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Genetics

Expression and regulation of a human metallothionein gene carried on an autonomously replicating shuttle vector

(gene expression/gene transfer/mammalian plasmid/bovine papillomavirus/heavy metals)

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ABSTRACT A human metallothionein (MT) gene was inserted into a bovine papillomavirus (BPV) vector. The chimeric vector (pMTII-BPV) transforms rodent fibroblasts to a cadmium-resistant phenotype. The resistance is due to the high level of expression of human *MT-II* in those cells. The vector is maintained in the cells as a free replicating plasmid, present at about 10–15 copies per cell. Transcription of the episomal human $MT\text{-}II_A$ gene is initiated from its authentic start sites and is regulated by the level of cadmium in the growth medium. The presence of the human $MT\text{-}II_A$ gene allows the BPV replicon to function even though it is ligated to an intact copy of pBR322. Due to the presence of plasmid origins of replication and dominantly acting selective markers functional in both *Escherichia coli* and mammalian cells, pMTII-BPV can be used as a shuttle vector.

Metallothioneins (MTs) are low molecular weight heavy metal-binding proteins. They are responsible for protection of living cells against the toxic effects of heavy metals (1). In cultured human cells, MTs are induced by exposure to heavy metal ions (2) or glucocorticoids (3). The increase in protein levels is mediated through increased synthesis of MT mRNA (4–6), which is due to increased transcription rates after treatment with either heavy metals or glucocorticoids (7, 8). To gain a better understanding of the molecular mechanisms controlling the expression of human *MT* genes, we have isolated a cDNA clone of human *MT-II* mRNA (9) and used it as a hybridization probe to isolate at least 12 different human *MT* genes from a genomic DNA library (10). We chose to study in detail the expression of the $MT\text{-}II_A$ gene (11) because it codes for the most abundantly expressed human MT subtype (12).

After introduction of the human $MT\text{-}II_A$ gene into Rat 2 cells by cotransfer, we noticed a wide variation in its level of expression and the type of regulation (unpublished data). The variation in levels of expression is probably due to random integration at variable distances from endogenous cellular "enhancer" sequences (13). To study the expression of the human $MT\text{-}II_A$ gene without interference from neighboring regulatory sequences, we have inserted the gene into a bovine papillomavirus (BPV) vector (14). BPV genomic DNA and various vectors derived from it have the unique property of being able to replicate autonomously as episomes in mammalian cells (15). After introduction of MT–BPV vectors into rodent fibroblasts, the human $MT\text{-}II_A$ gene is expressed and regulated in identical fashion in different transfected cell lines. Due to the high level of expression after heavy metal induction, the transfected cells containing the vector can be selected directly for cadmium resistance (Cd^r). In the past, it has been observed that physical linkage of pBR322 sequences to the subgenomic transforming fragment of BPV leads to a severe (to <1%) decrease in transformation efficiency by the virus (16). Interestingly, the presence of the human $MT\text{-}II_A$ gene in the vector allows replication, in mammalian cells, of BPV linked to pBR322.

MATERIALS AND METHODS

Restriction enzymes were used according to the manufacturer's recommendations (Bethesda Research Laboratories). A 3.0-kilobase (kb) *Hin*dIII fragment containing the human $MT\text{-}II_A$ gene, including 0.8 kb of the 5′ flanking region (11), was isolated by electrophoresis on a 1% agarose gel and electroelution. $pBPV_{69T}$DNA [kindly supplied by P. Gruss (National Institutes of Health); see ref. 14] was linearized with *Hin*dIII, dephosphorylated, and ligated to the *MT-II Hin*dIII fragment. Recombinant plasmids were used to transform *Escherichia coli* K-12 host (strain RRI). The desired recombinants were identified by restriction enzyme analysis of plasmid DNA isolated from individual colonies (17). All recombinant DNA procedures were performed according to National Institutes of Health guidelines.

C127 (18), NIH 3T3, or Rat 2 cells, at a density of 10^6 cells per 100-mm plate, were incubated with a calcium phosphate/DNA coprecipitate (19) for 16–18 hr and then for an additional 48 hr in normal growth medium (Dulbecco modified Eagle medium plus 10% fetal calf serum). Selection was in medium containing 20 μM Cd Cl_2 and $ZnCl_2$.

