A virus-encoded telomerase RNA promotes malignant T cell lymphomagenesis

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Telomerase is a ribonucleoprotein complex consisting of two essential core components: a reverse transcriptase and an RNA subunit (telomerase RNA [TR]). Dysregulation of telomerase has been associated with cell immortalization and oncogenesis. Marek's disease herpesvirus (MDV) induces a malignant T cell lymphoma in chickens and harbors in its genome two identical copies of a viral *TR* (*vTR*) with 88% sequence identity to chicken *TR*. MDV mutants lacking both copies of *vTR* were significantly impaired in their ability to induce T cell lymphomas, although lytic replication in vivo was unaffected. Tumor incidences were reduced by >60% in chickens infected with *vTR*$^-$ viruses compared with animals inoculated with MDV harboring at least one intact copy of *vTR*. Lymphomas in animals infected with the *vTR*$^-$ viruses were also significantly smaller in size and less disseminated. Constitutive expression of vTR in the chicken fibroblast cell line DF-1 resulted in a phenotype consistent with transformation as indicated by morphological alteration, enhanced anchorage-independent cell growth, cell growth beyond saturation density, and increased expression levels of integrin αv. We concluded that vTR plays a critical role in MDV-induced T cell lymphomagenesis. Furthermore, our results provide the first description of tumor-promoting effects of TR in a natural virus–host infection model.

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Abbreviations used: BAC, bacterial artificial chromosome; CEC, chicken embryo cell; chTR, chicken telomerase RNA; CR, conserved region; iNOS, inducible nitric oxide synthetase; MD, Marek's disease; MDV, MD virus; qPCR, quantitative real-time PCR; TERT, telomerase reverse transcriptase; TR, telomerase RNA; vTR, viral TR.

Telomeres are specialized nucleoprotein structures at the ends of linear chromosomes that protect chromosomal DNA from degradation, rearrangement, and detrimental fusion events (1). The ribonucleoprotein enzyme complex telomerase maintains telomeres by adding multiple hexameric repeats $(TTAGGG)_n$ to the end of chromosomes. Telomerase consists of two essential core components: telomerase reverse transcriptase (TERT) and a telomerase RNA (TR) that serves as the template for TERT (2). Vertebrate TRs have conserved secondary structures comprised of four structural domains: the pseudoknot (core) domain, the conserved region (CR)4 and 5 (CR4-5) domain, the boxH/ACA domain, and the CR7 domain (3). The pseudoknot domain contains the template region for synthesis of complementary DNA and is essential for stable assembly with TERT. The CR4-5 domain supports catalytic telomerase activity by enhancing the processivity of nucleotide addition. The boxH/ACA domain and the CR7 domain are essential for TR stability (3–5).

Telomerase activity is diminished in most somatic cells but commonly up-regulated in immortalized and cancer cells (6, 7). Unlike TERT, whose expression correlates with telomerase activity, TR is constitutively expressed in somatic cells (8); however, generally, cancer cells have elevated levels of TR. It has been speculated that telomere maintenance is necessary for unlimited cancer cell proliferation, but recent studies suggest that telomerase can promote tumorigenesis independently of telomere elongation (9–11).

Marek's disease (MD) is a fatal neoplastic disease of chickens caused by a lymphotropic alphaherpesvirus, MD virus (MDV). MD is characterized by neurological disorders, immune deficiency, and malignant T cell lymphomas that form as early as 12–14 d after infection (12).

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The online version of this article contains supplemental material.

The Journal of Experimental Medicine

MDV-transformed T cells and lymphoblastoid cell lines derived from primary lymphomas regularly harbor integrated viral genomes (13, 14). The rapid onset of virus-induced lymphoma suggests a direct involvement of virus-encoded oncogenes in MD tumorigenesis. The MDV Meq protein, a member of the Jun/Fos oncoprotein family, has been extensively characterized (15–17). Infection studies with a mutant MDV suggest that *meq* is the principal MDV oncogene (18, 19). Other viral genes, however, are suspected to serve important functions in MD tumorigenesis because Meq has only weak oncogenic potential in primary chicken cells (15).

MDV harbors in its genome a viral homologue of TR, termed viral TR (*vTR*), which exhibits 88% sequence identity to chicken TR (*chTR*) and was likely pirated from the chicken genome (20). The MDV genome is a 180-kbp double-stranded linear DNA consisting of two unique sequences, a long (unique-long [U_L]) and a short (unique-short [U_S]), each of which is bracketed by inverted internal (IR_L, IR_S) and terminal repeats (TR_L, TR_S; see Fig. 1 A). *vTR* is located in the TR_L and IR_L regions (see Fig. 1 B), and, consequently, two copies of *vTR* are present in the MDV genome. The four generic structural TR domains consisting of eight CRs are globally conserved in the vTR sequence (see Fig. 1 C). Compared with chTR, however, vTR exhibits several point mutations and deletions (20, 21), which are mainly located in the junction regions outside of the conserved structural domains, more specifically those of the pseudoknot domain (20, 22). Functional analyses have shown that vTR can reconstitute telomerase activity by interacting with TERT more efficiently than chTR (20, 21). The increased efficiency of vTR over chTR could be attributed to mutations affecting the pseudoknot core domain, likely by stabilization of the pseudoknot P2 helix (21). It is also notable that a single nucleotide substitution in the boxH region, which is present in the nononcogenic MDV vaccine strain CVI988, results in a substantial loss of functionality of vTR (21).

In this study, we aimed to elucidate the role of vTR in MDV-induced lymphomagenesis. Based on an infectious clone of the highly oncogenic MDV strain RB-1B (23), mutant MDV with functional deletions of either one or both copies of the diploid *vTR* gene were generated. Pathogenesis studies in chickens clearly showed that vTR is dispensable for early cytolytic replication but is required for efficient induction of T cell malignancies. Lymphoma incidences were drastically reduced in birds inoculated with mutant viruses lacking both copies of *vTR* when compared with parental virus or viruses with a deletion of only one copy of *vTR*. Lymphomas induced by the *vTR* double deletion viruses were significantly less disseminated and generally smaller in size than those induced by the parental virus or *vTR* single deletion viruses. Revertant viruses in which both copies of the previously deleted *vTR* gene were repaired showed oncogenic properties identical to those of the parental virus, confirming that vTR is required for efficient MDV-induced lymphomagenesis. Finally, we were able to show that vTR-overexpressing DF-1 cells exhibited characteristics of transformation. In summary, our results demonstrate that vTR plays an important role in MDV-induced malignant T cell lymphomagenesis and in promoting tumor dissemination, and provide further evidence that TR exhibits a pivotal function in telomerase-associated tumorigenesis.

