A novel BK virus-based episomal vector for expression of foreign genes in mammalian cells

Arrigo De Benedetti and Robert E.Rhoads*
Department of Biochemistry, University of Kentucky College of Medicine, Lexington, KY 40536, USA

Received October 16, 1990; Revised and Accepted March 12, 1991

ABSTRACT

A composite mammalian cell-*E. coli* shuttle vector was developed based on the human papova virus BK and pSV-neo. The vector contains a dioxin-responsive enhancer (DRE) controlling a mouse mammary tumor virus (MMTV) promoter for the inducible expression of inserted genes. In human cells the vector replicates episomally, presumably utilizing the BKV rather than the SV40 origin, and expresses the BK T/t antigens. A deletion in the late BK region precludes the expression of the core/capsid proteins VP1, VP2, and VP3, thereby preventing the infectious lytic cycle. HeLa cells which were transfected with this vector and selected for resistance to the antibiotic G418 maintained the construct primarily in episomal form during more than one year of continuous culture, with little or no integration into the host genome. Transformed cells cultured in higher concentrations of G418 contained higher copy numbers of the vector. This permits one to vary the dosage of an inserted gene easily and reversibly without the need of conventional amplification techniques and clonal analysis. Using a chloramphenicol acetyl transferase (CAT) reporter gene inserted downstream of the MMTV promoter, we found that CAT expression was greater in clones with higher vector copy number. CAT expression was inducible with 2,3,7,8-tetrachlorodibenzo-p-dioxin, but inducibility was found to be inversely proportional to the copy number. Transformation of bacteria with plasmid molecules retrieved from the mammalian host was efficient, making this vector well adapted for the screening of cDNA libraries for the ability to express a phenotype in mammalian cells. Moreover, DNA sequences were stable during long-term passage in mammalian cells; vector passaged continuously for more than one year retained fully functional bacterial genes for resistance to chloramphenicol and ampicillin.

INTRODUCTION

The development of expression vectors to introduce isolated genes and their derivatives into stable lines of cultured mammalian cells has provided one of the most powerful tools for the study of gene expression and regulation (1−3). Early vectors were based on bacterial plasmids which transformed mammalian cells by rare chromosomal integration events, and selection was generally accomplished with an antibiotic-resistance gene included in the plasmid. This type of plasmid/vector could not replicate within the mammalian cell. Modifications to improve the efficiency of expression were limited to those sequences around the inserted gene, *e.g.*, promoter region, termination-polyadenylation sites, processing signals, and in few instances, translational control sequences (reviewed in 4−6). Disadvantages of these vectors are that the integration process may disrupt the integrity of the foreign DNA segment of interest and that the site of integration may not be favorable for expression.

An alternative to vectors based on bacterial plasmids was provided by vectors of viral origin which replicate episomally. The first vectors of this type contained SV40 sequences, including the origin of replication and other control elements (reviewed in 7). Very high levels of amplification of the gene of interest could be obtained with these vectors in transfected cells (10^6-10^7 copies). Although the SV40 vector system offers a rapid and efficient way to introduce and express foreign DNA into cells, it also has limitations. First, the host range is limited to certain primate cells. Second, transfection of most permissive cells with recombinant SV40 vectors culminates in death, limiting studies to transient periods. One solution to this problem was to generate special cells in which the vector copy number could be regulated by the expression of the SV40 T antigen (T-Ag) under the control of the metallothionein promoter (8). A direct correlation was found between the level of T-Ag synthesis (which could be induced five- to ten-fold upon the addition of heavy metals) and the extent of episomal replication of transfected plasmids containing an SV40 origin. Thus, the lethal effects could be delayed.

Another virus-based expression vector was based on the bovine papilloma virus (BPV; ref. 9). A cloned subgenomic fragment of BPV representing 70% of the virus was very efficient in transforming rodent and other cell types without being infectious. Copies of the viral DNA were found predominantly as extrachromosomal molecules, thus offering a simple method of maintaining foreign DNA in the host cell in non-integrated form. However, the copy number was relatively low (10 to 100 per cell) and, in some cases, insufficient to obtain the desired level of expression.

* To whom correspondence should be addressed

An improvement was obtained in 'third generation' vectors consisting of *composites* of mammalian viruses. Roberts and Weintraub (10) developed vectors containing both SV40 and BPV origins and found that they replicated with characteristics that were roughly intermediate between the two viruses. Thus, cells transformed with these composites maintained approximately 400 copies of plasmid per cell. Composite vectors with SV40 elements were also obtained with subgenomic regions of Epstein Barr virus (11−13), vaccinia (14), and adenovirus (15).

In this article we describe the construction and properties of a different composite vector which is based on SV40 and the human papova virus BKV. After initial isolation (16) and sequencing (17) of BKV, the viral genome, lacking a portion of the late genes which encode the core/capsid proteins, was cloned into a pML plasmid (18). The resulting plasmid (pBK) was found to be maintained in episomal form in several human cell lines, resembling productive infection with the virus. We have combined this plasmid with the early SV40 promoter driving an antibiotic-resistance gene (G418; ref. 19) and the mouse mammary tumor virus (MMTV) promoter under the regulation of a cytochrome P-450 dioxin-responsive element (DRE; ref. 20). The resulting vector has several useful features. It replicates to high copy number, and the copy number can be varied by changing the G418 concentration in the culture medium. The vector remains episomal over long periods in cultured mammalian cells, with integration into the genome occurring only rarely. The vector DNA sequences are stable during long periods of culture in mammalian cells, as indicated by the fact that unselected bacterial antibiotic-resistance genes remain functional. Finally, inserted genes, as exemplified here by chloramphenicol acetyl transferase (CAT), are expressed from a regulated promoter.

