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Biochemistry

Transfer of the inducible *lac* repressor/operator system from *Escherichia coli* to a vaccinia virus expression vector

(mammalian expression vector/transcription regulation/*lac* repressor/*lac* operator)

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ABSTRACT **Cis- and trans-acting elements of the *Escherichia coli lac* operon were transferred to vaccinia virus and used to regulate gene expression. A recombinant virus that constitutively expresses a modified *lac* repressor gene (*lacI*) was constructed. We calculated that each infected cell contained ≈2 × 10^7 active repressor molecules (and 1–2 × 10^4 copies of the vaccinia virus genome). A strong vaccinia-virus late promoter was modified by insertion of the *lac* operator (*lacO*) at various positions. The ability of each modified promoter to regulate expression of β-galactosidase was tested by transient assays in cells infected with wild-type or *lacI*-containing vaccinia virus. Placement of the *lacO* just downstream of the conserved TAAAT sequence of a late promoter was consistent with a minimal effect on basal expression and good repressibility, whereas basal expression was severely inhibited when *lacO* overlapped or preceded the TAAAT motif. A single recombinant vaccinia virus containing *lacI* and the β-galactosidase gene under control of the optimal *lacO* promoter was constructed. In the absence of inducer, cells infected with this double recombinant virus synthesized little or no detectable β-galactosidase. Addition of isopropyl β-D-thiogalactoside restored expression to >20% of the unrepressed level. This inducible vector system has potential applications for expression of heterologous and homologous genes.**

Vaccinia virus has been developed as a vector for expression of heterologous genes (1–3). The availability of plasmid transfer vectors containing a vaccinia virus promoter, sites for insertion of foreign genes, and a variety of markers for selection and/or screening of recombinant plaques (4–6) has facilitated the use of this system. With the appropriate choice of vaccinia virus promoters, inserted genes can be expressed during the entire growth cycle of the virus or only during early or late phases. Expression of bacteriophage T7 RNA polymerase by vaccinia virus has allowed selective high-level transcription of genes that have been engineered to contain a T7 promoter and are present in either a transfected plasmid (7) or a second coinfected virus (8). Still missing from vaccinia virus vectors, however, is the capability of inducing specific gene expression on command. In the absence of any known naturally inducible vaccinia virus promoter, we considered the importation of one from eukaryotic or prokaryotic sources. Of the latter, we chose to explore the use of the operator/repressor system of the *Escherichia coli* lactose operon (9) because it is well characterized, exhibits both stringent repression and high inducibility, and has been adapted to mammalian cells (10–12).

Enzymes encoded within the *E. coli lac* operon are under the negative control of a repressor consisting of four identical 38.6-kDa subunits encoded by the *lacI* gene. The repressor can bind specifically and with high affinity to the *lacO* sequence, which overlaps the start point of transcription of the *lac* operon. The *lac* repressor can also bind to allolactose or a nonmetabolizable derivative such as isopropyl β-D-thiogalactoside (IPTG), which then decreases the affinity of the repressor for *lacO*. In this manner, IPTG can diminish the repression of *lac* operon transcription, resulting in an induction of expression.

Vaccinia virus, like other members of the poxvirus family, replicates in the cytoplasm and encodes its own transcriptional system (13). The RNA polymerase and specific factors for initiation and termination of transcription of the early class of genes (14, 15) are packaged within the infectious particle. A switch from early-to-late gene expression follows the replication of the vaccinia virus genome and involves the synthesis of new transcription factors (ref. 16; C. Wright and B.M., unpublished work). To meet our objective, we wished to insert the *lacI* gene into the genome of vaccinia virus so that sufficient amounts of biologically active repressor would be made and also to place a vaccinia virus promoter under control of *lacO*. In this communication, we describe a recombinant vaccinia virus that constitutively expresses *lac* repressor and can be induced with IPTG to express β-galactosidase.

