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Generation of Thymidine Kinase-Deficient Mutants of Infectious Laryngotracheitis Virus

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Current vaccines for the avian respiratory disease infectious laryngotracheitis consist of naturally attenuated strains of the causative agent — the herpesvirus infectious laryngotracheitis virus (ILTV). Due to the dissemination of these viruses from vaccinated chickens as well as their possible reversion to more pathogenic forms, the use of genetically engineered viral vaccines lacking virulence factors while retaining antigenicity is being considered. Since the thymidine kinase (TK) activity of herpesviruses has been associated with virulence, inactivation of the encoding gene in the ILTV genome should attenuate the virus. Moreover, by analogy to other TK$^-$ herpesviruses, the ability of such ILTV mutants to induce a protective response in chickens should not be compromised. Therefore, the deliberate genetic alteration of ILTV was attempted. In order to prevent reversion and also to enable identification of the modified virus, a "marker" transcriptional unit (*Escherichia coli lacZ* gene fused to a SV-40 3′-polyadenylation signal sequence and regulated by the pseudorabies virus gX gene promoter) was inserted via homologous recombination at one of two loci within the ILTV TK gene. Recombinant viruses were identified and plaque-purified on the basis of their ability to produce β-galactosidase. Retention of the foreign DNA at the predicted sites in the genomes of the recombinant ILTV was verified by Southern hybridization. Since their replication was unaffected by the thymine analog 1-(2-fluoro-2-deoxy-β-D-arabinofuranosyl)-5-methyluracil, the recombinants appeared to have a TK$^-$ phenotype. Despite this apparent deficiency, prior inoculation of either recombinant virus into chickens afforded the birds protection against a lethal challenge of virulent ILTV. Moreover, the degree of respiratory distress in the chickens vaccinated with the recombinants was relatively mild compared to the severe reaction in birds receiving the parental virus. Thus, ILTV can be genetically attenuated without an accompanying loss of immunogenicity.

INTRODUCTION

Infectious laryngotracheitis (ILT) is a highly contagious disease that is localized to the upper respiratory tract of chickens. Worldwide in distribution, this malady can cause reduced egg production and even result in mortality rates approaching 40% (Tripathy and Hanson, 1989; Hanson and Bagust, 1991). Current vaccines consist of biologically modified, live strains of the causative agent, infectious laryngotracheitis virus (ILTV). These attenuated alphaherpesviruses initially exhibit varying degrees of low to moderate pathogenicity in vaccinated chickens (Hilbink *et al.*, 1987; Guy *et al.*, 1990). However, it has been suggested that reversion to greater or parental-type virulence may occur as a result of bird-to-bird transmission of the virus (Guy *et al.*, 1989, 1990). Indeed, the predicted alteration took place during the continuous passaging in chickens of a vaccine virus previously propagated in chicken embryos (Guy *et al.*, 1991). This demonstration and the observed similarities in the restriction endonuclease digestion patterns of some of the genomes of field and vaccine viral isolates (Andreasen *et al.*, 1990; Guy *et al.*, 1989, 1990; Keller *et al.*, 1992) have given rise to concerns that field outbreaks of ILT may be due to the spread of vaccine virus to naive chickens. Although this theory is currently being disputed (Keeler *et al.*, 1993), the dissemination of vaccine viruses from immunized chickens has been demonstrated (Hilbink *et al.*, 1987; Mutalib, 1992). Moreover, like field strains (Hughes *et al.*, 1987, 1989), vaccine viruses can apparently establish a latent infection in chickens, presumably in the trigeminal ganglion (Williams *et al.*, 1992), and be reactivated at a later date (Bagust, 1986; Hughes *et al.*, 1991). Therefore, alternative methods, such as deliberate genetic impairment, should be considered when attenuating ILTV for use as a vaccine.

Among the potential targets for inactivation, the thymidine kinase (TK) gene is a likely choice. Elimination of this viral-encoded enzyme in other herpesviruses, such as bovine herpesvirus type 1 (Kit *et al.*, 1985b), herpes simplex virus (Marcialis *et al.*, 1975; Field and Wildy, 1978), and pseudorabies virus (Tatarov, 1968; Kit *et al.*, 1985a; McGregor *et al.*, 1985), resulted in a corresponding reduction in virulence. However, vaccination with such mutants still afforded protection against challenge with virulent virus. Moreover, all genetically engineered, live pseudorabies vaccine viruses produced against

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Aujeszky's disease have mutations in their TK genes (Mettenleiter, 1994). Therefore, similarly modified ILTV would probably retain antigenicity and also be acceptable for field use. Since the herpesvirus TK is nonessential for virus replication in tissue culture, vaccine production should not be affected by the replacement of currently used modified-live viruses with TK⁻ ones. In addition, by analogy to herpes simplex virus (Coen *et al.*, 1989; Efstathiou *et al.*, 1989), a loss of viral-encoded TK activity might cause a reduction in reactivation of ILTV from the latent state and thus spread of a TK⁻ virus would be more limited than that of presently used vaccine strains. In view of the potential practicality of such a mutant, this virus was created by inactivating its TK gene. Initially, to verify the viability of the TK⁻ ILTV and, later, to aid in its plaque purification, an expressible *lacZ* gene was inserted by homologous recombination into one of two sites within the coding sequence for TK. Both types of recombinants produced β-galactosidase (*lacZ* gene product) and appeared to lack TK activity. These viruses had become attenuated, yet apparently they could still induce a protective immune response in inoculated chickens. This success may signal the beginnings of a new generation of avian vaccines consisting of genetically engineered ILTV.