MATERIALS AND METHODS

Construction of the vector

The plasmid pSV2-neo (19) was obtained from E. Fuchs, University of Chicago. We modified it by replacing the single *Acc*I site (near the pBR322/SV40-origin boundary) with a *Sal*I linker (Fig. 1). The resulting plasmid harbors a *Sal*I-*Bam*HI fragment containing the SV40 control region, the neomycin-resistance gene and SV40 early/late polyadenylation sites. The BKV-containing plasmid (pBK; ref. 18) was obtained from G. Milanesi, Institute of Biochemical Genetics, National Research Council, Pavia, Italy, I-27100. These authors had cut BKV at the single *Eco*RI (nucleotide 1842) and *Bam*HI (nucleotide 1735) sites and cloned into the corresponding restriction sites of plasmid pML cut with, a deletion variant of pBR322 which does not include mammalian 'poison sequences' (21). We inserted the *Sal*I-*Bam*HI fragment of pSV2-neo between the corresponding sites of pBK and designated the resulting plasmid BK-SVneo.

The plasmid pMcat4.1 (22), which contains the DRE, the MMTV LTR promoter and the CAT gene, was donated by Dr. Phillip Jones, University of Kentucky. An *Eco*RV-*Bam*HI fragment containing these elements, but not the glucocorticoid-responsive element (GRE) of MMTV (22), was ligated into the same sites of Bluescript KS(−) (Stratagene), resulting in a plasmid we termed BS-DRE/CAT.

Next, the pML replicon was removed from BK-SVneo by digesting with *Eco*RI, filling in with DNA polymerase (Klenow), and digesting with *Sal*I. Then plasmid BS-DRE/CAT was digested with *Sal*I and *Eco*RV. The two linearized DNA fragments derived from BK-SVneo and BS-DRE/CAT were ligated together to form the final product, RDB-CAT (Fig. 1). The vector contains T3 and T7 phage promoters on opposite sides of the DRE promoter-CAT gene as well as an M13 origin of replication (not shown).

A similar vector containing the multiple cloning site (*Spe*I-*Sac*I) of the Bluescript polylinker but no CAT gene was constructed in parallel for use in cloning and expression of other genes. This vector is termed RDB-DRE.

Cell transfection, selection and isolation of transformants

HeLa cells (ATCC-CCL2) were obtained from the American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum in 100×20 mm dishes. The cells were washed twice with serum-free medium before addition of a 2-ml suspension containing 20−30 μg of RDB-CAT plasmid in Hepes-buffered saline (HBS; 20 mM Hepes/0.75 mM Na_2HPO_3/140 mM NaCl; adjusted to pH 7.05) and 0.08 M $CaCl_2$. After 20 min, 5 ml of serum-containing medium were added and the cells incubated for 5 h. They were then washed with HBS and allowed to recover for 24 h in fresh medium, after which they were re-plated in 75-cm^2 flasks. The next day, the antibiotic G418 (Gibco) was added at 0.2 mg/ml to select for cells that had acquired the vector. The medium was changed daily for four days (maintaining the G418 concentration) and thereafter once a week. Colonies were picked with Pasteur pipettes as soon as they were easily visible to the naked eye (about three weeks later) and grown in individual dishes. One particular colony was expanded to mass culture and was studied in detail. Cells resistant to higher concentrations of G418 were selected by plating 2×10^6 cells in 75-cm^2 flasks and culturing in higher concentration of G418 until a confluent monolayer was formed. Cell lines maintained at the initial G418 concentration of 0.2 mg/ml are referred to as basal.

Isolation of episomal DNA

Episomal DNA was separated from chromosomal DNA by the Hirt extraction protocol (23) with the following modifications. The cells were resuspended in HBS containing 2 mM EDTA. After pelleting by centrifugation at 800×g, they were resuspended in 0.5 ml lysis buffer [50 mM Tris/0.5% sodium dodecylsulfate (SDS)/0.1 mg/ml proteinase K; adjusted to pH 7.2] and incubated at 37°C for 6 h. The NaCl concentration was then adjusted to 1 M, and the samples were kept on ice overnight. Nucleic acids in the supernatant and pellet fractions (episomal and chromosomal DNA, respectively) were then precipitated and washed with 70% ethanol. The precipitated nucleic acid from the supernatant (episomal) fraction was resuspended in 0.1 ml of H_2O, made 3.5 M in KOAc, and kept on ice for 15 min. After centrifugation for 5 min in a microfuge, the supernatant was extracted with phenol, precipitated with ethanol and washed with 70% ethanol. DNA was finally resuspended in TE (50 mM Tris/2.5 mM EDTA; adjusted to pH 7.2) containing 10 μg/ml RNase A and incubated for 1 h at 37°C.

Isolation of RNA

Preparation of RNA for northern analysis was similar to the isolation of episomal DNA except that the last step was digestion of the sample with 30 ng/ml DNase in the presence of 200 units/ml RNasin (Promega) for 1 h at 37°C. The RNA was then extracted with an equal volume of water-saturated phenol and precipitated with ethanol.