RESULTS

Construction of MDV bacterial artificial chromosome (BAC) mutants

MDV mutants with a functional deletion of either one or both copies of the diploid *vTR* gene were generated from pRB-1B, an infectious BAC clone of the highly oncogenic MDV strain RB-1B (23). To generate *vTR* single deletion BAC mutants, pCR1-$2^{+/-}$ and pCR1-$4^{+/-}$, sequences encoding *vTR*, or more specifically the functionally essential CR1-2 or CR1-4, were deleted from the IR_L region of pRB-1B by Red mutagenesis (Fig. 1 B and Fig. S1 A, which is available at http://www.jem.org/cgi/content/full/jem.20052240/DC1). Mutant pCR1-$2^{-/-}$ and pCR1-$4^{-/-}$, in which both copies of the *vTR* gene in the IR_L and TR_L regions were disrupted by deletion of CR1-2 or CR1-4, respectively, were generated by two consecutive steps of Red mutagenesis and FLP recombination (24). FLP recombination resulted in removal of the previously introduced kanamycin-resistance gene (Fig. S1 A). Revertant genomes pCR1-$2^{-/-}$R1 and pCR1-$2^{-/-}$R2 from the pCR1-$2^{-/-}$ double deletion mutant were generated by a recently developed protocol using two-step Red mutagenesis that allows for markerless manipulation of BAC DNA (Fig. S1 B; reference 25). Genomic DNA of the generated mutant and revertant genomes was analyzed by PCR and by restriction fragment length polymorphism determination using BamHI digestion followed by Southern blotting using a *vTR*-specific probe. We confirmed that the expected genomic changes were present in the respective BACs and that no spurious rearrangements occurred during the genetic manipulations (Fig. 1, D and E).

vTR is expressed during lytic and latent MDV infection but is dispensable for MDV replication in vitro

To confirm that vTR is expressed during lytic virus infection and latency, total RNA was extracted from RB-1B–infected primary chicken embryo cells (CECs) or an RB-1B–transformed lymphoblastoid cell line (MDCC-UD14; reference 26), and RT-PCR was performed. Expression of vTR was detected in RB-1B–infected CECs and MDCC-UD14, but not in uninfected CECs or a retrovirus-transformed lymphoblastoid cell line (RECC-CU91; reference 27), which were used as controls (Fig. 2).

To assess growth properties of the generated recombinant viruses, parental vRB-1B and *vTR* mutant viruses, termed vCR1-$2^{+/-}$, vCR1-$4^{+/-}$, vCR1-$2^{-/-}$, and vCR1-$4^{-/-}$, were reconstituted from BAC DNA by transfection (28) and propagated on primary CECs. The in vitro replication properties of the mutant viruses were determined by analyzing

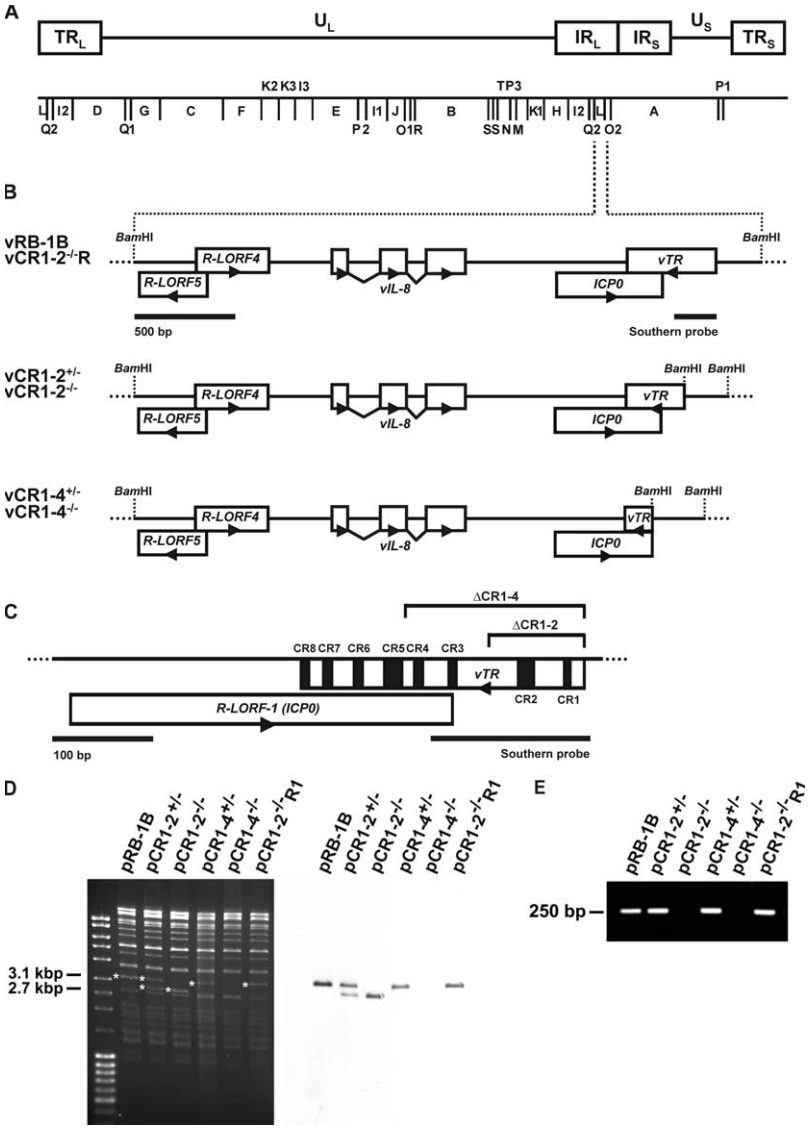

Figure 1. Genomic structure of *vTR* deletion and revertant viruses. (A) Schematic presentation of the genomic organization and the BamHI restriction map of MDV. The terminal and internal repeat long regions (TR_L, IR_L), the unique long region (U_L), the internal and terminal repeat short regions (IR_S, TR_S), and the unique short region (U_S) are shown. (B) Schematic presentation of the MDV BamHI-L fragment and the genes located therein. The parental (vRB-1B) and mutant viruses lacking either one (vCR1-$2^{+/-}$, vCR1-$4^{+/-}$) or both copies of *vTR* (vCR1-$2^{-/-}$, vCR1-$4^{-/-}$) are shown. BamHI restriction sites are given. (C) Detailed schematic presentation of the *vTR* gene and the putative *ICP0* ortholog. The eight CRs of *vTR* are given in black. Deletion of CRs 1 and 2 (ΔCR1-2) or 1 to 4 (ΔCR1-4) in the genome of *vTR* mutant viruses is indicated. Also shown is the location of sequences contained in the Southern blot probe. (D) Southern blot analysis of mutant MDV BACs. DNA of pRB-1B and mutant MDV BACs (pCR1-$2^{+/-}$, pCR1-$2^{-/-}$, pCR1-$4^{+/-}$, pCR1-$4^{-/-}$, and pCR1-$2^{-/-}$R1) was prepared, digested with BamHI, and separated on a 0.8% agarose gel. Southern blot analysis was performed using a PCR-generated digoxigenin-labeled probe using oligonucleotide primers vTRfw 5′-TGGCGGGTGGAAGGC-3′ and vTRrv 5′-CTGCGGGCGAGGACC-3′. Fragments detected by the *vTR* probe are indicated by asterisks, and sizes are given. (E) PCR analysis of the mutant BACs. *vTR*-specific sequences were amplified by using oligonucleotide primers vTRfw and vTRrv, and amplification products were separated on a 1% agarose gel. The size of the specific PCR product is given.

