested with *Dpn*I and *Bam*HI to linearize all replicated molecules and degrade any unreplicated input DNA. After agarose gel electrophoresis, the DNA was transferred to nitrocellulose and probed with nick-translated pS1*dl*2 DNA. The blot was exposed to Kodak XAR-5 film for 25 h to show the fainter bands.

Individual G418-resistant colonies were obtained at a low frequency on COS1, BOS4, and CMT cells (approximately 1 to 10 colonies per μg of DNA). In addition, many of the "colonies" which appeared on COS1, BOS4, and CMT cells did not survive clonal isolation and expansion of the culture under continued G418 selection, presumably because the colonies consisted of cells which were unstable for the propagation of the *neo* marker. Six clones of BOS4-derived *neo* transformants were obtained, but only two were obtained from COS1 cells and one was obtained from CMT4 cells. *neo*-transformants of CV-1 and BSC-1 origin were also obtained at a similar low frequency (approximately 10 colonies per μg of DNA), but these colonies were readily cloned

TABLE 2. Replication of SV40 *ori* DNA in cell lines producing T antigen[a]

Cell line	No.[b] of copies per cell obtained from[c]:				
	C[d]	I at time (days) posttransfection:			I/C[e]
		1	2	3	
COS1	6.5	<0.5	4.5	6.0	0.7
CMT1	4.0	<0.5	30	60	7.5
CMT3	5.0	0.75	33	40	6.6
CMT4	0.75	1.0	32	50	43
BMT10	<0.5	1.0	5.0	4.5	>10
BOS4	1.0	<0.5	0.5	0.75	0.5

[a] Measured by densitometric scanning of autoradiograms from the gel shown in Fig. 3 and comparison with a standard curve of known DNA concentrations.

[b] Numbers are expressed as thousands of copies per cell. Transfection efficiencies were not determined for the different cell lines, and therefore values may represent 2- to 10-fold underestimates of the number of copies per cell.

[c] C, Cells grown under control conditions; I, cells grown in the presence of heavy-metal inducer.

[d] Obtained at 2 days posttransfection.

[e] Ratio of induced to control values obtained at 2 days posttransfection.

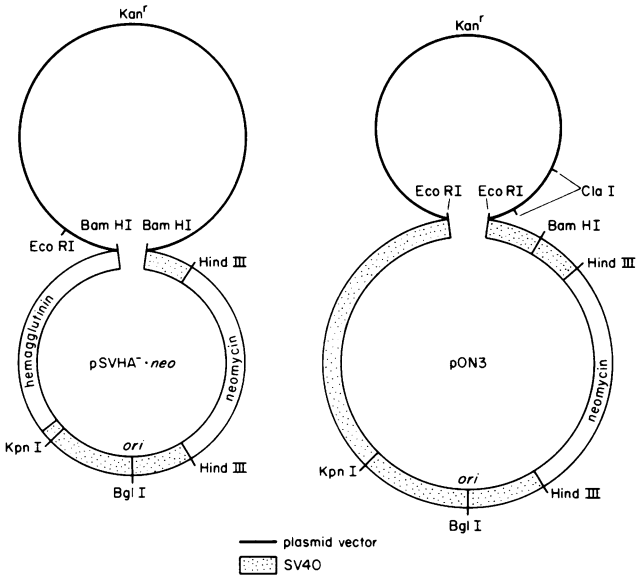

FIG. 4. *neo* plasmids used for transformation of T-antigen-producing monkey cells to G418 resistance. The SV40 *ori*$^+$ plasmids contain the *neo* gene cloned into the SV40 early region upstream of a *Hin*dIII-*Bam*HI fragment containing the SV40 small t antigen splice donor-acceptor and the polyadenylation signals (45). In pON3, the SV40 late region is interrupted by insertion of pMK16-*Bgl*I^r*Bam*HI^r at the *Eco*RI site (D. Hanahan, personal communication). In pSVHA$^-$*neo*, the SV40 late region has been replaced by influenza virus hemagglutinin structural sequences encoding a secreted form of the protein and is separated from the polyadenylation signal by pMK16-*Bgl*I^r sequences.

and propagated. However, transformation of BMT10 cells by pON3 resulted in 10 to 100 colonies per μg of DNA, and five of six colonies survived clonal isolation and expansion of the culture. Mass cultures of uncloned G418-resistant cells of COS1, CMT4, and BMT10 origin were also obtained for comparative purposes.

