Retroviral delivery of RNA interference against Marek’s disease virus in vivo

M. Chen,*1 W. S. Payne,* J. R. Dunn,† S. Chang,† H. M. Zhang,† H. D. Hunt,† and J. B. Dodgson*2

*Department of Microbiology and Molecular Genetics, Michigan State University, East Lansing 48824; and †USDA-Agricultural Research Service Avian Disease and Oncology Laboratory, East Lansing, MI 48823

ABSTRACT The process of RNA interference (RNAi) has been exploited in cultured chicken cells and in chick embryos to assess the effect of specific gene inhibition on phenotypes related to development and disease. We previously demonstrated that avian leukemia virus-based retroviral vectors are capable of delivering effective RNAi against Marek’s disease virus (MDV) in cell culture. In this study, similar RNAi vectors are shown to reduce the replication of MDV in live chickens. Retroviral vectors were introduced into d 0 chick embryos, followed by incubation until hatching. Chicks were challenged with 500 pfu of strain 648A MDV at day of hatch, followed by assays for viremia at 14 d postinfection. Birds were monitored for signs of Marek’s disease for 8 wk. A stem-loop PCR assay was developed to measure siRNA expression levels in birds. Delivery of RNAi co-targeting the MDV gB glycoprotein gene and ICP4 transcriptional regulatory gene significantly reduced MDV viremia in vivo, although to lesser extents than were observed in cell culture. Concomitant reductions in disease incidence also were observed, and the extent of this effect depended on the potency of the MDV challenge virus inoculum. Successful modification of phenotypic traits in live birds with retroviral RNAi vectors opens up the possibility that such approaches could be used to alter the expression of candidate genes hypothesized to influence a variety of quantitative traits including disease susceptibility.

Key words: RNA interference, retroviral vector, Marek’s disease virus, short hairpin micro-RNA, gallid herpesvirus type 2

INTRODUCTION

The process of RNA interference (RNAi) has been used widely to modify phenotypes and has the potential to be used similarly in chickens. Although RNAi has been used extensively to test the roles of various genes in chick embryos (Hu et al., 2002; Pekarik et al., 2003), there have been very few applications of RNAi in live chickens. One phenotype of particular interest is resistance-susceptibility to infectious diseases. Antiviral strategies based on RNAi have effectively inhibited several families of DNA and RNA viruses including retroviruses and herpesviruses (Haasnoot et al., 2007). Antiviral RNAi can be delivered into cells and animals by transient transfection of synthetic small interfering RNA (siRNA) or expression of short hairpin RNA (shRNA), modified primary micro-RNA hairpins (shRNA-mirs), and long hairpin RNA (lhRNA), which mimic various precursors of cellular micro-RNA (miRNA) and are processed in cells to siRNA oligonucleotides (Zamore and Haley, 2005). Depending on the degree of complementarity of the siRNA to the target sequence, RNAi can mediate gene silencing by means of direct degradation of mRNA, repression of translation, or recruitment of mRNA to cellular processing bodies where translation does not occur (Peters and Meister, 2007). Transfection of siRNA has been used to efficiently silence gene expression in chick embryos. Hu et al. (2002) demonstrated that siRNA against the Rous sarcoma virus gag gene can protect embryos from lethal infection; however, delivery of synthetic siRNA has only a transient effect in vertebrates. Plasmid or viral vector-delivered shRNA-lhRNA generally has been used to inhibit chronic viral infection (Akashi et al., 2005; Liu et al., 2007). More recently, RNAi based on naturally occurring miRNA primary transcripts (shRNA-mirs) has been developed and, in some cases, has proven more potent than shRNA in both transient and stable assays (Dickins et al., 2005; Silva et al., 2005; Stegmeier et al., 2005). Typically, shRNA-mirs are constructed by replacing the stem region of a miRNA gene with the target sequence and that of its guide RNA complement. The shRNA-mirs also can be expressed as
larger transcripts containing several stem-loop structures resembling polycistronic miRNA (multi-mirs). The potential advantage of using multi-mirs against viruses is to target multiple regions of a viral RNA or multiple viral RNA to enhance efficacy and prevent viral escape. We previously developed retroviral vectors based on the replication competent avian leukosis virus (ALV) backbone (Hughes et al., 1986) that express shRNA-mirs either as spliced RNA transcribed from the retroviral long-terminal repeat promoter or as RNA transcribed from internal promoters (Chen et al., 2007, 2008).