multistep growth kinetics and plaque sizes. Growth kinetics of all mutant viruses were virtually indistinguishable from those of parental vRB-1B (Fig. 3 A). Plaques induced by *vTR*$^-$ mutant viruses vCR1-$2^{-/-}$ and vCR1-$4^{-/-}$ were slightly, albeit insignificantly, smaller than those induced by vRB-1B or mutant viruses in which only one *vTR* copy was functionally deleted (vCR1-$2^{+/-}$ and vCR1-$4^{+/-}$; Fig. 3 B). The data clearly demonstrated that vTR is dispensable for lytic MDV replication in vitro and that no substantial growth defect in the absence of functional vTR was observed.

vTR is dispensable for efficient lytic MDV replication in vivo

In the next series of experiments, the growth properties of mutant MDV in chickens were assessed. To determine the

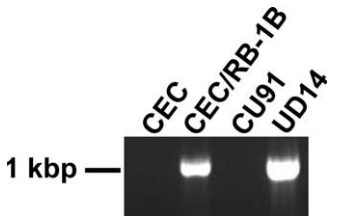

Figure 2. Expression of vTR in lytically infected CECs and latently infected and transformed lymphoma cells. An RT-PCR analysis of total RNA (2 μg) extracted from RB-1B–infected CECs or uninfected cells, as well as from lymphoblastoid cell lines RECC-CU91 and MDCC-UD14, is shown. RECC-CU91 is a lymphoblastoid cell line transformed by a retrovirus, reticuloendotheliosis virus. MDCC-UD14 is a T cell lymphoma cell line isolated from an RB-1B–infected bird. RT-PCR was performed using oligonucleotide primers, vTR(exp)fw 5′-GGCACACGTGGCGGGTGGAAGG-3′ and vTR(exp)Arv 5′-CAGTGCTGCGCCGATTCTAC-3′.

extent of lytic virus replication in the natural host, 1-d-old chickens were infected with either vRB-1B, vCR1-$2^{+/-}$, vCR1-$4^{+/-}$, vCR1-$2^{-/-}$, or vCR1-$4^{-/-}$. Whole blood samples were collected on 4, 7, 12, 15, 19, and 30 d after infection from all chickens of each group infected with the various viruses. Copy numbers of viral genomes were determined by quantitative real-time PCR (qPCR) amplification of a viral gene (gD) and normalized to chicken genome copies by the determination of the number of inducible nitric oxide synthetase (iNOS) copies (29). All viruses induced very similar levels of viremia until 15 d after infection, indicating that vTR is completely dispensable for the early cytolytic phase of MDV replication in vivo (Fig. 4). At 19 d after infection, viremia levels were higher in birds infected with vRB-1B, vCR1-$2^{+/-}$, or vCR1-$4^{+/-}$ when compared with those of birds infected with the *vTR* double deletion mutants. The differences, however, were not statistically significant and leveled off later in infection at 30 d after infection when virtually identical numbers of viral genome copies were detected in peripheral blood in all groups by qPCR (Fig. 4). From the results, we concluded that vTR is dispensable for lytic replication in vivo and that only minimal differences in normalized viral genome copy numbers were detected in chickens infected with either parental or vTR mutant viruses. In addition, the data clearly showed that vTR is also dispensable for the establishment of latency because sustained presence of MDV genomes in peripheral blood was observed >8 d after infection when MDV enters the latent state of infection (12).

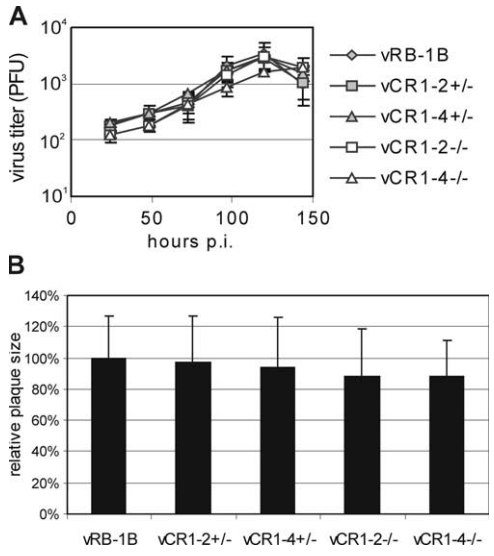

Figure 3. Growth properties in vitro of mutant viruses demonstrating that vTR is dispensable for MDV replication in vitro. (A) Growth kinetics of the indicated viruses. 10^6 CECs were infected with 100 PFU of vRB-1B or *vTR* mutant viruses. At the indicated times after infection, infected cells were dissociated by trypsinization and virus titers were determined by plating serial 10-fold dilutions of infected cells onto fresh CECs. Means and standard deviations (error bars) of three independent experiments are given. (B) Relative plaque sizes induced by the indicated viruses. Plaque areas were measured 7 d after infection after fixing cells with 90% acetone and performing indirect immunofluorescence using a convalescent MDV-specific chicken serum and Alexa Fluor 488 goat anti–chicken IgG (Invitrogen). For each virus, 100 randomly selected plaques were photographed with a digital camera (Zeiss Axiovert 25 and Axiocam; Carl Zeiss MicroImaging, Inc.), and plaque areas were determined using ImageJ software. Means and standard deviations (error bars) are given.

vTR is required for efficient MDV-induced lymphomagenesis

To determine whether vTR plays a role in MDV-induced lymphomagenesis, 1-d-old chickens were infected with parental or mutant viruses. Infected animals were monitored for development of disease, which is characterized by chronic wasting, paralysis, and lymphoma development, as well as mortality. Necropsied birds were scored for lymphoma incidence and dissemination, and the results of three independent animal experiments are summarized in Fig. 5. Significantly

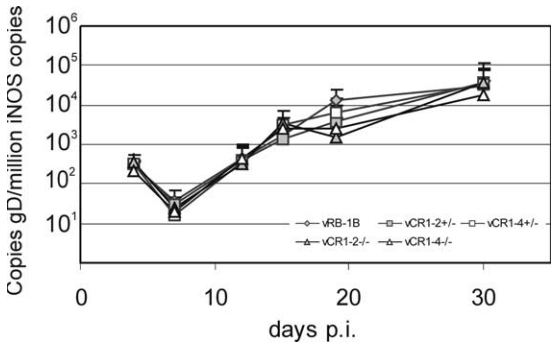

Figure 4. vTR is dispensable for lytic MDV replication in vivo and for the establishment of latency. A qPCR analysis of the viral *gD* gene and a host gene (*iNOS*) in total DNA isolated from whole blood samples, which were taken at days 4, 7, 12, 15, 19, and 30 after infection from virus-infected chickens, is shown. Mean viral load values and standard deviations (error bars) are given as copies of *gD* per 10^6 copies of chicken *iNOS*. No statistically significant differences in viremia levels were observed between groups infected with *vTR* single and double deletion viruses or parental vRB-1B.

