

Construction of Recombinant Avian Infectious Laryngotracheitis Virus Expressing the β -Galactosidase Gene and DNA Sequencing of the Insertion Region

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Avian infectious laryngotracheitis virus (ILTV), a herpesvirus, is a highly contagious pathogen that causes an upper respiratory tract infection in chickens. It is one of the major problems in the poultry industry worldwide. Current vaccines are not satisfactory due to the induction of latent infection. Here we describe a system for the construction of recombinant ILTV. A 4-kbp ILTV *Eco*RI DNA fragment was cloned into plasmid pUC13 and sequenced. Computer prediction revealed two potential open reading frames with 216 and 259 amino acid residues, respectively. The 259-residue polypeptide was serine-rich. The β -galactosidase (β -gal) gene of *E. coli* was cloned into the *Xho*I/*Bgl*II site of this DNA fragment, integrated into the ILTV genome via homologous recombination, expressed under the control of the immediate-early cytomegalovirus promoter, and caused the formation of blue plaques in the presence of X-gal. The insertion of a foreign gene into the ILTV genome and the successful expression of the incorporated gene demonstrated the potential for the construction of attenuated recombinant ILTV vaccines and the development of ILTV as vectors for polyvalent vaccines against avian upper respiratory tract infections. © 1994 Academic Press, Inc.

INTRODUCTION

Avian infectious laryngotracheitis virus (ILTV), a member of the α -subfamily of herpesviruses (Roizman *et al.*, 1981), contains a double-stranded DNA genome of approximately 155 kbp. It causes an upper respiratory tract infection in chickens that has a morbidity rate approaching 100% and a mortality rate of 10–40% (Roizman, 1982; Hanson, 1984). The disease occurs worldwide and is one of the major health and economic problems in the poultry industry (Bagust, 1986). Current attenuated ILTV vaccines are not satisfactory due to the induction of latent infection and recurrence or even spread of the disease (Bagust, 1986; Hughes *et al.*, 1991). Leib *et al.* (1986) have shown that the restriction endonuclease digests of nine European wild-type strains of ILTV were similar to those of the American vaccine strains, suggesting that the recurrence of the disease may in part result from reactivation or a lack of attenuation of the vaccine strains. Hughes *et al.* (1991) have reported that latent infection of ILTV can lead to shedding of the virus and induction of disease in susceptible birds similar to that seen in the field. There is an urgent demand for an attenuated

recombinant ILTV vaccine that does not induce latency in the bird.

Systems for the construction of recombinant DNA viruses have been established for vaccinia virus (Mackett *et al.*, 1982; Panicali and Paoletti, 1982; Guo *et al.*, 1989, 1990); adenovirus (Grunhaus and Horwitz, 1992); adeno-associated virus (Hermonat and Muzyczka, 1984); baculovirus (Bishop, 1992; Smith *et al.*, 1983); herpes simplex virus (Glorioso *et al.*, 1992; Weber *et al.*, 1987; Shih *et al.*, 1984; Smiley *et al.*, 1987; Palella *et al.*, 1988; Ho and Mocarski, 1988; Dobson *et al.*, 1989; Chiocca *et al.*, 1990); fowlpox virus (Boyle and Coupar, 1988; Tripathy and Schnitzlein, 1991; Taylor *et al.*, 1988); varicella-zoster virus (Lowe *et al.*, 1987); and pseudorabies virus (Kit, 1985; Leist *et al.*, 1989; Quint *et al.*, 1987; Katz *et al.*, 1990; Tatarov, 1968; Thomsen *et al.*, 1987). Development of recombinant ILTV vaccines, including polyvalent vaccines, has been hampered by the lack of an established system for this work. Although identified in 1931, the sequences of only five genes, equivalent to those coding for thymidine kinase, gB, P40, and ICP8 of herpes simplex virus as well as for the 32-kDa immunogenic protein, have been described for ILTV (Griffin and Boursnell, 1990; Griffin, 1990, 1991; Keeler *et al.*, 1991; Kongsuwan *et al.*, 1991, 1993; Sheppard and York, 1990). Recently we have described the cultivation of ILTV on an established line of avian hepatoma cells (Scholz *et al.*, 1993a,b; Guo *et al.*, 1993). The finding of this cell line has facilitated the development of an *in vitro* system for the construction of recombinant ILTV.

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This paper reports the nucleotide sequence of a 3962-bp ILTV DNA fragment and describes a system for the successful construction of a recombinant ILTV. A reporter bacterial β -galactosidase (β -gal) gene under the control of the human cytomegalovirus (CMV) immediate-early promoter was inserted into the *XhoI/BglII* site of the ILTV genome. Blue plaques were produced from recombinant ILTV harboring the β -gal gene. The nucleotide sequence of the insertion region is presented as well. The potential uses of this system are discussed.

MATERIALS AND METHODS

Cells

A chicken hepatoma cell line (Scholz *et al.*, 1993a) was used for all experiments described in this paper unless otherwise indicated. This cell line was established from a hepatoma induced in a chicken by diethylnitrosamine and provided by Solvay Animal Health, Inc. (Minnesota). Monolayer cultures were grown in DME-F12 medium (Sigma) supplemented with 10% fetal calf serum (FCS) and maintained in DME-F12 medium plus 3% FCS. Primary embryonic chicken hepatocytes were prepared as previously described (Scholz *et al.*, 1993a).

Virus

All experiments were performed with the USDA challenge strain NVSL that was generously provided by Dr. Robert Nordgren (Solvay Animal Health, Inc.). Adaptation of virus to the hepatoma cell line was obtained by 30 serial passages followed by three rounds of plaque purification and 3 subsequent passages in these cells. Virus titrations were carried out in hepatoma cells and measured as PFU (plaque-forming unit) per milliliter titrated on hepatoma cells as described previously (Scholz *et al.*, 1993a).