Cells were harvested by trypsinization and lysed with Nonidet P-40 (6). Nuclei were pelleted and used for isolation of either total cellular (20) or episomal DNA (21). Total cellular RNA was isolated from the cytosols as described (6). DNA was analyzed by electrophoresis on 1% agarose gels and blot hybridization (22). RNA was denatured by glyoxalation and analyzed on 1.5% agarose gels according to Thomas (23). Hybridization probes were prepared by nick-translation (24). Hybridization and washing procedures were as described by Wahl *et al.* (25). Mung bean nuclease mapping was performed as described (11).

RESULTS

Transformation and Selection of Cd^r Cells. For our initial experiments we chose the plasmid vector pMTII-BPV(−) (Fig. 1). In those experiments, vector DNA was digested with *Sal* I prior to transfection to achieve physical separation of the fragment containing both MTII and BPV sequences from pBR322 sequences in order to achieve efficient transformation (16). C127 mouse fibroblast cells were transfected with either *Sal* I-di-

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Abbreviations: BPV, bovine papillomavirus; MT, metallothionein; Cd^r, cadmium resistance or cadmium-resistant; kb, kilobase(s).

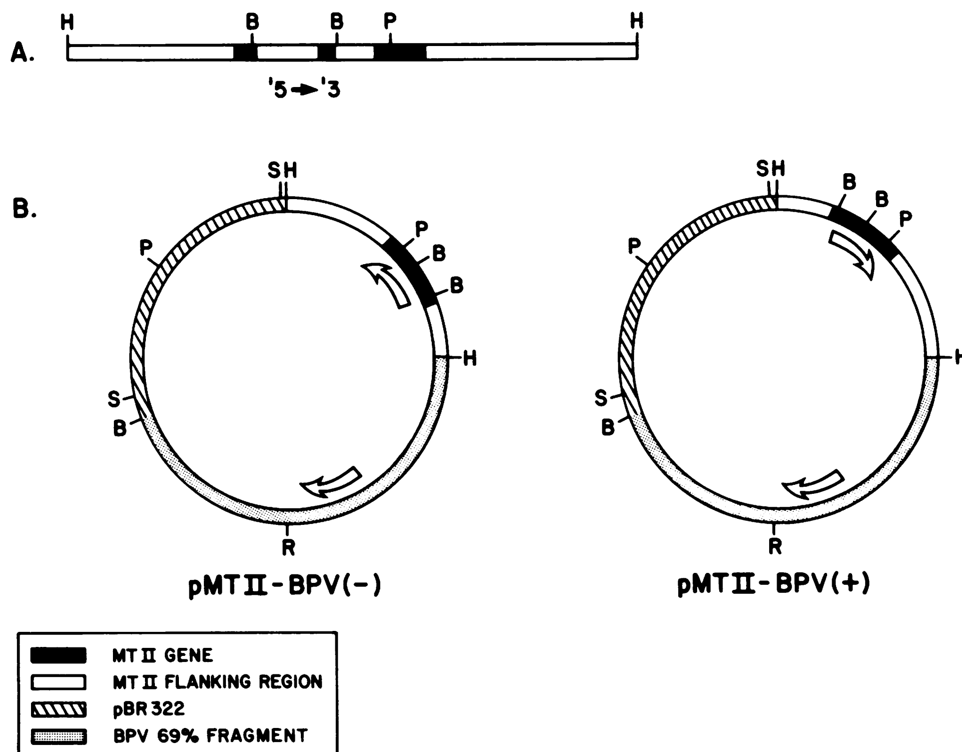

FIG. 1. (*A*) Structure of the human $MT\text{-}II_A$ gene. A 3.0-kb *Hin*dIII fragment containing the $MT\text{-}II_A$ gene is illustrated. The exons are indicated as solid black boxes. (*B*) Structure of pMTII-BPV vectors. Arrows indicate the direction of transcription of the $MT\text{-}II_A$ gene and the BPV genome. Restriction sites are indicated as follows: B, *Bam*HI; H, *Hin*dIII; P, *Pvu* II; S, *Sal* I; R, *Eco*RI.

gested pMTII-BPV(−) DNA or *Sal* I-digested $pBPV_{69T}$ serving as a control. Forty-eight hours after transfection the cells were subjected to selection in medium containing 20 μM Cd^{2+} and Zn^{2+}; 80–100 Cd^r colonies per μg of DNA were observed 10–14 days after transfection of cells with *Sal* I-digested pMTII-BPV(−) DNA, only slightly lower than the number of foci induced by the same vector. Microscopic examination of the Cd^r colonies indicated that all of them had a transformed phenotype (data not shown). Cultures transfected with $pBPV_{69T}$ did not give rise to any surviving colonies and were all dead by 1 week after the start of selection.

