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Recombinant Genomes Which Express Chloramphenicol Acetyltransferase in Mammalian Cells

CORNELIA M. GORMAN, LESLIE F. MOFFAT, AND BRUCE H. HOWARD*

Laboratory of Molecular Biology, Division of Cancer Biology and Diagnosis, National Cancer Institute, Bethesda, Maryland 20205

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We constructed a series of recombinant genomes which directed expression of the enzyme chloramphenicol acetyltransferase (CAT) in mammalian cells. The prototype recombinant in this series, pSV2-cat, consisted of the beta-lactamase gene and origin of replication from pBR322 coupled to a simian virus 40 (SV40) early transcription region into which CAT coding sequences were inserted. Readily measured levels of CAT accumulated within 48 h after the introduction of pSV2-cat DNA into African green monkey kidney CV-1 cells. Because endogenous CAT activity is not present in CV-1 or other mammalian cells, and because rapid, sensitive assays for CAT activity are available, these recombinants provided a uniquely convenient system for monitoring the expression of foreign DNAs in tissue culture cells. To demonstrate the usefulness of this system, we constructed derivatives of pSV2-cat from which part or all of the SV40 promoter region was removed. Deletion of one copy of the 72-base-pair repeat sequence in the SV40 promoter caused no significant decrease in CAT synthesis in monkey kidney CV-1 cells; however, an additional deletion of 50 base pairs from the second copy of the repeats reduced CAT synthesis to 11% of its level in the wild type. We also constructed a recombinant, pSV0-cat, in which the entire SV40 promoter region was removed and a unique *Hin*dIII site was substituted for the insertion of other promoter sequences.

As the number of putative mammalian promoter sequences isolated by recombinant techniques has increased over the past several years, the attention of many investigators has turned to ways in which the function of these sequences may be measured. The in vitro transcription systems developed by Manley et al. (17) and Weil et al. (38) offer one attractive approach. However, it is not yet clear that such in vitro transcription systems respond to regulatory signals. To confirm and extend in vitro results, the study of promoters after their introduction into tissue culture cells is crucial. Although RNA levels represent the most definitive gauge of promoter activity, quantitation of RNA is tedious and it is often difficult to obtain accurate measurements unless a given promoter is particularly strong or amplification of the template is achieved. It is often preferable to determine the function of a promoter by joining the promoter to a second gene segment which codes for a readily assayable enzymatic function. This is an approach frequently used to advantage in procaryotic systems.

In selecting an enzymatic activity to monitor promoter function, there are several relevant considerations. (i) The selected activity must be easily and completely distinguishable from any endogenous activities; ideally, corresponding endogenous enzymatic activities should be completely absent from the host cell. (ii) There should be no interference from other enzymatic activities which could compete for the substrate or cofactors. (iii) The assay should be rapid, sensitive, reproducible, and convenient and should not require the use of hazardous radionuclides or chemicals. With these considerations in mind, we developed a series of recombinants in which the enzyme chloramphenicol acetyltransferase (CAT) was used to measure promoter function in mammalian tissue culture cells.

MATERIALS AND METHODS

Preparation of plasmid DNAs. Plasmid DNAs were prepared by lysozyme–Triton X-100 lysis (13) and cesium chloride-ethidium bromide equilibrium gradient centrifugation (23). DNAs to be used in eucaryotic cell transfection experiments were further purified by a second equilibrium centrifugation step.

Enzymes. Restriction endonucleases were obtained from New England Biolabs or Bethesda Research Laboratories. DNA polymerase I was obtained from Boehringer-Mannheim, T4 polynucleotide kinase from P-L Biochemicals, bacterial alkaline phosphatase from

Worthington Diagnostics, and T4 DNA ligase from New England Biolabs.

Preparation of DNA fragments. Restriction endonuclease digestions were carried out as recommended by the supplier. Cohesive ends were converted to blunt ends by incubation with DNA polymerase I (28). The addition of synthetic oligonucleotide linkers was performed by the method of Mulligan et al. (21). DNA fragments were purified by agarose gel electrophoresis and visualized by ethidium bromide staining; fragments were eluted by dissolving agarose slices in 6 M sodium perchlorate and collecting the DNA on Whatman GF-C glass fiber filters (4).

