Conversion of the lac Repressor into an Allosterically Regulated Transcriptional Activator for Mammalian Cells

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A novel mammalian regulatory system was created by using the Escherichia coli lac repressor. The lac repressor was converted into a mammalian transcriptional activator by modifying the lac repressor coding region to include a nuclear localization signal from the simian virus 40 (SV40) large tumor antigen and the transcription activation domain from the herpes simplex virus type 1 virion protein 16. The lac activator protein (LAP) fusions were potent activators of several promoters containing lac operator sequences positioned either upstream or downstream of the transcription unit. A single lac operator allowed for transactivation, whereas multiple operators acted synergistically when separated by a small distance. Promoters containing 14 or 21 operator sequences were induced at least 1,000-fold in response to LAP, reaching levels of activity 20 to 30 times greater than that of the SV40 early promoter in HeLa cells. Activation was strongly inhibited by isopropyl-β-D-thiogalactoside (IPTG), indicating that LAP retained the functions needed for allosteric regulation. LAP was bifunctional, also acting as a repressor of expression of an SV40 promoter containing an operator immediately downstream of the TATA box. Finally, genetic selection schemes were developed such that LAP-expressing cell lines can be generated at high frequency from either established or primary cells in culture.

Inducible expression systems have been key tools in elucidating the function of a wide variety of genes in bacteria, yeasts, and Drosophila sp. It is only recently that similar systems for mammalian cells have been developed. These systems are of two types: promoters responsive to endogenous cellular transactivators and promoters regulated by exogenous bacterial regulatory proteins.

The first type includes promoters responsive to various treatments such as heat shock, heavy metals, or hormones (7, 22, 23, 47, 53). The main advantage to these systems is that expression can be induced greater than 100-fold in some cases. Such systems, however, have several inherent disadvantages. Because the promoters are responsive to cellular transactivators, induction presumably alters the expression of endogenous genes in addition to the gene being studied. Furthermore, these promoters often have relatively high basal activities and variable induced levels. Induction with hormone or heat shock also has pleiotropic effects on cell physiology, including effects on RNA stability and protein processing, in addition to their effect on transcription (13, 32).

The second approach has used bacterial repressor proteins to block transcription in mammalian cells. These bacterial proteins offer a distinct advantage as regulatory switches in mammalian cells in that the recognition sequence (the operator) for the repressor is relatively unique to the gene being studied. Both the lexA and lac repressors have been used in mammalian cells (8, 21, 49). The lac repressor, the product of the lacI gene, is particularly useful in that its DNA-binding activity is strongly inhibited by the non-hydrolyzable galactose analog isopropyl-β-D-thiogalactoside (IPTG) (43). Several groups have shown that the lac repressor can inhibit expression of the simian virus 40 (SV40) early promoter, a vaccinia virus promoter, or a T3 bacteriophage promoter in mammalian cells when the promoters contain appropriately placed operators and that repression can be largely relieved with IPTG (8, 11, 12, 15, 21). However, high levels of expression or inducibility have not been reported when such systems have been used in the context of stable cell lines.

An alternative approach would combine the unique specificity of bacterial repressors with the powerful induction provided by certain mammalian transactivators. Eucaryotic transcription factors are often composed of separate, independent DNA-binding and transcriptional activator domains (reviewed in reference 36). The independence of the domains has allowed for the creation of functional fusion proteins consisting of the DNA-binding and activating domains of heterologous proteins. The first chimeric procaryotic-eucaryotic regulatory protein consisted of the lexA DNA-binding protein and the activation domain of the yeast transcription factor, GAL4 (6).

The lac repressor has several properties which suggest that it could serve as the backbone of an ideal mammalian transactivator. First, extensive genetic and biochemical analyses have defined the functional domains of the repressor protein. The DNA-binding domain is contained in the first 60 amino acids of the repressor, the middle two-thirds of the protein are required for dimerization and inducer binding, and the very carboxyl-terminal amino acids have been suggested to be required for tetramerization (35). Second, the binding affinity and sequence specificity of lac repressor are extremely high. The Kd of the lac repressor for its operator sequence (10–11 M) is approximately 3 orders of magnitude greater than that of the lexA repressor for its operator (5, 44). The lac repressor recognizes a relatively large, complex operator of 25 base pairs (bp) (35). As few as three fortuitous lac operator sequences have been found to exist in various mammalian genomes (48) so that very few cellular sequences should be recognized by the lac repress-

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or. Third, it has already been shown that the lac repressor functions in mammalian cells; thus, the repressor can interact with DNA in the context of mammalian chromatin. In fact, packaging of operator-containing DNA by histone octamers has been reported to lower the nonspecific binding of DNA by the lac repressor without affecting its specific DNA-binding properties (10). Finally, as described above, the DNA-binding activity of the lac repressor is inhibited by IPTG in mammalian cells as well as in bacteria. Thus, a lac activator fusion protein, or lac activator protein (LAP), might also be allosterically regulated. In this case, however, the regulation would be reversed in that IPTG would repress expression by preventing activation.

One of the most efficient eucaryotic activation domains known is contained in the carboxy-terminal 100 amino acids of the herpes simplex virus type 1 (HSV) virion protein 16 (VP16) (45, 55). VP16, also known as Vmw65 or α-gene trans-inducing factor, is carried within the virion of HSV and activates transcription of the viral immediate early promotors, including those for ICPO and ICP4 (9, 28, 41). Although VP16 specifically activates promotors containing the canonical TAATGARAT element, the specificity is endowed by a cellular DNA-binding protein(s) which is complexed with the amino-terminal domain of VP16 (37, 42). The VP16 activation domain has also been previously shown to function efficiently when transplanted onto a heterologous DNA-binding domain (45).

The aim of the experiments reported here was to explore the use of the lac repressor as the basis for an inducible mammalian transactivator. LAP gene fusions between lacI and the 3' end of the VP16 gene were constructed and analyzed. The LAPs were IPTG-regulatable transactivators of promotors linked to lac operator sequences. Genetic selection for LAP function were also developed and should greatly facilitate the use of the LAP transactivators.

**MATERIALS AND METHODS**

**Construction of lacI derivatives.** Plasmids were constructed by standard procedures (33). Synthetic oligonucleotides were prepared on an Applied Biosystems automated DNA synthesizer, using phosphoramidite chemistry.