Later, we tested the ability of the intact vectors—both pMTII-BPV(−) and pMTII-BPV(+)—to transform either C127 or NIH 3T3 cells to the Cd^r phenotype. Five to 10 times more transformants were obtained by transfection with circular vectors compared to *Sal* I-digested vectors in spite of the presence of pBR322 sequences. The efficiency of transformation to Cd^r by the two MTII-BPV vectors was nearly identical, regardless of the orientation of the $MT\text{-}II_A$ gene (data not shown).

To determine the extent of resistance of the Cd^r cells relative to C127 cells transformed with $pBPV_{69T}$ (clone BPV3), we used a colony formation assay. Control plating efficiency in the absence of Cd^{2+} was identical for both cell types (70–72%). BPV3 cells were sensitive to Cd, and the number of surviving colonies decreased to 10% of the control value at 4 μM Cd^{2+} (Fig. 2). At this Cd^{2+} concentration, viability of the Cd^r cells was not affected. Their relative plating efficiency decreased to 10% of control only at 80 μM Cd^{2+}. Based on this criterion, Cd^r cells generated by transformation with pMTII-BPV(−) are 20-fold more resistant than BPV-transformed cells.

Expression of Human MT-II mRNA in the Cd^r Cells. To examine the expression of the human *MT-II* gene, three different Cd^r clones were grown in the presence of different Cd concentrations (10–350 μM) for 24 hr. At the end of this induction period, cytoplasmic RNA was extracted and analyzed for the presence of human MT-II mRNA by blot hybridization. To discriminate between mouse and human MT mRNAs, the 5′ noncoding region of the human MT-II cDNA (9) was used as a probe. Cd^r14 cells expressed the human *MT-II* gene carried on the

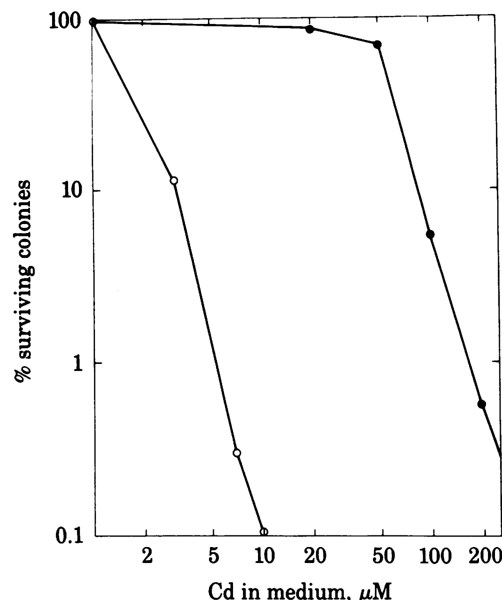

FIG. 2. Cd sensitivity of Cd^r and control BPV-transformed C127 cells. BPV-transformed C127 (clone BPV3, ○) and Cd^r C127 (clone Cd^r 14, ●) cells were seeded in various dilutions (10^4, 10^3, and 10^2 cells per plate) into 100-mm tissue culture plates. The cells were kept in growth medium containing the indicated Cd^{2+} (as Cd Cl_2) concentration for 2 weeks. Cells were fixed with ethanol/acetic acid, 3:1 (vol/vol) and stained with Giemsa stain. The number of colonies formed was determined and converted to percent of control value (the number of colonies formed in the absence of Cd^{2+}).