Analysis of low-molecular-weight DNA obtained from G418-resistant cell cultures demonstrated that sequences homologous to pON3 DNA were maintained episomally in T-antigen-producing cell lines (Fig. 5). *neo* transformants of CV-1 and BSC-1 origin contained no detectable pON3-hybridizing sequences in Hirt supernatant DNA (data not shown). In *neo* transformants derived from parental CMT4 or BMT10 cells, amplification of the episomal pON3 sequences occurred on induction of T-antigen synthesis. Inspection of the gel in Fig. 5 reveals the presence of circular DNA forms which are amplifiable to high copy number by heavy-metal induction. The magnitude of this amplification was 50- to 100-fold. Not surprisingly, the copy number of pON3-related sequences in COS1- and BOS4-derived *neo* transformants was unchanged by exposure to heavy metals (data not shown).

When *neo*-transformed cells were withdrawn from G418 selection, pON3 sequences were rapidly lost from cells of COS1, BOS4, and CMT4 origin (Fig. 6). Within 3 weeks after withdrawal of selection, no pON3-related sequences could be detected in *Bgl*I-digested Hirt supernatant DNA from CMT4-derived cell cultures. The episomal sequences in pON3-transformed BOS4 cells persisted for a longer period, but were not detectable at 6 weeks of G418 withdrawal. This is in agreement with previous observations (43) for COS1 cells transformed with pSV2-*gpt*. In contrast to this result, pON3 sequences in BMT10-derived *neo* transformants were relatively stable even after 6 weeks in the absence of G418 selection, although a decline was noted in the *neo*1 and *neo*6 lines.

The data in Fig. 5 and 6 indicate that rearrangements of the input pON3 sequences frequently occurred in the *neo* transformants. In many cases (Fig. 6, BOS4*neo*9, BMT10*neo*1, BMT10*neo*11, and BMT10*neo*M), the DNA isolated from the cells contained molecules of the same size as full-length pON3. However, all lines show significant changes in the sizes of the molecules, which indicates deletion and rearrangement of sequences. Multiple species of replicating molecules were present in many lines (Fig. 5, BMT10*neo*1, BMT10*neo*11, and CMT4*neo*25), although it is unknown whether these different molecules occur in the same or different cells within the population. Some *Bgl*I fragments in Fig. 6 are very small (prominent bands less than 1 kilobase long in BMT10*neo*1 and BMT10*neo*11) and presumably represent partial duplications containing the SV40 *ori* element.

To determine what rearrangements have occurred to generate the different species of pON3-related DNAs, Hirt supernatant DNA was used to transform *E. coli* DH-5 in a plasmid rescue experiment. The genomic arrangement of plasmid sequences in kanamycin-resistant bacteria obtained by this procedure was determined by restriction enzyme digestion of the plasmids and by Southern blotting to hybridization probes specific for SV40 and *neo* sequences (data not

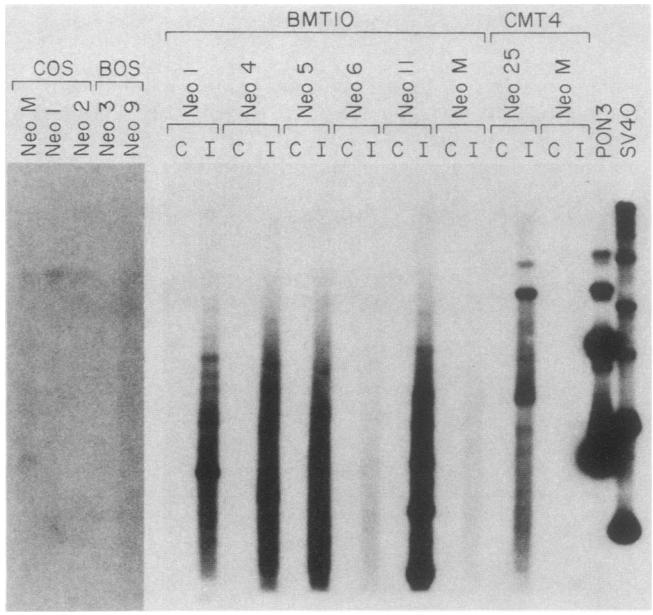

FIG. 5. Analysis of Hirt supernatant DNA in pON3-transformed cell cultures. G418-resistant mass cultures (Neo M) and individual clones were maintained in either normal selective medium (for COS, BOS, and lanes labeled C) or transferred to medium containing inducer (I) for 2 days before Hirt extraction. Uncut DNA from approximately 6×10^5 cells was run on a 0.8% agarose gel, blotted to nitrocellulose, and hybridized to nick-translated pON3 probe. pON3 and SV40 lanes contain the equivalent of about 1,600 copies per cell. The portion of the blot containing the BOS and COS lanes was exposed to film eight times longer to enhance the faint signal which would not otherwise be visible, as the amounts present are comparable to those in the control lanes in the right-hand portion of the figure.