Bacterial transformations. DNA fragments were ligated by incubation with T4 DNA ligase overnight at 14°C. Transformations of *Escherichia coli* HB101 were performed by the method of Mandel and Higa (16). Colonies were selected on plates containing ampicillin (50 μg/ml).

Mammalian cell transfections. Before the experiments, DNA preparations were routinely checked by agarose gel electrophoresis to ensure that greater than 50% of plasmid DNA was in the form I configuration. DNAs which had sustained sufficient random nicking to allow accumulation of linear molecules were assumed to contain numerous single-strand nicks; such DNAs gave unreproducible results and therefore were not used. On the day before transfection, cells were plated at a density of $10^4/cm^2$ in 100-mm plates, and 3 h before the addition of DNA cells were refed with fresh medium containing 10% fetal calf serum. Calcium phosphate-DNA precipitates were prepared by the method of Graham and van der Eb (11). Care was taken to prepare very fine precipitates: this could be accomplished reproducibly by mixing the DNA-$CaCl_2$ and HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-buffered sodium phosphate solutions under a gentle stream of nitrogen. Precipitates were allowed to stand for 30 min without agitation before being added to the tissue culture cells. Initially the amount of DNA added was varied between 1 and 25 μg per plate to determine the linear range of uptake and expression of the recombinants. Comparisons between the parental pSV2-cat and the modified vectors were made with 10 μg of DNA per plate.

Assay of CAT activity in mammalian cells. Cell extracts were made 48 h after transfection by sonicating washed, pelleted cells in 100 μl of 0.25 M Tris-hydrochloride (pH 7.8). After the cells were spun for 15 min in an Eppendorf microfuge at 4°C, the supernatants were removed and assayed for enzyme activity. The assay mixture contained (in a final volume of 180 μl) 100 μl of 0.25 M Tris-hydrochloride (pH 7.5), 20 μl of cell extract, 1 μCi of [^{14}C]chloramphenicol (50 μCi/mmol; New England Nuclear Corp.), and 20 μl of 4 mM acetyl coenzyme A. Controls contained CAT (0.01 U; P.L. Biochemicals, Inc.) instead of cell extract. All of the reagents except coenzyme A were preincubated together for 5 min at 37°C. After equilibration was reached at this temperature, the reaction was started by adding coenzyme A. In some experiments time points were taken during the 30-min incubation at 37°C to enhance the sensitivity and accuracy of the assay. The reaction was stopped with 2 ml of cold ethyl acetate, which was also used to extract the chloramphenicol. The organic layer was dried and taken up in 30 μl of ethyl acetate, spotted on silica gel thin-layer plates, and run with chloroform-methanol (95:5, ascending). After autoradiography of the separated acetylated chloramphenicol forms, spots were cut out and counted. Data are expressed as the amount of chloramphenicol acetylated by 20 μl of extract.

