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High-level expression of a foreign gene from the most 3′-proximal locus of a recombinant Newcastle disease virus

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A previous report showed that insertion of a foreign gene encoding chloramphenicol acetyltransferase (CAT) between the HN and L genes of the full-length cDNA of a virulent Newcastle disease virus (NDV) yielded virus with growth retardation and attenuation. The NDV vector used in that study was pathogenic to chickens; it is therefore not suitable for use as a vaccine vector. In the present study, an avirulent NDV vector was generated and its potential to express CAT protein was evaluated. The CAT gene was under the control of NDV transcriptional start and stop signals and was inserted immediately before the open reading frame of the viral 3′-proximal nucleocapsid protein gene. A recombinant NDV expressing CAT activity at a high level was recovered. The replication and pathogenesis of the CAT-expressing recombinant NDV were not modified significantly. These results indicate the potential utility of an avirulent NDV as a vaccine vector.

Introduction

Newcastle disease is a highly contagious virus disease affecting all species of birds. The disease can vary from asymptomatic infection to highly fatal disease, depending on the virus strain and the host species. Based on the severity of the disease produced in chickens, Newcastle disease virus (NDV) strains are grouped into three main pathotypes: lentogenic, mesogenic and velogenic. Lentogenic strains do not usually cause disease in adult chickens and are widely used as live vaccines. Viruses of intermediate virulence are termed mesogenic, while viruses that cause high mortality are termed velogenic (Alexander, 1997). The disease has a worldwide distribution and remains a major threat to the poultry industries of all countries.

NDV is a member of the genus *Rubulavirus* in the family *Paramyxoviridae* (Rima *et al.*, 1995). The genome of NDV is a non-segmented, single-stranded, negative-sense RNA of 15 186 nucleotides (Krishnamurthy & Samal, 1998; Phillips *et al.*, 1998; de Leeuw & Peeters, 1999). The genomic RNA contains six genes that encode in this order the nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), haemagglutinin–neuraminidase (HN) and large polymerase protein (L). Two additional proteins, V and W, are produced by RNA editing during P gene transcription (Steward *et al.*, 1993). NDV follows the same general model for transcription and replication as other non-segmented, negative-strand RNA viruses (Peeples, 1988; Lamb & Kolakofsky, 1996; Pringle, 1997). Like other non-segmented, negative-strand RNA viruses, there is a polar attenuation of transcription such that each downstream gene is transcribed less than its upstream neighbour (Peeples, 1988; Nagai, 1999).

The development of reverse-genetic techniques to recover negative-sense viruses from cloned cDNA (Conzelmann, 1996) provides a means not only to investigate the function of virus proteins and genetic elements (Palese *et al.*, 1996; Nagai, 1999) but also to express additional proteins by the insertion of new genes into the viral genome (Bukreyev *et al.*, 1996; Mebatsion *et al.*, 1996; Schnell *et al.*, 1996; Hasan *et al.*, 1997; He *et al.*, 1997). This provides a new method to generate improved vaccines and vaccine vectors. For NDV, reverse-genetic technology is currently available for avirulent strain LaSota (Römer-Oberdörfer *et al.*, 1999; Peeters *et al.*, 1999) and virulent strain Beaudette C (Krishnamurthy *et al.*, 2000).

Previously, we reported that virulent NDV strain Beaudette C could be used as an expression vector (Krishnamurthy *et al.*, 2000). This was achieved by introducing an extra transcription unit between the HN and L genes. Our previous results showed that expression of the foreign gene resulted in growth retardation and attenuation of the recombinant virus. Although these results indicated that recombinant NDV could be used as a vaccine vector, they raised concerns about the level of expression of the foreign gene and growth retardation of the avirulent vaccine strain after insertion of the foreign gene. In this report, we have addressed these concerns in studies of an

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avirulent NDV recombinant expressing a foreign gene. Here, we have recovered an avirulent NDV strain LaSota from cDNA and have inserted a foreign gene into a more upstream position, close to the 3′ end of the NDV genome. The recovered recombinant NDV allowed robust expression of the foreign gene due to polar gradient transcription. Moreover, the replication of the recombinant NDV expressing the foreign gene in cell culture and *in vivo* was not retarded. These results suggest that avirulent NDV recombinants expressing heterologous proteins could be used as multivalent vaccines.