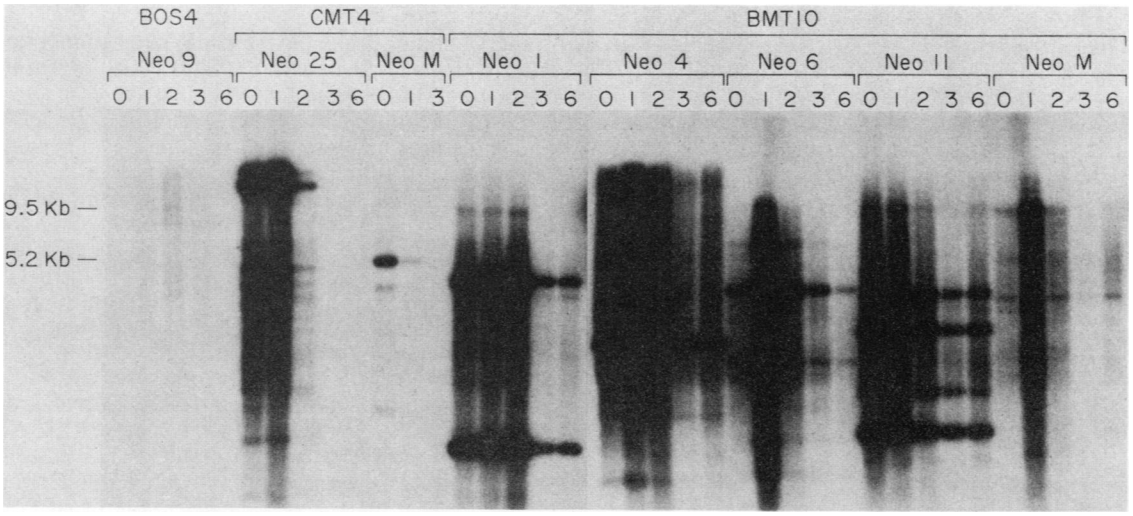

FIG. 6. Persistence of pON3-related episomal DNA in cell cultures after withdrawal from G418 selection. Cloned lines and mass cultures (Neo M) of G418-resistant cells were propagated for the indicated number of weeks in nonselective medium before Hirt supernatant DNA was prepared from cultures which were induced for 2 days with heavy metals. Purified DNA from about 6×10^5 cells was digested with *Bgl*I, run on a 1.1% agarose gel, blotted to nitrocellulose, and hybridized to nick-translated pON3 probe. The film was overexposed to show fainter bands. Quantitative estimates of the copy number range from about 300 copies per cell for BMT10*neo*M cells to about 4,400 copies per cell for BMT10*neo*11 cells. BOS4 lanes contain less than 100 copies per cell.

shown). The results indicate that 11 of 56 rescued plasmids had rearranged, but that most alterations occurred within plasmid vector sequences. This is probably an underestimate of the frequency of deletion of plasmid sequences, since a functional *ori* and kanamycin resistance gene are required for plasmid rescue. The *neo* gene was disrupted by deletion in only 2 of the 56 plasmids analyzed, and the SV40 *ori* was unaffected. There was no evidence for the selective mutation of specific sequences.

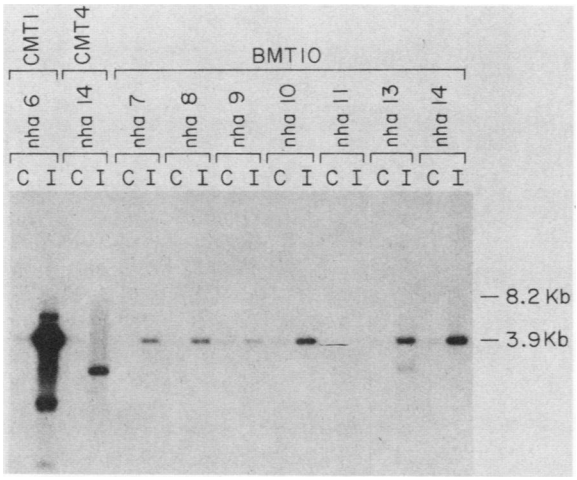

FIG. 7. *Bgl*I restriction analysis of Hirt supernatant DNA from SVHA$^-$*neo*-transformed cell lines. Clones of G418-resistant cells were maintained in either normal selective medium (C) or induced for two days (I) before Hirt extraction. Purified DNA from cells was restricted with *Bgl*I, run on a 1.2% agarose gel, blotted to nitrocellulose, and hybridized to nick-translated pSVHA$^-$*neo* probe. The 3.9-kilobase marker is intact SVHA$^-$*neo* DNA generated by *Bam*HI digestion of pSVHA$^-$*neo* DNA. The Hirt supernatant DNA run on this gel was prepared from the same cell culture used to measure the rate of hemagglutinin synthesis in Fig. 8.