MATERIALS AND METHODS

Viruses and cells

A severe vaccine strain, ILTV L608, (TRAVAX) originally provided by Schering–Plough Animal Health Corp. (Elkhorn, NE), was used as the parental virus in this study. This virus, as well as the derived recombinants, was propagated and titered by plaque assay in the chicken hepatoma cell line LMH as previously described (Schnitzlein *et al.*, 1994). In addition, a mild vaccine strain, LT-IVAX (Schering–Plough Animal Health Corp.), and the highly virulent USDA challenge strain of ILTV, NVSL (National Veterinary Services Laboratory, Ames, IA), were used in the safety trials in chickens. The former was grown in chicken embryo liver cells while the latter, as well as the TRAVAX ILTV used in the safety study, was propagated on the chorioallantoic membranes of developing chicken embryos. The number of infectious doses in preparations of these three viruses was determined using their respective hosts.

ILTV DNA to be used for cloning of the TK gene had been previously isolated from the LT-Blen vaccine strain (Schnitzlein *et al.*, 1994). This virus had been propagated in the chorioallantoic membranes of developing chicken embryos and then purified by centrifugation in a discontinuous sucrose gradient. Purified genomic DNA from the Rice strain of pseudorabies virus was kindly provided by Dr. G. Scherba, University of Illinois.

Isolation and construction of plasmids

For routine analysis, plasmids were prepared by a rapid, modified alkaline lysis procedure (Zhou *et al.*, 1990). However, when used in cloning or transfection protocols, plasmids were isolated by a more rigorous method (Lee and Rasheed, 1990). Plasmids were constructed and verified by standard techniques (Schnitzlein *et al.*, 1988; Schnitzlein and Tripathy, 1990), using enzymes obtained from GIBCO BRL Life Technologies, Inc. (Gaithersburg, MD) and Boehringer Mannheim Biochemicals (Indianapolis, IN). In some instances, DNAs were purified (GeneClean; BIO 101, La Jolla, CA) prior to use in ligation and transformation procedures.

Preparation of viral nucleocapsids

Nucleocapsids were released from ILTV-infected cells following solubilization of the cellular membranes with Triton X-100 (Schnitzlein *et al.*, 1994). Nuclei were removed by centrifugation of the cell lysate at 2000 rpm and 4° for 5 min. The resulting supernatant was layered over 5 ml of hypotonic buffer (10 m*M* Tris, pH 8.0, 10 m*M* KCl, 5 m*M* EDTA) containing 15% sucrose in a 30-ml Nalgene Teflon FEP tube and centrifuged at 4° and 11,000 rpm for 75 min in a Beckman JA-20 rotor. The pelleted cores were resuspended in 0.5 ml Waymouth's MB 752/1 medium containing 10 μg/ml gentamicin (GIBCO BRL Life Technologies, Inc.) and stored at −80°.

Transient expression assays

LMH cell monolayers in 24-well plates were transfected with 1 μg of intact plasmid by the calcium phosphate technique using a HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethane sulfonic acid)-buffered solution (Spandidos and Wilkie, 1984). After a 5.5-hr absorption period, the cells were washed twice and overlaid with fresh Waymouth's medium supplemented with 1% fetal calf serum, 10 μg/ml gentamicin, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (GIBCO BRL Life Technologies, Inc.). On the following day, the monolayers were infected with approximately 10 plaque-forming units (PFU) of ILTV per cell. After an additional 3 days, the monolayers were harvested and assayed for the presence of β-galactosidase (Schnitzlein and Tripathy, 1990).

Transfections

Prior to transfection, plasmids were digested with *Bam*HI, extracted with phenol–chloroform, and then ethanol precipitated. The linearized plasmids were resuspended in TE buffer (10 m*M* Tris, pH 8.0, 1 m*M* EDTA) at a concentration of approximately 0.5 μg/μl and stored at −20°. Plasmid DNAs were introduced into LMH cell monolayers in 12-well plates by the calcium phosphate

method using a BES [50 m*M* *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid]-buffered solution (Chen and Okayama, 1988). Either intact virions or viral nucleocapsids were used as the source of ILTV DNA. In the first case, transfection was initiated after a 1-hr period of infection by ILTV (approximately 15,000 PFU/monolayer). Alternately, nucleocapsids (50 μl) were combined with the transfection mixture immediately after its application to the cell monolayer. Following a 6 (nucleocapsid-infected cells)- or 16 (virus-infected cells)-hr absorption period, the monolayers were washed twice and overlaid with Waymouth's medium supplemented with 1% fetal bovine serum, 10 μg/ml gentamicin, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B. After an additional 5 (nucleocapsid-infected monolayers) or 6 (virus-infected monolayers) days, the transfected cells were stored at −80° until screened for the presence of recombinant ILTV.

Screen for recombinant virus

Monolayers of LMH cells were infected with the viral progeny released from transfected cells by a twofold freezing and thawing process. At 4 days postinfection, the monolayers were overlaid with Waymouth's medium supplemented with 1% fetal calf serum, 10 μg/ml gentamicin, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B and containing 0.4% agarose (Ultra Pure DNA Grade; Bio-Rad Laboratories, Inc., Richmond, CA). On the following day, a second agarose overlay (50% in volume relative to that of the first overlay) containing 300 μg/ml Bluo-gal (halogenated indolyl-β-D-galactoside; GIBCO BRL Life Technologies, Inc.) was applied. Generally, within 16 hr at ambient conditions, cells infected by the recombinant ILTV would turn blue due to hydrolysis of the Bluo-gal substrate by β-galactosidase. Such blue plaques were picked using a Pasteur pipette and stored at −80°. This cloning process was repeated until only blue plaques were detected in two consecutive rounds of infection. After the first two plaque purifications, the overlaying process was delayed one day in order that individual plaques could easily be visually observed. This delay helped in verifying a homogeneous virus population.

