MOLECULAR AND CELLULAR BIOLOGY, Apr. 1987, p. 1563–1567
0270-7306/87/041563-05$02.00/0

Vol. 7, No. 4

Context-Dependent Gene Expression: *cis*-Acting Negative Effects of Specific Procaryotic Plasmid Sequences on Eucaryotic Genes

DAVID O. PETERSON,* KATHERINE K. BEIFUSS, AND KIMBERLIN L. MORLEY

Department of Biochemistry and Biophysics, Texas Agricultural Experiment Station, Texas A & M University, College Station, Texas 77843

Received 6 August 1986/Accepted 23 December 1986

A sequence element within pBR322 DNA mediates a *cis*-acting negative effect on expression from eucaryotic genes in transient expression assays. The negative element overlaps with sequences that inhibit DNA replication, but its effect is observed in the absence of detectable replication of transfected DNA.

The ability to transfect recombinant gene constructs into cultured mammalian cells has been instrumental in the identification and analysis of *cis*-acting sequences required for eucaryotic gene expression. A component of such constructs is often DNA derived from bacterial plasmid sequences used to amplify the gene in *Escherichia coli*, and although effects of such plasmid sequences on expression of transfected DNA have been reported (1, 5), they have not been systematically investigated and are not usually considered in the design of transfection experiments.

Associated plasmid sequences affect expression of transfected genes in cultured mammalian cells. Plasmid pLC1 (Fig. 1) contains the coding sequences for the bacterial enzyme chloramphenicol acetyltransferase (CAT) linked to the glucocorticoid-responsive (9, 14, 20, 25) promoter contained within the mouse mammary tumor virus (MMTV) long terminal repeat (LTR); simian virus 40 (SV40) sequences provide a site that encodes poly(A) addition (8). Transcription from the MMTV promoter has been studied with transient expression assays of mouse Ltk$^-$ cells transfected with pLC1 (24), although several reports have indicated that activity of the MMTV promoter cannot be detected in the absence of an exogenous enhancer element (11, 16). Obvious differences between pLC1 and other plasmids in which exogenous enhancers have been reported to be required are the inclusion of different pBR322-derived sequences, the presence of DNA from bacteriophage M13, and the position and orientation of the MMTV-CAT transcription unit with respect to the pBR322 vector.

To directly test the effect of these differences on gene expression from the MMTV promoter, two related plasmids were constructed. The *Pvu*I-*Bam*HI fragment of pLC1 that contains the MMTV-CAT transcription unit was inserted into a *Bam*HI-*Pvu*I fragment derived from either pBR322 or pZ152 (27) to form pBLC1 and pZLC1, respectively (Fig. 1). Both of these plasmids contain undeleted pBR322 sequences from the *Bam*HI to the *Eco*RI sites and differ only in the presence of 455 base pairs of DNA derived from bacteriophage M13 in pZLC1. pLC1, pBLC1, and pZLC1 were purified by alkaline extraction (2) and CsCl-ethidium bromide density gradient centrifugation and were transfected into mouse Ltk$^-$ cells (10) by using the DEAE-dextran-dimethyl sulfoxide shock procedure (12) as described previously (24). At 40 h after the dimethyl sulfoxide shock, the synthetic glucocorticoid dexamethasone was added to some cultures to a final concentration of 10^{-7} M, and cell extracts were prepared 18 h later and assayed for total protein (3) and CAT activity (8). The CAT specific activities are reported relative to the specific activity of an extract of cells transfected with pLC1 (arbitrarily set to 100). Dexamethasone-induced CAT activity in cells transfected with both pBLC1 and pZLC1 was lower than that obtained with pLC1 by approximately seven- and fivefold, respectively (Fig. 2A, lanes 2, 4, and 6); similar results were observed in extracts of

