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

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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 (105–107 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.

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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. 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 AccI site (near the pBR322/SV40-origin boundary) with a SalI linker (Fig. 1). The resulting plasmid harbors a SalI-BamHI fragment containing the SV40 control region, the neomycin-resistance gene and SV40 early/late polyadenylation sites. The fragment containing these elements, but not the glucocorticoid-responsive element (GRE) of MMTV (22), was ligated into the fragment of pSV2-neo between the corresponding sites BamHI (nucleotide 1735) and 0.08 M CaCl₂ (near the pBR322/SV40-origin boundary) with a SalI linker (Fig. 1). The resulting plasmid was termed RDB-DRE/CAT.

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 H₂O, 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 then 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.

Preparation 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 [1,2-14C]chloramphenicol (10 μCi; 10^6 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 [3,4,5-3H]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-capped 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-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.
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 BamHI, and analyzed by Southern blotting. Serial dilutions of the BamHI-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 BamHI fragments reacting with the probe in chromosomal DNA revealed a small number of discrete bands. Two were the size of the BamHI 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 BamHI site in an integrated copy of RDB-CAT and the nearest BamHI 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.
Chloramphenicol
Kanamycin
Ampicillin

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.

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.

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 $2 \times 10^6$ 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 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

<table>
<thead>
<tr>
<th>Sample</th>
<th>Vector concentrationa copies/cell</th>
<th>Chloramphenicol Acetylatedb %</th>
<th>CAT in assayc ng</th>
<th>TCDD inductionid fold</th>
<th>[CAT] in cells e nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacterial CAT</td>
<td>1.3</td>
<td>3</td>
<td>180</td>
<td>245</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4.3</td>
<td>15</td>
<td>34</td>
<td>225</td>
<td></td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>57</td>
<td>53</td>
<td>900</td>
<td></td>
</tr>
<tr>
<td>RDB-CAT selected</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>in 0.2 mg/ml G418</td>
<td>1350</td>
<td>5.4</td>
<td>20</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>Induced w/ TCDD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RDB-CAT selected</td>
<td>26</td>
<td>180</td>
<td>245</td>
<td></td>
<td></td>
</tr>
<tr>
<td>in 0.4 mg/ml G418</td>
<td>2500</td>
<td>51</td>
<td>760</td>
<td>4.2</td>
<td>1025</td>
</tr>
<tr>
<td>Induced w/ TCDD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RDB-CAT selected</td>
<td>31</td>
<td>200</td>
<td>270</td>
<td></td>
<td></td>
</tr>
<tr>
<td>in 0.6 mg/ml G418</td>
<td>6500</td>
<td>48</td>
<td>600</td>
<td>3.0</td>
<td>810</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RDB-CAT selected</td>
<td>51</td>
<td>760</td>
<td>1025</td>
<td></td>
<td></td>
</tr>
<tr>
<td>in 0.8 mg/ml G418</td>
<td>8800</td>
<td>62</td>
<td>1140</td>
<td>1.5</td>
<td>1540</td>
</tr>
<tr>
<td>Induced w/ TCDD</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

aData derived from Fig. 2.
bExtracts of 1×10⁶ 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.
cA 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.
dRatio of TCDD-induced to uninduced CAT concentration, calculated from the results obtained in the second column.
eThe CAT concentrations in extracts was used to calculate the intracellular concentration, using a packed-cell volume of 30 µl for 10⁶ cells.

The level of CAT expression and inducibility vary with vector copy number. 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 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 comparable 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 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.
(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.

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