The lacI gene contained in the fl hybrid phage fl-Δ47 (generously provided by J. E. LeClerc), a derivative of fl-K12 (20), was altered by in vitro mutagenesis according to the procedure described by Zoller and Smith (58). fl-Δ47 differs from fl-K12 by a 47-bp deletion that removes the lac operator and creates an EcoRI site at the junction. The oligonucleotide 5'-CTGTTTTCATGTTAACCC-3' was used as a mutagenic primer to generate lacI<sup>ATG</sup> by changing the initiating GTG to ATG and by creating an HpaI site immediately 5' to the lacI coding region.

Two complementary oligonucleotides, 5'-GATATCATTG CCAAAAAAGAGGAGAAGGTA-3' and 5'-TACCTTTTC TCTTCTTTTGGGATGATATC-3', which contains an EcoRV site and encodes an initiator Met (ATG is underlined) and the SV40 large T-antigen nuclear localization signal (Pro-Lys-Lys-Lys-Arg-Lys-Val (25)), were phosphorylated, annealed, and inserted at the new HpaI site of lacI<sup>ATG</sup> to generate lacI<sup>5'N1</sup>. DNA containing lacI<sup>ATG</sup> was partially digested with HpaI, a BamHI linker was added, the DNA was excised by digestion with EcoRI, and the 1.2-kilobase-pair BamHI-EcoRI fragment was then subcloned into pBR322. The EcoRI site was converted to a SalI site, and the BamHI-SalI lacI<sup>ATG</sup> fragment was inserted into pExp1 (K. Leppard and T. Shenk, unpublished data), an expression vector containing the SV40 early promoter, the SV40 small t-antigen splice site, and the adenovirus type 5 E1B poly(A) addition site, to generate pExp1lacI<sup>ATG</sup>

pHCMVlacI<sup>ATG</sup> was constructed by ligation of the EcoRI-HindIII fragment from pBC123 (generously provided by L.-S. Chang), containing the human cytomegalovirus (CMV) promoter-enhancer between nucleotides -600 and +72 relative to the transcription start site (4), the HindIII-PvuII fragment from pBR322, and the EcoRI-NruI fragment from pExp1lacI<sup>ATG</sup>, containing the lacI<sup>ATG</sup> gene, the small-t splice site, and the E1B poly(A) site. The Smal-Apal fragment from pHCMVlacI<sup>ATG</sup> was replaced with the EcoRV-Apal fragment from lacI<sup>5'N1</sup> to create pHCMVlacI<sup>5'N1</sup>

pVP16-0 was created by insertion of the EcoRV-PstI fragment of the VP16 gene (41) into Smal-PstI-digested pBS-Avi1 (a derivative of pBSI; Stratagene). A 360-bp RasI fragment encoding the last 120 amino acids of VP16 was isolated from pVP16-0 and cloned into the EcoRV site of pBSI (Stratagene). The VP16-coding region was then removed by partial digestion with SmaI and complete digestion with HindIII. The small 400-bp fragment was isolated, and the HindIII overhang was filled in with the Klenow fragment of Escherichia coli DNA polymerase I and then ligated with pHCMVlacI<sup>5'N1</sup> which had been partially digested with PvuII, creating pCHMVLAP317 and pCHMV LAP348. The LAP348-coding region was removed from pCHMVLAP348 by partial digestion with SstI and complete digestion with BglII. The LAP348 gene was subcloned into pBSI and subsequently inserted into the human β-actin vector pLAP2 (19).

**Construction of reporter constructs.** pSVE<sup>CAT</sup> was created by insertion of the Sphl-BamHI fragment of pSV2CAT into Sphl-BamHI digested pFB69 (1), a derivative of pBR322. pLICAT was created by insertion of the Sphl-BamHI fragment of pSV2CAT into Smal-BamHI-digested pBSI (Bluescript vector KS+; Stratagene) such that the SV40 promoter was 150 bases downstream from the lac operator located in the vector. A synthetic lac operator DNA duplex with Sall cohesive ends was obtained by annealing the following two phosphorylated oligonucleotides: 5'-TCGACGGAAATTGGGACGATACATG-3' and 5'-TCGACATTGAATCCCGTACAAATCCG-3'.

pL1-1CAT and pL1-2CAT were created by insertion of one and two 29-bp synthetic promotors, respectively, into the SalI site of pLICAT. pL1-3CAT was created by inserting an additional operator into the XhoI site of pL1-2CAT. pL2CAT was created by insertion of the Xho1-BamHI fragment of pL1-2CAT into Xho1-BamHI digested pFB69. pL7CAT was created by ligation of a PvuII-EcoRV fragment containing the four lac operators of pL1-3CAT into the EcoRV site of pL1-2CAT. pL14CAT and pL21CAT were created by insertion of one or two copies of the operator-containing PvuII-EcoRV fragment of pL7CAT into the EcoRV site of pL7CAT. p3'E3CAT and p3'E7CAT were created by insertion of either the Apal-Clal or the PvuII-EcoRV operator-containing fragment of pL1-3CAT or pL7CAT, respectively, into the EcoRV site of pSVE<sup>CAT</sup>. pL1ICAT was created by insertion of the small EcoRI fragment of pIGA65 (16) into EcoRI-digested pCAT, which contains the chloramphenicol acetyltransferase (CAT) gene and SV40 splice and polyadenylation signals from pSV2CAT inserted into the EcoRV site of pBSI. pICP0 was created by insertion of the pICP0CAT gene without the vector-contained operator sequence into the EcoRV site of pFB69.