MATERIALS AND METHODS

Construction of Plasmids. Plasmid placI was constructed by inserting a 1.1-kilobase (kb) pair *Bgl* II fragment from pMTlacI (11) into pGS53 (8). The series of recombinant plasmids, placOZ-1, placOZ-2, and placOZ-3, were derived from a parent vector designated pMJ11 (received from A. J. Davison, University of Glasgow). Plasmid pMJ11 contains the vaccinia virus thymidine kinase (TK) DNA sequence inserted into a pUC-based vector. The TK sequence was bisected by cleavage with *Eco*RI into which a *lacZ* gene was inserted with the following polylinker sequence: 5′-GGATCC,GTCGAC,AAGCTT,GGTACC,CTCGAG,CATG-3′. The six nucleotide sets correspond to restriction endonucleases *Bam*HI, *Sal* I, *Hin*dIII, *Kpn* I, and *Xho* I, respectively. The ATG represents the first codon of the *lacZ* coding sequence. Plasmid pMJ11 was cleaved sequentially with *Hin*dIII and *Xho* I into which a set of oligonucleotides were inserted. These oligonucleotides reconstructed the vaccinia virus late promoter, P11, with *lacO* interposed in three different positions (see Fig. 3). The oligonucleotides used for placOZ-1 were PE1, PE2, PE3, and PE4; the oligonucleotides used for placOZ-2 were PE1, PE2, PE5, and PE6; and the oligonucleotides used for placOZ-3 were PE1,

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Abbreviations: IPTG, isopropyl β-D-thiogalactoside; TK, thymidine kinase; pfu, plaque-forming unit(s); moi, multiplicity of infection.

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PE2, PE7, and PE8. Sequences of these oligonucleotides were as follows:

PE1: 5′-AGCTTAAAAATATAGTAGAATTTCATTTTGTTTTTTT-3′;
PE2: 5′-TAGCATAGAAAAAAACAAAATGAAATTCTACTATATTTTTA-3′;
PE3: 5′-CTATGCTATAAATAGAATTGTGAGCGCTCACAATTC-3′;
PE4: 5′-TCGAGAATTGTGAGCGCTCACAATTCTATTTA-3′;
PE5: 5′-CTATGCTATAAATTGTGAGCGCTCACAATT-3′;
PE6: 5′-TCGAAATTGTGAGCGCTCACAATTTA-3′;
PE7: 5′-CTATGCTAGAATTGTGAGCGCTCACAATTCTAAATAC-3′; and
PE8: 5′-TCGAGTATTTAGAATTGTGAGCGCTCACAATTC-3′.

Plasmid placI/OZ-1 was constructed by cleaving placI with *Sma* I and *Sal* I and isolating a 1.4-kb DNA fragment containing the *lacI* gene downstream of the P7.5 promoter. This fragment was inserted between the *Bam*HI site made blunt with Klenow polymerase and *Sal* I site of placOZ-1. The promoters and coding sequences of *lacI* and *lacZ* were positioned in opposite orientation relative to each other.

RESULTS

Construction of a Recombinant Vaccinia Virus That Expresses the *lacI* Gene. A modified *lacI* gene, in which the natural GUG translation initiation codon was replaced by AUG (11), was inserted into the transfer vector pGS53 (8) so that it was (*i*) adjacent to the vaccinia virus P7.5 promoter, which would ensure both early and late expression (17) and (*ii*) between the left and right halves of the vaccinia virus TK gene (Fig. 1*A*). The resulting plasmid, placI, was used to transfect CV-1 cells that were also infected with vaccinia virus. Recombinant virus was then selected by plaque assay on TK^- cells in the presence of 5-bromodeoxyuridine and distinguished from spontaneous TK^- mutants by dot-blot hybridization to a *lacI* DNA probe (4). A recombinant virus, designated vlacI, was purified, and the correct insertion of the *lac* repressor into the TK gene was confirmed by Southern blot hybridization.

Evidence was obtained that *lacI* was expressed by the recombinant vaccinia virus. Extracts from cells that were infected with vlacI or wild-type vaccinia virus were dissociated with SDS and analyzed by polyacrylamide gel electrophoresis. An immunoblot was performed using an anti-*lac* repressor mouse monoclonal antibody (18). The autoradiogram (Fig. 1*B*) revealed a single immunoreactive band, of ≈38 kDa, which comigrated with authentic *lac* repressor.