Molecular analysis of ILTV DNA

ILTV DNAs were isolated from infected cells and digested with *Eco*RI and *Xho*I as previously described (Schnitzlein *et al.*, 1994). The resulting fragments were electrophoresed in a 0.9% agarose gel containing 1× TBE (89 m*M* Tris, 89 m*M* boric acid, 2 m*M* EDTA) at 20 V for 20 hr. The subsequent steps of alkaline transfer of the separated DNA fragments to a nylon membrane, hybridization, and autoradiography were performed as described previously (Tripathy and Schnitzlein, 1991). DNA probes for the ILTV TK gene and flanking sequences, pseudorabies virus gX gene promoter, and *Escherichia coli lacZ* gene were obtained by *Bam*HI and *Eco*RI digestion of pILTK1 (see Results), *Bam*HI and *Sal*I digestion of pXSB1 (Fig. 1), and *Bam*HI digestion of pSC8 (Chakrabarti *et al.*, 1985). All fragments were isolated by electrophoresis in low-melting-point agarose gels (GIBCO BRL Life Technologies, Inc.). The DNA probes were radioactively labeled with [α-^{32}P]dCTP using a Random Primers DNA Labeling System kit (GIBCO BRL Life Technologies, Inc.) either directly in the agarose (*lacZ* gene and gX gene promoter) or following removal from the agarose (ILTV TK gene) by using a QIAEX gel extraction kit (QIAGEN, Inc., Chatsworth, CA). After 6 hr at ambient temperature, the labeling reactions were terminated and unincorporated nucleotides were removed by passage through a CHROMA SPIN-100 column (CLONTECH Laboratories, Inc., Palo Alto, CA).

Effect of FMAU on ILTV replication

Monolayers of LMH cells in 12-well plates were infected with approximately 2000 PFU of virus. After absorption at ambient temperature for 2 hr, the inocula were removed. The monolayers were then washed twice and overlaid with Waymouth's medium containing 1% fetal bovine serum and 9.7 nmol/ml of the thymine analog 1-(2-fluoro-2-deoxy-β-D-arabinofuranosyl)-5-methyluracil (FMAU; Watanabe *et al.*, 1979), which had been kindly provided by Drs. J. Fox and K. Watanabe, Memorial Sloan-Kettering Cancer Center. At 5 days postinfection, the cells were frozen at −80° until assayed for the presence of infectious virus.

Safety testing of recombinant ILTV in chickens

Recombinant viruses were safety tested in chickens as per USDA regulation 9CFR 113.328 for evaluation of modified live ILTV vaccines. Groups of 15 4-week-old specific-pathogen-free (SPAFAS) chickens were inoculated intratracheally with the parental TRAVAX virus, one of the two recombinant viruses, or LT-IVAX. These, as well as a mock-vaccinated group, were housed in separate units. At 14 days postinfection, 10 chickens from each group (except those inoculated with TRAVAX) were challenged intratracheally with a lethal dose of NVSL ILTV challenge stock.

RESULTS

Generation of a foreign transcriptional unit cassette

In order to simplify the creation of insertion vectors, a transcriptional unit was constructed so that it could be easily excised intact and separated from its plasmid backbone. This cassette consisted of the pseudorabies virus glycoprotein gX gene promoter regulating the ex-