Marek’s disease virus (MDV) is a member of the Alphaherpesvirinae. There are 3 different serotypes of MDV designated as MDV1 (gallid herpesvirus type 2), MDV2 (gallid herpesvirus type 3), and MDV3 (herpesvirus of turkeys, HVT). It has been found that MDV1 is the only serotype that causes Marek’s disease (MD), a lymphoproliferative disorder in which aggressive T-cell lymphomas result from infection of susceptible chickens (Calnek and Witter, 1997). Although MD is currently well-controlled by commercial vaccines, MDV continues to evolve in vaccinated flocks and has shown the ability to generate new and more acute disease symptoms (Witter, 1997; Nair, 2005; Osterrieder et al., 2006), such that new control measures not based on vaccination warrant further research. The use of RNAi is an attractive antiviral strategy, as well as an option for general gene function analysis. First, RNAi is sequence-specific. Second, RNAi based on lhRNA or multi-mirs can deliver siRNA targeting several genes or sequences simultaneously to minimize the chances that viruses will mutate to resistant forms. Third, RNAi has the potential to be delivered by infection with nonpathogenic viruses. Finally, due to its sequence-specificity, RNAi potentially can be targeted against sequences of virulent viruses without inhibiting vaccine viruses themselves (Westehouw et al., 2006). We previously demonstrated that replication competent retroviral vectors are capable of delivering antiviral RNAi against MDV in cell culture (Chen et al., 2008). Cells expressing shRNA-mirs or multi-mirs targeting the MDV gB glycoprotein gene or the ICP4 transcriptional regulatory gene, or both, show significant inhibition of viral replication. Replication competent retroviral vectors also are attractive for RNAi studies in live chickens. Usually, a single proviral copy of the vector is stably integrated into the chicken genome, thus minimizing the possibility of saturating cellular miRNA biogenesis factors. Unlike many other delivery methods, replication competent vectors spread rapidly and efficiently in chickens and can deliver RNAi to tissues that are not readily transfected by DNA or RNA. We and others have demonstrated gene transfer studies in vivo using ALV-based vectors (Holmen et al., 1999; Das et al., 2006; Harpavat and Cepko, 2006). In this study, we demonstrate that RNAi delivered by replication competent ALV vectors can measurably inhibit pathogenic MDV replication in live chickens.

## MATERIALS AND METHODS

### Vector Constructions

The RNAi vectors used herein are based on the replication competent ALV vectors with a splice acceptor, the Bryan-strain pol gene and a subgroup(A) env gene [RCASBP(A)] developed by Hughes et al. (1986) and modified by Bromberg-White et al. (2004). These RCASBP(A) vectors are designed such that the inserted shRNA-mir sequence is expressed as a spliced RNA transcribed from the viral long-terminal repeat. The RCASBP(A)3mirs vector is used to simultaneously deliver RNAi against 3 different sequence targets (Chen et al., 2008). In trial 1, RCASBP(A)3mirs-1874-596-1518, which delivers 3 siRNA against the MDV gB gene, was used. Numbers indicate the first nucleotide of the RNAi target sequence within the viral gene coding sequence. In trials 2 and 3, RCASBP(A)3mirs delivering 2 siRNA (1874 and 1518) against the MDV gB and 1 siRNA against the ICP4 gene (6170) was used [RCASBP(A)3mirs-1874-6170-1518]. Details of the construction of these vectors and their efficacies in cell culture are provided in Chen et al. (2008).

### Cells and Viruses

The DF-1 cells (Schaefer-Klein et al., 1998) were maintained in 8.8 g/L of Leibovitz’s L-15, 5.0 g/L of McCoy’s 5A, and 1.5 g/L of NaHCO3 supplemented with 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA), 50 µg/mL of gentamicin (Invitrogen Corp., Carlsbad, CA), and 0.25 µg/mL of fungizone at 39°C. Chicken embryo fibroblasts (CEF) were maintained in the same media supplemented with 4% calf serum (Invitrogen Corp.). The serum content for MDV-infected CEF cells was reduced to 2% when cells reached confluence. The very virulent plus (VV+) 648A (39 or 40 passages in CEF) strain (Gimeno et al., 2001b) of serotype 1 MDV was obtained from Richard Witter and Mohammad Heidari, USDA-Agricultural Research Service, Avian Disease and Oncology Laboratory (East Lansing, MI). Propagation of RCASBP(A) vectors in DF-1 cells was initiated by transfection of plasmid DNA containing the retroviral provirus using SuperFect Transfection Reagent (Qiagen Inc., Valencia, CA) according to the protocol of the manufacturer. Viral spread was monitored by assaying culture supernatants for ALV p27 capsid protein by ELISA (Smith et al., 1979). Once the viral titer peaked, DF-1 cells were counted and prepared for direct egg injection.