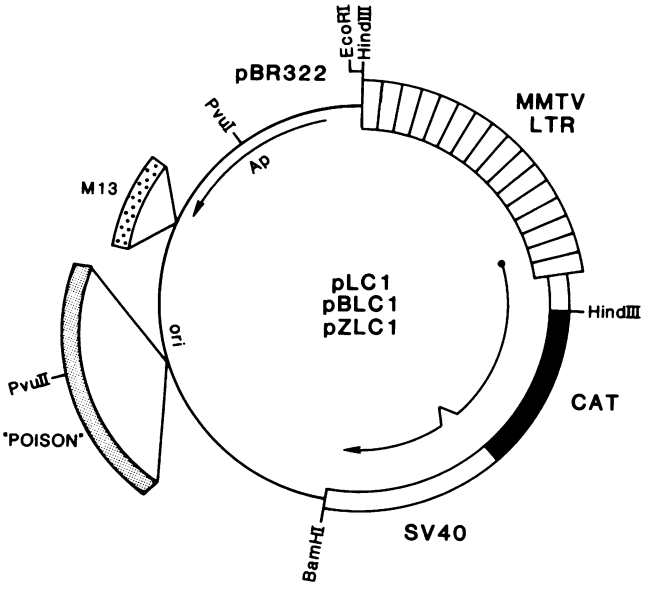

FIG. 1. Structure of pLC1 (6.5 kb), pBLC1 (7.0 kb), and pZLC1 (7.5 kb). The construction of pLC1 has been described previously (24). MMTV LTR sequences are contained within a 1.5-kb *Pst*I restriction fragment derived from the C3H strain of virus, the ends of which were converted to *Hin*dIII sites. The CAT gene, the SV40 small t intron, and sequences required for poly(A) addition are derived from pSV2CAT (8). The pBR322 sequences depicted as a single line are derived from pSVOd (15). The plasmid origin of replication (ori) and ampicillin resistance gene (Ap) are indicated. The sequences labeled Poison represent pBR322 DNA between coordinates 1424 and 2490; this region contains sequences that have been found to inhibit DNA replication (13, 21). The M13 origin of replication is derived from plasmid pZ152 (27). pLC1, pBLC1, and pZLC1 contain identical MMTV-CAT transcription units (indicated by the arrow); these plasmids differ in that pLC1 contains the M13 origin but not the poison region, pBLC1 contains the poison region but not the M13 origin, and pZLC1 contains both the M13 origin and the poison region.

* Corresponding author.

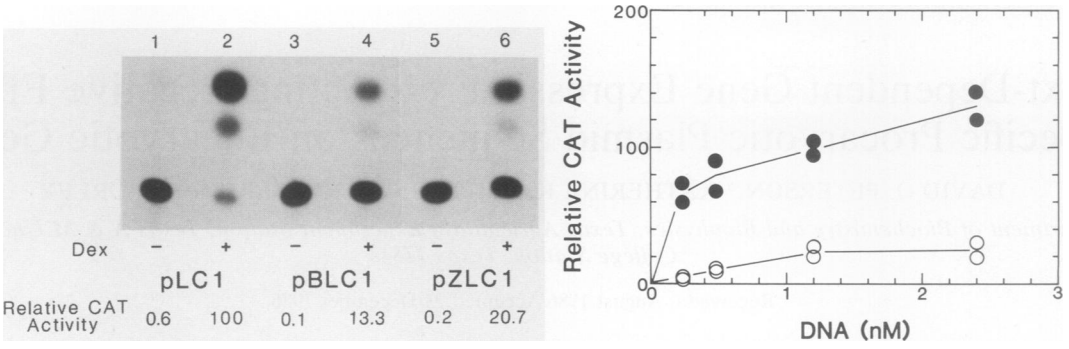

FIG. 2. (A) CAT gene expression from pLC1, pBLC1, and pZLC1. Ltk$^-$ cells were transfected with plasmid (1.2 nM, corresponding to 5 μg of pLC1 per ml), as described in the text. An autoradiogram of the thin-layer chromatography plate of a representative assay of extracts from cells transfected with pLC1 (lanes 1 and 2), pBLC1 (lanes 3 and 4), and pZLC1 (lanes 5 and 6) is shown. The two faster-migrating spots correspond to the 3-acetyl and 1-acetyl derivatives of chloramphenicol, while the slower-migrating spot is the unmodified substrate. The relative CAT activities (defined in the text) are the average of at least two (−) or three (+) independent transfection experiments. In the particular experiment shown, assays of extracts from cells not treated with dexamethasone (Dex) (lanes 1, 3, and 5) contained 80 μg of protein, whereas those from cells treated with hormone (lanes 2, 4, and 6) contained 40 μg; the assay in lane 2 was repeated (data not shown) for quantitation because the reaction shown had proceeded beyond the linear range to 93% conversion of chloramphenicol to its acetylated derivatives. (B) DNA concentration dependence of CAT expression. Ltk$^-$ cells were transfected with pLC1 or pZLC1 at various DNA concentrations. Symbols: ●, cells transfected with pLC1; ○, cells transfected with pZLC1.