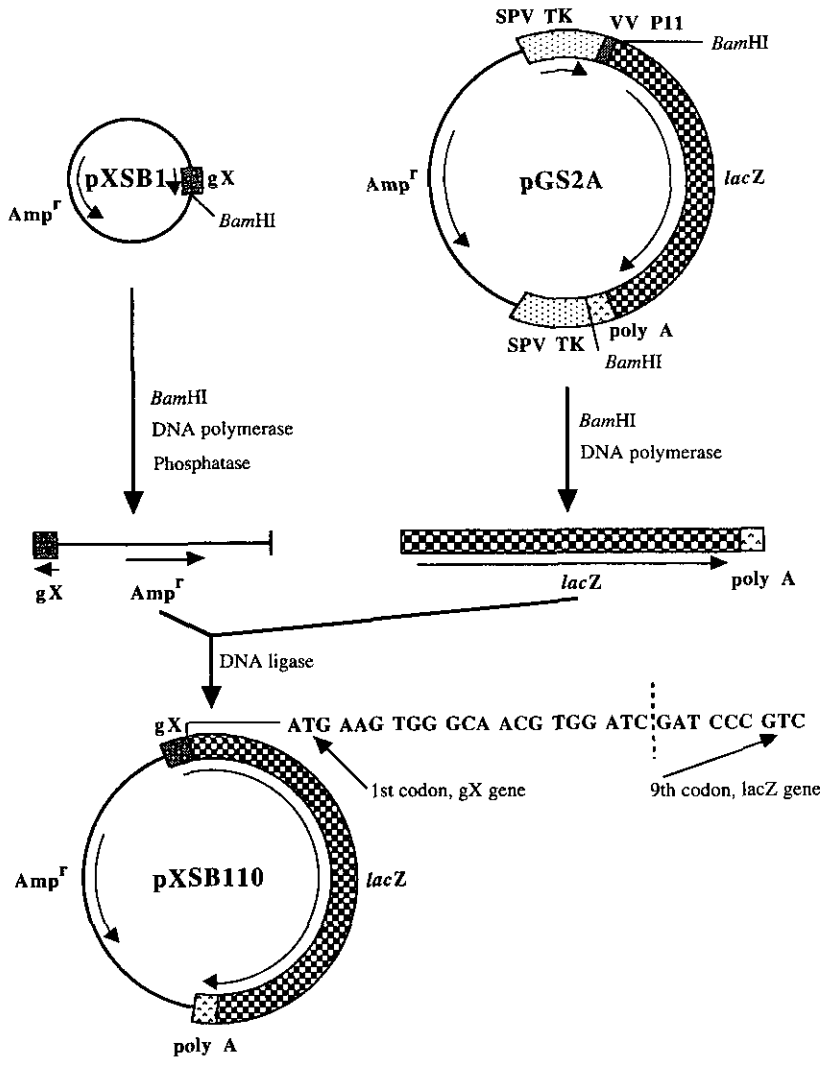

FIG. 1. Schematic representation of the construction of pXSB110. The relative positions and direction of transcription of the swinepox virus thymidine kinase (SPV TK) gene, SV-40 3′-polyadenylation signal sequence (poly A), and bacterial *lacZ* and β-lactamase (Amp^r) genes are shown. Transcription of the *lacZ* gene is regulated by either the vaccinia virus late P11 promoter (VV P11) or the pseudorabies virus glycoprotein X gene promoter (gX). Fusion of the gX and *lacZ* genes in pXSB110 is shown at the nucleotide level. The point of ligation is indicated by the vertical dotted line.

pression of the *E. coli lacZ* gene which was coupled to a SV-40 3′-polyadenylation signal sequence. This particular combination was chosen since the ninth codon of the *lacZ* gene could be fused in frame with the seventh codon of the gX gene. A similar construct has been used to generate pseudorabies virus recombinants (Mettenleiter and Rauh, 1990). However, in these viruses, polyadenylation of the *lacZ* mRNA was dependent on the nearest downstream signal sequence.

The gX gene promoter is contained in the *Bam*HI-10 fragment of the pseudorabies virus genome (Rea *et al.*, 1985). Thus, this DNA piece was cloned into the *Bam*HI site of pUC18 to produce pB105. Analysis of the published sequence of the region immediately upstream of the gX gene (van Zijl *et al.*, 1990) indicated that a 427-bp *Sal*I–*Bam*HI fragment should contain the entire gX gene promoter. Indeed, this piece has been shown to retain regulatory ability (Mettenleiter and Rauh, 1990). Since direct digestion of pB105 with *Bam*HI and *Sal*I produced several fragments similar in size to that of the desired piece, a unique 568-bp *Xho*I–*Bam*HI fragment was excised from pB105. After gel purification of this piece and digestion with *Sal*I, the resulting 427-bp fragment was inserted into the *Bam*HI and *Sal*I sites of pGEM3 to produce pXSB1.

In order to easily utilize the unique *Bam*HI site of pXSB1, the fusion of the *lacZ* gene and SV-40 polyadenylation recognition sequence should be flanked by *Bam*HI sites. This construct was made by ligating a 2.63-kb *Cla*I–*Bam*HI fragment excised from pCAL4 (Flanagan and

TABLE 1

Transient Expression of *lacZ* Gene[a]

Plasmid[c]	β-Galactosidase activity[b]	
	Mock	ILTV[d]
—	0.72	0.54
pXSB12	0.62	0.50
pXSB110	0.74	29.02

[a] Mock-infected and virus-infected LMH cell monolayers were assayed for β-galactosidase activity at 4 days post-transfection.

[b] Enzymatic activity is shown as μmol *o*-nitrophenyl-β-galactopyranoside (ONPG) cleaved per minute per monolayer.

[c] Plasmids contained the *lacZ* gene in the opposite (pXSB12) or some (pXSB110) orientation as the gX gene promoter.

[d] Monolayers were infected with approximately 10 PFU of ILTV at 16 hr post-transfection.

Wagner, 1987) into a 5.1-kb portion of pGS108 [a modified pGEM3 which contains the *lacZ* gene bounded by *Bam*HI sites and regulated by the vaccinia virus P11 promoter — the source of this transcriptional unit was originally pSC8 (Chakrabarti *et al.*, 1985)] generated by *Cla*I and partial *Bam*HI digestion. The smaller fragment contained a SV-40 3′-polyadenylation signal sequence which was immediately upstream of a *Bam*HI site and preceded by approximately 70% of the *lacZ* gene. The remainder of the *lacZ* gene (except for the first eight codons) terminated at the *Cla*I site of the deleted pGS108 and was preceded by an undigested *Bam*HI site. Thus, the resulting plasmid, pGS2A, contained *Bam*HI sites flanking the *lacZ* gene and polyadenylation recognition sequence.

An intact *lacZ* gene transcriptional cassette was then made by ligating the 3.45-kb *Bam*HI fragment from pGS2A into the *Bam*HI site in pXSB1 (Fig. 1). The correct orientation of the *lacZ* gene relative to the gX gene promoter and also recognition of this promoter by the ILTV enzymes were verified by analyzing transient expression of the *lacZ* gene in ILTV-infected cells which had been transfected with modified pXSB1 plasmids. β-Galactosidase (*lacZ* gene product) activity was only detected in those infected cells which had been transfected with the modified pXSB1 plasmid, pXSB110 (Fig. 1), predicted to have the *lacZ* gene correctly positioned (Table 1).

Cloning of the ILTV TK gene

Examination of the published sequences of the ILTV genome (Griffin and Boursnell, 1990; Keeler *et al.*, 1991) revealed that the TK gene is located within a 2.0-kb *Xho*I fragment. Thus, *Xho*I-generated ILTV DNA pieces of this magnitude were cloned into the *Sma*I site of pUC9 after their 5′ ends had been filled in through the use of Klenow DNA polymerase. A plasmid containing the desired fragment, as determined by restriction endonuclease analysis, was designated pILTK1. Following *Bam*HI and partial *Eco*RI digestion of this plasmid, the resulting 2.0-kb fragment was treated with Klenow DNA polymerase and then subcloned into the *Pvu*II and filled-in *Hin*dIII sites of pGEM3 (Promega Biotech, Madison, WI) to create pILTK5. This plasmid contains unique *Eco*RI, *Pvu*II, and *Sna*BI recognition sites which are located within the TK gene and thus might be suitable as loci for the insertion of foreign DNA. Moreover, the regeneration of a unique *Bam*HI site, located at one juncture of the ILTV and pGEM3 DNAs, provides a site for linearization of insertion vectors containing the novel *lacZ* transcriptional cassette. Such treatment of the plasmids, prior to transfection of virus-infected cells, would probably enhance the production of recombinant ILTV.