### In Vivo MDV Challenge Assay

Line 0 chickens (Astrin et al., 1979) were used. Fresh or stored embryos (stored up to 3 wk at 16 to 18°C) were somatically infected with RCASBP(A)3mirs vectors by injecting unincubated embryos near the blas-
todermin with about 1 million DF-1 cells producing the virus (Holmen et al., 1999). Viremic chicks were identified at hatch by ELISA for the ALV p27 protein (Smith et al., 1979). Chicks with low (optical density at 490 nm < 0.2) or negative ELISA readings were discarded from further studies (these were rare). Chicks were challenged with 500 pfu of 648A MDV, passaged 40× in CEF, by intraabdominal injection at day of hatch. The same MDV challenge stock was used in trials 1 and 2 but was unavailable for trial 3; therefore, passage 39 648A virus was grown once more in CEF to replicate the 40 passage state before use in this trial. Chickens were bled at 14 d postinoculation (dpi) for the determination of MDV viremia using peripheral blood cells (PBL) as described below. A second test for MDV-infected PBL at 28 dpi was performed in trial 1. In trials 2 and 3, chickens were also bled at 21 dpi for siRNA expression analysis as described below. (The volume of blood required for these assays prevented sampling more than once per week.)

Chickens were examined daily up to 56 dpi. Mortality was recorded, and tumor samples were collected for immunohistochemical examination. Peripheral nerves with undetermined enlargement were collected in selected chickens for histological analysis to identify inflammatory or neoplastic lesions using hematoxylin and eosin staining. Chickens that died during the experiment or that were killed at the termination of the experiment at 56 dpi were necropsied and evaluated for gross MD lesions, which included enlarged peripheral nerves, visceral lymphomas, and bursal-thymic atrophy. Chickens with nerve enlargements or MDV-induced lymphomas (meq-positive), or both, were diagnosed as chickens positive for MD.

Plaque Assays to Determine MDV Viremia

Blood was collected from chicken jugular veins in the presence of heparin, and PBL were obtained by centrifugation. About 1.5 mL of blood was collected from each bird, if possible, to obtain at least 2 × 10^6 PBL for the plaque assay. Blood was spun at 1,000 rpm for 5 min, and the layer containing plasma along with PBL were collected and transferred into new 15-mL tubes. After a second spin at 1,500 rpm for 5 min, PBL were suspended in 2 mL of Leibovitz-McCoy medium as described above with 4% calf serum. The PBL were identified and counted using a hemocytometer chamber. Two million PBL were added to a CEF monolayer (6.6 × 10^5 CEF cells were seeded on 6-well plates and allowed to attach overnight). Calf serum was decreased to 1% on the day after PBL seeding. Viral plaques were counted after 5 to 6 d. In trial 3, CEF plates were fixed and plaques were stained using an immunoperoxidase-based technique (Silva et al., 1997). In rare cases (less than 5%) where fewer than 2 × 10^6 PBL were available, all PBL were plated and the plaque number adjusted [multiplied by (2 × 10^6)/number of PBL used].