Southern and northern blotting

After electrophoretic separation on 1.2% agarose gels, nucleic acids were affixed to Hybond membranes (Amersham) by forced capillary transfer under reduced pressure in 5×SSC buffer (1×SSC is 0.15 M NaCl, 0.015 M sodium citrate). For Southern blotting analysis, this was immediately followed by a denaturation step in 0.2 M NaOH for 30 min. Annealing or hybridization were carried out at 43°C with nick-translated probes (6) in 6×SSC, 30% formamide, 0.5% SDS, 10 mg/ml dry milk, and 1 mg/ml sonicated salmon sperm DNA. Four washes were carried out for 30 min each in 2×SSC and 0.1% SDS at 65°C followed by a final wash for 15 min in 0.2×SSC at room temperature.

CAT assays

Cell extracts from 1×10^6 RDB-CAT-transformed cells were incubated for 1 h with [1,2-^{14}C]chloramphenicol (10 μM; 10^5 cpm) and 10 mM acetyl coenzyme A in a final volume of 0.1 ml as described by Jones *et al.* (20). Standards for CAT activity (purified bacterial enzyme) were purchased from Boehringer Mannheim (Indianapolis, IN). Acetylated products were separated by silica gel thin layer chromatography and quantitated by scintillation spectrometry.

Protein labeling and affinity chromatography

Control HeLa cells and transformed cells selected with 0.6 mg/ml G418 (10^7 in each case) were incubated with and without 10 nM 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) for 3 h. Proteins were then labeled for 24 h with [3,4,5-^{3}H]leucine (50 μCi/ml). Cells were collected and lysed in 0.15 ml lysis buffer (20 mM Tris-HCl, pH 7.6, 2 mM DTT, 0.3% Brij-58, and 2 μg/ml each of leupeptin and pepstatin). CAT was isolated by a modification of the protocol described by Zaidenzaig and Shaw (24). Cell extract (30 μl, corresponding to 420,000 cpm and 0.15 mg protein) was mixed with an equal volume of chloramphenicol-caproate-agarose beads (Sigma), which had been pre-equilibrated in lysis buffer, and kept on ice for 1 h. Unadsorbed protein was removed with 3 washes of 0.3 ml lysis buffer containing 0.3 M NaCl. Bound CAT was eluted with 20 μl of 50 mM Tris-HCl, pH 7.6, and 5 mM chloramphenicol. The eluted proteins were separated by SDS/PAGE, visualized by fluorography, and the major band (25 kDa) excised and quantitated by scintillation spectrometry.

RESULTS

Elements of the vector which determine copy number

The RDB-CAT vector is depicted in Fig. 1. Several elements contribute in either a positive or negative fashion to determining the extent of vector replication, and hence, to the copy number. First, the vector contains two similar cis-acting sequences containing the origins of replication of BKV and SV40. In bacterial systems it has been shown that plasmids containing more than one origin of replication are frequently inactive; in a few cases one dominant replicon is functional while the other interferes with plasmid replication and decreases copy number (reviewed in ref. 25). A similar negative control of DNA replication was described for BPV-SV40 composites in mammalian cells (10). Presumably this phenomenon is operative in the RDB-CAT vector and prevents excessive (and lethal) replication of the plasmid. Another feature which may limit replication is the metabolic load placed on the cell by the vector-

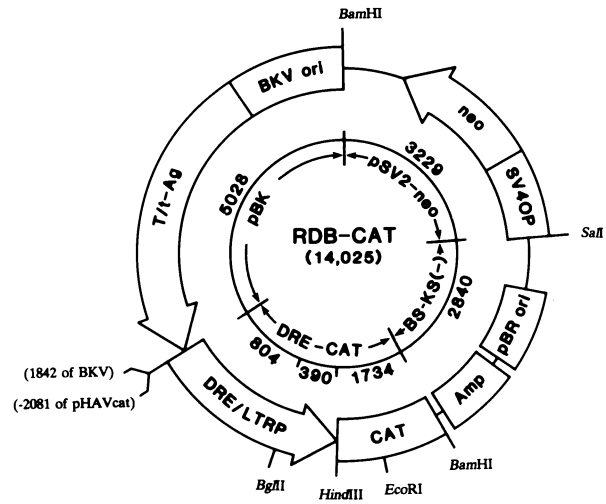

Figure 1. The RDB-CAT vector. Large arrows indicate the direction of transcription. Abbreviations in boxes are: BKV ori, BK virus origin of replication; T/t-Ag, BK virus large and small tumor antigens; DRE-LTRP, dioxin-responsive enhancer and promoter region of the long terminal repeat of mouse mammary tumor virus; CAT, chloramphenicol acetyltransferase gene; Amp, ampicillin-resistance gene; pBR ori, bacterial origin of replication; SV40P, SV40 early promoter; and neo, neomycin-resistance gene (aminoglycoside phosphotransferase). Selected restriction sites at the boundaries of various components are shown. The *Eco*RI and *Eco*RV sites at the junction of the BKV and DRE elements were lost upon fusion. The *Eco*RI site in the CAT gene was used to linearize the construct. The inner circle gives the lengths in nucleotides between the indicated restriction sites and the origins of the various portions.

associated synthesis of DNA, RNA and protein. Hence, cells carrying very high copy numbers of vector will be at competitive disadvantage for growth. Offsetting this disadvantage is a positive selection feature: the expression of the neomycin phosphotransferase gene. This enzyme is poorly expressed from the pSV2-neo plasmid since its mRNA contains several AUG codons in the 5′ untranslated region (26). Therefore, only cells expressing high levels of this mRNA will accumulate sufficient amounts of enzyme. This feature allows for the selection of cell subsets containing a high number of vector copies. Finally, an element working in favor of high copy number is the synthesis of T-Ag. Cells making high levels of vector make high levels of T-Ag, which binds to the BKV origin to further activate DNA replication. The combination of these elements should result in a copy number which allows high level expression of foreign genes but does not cause deleterious effects on the cell. Furthermore, it should allow one to vary the cellular level of a transfected gene in order to observe a graded effect on cell phenotype.