The ability of the *lac* repressor, made by recombinant vaccinia virus, to bind to its cognate operator sequence *in vitro* was tested by a gel-mobility-band shift assay. A radioactively labeled synthetic operator containing the sequence GAATTGTGAGCGCTCACAATTC (19) and its complement were prepared by hybridizing oligonucleotides PE7 and PE8 together and filling in the nonoverlapping nucleotides with [α-^{32}P]dCTP. The 41-base-pair (bp) probe was incubated with varying dilutions of extracts made from cells that had been infected with vlacI or wild-type virus. In the presence of extracts from vlacI-infected cells, the mobility of the probe was retarded (Fig. 2). Moreover, the latter protein–DNA complex comigrated with the complex formed from the association of authentic *lac* repressor with the probe. By use of the purified repressor as a standard, the amount of repressor made in recombinant vaccinia-virus-infected cells could be calculated from densitometer scans of the autoradiogram. We estimated from the amount of DNA shifted in mobility that ≈6 μg of biologically active repressor is present per 10^6 cells; the latter is equivalent to 2×10^7 repressor tetramers per cell.

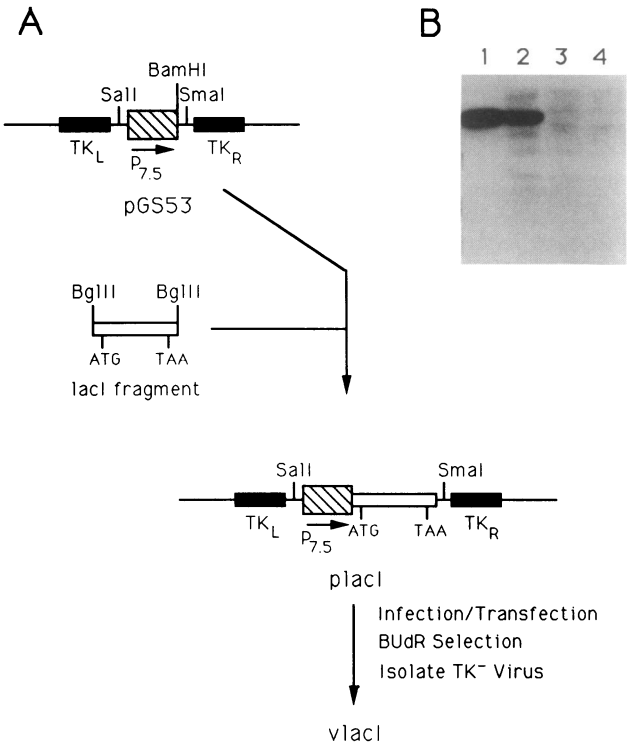

FIG. 1. Construction of a recombinant vaccinia virus that expresses *lacI*. (*A*) A *Bgl* II fragment containing the *lacI* gene was inserted into the unique *Bam*HI site of pGS53 to form placI. In the latter plasmid *lacI* is downstream of the vaccinia P7.5 promoter, and the hybrid gene is flanked by the left (TK_L) and right (TK_R) halves of the vaccinia TK gene. CV-1 cells were infected with vaccinia virus and transfected with placI. After 48 hr, the cells were harvested and tested for plaques on TK^- cells with 5-bromodeoxyuridine (BUdR). Virus plaques were picked and screened by dot-blot hybridization to *lacI* DNA; the recombinant virus vlacI was then purified. (*B*) Immunoblot analysis for detection of *lac* repressor. CV-1 cells were infected with either recombinant (vlacI) or wild-type vaccinia viruses at a multiplicity of 15 plaque-forming units (pfu) per cell. Cell extracts were prepared 24 hr after infection, and samples were analyzed by SDS/polyacrylamide gel electrophoresis, blotted to nitrocellulose, and probed with anti-*lac* repressor monoclonal antibody B-2, followed by ^{125}I-labeled protein A. Lanes: 1, 1 μg of purified *E. coli lac* repressor protein; 2, extract from cells infected with vlacI; 3, extract from cells infected with wild-type virus; and 4, extract from uninfected cells.