Stem-Loop PCR Assay of siRNA Expression

Expression levels of siRNA targeting MDV genes were measured using a stem-loop PCR technique specifically designed to assay small RNA levels (Chen et al., 2005). Briefly, stem-loop PCR employs a hairpin loop with a short 3′ extension complementary to the 3′ end of the siRNA as the reverse transcriptase primer, followed by PCR with 1 primer in the siRNA sequence and 1 primer in the stem-loop sequence to ensure a product of adequate size and specificity. Details of this approach are described in Chen et al. (2005). In trials 2 and 3, chickens were bled at 21 dpi. Blood was carefully layered on Histopaque solution (Invitrogen Corp.) and centrifuged at 3,400 × g for 10 min. White blood cells were removed, washed with PBS, and resuspended in RNAlater solution (Ambion Inc., Austin, TX) at −20°C. The RNA was collected using the Ribopure-Blood kit (Garcia-Effron et al., 2007) with an alternate protocol for small RNA isolation according to the instructions of the manufacturer (Ambion Inc.). One micromgram of RNA was reverse-transcribed with Moloney murine leukemia virus reverse transcriptase (Invitrogen Corp.) in a 20-µL reaction volume. Amplifications of cDNA corresponding to siRNA 1874, 6170, or 1518 were carried out in separate reactions using the following primers, respectively: GTGCATATCCA-GTGCAGGGTGCCAGGTATTGCGACTGATACA-GACGACAT; GTGCATATCCAGTCAGGGTCCAGGTATTGCGACTGATACGTCA; or GTGCATATCCAGGTCAGGGTGCCAGGTATTGCGACTGATACGTCA. Forward PCR primers were CGCGGAGATTAAGCTCGACAAAT, CGGCGTTTGTTGCGGTCTGACCA; or CGGCGTTTGTTGCGGTGCAATTTG. Reverse PCR primers were GACGCACAT; GTCGTATCCAGTGCAGGGTC-GTTCCGAGGTATT). Forward PCR primers were CGCGGAGATTAAGCTCGACAAAT, CGGCGTTTGTTGCGGTCTGACCA; or CGGCGTTTGTTGCGGTGCAATTTG. Reverse PCR primers were GACGCACAT; GTCGTATCCAGTGCAGGGTC-GTTCCGAGGTATT. The PCR products were run on a 1.5% wt/vol agarose gel. The cDNA prepared from chickens that received neither a retroviral vector nor MDV challenge were measured using a stem-loop PCR technique specifically designed to assay small RNA levels (Chen et al., 2005). Forward PCR primers were CGCGGAGATTAAGCTCGACAAAT, CGGCGTTTGTTGCGGTCTGACCA; or CGGCGTTTGTTGCGGTGCAATTTG. Reverse PCR primers were GACGCACAT; GTCGTATCCAGTGCAGGGTC-GTTCCGAGGTATT. The PCR products were run on a 1.5% wt/vol agarose gel. The cDNA prepared from chickens that received neither a retroviral vector nor MDV challenge viruses was used as a negative control as well as a no reverse transcriptase negative control, whereas cDNA prepared from CEF expressing RCASBP(A)3mir was used as a positive control. Polymerase chain reaction assays were done in 50 µL containing 25 µL of 2 × PCR master mix (Promega Corp., Madison, WI), 2 µL of cDNA, and primers at 25 pmol each for 2 min at 95°C followed by 20 cycles (cycle number chosen to ensure measurement in the linear range of the assay) of 1 min at 95°C, 30 s at 57°C, and 30 s at 72°C. The reverse PCR primer was the same for detection of all 3 siRNA (GTGCAGGGTCCAGGATATT). Forward PCR primers were CGCGGAGATTAAGCTCGACAAAT, CGGCGTTTGTTGCGGTCTGACCA; or CGGCGTTTGTTGCGGTGCAATTTG. Reverse PCR primers were GACGCACAT; GTCGTATCCAGTGCAGGGTC-GTTCCGAGGTATT. The PCR products were run on a 1.5% wt/vol agarose gel. The cDNA prepared from chickens that received neither a retroviral vector nor MDV challenge viruses was used as a negative control as well as a no reverse transcriptase negative control, whereas cDNA prepared from CEF expressing RCASBP(A)3mir was used as a positive control. Polymerase chain reaction images were captured and analyzed using ImageJ (fro
**Immunofluorescence Assays**

An immunohistochemistry assay based on the avidin-biotin-peroxidase complex method (Hsu et al., 1981; Vectastain ABC kit, Vector Laboratories, Burlingame, CA) was used to differentiate vector-induced tumors (B-cell lymphomas) from MDV-induced tumors (T-cell lymphomas). The protocol of Gimeno et al. (2001a) was employed. Fresh tumors were collected and snap-frozen in liquid nitrogen. Samples were stored at −70°C until they were processed. A polyclonal antibody specific to MDV meq protein (a gift from Lucy Lee, Avian Disease and Oncology Laboratory, East Lansing, MI) was used at a working dilution of 1:2,500. The monoclonal antibody specific to Bu1, an antigen mostly expressed by chicken B cells, was used at a working dilution of 1:500. (Bu1-positive B-cell lymphomas were observed, on average, in 7% of the RCASBP vector-treated birds in trials 2 and 3.)