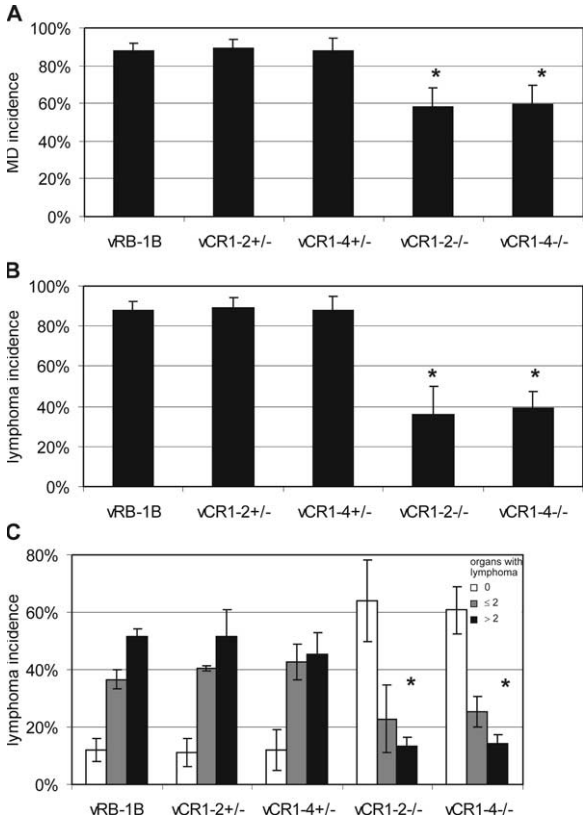

Figure 5. Mutant viruses lacking both copies of *vTR* are attenuated and severely impaired in their ability to induce lymphoma. In three independent experiments, chickens were inoculated with 500 PFUs of vRB-1B or *vTR* mutant viruses. During the course of the experiments, moribund birds were killed and necropsied. After a 9-wk observation period, all surviving birds were necropsied and evaluated for MD. Morbidity and lymphoma incidences were recorded. (A) MD incidence (percentage of birds showing clinical symptoms and/or lymphomatous lesions) in animal groups infected with the indicated viruses. Means and standard deviations (error bars) are given. The decreased MD incidences observed in animal groups infected with vCR1-$2^{-/-}$ and vCR1-$4^{-/-}$ relative to groups infected with vRB-1B, vCR1-$2^{+/-}$, or vCR1-$4^{+/-}$ were statistically significant (*) as follows: vRB-1B versus vCR1-$2^{-/-}$, $P = 0.0117$; vRB-1B versus vCR1-$4^{-/-}$, $P = 0.0117$; vCR1-$2^{+/-}$ versus vCR1-$2^{-/-}$, $P = 0.0055$; vCR1-$2^{+/-}$ versus vCR1-$4^{-/-}$, $P = 0.0055$; vCR1-$4^{+/-}$ versus vCR1-$2^{-/-}$, $P = 0.0055$; vCR1-$4^{+/-}$ versus vCR1-$4^{-/-}$, $P = 0.0055$. (B) Lymphoma incidences (percentage of birds showing lymphomatous lesions) in groups infected with the indicated viruses are given as means and standard deviations (error bars). The decreased lymphoma incidences detected in groups infected with vCR1-$2^{-/-}$ and vCR1-$4^{-/-}$ relative to groups infected with vRB-1B, vCR1-$2^{+/-}$, or vCR1-$4^{+/-}$ were statistically significant (*) as follows: vRB-1B versus vCR1-$2^{-/-}$, $P = 0.0117$; vRB-1B versus vCR1-$4^{-/-}$, $P = 0.0117$; vCR1-$2^{+/-}$ versus vCR1-$2^{-/-}$, $P = 0.0055$; vCR1-$2^{+/-}$ versus vCR1-$4^{-/-}$, $P = 0.0055$; vCR1-$4^{+/-}$ versus vCR1-$2^{-/-}$, $P = 0.0055$; vCR1-$4^{+/-}$ versus vCR1-$4^{-/-}$, $P = 0.0055$. (C) Lymphomas induced by mutant viruses lacking both copies of *vTR* have an altered dissemination pattern within individual chickens. Organ manifestations of lymphomatous lesions in individual birds were recorded in three independent animal experiments, and percentages are given of birds infected with the indicated viruses, which failed to develop lymphoma (0) or in which less than or more than two sites of lymphoma manifestation were observed. Means and standard deviations (error bars) are given. The reduced disseminations of lymphomas detected in groups infected with vCR1-$2^{-/-}$ and vCR1-$4^{-/-}$ relative to groups infected with vRB-1B, vCR1-$2^{+/-}$ or vCR1-$4^{+/-}$ were statistically significant (*) as follows: vRB-1B versus vCR1-$2^{-/-}$, $P = 0.0025$; vRB-1B versus vCR1-$4^{-/-}$, $P = 0.0033$; vCR1-$2^{+/-}$ versus vCR1-$2^{-/-}$, $P = 0.0022$; vCR1-$2^{+/-}$ versus vCR1-$4^{-/-}$, $P = 0.0029$; vCR1-$4^{+/-}$ versus vCR1-$2^{-/-}$, $P = 0.0143$; vCR1-$4^{+/-}$ versus vCR1-$4^{-/-}$, $P = 0.0188$.

reduced incidences of MD were observed in groups infected with the *vTR* double deletion mutants vCR1-$2^{-/-}$ (57.8%) and vCR1-$4^{-/-}$ (59.7%), relative to groups infected with vRB-1B (88.1%) or either of the *vTR* single deletion mutants (vCR1-$2^{+/-}$, 89.4%; vCR1-$4^{+/-}$, 88.0%; Fig. 5 A). As expected, no clinical or pathological signs of MD were observed in mock-infected birds used as a negative control in experiment 1 (not depicted). All animals infected with vRB-1B, vCR1-$2^{+/-}$, or vCR1-$4^{+/-}$ that developed MD also showed lymphoma development. In stark contrast, in both the vCR1-$2^{-/-}$ and vCR1-$4^{-/-}$ groups, some of the birds that presented with clinical signs of MD, such as neurological signs, reduced weight gain, and wasting, did not develop cancerous lesions. Accordingly, only 36.1 and 39.3% of birds infected with vCR1-$2^{-/-}$ and vCR1-$4^{-/-}$, respectively, developed lymphomas compared with 88.1, 89.4, and 88.0% of birds infected with vRB-1B, vCR1-$2^{+/-}$, or vCR1-$4^{+/-}$ (Fig. 5 B). Furthermore, lymphomas induced by viruses harboring at least one intact copy of *vTR* predominantly disseminated to multiple organ sites, whereas the majority of lesions induced by vCR1-$2^{-/-}$ and vCR1-$4^{-/-}$ affected no more than two organs (Fig. 5 C and Fig. S3, which is available at http://www.jem.org/cgi/content/full/jem.20052240/DC1). Generally, lesions induced by vCR1-$2^{-/-}$ and vCR1-$4^{-/-}$ were considerably smaller than those induced by vRB-1B, vCR1-$2^{+/-}$, or vCR1-$4^{+/-}$ (Fig. S3).