Expression of a protein product from an episomal vector. The influenza virus hemagglutinin gene was cloned into the SV40 late transcription unit to test the expression of a gene carried on an episomally maintained vector. In this recombinant, designated pSVHA$^-$*neo* (Fig. 4), the *neo* gene is expressed off the SV40 early promoter, and an anchor-minus secreted form of hemagglutinin (HA$^-$) is expressed off the SV40 late promoter. Efficient expression of the HA$^-$ protein by pSVHA$^-$*neo* could only be expected to occur if the plasmid vector sequences which separate the HA$^-$ structural gene sequences from the poly(A) addition signal located at the 3′ end of the *neo* gene were first removed. This was accomplished by digestion with *Bam*HI and ligation of the DNA in dilute solution to promote intramolecular ligation of SVHA$^-$*neo* sequences. T-antigen-producing monkey cells were transfected with SVHA$^-$*neo* DNA by calcium phosphate precipitation, and *neo* transformants were selected by G418 treatment without heavy-metal induction. No G418-resistant colonies were observed in COS1 and BOS4 cell cultures. CMT cultures contained a small number of G418-resistant colonies which grew very poorly and proved difficult to establish as clonal populations (only two clones were obtained). By contrast, BMT10 cultures transfected with SVHA$^-$*neo* DNA efficiently produced G418-resistant colonies which were easily propagated as clonal lines.

Hirt supernatant DNAs from the SVHA$^-$*neo* transformants were analyzed by restriction digestion and Southern blotting to determine the state of the transfected DNA (Fig. 7). In the *neo* lines derived from CMT cells, CMT1*nha*6 contained a relatively large amount of episomal DNA represented by several *Bgl*I fragments and CMT4*nha*14 contained predominantly a single, rearranged form. BMT-derived *neo* transformants contained predominantly unrearranged SVHA$^-$*neo* DNA at low copy number. Induction of T-antigen synthesis by heavy metals resulted in the amplification of SVHA$^-$*neo* sequences to higher copy number. Densitometric scanning of the autoradiogram shown in

TABLE 3. Quantitation of episomal SVHA$^-$*neo* DNA and hemagglutinin synthesis in SVHA$^-$*neo*-transformed lines

Cell line	Amt of DNA from[a]:			Amt of HA secreted[b] from:		
	C[c]	I[c]	I/C	C	I	I/C
CMT1*nha*6	25	2,500	100	2	9	4
CMT4*nha*14	15	450	30	ND[d]	ND	
BMT10*nha*7	8	60	7	1	4	4
BMT10*nha*8	8	65	8	2	7	3
BMT10*nha*9	11	35	3	1	5	5
BMT10*nha*10	15	120	8	2	13	6
BMT10*nha*11	ND	ND		ND	ND	
BMT10*nha*13	9	100	11	3	13	4
BMT10*nha*14	15	240	16	3	16	5

[a] Amount of total Hirt DNA expressed as copies per cell. Values were obtained by densitometric scanning of autoradiograms of the gel shown in Fig. 7 and comparison with the signal obtained from a known amount of pSVHA$^-$*neo* DNA.

[b] The relative amount of hemagglutinin labeled and secreted was obtained by scanning the gel shown in Fig. 8 and normalizing to the amount from the BMT10*nha*7 uninduced control.

[c] C, Cells grown under control conditions; I, cells grown in the presence of heavy-metal inducer.

[d] ND, Not detected.

Fig. 7 allowed a quantitative estimate of the copy number increase in these cell lines (Table 3). Even though all cell lines examined contained similar amounts of episomal SVHA$^-$*neo* DNA when uninduced, CMT1*nha*6 and CMT4*nha*14 showed a 30- to 100-fold amplification of the episomal DNA on induction, whereas the increase in BMT10-derived cells was only 3- to 16-fold.

The amount of hemagglutinin protein synthesized and secreted by SVHA$^-$*neo* transformants was determined by immunoprecipitation of [^{35}S]methionine-labeled cell culture medium and gel electrophoresis (Fig. 8). Influenza hemagglutinin was efficiently expressed by all clones except CMT4*nha*4 and BMT10*nha*11, presumably because the sequences are rearranged in CMT4*nha*14 cells and absent from BMT10*nha*11 cells (Fig. 7). The level of expression of the hemagglutinin protein could be increased by heavy-metal induction of T-antigen synthesis. Table 3 shows the quantitation of this increase in HA$^-$ expression determined by densitometric scanning of the autoradiogram. The magnitude of the increase in protein synthesis was three- to sixfold.

DISCUSSION

Fusion of the mouse metallothionein promoter to the early region of SV40 renders expression of T antigen inducible by heavy metals. In a transient assay, T antigen expressed from the chimeric gene was readily detected by immunofluorescence in the presence, but not the absence, of heavy metals. This indicates that cellular controls over mouse metallothionein expression are operative in monkey cells and that the hybrid gene is capable of being efficiently transcribed and translated. This result was anticipated, as previous results (31) have shown that a mouse metallothionein promoter-thymidine kinase gene is transcriptionally regulated by cadmium in human cells.