**Statistical Analysis**

The reduction in MDV viremia due to RNAi treatment was analyzed with a simple GLM in which the RNAi treatment was treated as a fixed variable. The statistical analysis was accomplished with SAS for Windows v9.1.3 (SAS Institute Inc., Cary, NC). In some cases, the raw plaque number was inversely or logarithmically transformed to meet the assumptions of the GLM (i.e., normal distributions). The differences in average viral load among the RNAi treatments were compared with Fisher protected least significant difference tests. The differences in the percentage of gross MD among the treatment groups were compared using 2-proportion z-tests. Cumulative survival rates were calculated to assess the protective efficacy of RNAi treatment, and the univariate comparison of survival for control versus treated groups was tested using a log-rank test, comparing 2 groups at a time (Mantel, 1966).

**RESULTS**

**Protocol for RNAi Delivery and MDV Challenge**

All experiments employed line 0 chickens (Astrin et al., 1979; Bacon et al., 2000), which lack endogenous retroviral loci similar to ALV. This minimizes the chances of recombination between the retroviral RNAi delivery vector and the host genome, potentially leading to the loss of the RNAi cassette, and it allows the presence of the retroviral vector to be monitored by a simple ELISA assay (Smith et al., 1979). Unincubated line 0 embryos were injected near the blastoderm with DF-1 cells producing the RNAi retroviral delivery vector, sealed, and incubated to hatch. Newly hatched chicks were challenged with MDV by intraabdominal injection and monitored for MD for up to 8 wk. An initial pilot test demonstrated that line 0 chicks are relatively resistant to the widely used, very virulent Md11 strain of MDV (data not shown). Thus, all experiments described here employed a challenge with 500 pfu of vv+ strain 648A, passage 40, that was shown to cause MD in a high percentage of injected line 0 chicks (H. M. Zhang, unpublished data).

**RNAi Targeted at 3 gB Sequences Inhibits 648A Strain MDV Replication In Vivo**

In trial 1, the RNAi delivery vector [RCASBP(A)3mirs-1874-596-1518] targeting 3 different sequences in the MDV gB gene (Chen et al., 2008) was used. Although this multimir vector was not significantly more effective than a vector with a single target in gB [RCASBP(A)miRNA-1874] in cell culture (Chen et al., 2008), it potentially could be more resistant to MDV evolving resistance to the RNAi effect. A comparable control vector [RCASBP(A)3mirs-scrambled] in which all 3 sequences were randomly rearranged was used as the primary negative control, along with embryos that received no retrovirus. Marek’s disease virus replication was monitored by plaque assay of infected PBL at 14 and 28 dpi. Treatment with RNAi reduced MDV viremia reduced MDV viremia at 14 dpi by 70 to 75% relative to the scrambled sequence and no vector controls, respectively (Table 1), and by 35 to 71% at 28 dpi. Due to the complex in vivo life cycle of MDV, with many viruses going latent in later stages, we chose to use only 14 dpi viremia tests in subsequent trials. (As noted in Materials and Methods, the volume of blood needed for viremia and stem-loop PCR assays limits the frequency with which either assay can be performed without weakening the chicks.) The antiviral RNAi effect was significant when comparing RNAi treatment vs. no vector (P < 0.02, ANOVA, Fisher protected least significant difference) but not RNAi treatment vs. scrambled sequence control, primarily due to the fact that too few birds hatched in the scrambled sequence group. Previous experience (Holmen et al. 1999; the pilot trial discussed above) showed that injection with ALV vectors often reduces hatch rates. Both the injection process by itself and ALV replication appear to contribute to this. However, the extent of that reduction is highly variable and also depends on the age of the hens producing the embryos (relative to their laying cycle) and the storage time of embryos. Hatch rates were not significantly reduced in later trials.