Generation of insertion vectors

The genomic structure of herpesviruses is such that genes are transcribed in both directions and, as a result, transcriptional units may overlap. Indeed, the TK gene of ILTV has a 37-codon head–head overlap with an upstream open reading frame and terminates 33 bp from the initiation codon of a downstream gene (Griffin and Boursnell, 1990). Since the essentiality of the products of these adjacent open reading frames is unknown, any location chosen for inserting the foreign transcriptional unit should not interfere with the expression of these genes. Therefore, insertion sites closer than 290 bp from the initiation codon of either of the two unknown genes were not considered. At this distance, even the promoters regulating these genes probably should not be affected.

Two distinct insertion plasmids were created by blunt-end ligating the 3.88-kb *Sal*I–*Sma*I *lacZ* transcriptional cassette excised from pXSB110 (after treatment of the *Sal*I-generated termini with Klenow DNA polymerase) into either the *Pvu*II or the *Sna*BI site of pILTK5 (Fig. 2). The resulting plasmids were designated pLTX24 and pLTX36, respectively. Expression of the *lacZ* gene was not affected by the flanking TK gene sequences, since β-galactosidase activity was found in ILTV-infected cells which have been transfected with either plasmid (data not shown).

Generation and characterization of recombinant ILTV

For the *lacZ* transcriptional unit to be inserted into the ILTV genome, homologous recombination between the plasmid and viral DNAs must occur. Although the plasmid must be transfected into the cell, the optimum choice for introduction of the viral genome is not known. Therefore, LMH cells either were transfected with linearized pLTX24 in the presence of viral nucleocapsids or were first infected with intact virions and then transfected with linearized pLTX36. Both approaches were successful,

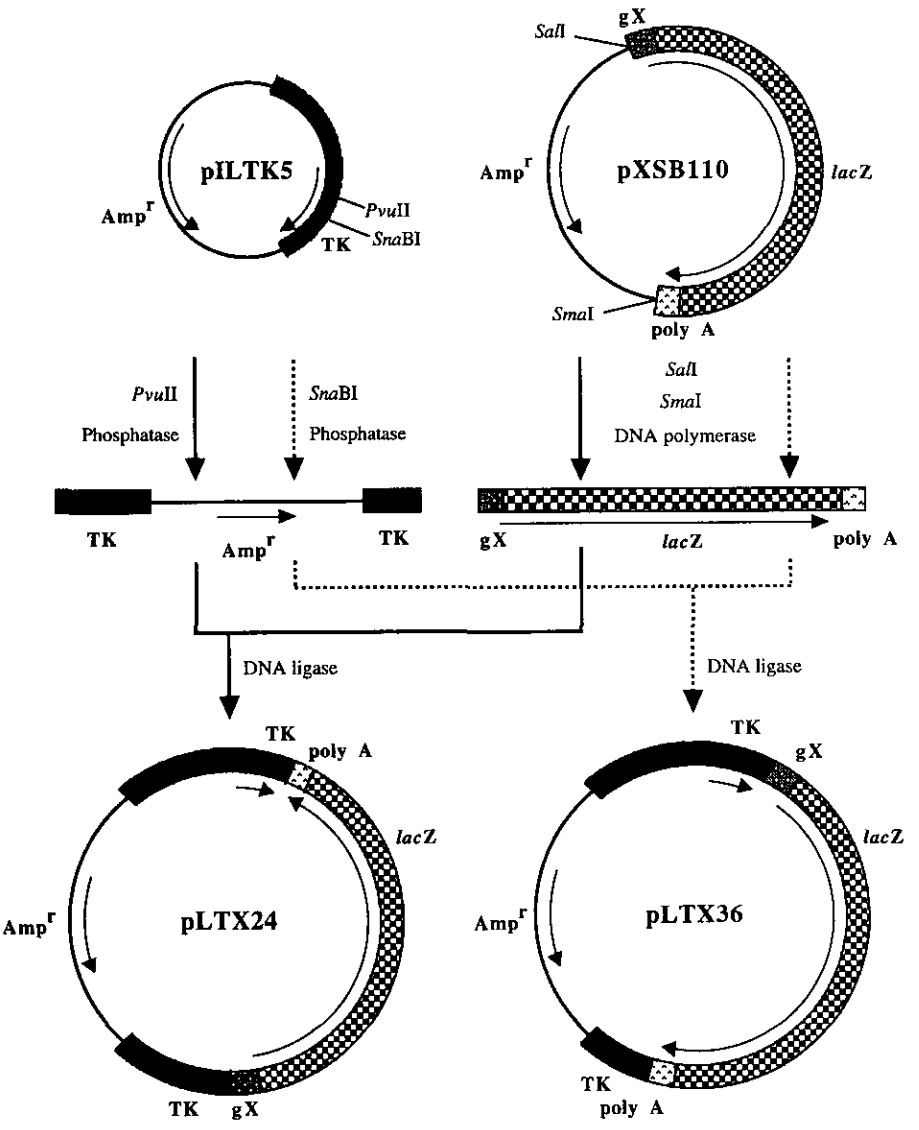

FIG. 2. Schematic representation of the construction of pLTX24 and pLTX36. The relative positions and direction of transcription of the ILTV thymidine kinase (TK) gene, SV-40 3′-polyadenylation signal sequence (poly A), and bacterial *lacZ* and β-lactamase (Amp^r) genes are shown. Transcription of the *lacZ* gene is regulated by the pseudorabies virus glycoprotein X gene promoter (gX).

since screening of either progeny for the ability to produce β-galactosidase (blue plaques) demonstrated the presence of recombinant viruses. A representative of each recombinant (ILTV-*lacZ*-24 and ILTV-*lacZ*-36) was plaque-purified and then propagated without further visual selection for the ability to express the *lacZ* gene.