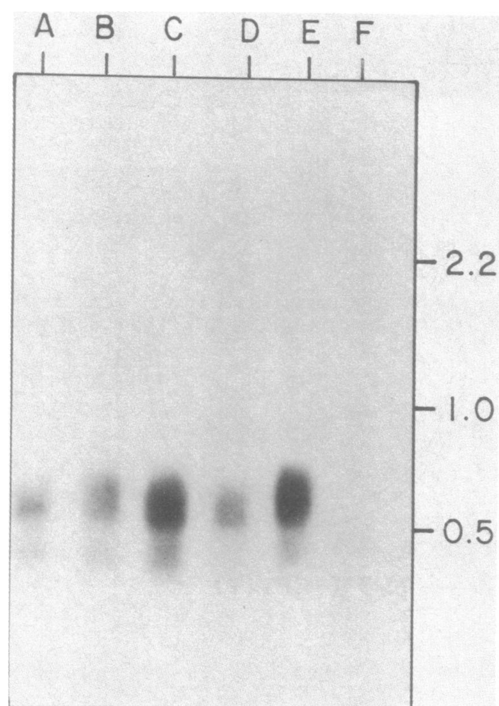

FIG. 3. Expression and regulation of human MT-II mRNA in Cd^r C127 cells. Total cytoplasmic RNA (10 μg) from Cd^r C127 cells (clone Cd^r 14) grown in the presence of Cd for 24 hr was analyzed by RNA blot hybridization (23). Lanes: A, 10 μM; B, 35 μM; C, 100 μM; D, 350 μM. Also analyzed on the same gel were 20 μg of total cellular RNA from Cd^{2+}-induced HeLa cells (5 μM Cd for 8 hr) (lane E) and Cd^{2+}-induced BPV-transformed C127 cells (lane F). The probe was the 5′ untranslated region of a *MT-II* cDNA clone (9). Numbers at the right indicate the size (in kb) of restriction fragments of bacteriophage PM2 used as markers.

vector (Fig. 3). Moreover, expression of the human *MT-II* gene in its new environment was regulated by the concentration of Cd^{2+} in the growth medium (lanes A–D). Two other Cd^r clones (Cd^{r}15 and Cd^{r}18) were found to exhibit levels of expression and dose–response relationships identical to those of Cd^{r}14. Maximal induction of MT-II mRNA was observed at 100 μM Cd^{2+}, a concentration 20-fold higher than the optimal concentration for induction of MT in HeLa cells (unpublished data). This higher requirement for Cd for maximal induction of the *MT-II*$_A$ gene probably results from its higher copy number in the Cd^r cells. Mouse cells in which the endogenous *MT* genes are amplified also exhibit a higher requirement for Cd to achieve optimal induction (26). The induction response to Cd is biphasic, and the level of MT-II mRNA actually declines at high Cd^{2+} concentrations due to a cytotoxic effect.

The copy number of pMTII-BPV in the cells was not affected by 24-hr incubation in various Cd^{2+} concentrations (data not shown). Therefore, the apparent induction of human MT-II mRNA does not result from changes in template concentration and most likely reflects transcriptional activation of the episomal *MT-II*$_A$ gene.

The level of MT-II mRNA and its regulation were identical in eight different cell lines examined. The relative orientation of the *MT-II*$_A$ gene within the vector had no effect on its expression (data not shown).

The start sites of transcription of the *MT-II*$_A$ gene carried on the BPV vector in mouse cells were determined by using a modification of the nuclease mapping technique (27). The coding strand of the *MT-II*$_A$ gene was 5′ end labeled at the 5′ *Bam*HI site (Fig. 1). The *Bam*HI–*Hin*dIII fragment containing 740 nucleotides of 5′ flanking DNA and 76 nucleotides of the 5′ untranslated region (11) was isolated to be used as a probe. Hybridization to RNA from HeLa cells yielded three protected DNA fragments starting at the *Bam*HI site and ending at the following residues: T at −3, A at +1, and A at +3 (Fig. 4, lane H). Hybridization of the probe to RNA from Cd^r C127 cells (clone Cd^{r}15) resulted in two protected bands, corresponding to fragments starting at the *Bam*HI site and ending at the A at +1 and +3 (Fig. 4, lane CdR).

These results indicate that two of the three authentic initiation sites of the human *MT-II*$_A$ gene are used in MTII-BPV-transformed mouse cells.

Cd^r-Cells Contain Multiple Free Copies of the MTII-BPV Vector. BPV is known to induce transformation of mouse cells without integrating into the host DNA (16). This property of the virus or of its subgenomic transforming fragment has led to the development of BPV as a vector system by Howley and coworkers (14). Chimeric BPV vectors containing various genes also exist in cells as episomes (14, 28–30). In all those cases, the cells containing the vector were selected on the basis of their transformed phenotype. It was of interest to determine whether cells selected for a Cd^r phenotype also contain free copies of the vector. Analysis of *Eco*RI-digested total cellular DNA revealed that C127, NIH 3T3, and Rat 2 cells selected for Cd^r (at 20 μM Cd^{2+}) after transfection with either pMTII-BPV(−) or pMTII-BPV(+) all contained approximately 10–15 copies of the vector (Fig. 5 *A* and *D*). The presence or absence of pBR322 sequences in the vector had no effect on its copy number in the resistant cells.