The MDV meq protein has been shown to be consistently expressed in MD tumors (Gimeno et al., 2005). Avian leukosis virus does not induce nerve enlargements; therefore, birds with nerve enlargements were diagnosed as exhibiting MD without further staining of tumors. Tumors from birds at risk of MD with no nerve enlargements were sectioned and tested for meq expression. The percentage of birds in the RNAi-treated group that exhibited MD (birds with nerve lesions or meq-positive tumors, or both) was 57% vs. 67% and
74% in the scrambled sequence and no vector control groups, respectively (Table 2). Although these results are in line with the RNAi-induced reduction in viremia, RNAi did not reduce MD (2-proportion z-tests) or increase overall survival rate (log-rank test) to a statistically significant level in this trial.

RNAi Targeted at gB and ICP4 Sequences Inhibits 648A Strain MDV Replication In Vivo

By the onset of trials 2 and 3, a retroviral vector containing 2 RNAi targets against the MDV gB gene and one against the MDV ICP4 gene was available [RCASBP(A)3mirs-1874-6170-1518]. This construct showed the greatest reduction in MDV replication in vitro (RNAi treatment reduced MDV titers in cell culture to 6% of the no vector treatment with this construct, Chen et al., 2008). In trial 2, RNAi treatment using RCASBP(A)3mirs-1874-6170-1518 significantly (P < 0.05) reduced MDV-infected PBL titers at 14 dpi when compared with either the scrambled sequence or no vector controls (Table 1). The reduction was about 50% relative to the scrambled control-treated birds and nearly 80% relative to the untreated birds. In this case, the scrambled control birds showed a reduction in viremia relative to no vector treatment; however, this effect was not observed generally in either trials 1 or 3 or in 2 other pilot trials. The MDV-induced tumors were differentiated from RCASBP(A)-induced B cell lymphomas by staining for meq and the B cell marker Bu1. In this trial, the percentage of birds in the RNAi group that exhibited MD was 54%, which was significantly different (P < 0.001) from that in the no vector control group and from that of the scrambled insert control group (both 83%, Table 2). Treatment by RNAi also significantly increased the survival rate in this trial by reducing MD-induced death compared with the scrambled sequence control treatment but not compared with the no vector control group (log-rank test: P < 0.01; data not shown).

One concern with retroviral delivery is incomplete spread of the viral vector or failure to express siRNA from the RNAi cassette. The ELISA assays (for ALV p27) demonstrated that 100% of the birds injected contained the retroviral vector (data not shown). However, the birds are a mosaic of infected and uninfected cells and even those cells (or birds) that contain the vector provirus might fail to produce siRNA due to genetic (e.g., internal deletions) or epigenetic (e.g., DNA methylation) silencing of proviral expression. We therefore developed stem-loop PCR assays to measure the levels of target-specific siRNA in white blood cells from experimental birds. Figure 1 shows that the average siRNA level as measured by stem-loop PCR varied among the birds. However, there was not a statistically significant correlation of the average siRNA expression level with MDV titer at 14 dpi (data not shown) or with MD or tumor incidence. It’s unclear what this lack of correlation may mean, however, because there are other examples in which the measured level of siRNA does not correlate well with the RNAi effect achieved (e.g., Dickins et al., 2005; Liu et al., 2008). Furthermore, the siRNA expression level is only an average across all harvested cells, and it’s unclear what the cell-to-cell variance may be and how this might affect MDV infectivity. In this regard, as a strictly cell-associated virus, the course of MDV replication may be less reflective of average cell properties than that of a lytic virus that releases bursts of extracellular infectious viral particles.

Trial 3 replicated trial 2 except that the passage 40, 648A strain MDV stock used in trial 2 was no longer available; therefore, a sample of passage 39, 648A MDV was obtained and then passed once more in CEF before the challenge. In addition, trial 3 employed a

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Trial 1 Bird number</th>
<th>pfu ± SD</th>
<th>Trial 2 Bird number</th>
<th>pfu ± SD</th>
<th>Trial 3 Bird number</th>
<th>pfu ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>No vector</td>
<td>15</td>
<td>100 ± 120</td>
<td>50</td>
<td>97 ± 120</td>
<td>72</td>
<td>183 ± 150</td>
</tr>
<tr>
<td>Scrambled control</td>
<td>7</td>
<td>84 ± 110</td>
<td>49</td>
<td>41 ± 58</td>
<td>70</td>
<td>198 ± 200</td>
</tr>
<tr>
<td>RNAi treatment</td>
<td>12</td>
<td>25 ± 44*</td>
<td>88</td>
<td>20 ± 39**</td>
<td>78</td>
<td>111 ± 89**</td>
</tr>
</tbody>
</table>

*Statistical difference at P < 0.05 from the no vector treatment within a column.