cells not treated with hormone (Fig. 2A, lanes 1, 3, and 5). These results indicate that the M13 sequences in pLC1 and pZLC1 had little or no effect on CAT gene expression but that there were negative effects of BR322 DNA present in pBLC1 and pZLC1 but absent in pLC1. This region corresponds to 1,066 base pairs of DNA between pBR322 coordinates 1424 and 2490 (17, 23). The observed negative effect was not dependent on DNA concentration, and saturation levels of input DNA were similar for different plasmids regardless of the presence of the pBR322 negative element (Fig. 2B).

Effects of pBR322 sequences are not related to DNA replication. The region of pBR322 implicated as having a negative effect on CAT gene expression overlaps the pBR322 "poison" sequences that have been shown to inhibit DNA replication in several cultured cell systems (13, 21). Although none of the plasmids used in our studies contain known eucaryotic replication origins and were thus not expected to be replicated in transfected mouse cells, we directly investigated this possibility.

Nuclei were isolated from pLC1-transfected Ltk$^-$ cells at the time extracts would normally be prepared for CAT enzyme assay, and total nuclear DNA was purified (18). Isolation of nuclei before DNA purification assured that only DNA present in the nucleus and therefore a potential transcription template would be observed in the subsequent analysis. The pLC1 plasmid DNA used in our transfections had been amplified in a *dam*$^+$ strain of *E. coli*, and thus all adenines present in the sequence 5′-GATC-3′ contained a methyl group at the N-6 position (7). This methylation made the DNA insensitive to cleavage by the restriction endonuclease *Mbo*I, which recognizes the 5′-GATC-3′ sequence. However, the modified DNA was cleaved by the isoschizomer *Sau*3A, which is not sensitive to adenine methylation. Total nuclear DNA was treated with either *Mbo*I or *Sau*3A and subjected to agarose gel electrophoresis and Southern blot (22) analysis in which the probe was nick-translated (6) pMT2, a plasmid identical to pLC1 (Fig. 1) but containing no MMTV sequences. If transfected DNA had been replicated, sites of adenine methylation would have been lost, and plasmid recovered from transfected cells would have become sensitive to *Mbo*I cleavage. If, on the other hand, no replication occurred, *Mbo*I resistance would be retained after transfection. pLC1 DNA recovered from nuclei of transfected cells retained full resistance to *Mbo*I, while the bulk genomic DNA was cleaved by this enzyme (Fig. 3). In addition, the results in Fig. 3 indicate that the supercoiled topology of the plasmid DNA was largely maintained after transfection; this observation is in contrast to a recent report in which supercoiled molecules were shown to become nicked and linearized after transfection by a similar, though not identical, protocol (26).

Plasmid copy number in the nuclei of transfected cells. Although the experiment described above indicated that