Isolation of ILTV DNA

ILTV DNA was isolated as previously described (Scholz *et al.*, 1993b) from (1) the supernatant of ILTV-infected cells, (2) the cytoplasmic fraction of ILTV-infected cells, and (3) whole infected cells. DNA derived from cell culture supernatant was obtained from primary hepatocytes and was used for *EcoRI* digestion and cloning. For transfection experiments, ILTV DNA from the whole infected cells or the cytoplasmic fraction of infected cells was used.

To isolate the DNA from the cell culture supernatant, ILTV particles were pelleted by ultracentrifugation at 20,000 *g* for 60 min at 4° and then digested with proteinase K at 0.5 mg/ml (final) in the presence of 1% SDS (final) at 37° for 30 min. After phenol and chloroform extraction the ILTV DNA was precipitated in 70% ethanol

and resuspended in TE buffer (1 mM EDTA, 10 mM Tris-HCl, pH 8.0).

Infected hepatoma cells were treated with NP-40 (Sigma) at a final concentration of 1% in sterile H₂O to isolate ILTV DNA from the cytoplasmic fraction of infected cells. Unbroken nuclei were removed by centrifugation at 1000 *g* for 10 min at 4°. DNA was isolated from the supernatant as described above.

To isolate ILTV DNA from whole infected cells, the NaI gradient method of Walboomers and Schegget (1976) was modified. Briefly, confluent monolayers of hepatoma cells were infected with ILTV at an m.o.i. of 1. Five hours postinfection (p.i.), half the cells were radiolabeled with [³H]thymidine. When the cells showed maximal cytopathic effect, the monolayers were washed twice with ice-cold PBS and detached with a cell scraper. Labeled and unlabeled cells were collected separately by centrifugation at 700 *g* for 3 min. The pellets were resuspended in TE buffer and 1% SDS (final). Proteinase K stock solution was added to the lysate to a final concentration of 2 mg/ml and incubated at 54° for 3 hr. After 3 hr, pronase was added to a final concentration of 2 mg/ml and incubation was continued for another 12 hr at 37°. Saturated NaI solution was added to the samples to a final ratio of 6 to 4 (final density of 1.525 g/ml). After mixing, the samples were centrifuged at 200,000 *g* for 44 hr at 15°. Both gradients were fractionated from the bottom of the tube. The fractions from the ³H-labeled sample were counted in a scintillation counter. Peaks of radioactivity indicated the location of ILTV DNA. Corresponding fractions of the unlabeled gradient were dialyzed against TE buffer at 4° and examined for the presence of DNA by CHEF (Bio-Rad) pulse-field electrophoresis.

DNA sequencing of the 4-kbp ILTV-*EcoRI* fragment

DNA was sequenced with the Genesis 2000 automatic DNA analysis system (DuPont) and manually by the Sanger dideoxy chain-termination method with the Sequenase-2.0 kit (USB). Sequencing was done in parallel by both methods using identical primers walking through the entire DNA fragment from both directions. The results from both methods were compared. If the same sequence was identified by both methods, the sequence was recorded. If the sequence was not identical in both methods, the analyses were repeated until there was agreement between the two methods. With the DuPont Genesis 2000 automatic DNA analysis system, the results were automatically recorded in GCG (see below) software package format on a VAX cluster (Devereaux *et al.*, 1984). The Genesis 2000 system uses a modification of the Sanger DNA sequencing technique. Each of the four dideoxynucleotides has a unique fluorescent dye attached that, when excited by a laser beam, emits light of a specific and easily-detected wavelength. Two photo-

multiplier tube (PMT) detectors produce a signal corresponding to the transmitted light intensities. The unique emission wavelength of a given terminating nucleotide can then be determined by the ratio of the PMT signals.

The nucleotide sequence was further confirmed with a number of random subclones. Fragments produced by ultrasonic shearing in an XL sonicator (Heat Systems), at setting 3.5 for 2.5 min at 4° using a microtip, were filled at the ends with T4 DNA polymerase. *EcoRI* linkers were added and, after digestion with *EcoRI*, the fragments were ligated into plasmid pUC13. M13 forward and reverse primers were used for sequencing. Sequences obtained from these subclones were used to verify the final sequencing results.

Construction of plasmid p59gal

The basic method of our previous work in DNA cloning and plasmid construction was followed (Guo and Moss, 1990; Guo *et al.*, 1986, 1987a, 1991). ILTV DNA from virus isolated from the supernatant of infected primary chicken liver cells was digested with *EcoRI*. A 4-kbp fragment was designated No. 59 and was cloned into pUC13 to produce plasmid p59 (Fig. 4). The origin of the fragment was tested by PCR using two sequencing primers, TTC-CCGCCGGACCGCTG and ACTGCCACCAGTTACA, derived from the sequence of the 4-kbp DNA fragment.

To construct plasmid p59gal, plasmid p59 was double digested with *XhoI* at position 660 and *BglII* at position 573, counting the first "A" of the 5' *EcoRI* site of the insert as position 1, eliminating a fragment of 87 bp. A *SaII/BamHI* fragment of plasmid pON249 (kindly provided by Dr. R. Silva, Avian Diseases and Oncology Laboratory, USDA, East Lansing, MI) (Geballe *et al.*, 1986; Silva and Finkelstein, 1990; Scholz *et al.*, 1993b) carrying the bacterial β -gal gene driven by CMV promoter, and carrying an SV40 poly(A) addition signal, was inserted (Fig. 4) at the *XhoI/BglII* site of p59. Ligation of the compatible ends of *SaII* and *XhoI* as well as the compatible ends of *BamHI* and *BglII* resulted in plasmid p59gal.

Plasmid pON249 is a derivative of pON239 which was constructed by Spaete and Mocarski (1985) with the insertion of a 2.1-kbp *PstI* DNA fragment into plasmid pON1 upstream of the *lacZ* coding sequence. This *PstI* fragment contains 1.1 kbp of DNA sequence upstream to the transcription start site of the CMV α -gene. Plasmid pON1 (Spaete and Mocarski, 1985) was derived from pCH110 (Hall *et al.*, 1983) (Pharmacia, NJ).