Plasmids pX-8, pX-58, pX-100, pS-312, and pS-232, de-
scribed by Fromm and Berg (14), were generously provided by P. Berg. The synthetic lac operator was cloned into the XhoI site of pX-8 (deletion endpoints 3 and 5237 in the SV40 DNA sequence) to generate pSVL-1p. Deletion mutants pX-8 (deletion endpoints 53 and 5237) and pS-312 (deletion endpoints 346 and 34) or X-100 (deletion endpoints 108 and 5237) and pS-232 (deletion endpoints 346 and 114) were recombined by ligation in the presence synthetic lac operator, and single insertions of lac operator were identified to construct pSVL-1p-2 and pSVL-1p-3. Insertion of operators was first detected by the appearance of blue colonies on plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; Boehringer Mannheim Biochemicals) after transformation of HB101 cells, due to titration of the endogenous lac repressor with a high-copy-number plasmid bearing the operators. Various CAT reporters were constructed by excising the Sau3AI-HindIII fragment encompassing the SV40 early promoter from pX-8, pSVL-1p, pSVL-2p, pSVL-3p, and pSVL-4p-3 and then inserting each fragment into pBlII-HindIII-digested pA10CAT2 (30).

pL1-3neo was created by insertion of the SfiI-BamHI fragment of pSV2neo into SfiI-BamHI-digested pL1-3CAT. pL2T-antigen was created by insertion of the SphI-BamHI fragment of pSVL2T-antigen into SphII-BamHI-digested pFB69.

Cell culture and transfections. Monolayer HeLa and Ltk- cell lines were maintained in Dulbecco modified Eagle medium supplemented with 10% calf serum. Primary rat embryo fibroblasts (REF) cells (generously provided by C. Finlay) and transformants were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum.

Cells were transfected by the calcium phosphate precipitation method as previously described (56). For transient expression assays, 50 to 70% confluent 100-mm-diameter plates of cells were incubated overnight after addition to the medium of a 1-ml precipitate containing 2 μg of reporter and 1 μg of effector plasmids and carrier DNA (high-molecular-weight calf thymus DNA) for a total of 25 μg of DNA. The medium and precipitate were removed and replaced with fresh medium the next morning.

For stable transformation assays, G418 HeLa and Ltk- cells were selected with 0.5 and 1 mg, respectively, of G418 per ml. Individual G418 colonies and transformed REF foci were isolated with cloning cylinders and propagated in appropriate media.

Cell labeling and immunoprecipitation analysis. Antibodies were raised in rabbits against purified lac repressor (generously provided by J. Borowiec and J. Gralla) by standard procedures.

For immunoprecipitation analysis, HeLa cells (106 cells per 10-cm-diameter dish) were transfected by using 10 μg of each lac derivative and 2 μg of pAdSVAl-II, a plasmid encoding adenovirus type 5 VA RNA genes which has been shown to enhance expression of cotransfected genes (26). The DNA precipitates were left on the cells for 4 h, followed by incubation for 3 min with 15% glycerol in phosphate-buffered saline (PBS). Cells were labeled with [35S]methionine 46 to 48 h after transfection.

For metabolic labeling with [35S] HeLa cells were washed with methionine-free Dulbecco modified Eagle medium and then incubated for 2 h in methionine-free medium containing 2% calf serum and 100 μCi of [35S]translabel (ICN Pharmaceuticals) per ml. Labeled cell lysates were prepared, and immunoprecipitations were performed as described previously (46) except for the use of RIPA buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 0.1% sodium dodecyl sulfate [SDS], 0.1% Triton X-100, 0.1% deoxycholate). Samples of precleared lysates (107 cpm) were incubated with 2 μl of rabbit preimmune or immune serum for 1 h on ice. Fixed, prewashed Staphylococcus aureus cells (Pansorbin; Calbiochem-Behring) were added (25 μl of 10% [wt/vol] suspension), and incubation continued for 10 min on ice. Cells were pelleted and washed three times in RIPA buffer and once in TEN (10 mM Tris [pH 7.4], 1 mM EDTA, 50 mM NaCl). Samples were suspended in 15 μl of loading buffer (2.5 mM Tris [pH 6.8], 2.5% SDS, 100 mM dithiothreitol, 10% glycerol, 0.005% pyronin Y), boiled for 5 min, and loaded onto a 10% SDS-polyacrylamide gel. After electrophoresis, gels were fluorographed with Resolution (E-M Corp.), dried, and exposed to Kodak XAR film at −70°C.

Indirect immunofluorescence. Subconfluent cells (LAP neo2b and LAPneo6b) and transfected HeLa cells grown in 35-mm-diameter dishes were washed three times with PBS, fixed with PBS containing 4% formaldehyde for 10 min, and permeabilized with 0.1% Triton X-100 in PBS for 2 min. Cells were washed five times with PBS and then incubated for 1 h with rabbit immune serum against lac repressor diluted 1:100 with 5% goat serum in PBS. Cells were washed five times with PBS containing 0.5% bovine serum albumin and 0.1% Tween 20 and then incubated for 1 h with fluorescein-conjugated goat anti-rabbit antibody (Jackson Immunoresearch Laboratories, Inc.) diluted to 25 μg/ml with 0.5% bovine serum albumin and 0.1% Tween 20. Cells were washed five times with PBS containing 0.1% Tween 20 and once with PBS and then analyzed by fluorescence microscopy.

RNA analysis. Cytoplasmic RNA was isolated as previously described (29) except that the samples were also digested with DNase I (Promega Inc.). RNase protection assays were carried out as previously described (34), using 20 μg of cytoplasmic RNA, and hybridized with approximately 107 cpm of RNA probe prepared with [32P]UTP (>2,000Ci/mmol; Dupont, NEN Research Products). RNase-digested samples were fractionated on a denaturing 5% polyacrylamide sequencing gel, dried, and exposed to XAR-5 film for autoradiography.

CAT assays. Cells were harvested 44 to 48 h after transfection, and CAT assays were performed essentially as described previously (18). Equal amounts (30 to 200 μg) of protein were used in each experiment. CAT activity was quantitated by thin-layer chromatography and liquid scintillation counting of spots. Extracts that converted greater than 80% of the chloramphenicol in an assay were diluted appropriately to ensure linearity of the assay.

RESULTS

Construction of lacI-VP16 fusion proteins. Structures of the lac derivatives used are shown in Fig. 1A. To facilitate expression in mammalian cells, the bacterial initiator GTG was changed to ATG, creating lacIATG. The 5′ end of lacI was further modified to include the nuclear localization signal from the SV40 large T antigen (25), generating lacIATG. When expressed in mammalian cells, both lacIATG and lacI5′N1 functioned to efficiently repress transcription from promoters containing appropriately positioned lac operator sequences (data not shown; see below). The results
with lacI5'N1 demonstrate that extending the amino terminus of lac repressor with the nuclear localization signal does not significantly interfere with operator binding.