The copy number can be controlled by selection in G418

Transformation of HeLa cells with RDB-CAT typically resulted in 50−200 G418-resistant colonies from a plate containing 3×10^6 cells. One of these colonies was expanded to form a stable line. When this line was cultured in progressively higher concentrations of G418, many of the cells died, but the remaining cells formed new colonies which were resistant to higher G418 concentrations. This observation could best be explained by a natural selection process in which cells maintaining a higher vector copy number were more resistant to the drug than those with fewer copies. This idea was confirmed by Southern analysis

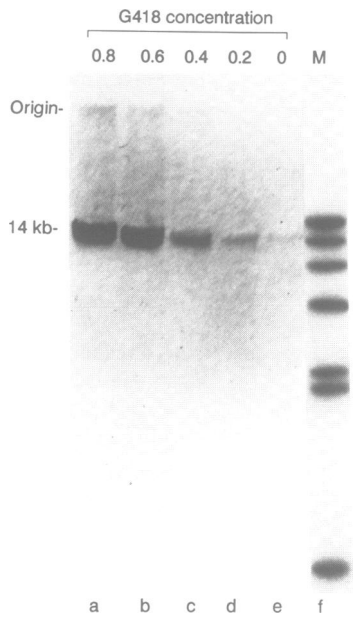

Figure 2. Vector copy number as a function of G418 concentrations. RDB-CAT-transformed cells which had been maintained at the basal G418 concentration (0.2 mg/ml) for several months were subcloned in medium containing 0.4–0.8 mg/ml of G418 (lanes a-c), and thereafter maintained for 1–1.5 months in culture. Other basal cells were removed from G418 selection for three weeks (lane e). In each case, plasmid was isolated from 2×10^7 cells, linearized at the single *Eco*RI site and quantitated by Southern blotting using nick-translated RDB-CAT as probe. The amount of DNA in each lane was estimated by comparison with known quantities of linearized RDB-CAT (grown in bacteria), run in parallel lanes (not shown). From this it was estimated that cells in lane d contained 0.4 μg of plasmid, whereas those in lane a contained 2.6 μg. Based on a molecular weight of 8,864,000, this corresponds to 1350 and 8800 copies per cell, respectively. The positions of the wells (Origin) and of linearized RDB-CAT (14 kb) are indicated. 'M' lane shows the fragments of λ DNA (New England Biolabs) cut with *Hin*dIII and end-labeled with DNA polymerase and [α^{32}P]dATP.

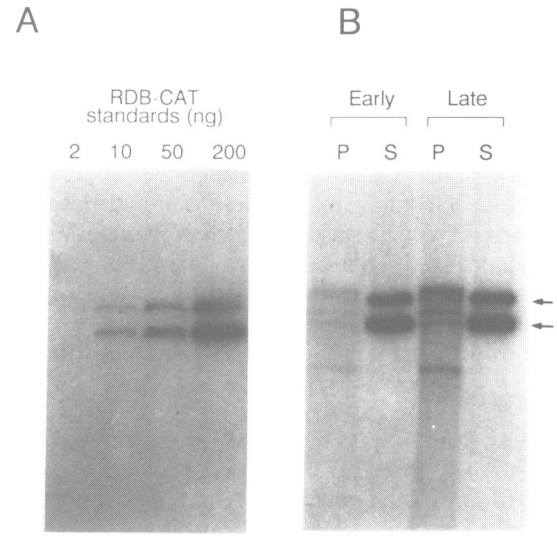

Figure 3. Distribution of RDB-CAT in HeLa cells between episomal and integrated forms. Samples of basal RDB-CAT-transformed cells were frozen after the culture reached confluence ('early'), whereas others were maintained continuously in culture for one year ('late'). DNA was isolated from the Hirt supernatant fraction (S), representing the episomal molecules, and the pellet fraction (P), representing the chromatin, as described in Materials and Methods. Samples were digested with *Bam*HI, which cuts RDB-CAT twice to generate fragments of 7.8 and 6.1 kb (arrows), and analyzed by Southern blotting using nick-translated RDB-CAT as probe. The reason why the 7.8-kb bands in lanes P did not exactly co-migrate with those in lanes S is not known; in other gels the migrations were identical (not shown). A, purified RDB-CAT (grown in bacteria) used as standards. B, chromosomal and episomal fractions. Quantitation of the latter was achieved by comparison to the standards, using several autoradiographic exposures.

of Hirt supernatants of cells maintained in culture with concentrations of G418 ranging from 0 to 0.8 mg/ml (Fig. 2). The results indicated that the vector levels could be varied from 1,350 to 8,800 copies per cell using this G418 selection protocol. When selection was removed, the vector level decreased 3- to 6-fold (Fig. 2, lanes d *vs.* lane e). At least some cells, however, remained resistant to basal concentrations of G418 even after months of culture in non-selective medium (data not shown). It is not known whether this was due to a persistent low copy number of episomal vector or to its integration into the chromosome.