RESULTS

Construction of pSV2-cat^r and pSV2-cat^s. The *E. coli* transposable element Tn*9* (15), which confers resistance to the antibiotic chloramphenicol, consists of a 1,102-base-pair (bp) CAT cistron flanked by two 768-bp IS*1* elements (1). Tn*9* was transferred from an R factor via bacteriophage P1 to bacteriophage lambda derivatives and subsequently to the *E. coli* plasmid pBR322 (27). An *E. coli* strain carrying one of these pBR322-Tn*9* constructs, N6302, was constructed and provided to us by J. L. Rosner, National Institutes of Health. We prepared two segments of the CAT gene from N6302 (Fig. 1). A 955-bp fragment was generated by cleavage of pBR322-Tn*9* at an *Hha*I site upstream from the CAT bacterial control sequences, followed by a *Taq*I partial digestion to cleave downstream from the CAT gene. A 773-bp fragment deleted of CAT promoter sequences was generated by a complete digestion of pBR322-Tn*9* with *Taq*I. Both of these fragments were modified by incubation with DNA polymerase I to create blunt ends and by the addition of mixed *Hin*dIII and *Bam*HI synthetic oligonucleotide linkers and then separated from other products by agarose gel electrophoresis. In this procedure about 25% of the fragments to which synthetic linkers were successfully ligated at both ends will carry a *Hin*dIII site 5′ to the CAT gene sequences and a *Bam*HI site 3′ to the gene. The modified 955-bp and 773-bp fragments were joined with the procaryotic-eucaryotic vector pSV2 (B. Howard, R. Mulligan, P. Southern, M. Yaniv, A. Geller, and P. Berg, manuscript in preparation), which consists of the origin of replication and ampicillin resistance genes of pBR322 and a simian virus 40 (SV40) early region transcription unit modified to accept foreign polypeptide coding sequences between *Hin*dIII and *Bgl*II sites (*Bam*HI and *Bgl*II produce identical cohesive ends). Recombinants were isolated as ampicillin-resistant colonies in *E. coli* HB101. In the first recombinant, pSV2-cat^r, the 955-bp bacterial insert consisted of a 210-bp CAT gene promoter segment, the entire CAT coding sequence, and 86 bp 3′ to the UAA stop codon. As denoted by the superscript r, this recombinant in bacteria confers chloramphenicol resistance. In the second recombinant, pSV2-cat^s, the 773-bp bacterial insert consisted of a 29-bp 5′ untranslated segment, the CAT coding sequence, and 86 bp 3′ to the translation stop codon. Since the promoter and 32 bp corresponding to the 5′ untranslated region of the