Synthesis of T antigen in monkey cells transformed with the hybrid gene was inducible to high levels by heavy metals. The rate of synthesis of T antigen by CMT cell lines was higher than that by BMT10 cells, probably because the CMT lines carry five or six integrated copies of the chimeric gene, whereas the BMT10 line contains a single copy (unpublished observations). As mentioned above, the CMT lines are all derived from a single transformed cell. Presumably, the multiple copies in the different CMT lines function independently to give a cumulatively higher rate of T-antigen synthesis that differs from line to line.

CMT and BMT10 cells are examples of a new type of inducible host cell line which synthesizes a gene product in a controlled fashion. Recently, Klessig et al. reported the construction and characterization of human cell lines which inducibly synthesize the adenovirus DNA-binding protein from the glucocorticoid-responsive mouse mammary tumor virus promoter (27). It was necessary to control the synthesis of this gene product, because the protein appears to be toxic to dividing cells when produced at a high level. There is some evidence that high levels of T antigen are also toxic to cells. The *ts*COS cells recently isolated by Rio et al. (38) produce amounts of T antigen similar to that produced by COS7 cells, even though the Rous sarcoma virus promoter driving the *ts*A gene is severalfold stronger than the SV40 early promoter used to express T antigen in COS cells (15). It may be that there was some selection for *ts*COS cell lines which produce a lower level of T-antigen protein. Our unpublished observations about the survival of CMT and BMT10 cells on heavy-metal induction also suggest that T antigen is toxic when synthesized at high levels. After 4 to 5 days of induction, CMT lines exhibit massive cell death, whereas BMT10, COS1, and BOS4 cell lines show no ill effects. Apparently, a rather high level of T antigen can be tolerated, but the level must be controlled if the cells are to remain viable.

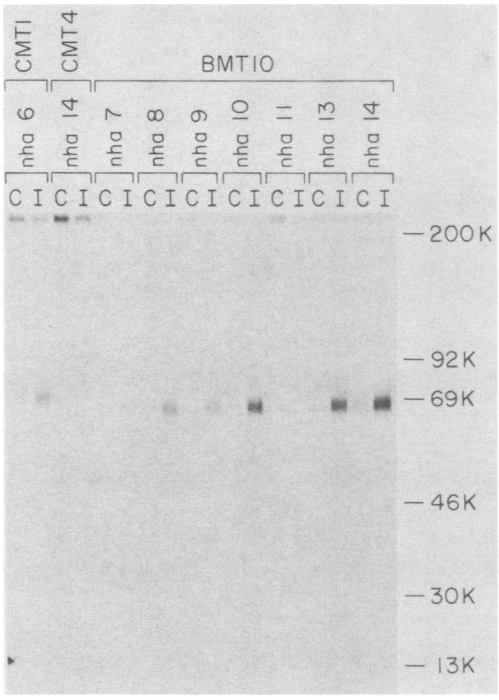

FIG. 8. Rate of hemagglutinin synthesis by SVHA$^-$*neo*-transformed cell lines. Cells maintained in either normal selective medium (C) or medium containing heavy-metal inducer for 2 days (I) were labeled with [^{35}S]methionine as described in Materials and Methods. Cell culture medium was collected, and the secreted hemagglutinin protein was immunoprecipitated and subjected to gel electrophoresis. Each lane represents the HA$^-$ synthesized and secreted by approximately 10^6 cells.

In the experiments described in this paper, there is a direct correlation between the level of T-antigen synthesis and the level of DNA replication. Cell lines which expressed higher levels of T antigen replicated DNA to higher levels than did cell lines synthesizing less T antigen. This was true for the different CMT cell lines and for CMT and BMT10 cell lines which were induced by comparison with uninduced controls. This positive correlation also holds for cell lines stably transformed with SV40 *ori*-containing vectors. Apparently, the amount of T antigen in permissive monkey cells is the limiting factor in terms of the extent of SV40 DNA replication.

As CMT cells replicated SV40 DNA to a much greater extent than did any other cell type examined, they represent an improved system for the transient replication of SV40 *ori*-containing vectors. The increase in replication in CMT cells will facilitate transient expression assays, and these cells may well produce higher titers of early-region defective virus stocks than COS1 cells do because of their more efficient replication of SV40 DNA.