To confirm that the observed phenotype of the *vTR*$^-$ viruses, characterized by a reduction in lymphoma incidence and dissemination, was actually caused by the functional deletion of *vTR*, oncogenic properties of two independently isolated revertant viruses in which both copies of *vTR* were repaired to parental sequences were investigated. Oncogenicity of revertant viruses vCR1-$2^{-/-}$R1 and vCR1-$2^{-/-}$R2 was assessed in an additional animal experiment and compared with parental vRB-1B as well as the respective CR1-2 single and double deletion mutants, vCR1-$2^{+/-}$ and vCR1-$2^{-/-}$. The results of the experiment clearly demonstrated that both revertant viruses were indistinguishable from parental vRB-1B virus with respect to viremia levels as well as lymphoma incidence and dissemination (Fig. 6 and Fig. S2, which is available at http://www.jem.org/cgi/content/full/jem.20052240/DC1).

Collectively, the results of the animal experiments clearly demonstrated that expression of vTR is critical for efficient MDV-induced T cell lymphomagenesis and substantially promotes invasiveness of MD lymphomas.

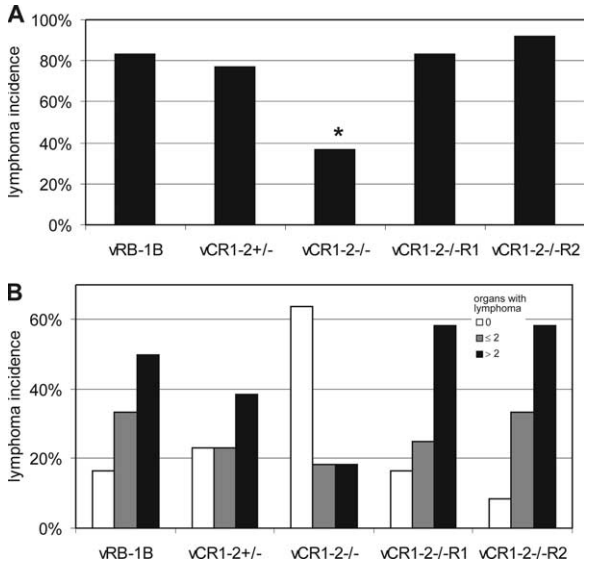

Figure 6. Lymphoma incidences (A) and dissemination patterns (B) in chickens infected with parental, *vTR* mutant, and *vTR* revertant viruses. Lymphoma incidences are given as the percentage of birds having lymphomatous lesions. Dissemination patterns are shown as the percentages of birds that failed to develop lymphoma (0) or in which less than or more than two sites of lymphoma manifestation were observed. The decreased lymphoma incidence detected in groups infected with vCR1-$2^{-/-}$ relative to groups infected with vRB-1B, vCR1-$2^{+/-}$, vCR1-$2^{-/-}$R1, or vCR1-$2^{-/-}$R2 were statistically significant (*) as follows: vCR1-$2^{-/-}$ versus vRB-1B, $P = 0.0361$; vCR1-$2^{-/-}$ versus vCR1-$2^{+/-}$, $P = 0.0453$; vCR1-$2^{-/-}$ versus vCR1-$2^{-/-}$R1, $P = 0.0361$; vCR1-$2^{-/-}$ versus CR1-$2^{-/-}$R1, $P = 0.0094$.

DF-1 cells overexpressing vTR exhibit a partially transformed phenotype

The results of the animal experiments led us to explore the transforming potential of vTR in vitro. A DF-1–based cell line constitutively overexpressing vTR (DF-1vTR) was generated. Growth characteristics of DF-1vTR were analyzed and compared with those of a vector control cell line (DF-1vector) and a cell line constitutively expressing Meq (DF-1Meq). The DF-1Meq cell line was used as a positive control because the Meq oncoprotein was shown to possess transforming and mitogenic properties (15, 19, 30).

First, growth rates of the different recombinant cell lines were determined (Fig. 7 A). Until 72 h after seeding, DF-1Meq cells showed the highest population doubling levels of all three cell lines analyzed. At 96, 120, and 144 h after seeding, however, DF-1vTR cells exhibited the highest population doubling levels, and cells overexpressing vTR grew to a significantly higher saturation density than both DF-1vector and DF-1Meq cells. Compared with DF-1vTR, saturation densities of DF-1vector and DF-1Meq cells were reduced by 38 and 7%, respectively.

Second, soft agar colony formation assays were performed (Fig. 7, B and C). All three cell lines were able to form colonies in nutrient agar with the same cloning efficiency of ~3% (Fig. 7 B). Expression of Meq resulted in significantly enlarged soft agar colonies, which have been described (15, 19), and average colony sizes formed by DF-1Meq cells were increased by 64.6% relative to those formed by DF-1vector (Fig. 7 C). Similarly, DF-1vTR cells formed significantly enlarged colonies (21.2%) when compared with those formed by the DF-1vector cells (Fig. 7 C).

Third, morphological phenotypes of DF-1vector, DF-1vTR, and DF-1Meq cells were analyzed by phase contrast microscopy. Overexpression of vTR in DF-1vTR cells was accompanied by clearly identifiable changes in cellular morphology toward a more refractile and spindle-shaped phenotype as compared with DF-1vector cells. These morphological alterations of DF-1vTR cells were very similar to those seen in DF-1Meq cells (Fig. 7 D).

We also asked whether vTR overexpression would result in up-regulation of telomerase activity, resulting in altered telomere lengths in DF-1vTR cells. Terminal restriction fragment lengths of chromosomal DNA prepared from DF-1vector, DF-1vTR, and DF-1Meq cells were compared by Southern blot hybridization using a telomere-specific probe. The results of the terminal restriction fragment analysis clearly indicated that the lengths of telomeres in these different cell lines were indistinguishable from one another (Fig. S4 C, available at http://www.jem.org/cgi/content/full/jem.20052240/DC1). Prompted by a recent report suggesting that TR may have a malignancy-promoting function mediated by the up-regulation of integrin αv (31), we examined expression of this cell adhesion receptor molecule and that of MHC class I as a control in the generated DF-1vTR cell line. On average, integrin αv expression was twofold higher (range: 1.55- to 2.69-fold in three independent experiments) in DF-1vTR cells relative to DF-1vector or DF-1Meq cells (Fig. 7 E). In contrast, MHC class I expression was virtually identical in DF-1vTR and DF-1vector cells, whereas it was up-regulated 1.84-fold (range: 1.68- to 1.94-fold) in Meq-expressing cells when compared with DF-1vector or DF-1vTR cells (Fig. 7 E). These latter results are in agreement with microarray data of an independently generated DF-1Meq cell line for which up-regulation of MHC class I transcription was reported (19).

From the results of the cell proliferation and soft agar colony formation assays as well as the examination of cellular morphologies, we concluded that MDV vTR is clearly able to promote cell growth, induces increased levels of integrin αv expression, and exhibits a potential for transformation, which is comparable to that of the MDV Meq oncoprotein.