We obtained similar results with human osteosarcoma (HOS) and African monkey (COS1) cells (data not shown). With HOS cells, the copy number was somewhat lower (400 copies per cell) than with HeLa cells. The copy number was not determined for COS1 cells, but a transformation efficiency of 30% was observed, which is remarkably higher than that obtained with HeLa cells, presumably due to the endogenous expression of SV40 T-Ag.

Distribution of the vector between integrated molecules and free episomes

A problem with some other composite papova-based vectors has been integration into the host chromosomal DNA (13,27). To test whether the RDB-CAT vector was maintained in episomal or integrated form, the experiment summarized in Fig. 3 was performed. Early cells, frozen as soon as a stable line was obtained, were compared with late cells, which had been maintained continuously in culture for one year. Episomal and chromosomal DNA were prepared, digested with *Bam*HI, and analyzed by Southern blotting. Serial dilutions of the *Bam*HI-digested RDB-CAT plasmid provided a standard for quantitation (panel A). As shown in panel B, the same amount of plasmid was obtained from early and late cells, indicating that the copy number of free episomes remains constant for at least one year. Furthermore, in early cells, approximately 95% of the RDB-CAT sequences were episomal ('Early', *cf.* lanes P and S). Even in late cells, the great majority of RDB-CAT sequences (80%) remained episomal.

Examination of the *Bam*HI fragments reacting with the probe in chromosomal DNA revealed a small number of discrete bands. Two were the size of the *Bam*HI fragments of RDB-CAT. These fragments are presumably derived from two types of integration events, one in which a random break occurred in the 7.8-kb fragment, yielding an integrated copy containing an intact 6.1-kb fragment, and one in which the break occurred in the 6.1-kb fragment, yielding an intact 7.8-kb fragment. The other fragments seen in Fig. 3B, lanes P, are likely to reflect the distance between one *Bam*HI site in an integrated copy of RDB-CAT and the nearest *Bam*HI site in the chromosomal DNA. The fact that the pattern of bands is so simple indicates that only a small number of integration events occurred. Also, the pattern is no more complex in late cells than in early cells, which means that integration did not occur continuously over the one-year culture period. There are several possible explanations for the fact that late cells apparently contain more integrated RDB-CAT sequences

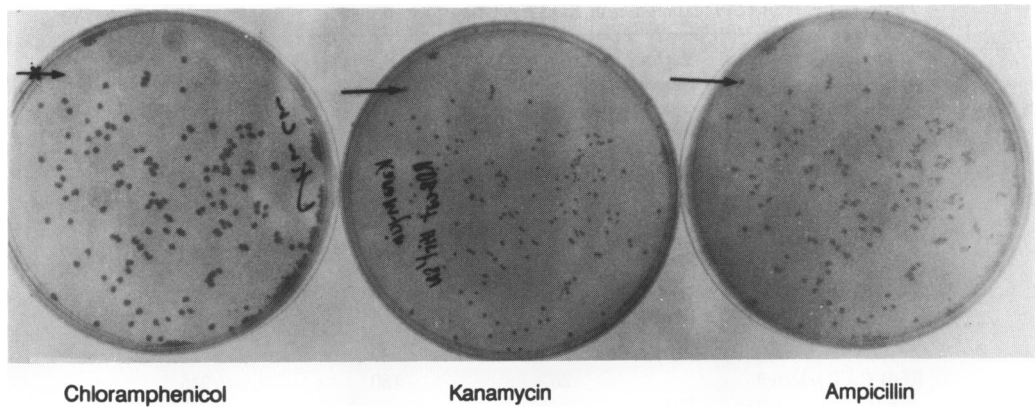

Figure 4. Transformation of bacteria with Hirt supernatants of RDB-CAT transformed HeLa cells. Basal line cells (10^7) were lysed and plasmid DNA was isolated. Competent JM101 cells were transformed with 1/5 of the preparation (6) and colonies were scored on plates containing 50 μg/ml kanamycin. The colonies were replica-plated on ampicillin (150 μg/ml) and chloramphenicol (15 μg/ml) plates. The arrow indicates the position of the single colony present on kanamycin and ampicillin plates but missing from the chloramphenicol plate.

than early cells: (i) the progenitor cell giving rise to the line did not originally contain integrated copies, but this happened subsequently in a daughter cell, and its progeny containing integrated copies outgrew those cells with only episomal copies; ii) the progenitor cell contained integrated copies which were amplified by tandem duplication in subsequent generations (28,29); iii) an increase in a population of large, rearranged episomal molecules wihch arose by head-to-tail ligation, and partitioned with the chromosomal DNA in the Hirt procedure (29). In the latter situation, the pattern in Fig. 3B, lanes P, would not represent integrated material. In any case, the implication is that integration, if it occurred at all, occurred rarely, at an early stage, and did not continue throughout the one-year culture period.