Both CMT and BMT10 cell lines could be transformed with a vector that contains the dominant selectable marker for neomycin resistance and an SV40 *ori*. The higher frequency of transformation of BMT10 cells by *neo* vectors probably reflects the much lower level of T-antigen synthesis compared with that in CMT cells (Fig. 2). COS1 and BOS4 cells are also rarely transformed to G418 resistance, even though they synthesize only about twofold more T antigen than do uninduced BMT cells. This block to stable transformation may be explained by the heterogeneous levels of T-antigen synthesis in the COS1 and BOS4 cell populations (Fig. 1). The level of T-antigen expression in a single cell may vary and allow deleterious replication of *ori*-containing DNA when high levels of T antigen are produced. This may also explain why even the "stable" G418-resistant cell lines that are obtained will rapidly lose their episomal copies on withdrawal of the selective pressure to maintain them (see above) (43). The uniform low level of T-antigen synthesis observed in BMT10 cells is an obvious advantage in obtaining *neo* transformants for gene expression experiments.

The *neo*-transformed cell lines usually maintained the vector episomally as a freely replicating molecule. We observed very few, if any, high-molecular-weight concatemers of the episomal DNA, in contrast to the large quantities of concatemeric DNA observed in pSV2-*gpt*-transformed COS1 cells (44). This difference may be due to the use of different selectable markers or to structural differences in the DNA constructions. The presence of limited quantities of tandemly repeated DNA in SVHA$^-$*neo* transformants was noted. Although we do not know what role these sequences play, it is possible that they can excise intact SVHA$^-$*neo* DNA by homologous recombination and contribute to the pool of episomal molecules.

Rearrangements of the episomal sequences were frequently observed in the pON3-transformed cell lines, but were rarely observed in the SVHA$^-$*neo*-transformed lines. The high frequency of pON3 rearrangements may be due to the larger size of the vector (including more dispensible sequences) or to the presence of bacterial vector sequences. The low frequency of SVHA$^-$*neo* rearrangements could also result from a suppression of recombination by the HA$^-$ sequences.

The episomal *neo* sequences were amplifiable in BMT10- and CMT-derived cells by induction of T-antigen synthesis with heavy metals. Two days of induction resulted in the accumulation of 50- to 100-fold more pON3-related DNA (quantitation not shown). Curiously, the accumulation of episomal DNA in SVHA$^-$*neo* transformants was generally only about 5- to 15-fold greater after 2 days of induction. In a series of experiments designed to understand this discrepancy, it was determined that T-antigen synthesis and function were normal in the SVHA$^-$*neo*-transformed cell lines. Cellular "permissivity factors" were also normal, as the cells could replicate SV40 DNA to high levels. There were also no *cis*-acting defects in the original SVHA$^-$*neo* construction as determined by transient replication of SVHA$^-$*neo* DNA. It appears as if the low level of SVHA$^-$*neo* DNA replication in the BMT transformants is due to a *cis*-acting modification (e.g., methylation) of the DNA which does not permit efficient replication in the transformed cell. Experiments are in progress to determine the nature of this modification. Even though the cells did not replicate large amounts of SVHA$^-$*neo* DNA, expression of the HA$^-$ sequences in the late transcription unit of the episomal vector was observed. Since the expression is dependent to a large extent on the amplification of the vector, only a modest increase in influenza virus HA$^-$ protein was observed on heavy-metal induction. An understanding of the limited replication of the SVHA$^-$*neo* sequences in these cells may enable us to overcome the limitation and allow higher levels of replication and HA$^-$ expression.

Inducible host cells such as those described here should prove valuable for the high-level expression of products deleterious to the cell. CMT and BMT10 cells are a useful host cell system for the propagation of SV40 promoter-enhancer mutations, as there is no potential for recombination with homologous sequences in the genome of these cells (W. Herr, personal communication). In addition, BMT10 cells may be a useful system for establishing permanent cell lines containing a variety of cloned genes in an amplifiable vector.

ACKNOWLEDGMENTS

We thank Joan Cossetti for technical assistance, Mary Jane Gething for pSVEHA20A and rabbit anti-hemagglutinin antiserum, Dean Hamer for pBRMT, Doug Hanahan for *E. coli* DH-5 and pON3, and Ed Harlow for monoclonal antibodies PAb416 and PAb419. We thank Victoria Bautch, Howard Fox, and Winship Herr for a critical reading of the manuscript and Marilyn Goodwin for assistance in its preparation.

This work was supported by Public Health Service grant CA13106 to Cold Spring Harbor Laboratory from the National Cancer Institute. R.D.G. was supported by fellowships from the Damon Runyon-Walter Winchell Cancer Fund (DRG 663) and by National Research Service Award CA07493 from the National Cancer Institute.