Methods

■ **Assembly of the full-length clone of NDV lentogenic strain LaSota and construction of support plasmids.** NDV strain LaSota was grown in 10-day-old embryonated, specific-pathogen-free (SPF) eggs. The virus was purified from allantoic fluid as described previously (Kingsbury, 1966). Viral RNA was extracted from the purified virus by using TRIzol according to the manufacturer's protocol (Life Technologies). The extracted RNA was subjected to RT–PCR with virus-specific primer pairs (Table 1) to generate seven overlapping PCR fragments of the entire viral genome with high-fidelity *Pfx* DNA polymerase (Life Technologies). A low-copy-number plasmid, pBR322, was modified as pBR322/dr to contain a 72 nt linker between the *Eco*RI and *Pst*I sites for subsequent assembly of full-length NDV strain LaSota. The LaSota strain cDNA was placed in the antigenomic orientation between the T7 promoter and a self-cleaving hepatitis delta virus (HDV) antigenome ribozyme, followed by a T7 RNA polymerase terminator (Fig. 1). Two genetic markers were introduced into the full-length NDV cDNA for the purpose of identifying the recovered virus. An *Mlu*I site was created in the F–HN intergenic region and a *Sna*BI site was created in the HN–L intergenic region. For the construction of NP, P and L expression plasmids, the open reading frame (ORF) of each gene was amplified from the above full-length clone by PCR. The NP gene was cloned in the plasmid pGEM-7Z (Promega) between *Eco*RI and *Bam*HI sites. The P and L genes were cloned in an expression plasmid that has an encephalomyocarditis virus internal ribosome entry site (IRES) downstream of the T7 RNA polymerase promoter, and they make use of the translation start codon contained in the *Nco*I site of the IRES. The assembled full-length cDNA clone and the support plasmids encoding LaSota NP, P and L proteins were sequenced in their entirety. The resulting full-length clone and support plasmids were designated pLaSota, pNP, pP and pL, respectively.

■ **Construction of a full-length plasmid containing the chloramphenicol acetyltransferase (CAT) gene.** For the convenience of inserting CAT into the most 3′-proximal locus, an *Asc*I–*Sac*II fragment of the full-length cDNA clone was subcloned into plasmid pGEM-7Z between the *Xba*I and *Hin*dIII sites by using a specific primer pair with *Xba*I and *Hin*dIII site overhangs. An 18 nt insert with a unique *Pme*I site was then introduced just before the NP ORF by the method described previously (Byrappa *et al.*, 1995). To insert the CAT gene into the *Pme*I site, the CAT gene ORF was amplified by primers (5′ gctagtttaaac-ATGGAGAAAAAAATCACTGGATATACC 3′, positive sense, and 5′ gctagtttaaacttctacccgtgtttttctaatctgcagTTACGCCCCGCCCTGCC-ACTCAT CGC 3′, negative sense; *Pme*I site and NDV gene start and gene end signal in lower case, CAT-specific sequence in capitals), digested with *Pme*I and placed into the NP non-coding region in pGEM-7Z (Fig. 2). Clones with the CAT gene in the correct orientation were chosen for sequencing. The *Asc*I–*Sac*II fragment containing the CAT gene was used to replace the corresponding fragment in pLaSota. Thus, an additional transcriptional unit, the CAT ORF flanked by NDV gene start and gene end signals, was inserted into pLaSota. The total number of nucleotides was adjusted by inserting nucleotides after the CAT gene stop codon to maintain the 'rule of six'. The resulting clone was designated pLaSota/CAT.

■ **Transfection and recovery of recombinant NDV.** Transfection was carried out as described previously (Krishnamurthy *et al.*, 2000). Briefly, HEp-2 cells (6-well plates) were infected at 1 p.f.u. per cell with modified vaccinia virus (MVA/T7) expressing T7 RNA polymerase. A mixture of three plasmids containing NDV NP, P and L gene ORFs under the control of the T7 promoter (2·5, 1·5 and 0·5 μg per well, respectively) and a fourth plasmid encoding either the NDV or NDV plus CAT antigenome (5 μg) was transfected with LipofectAMINE Plus (Life Technologies). Four h after transfection, cells were washed and the

Table 1. Oligonucleotide primers used for RT–PCR and assembly of full-length cDNA

The cDNA fragments correspond to the fragments shown in Fig. 1. The T7 promoter sequence is in italics and virus sequences are underlined. Restriction sites are in bold. The partial HDV ribozyme sequence (24 nt) overhang is shown in lower case.