In order to verify retention of the foreign transcription unit at the predicted locus, the two recombinant viruses were separately passaged six times and then their genomes were analyzed by Southern hybridization. For this purpose, the DNAs of the parental virus and both recombinants were digested with *Xho*I to release the TK gene and flanking sequences and with *Eco*RI to determine the orientation and location of the foreign transcriptional unit (Fig. 3). Under these conditions, the gX gene promoter and the majority of the *lacZ* gene are retained in the same fragment. Therefore, to enable detection of a carryover signal due to incomplete stripping of the membrane, the viral DNA fragments were sequentially hybridized with the *lacZ* gene, TK gene, and gX gene promoter probes. As anticipated, the *lacZ* gene and gX gene promoter are contained in an approximately 3.5-kb fragment derived from the genome of either recombinant (Fig. 4, lanes 5 and 6, and lanes 8 and 9, respectively). Although the remainder of the bacterial gene was not detected due to its relatively small overall percentage (2.3%) of the *lacZ* gene probe, retention of this piece and the polyadenylation site was indicated by the decreased mobilities of the corresponding fragments of the TK gene to which they were fused (Fig. 3). Thus, the 1.4-kb TK-containing

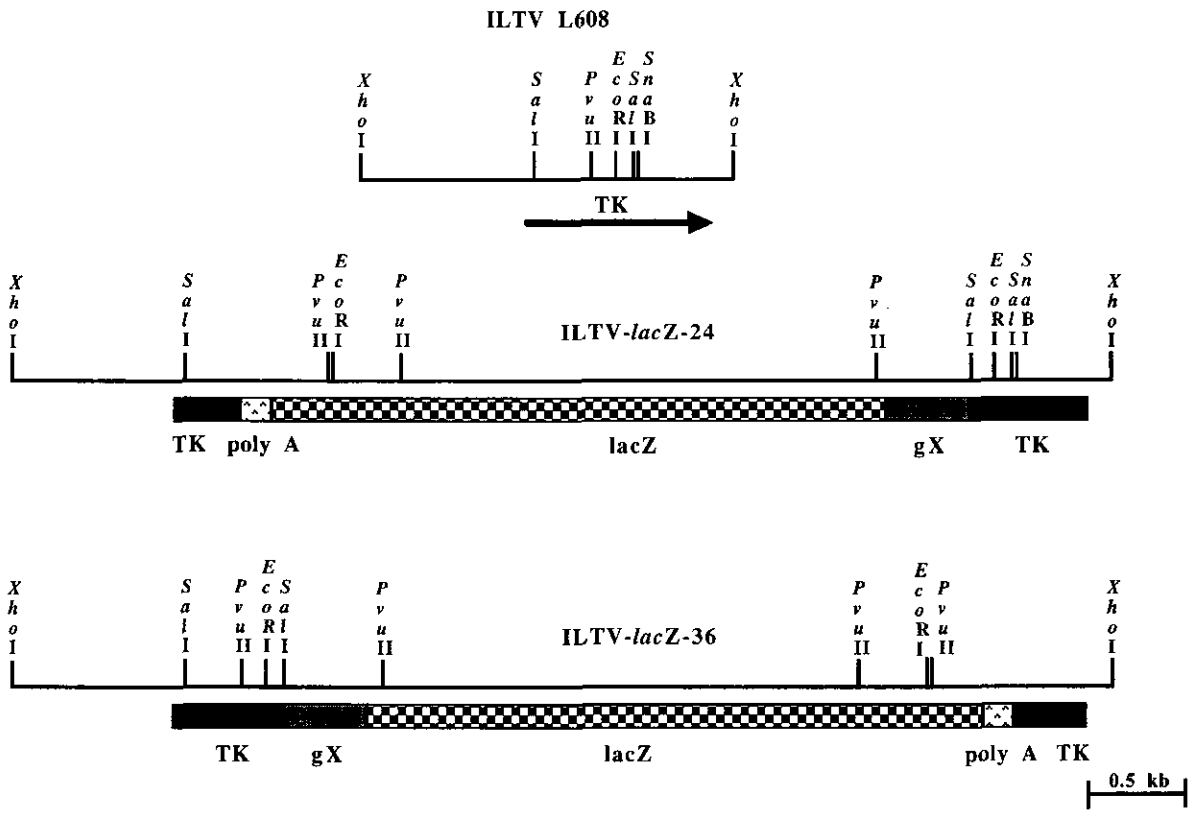

FIG. 3. Restriction endonuclease maps of a *Xho*I fragment of the ILTV L608, ILTV-*lacZ*-24, and ILTV-*lacZ*-36 genomes containing the thymidine kinase (TK) gene. The relative positions of the ILTV TK gene, pseudorabies virus glycoprotein X gene promoter (gX), *E. coli lacZ* gene (*lacZ*), and SV-40 3′-polyadenylation signal sequence (poly A) are shown. All sites recognized by restriction enzymes *Eco*RI, *Pvu*II, *Sal*I, *Sna*BI, and *Xho*I are indicated.

piece of the parental DNA (Fig. 4, lane 1) increased to approximately 1.7 kb in the genome of ILTV-*lacZ*-24 (Fig. 4, lane 2). Likewise, the 0.64-kb TK-containing fragment of the parental DNA (Fig. 4, lane 1) shifted to approximately 1.0 kb in the genome of ILTV-*lacZ*-36 (Fig. 4, lane 3). The increase in the size of these fragments also verified the predicted orientations of the foreign transcriptional unit in the genomes of both recombinants (Fig. 3).