Two LAP constructs were generated by modification of lacI5'N1. A cassette encoding the carboxyl-terminal acidic domain between amino acids 369 and 488 of VP16 was inserted in frame either into two PvuII sites, after the codons for amino acids 317 or 348 of the lacI gene, creating LAP317 and LAP348, respectively (Fig. 1A). This strategy was chosen for two reasons. First, the two PvuII sites were within a region of the lacI gene with very few mapped mutations, suggesting that this region might be dispensable or independent from the domains involved in DNA binding, dimerization, and inducer binding (reviewed in references 3 and 35). Furthermore, previous experiments demonstrated that insertion of a nuclear localization signal at amino acid 348 did not appear to alter the activity of the repressor (data not shown). Second, genetic analysis of the lacI gene suggested that the carboxyl-terminal 40 amino acids including the last 8 amino acids of the repressor might be required for tetramerization. Thus, the fusion proteins were constructed such that every amino acid of lacI was conserved and the carboxyl terminus remained intact. Each coding region was placed under control of the human CMV immediate-early enhancer-promoter (4), and these constructs were used in all transient assays described.

lacI-related proteins were detected by immunoprecipitation of [35S]methionine-labeled proteins with anti-lac rabbit polyclonal antiserum 48 h after transfection of HeLa cells (Fig. 1B). A predominant 41-kilodalton protein was detected after transfection with lacI16, whereas the protein detected after transfection with lacI5'N1 was slightly larger because of the nuclear localization signal. Both LAP317 and LAP348 migrated more slowly than expected, with apparent molecular sizes of 68 and 61 kilodaltons, respectively. This observation is not surprising in that many proline-rich nuclear proteins, such as p53 and VP16, have much slower mobilities on SDS-polyacrylamide gels than predicted by amino acid sequence (41, 57). The reason for the different mobilities of LAP317 and LAP348 is not known but is most likely related to the location of the VP16 insertion within the lacI coding region. Consistently less LAP than lac repressor protein was detected in these transient assays, even though equal amounts of DNA were transfected and equal amounts of [35S]labeled extracts were used for immunoprecipitations. This result might be due either to changes in protein stability or to effects of the LAPs on the CMV promoter (see below).

The intracellular location of lacI derivatives was determined by indirect immunofluorescence. The fluorescent micrographs of HeLa cells transfected with either lacIATG, lacI5'N1, or LAP348 are compared in Fig. 1C. lacIATG was present in approximately equal amounts in both the nuclear and cytoplasmic compartments. In contrast, the majority of the fluorescent signal observed after transfection with either lacI5'N1 or LAP348 was in the nucleus. Immunoprecipitation of fractionated cell extracts from transfected HeLa cells confirmed that almost all of the lacI5'N1 was present in the nuclear compartment, whereas lacIATG was distributed equally in both cytoplasm and nucleus (data not shown).

LAP317 and LAP348 activate expression from an SV40 promoter containing lac operator sequences. The ability of the LAPs to activate a suitable promoter was tested by cotransfection assays in HeLa cells. The first reporter tested, pL1-2CAT, consisted of a CAT gene under control of the SV40 promoter, lacking its enhancers but linked to three lac operators. As a control for specificity, similar cotransfections were carried out with pSV2CAT (18), which contains the endogenous SV40 enhancer but no lac operator sequences. Cellular extracts were prepared 48 h after transfection and assayed for CAT activity (Fig. 2A). Expression from pSV2CAT was not significantly affected by cotransfection with either LAP construct. Very low levels of activity were detected after cotransfection of pL1-2CAT with either pBR322 or pHCMVlacI5'N1; however, high levels of CAT activity were observed after cotransfection of pL1-2CAT with either pHCMVLAP317 or pHCMVLAP348. Cotransfection with pHCMVLAP348 resulted in the production of significantly higher amounts of CAT activity as compared with pHCMVLAP317 and was therefore used in all of the following experiments.

The effects of LAP348 on expression from the SV40 promoter with or without the enhancer were further examined by cotransfection assays in HeLa cells (Fig. 2B). Although LAP348 had no significant effect on pSV2CAT expression (lanes 1 and 2), LAP348 activated expression from pSVLpCAT (lanes 3 and 4), which contains the SV40 enhancer and a single operator sequence between the enhancer and the Sp1-binding sites (see below). Thus, operator-dependent activation occurred irrespective of the SV40 enhancer. The effects of LAP348 on expression of enhancerless SV40 CAT genes containing single or multiple operators were also examined. LAP348 had no effect on expression of an enhancerless SV40 construct, pSVE-CAT (lanes 5 and 6). The presence of a single operator located approximately 150 bp upstream of the SV40 promoter in pl1CAT allowed for activation (lanes 7 and 8). The insertion of one and two additional operators at 40 bp upstream of the Sp1-binding sites of the SV40 promoter present in pl1-1CAT and pl1-2CAT, respectively, greatly enhanced activation by LAP348 (compare lanes 8, 10, and 12). Thus, activation was dependent on

FIG. 1. Structures and characterization of lac and LAP genes. (A) Structures of the lacI and modified lacI genes. The amino acid-coding regions of various lacI constructs are shown. The first and last amino acids (1 and 360) of the wild-type lacI repressor are shown above the lacI gene. The nuclear localization signal (NLS) and the initiator ATG added to create lacI5'N1 and LAP genes (●) and the location of the VP16-coding region (□) are indicated. The amino acids of lac repressor at the junctions of the lac and VP16-coding regions and the amino acids of VP16 added (underlined) are indicated above the box (41). The amino acid numbers given for lac repressor refer to that of the wild-type allele (35). The amino acids indicated for VP16 do not include eight amino acids encoded by the vector polylinker. (B) Immunoprecipitation analysis of lac repressors and LAP proteins. HeLa cells were transfected with pHCMVlacIATG (lane 1), pHCMVlacI5'N1 (lane 2), PHCMVLAP317 (lane 3), or PHCMVLAP348 (lane 4) and pAdSV5A1/II and were labeled with [35S]methionine as described in Materials and Methods. Cell lysates were immunoprecipitated with either rabbit preimmune (P) or anti-lac repressor immune (I) serum and analyzed by electrophoresis on a 10% SDS-polyacrylamide gel. All genes were under control of the CMV promoter-enhancer as described in the text. The sizes (in kilodaltons [kD]) and positions of molecular weight standards are shown on the left, and the positions of the various lacI derivatives are shown on the right. (C) Intracellular location of lacI derivatives. HeLa cells transfected with the lacI genes described as indicated were fixed and stained with lacI antibodies as described in Materials and Methods. No true fluorescent signal was seen in experiments with mock-transfected HeLa cells (data not shown). An approximate size scale (20 μm) is represented by the bar.
the number of operators and multiple operators acted synergistically.