Transfection of hepatoma cells with plasmid DNA for transient β -gal expression

The procedure developed by Graham and Van der Eb (1973) and modified by Scholz *et al.* (1993b) was used for transfection. Two micrograms plasmid DNA and 2 μ g herring sperm DNA as carrier were diluted in HEPES-

buffered saline (0.14 M NaCl, 5 mM KCl, 1 mM Na₂HPO₄, 0.1% dextrose, 20 mM HEPES, adjusted to pH 7.05 and sterilized by filtration) to a total volume of 240 μ l. After adding 12 μ l of 2.5 M CaCl₂, the solution was carefully mixed and incubated at room temperature for 40 min. Monolayers of hepatoma cells grown in 9.4-cm² wells at a confluency of 80–90% were prepared for transfection. After removal of the medium, the DNA/CaCl₂ mixture was added to the cell monolayer. After incubation at room temperature for 30 min, DME-F12 medium plus 3% FCS was added to make the final CaCl₂ concentration of 14 mM. The cells were then incubated at 37° for 5 hr, at which time the transfection complex was removed and fresh DME-F12 with 3% FCS was added. The staining for transient β -gal expression was carried out 30 hr after transfection with the procedure described by Sanes *et al.* (1986).

Cotransfection of ILTV and donor plasmid DNA as well as ILTV superinfection for the generation of recombinant virus

One microgram of NaI-purified ILTV DNA derived from infected hepatoma cells was cotransfected with 2 μ g plasmid p59gal DNA plus 7 μ g herring sperm DNA into 5 \times 10⁶ hepatoma cells. Fifteen hours post-transfection, infectious ILTV was added at an m.o.i. of 1. The infected cells were harvested with the supernatant 30 hr postinfection, frozen and thawed three times, and then sonicated for 30 sec at setting 4 to 5 of an XL sonicator (Heat System), using the cup-horn. The suspension was tested for the presence of recombinant virus as described below.

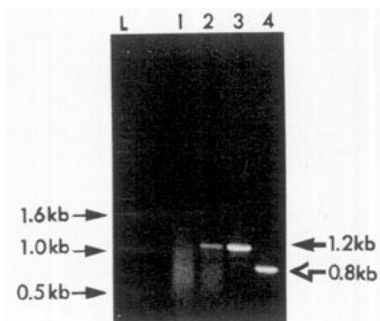


Fig. 1. Agarose gel identifying the origin of the 4-kbp *EcoRI* DNA fragment by PCR with two sequencing primers used for the sequencing of this DNA fragment. The PCR fragment is predicted to be 1.1 kbp calculated from the distance of the two primers. Lane L is a 1-kb DNA ladder as size marker. Both ILTV DNA (lane 2) and p59 DNA (lane 3) rendered positive results as indicated by the appearance of the 1.2-kbp fragment. PCR with chicken DNA (lane 1) did not produce a positive result, indicating that the fragment-derived primers correspond uniquely to ILTV DNA. Lane 4 is a positive control for PCR reaction that was expected to produce a 0.8-kbp DNA fragment.