**Statistical difference at P < 0.05 from both the no vector and scrambled control within a column.

Table 2. Levels of Marek’s disease (MD) observed after treatment by RNA interference (RNAi) retroviral vectors

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Trial 1 Bird number</th>
<th>MD (%)</th>
<th>Trial 2 Bird number</th>
<th>MD (%)</th>
<th>Trial 3 Bird number</th>
<th>MD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No vector control</td>
<td>19</td>
<td>74</td>
<td>81</td>
<td>83</td>
<td>93</td>
<td>87</td>
</tr>
<tr>
<td>Scrambled control</td>
<td>9</td>
<td>67</td>
<td>46</td>
<td>83</td>
<td>89</td>
<td>91</td>
</tr>
<tr>
<td>RNAi treatment</td>
<td>14</td>
<td>57</td>
<td>112</td>
<td>54**</td>
<td>80</td>
<td>81*</td>
</tr>
</tbody>
</table>

*Statistical difference at P < 0.05 from scrambled control treatment within a column.

**Statistical difference at P < 0.0001 from both no vector and scrambled controls within a column.
more sensitive immunoperoxidase-based staining technique (Silva et al., 1997) that allowed for more accurate detection of smaller MDV plaques than did the direct microscopic examination used previously. These 2 changes probably explain the fact that MDV titers were higher among all treatment groups compared with those in trial 2 (Table 1). The RNAi treatment again significantly reduced MDV titer in trial 3 compared with either the scrambled sequence control treatment or no vector treatment ($P < 0.01$, ANOVA, Fisher protected least significant difference), in both cases by about 40% (Table 1). The lower percentage reduction in trial 3 vs. trial 2 may relate to the improved ability of the new assay to detect small plaques. Our in vitro results previously demonstrated that retroviral-delivered RNAi reduced MDV plaque size as well as plaque number (Chen et al., 2008). Marek’s disease incidence was also slightly reduced by RNAi treatment; however, statistical significance was only achieved when comparing the RNAi treatment group with the scrambled control group ($P < 0.05$, 2-proportion z-tests, Table 2). Compared with trial 2, MD incidence increased in all treatment groups in trial 3 (Table 2), and RNAi treatment did not reduce the MD survival rate in trial 3. This likely relates to the new viral stock used in the challenge. As in trial 2, there was no correlation between the average siRNA expression level measured by stem-loop PCR and MDV titers at 14 dpi. There was a modest inverse correlation between siRNA expression and MD incidence because eliminating birds with less than 10, 20, and 50% siRNA expression (relative to the infected CEF control) reduced average MD incidence to 74, 70, and 61%, respectively.

**DISCUSSION**

We describe the use of retroviral vector-based RNAi against MDV in vivo and demonstrate that the RCASBP(A)3mirs vector targeting both the MDV $gB$ and $ICP4$ genes can significantly reduce MDV viremia, measured as infected PBL at 14 dpi. In trial 1, a RCASBP(A)3mirs vector with 3 targets solely in the MDV $gB$ gene reduced viremia compared with no vector treatment but not compared with a scrambled insert. Although this could be due to the lesser inhibition observed with this vector in cell culture (Chen et al., 2008), it more likely results from the low statistical power in trial 1 due to an unusually low hatch rate. (RNAi-treated viremia was 30% of that in the scrambled insert-treated group.) Although consistent reductions in MDV viremia have been observed, to date, only modest, if any, reductions in disease (or increases in survival) have been observed in RNAi-treated birds. In trial 2, RNAi significantly

![Figure 1](link)
reduced MD incidence (30% less than the no vector and scrambled sequence controls over all birds; 40% less than no treatment if one eliminates birds with low or no siRNA expression). A more modest reduction in MD incidence was observed in trial 3 \((P < 0.05\) relative to the scrambled sequence control). The apparent greater virulence of the MDV challenge stock in trial 3 vs. trial 2 may have impaired our ability to observe an RNAi effect on MD. In general, survival rates showed fewer significant RNAi effects than did MD incidence, probably because they are more inherently variable, regardless of RNAi treatment.