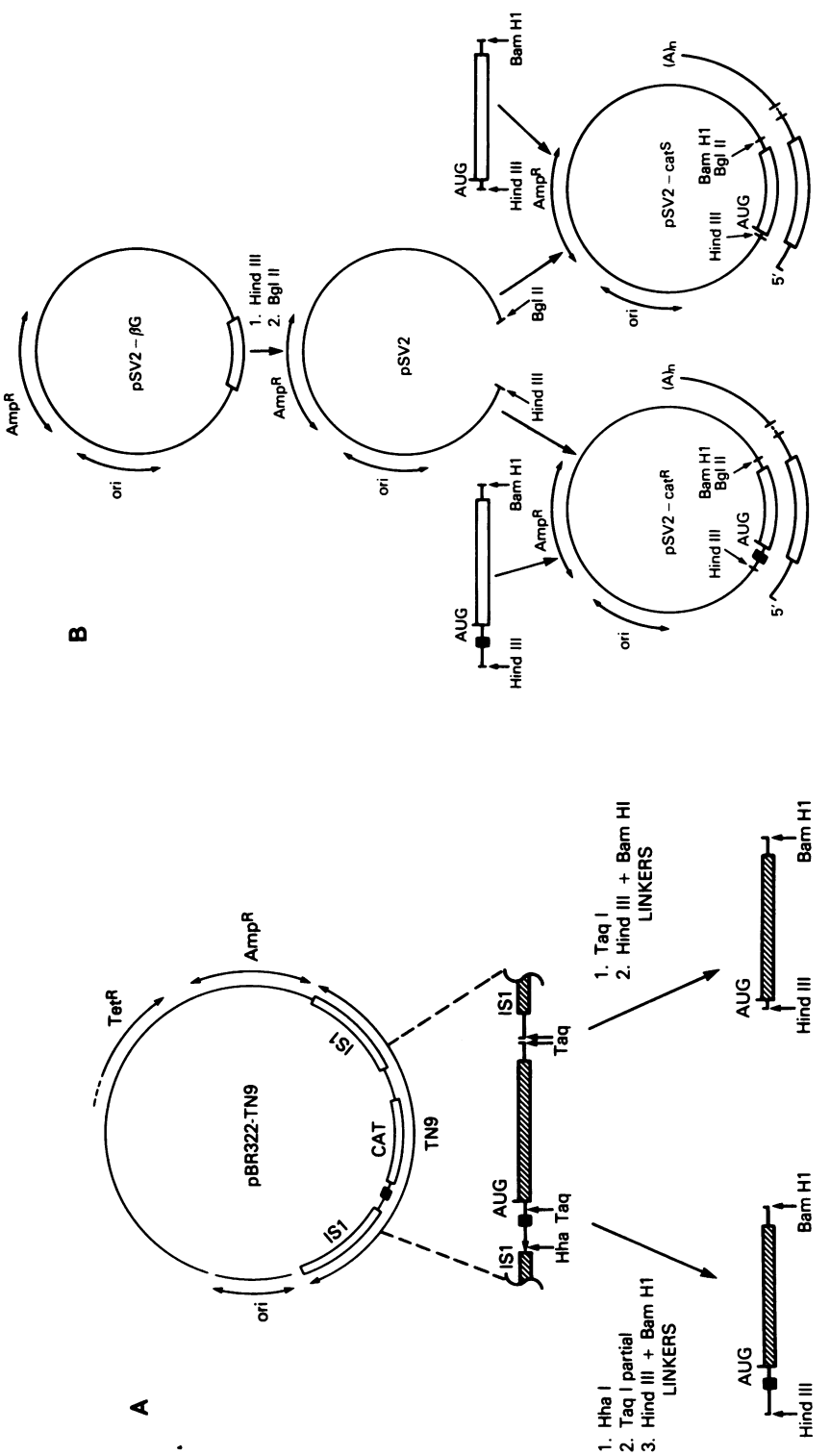

FIG. 1. (A) Preparation of CAT segments from the pBR322-Tn9 plasmid in *E. coli* N6302. The blackened box represents the CAT core promoter region, including the transcription start site. (B) Insertion of CAT fragments into pSV2. pSV2-βG contains the following functional elements (counterclockwise from 12 o'clock on the circular map): the Amp^r cistron and the origin of replication from pBR322 (2,295 bp) and segments containing the SV40 early promoter region (400 bp), a rabbit beta-globin cDNA insert (485 bp), the SV40 small t intron (610 bp), and the SV40 early region polyadenylate addition site (988 bp). Prototype transcripts associated with the PSV2-cat^r and PSV2-cat^s structures show the locations of the SV40 transcriptional control elements.

CAT mRNA were deleted from the bacterial insert in pSV2-cat^s, *E. coli* cells carry this recombinant are sensitive to chloramphenicol. Since all of our subsequent constructions were derived from pSV2-cat^s, it will frequently be referred to in the text simply as pSV2-cat.

Assay for expression of CAT in mammalian cells. CAT inactivates chloramphenicol by the formation of mono- and diacetylated derivatives (29). A number of assays have been developed to measure this activity (25, 29–32); we used the assay described by Cohen et al. (5) and Shaw and Brodsky (31), in which the acetylation of chloramphenicol is measured by silica gel thin-layer chromatography. This assay for CAT is very sensitive and highly specific, easily separating the parent, mono-, and diacetylated forms of chloramphenicol.

We tested the expression of the CAT gene in mammalian cells by introducing pSV2-cat^r and pSV2-cat^s DNAs into CV-1 monkey kidney cells as calcium phosphate precipitates (11). At 48 h after transfection, the cells were harvested and sonicated. Extracts from the cells transformed with pSV2-cat^s (Fig. 2, lane 4) and pSV2-cat^r (lane 5) contained readily measured CAT activity. In this experiment, 5×10^6 cpm (6 nmol) of [^{14}C]chloramphenicol was converted to acetylated derivatives by an extract from about 5×10^6 pSV2-cat^s-transfected cells. The autoradiogram (Fig. 2) was developed after 24 h. We detected no CAT activity in calf thymus DNA-transfected cell extracts at this level of sensitivity (lane 3) or after much longer exposures (data not shown). Several additional controls were tested: pBR322 carrying the Tn*9* element (lane 6), the pSV2 vector component carrying a xanthine-guanine phosphoribosyltransferase coding sequence (data not shown) (19), and a pSV2 derivative carrying the CAT gene 2.2 kilobases downstream from the SV40 early promoter region, near the Amp^r gene (data not shown; see Fig. 1B). None of these DNAs induced CAT activity, indicating that this enzyme is expressed only when the CAT gene is under SV40 early region control. It is of interest that the additional bacterial control sequences present in pSV2-cat^r (which include an AUG followed by a stop codon upstream from the CAT start codon) did not strongly inhibit expression.