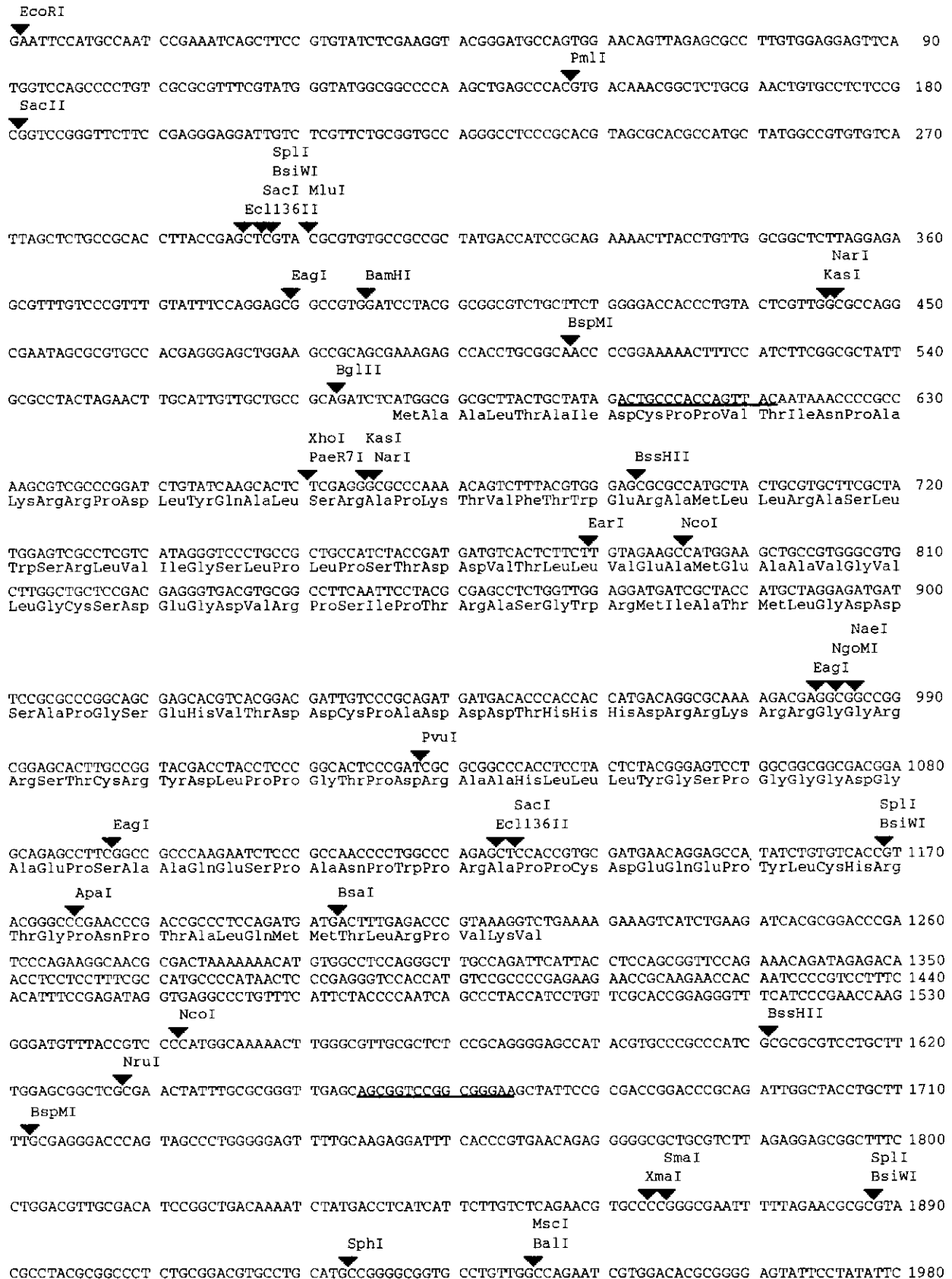
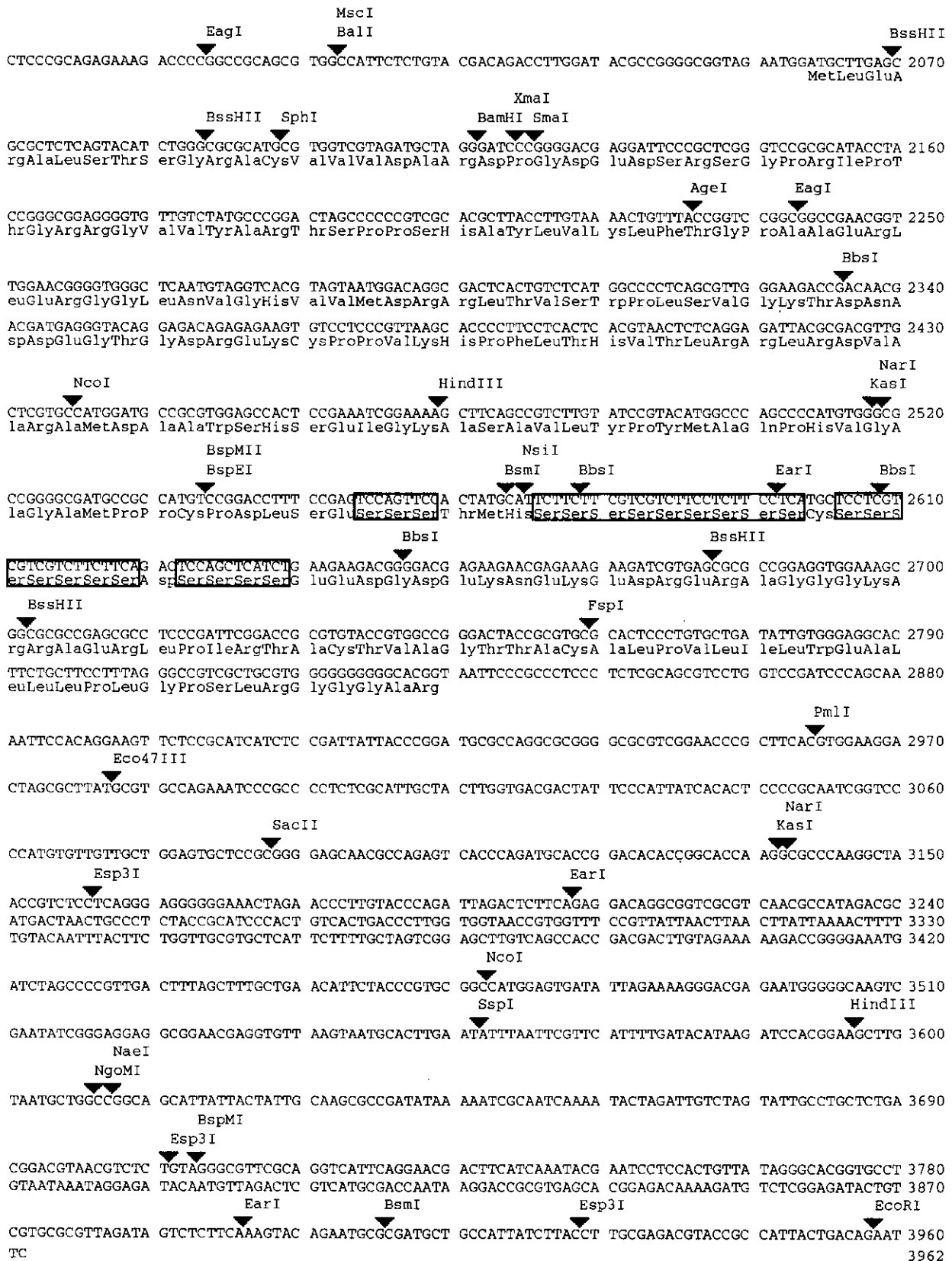


Fig. 2. The nucleotide sequence of the ILTV DNA fragment with 3962 bp. Restriction sites of all nonambiguous six-cutters are indicated in the sequence. The computer-predicted amino acid sequence of the two larger ORF (see Fig. 3) is included. The locations of the two primers used for PCR in Fig. 1 are underlined. The serine-rich regions are enclosed in rectangles.