LITERATURE CITED

1. **Benchimol, S., D. Pim, and L. Crawford.** 1982. Radioimmunoassay of the cellular protein p53 in mouse and human cell lines. EMBO J. **1**:1055–1062.
2. **Bergsma, D. J., D. M. Olive, S. W. Hartzell, and K. N. Subramanian.** 1982. Territorial limits and functional anatomy of the simian virus 40 replication origin. Proc. Natl. Acad. Sci. USA **79**:381–385.
3. **Brady, J., J. Bolen, M. Radonovich, N. Salzman, and G. Khoury.** 1984. Stimulation of SV40 late gene expression by SV40 T-antigen. Proc. Natl. Acad. Sci. USA **81**:2040–2044.
4. **DiMaio, D., and D. Nathans.** 1982. Regulatory mutants of SV40. Effect of mutations at a T antigen binding site on DNA replication and expression of viral genes. J. Mol. Biol. **156**:531–548.
5. **Durnam, D. M., and R. D. Palmiter.** 1981. Transcriptional regulation of the mouse metallothionein-I gene by heavy metals.

J. Biol. Chem. **256**:5712–5716.
6. **Fromm, M., and P. Berg.** 1983. Simian virus 40 early- and late-region promoter functions are enhanced by the 72-base-pair repeat inserted at distant locations and inverted orientations. Mol. Cell. Biol. **3**:991–999.
7. **Gerard, R. D., B. A. Montelone, C. F. Walter, J. W. Innis, and W. A. Scott.** 1985. Role of specific simian virus 40 sequences in the nuclease-sensitive structure in viral chromatin. Mol. Cell. Biol. **5**:52–58.
8. **Gerard, R. D., M. Woodworth-Gutai, and W. A. Scott.** 1982. Deletion mutants which affect the nuclease-sensitive site in simian virus 40 chromatin. Mol. Cell Biol. **2**:782–788.
9. **Gething, M. J., and J. Sambrook.** 1981. Cell surface expression of influenza haemagglutinin from a cloned cDNA copy of the RNA gene. Nature (London) **293**:620–625.
10. **Gething, M. J., and J. Sambrook.** 1982. Construction of influenza haemagglutinin genes that code for intracellular and secreted forms of the protein. Nature (London) **300**:598–603.
11. **Glanville, N., D. M. Durnam, and R. D. Palmiter.** 1981. Structure of mouse metallothionein-I gene and its mRNA. Nature (London) **292**:267–269.
12. **Gluzman, Y.** 1981. SV40-transformed simian cells support the replication of early SV40 mutants. Cell **23**:175–182.
13. **Gluzman, Y., and B. Ahrens.** 1982. SV40 early mutants that are defective for viral DNA synthesis but competent for transformation of cultured rat and simian cells. Virology **123**:78–92.
14. **Gluzman, Y., R. Frisque, and J. Sambrook.** 1980. Origin-defective mutants of SV40. Cold Spring Harbor Symp. Quant. Biol. **44**:293–298.
15. **Gorman, C. M., G. T. Merlino, M. C. Willingham, I. Pastan, and B. H. Howard.** 1982. The Rous sarcoma virus long terminal repeat is a strong promoter when introduced into a variety of eukaryotic cells by DNA-mediated transfection. Proc. Natl. Acad. Sci. USA **79**:6777–6781.
16. **Gruss, P., and G. Khoury.** 1981. Expression of simian virus 40-rat preproinsulin recombinants in monkey kidney cells: use of preproinsulin RNA processing signals. Proc. Natl. Acad. Sci. USA **78**:133–137.
17. **Hamer, D. H., M. Kaehler, and P. Leder.** 1980. A mouse globin gene promoter is functional in SV40. Cell **21**:697–708.
18. **Hamer, D. H., and P. Leder.** 1979. Expression of the chromosomal mouse β^{maj} globin gene cloned in SV40. Nature (London) **281**:35–40.
19. **Hamer, D. H., and M. J. Walling.** 1982. Regulation *in vivo* of a cloned mammalian gene: cadmium induces the transcription of a mouse metallothionein gene in SV40 vectors. J. Mol. Appl. Genet. **1**:273–288.
20. **Hanahan, D.** 1983. Studies on transformation of Escherichia coli with plasmids. J. Mol. Biol. **166**:557–580.
21. **Harlow, E., L. V. Crawford, D. C. Pim, and N. M. Williamson.** 1981. Monoclonal antibodies specific for simian virus 40 tumor antigens. J. Virol. **39**:861–869.
22. **Harlow, E., D. C. Pim, and L. V. Crawford.** 1981. Complex of simian virus 40 large-T antigen and host 53,000-molecular-weight protein in monkey cells. J. Virol. **37**:564–573.
23. **Hirt, B.** 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. **26**:365–369.
24. **Innis, J. W., and W. A. Scott.** 1984. DNA replication and chromatin structure of simian virus 40 insertion mutants. Mol. Cell. Biol. **4**:1499–1507.