A major concern for the in vivo application of RNAi is the possibility of stimulating interferon responses through the toll-like receptor (TLR) pathways. Small interfering RNA can stimulate the type II interferon response through TLR3 in the TRIF-NF-κB cascade (Kleinman et al., 2008). However, we think it unlikely that the antiviral effects we observed in vivo are due to RNAi-induced interferon. First, shRNA-mirs based on the cellular miR-30a gene were shown not to stimulate interferon responses in a different study (Bauer et al., 2009). Second, we demonstrated that the vectors we used were able to significantly inhibit MDV replication both in vitro and in vivo. Our siRNA lack immunostimulatory sequences that would be predicted to trigger type I interferon responses through TLR7/8. The TLR3 activation is based on a siRNA-class effect, which is independent of both siRNA sequence and chemistry. If our RNAi constructs were able to activate TLR3, then both effective siRNA and scrambled sequence control insert siRNA should show equivalent antiviral effects. In one case, we did see a significant difference in MD viremia in the scrambled controls vs. the untreated birds (trial 2, Table 1). However, no similar significant effect was observed in either trials 1 or 3 (or in 2 pilot trials, data not shown), nor was any effect of the scrambled control treatment noted on disease incidence (Table 2). Other than the low (~7%) incidence of B-cell lymphomas mentioned above, we did not observe any consistent vector-alone influence on the health of the birds. It is unlikely that there are RCASBP vector-derived changes in the immune system because we injected d 0 chicken embryos, which results in congenitally infected birds that are immunologically tolerant to the retroviral vectors we employ (Payne, 1998).

Despite the concerns discussed above, to our knowledge, these results represent the most dramatic RNAi-driven antiviral effect observed to date in live chickens. Lambeth et al. (2009) recently described modified HVT vaccine strains that also generate RNAi targeted at MDV qB or UL29 genes. In their studies (10 birds per group), there was no significant increase in MD survival compared with the nonspecific sequence control vector and a significant reduction in viral copy number at only 1 time point for 1 of 4 RNAi-expressing vectors. As in some of our trials, these studies were limited by their size and the relative pathogenicity of their challenge virus (they used 1,000 pfu of vv strain 595 compared with our 500 pfu of vv+ strain 648A). Although the use of an existing vaccine virus for RNAi delivery is attractive for commercial applications, a variety of RNAi approaches should be tested for use in chickens. Retroviral delivery has the potential to infect a relatively large proportion of the cells in the bird, each of which will retain a proviral copy of the vector in their genome, whereas the HVT vaccine virus is lytic and will kill most of the cells to which it delivers RNAi. Furthermore, given the inherent variability of MDV virulence, it may be difficult to assess significant RNAi-driven changes, if any, above the natural antiviral effect of the HVT vector. We recognize that ALV-based vectors are not suitable for application in the field due to their oncogenic properties; however, they remain an excellent system to test for phenotypic changes due to RNAi or other ectopic gene delivery methods (Holmen et al., 1999; Das et al., 2006; Harpavat and Cepko, 2006). It should be feasible to adapt successful RNAi based on ALV vectors to a variety of other delivery systems. In any case, our results suggest that retroviral vector-driven RNAi may be a general method by which to reduce the expression of specific genes in live chickens to test the resulting effect on phenotypic traits of interest. Successful modification of phenotypic traits in live birds with retroviral RNAi vectors opens up the possibility that such approaches could be used to alter the expression of candidate genes hypothesized to influence a variety of quantitative traits including disease susceptibility.

ACKNOWLEDGMENTS

This work was supported in part by the USDA National Research Initiative Competitive Grants Program (2004-35204-14780). We thank the following members of the USDA-Agricultural Research Service Avian Disease and Oncology Laboratory (East Lansing, MI): Lucy Lee, Robert Silva, Taejoong Kim, Mohammad Heidari, and Richard Witter for gifts of antibodies, PCR primers, virus stocks, and assistance in viral plaque assays and Evelyn Young for excellent support in animal care and viral challenges. We thank Sheri L. Holmen of the Nevada Cancer Institute (Las Vegas) for advice on vector construction.

REFERENCES