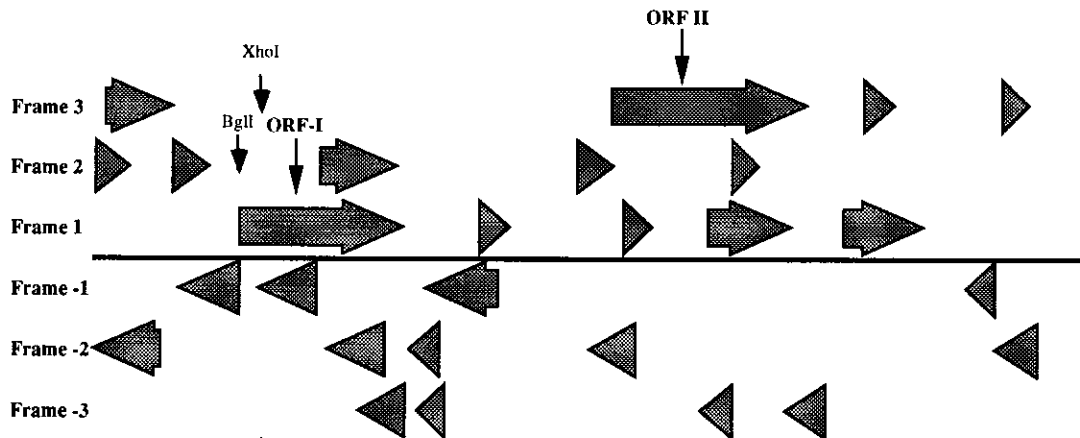


FIG. 3. Predicted ORF map of the 4-kbp *EcoRI* DNA fragment predicted via the GeneWorks analysis package (IntelliGenetics, Inc.) (Glynias, 1991). All six reading frames are presented.

Screening for recombinant virus expressing the β -gal gene

The procedures for plaque staining of recombinant viruses expressing the β -gal gene have been described (Klebe and Harris, 1984; Panicali *et al.*, 1986; Chakrabarti *et al.*, 1985). Hepatoma cells were infected with the above suspension and incubated at 37° and 4% CO₂ for 15 hr. The inoculum was removed and the cells were overlaid with 1% low-melting-point agarose, containing 0.3 mg/ml X-gal in half DME-F12, half Eagle's basal medium (Gibco) containing 3% FCS. Viruses carrying the β -gal gene formed blue plaques within 15–40 hr after staining.

Computer analysis

Both GCG (Genetics Computer Group, WI) and GeneWorks (IntelliGenetics, CA) programs were used for DNA and protein analysis (Devereaux *et al.*, 1984; Glynias, 1991; Guo *et al.*, 1987b).

Polymerase chain reaction (PCR)

Primer β gal1 (5'-TGATGCGGTGCTGATTACG) was derived from the β -gal coding sequence. Primer EK1 (5'-ACGTAGCGCAGCCATGC) was derived from ILTV DNA sequence. Primer PRFA (5'-GGGTTTTCCAGTCACGACGTTGT) was derived from the plasmid vector pUC13.

RESULTS

Construction of plasmid p59 carrying a 4-kbp ILTV insert

ILTV DNA from virus isolated from cell culture supernatant of infected primary chicken hepatocytes was digested with *EcoRI* and cloned into pUC13. Clone No. 59 had an insert of 4 kbp. The origin of the fragment was subsequently confirmed by PCR with ILTV or chicken DNA as template, using primers derived from the se-

quence of the 4-kbp DNA fragment. ILTV DNA and p59 showed a positive signal while total DNA derived from chicken cells gave a negative result (Fig. 1), indicating that the DNA fragment originated from the ILTV genome. The plasmid carrying the 4-kbp insert was called p59.

Sequencing of the 4-kbp ILTV DNA fragment

The complete nucleotide sequence of the 3962-bp ILTV DNA fragment and the sites of some common restriction enzymes are shown in Fig. 2. Restriction map analysis revealed that this sequence was the *EcoRI*-R restriction fragment located in the unique short region (Us) of the ILTV genome (Johnson *et al.*, 1991). The *EcoRI*-R fragment has been determined to be 3.9 kbp in length and to contain two *SmaI* sites and no *KpnI* site (Johnson *et al.*, 1991), which is in perfect agreement with our DNA sequencing data. The sequence has been deposited in the GenBank under Accession No. UO8845. Open reading frames (ORF) (Fig. 3) were predicted by the GeneWorks sequence analysis package (IntelliGenetics). Two potential ORF with 216 (ORF-I) and 259 (ORF-II) amino acids, respectively, were predicted (Figs. 2 and 3). DNA sequence and amino acid searches through GenBank with the sequence analysis software package of GCG (Devereaux *et al.*, 1984) did not reveal any significant homologies of the 4-kbp fragment or any potential ORF with other herpesvirus genes or gene products within a 5% homology range for either DNA or amino acid. Furthermore, DD-matrix analysis (Guo, 1990) of these two ORF with more promising GenBank candidates also revealed very little homology (data not shown). A salient feature of ORF-II is that it contained a serine-rich (80% serine) region from residue 164 to residue 193. However, amino acid sequence alignment of ORF-II with several serine-rich proteins of other herpesviruses, such as vmw118(ICPO) and vmw175 of HSV-1 revealed that the homologies were restricted to the serine-rich regions