Transformation of bacteria with Hirt supernatants of RDB-CAT cells

The Southern blotting experiment in Fig. 2 indicated that the basal line of RDB-CAT-transformed HeLa cells contained approximately 0.2 g of vector DNA per 10^7 cells. Thus, it should be possible to obtain significant numbers of transformed bacterial cells from a Hirt supernatant of mammalian cells. This is demonstrated in the experiment shown in Fig. 4. Transformation of *E. coli* JM-101 with the Hirt supernatant from 2×10^6 RDB-CAT-transformed HeLa cells produced approximately 100 colonies on kanamycin plates (Fig. 4, center). As a control, bacteria were transformed in parallel with purified RDB-CAT, resulting in an efficiency of 10^3 colonies/μg DNA (data not shown). From these results, one can calculate that the Hirt supernatant from 10^7 mammalian cells contains 0.5 μg of vector DNA. This is similar to the estimate of 0.2 μg obtained by Southern blotting (Fig. 2). This experiment indicates the ease of obtaining transformed bacteria using the vector from relatively few mammalian cells, and it also provides a rapid and non-radioactive method for assaying vector copy number.

The ability to transform bacteria with plasmid molecules extracted from mammalian cells also provided a way to test the structural integrity of the vector, since bacterial elements of the construct other than the *neo* gene were not selected for. The results shown in Fig. 4 indicate that replica-plating on ampicillin and chloramphenicol yielded the same colonies as were observed

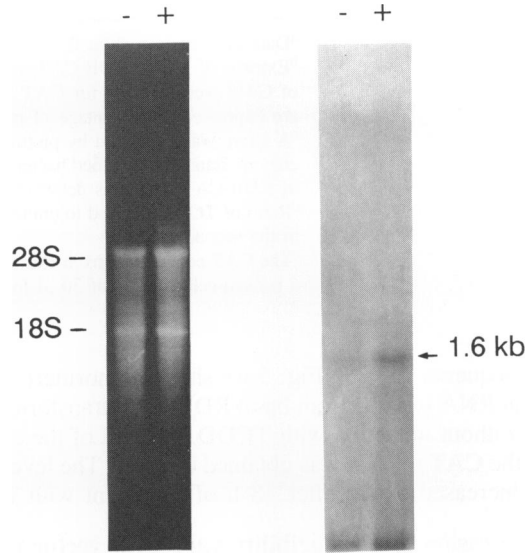

Figure 5. Northern analysis of CAT mRNA. Basal line cells (10^7) were incubated with (+) and without (−) 10 nM TCDD for 18 h. Total RNA was extracted and 25 μg were denatured with glyoxal/DMSO (31) and separated on a 1.3% agarose gel. RNA was transferred to a HyBond membrane (Amersham) and probed with nick-translated BS-DRE/CAT plasmid. *Left*, gel stained with ethidium bromide. *Right*, northern analysis. The position of the 1.6-kb CAT mRNA, based on the mobility of rRNAs, is indicated.

on the kanamycin plate, with the exception of one colony missing on the chloramphenicol plate (arrow). This experiment was repeated with cells grown continuously for various lengths of time, some more than one year, with similar results. Thus, the vast majority of episomal molecules in the RDB-CAT-transformed HeLa cells were stable. In the rare cases where colonies failed to grow on one antibiotic, restriction analysis revealed that rearrangements, rather than point mutations, had occurred.

Expression of CAT mRNA in transfected HeLa cells

The CAT gene in the RDB-CAT construct would be expected to encode a 1.6-kb mRNA terminating with the SV40 early

Table 1. Level of CAT expression in RDB-CAT-transformed cells

Sample	Vector concentration[a] *copies/cell*	Chloramphenicol Acetylated[b] %	CAT in assay[c] *ng*	TCDD induction[d] fold	[CAT] in cells[e] *nm*
Bacterial CAT		1.3	3		
		4.3	15		
		13	57		
		34	225		
		53	900		
RDB-CAT selected in 0.2 mg/ml G418	1350	5.4	20		27
Induced w/ TCDD		23.5	130	6.5	175
RDB-CAT selected in 0.4 mg/ml G418	2500	26	180		245
Induced w/ TCDD		51	760	4.2	1025
RDB-CAT selected in 0.6 mg/ml G418	6500	31	200		270
Induced w/ TCDD		48	600	3.0	810
RDB-CAT selected in 0.8 mg/ml G418	8800	51	760		1025
Induced w/ TCDD		62	1140	1.5	1540

[a]Data derived from Fig. 2.
[b]Extracts of 1×10^6 RDB-CAT-transformed HeLa cells cultured in the indicated concentrations of G418 were assayed for CAT activity as described in Materials and Methods. The results are expressed as percentage of input radioactivity.
[c]A curve was generated by plotting% acetylated chloramphenicol *vs.* the indicated amount of enzyme standards (purified bacterial CAT). From this curve the CAT level in different samples of RDB-CAT cells was determined by interpolation.
[d]Ratio of TCDD-induced to uninduced CAT concentration, calculated from the results obtained in the second column.
[e]The CAT concentrations in extracts was used to calculate the intracellular concentration, using a packed-cell volume of 30 μl for 10^6 cells.

poly(A) sequence (30). In Fig. 5 we show the northern analysis from total RNA isolated from basal RDB-CAT-transformed cells with or without induction with TCDD. A band of the expected size for the CAT mRNA was obtained (arrow). The level of this mRNA increased 5-fold after 18 h of treatment with TCDD.