The structures of the promoters used to drive CAT expression are shown in Fig. 3 along with their relative activities after cotransfection with pBR322 DNA or with LAP348. The relative activities represent the averages derived from three to five independent transfection experiments normalized to the CAT activity obtained with pSV2CAT in parallel transfections. The basal SV40 promoter used for these experiments contains the 21-bp repeats of the SV40 promoter, which includes multiple binding sites for the transcription factor Sp1, the TATA box, origin of replication, and normal transcription start sites. The basal activity of the enhancerless SV40 promoter (pSVE- CAT) and derivatives were normally undetectable in these assays (less than 0.02 of the activity produced by the SV40 enhancer-promoter).

pL1CAT, which contains a single operator about 150 bp upstream of the SV40 promoter, produced about 1/10 the CAT activity of pSV2CAT upon activation by LAP348. pL2CAT, which contains two tandem operators about 40 bp upstream of the SV40 promoter, produced two- to threefold higher levels of CAT than did pL1CAT. In contrast, reporters containing the 150-bp upstream operator and either one, two, or three tandem operators (pL1-1CAT, pL1-2CAT, or pL1-3CAT, respectively) inserted 40 bp upstream of the SV40 promoter produced significantly higher levels of CAT, 1.6 to 3 times that produced by pSV2CAT. Thus, there was a synergistic effect of multiple operators on activation when the operators were separated by a small distance. The reason for the spacing requirement is unclear, especially in light of the observations made by others that tandem operators increase the ability of lac repressor to inhibit expression from appropriate promoters in mammalian cells (21). One explanation for this observation is that the increased size of LAP compared with lac repressor protein or the recruitment of additional cellular proteins by the VP16 sequences might

FIG. 2. Transactivation of operator-containing promoters by LAPs. (A) Transactivation of pL1-2CAT by LAP317 and LAP348. HeLa cells were transfected with 2 μg of either pSV2CAT or pL1-2CAT and 1 μg of pBR322 or lac derivatives as indicated. The total amount of DNA in each transfection was adjusted to 25 μg with carrier calf thymus DNA. Cell extracts were made 48 h posttransfection, and CAT assays were carried out as described in Materials and Methods. (B) Operator-dependent activation by LAP348. HeLa cells were transfected with 1 μg of either pSV2CAT (lanes 1 and 2), pSV1+CAT (a derivative of pSV2CAT containing an operator at position 3 between the enhancer and 21-bp repeats; lanes 3 and 4), or enhancerless SV40 promoter CAT vectors containing no (pSVE- CAT; lanes 5 and 6), one (pL1CAT; lanes 7 and 8), two (pL1-1CAT; lanes 9 and 10), or three (pL1-2CAT; lanes 11 and 12) operators with pBR322 (−) or with 1 μg of pHCMVLAP348 (+). Transfections and CAT assays were performed as described for panel A.
prevent binding of two LAP tetramers to adjacent operators. Alternatively, the spacing of operators might be important only for efficient activation.

These data suggested that a functional unit for highly efficient activation consists of a set of spaced operators. On the basis of this information, reporter plasmids containing tandem arrays of the spaced operators used in pL1-2CAT and pL1-3CAT (A and B repeats, respectively) were constructed and analyzed (Fig. 3). Although little difference in CAT expression was observed when pL1-2CAT and pL1-3CAT were compared, constructs containing tandem arrays of the spaced sets of operators (pL7CAT, pL14CAT, and pL21CAT) showed enhanced levels of activation by LAP348. pL7CAT, consisting of the tandem A and B operator repeats, produced approximately 10 times as much CAT activity as did pSV2CAT. A reporter containing 14 operators (pL14CAT), consisting of a tandem duplication of the L7 operators (with the structure ABAB), produced two- to threefold-greater amounts of CAT activity than did pL7CAT, resulting in production of approximately 20 times more CAT than produced by pSV2CAT. Finally, pL21CAT, containing three tandem copies of the pL7CAT operators, produced on average higher levels of CAT, approximately 30 times that by pSV2CAT. Because expression of pL14CAT and pL21CAT was not readily detectable in the absence of LAP, we infer from these experiments that expression was induced at least 1,000- to 1,500-fold by LAP348 under these transfection conditions (20.3 or 31.0 divided by 0.02, the lowest detectable level of expression; Fig. 3).

LAP348 also activated expression of the SV40 promoter when operator sequences from pL7CAT were placed 3' to the CAT gene of pSVE-1CAT (p3'L7CAT), although the amount of CAT produced was significantly lower than observed with the reporter containing the same operators just upstream of the promoter (pL7CAT). Thus, the operators act as a LAP-inducible enhancer. A reporter construct (p3'L3) containing a tandem array of three operator sequences 3' to the CAT gene produced low amounts of CAT upon activation by LAP348. This observation supports the conclusion stated above that activation through spatially separated operators is significantly more efficient than that achieved through tandem operators.

To confirm that LAP348 did not retain any specificity for VP16 responsive elements (the TAATGARAT elements),
induction assays were also carried out with CAT genes under the control of the HSV ICP0 promoter. Expression from the ICP0 promoter of pICP0CAT, which contains multiple TAATGARAT elements, was not affected by LAP348 (Fig. 3). Thus, the transplanted activation domain of VP16 did not retain any of its original sequence specificity. An ICP0 promoter construct containing a single operator placed downstream of the CAT gene (pICP0L1CAT) was activated by LAP348, demonstrating that activation by LAP is not restricted to the SV40 promoter.