We used two different criteria to demonstrate that the MTII-

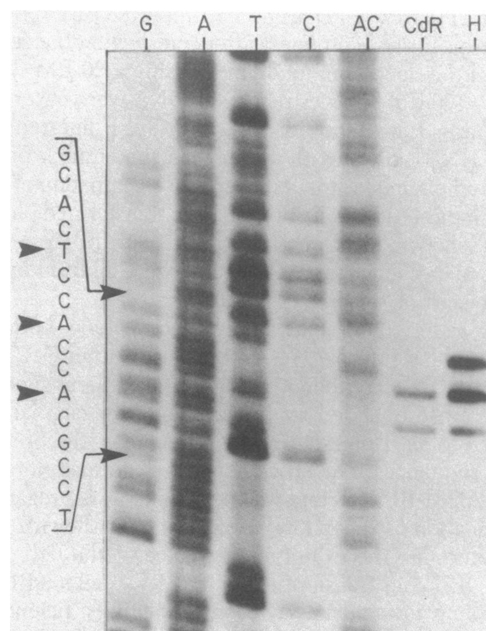

FIG. 4. Mapping of initiation sites for transcription of the human *MT-II* gene in Cd^r C127 and in HeLa cells. Total cellular RNA (60 μg) from Cd^{r}14 cells (lane Cd^R) grown for 24 hr in 50 μM Cd^{2+} and from Cd^{2+}-induced HeLa cells (lane H, 150 μg) were hybridized to a 5′ end labeled *Bam*HI–*Hin*dIII fragment that contained the 5′ untranslated region of the human *MT-II*$_A$ gene (see Fig. 1). The hybrids were digested with mung bean nuclease. Nuclease-resistant fragments were displayed on an 8% sequencing gel, together with the chemical degradation products of the *Bam*HI–*Hin*dIII fragment, serving as size markers. Lanes G, A, T, C, and AC are the corresponding sequencing ladders. The nucleotide sequence of the sense strand at the region of interest is indicated on the left and the putative transcripton start sites are indicated by arrows.