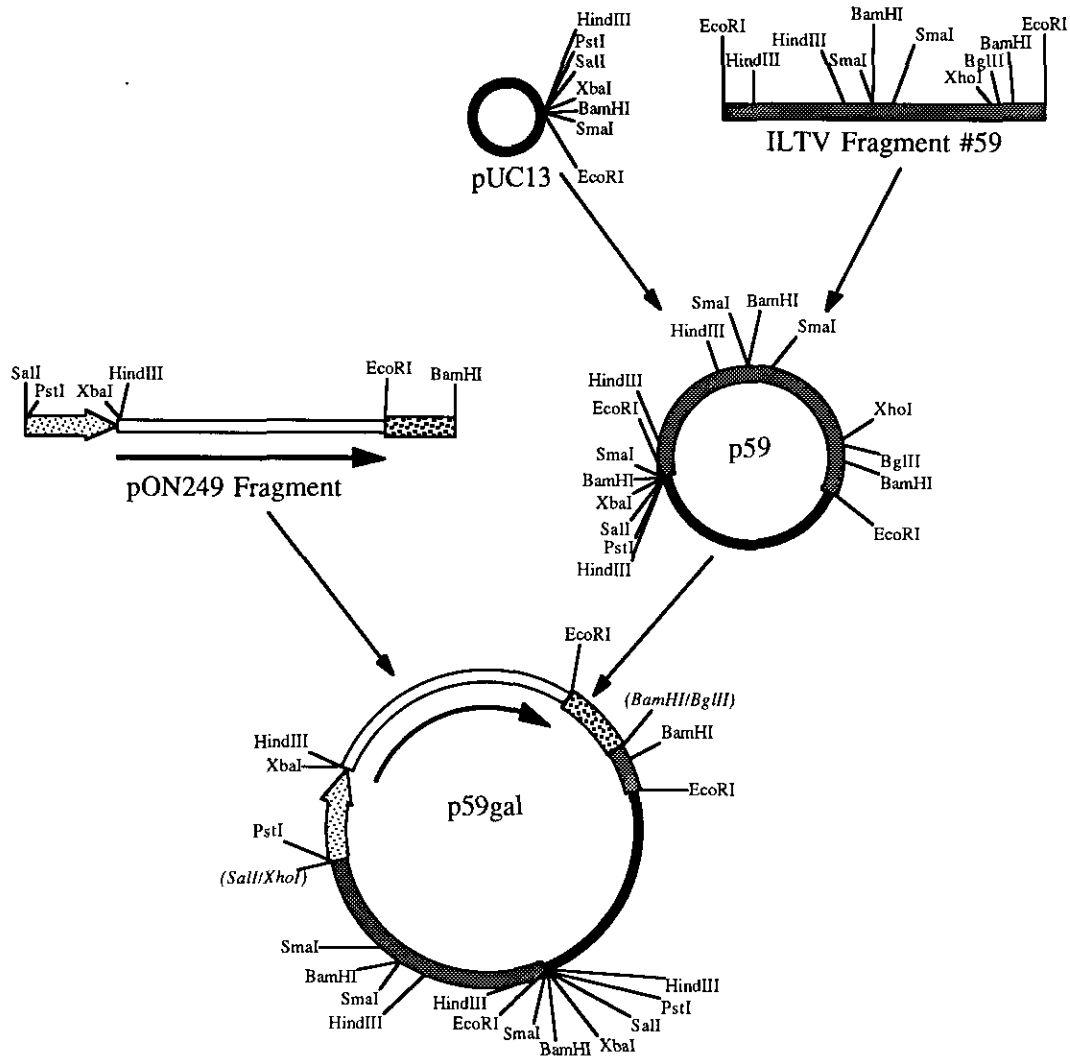


Fig. 4. Construction of plasmid p59 and donor plasmid p59gal. A 4-kbp ILTV *EcoRI* DNA fragment was cloned into plasmid pUC13 to generate plasmid p59. The *SmaI/BamHI* fragment derived from plasmid pON249 was ligated via compatible ends into the *XhoI/BglIII* site of plasmid p59. The insert contained the β -gal gene driven by both gpt and CMV promoters and followed by an SV40 poly(A) addition signal.

only (data not shown). The function of these two predicted polypeptides and the question of whether these are true ORF could not be confirmed.