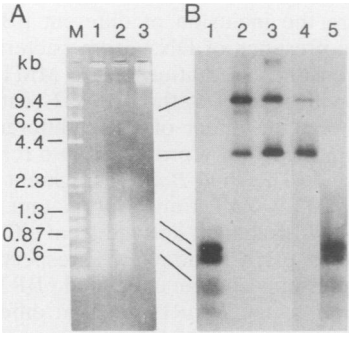

FIG. 3. Sensitivity of transfected DNA to *Mbo*I cleavage. Ltk$^-$ cells were transfected with pLC1, and DNA was purified from isolated nuclei. Samples of total nuclear DNA (5 μg) were digested with either *Sau*3A or *Mbo*I and subjected to agarose gel electrophoresis and Southern blot analysis with a probe of nick-translated pMT2, a plasmid identical to pLC1 (Fig. 1), but containing no MMTV sequences. (A) Agarose gel stained with ethidium bromide. Molecular size markers (lane M), DNA digested with *Sau*3A (lane 1) or *Mbo*I (lane 2), and uncut DNA (lane 3) are shown. (B) Southern blot analysis. Lanes 1 to 3 are as in panel A. Lanes 4 and 5 contain purified pLC1 (0.1 and 0.5 ng, respectively) uncut by restriction endonucleases (lane 4) or digested with *Sau*3A (lane 5).

differential CAT gene expression was not due to preferential replication of pLC1, it remained possible that the pBR322 element was affecting gene expression by affecting the number of plasmid molecules maintained in the nuclei of transfected cells. To directly test this idea, total nuclear DNA was purified from cells transfected with pLC1, pBLC1, and pZLC1. This DNA was then digested with *Hin*dIII, an enzyme that cleaves each plasmid to generate a 1.5-kilobase (kb) LTR-containing fragment (Fig. 1). Serial twofold dilutions of this digested DNA were subjected to agarose gel electrophoresis and Southern blot analysis with an LTR-specific probe (Fig. 4), and autoradiograms were scanned with a Zeineh soft laser scanning densitometer (Biomed Instruments, Inc., Fullerton, Calif.). The relative copy number of transfected plasmid sequences was measured by determining the ratio of the integrated intensity of the 1.5-kb LTR fragment from the plasmid (determined in one of the serial dilutions) to that of the 9.4-kb fragment containing endogenous MMTV LTR sequences derived from cellular DNA (determined in the most concentrated sample). For cells transfected with pLC1, this ratio was between 8 and 16, whereas for cells transfected with either pBLC1 and pZLC1, it was between 4 and 8. Thus, while this experiment does not rule out some contribution of plasmid copy number to the observed effects on gene expression, copy number effects apparently do not fully account for the differential gene expression of five- to sevenfold (Fig. 2). The DNA concentration dependence of transfected gene expression (Fig. 2B) is consistent with this interpretation. If the negative element served only to decrease the number of transcription templates in the nuclei of transfected cells, gene expression from plasmids with and without the element might have been expected to be similar at a sufficiently high DNA concentration, especially in consideration of the report that plasmid copy number increases with increasing DNA concentration

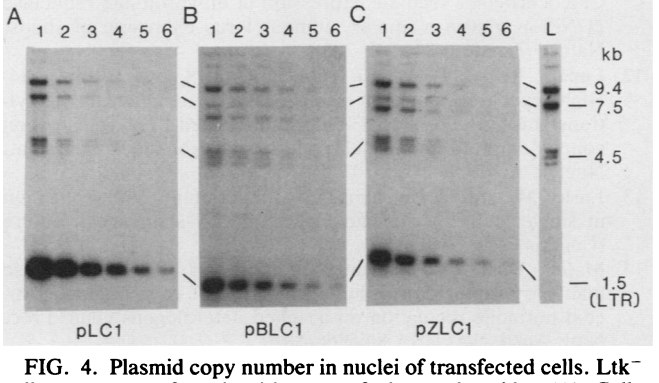

FIG. 4. Plasmid copy number in nuclei of transfected cells. Ltk$^-$ cells were transfected with one of three plasmids. (A) Cells transfected with pLC1. (B) Cells transfected with pBLC1. (C) Cells transfected with pZLC1. DNA was purified from isolated nuclei. Samples of total nuclear DNA were digested to completion with *Hin*dIII and subjected to agarose gel electrophoresis and Southern blot analysis with a single-stranded RNA probe specific for the MMTV LTR and synthesized with T7 RNA polymerase as previously described (24). In panels A to C, either 5 μg of total nuclear DNA (lane 1) or serial twofold dilutions (lanes 2 to 6) of the samples in lane 1 were used. *Hin*dIII-digested DNA (5 μg) from untransfected Ltk$^-$ cells is shown (lane L). We have not determined the reason for the variable yield of the 7.5-kb fragment. The 1.5-kb LTR fragment is derived from the transfected plasmid. The band that migrates just below the 7.5-kb fragment in panels B and C represents an internal control plasmid added to the purified DNA samples to monitor the completion of the *Hin*dIII digestion.