In addition to experiments with CV-1 monkey kidney cells, we introduced pSV2-cat^s DNA into NIH3T3 mouse, Chinese hamster ovary, and chicken embryo fibroblast cells. In each cell type the recombinant was expressed but no background CAT activity was detected in the mock-transfected control (data not shown). The intensity of the signal in nonprimate cells varied between 20 and 70% of the signal obtained in CV-1 cells. Further experiments will be required to determine whether the weaker signal in nonprimate cells is due to less efficient DNA uptake, lower SV40 early promoter activity, or both.

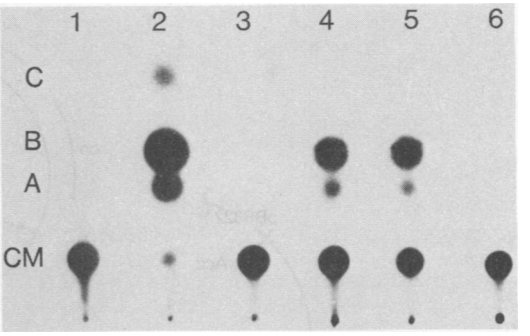

FIG. 2. Assay of CAT activity in mammalian cells. The appropriate DNA (10 μg as a calcium phosphate precipitate) was applied to 5×10^5 CV-1 monkey kidney cells plated the day before at a density of 10^4/cm^2. After 48 h, cell extracts were prepared for assay (see the text); 10- to 50-μl samples were then spotted on thin-layer silica gels. Chloramphenicol (CM) and its acetylated forms (in order of increasing mobility: [A] 1-acetate chloramphenicol, [B] 3-acetate chloramphenicol, and [C] 1,3-diacetate chloramphenicol) were detected by autoradiography. (Lane 1) The chloramphenicol standard. Remaining lanes show the products of chloramphenicol after incubation with (lane 2) CAT, (lane 3) extract of CV-1 cells transfected with calf thymus DNA, (lane 4) extract of CV-1 cells transfected with pSV2-cat^s, (lane 5) extract of CV-1 cells transfected with pSV2-cat^r, and (lane 6) extract of CV-1 cells transfected with pBR322-Tn*9*.

Construction of pSV(Sph)-cat, pSV1-cat, and pSV0-cat. Other investigators have reported that two elements, a Goldberg-Hogness TATA box and two tandemly repeated 72-bp sequences, are determinants of SV40 early promoter function (2, 3). The 72-bp repeat sequences appear to function as activators of the early promoter (18). To apply the relatively sensitive CAT assay to evaluating and extending these previous reports, we derived from pSV2-cat a set of recombinants which carried deletions in the SV40 early promoter region (Fig. 3). pSV(Sph)-cat was constructed by first cleaving pSV2-cat with the enzyme *Sph*I, which cuts once within each copy of the tandemly repeated 72-bp sequence. The resulting 72-bp *Sph*I fragment was then removed by agarose gel electrophoresis, and the remainder of the recombinant genome was recircularized. The SV40 early promoter in pSV2(Sph)-cat thus retained the Goldberg-Hogness TATA box and a single copy of the 72-bp activator sequence. Construction of pSV1-cat involved the restriction of pSV2-cat with *Sph*I and *Acc*I. The latter enzyme cuts pSV2-cat once within the pBR322 sequences, 180 bp from the SV40-pBR322 junction and 250 bp from the distal *Sph*I