It should be noted that LAP348 consistently inhibited expression, apparently nonspecifically, of pSV2CAT. Under the conditions used in the above transfections (2 μg of CAT vector and 1 μg of LAP348), LAP348 inhibited expression from pSV2CAT approximately twofold (Fig. 3). Increasing the amount of LAP greatly inhibited pSV2CAT expression compared with cotransfections with lacI5'N1 (data not shown). One possible explanation for this observation is that overproduction of the strong activator domain of VP16 may sequester cellular transcription factors, poisoning the transcription machinery. This phenomenon, previously referred to as squelching (17), may account for the observation that less LAP was produced in transient assays than lacI5'N1 (Fig. 1B), perhaps reflecting an autoinhibitory effect on transcription from the CMV promoter.

LAP348 acts by increasing the level of mRNA. The specificity for activation by LAP348 and the characterized transcriptional activation function of the VP16 domain are consistent with LAP acting at the level of transcription. To confirm this, the level of cytoplasmic RNA produced by pL1-3neo, a neomycin phosphoribosyltransferase gene placed under control of the pL1-3CAT promoter (diagrammed in Fig. 7A), was measured after transfection alone or with LAP348, using an RNase protection assay (34) (Fig. 4A). The neo gene was used in this experiment because detection of the neo mRNA was more reproducible than that of the CAT mRNA (data not shown). This observation was likely due to the reported instability of the CAT message (31). The neo probe was used from p7neo-320 (24) and is approximately 360 nucleotides long, consisting of neo sequences from the HindIII-BglII sites of pSV2neo flanked by vector sequences, and protects an RNA fragment of approximately 320 nucleotides. Although extremely low amounts, if any, of the protected neo RNA were detected after transfection of pL1-3neo alone (lane 2), the protected neo RNA was readily detected when pL1-3neo was cotransfected with LAP348 (lane 3), supporting the notion that LAP acts at the transcriptional level. The protected neo RNA was not detected after transfection either with carrier DNA or with the LAP348 vector alone (lanes 1 and 4). Parallel protection assays carried out with a human β-actin probe (Fig. 4B) indicated that all RNAs were intact and that similar amounts of RNA were used.

LAP348 acts as a repressor or an activator in a position-dependent fashion. The lac repressor has been shown to block transcription of mammalian promoters containing suitably placed operator sequences. Thus, it was of interest to determine whether LAP348 could also specifically repress gene expression. The effects of LAP348 on expression of a set of SV40 enhancer-promoter CAT constructs containing single lac operators at various positions after transfection into HeLa cells were determined. The promoters contained operators inserted at the SV40 origin of replication immediately downstream of the TATA box (Fig. 5; position 1 [p1]; pSVLp3CAT), between the 21-bp repeats and the TATA box (p2; pSVLp2CAT), between the 72-bp repeats and the 21-bp repeats (p3; pSVLp3CAT), or at both p1 and p3 (pSVLp1-p3 CAT). Transfections were carried out with 2 μg of CAT vector and 5 μg of plasmids expressing the lac derivatives, concentrations of plasmids which were determined to be optimal for repression (data not shown). Also, pX-8CAT (14), a construct containing an SV40 promoter with a XhoI linker at p1 (14), was used as a control (pSVLp3CAT was derived from pX-8). Both lacI5'N1 and LAP348 inhibited
expression from pSV^{Lp1}CAT (Fig. 5). LAP348 inhibited expression from pSV^{Lp1}CAT to an approximately fivefold-greater extent than that observed for pX-8CAT. Although lac{\textit{IS}}'N1 had less of an inhibitory effect on constructs with operators at p2 and p3, LAP348 enhanced expression from these constructs two- and fivefold, respectively. Whereas an operator at p3 allowed for transactivation by LAP, a construct containing operators at both p1 and p3 was repressed by LAP. Thus, the ability of LAP to activate or repress the SV40 promoter was dependent on the position of the operator.

LAP is allosterically regulated by IPTG. IPTG regulates the lac repressor by greatly lowering the binding affinity for its specific operator sequence (43). The ability of IPTG to regulate LAP activity was examined by cotransfection assays with or without 10 mM IPTG in the growth medium. LAP348 activated expression from pL1-2CAT (Fig. 6, lanes 5 and 7) but not from pSV2CAT (lanes 1 and 3). Although IPTG had no effect on expression of pSV2CAT either in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of LAP348, IPTG reduced the level of activation of pL1-2CAT by greater than 10-fold (lanes 7 and 8). Thus, LAP348 retained functions needed for allosteric regulation. A low level of activation of pL1-2CAT was still observed, however, even in the presence of IPTG. These data may indicate that LAP348 is not as responsive to IPTG as the wild-type repressor or that intracellular concentration of IPTG were not great enough to inactive LAP348. In this regard, other workers have noted that the response of the wild-type lac repressor to IPTG in mammalian cells is also slower and more incomplete than observed in bacteria (12, 21).

Genetic selections for LAP-producing cell lines. To facilitate the installation and maintenance of the LAP system into a desired cell type, positive selection schemes for LAP expression were developed. Two of the selectable vectors are diagrammed in Fig. 7A. The first, pL1-3neo, contains the operators and SV40 early promoter from pL1-3CAT as described above (Fig. 3) linked to the neo gene from pSV2neo (50) and can be used to select for G418" clones from established cell lines. The second vector, pL2T-antigen, consists of the SV40 large and small tumor antigen genes linked to an enhancerless early promoter and two tandem operators. This vector was used for selection of transformed foci in REF cells. The vectors were transfected alone or cotransfected with pHBa348 (LAP348 under the control of the human β-actin promoter) into the appropriate cell lines (Table 1). Transfection of pL1-3neo into HeLa cells or pL2T-antigen into REF cells resulted in very few G418" colonies or transformed foci, respectively; however, cotransfection with pHBa348 resulted in production of sig-
nificantly higher numbers of G418<sup>+</sup> colonies and transformed foci. No effects on colony number were observed after cotransfection with <i>lac</i> repressor constructs (data not shown), indicating that the activation function of LAP was required for this enhancement. These data demonstrated a strong selection for LAP348 expression in the HeLa cell and REF cell assays.