CAT expression and inducibility vary with vector copy

The activity and inducibility of CAT expression by TCDD were determined as a function of vector copy number (Table 1). The levels of CAT activity were greater in extracts of cells maintained at higher G418 concentrations. As demonstrated above (Fig. 2), these cells contained more copies of the RDB-CAT vector. CAT activity increased in cells treated with TCDD for 24 h. Unexpectedly, however, the TCDD inducibility was different in cells maintained on different concentrations of G418. CAT activity in cells with the lowest vector copy number was induced 6.5-fold, whereas that in cells with the highest vector copy number was induced only 1.5-fold.

We also quantitated the amount of CAT protein as percentage of total cellular protein in RDB-CAT cells grown in 0.6 mg/ml G418. CAT from labeled cell extracts was purified by affinity chromatography and SDS/PAGE as described in Material and Methods. This purification yielded 2280 cpm of CAT for uninduced cells, and 7010 cpm for TCDD-induced cells. Since the labeling protocol was to equilibrium, all the proteins had the same specific radioactivity. Assuming that recovery from the affinity resin was quantitative, CAT represented 0.6% and 1.7% of total cellular protein for uninduced and induced cells, respectively. This level of induction (3-fold) is consistent with the results obtained by enzymatic assays (Table 1; 0.6 mg/ml G418).

DISCUSSION

The composite episomal shuttle vector described here has several favorable properties. One of these is the capacity to regulate the plasmid copy number simply by changing the concentration of G418 in the culture medium. Gerard *et al.* (8) were also able to demonstrate regulation of vector copy number with an SV40 construct in which T-Ag expression was under control of a metallothionein promoter. That system is somewhat more limited than the one described here, since it can be expressed only in COS cells and requires the use of heavy metals for induction, which may have additional effects on cellular metabolism. Similarly, vectors using GRE-inducible promoters may produce unexpected effects because of the large number of genes regulated by glucocorticoids. The RDB vector, by contrast, has been used effectively in three types of mammalian cells, and the inducer of its regulated promoter, TCDD, has no detectable effect on cultured cells other than inducing cytochrome P-450-like genes (32). The strength and inducibility of this promoter were directly compared to those of the GRE-driven promoter of MMTV in plasmids containing both elements (22). In all cases, TCDD inducibility was found to be 5- to 10-fold, the overall expression being comparable to or better than that obtained with dexamethasone. The use of TCDD may initially appear to be a disadvantage because of this compound's hazard to health (33). However, the amounts needed for full induction of the promoter

(1–10 nM) are very low. We carried out experiments continuously over a two-year period with only 1 mg of TCDD. The level of TCDD which is hazardous to humans is currently in question (34). Extrapolation from animal studies indicates that humans may safely ingest 70 ng of TCDD per day. This corresponds to daily ingestion of 20–200 ml of cell culture medium containing TCDD. Even this is likely to be an underestimate of the maximum safe dose since humans appear to be less sensitive to TCDD than the test animals (34). Therefore, the use of TCDD under controlled laboratory conditions for this type of research can hardly be considered hazardous.

Another advantage of the vector described here is the high level of free episomal molecules and low degree of integration, as compared to other episomal vectors. This property makes the vector ideally suited for preparation of cDNA libraries in bacteria followed by phenotype screening in mammalian cells. The gene of interest could then be re-cloned in bacteria by transformation with Hirt supernatants of selected mammalian clones. For this purpose, the M13 origin should also enable one to generate ssDNA from isolated clones for direct sequencing of expressed cDNAs, and the T7 and T3 promoters would allow the production of strand-specific probes and synthetic mRNAs. Several vectors have been employed in the past for phenotype screening (13,35,36), but in most cell types they integrate after a few generations and do not permit retrieval of episomal molecules (27).

The RDB vector also displays remarkable structural integrity, based on the fact that nonselected bacterial resistance markers were preserved in HeLa cells, even after prolonged culture. This is an important property for both the screening of cDNA libraries by expression in mammalian cells, and for maintenance of a given phenotype over long periods of time in culture.

The finding that the DRE-MMTV promoter became progressively more 'leaky' as vector copy number increased (Table I) may be due to properties of the DRE itself. TCDD activates transcription through a receptor protein(s) designated Ah (37). Recently, several groups have shown that deletion of sequences within the DRE caused enhancement of constitutive synthesis from a linked promoter, suggesting that this system may be under repression (37–39). Direct evidence that cytochrome P-450IA1 is under repression was recently shown for human keratinocytes (40). Thus, TCDD may act as a derepressor. If so, DRE sequences in cells containing high levels of RDB-CAT may titrate out a putative repressor protein, leading to derepression of the promoter and constitutive synthesis of CAT. Alternatively, the basal constitutive expression of CAT may simply be a function of the vector copy number. The level of expression in the presence of TCDD, however, may be limited because transcription of the promoter is saturated. Regardless of the explanation, it is clear that one may obtain immediate and substantial increases in expression upon TCDD addition, especially if the vector copy number is relatively low.

ACKNOWLEDGEMENTS

The authors are indebted to Drs. E. Fuchs, G. Milanesi, and P. Jones for donating vector elements and to Dr. L. Robertson, Graduate Center for Toxicology of the University of Kentucky, for helpful suggestions on the use of TCDD and its potential hazards. This work was supported by Grant No. GM20818 from the National Institute of General Medical Sciences and by funds from the Office of the Vice Chancellor for Research and Graduate Studies, University of Kentucky Medical Center.