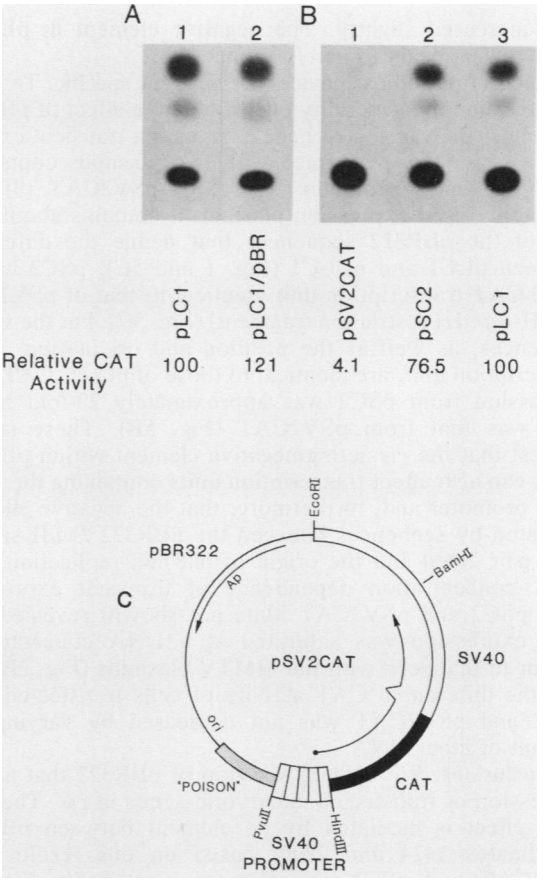

FIG. 5. *cis* and *trans* action and gene specificity of the negative element. Ltk$^-$ cells were transfected with plasmid, and 5 μg of protein from a crude cell extract was assayed to determine CAT specific activity. Representative CAT assays are shown. (A) Cells were transfected with either pLC1 (lane 1) or pLC1 plus pBR322 (lane 2). The concentration of each plasmid was 1.2 nM during the transfection, corresponding to 5 μg of pLC1 per ml. Cells were treated with dexamethasone before the preparation of extracts. The reported relative CAT activities are the average of five independent transfection experiments. (B) Cells were transfected with either pSV2CAT (lane 1), pSC2 (lane 2), or pLC1 (lane 3). pLC1-transfected cells were treated with dexamethasone before the preparation of extracts. The reported relative CAT activities are the average of three independent transfection experiments. (C) Structure of pSV2CAT (8). The position and orientation of the SV40-CAT transcription unit relative to the poison sequences are indicated.

even after transfected gene expression has become saturated (26).

Most endogenous MMTV proviral units contain a single *Hin*dIII site (19); thus, assuming that the 9.4-kb fragment from cellular DNA contains a single endogenous LTR, that Ltk$^-$ cells are essentially diploid, and that the labeled probe hybridizes with equal efficiency to the endogenous and plasmid-derived LTR sequences, the average number of transfected plasmid molecules per cell was approximately 15 to 30.

Effects of plasmid sequences are *cis* acting. To determine whether the negative effect of pBR322 sequences could be mediated in *trans*, cotransfections were performed in which pLC1 was introduced into cells in the presence of an equal molar concentration of pBR322. Crude cell extracts were prepared and assayed for CAT activity (Fig. 5A). Relative CAT activity was not decreased in the cotransfection and, in

fact, increased slightly. The negative element in pBR322 DNA thus appears to be *cis* acting.