cDNA fragment	Primer		Order of cloning
	Sense	Antisense	
I	5′ CTGA**GGCGCGCC***TAATACGACTCACTATAGG* ACCAAACAGAGAATCCGTGAGTTAG 3′	5′ GTTT**CCGCGG**CTGGGTTGACTCCCCT 3′	4
II	5′ GGTG**CCGCGG**AAACAGCCAGG 3′	5′ GAGCT**GCGGCCGC**TGTTATTTG 3′	6
III	5′ AACA**GCGGCCGC**AGCTCTGAT 3′	5′ TACA**ACGCGT**AGTTTTTTCTTAACTC 3′	7
IV	5′ AACT**ACGCGT**TGTAGATGACCAAAG 3′	5′ GCAC**TACGTA**TTTTGCCTTGTATCTC 3′	5
V	5′ CAAAA**TACGTA**ATGGTAAATAATACGGGT AGGACATG 3′	5′ TTCA**GCTTAGC**GAAGATCCGTCCATTAACT 3′	3
VI	5′ TTCA**GCTAAGC**TGACAAAGAAGTTAAGG AACTG 3′	5′ GTCT**AGGCCT**CTTACTCTCAGGTAATAG 3′	1
VII	5′ TCAG**AGGCCT**AGACAATATTGTCT 3′	5′ GATC**CGGACCG**cgaggaggtggagatgccatgccg ACCAAACAAAGATTTGGTGAATGACGAG 3′	2

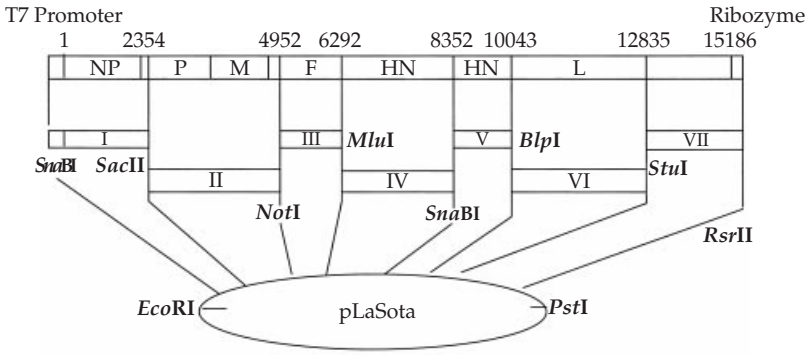

Fig. 1. Assembly of full-length cDNA of NDV strain LaSota. Seven subgenomic cDNA fragments generated by high-fidelity RT–PCR were assembled in pBR322/dr (not to scale). Plasmid pBR322 was modified to include a 72 nt oligonucleotide linker between the *Eco*RI and *Pst*I sites, an 84 nt HDV antigenome ribozyme sequence and a T7 RNA polymerase transcription-termination signal. Transcription of the plasmid pLaSota by the T7 RNA polymerase results in NDV antigenomic RNA with three non-viral G residues at the 5′ terminus.

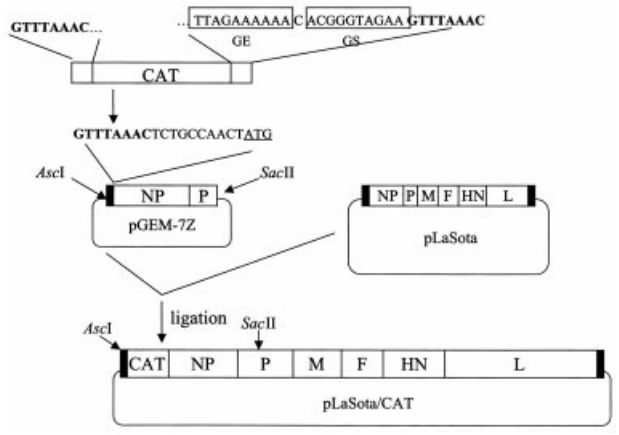

Fig. 2. Construction of pLaSota/CAT. An 18 nt fragment containing a *Pme*I site was inserted into the non-coding region immediately before the NP ORF. The ORF of the CAT gene was amplified by PCR with *Pme*I-tagged primers, digested with *Pme*I and introduced into the newly created *Pme*I site of the NP gene. A set of NDV gene-end (GE) and gene-start (GS) signals connected to the NP–P intergenic sequence was placed at the end of the CAT gene. The resulting plasmid pLaSota/CAT gives rise to an antigenomic RNA of 15900 nt, which is a multiple of six.

medium was replaced with 2 ml fresh medium (DMEM with 0% foetal calf serum and 1 μg/ml acetyltrypsin). Three days post-transfection, the supernatant was harvested, clarified and used to infect fresh HEp-2 cells. Three days later, 100 μl supernatant was taken to inoculate into the allantoic cavity of 10-day-old embryonated SPF eggs. After 96 h, allantoic fluid was harvested and tested for haemagglutinating (HA) activity. The recovered viruses were designated rLaSota and rLaSota/CAT.