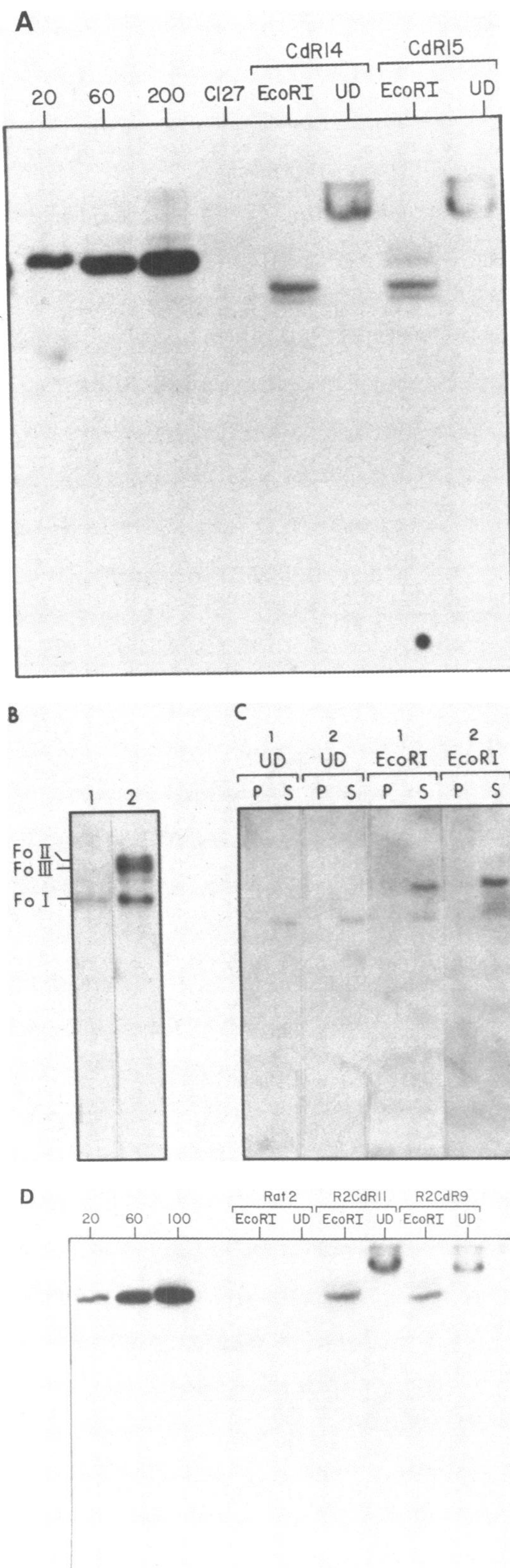

FIG. 5. Presence of the MTII-BPV vector molecules in genomic DNA of Cd^r cells. (*A*) Undigested (UD) and *Eco*RI-digested (EcoRI) total genomic DNA (10 μg) from two Cd^r C127 lines (CdR14 and CdR15) transfected with *Sal* I-digested pMTII-BPV(−) were analyzed by Southern blot hybridization (22); 0.66, 2.0, and 6.6 ng of *Eco*RI-digested pMTII-BPV(−) mixed with 10 μg of high molecular weight salmon sperm DNA was used as copy number standards. The numbers of copies per cell are indicated above the first three lanes. *Eco*RI-digested DNA (10 μg) from nontransformed C127 cells was used as a negative control (C127). (*B*) Total genomic DNA from Cd^{r}14 was either mechanically sheared (lane 1) or both mechanically sheared and partially digested with *Eco*RI (lane 2) and analyzed as described above. Fo I, Fo II, and Fo III indicate the migration positions of the three forms of MTII-BPV DNA (without pBR322 sequences and religated at the *Sal* I site). (*C*) Cd^r C127 cells (transfected with intact pMTII-BPV) were subjected to Hirt extraction (21). The supernatant (S) and the pellet (P) DNAs were analyzed either after mechanical shearing (UD) or after *Eco*RI digestion as described above. Lanes: 1, DNA from cell line Cd^{r}23; 2, DNA from line Cd^{r}27. (*D*) Undigested (UD) and *Eco*RI-digested (EcoRI) total genomic DNAs from parental Rat 2 cells and two Cd^r lines [transfected with intact pMTII-BPV(−)] were analyzed for presence of MTII-BPV genomes as described in *A*.

BPV vectors exist in Cd^r cells as free replicating plasmids, as reported originally by Law *et al.* (15). First, digestion of total cellular DNA with an enzyme that cuts only once within the vector (i.e., *Eco*RI) generated mostly full length linear molecules. The size of the vector DNA detected by hybridization corresponded to the size of the vector used for transfection: either 9.1 kb in the case of the large *Sal* I fragment (Fig. 5*A*) or 12.5 kb in the case of the intact circular vector (Fig. 5*D*). In undigested cellular DNA, MTII-BPV was present as a high molecular weight, probably catenated, form (Fig. 5 *A* and *D*). Mechanical shearing and partial digestion with *Eco*RI released forms I, II, and III DNA from those complexes (Fig. 5*B*). Migration of the vector as a high molecular weight form was not due to trapping within the genomic DNA because the plasmid mixed with high molecular weight carrier DNA did migrate properly (Fig. 5 *A* and *D*). Second, we subjected several Cd^r lines to Hirt extraction (21), to separate free replicating circular DNA from high molecular weight chromosomal DNA. pMTII-BPV DNA was present mostly in the supernatant, as expected of episomal DNA (Fig. 5*C*).

In addition to C127 and NIH 3T3 cells, Rat 2 cells also were transformed to a Cd^r phenotype by transfection with pMTII-BPV with the same efficiency as mouse C127 cells. After several of the Cd^r colonies were propagated into mass cultures, they no longer exhibited contact inhibition of growth, and multiple foci appeared at high cell densities. Therefore, we conclude that Rat 2 cells can be oncogenically transformed by BPV. Cellular DNA from several of the Rat 2 Cd^r lines was found to contain approximately 10–15 copies of free replicating pMTII-BPV (Fig. 5*D*).