Construction of Hybrid Vaccinia Virus Promoters Containing *lacO*. We wished to place *lacO* within or adjacent to a late promoter so that repressor binding would block transcription; however, it was important that the operator sequence not severely disturb transcription in the absence of repressor. Previous data (20, 21) suggested that only a relatively short sequence preceding the highly conserved TAAAT motif (22) of late promoters was required for transcription. The RNA start site is apparently located within the TAAAT sequence, and any variation of these nucleotides impairs expression (A. J. Davison and B.M., unpublished work). It seemed likely, therefore, that binding of repressor close to the TAAAT sequence would have the desired regulatory effect. β-galactosidase (encoded by the *E. coli lacZ* gene) was chosen as a reporter because there is no detectable background activity in mammalian cells and the assay is quantitative (5).

The late promoter for the gene encoding the 11-kDa structural protein is typical in that the conserved TAAAT motif is followed immediately by a guanosine that completes

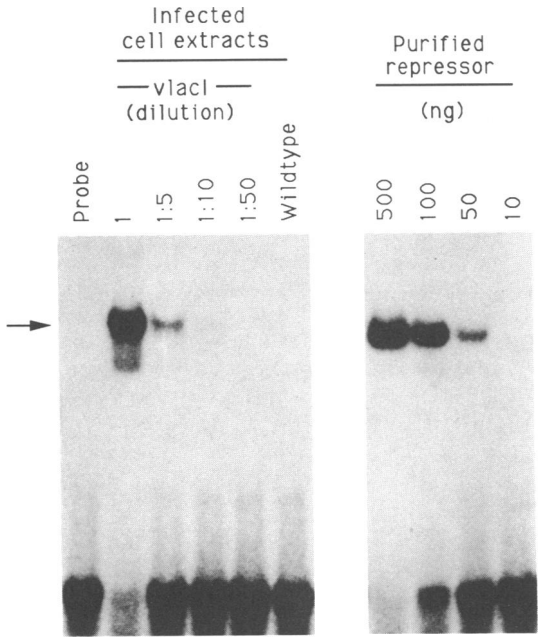

FIG. 2. Binding of *lac* repressor to *lacO*. Approximately 2×10^7 CV-1 cells were infected with vlacI or wild-type vaccinia virus at a multiplicity of infection (moi) of 15. Cells were harvested 24 hr after infection, and cytoplasmic fractions were prepared by Dounce homogenization in hypotonic buffer (10 mM Tris·HCl, pH 7.6/10 mM NaCl/1.5 mM $MgCl_2$). Nuclei and cell debris were removed by centrifugation, and either undiluted or diluted samples (1 μl) of vlacI extracts were mixed with ^{32}P-labeled, double-stranded oligonucleotide probe and 50 mM poly(dIdC). Similarly, undiluted wild-type virus-infected cell extract and various amounts of purified *E. coli lac* repressor protein were mixed with probe and poly(dIdC) to serve as negative and positive controls, respectively. The samples were separated by 8% polyacrylamide gel electrophoresis and exposed to x-ray film. Arrow, *lac* repressor DNA.

the translation initiation codon. To facilitate construction, it was important to move the translation initiation codon further downstream. Although the presence of a guanosine immediately following TAAAT is not essential for late transcription, substitution of other nucleotides may lower expression (A. J. Davison and B.M., unpublished work). The TAAATG sequence was retained in the *lacZ* fusion gene of plasmid pSC11 (Fig. 3). In plasmid placOZ-1, however, the guanosine was changed to an adenosine, and this was followed by a 22-bp *lacO* palindrome, a new translation initiation codon, and the *lacZ* open reading frame. Plasmid placOZ-2 is similar, except that *lacO* overlaps the TAAAT motif, which is followed by a ribosylthymine. In plasmid placOZ-3, *lacO* precedes the TAAAT motif, which is followed by an adenosine and the *lacZ* open reading frame.

The effects of the above mutations on promoter function were tested by transfecting the plasmids into CV-1 cells that had been infected with wild-type vaccinia virus and subsequently assaying lysates for β-galactosidase activity. In Fig. 3, the values are presented as the percent of activity obtained with plasmid pSC11 in which the late promoter has been unperturbed. Placement of the operator immediately downstream of the TAAAT box without (placOZ-1) or with (placOZ-2) a slight overlap resulted in 66% and 20% of β-galactosidase activity, respectively. These differences might be due, at least in part, to the change in the nucleotide following the ribosylthymine from guanosine to adenosine in one case and to ribosylthymine in the other. Placement of the operator immediately upstream of the TAAAT box (placOZ-3) completely abolished the expression of β-galactosidase, presumably by interrupting the promoter sequence.