Effects of plasmid sequences are not gene specific. To begin to determine the generality of the negative effect of pBR322 sequences on transfected gene expression, transient expression assays were performed with two plasmids containing the SV40 early promoter (Fig. 5B). pSV2CAT (8) is a commonly used expression plasmid; it contains about one-half of the pBR322 sequences that define the difference between pLC1 and pZLC1 (Fig. 1 and 5C). pSC2 has an SV40-CAT transcription unit identical to that of pSV2CAT (*Pvu*II-*Bam*HI restriction fragment) (Fig. 5C), but the vector sequences, as well as the position and orientation of the transcription unit, are identical to those of pLC1. CAT gene expression from pSC2 was approximately 20-fold higher than was that from pSV2CAT (Fig. 5B). These results suggest that the *cis*-acting negative element within pBR322 DNA can also affect transcription units containing the SV40 early promoter and, furthermore, that the negative effect is mediated by sequences between the pBR322 *Pvu*II site (at base pair 2066) and the origin of plasmid replication. The DNA concentration dependence of transient expression from pSC2 and pSV2CAT (data not shown) revealed that gene expression was saturated at a DNA concentration similar to that seen with the MMTV plasmids (Fig. 2B) and that the differential CAT activity of cells transfected with pSC2 and pSV2CAT was not decreased by varying the amount of input DNA.

Conclusions. We defined a region of pBR322 that affects expression of transfected eucaryotic genes in *cis*. The negative effect is mediated by an element between pBR322 coordinates 1424 and 2490; based on our results with pSV2CAT and pSC2, the element is probably between pBR322 coordinates 2066 and 2490. This region overlaps the poison sequence that has been defined as inhibiting DNA replication (13, 21), but there is no evidence that replication is related to the observed effect on gene expression. Courey et al. (5) have reported that certain pBR322 sequences can attenuate activation of the human β-globin promoter by the SV40 enhancer, but the implicated region contains DNA between pBR322 coordinates 2520 and 3103 and thus does not overlap with the negative element described here.

We have not specifically addressed the process that is affected in *cis* by the pBR322 sequence element. We have previously reported, however, that CAT activity in cells transfected with plasmids such as pLC1 is directly correlated with the abundance of MMTV-CAT transcripts (24), strongly suggesting that a decreased number of such transcripts is the basis of the observed negative effect. By whatever mechanism the negative element functions, it appears to be able to decrease the expression of genes containing at least two different promoters when located at multiple positions and in both orientations with respect to the affected gene. Eucaryotic negative regulatory elements that mediate similar effects by reducing promoter activity have been termed transcriptional silencers (4); it is possible that the negative element within pBR322 DNA bears a fortuitous homology to eucaryotic sequences that mediate silencerlike effects.

We thank Bob Hathorn for technical assistance, Mark Toohey for kindly providing us with pMT2, and Wen-Liang Kuo and Mark Toohey for helpful comments on the manuscript.

This work was supported by Public Health Service grant CA32695 from the National Cancer Institute and grant A-904 from the Robert A. Welch Foundation. The support of the Texas Agricultural Experiment Station is also gratefully acknowledged.