25. **Jongstra, J., T. L. Rendelhuber, P. Oudet, C. Benoist, C.-B. Chal, J.-M. Jeltsch, D. J. Mathis, and P. Chambon.** 1984. Induction of altered chromatin structures by simian virus 40 enhancer and promoter elements. Nature (London) **307**: 708–714.
26. **Keller, J. M., and J. C. Alwine.** 1984. Activation of the SV40 late promoter: direct effects of T antigen in the absence of viral DNA replication. Cell **36**:381–389.
27. **Klessig, D. F., D. E. Brough, and V. Cleghon.** 1984. Introduction, stable integration, and controlled expression of a chimeric adenovirus gene whose product is toxic to the recipient human cell. Mol. Cell. Biol. **4**:1354–1362.
28. **Lassar, A. B., D. H. Hamer, and R. G. Roeder.** 1985. Stable transcription complex on a class III gene in a minichromosome. Mol. Cell. Biol. **5**:40–45.
29. **Lusky, M., and M. Botchan.** 1981. Inhibition of SV40 replication in simian cells by specific pBR322 DNA sequences. Nature (London) **293**:79–81.
30. **Luthman, H., and G. Magnusson.** 1983. High efficiency polyoma DNA transfection of chloroquine-treated cells. Nucleic Acids Res. **11**:1295–1308.
31. **Mayo, K. E., R. Warren, and R. Palmiter.** 1982. The mouse metallothionein-1 gene is transcriptionally regulated by cadmium following transfection into human or mouse cells. Cell **29**:99–108.
32. **McCutchan, J. H., and J. S. Pagano.** 1968. Enhancement of the infectivity of simian virus 40 deoxyribonucleic acid with diethylaminoethyl-dextran. J. Natl. Cancer Inst. **41**:351–357.
33. **Mellon, P., V. Parker, Y. Gluzman, and T. Maniatis.** 1981. Identification of DNA sequences required for transcription of the human α1-globin gene in a new SV40 host-vector system. Cell **27**:279–288.
34. **Moriarty, A. M., B. H. Hoyer, J. W.-K. Shih, J. L. Gerin, and D. H. Hamer.** 1981. Expression of the hepatitis B virus surface antigen gene in cell culture by using a simian virus 40 vector. Proc. Natl. Acad. Sci. USA **78**:2606–2610.
35. **Mulligan, R. C., B. H. Howard, and P. Berg.** 1979. Synthesis of rabbit β-globin in cultured monkey kidney cells following infection with a SV40-β-globin. Nature (London) **277**:108–114.
36. **Myers, R. M., D. C. Rio, A. K. Robbins, and R. Tjian.** 1981. SV40 gene expression is modulated by the cooperative binding of T antigen to DNA. Cell **25**:373–384.
37. **Peden, K. W. C., J. M. Pipas, S. Pearson-White, and D. Nathans.** 1980. Isolation of mutants of an animal virus in bacteria. Science **209**:1392–1396.
38. **Rio, D. C., S. G. Clark, and R. Tjian.** 1985. A mammalian host-vector system that regulates expression and amplification of transfected genes by temperature induction. Science **227**:23–28.
39. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
40. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by agarose gel electrophoresis. J. Mol. Biol. **98**:503–515.
41. **Sveda, M. M., and C.-J. Lai.** 1981. Functional expression in primate cells of cloned DNA coding for the hemagglutinin surface glycoprotein of influenza virus. Proc. Natl. Acad. Sci. USA **78**:5488–5492.
42. **Tooze, J. (ed.).** 1981. DNA tumor viruses. Molecular biology of tumor viruses, 2nd ed. (part 2). Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
43. **Tsui, L.-C., and M. L. Breitman.** 1985. Replication of pSV2-gpt in COS-1 cells: stability of plasmid DNA in the presence and absence of biochemical selection. Somat. Cell Mol. Genet. **11**:167–176.
44. **Tsui, L.-C., M. L. Breitman, L. Siminovitch, and M. Buchwald.** 1982. Persistence of freely replicating SV40 recombinant molecules carrying a selectable marker in permissive simian cells. Cell **30**:499–508.
45. **Van Doren, K., D. Hanahan, and Y. Gluzman.** 1984. Infection of eucaryotic cells by helper-independent recombinant adenoviruses: early region 1 is not obligatory for integration of viral DNA. J. Virol. **50**:606–614.
46. **Wigler, M., A. Pellicer, S. Silverstein, and R. Axel.** 1978. Biochemical transfer of single-copy eukaryotic genes using total cellular DNA as donor. Cell **14**:725–731.
47. **Wigmore, D. J., R. W. Eaton, and W. A. Scott.** 1980. Endonuclease-sensitive regions in SV40 chromatin from cells infected with duplicated mutants. Virology **104**:462–473.