■ **RT–PCR and demonstration of genetic marker.** RNA was isolated from recovered virus by using TRIzol reagent. RT–PCR was performed with primers P1 (5′ TCCCCTGGTATTTATTCCTGC, positions 5609–5629) and P1R (5′ GTTGGCCACCCAGTCCCCGA, negative sense, positions 7286–7305) to amplify a fragment including the introduced *Mlu*I site in the intergenic region between the F and HN genes. Similarly, a fragment containing the *Sna*BI site within the HN–L intergenic region was amplified with primers P2 (5′ CGCATACAG-CAGGCTATCTTATC, positions 7513–7535) and P2R (5′ GGGTCA-TATTCTATACATGGC, negative sense, positions 9739–9759). The RT–PCR products were then subjected to restriction enzyme digestion, the first product with *Mlu*I, the second with *Sna*BI. The restriction patterns were analysed by agarose gel electrophoresis. RT–PCR was also performed to demonstrate the location of the CAT gene insert in the recombinant NDV expressing the CAT gene.

■ **CAT assay and analysis of the stability of CAT expression.** Chicken embryo fibroblast DF1 cell pellets were lysed by three freeze–thaw cycles and 1% of the lysed pellet from a 25 cm^2 flask was analysed by TLC for the ability to acetylate [^{14}C]chloramphenicol (Amersham Pharmacia). To study the stability of CAT expression by the recombinant virus, a total of 12 serial passages were performed at a passage interval of 4 days. At each passage, 100 μl of the medium supernatant was used for passing to fresh DF1 cells in a 25 cm^2 flask. Acetyltrypsin (1 μg/ml) was included in the medium of DF1 cells for cleavage of the F protein of rLaSota and rLaSota/CAT.

■ **Northern blot hybridization.** The protocol for Northern blot hybridization was described previously (Krishnamurthy *et al.*, 2000). Briefly, RNA was isolated from cells infected with either rLaSota or rLaSota/CAT at an m.o.i. of 1. Total RNA was extracted with TRIzol reagent and poly(A)$^+$ mRNA was selected by using an mRNA isolation kit (Promega). mRNA samples were subjected to electrophoresis on 1·5% agarose gels containing 0·44 M formaldehyde, transferred to nitrocellulose membrane and used for hybridization with [^{32}P]CTP-labelled riboprobes. The negative-sense CAT and NP probes where synthesized by *in vitro* transcription of linearized plasmids containing these genes.

■ **Determination of the intracerebral pathogenicity index (ICPI) in 1-day-old chicks.** ICPI was used to determine the virulence of wild-type and recombinant NDVs in 1-day-old chicks. For each ICPI test, 15 1-day-old SPF chicks were used (ten birds for test and five birds for control). The inoculum consisted of fresh, infective allantoic fluid with an HA titre > 24 (1:16) for the test birds and allantoic fluid from uninfected embryonated chicken eggs for control birds. Both inocula were diluted 1:10 in sterile PBS. Each bird was inoculated intracerebrally with 0·05 ml inoculum. The birds were observed for clinical signs and mortality every 24 h for a period of 8 days. The scoring and determination of ICPI were done according to the method described by Alexander (1997).

Results

Construction of cDNA encoding NDV antigenomic RNA and recovery of recombinant virus

A cDNA clone encoding NDV strain LaSota antigenomic RNA was assembled from seven cDNA fragments, as shown in Fig. 1. This plasmid, termed pLaSota, positioned the NDV cDNA between the T7 promoter and the HDV ribozyme sequence. During its construction, the antigenomic cDNA was