LITERATURE CITED

1. **Banerji, J., S. Rusconi, and W. Schaffner.** 1981. Expression of a β-globin gene is enhanced by remote SV40 DNA sequences. Cell **27**:299–308.
2. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
3. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
4. **Brand, A. H., L. Breeden, J. Abraham, R. Sternglanz, and K. Nasmyth.** 1985. Characterization of a "silencer" in yeast: a DNA sequence with properties opposite to those of a transcriptional enhancer. Cell **41**:41–48.
5. **Courey, A. J., S. E. Plon, and J. C. Wang.** 1986. The use of psoralen-modified DNA to probe the mechanism of enhancer action. Cell **45**:567–574.
6. **Feinstein, S. C., S. R. Ross, and K. R. Yamamoto.** 1982. Chromosomal position effects determine transcriptional potential of integrated mouse mammary tumor virus DNA. J. Mol. Biol. **156**:549–566.
7. **Geier, G. E., and P. Modrich.** 1979. Recognition sequence of the *dam* methylase of *Escherichia coli* K12 and mode of cleavage of *Dpn*I endonuclease. J. Biol. Chem. **254**:1408–1413.
8. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2**:1044–1051.
9. **Hynes, N., A. J. J. van Ooyen, N. Kennedy, P. Herrlich, H. Ponta, and B. Groner.** 1983. Subfragments of the large terminal repeat cause glucocorticoid-responsive expression of mouse mammary tumor virus and of an adjacent gene. Proc. Natl. Acad. Sci. USA **80**:3637–3641.
10. **Kit, S., D. R. Dubbs, L. Piekarski, and T. C. Hsu.** 1963. Deletion of thymidine kinase activity from L cells resistant to bromodeoxyuridine. Exp. Cell. Res. **31**:297–312.
11. **Lee, F., R. Mulligan, P. Berg, and G. Ringold.** 1981. Glucocorticoids regulate expression of dihydrofolate reductase cDNA in mouse mammary tumour virus chimaeric plasmids. Nature (London) **294**:228–232.
12. **Lopata, M. A., D. W. Cleveland, and B. Sollner-Webb.** 1984. High level transient expression of a chloramphenicol acetyltransferase gene by DEAE-dextran mediated DNA transfection coupled with a dimethylsulfoxide or glycerol shock treatment. Nucleic Acids Res. **12**:5707–5717.
13. **Lusky, M., and M. Botchan.** 1981. Inhibition of SV40 replication in simian cells by specific pBR322 DNA sequences. Nature (London) **289**:79–81.
14. **Majors, J., and H. E. Varmus.** 1983. A small region of the mouse mammary tumor virus long terminal repeat confers glucocorticoid hormone regulation on a linked heterologous gene. Proc. Natl. Acad. Sci. USA **80**:5866–5870.
15. **Mellon, P., V. Parker, Y. Gluzman, and T. Maniatis.** 1981. Identification of DNA sequences required for transcription of the human α1-globin gene in a new SV40 host-vector system. Cell **27**:279–288.
16. **Ostrowski, M. C., A. L. Huang, M. Kessel, R. G. Wolford, and G. L. Hager.** 1984. Modulation of enhancer activity by the hormone responsive element from mouse mammary tumor virus. EMBO J. **3**:1891–1899.
17. **Peden, K. W. C.** 1983. Revised sequence of the tetracycline resistance gene of pBR322. Gene **22**:277–280.
18. **Peterson, D. O.** 1985. Alterations in chromatin structure associated with glucocorticoid-induced expression of endogenous mouse mammary tumor virus genes. Mol. Cell. Biol. **5**:1104–1110.
19. **Peterson, D. O., K. G. Kriz, J. E. Marich, and M. G. Toohey.** 1985. Sequence organization and molecular cloning of mouse mammary tumor virus DNA endogenous to C57BL/6 mice. J.

Virol. **54**:525–531.

20. **Ponta, H., N. Kennedy, P. Skroch, N. E. Hynes, and B. Groner.** 1985. Hormonal response region in the mouse mammary tumor virus long terminal repeat can be dissociated from the proviral promoter and has enhancer properties. Proc. Natl. Acad. Sci. USA **82**:1020–1024.
21. **Sarver, N., J. C. Byrne, and P. M. Howley.** 1982. Transformation and replication in mouse cells of a bovine papillomavirus-pML2 plasmid vector that can be rescued in bacteria. Proc. Natl. Acad. Sci. USA **79**:7147–7151.
22. **Southern, E. M.** 1975. Detection of specific DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
23. **Sutcliffe, J. G.** 1978. Complete nucleotide sequence of the *Escherichia coli* plasmid pBR322. Cold Spring Harbor Symp. Quant. Biol. **43**:77–90.
24. **Toohey, M. G., K. L. Morley, and D. O. Peterson.** 1986. Multiple hormone-inducible enhancers as mediators of differential transcription. Mol. Cell. Biol. **6**:4526–4538.
25. **Ucker, D. S., G. L. Firestone, and K. R. Yamamoto.** 1983. Glucocorticoids and chromosomal position modulate murine mammary tumor virus transcription by affecting efficiency of promoter utilization. Mol. Cell. Biol. **3**:551–561.
26. **Weintraub, H., P. F. Cheng, and K. Conrad.** 1986. Expression of transfected DNA depends on DNA topology. Cell **46**:115–122.
27. **Zagursky, R. J., and M. L. Berman.** 1984. Cloning vectors that yield high levels of single-stranded DNA for rapid DNA sequencing. Gene **27**:183–192.