Transient expression of the β -gal gene in *E. coli* and avian hepatoma cells

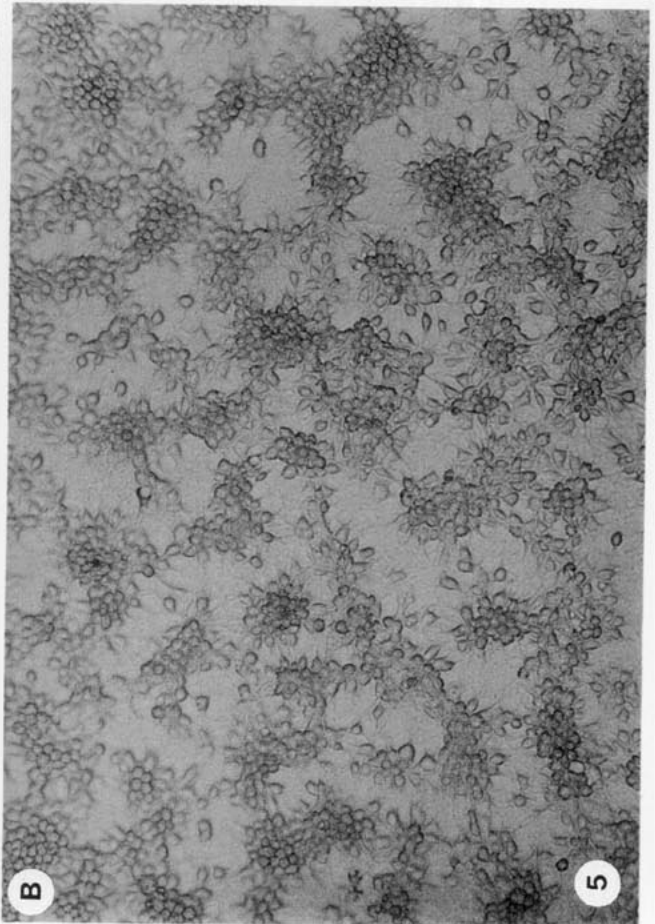
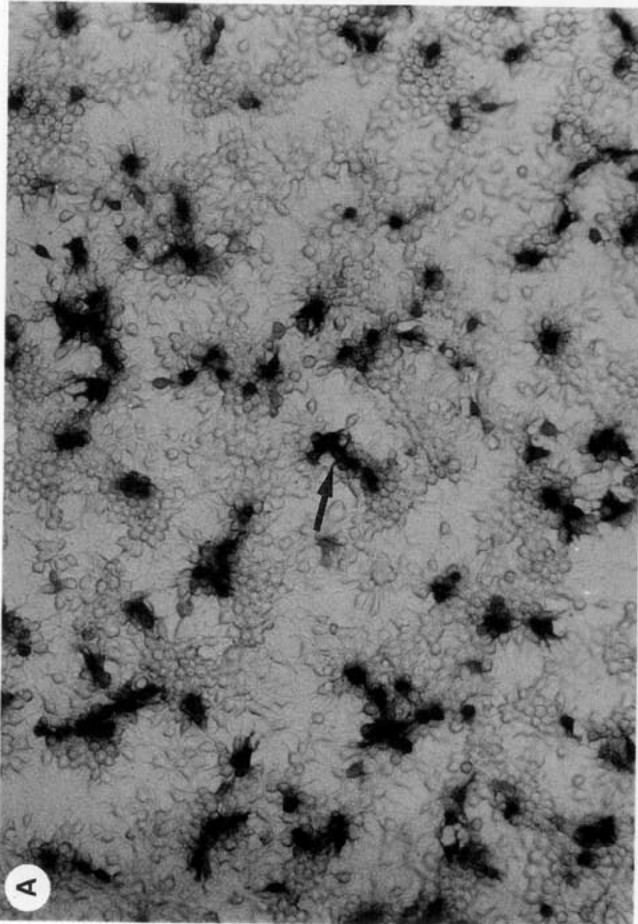
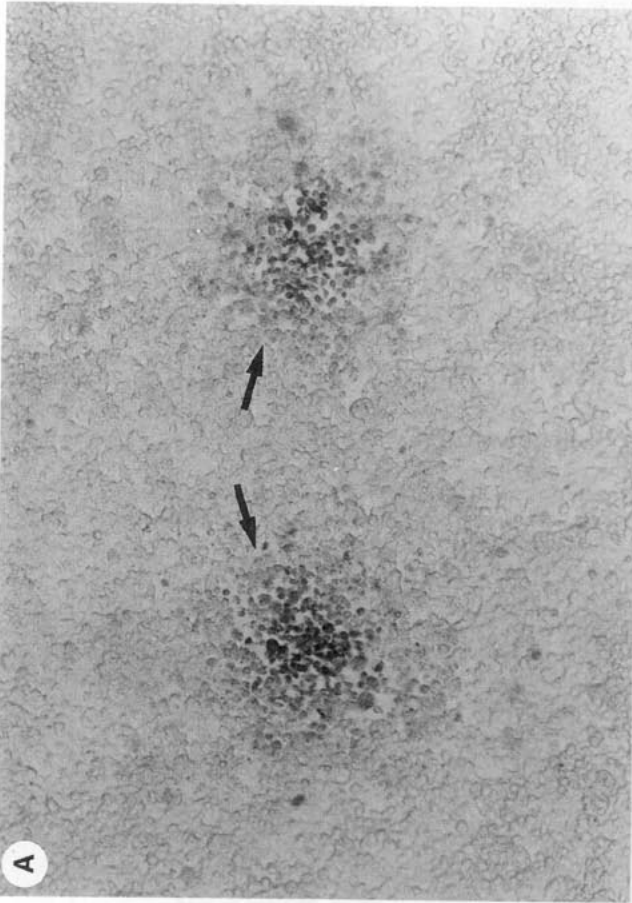
A *SmaI/BamHI* fragment from plasmid pON249 carrying the bacterial β -gal gene was inserted into plasmid p59 at the *XhoI/BglIII* site, eliminating a fragment of 87 bp and generating plasmid p59gal (Fig. 4).

The *SmaI/BamHI* fragment (Geballe *et al.*, 1986) was originally derived from plasmid pCH110 (Hall *et al.*, 1983) (Pharmacia), which contains a functional *lacZ* gene driven by both the *E. coli* gpt and SV40 early promoters (Hall *et al.*, 1983). In plasmid pON249 the SV40 promoter preceding the β -gal gene is replaced by the mammalian CMV promoter (Geballe *et al.*, 1986; Silva and Finkelstein, 1990;

Scholz *et al.*, 1993b). Therefore, the β -gal gene in plasmid p59gal was preceded by both eukaryotic and prokaryotic promoters. Tested for its blue color reaction on X-gal plates, the β -gal gene was expressed in both *E. coli* harboring p59gal (data not shown) and avian hepatoma monolayers transfected with CsCl-purified p59gal DNA using the calcium phosphate procedure (Fig. 5). The blue spots in Fig. 5A indicate that the β -gal gene under the control of CMV promoter was transiently expressed in the avian hepatoma cells. Control cells that had not received the plasmid p59gal and had only received carrier DNA showed no blue staining (Fig. 5B). The β -gal was easily assayed in both prokaryotic and eukaryotic cells.

Construction of recombinant ILTV expressing the β -gal gene

The mechanism for the construction of recombinant ILTV was homologous recombination (Post and Roizman,



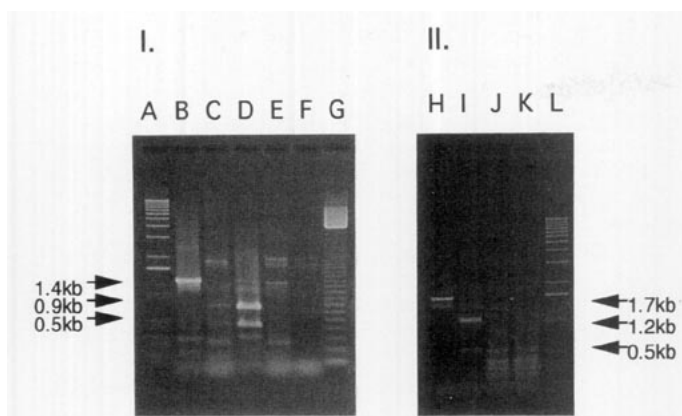


Fig. 7. Agarose gel electrophoresis showing the DNA fragments from PCR with the primer pairs of β gal1/EK1 (I) and β gal1/PRFA (II) and with the following DNAs as templates: B, plasmid p59gal as positive control; C, recombinant ILTV DNA with subsequent *Eco*RI digestion of the resulting PCR fragments; D, plasmid p59gal with subsequent *Eco*RI digestion of the resulting PCR fragments; E, recombinant ILTV DNA; F, wild-type ILTV DNA as negative control; H, plasmid p59gal as positive control; I, plasmid p59gal with subsequent *Eco*RI digestion of the resulting PCR fragments; J, recombinant ILTV DNA; K, recombinant ILTV DNA with subsequent *Eco*RI digestion of the resulting PCR fragments; A and L, 1-kb DNA ladders; and G, 123-bp ladder.