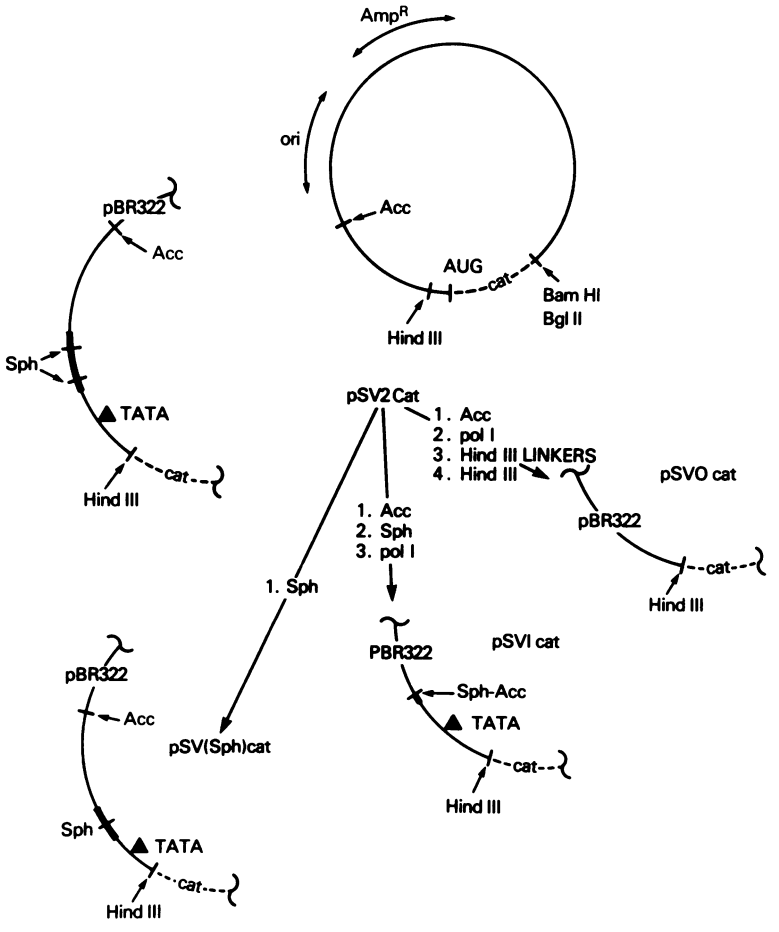

FIG. 3. Construction of modified pSV2-cat vectors. The complete circle and the uppermost arc represent the original pSV2-cat[5] structure. The wild-type SV40 early promoter region in pSV2-cat contained two 72-bp repeats (heavy line) and a Goldberg-Hogness TATA box; pSV(Sph)-cat contained one complete 72-bp repeat and an intact TATA box; pSV1-cat contained 30% of one 72-bp repeat and the TATA box; pSV0-cat had the entire early promoter region removed and was reclosed with a *Hin*dIII site for easy insertion of additional promoter sequences. Acc, Cleavage site for the restriction endonuclease *Acc*I; Sph, cleavage site for the restriction endonuclease *Sph*I.

site. After the removal of two small fragments by gel electrophoresis and incubation of the recombinant with DNA polymerase I, the deleted recombinant genome was recircularized by blunt-end ligation. The SV40 promoter region in pSV1-cat retained the Goldberg-Hogness box and 22 bp from one of the 72-bp repeat sequences. Construction of pSV0-cat required restriction of pSV2-cat with *Acc*I, blunting of this cohesive end, addition of *Hin*dIII oligonucleotide linkers, restriction with *Hin*dIII, and recircularization. All of the SV40 early promoter sequences were thus deleted in the construction of pSV0-cat.

Comparison of SV40 early promoter deletions by the CAT assay. CAT assays were performed on extracts prepared 48 h after the introduction of pSV2-cat, pSV(Sph)-cat, pSV1-cat, and pSV0-cat DNAs (10 μg per 10-cm plate) into subconfluent CV-1 cells. The formation of 3-acetyl chloramphenicol was plotted as a function of time to ensure that all assays were in the linear range and to minimize random variation. We detected no decrease in CAT synthesis when one 72-bp repeat sequence was deleted [pSV(Sph)-cat; Fig. 4]. In contrast, when 70% of the second repeat sequence was removed (pSV1-cat), CAT synthesis decreased to 11% of that obtained with the wild-type promoter. The sensitivity of the system was demonstrated by the finding that the signal from pSV1-cat, which retains the Goldberg-Hogness box and only 30%