Attempts to demonstrate the expected TK^- phenotype of the recombinants by directly assaying for enzymatic activity yielded ambiguous results due to the presence of cellular TK. Therefore, a variety of thymine analogs, including 5-(2-bromovinyl)-2′-deoxyuridine and thymine 1-β-D-arabinofuranoside, were examined for their ability to inhibit virus replication without a concomitant loss in cell viability. Among these potential inhibitors, only FMAU fit the criteria. At a concentration of 9.7 μM, the production of the parental ILTV was reduced by almost 4 orders of magnitude (Table 2). In contrast, this nucleoside only marginally affected the replication of either recombinant. Moreover, based on the comparable yields of all three viruses in the absence of FMAU (Table 2), even the loss of virus-encoded TK activity was not detrimental to ILTV replication.

Evaluation of virulence and protective ability of recombinant ILTV

In order to determine whether the genetic modification of the ILTV genome affected its pathogenicity or immunogenicity, the recombinants were safety tested in chickens according to USDA regulation 9CFR 113.328 (Table 3). For comparison, both the highly virulent parental vaccine virus (TRAVAX) and a mildly virulent vaccine virus (LT-IVAX) were included. During the 2- to 4-day interval following intratracheal inoculation, all 15 birds receiving TRAVAX exhibited moderate–severe respiratory reactions. Moreover, 6 animals in this group had died by the fourth day. In contrast, the chickens in all the other groups survived and a reduced number of them had moderate–severe respiratory distress during this time period. In fact, only mild reactions were seen in the birds inoculated with ILTV-*lacZ*-36. The respiratory reaction index, obtained using this recombinant, was 10-fold lower than that determined for the parental virus. Although this value was higher for the other recombinant, it was comparable to that obtained using the mild vaccine, LT-IVAX. Regardless of the severity of the reactions induced by either recombinant or LT-IVAX, complete protection against a

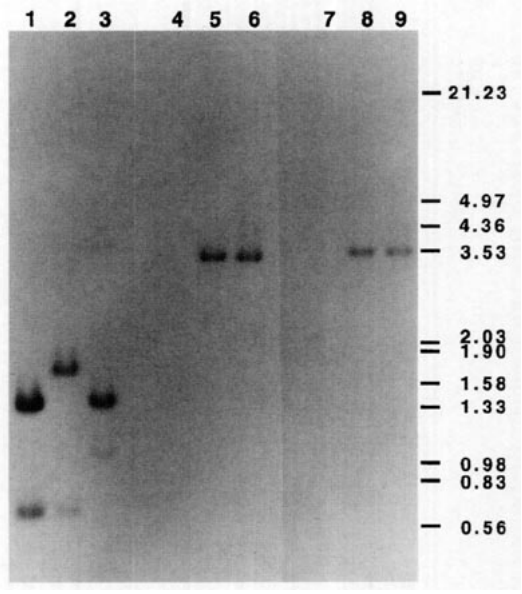

FIG. 4. Analysis of parental and recombinant ILTV DNAs. ILTV L608, ILTV-*lacZ*-24, and ILTV-*lacZ*-36 genomes were digested with *Eco*RI and *Xho*I, loaded in the above respective order into a 0.9% agarose gel, and then electrophoresed at 20 V for 20 hr. Following transfer to a nylon membrane under alkaline conditions, the DNA fragments were consecutively hybridized with ^{32}P-labeled *E. coli lacZ* gene (lanes 4–6), ILTV TK gene (lanes 1–3), and pseudorabies virus gX gene promoter (lanes 7–9) after the preceding probe had been stripped off of the membrane. The relative mobilities of the molecular weight markers (λ DNA *Eco*RI/*Hin*dIII fragments) are indicated on the right.

lethal challenge of virulent ILTV was afforded to these vaccinated birds.

DISCUSSION

Although a variety of herpesviruses have been genetically engineered to express foreign genes, the creation of recombinant ILTV has been hampered by the lack of a cell line in which to propagate the virus. Recently, this difficulty was overcome as evidenced by the demonstrations of ILTV replication in chicken hepatoma cell lines (Scholz *et al.*, 1993a; Schnitzlein *et al.*, 1994) and the use of these cells to produce an ILTV recombinant (Guo *et al.*, 1994). Indeed, LMH cells were a key factor in the generation of TK⁻ ILTV. However, the subsequent purification of these mutants was also dependent on expression of an inserted "marker." In order to prevent homologous intragenomic recombination, this insert consisted of the *E. coli lacZ* gene which was transcriptionally regulated by a mammalian herpesvirus promoter and whose mRNA was polyadenylated due to a SV-40 signal sequence. Interestingly, in contrast to the early Rous sarcoma virus and immediate-early cytomegalovirus promoters (Scholz *et al.*, 1993a,b), the pseudorabies virus gX gene promoter was not detectably active in chicken hepatoma cells in the absence of ILTV (Table 1). Presumably, like the SV-40 early promoter/enhancer and the ILTV TK gene promoter (Scholz *et al.*, 1993b), viral-encoded enzymes are required for enhanced activity of this transcriptional regulatory element.