To see whether the promoters were under *lacO* control, similar transfections were performed with cells that were infected with the recombinant vaccinia virus, vlacI, which expresses the *lac* repressor. In the absence of IPTG, no β-galactosidase activity was detected, suggesting stringent repression. With IPTG the β-galactosidase activity obtained with placOZ-1 was 8.3% of pSC11 or 12.5% of the value obtained with placOZ-1 in cells infected with wild-type vaccinia virus (Fig. 3). Significant, although low, β-galactosidase levels were achieved with placOZ-2 and vlacI in the presence of IPTG (Fig. 3).

Coinfection of Cells with Recombinant Viruses Containing *lacI* and *lacO* Regulatory Elements. The previous experiments indicated that the placOZ-1 construct, in which the operator is immediately downstream of TAAATA, provided the best combination of stringent repression and inducibility; however, in those preliminary experiments, the operator was present in a transfected plasmid instead of the virus genome. The plasmid had been engineered with TK sequences flank-

Plasmids	Position of Lac-Operator Insertion	% Expression		
	Virus:	WT	vlacI	vlacI
	IPTG:	-	-	+
pSC11	P11 - TAAATG - lacZ	100	100	100
placOZ-1	P11 - TAAATA GAATTGTGAGC:GCTCACAATTC TCGAGCATG - lacZ	66	<0.1	8.3
placOZ-2	P11 - TA AATTGTGAGC:GCTCACAATT TCGAGCATG - lacZ	20	<0.1	1.4
placOZ-3	P11 - GAATTGTGAGC:GCTCACAATTC TAAATACTCGAGCATG - lacZ	<0.1	<0.1	<0.1

FIG. 3. Effect of *lacO* insertion on vaccinia virus late-promoter function. The *lacO* (boxed region) was inserted either downstream, overlapping, or upstream of the canonical TAAAT (bracket) of the P11 late promoter in plasmid constructions placOZ-1, placOZ-2, and placOZ-3, respectively. The translation initiation codon is underlined, and the nucleotide replacing the guanosine of TAAATG is marked with a black square. β-Galactosidase activity was measured after transfection of CV-1 cells that were infected with wild-type (WT) vaccinia virus or vlacI (7); IPTG was used at 5 mM. The percentage of expression is relative to that of WT-infected cells transfected with pSC11.

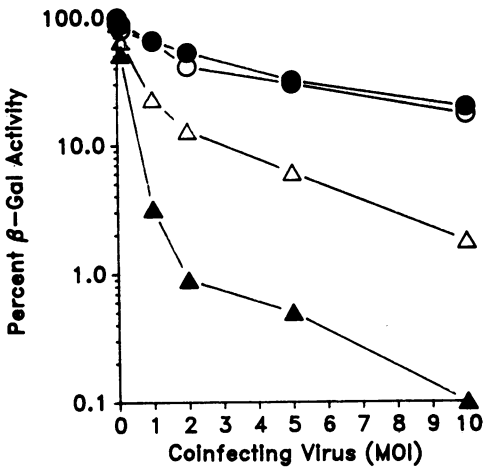

FIG. 4. Effect of moi of vlacI on repression. CV-1 cells were infected with 1 pfu of vlacOZ-1 and coinfected with either wild-type virus (○, ●) or recombinant virus vlacI (△, ▲) over a range of moi with (○, △) or without (●, ▲) 5 mM IPTG. At 24 hr after infection, cells were harvested and assayed for β-galactosidase. All values are expressed relative to a 100% value set for the vlacOZ-1 single infection. MOI, moi.