No enhancement of colony formation was seen in mouse Ltk<sup>-</sup> cells. In fact, cotransfection of pHB3A438 lowered the number of G418<sup>+</sup> Ltk<sup>-</sup> colonies. These data suggest that the basal activity of pL1-3neo is sufficient in Ltk<sup>-</sup> cells for transformation without transactivation. The decrease in colony number most likely indicates some toxicity of the LAP construct in Ltk<sup>-</sup> cells. Thus, transformation assays in some cell lines may require alternative basal promoters for the chosen selectable marker and test genes.

G418<sup>+</sup> colonies and transformed foci resulting after cotransfection with the LAP vector were isolated, propagated, and screened for the presence of LAP activity. A cell line was judged to be LAP<sup>+</sup> if it supported high levels of CAT expression from a supertransfected LAP-inducible CAT gene such as pL7CAT (Table 1 and Fig. 7C). The presence of LAP in the cells was also verified in most cases by immunoprecipitation and immunofluorescence (data not shown and Fig. 7B). All of the HeLa and REF lines (six of six) expressed LAP activity (Table 1); however, only one of seven G418<sup>+</sup> Ltk<sup>-</sup> lines expressed LAP. Figure 7B shows the fluorescent micrographs of two representative LAP<sup>+</sup> HeLa lines (LAPneo2b and LAPneo6b cells) stained by using anti-<i>lac</i> repressor antisera as described above. Virtually all of the cells showed distinct nuclear fluorescence, indicating that LAP348 was efficiently sequestered in the nucleus.

Figure 7C illustrates the CAT activity produced after transfection of several representative LAP<sup>+</sup> cell lines with pL7CAT or pSV2CAT. pL7CAT was expressed at low levels in control HeLa cells or MSV-T cells, an REF cell line transfected with T antigen under control of the murine sarcoma virus long terminal repeat. The LAP<sup>+</sup> cell lines, however, expressed pL7CAT at greatly enhanced levels (5- to 10-fold greater than with pSV2CAT; data not shown) comparable to that observed in the transient assays. Preliminary experiments indicate that reporters such as pL14CAT and pL21CAT are expressed greater than 30-fold more efficiently than pSV2CAT in LAP<sup>+</sup> cell lines (data not shown). Thus, high levels of LAP-inducible expression by operator-containing promoters does not require high amounts of LAP that might be produced in the transient assays.

One Ltk<sup>-</sup> line stably expressing LAP activity was identified even though the selection assay did not suggest a requirement for LAP for G418<sup>+</sup>. The isolation of this line may have been fortuitous or may indicate that some transformants require LAP for expression of the neo gene. In either case, it should be noted that this and the other LAP<sup>+</sup> lines have stably produced LAP348 for 3 months of continuous passage, indicating little selection against LAP expression.

Experiments were also carried out to determine whether the LAP activity in the stable cell lines was regulatable by IPTG. HeLa cells or LAPneo2b cells were transfected with either pSV2CAT or pL1-2CAT, with or without IPTG in the medium (Fig. 8). pL1-2CAT was efficiently expressed in LAPneo2b cells but not in HeLa cells (lanes 3 and 7). Addition of IPTG to the medium 2 days before transfection reduced expression of pL1-2CAT greater than 30-fold (lane 8) without significantly affecting expression of pSV2CAT (lane 2 and 6). Similar results were observed in six of six independent LAP<sup>+</sup> cell lines tested (data not shown).

### TABLE 1. Number of colonies and foci<sup>a</sup>

<table>
<thead>
<tr>
<th>Cell</th>
<th>Marker</th>
<th>No. of colonies and foci</th>
<th>LAP&lt;sup&gt;+&lt;/sup&gt; clones&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Marker gene alone</td>
<td>LAP348</td>
<td>LAP&lt;sup&gt;+&lt;/sup&gt; clones</td>
</tr>
<tr>
<td>HeLa</td>
<td>pL1-3neo</td>
<td>6</td>
<td>47</td>
</tr>
<tr>
<td>Ltk&lt;sup&gt;-&lt;/sup&gt;</td>
<td>pL1-3neo</td>
<td>&gt;200</td>
<td>75</td>
</tr>
<tr>
<td>REF</td>
<td>pL2T-antigen</td>
<td>0</td>
<td>31, 34</td>
</tr>
</tbody>
</table>

<sup>a</sup> The indicated cells were transfected with 1 μg of the marker gene alone or with 1 μg of pHB3A438 and calf thymus DNA for a total of 25 μg of DNA. The number of G418<sup>+</sup> colonies and transformed foci was determined by visually counting the plates before and after fixation and staining with formaldehyde and cresyl violet.

<sup>b</sup> Cell lines were cloned and determined to be LAP<sup>+</sup> by their ability to support high levels of expression of pL7CAT (Fig. 7C). The expression of LAP348 protein was verified by immunofluorescence-immunoprecipitation analysis (Fig. 7B and data not shown).

**FIG. 7.** Genetic selections for LAP-producing cell lines. (A) Vectors for selection of LAP-producing cell lines. Shown are the SV40 early promoter (□□), the direction of transcription (→), operators (□□), the neo gene from pSV2neo (□□□□), and the SV40 T-antigen gene (□□□□). The caret indicates the SV40 early intron, and the short gray box within the neo construct indicates the SV40 polyadenylation signal from pSV2neo. (B) Indirect immunofluorescence of HeLa cell lines expressing LAP348. HeLa cell lines LAPneo2b and LAPneo6b were stained and stained for LAP348 as described in Materials and Methods. No true fluorescent signal was observed with naive HeLa cells (data not shown). (C) Expression of inducible CAT vectors in LAP<sup>+</sup> cell lines. HeLa cells, MSV-T cells (see Results), or cell lines derived from cotransfections described in Table 1 were transfected with either pSV2CAT or pL7CAT as indicated. Cells derived from cotransfection of HeLa or REF cells are indicated.
DISCUSSION

This report describes the development of a novel transcriptional regulatory system for mammalian cells. Central to this system is the conversion of the lac repressor into a transcriptional transactivator (LAP). The LAP retains the ability to specifically bind operator DNA and to be allosterically regulated by IPTG even though both the very 3' and 3' domains of the lacI coding region have been modified. These results are consistent with the observations from lacI-lacZ and lacI-T7 endonuclease fusions that show that the extreme carboxyl terminus of lac repressor can be modified and still retain specific DNA-binding activity (39, 40). The LAPs, however, were designed such that the carboxyl terminus was left intact in case it was required for tetramerization. Although LAP348 has not been tested for tetramerization, the synergistic effect of spaced operators on transactivation by LAP (Fig. 2B and 3) suggests that tetramers of LAP may be binding separated operators (see below). Although the actual affinities of LAPs for operator DNA have not been measured, the complex between LAP348 and operator DNA in mammalian cells must be quite stable, since LAP348 was also able to block expression of an SV40 promoter containing an operator downstream from the transcriptional start site even if the promoter also contained an upstream, activating operator (Fig. 5).