Recognition of the gX gene promoter by ILTV should enable the production of a variety of recombinants, including some potential immunizing agents. First, the foreign transcriptional unit, used to insertionally inactivate the ILTV TK gene, could be utilized in a similar manner to identify other nonessential sites within the virus genome. Genes coding for enzymes, which are associated with virulence in other herpesviruses, such as dUTPase (Pyles *et al.*, 1992), ribonucleotide reductase (Cameron *et al.*, 1988; de Wind *et al.*, 1993), and uracil DNA glycosylase (Liang *et al.*, 1993; Pyles and Thompson, 1994), would be obvious locations to target. Second, the *lacZ* gene or the entire foreign transcriptional unit in the recombinant viral genome could be replaced by other nucleotide sequences, consisting of the coding regions for antigens of distinct poultry pathogens and possibly their respective promoters. The resulting multivalent vaccine viruses would be visually identified and plaque-purified based on the inability to produce β-galactosidase. Since the utility of such heterologous promoters in generating ILTV recombinants is unknown at this time, their recognition by the virus would first have to be assessed by a transient expression system like that described in this report.

Current vaccine strains of ILTV have been chosen on the basis of their relatively reduced virulence. Although the underlying genetic factors responsible for these phenotypic differences are unknown, the pathogenicity of these viruses can be ameliorated by the selected route of inoculation. Thus, the mild LT-IVAX is introduced into chickens by eye drops, whereas the severe TRAVAX virus is inoculated into the birds' vents. Since transmission of the virus to naive birds is of concern, the safety of vac-

TABLE 2

Effect of FMAU on the Replication of ILTV[a]

Virus	FMAU[b]	Yield (PFU/ml)[c]
ILTV L608	–	5.6×10^5
	+	6.8×10^1
ILTV-*lacZ*-24	–	9.1×10^6
	+	6.8×10^5
ILTV-*lacZ*-36	–	9.0×10^5
	+	8.1×10^5

[a] Monolayers were infected with parental or recombinant ILTV for 2 hr at 25° and then washed twice before placement at 37°.

[b] Where indicated, medium contained 9.4 μ*M* of the thymine analog, FMAU.

[c] Virus yields at 5 days postinfection represent the average of duplicate plaque assays.

TABLE 3

Safety Testing of Recombinant and Vaccine ILTV in Chickens[a]

Virus	Dosage[b]	Respiratory reaction: Days postinoculation[c] 2	3	4	Index[d]	Protection
TRAVAX	4.3	15 (15)	15 (15)	15 (15)[e]	2.3	ND[f]
ILTV-*lacZ*-24	4.8	9 (3)	11 (4)	7 (1)	0.8	10/10
ILTV-*lacZ*-36	4.6	3	4	3	0.2	10/10
LT-IVAX	4.1	4 (1)	10 (2)	9 (3)	0.6	10/10
None	—	—	—	—	—	1/10

[a] Chickens were inoculated intratracheally with the indicated doses of various ILTV strains and then challenged 14 days later with a lethal amount of NVSL ILTV.

[b] Dosage represents either the $\log_{10}$ EID_{50} or the $\log_{10}$ $TCID_{50}$ per 0.2 ml.

[c] Both the number of all reactions, including mild ones, and the number of just those that were moderate–severe (in parentheses) are shown.

[d] Index represents a weighted average whereby normal, mild, moderate, and severe reactions and mortality are assigned values of 0, 1, 2, 3, and 4, respectively.

[e] Six deaths are included.

[f] Not done.

cines is evaluated by intratracheal inoculation of chickens. Using this procedure, the severity of infection by TRAVAX ILTV was indicated by the nearly fourfold increase in its respiratory reaction index compared to that for LT-IVAX (Table 3). Moreover, the only mortalities were in birds inoculated with TRAVAX vaccine. In contrast, the indexes for the recombinants were either slightly higher or threefold lower than that obtained using LT-IVAX vaccine. Despite a fourfold difference in their indexes, both recombinants still provided adequate protection to chickens challenged with a lethal dose of virulent ILTV. Whether the point of insertion into the TK gene influenced the extent of reduction of the recombinants' virulence is unclear at this time. Regardless, these altered viruses represent the first ILTV vaccines that are genetically, rather than biologically, modified to exhibit low pathogenicity in poultry.

Insertional inactivation of the ILTV TK gene at either of two sites, located 258 bp apart, did not impair the replicative ability of the virus or affect its immunogenicity, although differences in virulence were observed. Whether expression of the adjacent genes was affected by this mutagenesis is not presently known. However, in the case of the open reading frame that head–head overlaps with the TK gene, mutations in the homologous gene of herpes simplex virus and herpesvirus of turkeys have resulted in titer reductions exceeding two orders of magnitude (Jacobson *et al.*, 1989). In anticipation of a similar phenomenon occurring for ILTV, the nearest insertion site was 290 bp downstream of the initiation codon of this gene. Moreover, a third insertion vector (pLTX13) had been constructed by ligating the *lacZ* transcriptional unit with *Eco*RI-digested pILTK5. Use of this plasmid would effectively have moved the point of mutation an additional 130 bp downstream. This distance would have then been comparable to that from the other insertion locus to the beginning of the downstream open reading frame (468 bp). Since one objective of this study was to identify endpoints from which a viable TK-deletion mutant could be created, the successful generation of both recombinants rendered the production of ILTV-*lacZ*-13 unnecessary.

Although attenuation of ILTV was accomplished by insertional inactivation of the viral TK gene, the genomes of future vaccine viruses may only lack the 258 bp between the *Pvu*II and *Sna*BI sites of this gene. Such deletion mutants could probably be selected on the basis of their resistance to the thymine analog, FMAU. Alternatively, the mutants could be visually detected if the deleted region was replaced with the *lacZ* transcriptional unit. In either case, use of such genetically altered viruses would enable determination of whether outbreaks of infectious laryngotracheitis are due to an invading field isolate or to the vaccine.

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