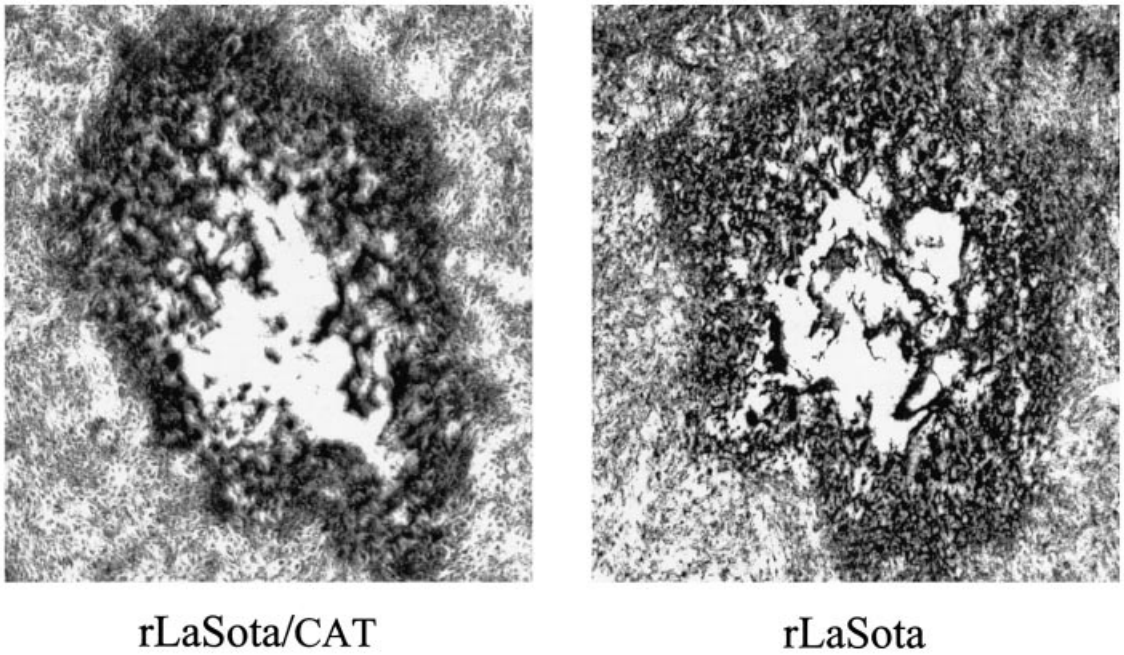

Fig. 3. Plaques produced by rLaSota and rLaSota/CAT on DF1 cells. Infected cells overlaid with 1% methylcellulose were incubated for a period of 4 days. Plaques were visualized by immunostaining using a monoclonal antibody against the NDV HN protein.

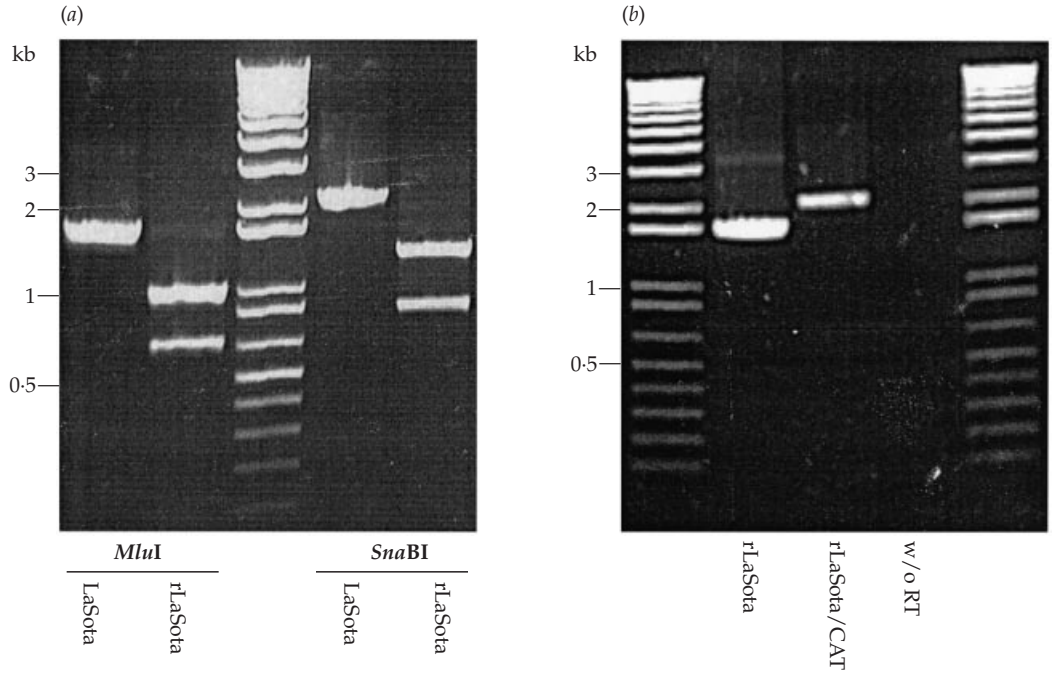

Fig. 4. Identification of genetic markers in the genome of rLaSota and confirmation of the presence of the CAT gene in the genome of rLaSota/CAT. RT–PCR was performed from genomic RNA extracted from purified viruses. (*a*) Identification of genetic markers in the genome of rLaSota. Primers spanning the corresponding regions were used for PCR and the products were subjected to restriction enzyme digestion. Wild-type LaSota was used as control. (*b*) Confirmation of the presence of the CAT gene in the recovered rLaSota/CAT by PCR with specific primers. The larger RT–PCR product (2·3 kb) from rLaSota/CAT confirmed the presence of the CAT gene compared with the smaller RT–PCR product (1·6 kb) from rLaSota.

modified to generate two new restriction sites as markers. A genetic marker was introduced into the intergenic region between the F and HN genes by changing two nucleotides to mutate the original *Age*I site to a unique *Mlu*I site (positions 6292–6297 in the full-length cDNA clone). Similarly, a unique *Sna*BI site was generated in the HN and L intergenic region by changing four nucleotides (positions 8352–8357). To facilitate transcription by T7 RNA polymerase, three G residues were included before the NDV leader sequence.