One or two tandem operators allowed for activation by LAP, and multiple spaced operators acted synergistically (Fig. 3). Similarly, many other eucaryotic transactivators appear to require multiple sites for efficient activation and appear to interact synergistically. Although multiple glucocorticoid response elements or metal response elements have additive effects on transcription, at least two response elements are required for efficient induction (47, 52). Furthermore, there is a synergistic effect between single glucocorticoid or estrogen response elements and other transcription factor-binding sites (51, 54). There is also a synergistic effect of tandem GAL4 sites on activation by GAL4 and GAL4-VP16 fusions (54). In contrast to these systems, strong synergistic effects on activation by LAP were only observed when tandem operators were separated by some distance. Promoters containing two operators separated by about 100 bp (pL1-1CAT) produced approximately 5 to 10 times more CAT activity than did promoters containing one or two tandem operators (Fig. 3). Also, little difference was observed between promoters containing two or four spaced operators (pL1-1CAT and pL1-3CAT), whereas a promoter containing seven operators (pL7CAT), consisting of the two spaced operator arrays A and B, produced about twice the CAT activity expected for an additive effect of the operators. Multimerizing the pL7 operators, however, in pL14CAT and pL21CAT displayed an additive effect, respectively doubling and tripling the amount of CAT produced by pL7CAT.

These data are consistent with studies showing that spaced operators greatly enhance the ability of the lac repressor to block transcription in E. coli (2, 38). Indeed, the affinity of lac repressor for tandem operators has been shown to increase as the distance between operators is increased. This relationship between spacing length and affinity parallels a relationship between length and the ability of the DNA to form a circle. The increase in repressor affinity was suggested to be related to the ability to form stable loops of DNA. Supporting this view is the observation that a repressor tetramer bound to spatially separated operators can loop out intervening DNA in a length-dependent fashion (27). Thus, two predictions can be made concerning the interaction of LAP with spaced operators. The first is that LAP tetramers will bind with increasing affinity to these arrays, and the second is that LAP tetramers are probably forming loops of DNA between these arrays. Further experiments will be needed to determine whether either of these situations is necessary for the synergistic activation by LAP.

The LAPs offer several advantages for use as an inducible expression system in mammalian cells. First, LAP-inducible promoters can be activated to very high levels. Vectors such as pL7CAT, pL14CAT, and pL21CAT were induced over 1,000-fold in response to LAP, producing 10 to 30 times as much CAT as did pSV2CAT either in transient cotransfections or in transfections into stable, LAP' cell lines (Fig. 3 and 7). Thus, the LAP system has the potential to regulate transcription over 3 orders of magnitude, reaching levels comparable to bacterial regulatory systems. This is in contrast to cell lines expressing the lac repressor, which typically display induced levels of expression lower than that for the parent promoters (8, 21). This observation has been attributed to the inability of IPTG to completely relieve repression and the finding that insertion of operators around the start site of transcription appears to lower the basal expression of the promoter. An additional disadvantage in the use of lacI as a repressor is that cell lines must produce large amounts of repressor to saturate the operator and block expression. On the other hand, there is no theoretical reason that LAP need saturate operators in order to significantly activate expression. The addition of a nuclear localization signal also concentrates lac repressor and LAP in the nucleus (Fig. 1 and 7) and should lower the amount of protein needed to affect regulation.

Second, the LAP system is highly specific. The lac operator is a complex 25-bp sequence which appears extremely infrequently in mammalian genomes. Furthermore, the observation made here that multiple spaced operators are required for efficient activation suggests that virtually no cellular genes would be significantly affected by LAP expression. In contrast, heat shock, heavy metal, or hormone induction of promoters is likely to alter expression of a variety of genes. This specificity may make LAP an ideal transactivator for use in transgenic animals.

Third, LAP retains the ability to act as a repressor (Fig. 5). Thus, LAP may be used as a bidirectional switch to turn off one gene while turning on another. It should be noted, however, that our experiments to date indicate that LAP is not as efficient a repressor as lacI'S'N1. This observation may simply be due to accumulation of lower amounts of LAP than lacI'S'NI in transient assays (Fig. 1).

Fourth, LAP is allosterically regulated by IPTG (Fig. 6 and 8). IPTG in the medium inhibited LAP activity both in transient assays and in stable cell lines. Preliminary experiments have also demonstrated that expression from the integrated LAP-inducible neo and T-antigen genes in transformed cell lines described in Fig. 7 are more stringently regulated by IPTG than the supertransfected CAT gene (Fig. 8) and that the transformed phenotype of the pL2T-antigen-transformed cells can be largely reversed by IPTG (data not shown). Thus, IPTG provides a useful switch for regulating LAP-inducible genes in stable cell lines. As pointed out by others, however, IPTG induction in mammalian cells is slow and occurs over hours or days, as compared with minutes in bacteria; indeed, IPTG never completely eliminated activation by LAP in these studies. In addition, preliminary experiments indicate that regulation by IPTG is less efficient for the pL14 and pL21 promoters in transient transfection assays than for the pL1-2 or pL7 promoters (data not
shown). Although we are currently optimizing conditions for regulation by IPTG, alternative methods of induction may be required. In this regard, we are developing temperature-sensitive alleles of LAP with the expectation that regulation by temperature will be significantly more stringent than by IPTG.

The final advantage to the LAP system is the ability to easily select for LAP* cell lines by using LAP-inducible markers (Fig. 7 and Table 1). These selections should be useful not only in generating and maintaining LAP* lines but also as phenotypic tags to coselect or identify cells also expressing test genes under the regulation of LAP.

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LITERATURE CITED