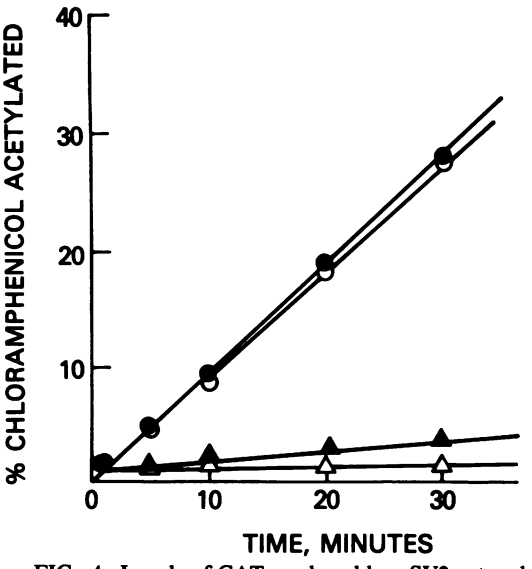

FIG. 4. Levels of CAT produced by pSV2-cat and modified vectors. Shown are the enzyme activity levels produced from 20 μl of extract (see the text). Counts were converted to the percentage of chloramphenicol acetylated as a function of time. pSV2-cat (●); pSV(Sph)-cat (○); pSV1-cat (▲); pSV0-cat (△).

of one 72-bp repeat sequence, was three times greater than the background signal obtained from pSV0-cat, which carries no SV40 early promoter. In previous studies, no SV40 early promoter activity was detected in early promoter mutants retaining less than 50% of one 72-bp repeat sequence (3, 12).

DISCUSSION

The probability that appropriate regulation of a cloned gene will be reproduced is enhanced when that cloned gene is reintroduced into the parental cell type. However, in the parental or homologous cell the problem of distinguishing between expressions of the cloned and endogenous genes is most pronounced. One solution to this problem is to combine the transcription start site or putative regulatory region(s) of the cloned gene of interest with a second gene segment which provides an easily assayable and readily distinguished function. In this paper we describe the combination of just such an assayable function, the CAT gene, with derivatives of the eucaryotic vector pSV2. We also demonstrated that the resulting vectors may be used to measure eucaryotic promoter function in a variety of cell types.

Several characteristics of the pSV2-cat vectors make them uniquely useful for the study of eucaryotic promoters. Most important is the absence of CAT activity in mammalian and avian cells. We found no evidence of chloramphenicol-modifying enzymes in any of the tissue culture cells we examined, African green monkey kidney CV-1, mouse NIH3T3, Chinese hamster ovary, or chicken embryo fibroblast cells; furthermore, since the pharmacological metabolism of chloramphenicol in mammalian cells consists primarily of its inactivation by glucuronyl transferase, it seems highly unlikely that examination of other mammalian cells would reveal endogenous CAT activity. The absence of interfering endogenous enzyme activities becomes particularly important in experiments in which the signal from the exogenous DNA is relatively weak and accurate quantitation is desired. This is evident in our data which show that an SV40 early promoter retaining only 30% of one 72-bp repeat sequence still induced about 11% as much CAT synthesis as did the wild-type promoter (about three times the background activity attributable to pSV0-cat, which retained neither the repeat sequences nor the TATA box sequences).

Other eucaryotic vector systems have been described as being potentially useful for the study of promoter activity in tissue culture cells. Examples are the pSV2 derivatives pSV2-gpt and pSVKgal, which express *E. coli* guanine phosphoribosyltransferase (19) and *E. coli* galactose kinase (26), respectively, in mammalian cells. In these systems, acrylamide or starch gel electrophoresis followed by an in situ assay is used to separate the vector-coded *E. coli* enzymes from the corresponding endogenous eucaryotic activities. In our experience, such procedures are considerably more complicated and difficult to quantitate accurately than is the CAT assay. In addition, the separation of exogenous and endogenous activities may vary depending upon the eucaryotic cell line used (19, 26). It has been reported that thymidine kinase from herpes simplex virus may be distinguished from endogenous thymidine kinase activities if iododeoxycytidine is employed as a substrate, although in some cell types it is necessary to add the inhibitor tetrahydrouridine to prevent a high background signal (9, 34). Currently the only commercially available radionuclide form of this substrate is the ^{125}I derivative. We feel that ^{125}I is in general more difficult to work with than is the ^{14}C-labeled chloramphenicol presently available.