A recombinant vaccinia virus-based transfection system was used to recover recombinant NDV from cDNA. HEp-2 cells were infected with vaccinia virus strain MVA, capable of synthesizing T7 RNA polymerase. Simultaneously, the cells were transfected with plasmids pLaSota, pNP, pP and pL, to provide ribonucleoproteins and allow synthesis of full-length antigenomic RNA. Four h after transfection, the cells were washed twice and the medium was replaced with medium containing acetyltrypsin. After two passages in HEp-2 cells, 100 µl clarified supernatant was inoculated into the allantoic cavity of 10-day-old embryonated chicken eggs. The allantoic fluid was harvested 4 days after inoculation and tested for HA. After two passages in eggs, the virus was plaque-purified to eliminate vaccinia virus. The plaques produced by the virus were stained with monoclonal antibodies specific to the NDV HN protein to confirm the specificity of the recovered virus (Fig. 3). To identify the recovered virus, two genetic markers (*Mlu*I and *Sna*BI) were introduced in the full-length cDNA clone. In order to verify the presence of these markers, RNA from recovered virus was subjected to RT–PCR. DNA fragments encompassing the regions containing the *Mlu*I and *Sna*BI sites were subjected to restriction enzyme digestion with the respective enzymes. Analysis of the restriction enzyme patterns revealed the presence of both genetic markers in rLaSota, as calculated from the sizes of the bands, while RT–PCR products from wild-type LaSota were not digested by the enzymes (Fig. 4*a*). Nucleotide sequence analysis of RT–PCR products also confirmed the presence of the genetic markers.

Construction of a cDNA encoding NDV antigenomic RNA containing the CAT gene and recovery of recombinant virus

The CAT gene ORF, flanked by NDV gene start and gene end sequences, was inserted into the non-coding region of the NP gene immediately before the NP ORF (Fig. 2). The resulting plasmid would encode an antigenome of 15 900 nt, obeying the 'rule of six' (Peeters *et al*., 2000). In the recovered virus, the inserted CAT gene would be expressed as a monocistronic mRNA under the control of the NDV transcription system. The method for recovery of recombinant NDV was the same as described above. Plaques produced by rLaSota/CAT were immunostained with HN-specific monoclonal antibody and were of a size and morphology similar to those produced by rLaSota. The presence of the CAT gene in the genome of recovered virus was verified by RT–PCR with oligonucleotide primers spanning the CAT gene. The size of the RT–PCR product from recovered rLaSota was 1·6 kb, while that from rLaSota/CAT was 2·3 kb (Fig. 4*b*). Direct PCR from extracted RNA without RT did not yield any product. Nucleotide sequence analysis of the RT–PCR product confirmed the presence of the CAT gene in the genome of the recovered virus rLaSota/CAT.

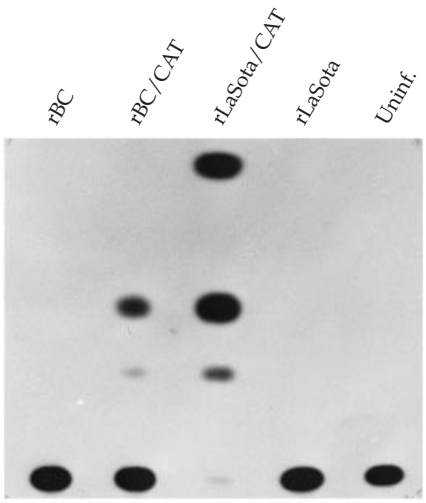

Fig. 5. Comparison of CAT expression by rBC/CAT and rLaSota/CAT at passage 6. The CAT gene was expressed from the sixth position in rBC/CAT while it was expressed from the first position in rLaSota/CAT. Equal cell equivalents of lysates were analysed for acetylation of [^{14}C]chloramphenicol as visualized by thin-layer chromatography. Relative CAT activity was quantified by densitometry.