DISCUSSION

Telomerase activity is strongly correlated with cancer development and implicated in the process of cellular immortalization and oncogenesis. An association of TR up-regulation and malignancy has been described in several reports (32–34). Recently, it was shown that TR is required for the tumor-promoting effects of TERT overexpression (35), and that RNAi-mediated depletion of TR is effective in inhibiting cancer cell growth independently of its function in maintaining telomere

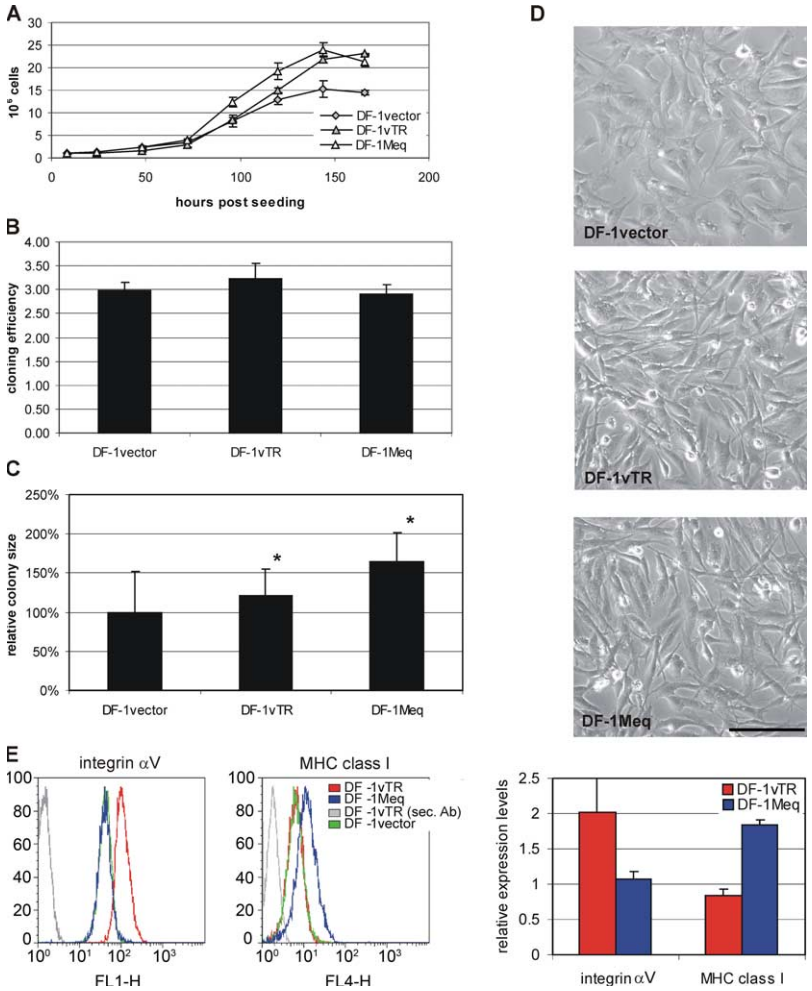

Figure 7. DF-1 cells overexpressing vTR exhibit a partially transformed phenotype and elevated integrin αv expression levels. (A) Growth rates of DF-1vector, DF-1vTR, and DF-1Meq cell lines. Cells were seeded in duplicate at a density of 10^5 in 12-well dishes. At 8, 24, 48, 72, 96, 120, and 144 h after plating, cells were trypsinized and viable cells were counted using a hemocytometer. Means and standard deviations (error bars) of three independent experiments are given. At 144 h after plating, the average numbers of viable cells of the different recombinant cell lines were statistically significantly different as follows: DF-1vector versus DF-1vTR, $P < 0.0001$; DF-1vector versus DF-1Meq, $P < 0.0001$; DF-1vTR versus DF-1Meq, $P = 0.0155$. (B) Anchorage-independent growth of DF-1vector, DF-1vTR, and DF-1Meq cells in soft agar as determined by colony formation after 4 wk of incubation at 37°C. The cloning efficiencies (percentages of cells forming colonies after 3 wk) of the indicated cell lines are shown. Means and standard deviations (error bars) of three independent experiments are given. No statistically significant differences in cloning efficiencies between the cell lines were observed. (C) Relative sizes of soft agar colonies formed by DF-1vector, DF-1vTR, and DF-1Meq. For each of the indicated cell lines, 100 randomly selected colonies were photographed with a digital camera and colony diameters were determined using ImageJ software. Means and standard deviations (error bars) are given. The average colony sizes formed by DF-1vector, DF-1vTR, and DF-1Meq cells were statistically significantly different as follows: DF-1vector versus DF-1vTR, $P = 0.0021$; DF-1vector versus DF-1Meq, $P < 0.0001$; DF-1vTR versus DF-1Meq, $P = 0.0007$. (D) Morphological phenotypes of DF-1vector, DF-1vTR, and DF-1Meq cells photographed under an inverted microscope (Zeiss Axiovert 25; Carl Zeiss MicroImaging, Inc.). Bar, 50 μm. (E) Integrin αv expression was higher in DF-1vTR relative to DF-1vector or DF-1Meq cells, whereas MHC class I expression was up-regulated in DF-1Meq cells. Cell lines were trypsinized and incubated with a mixture of a 1:2,000 dilution of a rabbit polyclonal antibody directed against integrin αv (Chemicon) and a 1:200 dilution of anti–chicken MHC class I monoclonal antibody C6B12 (Developmental Study Hybridoma Bank, University of Iowa). The secondary antibodies used were anti–rabbit IgG Alexa 488 (Invitrogen) and anti–mouse IgG Cy5 (Jackson ImmunoResearch Laboratories), used at 1:200 dilutions, respectively. Cells were examined using FACScan (Becton Dickinson), and data were analyzed using FlowJo, version 5.7.2 for Windows (Tree Star). A representative experiment of integrin αv expression (left) and MHC class I expression (middle) is shown. Means and standard deviations of mean channel of fluorescence ratios of integrin αv and MHC class I expression of DF-1vTR and DF-1Meq cells relative to DF-1 vector cells of three independent experiments are also given (right).

length (31, 36). A direct involvement of TR in tumorigenesis, however, has not yet been reported. Here we describe a critical role for a virus-encoded TR in malignant T cell lymphomagenesis induced by the herpesvirus MDV. This, to our knowledge, is the first evidence for tumor-promoting effects of TR activity in a natural small animal model.