REFERENCES

1. Goff, S.P. and Berg, P. (1979) *J. Mol. Biol.* **133**, 359–383.
2. Hamer, D.H., Smith, K.D., Boyer, S.H. and Leder, P. (1979) *Cell* **17**, 725–735.
3. Mulligan, R.C. and Berg, P. (1980) *Science* **209**, 1423–1427.
4. Rigby, P.W.J. (1983) *J. Gen. Virol.* **64**, 255–266.
5. Pouwels, P.H., Enger-Valk, B.E., Brammar, W.J., eds. (1985) *Cloning Vectors*, Elsevier, New York.
6. Sambrook, J., Fritsch, E.F., Maniatis, T., eds. (1989) *Molecular Cloning*, Cold Spring Harbor Laboratory Press, New York.
7. Gluzman, Y., ed. (1982) *Eukaryotic Viral Vectors*, Cold Spring Harbor Laboratory Press, New York.
8. Gerard, R.D. and Gluzman, Y. (1985) *Mol. Cell. Biol.* **5**, 3231–3237.
9. Saver, N., Grus, P., Law, M.F., Khoury, G. and Howley, P. (1981) *Mol. Cell. Biol.* **6**, 486–496.
10. Roberts, J.M. and Weintraub, H. (1986) *Cell* **46**, 741–752.
11. Sugden, B., Marsh, K. and Yates, J. (1985) *Mol. Cell. Biol.* **5**, 410–413.
12. Heinzel, S.S., Krysan, P.J., Calos, M.P. and DuBridge, R.B. (1988) *J. Virol.* **62**, 3738–3746.
13. Margolskee, R.F., Kavathas, P. and Berg, P. (1988) *Mol. Cell. Biol.* **8**, 2837–2847.
14. Fuerst, T.R., Earl, P.L. and Moss, B. (1986) *Mol. Cell. Biol.* **7**, 2538–2543.
15. Kaufman, R.J., Davies, M.V., Pathak, V.K. and Hershey, J.W.B. (1989) *Mol. Cell. Biol.* **9**, 946–950.
16. Gardner, S.D., Field, A.M., Coleman, D.V. and Hulme, B. (1971) *Lancet* **1**, 1253–1257.
17. Seif, I., Khoury, G. and Dhar, R. (1979) *Cell* 18, 963–977.
18. Milanesi, G., Barbanti-Brodano, G., Negrini, M. Lee, D., Corallini, A., Caputo, A., Grossi, M.P. and Ricciardi, R.P. (1984) *Mol. Cell. Biol.* **4**, 1551–1560.
19. Southern, P.J and Berg, P. (1982) *J. Mol. & App. Gen.* 1, 327–341.
20. Jones, P.B.C., Galeazzi, D.R., Fisher, J.M., and Whitlock, J.P. (1985) *Science* **227**, 1499–1502.
21. Lusky, M. and Botchan, M. (1981) *Nature* **293**, 79–81.
22. Jones, P.B.C., Durrin, L.K., Galeazzi, D.R., and Whitlock, J.P. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 2802–2806.
23. Hirt, B. (1967) *J. Mol. Biol.* **26**, 365–369.
24. Zaidenzaig, Y. and Shaw, W.V. (1976) *FEBS Letters* **62**, 266–271.
25. Austin, S. and Nordstrom, K. (1990) *Cell* **60**, 351–354.
26. Chen, C. and Okayama, H. (1987) *Mol. Cell. Biol.* **7**, 2745–2752.
27. Heller, R.A., Song, K., Villaret, D., Margolskee, R., Dunne, J., Hayakawa, H. and Ringold, G.M. (1990) *J. Biol. Chem.* **265**, 5708–5717.
28. Della Valle, G., Fenton, R.G. and Basilico, C. (1981) *Cell* 23, 347–355.
29. Steinberg, V.I. and Norkin, L.C. (1988) *Virology* **163**, 420–428.
30. Carswell, S. and Alwine, J.C. (1989) *Mol. Cell. Biol.* **9**, 4248–4258.
31. De Benedetti, A., Williams, G.H. and Baglioni, C. (1985) *J. Virol.* **54**, 408–413.
32. Knutson, J.C. and Poland, A. (1980) *Toxicol. & Appl. Pharm.* 54, 377–383.
33. Kimbrough, R.D. (1987) *Annu. Rev. Pharmacol. Toxicol.* **27**, 87–111.37.
34. Cordle, F., in *Accidental exposure to Dioxins. Human Health Aspects*, Coulston, F., and Pocchiari, F., eds., 1983, Academic Press, New York, NY, p. 253.
35. Okayama, H. and Berg, P. (1983) *Mol. Cell. Biol.* **3**, 280–289.
36. Vasavada, H.A., Ganguly, S., Chorney, M., Mathur, R., Shukla, H., Swaroop, A. and Weissman, S.M. (1990) *Nucl. Acid Res.* **18**, 3668.
37. Fisher, J.M., Wu, L., Denison, S. and Whitlock, J.P. (1990) *J. Biol. Chem.* **265**, 9676–9681.
38. Neuhold, L.A., Shirayoshi, Y., Ozato, K., Jones, J., and Nebert, D.W. (1989) *Mol. Cell. Biol.* **9**, 2378–2386.
39. Yanagida, A., Sogawa, K., Yasumoto, K.I and Fujii-Kuriyama, Y (1990) *Mol. Cell. Biol.* **10**, 1470–1475.
40. Berhard, A., Gradin, K. and Toftgard, R. (1990) *J. Biol. Chem.* **265**, 21086–21090.