Expression of the CAT gene from the virus rLaSota/CAT

To examine the expression of the CAT protein from rLaSota/CAT, cell lysates from 12 passages, beginning with the third, were tested for CAT activity. For rLaSota/CAT, all passages showed similar CAT enzyme activity by CAT assay (data not shown). These results suggested that the inserted CAT gene was stable, at least up to passage 12. In our previous work, an NDV–CAT chimeric transcription cassette was inserted between the HN and L genes of the full-length cDNA of virulent NDV strain Beaudette C and infectious CAT-expressing recombinant NDV (rBC/CAT) was recovered (Krisnamurthy *et al*., 2000). In order to compare the level of expression of the CAT genes from rLaSota/CAT and rBC/CAT, replicate monolayers of DF1 cells were infected with each virus separately at an m.o.i. of 0·1. Four days after infection, CAT enzyme activities in the cell lysates were examined (Fig. 5). Our results showed that the CAT enzyme activity was about 11-fold higher in cells infected with rLaSota/CAT than in cells infected with rBC/CAT. To

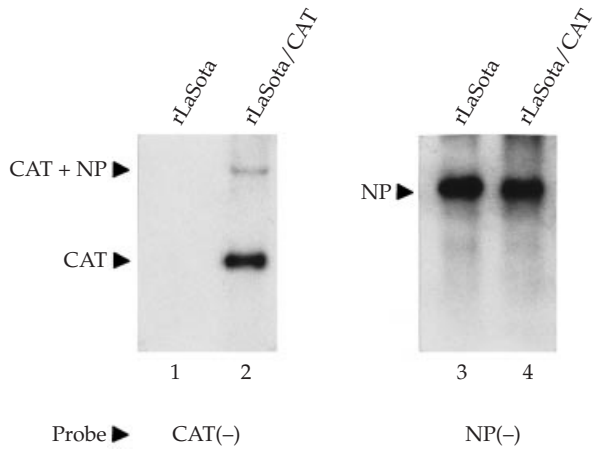

Fig. 6. Northern blot hybridization of intracellular mRNAs encoded by rLaSota and rLaSota/CAT. Poly(A)$^+$ mRNAs were isolated from total intracellular RNA by oligo(dT) chromatography and were electrophoresed on formaldehyde–agarose gels. The gels were transferred to nitrocellulose membrane and probed with the negative-sense riboprobes.

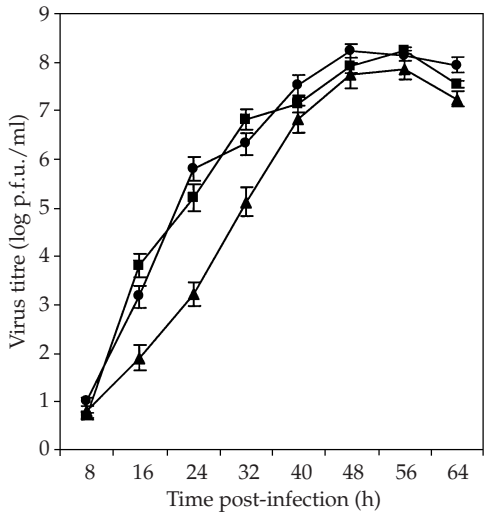

Fig. 7. Multi-step growth curve for wt LaSota (●), rLaSota (■) and rLaSota/CAT (▲) in DF1 cells. Cell monolayers in 25 cm^2 flasks were infected with 0·005 p.f.u. per cell with three replicate flasks per virus. Samples were taken every 8 h for 56 h. The virus in the supernatant was titrated by plaque assay. The log titre was derived from the mean virus titre and error bars indicate standard deviations.

examine the presence of CAT mRNA and the level of synthesis of the immediate downstream NP mRNA, Northern blot hybridization was performed with poly(A)$^+$ RNA from cells infected with rLaSota or rLaSota/CAT, each at passage 6. Hybridization of the mRNA extracted from rLaSota/CAT-infected cells with a negative-sense CAT-specific riboprobe detected a single major band of the size predicted for CAT mRNA (Fig. 6). Hybridization with a negative-sense riboprobe specific for the NP gene showed a single major band at the size predicted for NP mRNA in both rLaSota and rLaSota/CAT blots. Densitometry scanning did not show a significant difference in the level of NP mRNA synthesis between rLaSota and rLaSota/CAT. This result indicated that insertion of the CAT gene at the most 3′-proximal locus did not affect mRNA synthesis of the immediate downstream NP gene significantly.

Growth characteristics of the recombinant viruses

The efficiency of replication in tissue culture of rLaSota, rLaSota/CAT and wild-type NDV LaSota was compared in a multiple-step growth cycle. Triplicate monolayers of DF1 cells were infected with each virus at an m.o.i. of 0·005 and samples were collected at 8 h intervals. The virus titres of these samples were quantified by plaque assay (Fig. 7). Both the kinetics and the magnitude of replication of the three viruses were very similar. However, the production of rLaSota/CAT was delayed slightly compared with rLaSota and wild-type NDV strain LaSota.