Four different mutant MDVs lacking either one or both copies of the diploid *vTR* gene showed virtually no defect in viral replication in vitro when compared with parental virus. We also discovered that vTR is completely dispensable for the early lytic phase of viral replication in the natural chicken host. MDV infection switches from the cytolytic to latent phase from around 8 d after infection (12). Because parental and mutant viruses replicated to similar levels until at least 15 d after infection, our data clearly indicate that vTR is dispensable not only for early lytic replication of MDV, but also for establishment of latency, an essential prerequisite for MDV-induced transformation and lymphomagenesis. A trend of higher levels of viral genomic copy numbers was observed in birds infected with the parental virus compared with those infected with *vTR* double deletion viruses at 19 d after infection. Although the differences were not statistically significant, the lower MDV copy numbers may reflect a difference in the number of transformed T cells between animals that were inoculated with vTR^+ or vTR^- viruses. MDV-induced transformation of T cells results in massive proliferation between 14 and 20 d after infection. Viremia levels induced by all five viruses tested were very similar at 30 d after infection. This finding seems to suggest that absence of vTR does not result in significantly reduced T cell proliferation; however, it is known that the later phases of MDV infection are characterized by alternating stages of latent infection and reactivation, and the detection of relatively high viral genome numbers in birds infected with the vTR^- viruses may reflect lytic replication. This latter interpretation is supported by the fact that birds in these groups—although clearly less afflicted by lymphoma formation and particularly tumor dissemination—still developed MD characterized by immunosuppression and chronic wasting that is associated with lytic destruction of B and T lymphocytes by reactivated virus (12). Collectively, the data clearly indicate that the tumor-promoting effects of vTR expression cannot be attributed to a functional role in MDV replication per se, or in the establishment or reactivation from latency, but are caused by events downstream of primary establishment of infection.

Although lytic virus replication and entry of the latent state did not seem grossly affected in the absence of vTR, mutant viruses lacking both copies of either CR1 and CR2 or CR1 to CR4 of *vTR* were significantly impaired in their ability to induce lymphoma and displayed a virtually identical phenotype in vivo. This finding is remarkable because deletion of the CR1 to CR4 in the vCR1-$4^{-/-}$ mutant also affected the 3′ end of a predicted open reading frame (R-LORF1, MDV002), which shares homology to herpesviral ICP0 orthologs. Therefore, the putative MDV ICP0 ortholog, if expressed, is not required for virus lytic replication and reactivation from latency, functions for which ICP0 proteins of other herpesviruses are essential (37–39).

Lymphoma incidences were reduced by ∼60% in the case of the double deletion viruses, but the number of birds with MD symptoms was only reduced by ∼40% compared with groups infected with viruses that still expressed one intact copy of *vTR*. MD in chickens can develop without formation of lymphoma, which was in fact associated with the disease syndrome only >20 yr after the first description of the disease (40, 41). Impaired lymphoma formation in animals infected with vTR^- viruses in the presence of unabated lytic and latent infection strongly suggests a direct role for vTR in MDV-induced lymphomagenesis.

A functional telomerase complex consisting of vTR and chicken TERT was shown to have threefold enhanced telomere amplification activity over the chTR–chicken TERT complex (21). It is therefore possible that the tumor-promoting function of vTR is linked to its ability to form a functional telomerase complex with chicken TERT. It was also shown, however, that telomere elongation is not an absolute requirement for tumor formation in some cases and that activation of telomerase can promote tumorigenesis independently of this function of telomerase (9–11). We currently favor a model in which the lymphomagenic activity of vTR is independent of telomere length for the following main reasons. First, MDV infects young birds and causes tumors starting from 14 d after infection when the condition of telomeres should not be a factor for cell survival. Second, telomerase is commonly up-regulated in activated T cells, which are the target for MDV-induced oncogenic transformation (42–44). Third, we did not observe obviously altered telomere lengths either in birds infected with the vTR^- viruses at later times after infection (unpublished data) or in DF-1vTR cells constitutively expressing vTR (Fig. S4 C). It is worthwhile to note in this context that it was recently described that expression of TR is vital for the tumor-promoting effects of TERT overexpression independent of telomere maintenance (35). In a murine skin tumorigenesis model, mice overexpressing TERT in a TR^- background (K5-TERT/$TERC^{-/-}$) were significantly impaired in their ability to develop papillomas (35). Moreover, the size and number of cancerous lesions per animal were significantly reduced in *TR* knockout mice when compared with *TR*-expressing K5-TERT mice (35). These findings are fully consistent with our results on the incidence, dissemination, and size of tumors induced by MDV that are unable to express vTR. Lymphomas caused by VTR^- viruses exhibited significantly reduced dissemination compared with that induced by VTR^+ viruses. Furthermore, we found that neoplastic lesions induced by the latter viruses were more pronounced than those induced in the absence of *vTR*.

A positive correlation between TR expression and cancer malignancy has been reported in numerous clinical studies (32–34, 45), and TR expression levels are considered a valuable diagnostic and prognostic marker in clinical oncology (46, 47). Although a direct causal connection between TR expression and cancer malignancy is not yet conclusively established, our data in the MDV-chicken model clearly indicate that aggressiveness of MDV-induced lymphomas is closely related to a functional role of TR in promoting lymphoma dissemination and cancer cell growth. One possible mechanistic explanation of the modulation of tumor

dissemination by (v)TR may be the up-regulation of integrin αv expression, which is an important factor in progression and metastasis of several different malignancies (48). We were able to establish a link between MDV vTR and integrin αv expression by demonstrating increased levels of the protein in a vTR-expressing cell line. These results, in the context of findings in EBV-transformed lymphomblastoid cell lines in which latent gene products were shown to transactivate integrin αv promoters and up-regulate expression (49), may indicate that cell adhesion receptors containing this particular α subunit are important for lymphomagenesis, more specifically for dissemination and homing of transformed lymphocytes. It was also demonstrated that RNAi-mediated TR knockdown induced decreased expression levels of cyclin G2 and integrin αv, and resulted in a rapid inhibition of cancer cell growth (31). In future studies, we will seek to corroborate the findings of vTR-mediated up-regulation of integrin αv by examination of peripheral blood mononuclear cells and lymphoma cells derived from animals infected with the various *vTR* mutant viruses and elucidate the signaling pathways involved in this putative function of MDV-encoded vTR.

Our observation that vTR is directly involved in T cell lymphomagenesis as well as the spread and homing of transformed cells was further supported by data on the growth-promoting effects of vTR in DF-1 cells in vitro. DF-1 is a continuous nontransformed chicken cell line that has been extensively used for studying transformation by avian retroviral oncogenes (50, 51). We observed that DF-1vTR cells exhibited growth rates similar to those of DF-1 cells transformed by the MDV oncoprotein Meq, a member of the Jun/Fos oncoprotein family that has previously been described to exhibit transforming properties (15, 19). DF-1 cells expressing vTR or the Meq oncoprotein grew to significantly increased saturation densities compared with control cells. In addition, we noticed that they both displayed a more refractile and spindle-shaped morphological phenotype and formed larger colony sizes in soft agar, which are common indicators for transformed cells. Unlike expression of Meq, expression of vTR exerted only a minor effect on anchorage-independent DF-1 cell growth, but it is possible that an effect of vTR was partially masked by the inherent potential of DF-1 cells to form colonies in nutrient agar (51). However, the data support the hypothesis that assigns to vTR a role in promoting anchorage-independent cell growth that is possibly, at least in part, mediated by increased levels of integrin αv. Future experiments will focus on a more detailed functional characterization of the effects of vTR in vitro and in vivo, and a possible cooperation of vTR and the MDV oncoprotein Meq in lymphoma formation and dissemination.