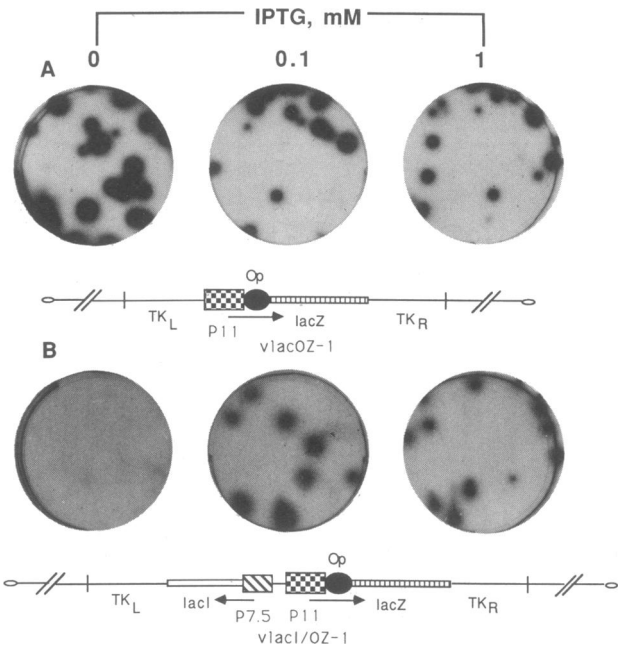

FIG. 5. Induction of blue-plaque formation from a single vaccinia-virus recombinant containing both *lacI* and *lacO* genetic elements. Recombinant virus vlacOZ-1 has a cassette containing the vaccinia-virus late promoter (P11) coupled to the *lacO* (Op) and *lacZ* gene inserted into the TK locus of the vaccinia-virus genome with flanking left (TK_L) and right (TK_R) TK DNA sequences. Recombinant virus vlacI/OZ-1 contains the same cassette described above, plus the vaccinia virus early/late promoter (P7.5) regulating the expression of *lacI*. BSC-1 cell monolayers were infected with ≈10 pfu of vlacOZ-1 (*A*) or vlacI/OZ-1 (*B*) without or with the indicated concentration of IPTG. After 2-day incubation, the cell monolayers were stained with 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal). *A* was stained for 24 hr and *B* was stained for 48 hr.

ing the transcriptional regulatory cassette so that recombination with wild-type virus could be carried out to form vlacOZ-1 as the next step.

We tested repression and induction by coinfecting cells with vlacI and vlacOZ-1. The level of repression was affected by the ratio of viruses containing *lacI* and *lacO* (Fig. 4). At 1:1 repression was 97% and increased to >99% at a ratio of 2:1. At 10:1 no β-galactosidase activity could be detected. IPTG, however, increased β-galactosidase expression >15-fold. As anticipated, only a minor diminution in expression occurred when the ratio of wild-type vaccinia virus to vlacOZ-1 was increased, and IPTG had no effect on this.

Construction of a Single Recombinant Vaccinia Virus Containing *lacI* and *lacO* Elements. The next step in the development of vaccinia virus as an inducible vector was to incorporate both the repressor and operator sequences into the same virus. A new transfer vector was constructed and used to prepare a TK^- recombinant virus designated vlacI/OZ-1 (Fig. 5*B*). Initial experiments revealed that vlacI/OZ-1 plaques did not stain blue when the chromogenic β-galactosidase substrate 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) was added to the agar, whereas vlacOZ-1 plaques were intensely blue when incubated for half the vlacI/OZ-1 time exposure (Fig. 5). The vlacI/OZ-1 plaques formed in the presence of IPTG, however, stained dark blue under the same conditions. These results suggested a high degree of repression and considerable induction even at relatively low IPTG concentrations. Quantitative analysis was obtained by measuring β-galactosidase activity in lysates of CV-1 cells infected 24 hr earlier with 1 or 10 pfu per cell of vlacI/OZ-1. In the absence of IPTG, β-galactosidase synthesis was repressed by >97% during a low moi and was undetectable at higher multiplicities (Table 1). This difference probably reflects a multiplicity dependence on early gene (i.e., repressor) expression before DNA replication. With optimal amounts of IPTG, the levels of β-galactosidase derepression were 21–28% of maximum values obtained in the absence of *lac* repressor (Table 1). By contrast, IPTG had negligible effect on β-galactosidase expression by vlacOZ-1 (Table 1).