1981). The ILTV DNA sequences flanking the β -gal gene and CMV promoter served as arms in recombination. The avian hepatoma cells were cotransfected with p59gal and purified ILTV DNA and infected with infectious ILTV at an m.o.i. of 1 at several hours post-transfection. The resulting virus was harvested, freeze-thawed three times, sonicated, and replated. After the cell culture monolayer was overlaid with agarose containing X-gal, blue plaques of recombinant viruses appeared (Fig. 6A), indicating the successful insertion of the β -gal gene into the ILTV genome and expression of β -gal gene in ILTV-infected cells. The blue plaques were picked with a Pasteur pipette and amplified on avian hepatoma cells. Blue plaques were identified after five passages. Insertion of β -gal into the *Xho*I/*Bgl*II site of the ILTV genome and the removal of 87 bp at the 5' end of the coding sequence for the predicted 216-residue polypeptide eliminated this potential ORF.

Integration of the β -gal gene into the ILTV genome was verified by PCR. Primers β gal1, EK1, and PRFA were derived from the β -gal coding sequence, ILTV DNA sequence, and the plasmid vector pUC, respectively. The DNA sequence of the primer PRFA was not expected to be present in the recombinant ILTV genome. With the primer pair β gal1/EK1, a DNA fragment with an expected

size of 1.4 kbp was revealed from the recombinant ILTV DNA template (Fig. 7, lane E) as well as from the positive control plasmid (Fig. 7, lane B). The 1.4-kbp fragment was not found when wild-type ILTV DNA was used (Fig. 7, lane F). *Eco*RI digestion of the PCR fragments from both the recombinant ILTV DNA and positive control plasmid template produced DNA fragments of expected sizes, which were 0.9 and 0.5 kbp in each digestion (Fig. 7, lanes C and D). With the primer pair β gal1/PRFA, a DNA fragment with an expected size of 1.7 kbp was obtained (Fig. 7, lane H) and verified by *Eco*RI digestion (Fig. 7, lane I) from the positive control plasmid. This fragment, however, was not found when recombinant ILTV DNA was used as PCR template (Fig. 7, lanes J and K), indicating that the DNA fragment resulting from the β gal1/EK1 primer pair did not originate from the contaminating plasmid. The conclusion of an inheritable insertion of the foreign gene into ILTV genome at the expected location was further documented extensively in another report (E. Scholz *et al.*, submitted for publication).

DISCUSSION

This is the first report of a successful attempt to create a recombinant ILTV expressing a foreign gene. The bacterial β -gal gene was expressed in recombinant ILTV-infected avian hepatoma cells under the control of the CMV promoter (Scholz *et al.*, 1993b). This demonstrates that ILTV has the capacity to incorporate additional DNA into its genome. Neither the presence of this extra DNA nor its expression appeared to interfere with the ability of the virus to produce plaques in cell culture. Therefore, ILTV could possibly serve as an expression vector for other genes that code for highly immunogenic proteins of avian pathogens and might be useful as a vaccine vector. The stability of the recombinant ILTV was also tested by five generations of plaque purification. The production of blue plaques after five passages indicated that the foreign insert was inheritable. White plaques were also found in each generation. The white plaques may have resulted from the partial loss or mutation of the β -gal coding sequence due to its large size (more than 3 kbp). Consequently, because of its smaller size and selection pressure, the neomycin resistance gene is currently being tested in our laboratory for its usefulness as a selection marker. The plaque morphology of recombinant ILTV expressing the β -gal gene was different from that of the wild type. Fewer syncytial cells were found around plaques from recombinant viruses. The possible

Fig. 5. Transient expression of the bacterial β -gal gene after transfection of hepatoma cells with (A) and without (B) the plasmid p59gal. Expression of the β -gal gene was visualized (arrow) by staining with X-gal.

Fig. 6. Plaque assay for the expression of the β -gal gene in avian hepatoma cells. After X-gal staining, blue plaques were visible (arrows) from monolayers infected with recombinant ILTV harboring and expressing the β -gal gene (A). Wild-type ILTV produced uncolored plaques (arrows) in the presence of X-gal (B).

involvement of β -galactosidase, of which β -galactoside is a substrate, in the change of plaque morphology is under investigation.

Respiratory tract infections, such as Newcastle disease, avian infectious laryngotracheitis, avian influenza, infectious bronchitis, infectious coryza, and mycoplasmosis, are major problems in the poultry industry. Mucosal surface immunity plays a crucial role in the protection and recovery of birds from these respiratory tract infections. Many current vaccines against these diseases do not satisfactorily stimulate mucosal immunity. One objective is to develop a polyvalent vaccine that is effective in stimulating high levels of mucosal immunity and is easy to administer to large flocks of birds by aerosol spray. Several attenuated viruses, including vaccinia virus, fowlpox virus, adenovirus, herpes simplex virus, EB virus, varicella-zoster virus, and pseudorabies virus, have been shown to be useful vectors for recombinant vaccines and for the expression of recombinant proteins, but none of these vectors are effective in stimulating mucosal immunity. Vaccination against respiratory disease requires the stimulation and production of IgA-like secretory antibody. To establish active immunity at mucosal surfaces, the ideal vector should fulfill the following requirements: (1) contain an envelope to facilitate the expression and translocation of glycoproteins and membrane proteins essential for stimulating cellular and humoral immunity; (2) replicate significantly in the respiratory tract; (3) be of low pathogenicity; (4) have sufficient genome capacity to accommodate foreign DNA inserts; (5) be eukaryotic; and (6) be simple in construction. An attenuated ILTV could possess these properties and be useful as a vector for the construction of polyvalent recombinant vaccines against multiple avian respiratory tract infections.

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