Pathogenicity of the recombinant viruses

In order to compare the pathogenicity of rLaSota, rLaSota/CAT and wild-type NDV strain LaSota, ICPI tests in 1-day-old chicks were performed by scoring clinical signs and mortality. The most virulent NDV strains give indices close to 2·0, while avirulent viruses give values close to 0. In our experiment, the results of ICPI were 0·27 for wild-type NDV LaSota, 0·29 for rLaSota and 0·24 for rLaSota/CAT. These results show that the recombinant viruses were similar in virulence to wild-type NDV strain LaSota.

Discussion

Negative-strand RNA viruses with non-segmented genomes have potential for use as vectors for several reasons. Firstly, in most cases, the foreign gene is expressed stably even after many passages *in vitro* (Bukreyev *et al.*, 1996; Mebatsion *et al.*, 1996; Schnell *et al.*, 1996; Hasan *et al.*, 1997; He *et al.*, 1997). Secondly, negative-strand RNA viruses do not show a measurable rate of homologous RNA recombination, which contributes to the stability and safety of these vectors (Lamb & Kolakofsky, 1996; Palese *et al.*, 1996). Thirdly, in most cases, these viruses have accommodated large foreign genes without drastic reductions in replication (Sakai *et al.*, 1999; Haglund *et al.*, 2000). Finally, attenuated vaccine strains of many negative-strand RNA viruses pathogenic to humans and animals are available for use as safe vectors. In these cases, immunity would be induced against the vector as well as the expressed foreign protein. This new technology for using negative-strand RNA viruses as vaccine vectors against human and veterinary pathogens is currently being pursued in several laboratories.

NDV is particularly well suited to the development of a negative-strand RNA virus vector for avian pathogens for several reasons. (i) Live NDV vaccines are widely used in the poultry industry with a proven track record of efficacy and

safety (Alexander, 1997). (ii) NDV grows to high titres and expresses proteins encoded within its genome at high levels; NDV recombinants expressing foreign proteins are therefore likely to grow to high titres and to produce the foreign proteins at high levels. (iii) NDV naturally infects the respiratory tract, leading to the induction of both mucosal and systemic immunity. Thus, NDV would be a suitable vector for antigens of agents infecting the respiratory tract.

In this report, we have constructed a recombinant, attenuated NDV expressing a foreign gene, CAT. The CAT gene was inserted into the NDV antigenomic cDNA in the upstream region of the 3′-proximal NP gene ORF. We deduced that insertion of a new cistron in the first locus would lead to the highest level of foreign gene expression, due to the polarity of transcription (Hasan *et al.*, 1997; Wertz *et al.*, 1998). The total length of the recombinant virus genome was maintained as *6n* nucleotides to maximize virus replication (Calain & Roux, 1993; Peeters *et al.*, 2000). Both recovered viruses (rLaSota and rLaSota/CAT) were viable, with multiple-step growth curves very similar to that of the wild-type NDV strain LaSota. rLaSota/CAT expressed a high level of the CAT protein, and expression of the CAT protein was stable over 12 low-dilution passages. The pathogenesis of the recombinant viruses was not augmented in a natural avian host.

Previous characterization in our laboratory of a virulent NDV recombinant expressing CAT protein had shown a lower level of expression of the CAT gene and growth retardation of the recombinant virus (Krishnamurthy *et al.*, 2000). In that report, the CAT gene was inserted into a 5′ position between the HN and L genes. In this study, it was demonstrated that the level of expression of the CAT gene was much higher when the cistron was inserted in the most 3′-proximal locus, probably due to the polarity of transcription. Furthermore, it was shown that growth of the recombinant virus was not affected significantly when the foreign gene was expressed from the first locus. This result indicated that maintenance of the normal transcriptional gradient of NDV is important for replication of the virus; replication is not disrupted after the foreign gene is expressed from the most 3′-proximal locus but is disrupted after the foreign gene is expressed from an internal locus. Results from this study suggest that high-level expression of the CAT protein from an upstream position did not interfere with NDV replication. However, expression of antigens from other pathogens might interfere with NDV replication; thus, high-level expression of such antigens from a locus upstream of the NP gene might lead to attenuation and even instability of the vector.

The results described here show that it is possible to use attenuated NDV as a vaccine vector to express a foreign gene. Development of recombinant NDV as a vaccine vector has several applications. Based on studies with other non-segmented, negative-strand RNA viruses, it should be possible to insert and express several foreign genes in the same virus and to obtain simultaneous immune responses to the expressed antigens in inoculated animals. For example, a single recombinant NDV could be generated that expressed the immunogenic proteins of multiple avian pathogens. Alternatively, several NDVs, each expressing various heterologous antigens, could be administered as a multivalent vaccine. A further extension would be to use NDV vectors in non-avian species, where NDV is capable of undergoing incomplete replication to the extent necessary to express inserted genes. Thus, development of NDV as a vector should prove to be useful against avian and non-avian diseases for which suitable vaccines are not currently available.

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