MATERIALS AND METHODS

MDV reconstitution and propagation. CECs were prepared from 11-d-old specific pathogen-free embryos by standard methods (28). Cells were maintained in DMEM supplemented with 10% FBS and grown at 37°C under a 5% CO_2 atmosphere. Parental vRB-1B, *vTR* mutant, and revertant viruses were generated from pRB-1B, an infectious full-length BAC clone of the highly oncogenic MDV strain RB-1B (23). Recombinant viruses were reconstituted by $CaPO_4$ transfection of BAC DNA into CECs (28). Low-passage virus stocks were aliquoted and frozen in liquid nitrogen until use. Virus propagation as well as determination of virus growth kinetics and plaque sizes were performed exactly as described previously (52, 53).

Red mutagenesis and Flp recombination of MDV BACs. One- or two-step Red mutagenesis (54, 55) was used for site-directed deletion or repair of *vTR* in pRB-1B. Details of the performed recombinations are shown in Fig. S1. *Escherichia coli* EL250 cells (provided by N. Copeland, National Cancer Institute, Frederick, MD) harboring pRB-1B or its derivatives were used for all manipulations. Generally, 100 ng of a linear DNA was electroporated (1.25 kV/cm, 200 Ω, 25 μF) into 50 μl of recombination-competent cells exactly as described previously (53). For deletion of *vTR*, a PCR product comprising a kanamycin-resistance gene (*kan*^{R}) flanked by Flp recombinase recognition target (*FRT*) sites was used. To allow subsequent removal of the selectable marker by homologous recombination of the flanking *FRT* sites, FLP expression in EL250 cells was induced by growing bacterial clones for 12 h at 30°C in liquid Luria-Bertani medium containing chloramphenicol and 0.2% arabinose.

Previously deleted *vTR* sequences were reintroduced into double deletion mutant pCR1-2$^{-/-}$ by two-step Red mutagenesis. The procedure was performed exactly as described recently for introduction of long sequence stretches (Fig. S1 B; reference 25). A transfer cassette containing *vTR* sequences, *kan*^{R}, and an I-SceI site was released from recombinant plasmid pCUC*vTR* by cleavage with I-CeuI and introduced into pCR1-2$^{-/-}$ by a first Red recombination. The insertion of the transfer cassette resulted in duplication of a short sequence stretch within *vTR*, which was used to remove the resistance gene by a second Red recombination and, ultimately, scarless repair of authentic *vTR* sequences (25). Using this two-step ("en passant") mutagenesis protocol, two independently generated revertant BACs were generated and termed pCR1-2$^{-/-}$R1 and pCR1-2$^{-/-}$R2, respectively.

Animal experiments. All animal experiments were approved by Cornell's Institutional Animal Care and Use Committee (internal approval no. 2002-85). 1-d-old P2a chickens (56) were inoculated by i.m. injection with 500 PFUs of the various viruses. Mock-infected birds received an injection of uninfected CECs in culture medium. Experiment 1 included five groups (vRB-1B, vCR1-2$^{+/-}$, vCR1-2$^{-/-}$, vCR1-4$^{+/-}$, and vCR1-2$^{-/-}$) of 15 birds as well as an additional mock-infected group. In experiments 2 and 3, 10 and 12 birds per group, respectively, were infected. In experiment 3, whole blood was obtained from all birds at days 4, 7, 12, 15, 19, and 30 after infection by wing vein puncture to examine in vivo virus replication by qPCR exactly as described previously (29, 54). Experiment 4 included five groups (vRB-1B, vCR1-2$^{+/-}$, vCR1-2$^{-/-}$, vCR1-2$^{-/-}$R1, and vCR1-2$^{-/-}$R2) of 12 birds per group.

Establishment of recombinant cell lines. DF-1 cells (50) were maintained in M199 medium supplemented with 10% FBS, 2 mM L-glutamine, and 2 mM sodium pyruvate and grown at 37°C under a 5% CO_2 atmosphere. Recombinant cell lines DF-1vector, DF-1vTR, and DF-1Meq were generated by transfecting expression vectors pBKCMV (Stratagene), pBKCMV-vTR, or pBKCMV-Meq into DF-1 cells using lipofectamine (Invitrogen). pBKCMV-vTR and pBKCMV-Meq were constructed by cloning the respective full-length coding regions of MDV strain RB-1B into pBKCMV. Stable transfectants were selected and maintained by the addition of 300–700 μg/ml G418 (Invitrogen) to the culture medium.

Cell growth rates. To analyze cellular growth, cells of each of the recombinant lines were seeded in 12-well culture plates at a density of 10^5 per well. At 8, 24, 48, 72, 96, 120, and 144 h after plating, cells were dissociated by trypsinization, and the total number of viable cells was determined using a Neubauer hemocytometer under a light microscope (CK 2; Olympus). Cell viability was assessed by the trypan blue exclusion method.

Soft agar colony formation assays. To analyze anchorage-independent cell growth in soft agar, 10^3–10^4 cells of each of the DF-1–based cell lines were suspended in 3 ml M199 complete and 0.35% Bacto agar and plated in triplicate onto a single dish of a six-well culture plate containing a 3-ml bottom layer of the same medium containing 0.5% Bacto agar. Cultures were incubated at 37°C under a 5% CO_2 atmosphere and supplemented with nutrient agar every 4 d. After 4 wk of incubation, colonies of >50 cells were counted under an inverted microscope (CK 2; Olympus). For each of the cell lines, 100 randomly selected colonies were photographed with a digital camera and colony diameters were determined using ImageJ software (http://rsb.info.nih.gov/ij/index.html).

Statistical analyses. All statistical calculations were performed using SAS v8.2 for Windows (SAS Institute). Animal experiments performed in triplicate (experiments 1–3) were evaluated with nonparametric analyses, and the determination of globally significant differences between groups was done using the Kruskal-Wallis test before individual comparisons of groups were done by pairwise testing with adjusted p-values. Fisher's exact test was used to analyze animal experiment 4.

Online supplemental material. Fig. S1 shows the construction of *vTR*$^-$ and revertant MDV genomes. Fig. S2 summarizes viremia levels determined in animal experiment 4 in which revertant viruses were compared with parental and *vTR* mutant viruses. Fig. S3 displays representative gross pathological findings of chickens infected with *vTR*$^+$ or *vTR*$^-$ viruses, respectively. Stable expression of vTR and Meq in recombinant DF-1 cells analyzed by RT-PCR and indirect immunofluorescence, respectively, is shown in Fig. S4, as is a Southern blot determining telomere lengths. Figs. S1–S4 are available at http://www.jem.org/cgi/content/full/jem.20052240/DC1.

S. Trapp and N. Osterrieder would like to dedicate this paper to the memory of their late mentor, Oskar-Rüger Kaaden. We thank Kerstin Osterrieder for statistical analyses and advice as well as Laetitita Fragnet (Centre de Recherche INRA de Tours, France) for inspiring discussions. We are indebted to Barry L. Wanner and Neal G. Copeland for providing recombinant plasmids and *E. coli* strains.

This study was in part supported by United States Department of Agriculture grants 2003-02234 and 2005-01806 to N. Osterrieder, as well as 2004-01577 to J.P. Kamil.

The authors have no conflicting financial interests.

Submitted: 7 November 2005
Accepted: 6 April 2006

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