DISCUSSION

Successful transfer of the *E. coli lac* operator/repressor system to eukaryotic cells was carried out previously (10–12). The presence of 4×10^4 tetramer repressor molecules per cell was sufficient to stringently repress a single chromosomal operator site (12). Nevertheless, no detectable repression was seen when we infected such cells with a recombinant vaccinia virus containing the *lacZ* gene under *lacO* control. Although only a single copy of the operator was inserted into the vaccinia virus genome, ≈$1–2 \times 10^4$ DNA molecules are synthesized in infected cells. Thus, in the *lacI*-transformed cells that were infected with vaccinia virus, the ratio of repressor to operator was, at most, 4:1. That number might even be lower, depending on the stability of *lac* repressor,

Table 1. Regulation of β-galactosidase expression using a vaccinia recombinant containing both *lac* repressor and operator elements

	β-galactosidase expression, %			
	vlacOZ-1		vlacI/OZ-1	
IPTG, mM	1 moi	10 moi	1 moi	10 moi
0	100.0	100.0	2.8	<0.1
0.1	105.3	106.4	19.0	16.2
1	103.2	103.2	27.0	19.9
5	95.8	99.0	28.3	20.8

CV-1 cells (≈2×10^6) were infected with either recombinant virus vlacOZ-1 or vlacI/OZ-1, and after 2 hr the virus inoculum was replaced with 2 ml of Eagle's medium (no phenol red) containing the indicated concentrations of IPTG and 2.5% fetal bovine serum. Cells were collected at 24 hr and assayed for β-galactosidase activity. Values are expressed as % β-galactosidase activity relative to the value obtained for vlacOZ-1 without IPTG at each moi tested. The 100% values for vlacOZ-1 infection at moi values of 1 and 10 correspond to 4.5×10^4 and 4.8×10^4 nmol of product formed in 30 min per 2×10^6 cells, respectively.

because vaccinia virus inhibits host-cell protein synthesis. To increase the amount of repressor, as well as to enhance the portability of the system for any permissive cell line, we chose to also insert the *lacI* gene into the genome of vaccinia virus. By using the vaccinia virus P7.5 promoter, the *lacI* gene was expressed at both early and late times in infection. At 24 hr after infection, $\approx 2 \times 10^7$ active tetramer molecules were present or $\approx$1000 for each replicated vaccinia virus genome. Hu and Davidson (10) had shown that most of the *lac* repressor made in mammalian cells remains in the cytoplasm, which is also the site of vaccinia virus DNA replication. Sufficient repressor was made in infected cells to inhibit a *lacO*-controlled reporter gene in vaccinia virus by >1000-fold. Addition of IPTG, however, induced expression of the reporter gene to values at least 20% of maximum.

The effect of *lacO* on the basal (unrepressed) level of promoter activity depended on its location. When the operator was placed just downstream of the conserved TAAAT late-promoter sequence, expression was reduced 34%, whereas overlapping of the TAAAT sequence with *lacO* led to an 80% reduction. Transient expression experiments demonstrated that *lac* repressor inhibited expression from both of these hybrid promoters and that IPTG partially overcame this inhibition. Placement of the operator just upstream of the TAAAT sequence evidently destroyed the promoter, because no expression was detected. Based on these experiments, we concentrated on the hybrid promoter in which the operator was located just downstream of the TAAAT motif. The role, if any, of the weak secondary operator element within the *lacZ* gene (23) was not evaluated. In *E. coli*, operator binding of *lac* repressor appears to inhibit the formation of a stable open complex between RNA polymerase and the *lac* promoter (24). Preliminary nuclease S1 analysis indicated that β-galactosidase transcripts were not detected in cells infected with the recombinant vaccinia virus expressing repressor unless IPTG was added (T.R.F., unpublished work); however, additional studies are needed to prove the mechanism of repressor action in the vaccinia-virus system.

We can envision several important uses of the *lac* operator/repressor system in vaccinia virus. It should now be possible to clone into vaccinia virus toxic genes that will be expressed only on command—e.g., upon addition of inducer. Another use may be to regulate the expression of essential genes of vaccinia virus and thereby to study their roles. In this manner, it should be possible to make conditionally lethal (IPTG-dependent) virus mutants. The *E. coli lac* repressor/operator system could be readily transferred to other large DNA viruses, such as adenoviruses or herpesviruses; however, these viruses replicate in the nucleus and therefore it might be advantageous to add nuclear localization sequences to the repressor.

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