The DNA sequence information available for the pSV2-cat vectors also contributes to their usefulness. pSV2-cat is assembled from elements of pBR322, SV40 (8, 24), and the CAT cistron (1), the DNA sequences of which have all been determined. Accordingly, working versions of pSV2-cat or its derivatives may be readily assembled and analyzed by computer programs such as that published by Queen and

Korn (22). The information obtained is extremely helpful in constructing and characterizing derivatives of pSV2-cat which carry alternate promoter segments. The nucleotide sequence has not been published for either the *E. coli* guanine phosphoribosyltransferase gene or the *E. coli* galactose kinase gene. The herpes simplex thymidine kinase DNA sequence is known (36); however, to our knowledge there are no published reports of eucaryotic vectors comparable to pSV0-cat which carry unique restriction sites immediately upstream of the thymidine kinase coding region.

Studies are in progress with pSV2-cat derivatives in which various eucaryotic promoters have been substituted for the SV40 early promoter region. We found that the herpes thymidine kinase promoter (36) drives the expression of CAT both in CV-1 monkey kidney cells and in mouse NIH3T3 cells (C. Gorman, M. Dobson, B. Howard, and G. Khoury, unpublished data). Similarly, the promoter for the chicken α2 type I collagen gene (35) directs the synthesis of CAT in CV-1 and chicken embryo fibroblasts (H. Ohkubo, C. Gorman, B. Howard, and B. de Crombrugghe, unpublished data). Less CAT is produced when these promoters replace the SV40 early promoter (less than 10% of pSV2-cat activity). In contrast, the promoter contained in the 3′ long terminal repeat from Rous sarcoma virus (39) directs three to five times as much CAT synthesis as does the SV40 early promoter in both chicken embryo fibroblasts and CV-1 cells. This difference between Rous sarcoma virus and SV40 early promoter strengths was verified by measuring cytoplasmic CAT mRNA after transfection of CV-1 cells (C. Gorman, G. Merlino, I. Pastan, and B. Howard, manuscript in preparation). Our experience in these and other experiments is that the CAT system provides a rapid and accurate method for comparing different promoters, measuring the apparent strength of one promoter in a number of cell types, comparing transfection protocols, and evaluating other parameters which affect exogenous gene expression. Particularly interesting results obtained by the CAT screening assay were confirmed, where necessary, by determining RNA levels.

The emphasis of the experiments presented in this study was on the measurement of CAT activity within 48 h after the introduction of exogenous DNAs. We also constructed a recombinant genome, pSV2-cat(SV-gpt), in which the SV40-gpt early region transcription unit from pSV2-gpt (19) was inserted at the *Bam*HI site in pSV2-cat. After transfection of pSV2-cat(SV-gpt) into mouse NIH3T3 cells, colonies resistant to mycophenolic acid-xanthine-HAT (hypoxanthine-aminopterin-thymidine) selective medium (20) were picked, amplified, and assayed for CAT activity. A signal was readily detected (data not shown), showing that CAT may also be used to monitor the expression of exogenous gene copies in stably transformed cell lines.

Growth of mammalian cells in tissue culture may be inhibited by chloramphenicol (6, 33, 40). Studies on chloramphenicol-resistant cell lines suggest that the mechanism of growth suppression is inhibition of mitochondrial protein synthesis (7, 10, 14, 37). Cells which carry vectors such as pSV2-cat should rapidly acetylate intracellular chloramphenicol; if acetylation is sufficiently complete and prevents the chloramphenicol from binding to mitochondrial ribosomes, cells carrying CAT vectors should be protected from chloramphenicol toxicity. Our experiments show that CAT expression after DNA transformation does confer at least limited resistance to this antibiotic. The details of selection protocols and results will be presented in a separate communication (C. Gorman and B. Howard, manuscript in preparation).

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