Rescue of Vector DNA from Mammalian Cells. Because pMTII-BPV vectors replicated in mammalian cells while still linked to pBR322 DNA, and no deletions or rearrangements were found to occur within those sequences (Fig. 5*D*), it should be possible to rescue the vector DNA from mammalian cells by transformation of *E. coli*. Between 355 and 685 ampicillin-resistant colonies were detected after transformation of *E. coli* (RRI) with either Hirt (21) or alkaline extracts (31) of about 10^7 mammalian cells. Some of the bacterial colonies were analyzed by rapid plasmid DNA preparation (17) and were found to contain the original pMTII-BPV DNA (data not shown).

DISCUSSION

The data we have presented indicate that a cloned human *MT-II* gene is properly expressed and regulated after introduction into rodent fibroblasts on a BPV-derived vector which is maintained in those cells as an episome.

The regulatory signals for heavy metal ion induction and initiation of transcription of the *MT-II* gene are properly recognized by the mouse transcriptional apparatus. Production of human MT-II mRNA in Cd^r cells is regulated by heavy metal ions, and transcripts are properly initiated and processed to produce normal size MT-II mRNA which is translated to yield functional protein.

In other experiments, in which the human *MT-II* gene was introduced into either Ltk$^-$ or Rat 2 cells by cotransfer with the herpes simplex thymidine kinase gene, the level of expression and its regulation varied considerably from one clone to another (unpublished data). This probably was due to random integration into various sites in the host genome. All of the Cd^r clones generated by transformation with the pMTII-BPV vectors that we have analyzed exhibited similar levels of human MT-II mRNA expression, most likely as a result of the episomal state of such vectors and the presence, on the BPV_{69T} fragment, of an enhancer sequence (32). Therefore, such episomal vectors would facilitate studies correlating chromatin structure and gene expression of isolated and *in vitro* mutagenized genes without integration site interference.

We have been unable to detect any response to glucocorticoid hormones of the $MT\text{-}II_A$ gene in the episomal state. At the present time, we do not know the reason for this because the same gene introduced into Rat 2 or Ltk$^-$ cells by cotransfer is transcriptionally activated by glucocorticoids (unpublished data).

The Cd^r cells contain about 10–15 copies of the MTII-BPV plasmid. They also produce considerably higher levels of MT protein and are 20-fold more resistant to Cd than are control cells. Taken together, the data suggest that most of the copies of the human *MT-II* gene are functional in the transformed mouse cells and Cd^r is a consequence of the increased *MT* gene copy number. This is in contrast to the efficiency of expression of *MT* genes carried on simian virus 40 vectors. Hamer and Walling (33) recently reported that, after infection of monkey kidney cells with a simian virus 40–mouse *MT-I* recombinant, the infected cells, which contained about 100,000 copies of the gene, accumulated less MT mRNA than did mouse fibroblasts, which contained only 2 copies of that gene. Although BPV-derived episomes do not reach as high copy number as replicating simian virus 40 vectors, they seem to be more efficient in expression of foreign genes and do not damage the host cells. While this manuscript was being prepared, results similar to ours were obtained by Pavlakis and Hamer (34) by inserting the mouse *MT-I* gene into a BPV vector. In this case, the gene was more active transcriptionally than when carried on a simian virus 40 vector (33).

Due to increased expression of MT, the transformed cells become resistant to Cd and can be easily selected in Cd^{2+}-containing medium. Thus, the newly constructed MTII-BPV vectors can be utilized to introduce other genes into various cell types. Using the Cd selection, we have obtained Cd^r clones of various cell lines including C127, NIH 3T3, Rat 2, and F9 mouse teratocarcinoma stem cells, indicating a potentially wide host range of those vectors and possible future application for gene transfer into animals.

Besides being an easily selectable, dominantly acting, genetic marker, the human $MT\text{-}II_A$ gene has another highly desirable property: it can counteract the deleterious effect of pBR322 sequences on BPV replication (16). Several other genes have such an effect (28, 30, 35). This property of the $MT\text{-}II_A$ gene can be extended, and other genes can be inserted into the pMTII-BPV vectors and propagated extrachromosomally without removal of pBR322 sequences (unpublished data).

Because the pMTII-BPV vectors contain plasmid origins of replication and selectable markers for both *E. coli* and mammalian cells, they can serve as shuttle vectors. Such vectors can be used for gene cloning and for isolation of mutants of already cloned genes present on such vectors.

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