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A single amino acid substitution in the mRNA capping enzyme λ2 of a mammalian orthoreovirus mutant increases interferon sensitivity

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ARTICLE INFO

Article history:
Received 2 December 2014
Returned to author for revisions 16 December 2014
Accepted 23 April 2015
Available online 15 May 2015

Keywords:
Reovirus
Interferon
Reverse genetics
RNA capping
RNA methyltransferase

ABSTRACT

In the last few years, the development of a plasmid-based reverse genetics system for mammalian reovirus has allowed the production and characterization of mutant viruses. This could be especially significant in the optimization of reovirus strains for virotherapeutic applications, either as gene vectors or oncolytic viruses. The genome of a mutant virus exhibiting increased sensitivity to interferon was completely sequenced and compared with its parental virus. Viruses corresponding to either the parental or mutant viruses were then rescued by reverse genetics and shown to exhibit the expected phenotypes. Systematic rescue of different viruses harboring either of the four parental genes in a mutant virus backbone, or reciprocally, indicated that a single amino acid substitution in one of λ2 methyltransferase domains is the major determinant of the difference in interferon sensitivity between these two viruses.

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Introduction

Mammalian reoviruses are among a plethora of viruses that are presently under investigation for their ability to preferentially infect and destroy transformed/cancer cells. This has led to recent progress in their clinical use against various forms of human cancers (briefly reviewed among others by Kelly et al., 2009; Black and Morris, 2012). In contrast to most other “oncolytic” viruses presently under consideration for clinical use, reoviruses are naturally oncolytic and have not been submitted to genetic manipulation for their clinical use. In fact, it was not possible until recently (Kobayashi et al., 2007) to easily manipulate the reovirus genome using a plasmid-based reverse genetics system.

The initial observations that led to the utilization of reovirus as an “oncolytic” virus suggested that a decreased activity of the interferon-inducible double-stranded RNA-dependent protein kinase (PKR), which occurs upon Ras-induced cell transformation, was responsible for the preferential infection and destruction of cancer cells by reovirus (Strong et al., 1998). Despite the fact that multiple steps of the reovirus replicative cycle are likely involved in preferential replication and virus-induced cell death (Lemay et al., 2007; Marcato et al., 2007), further work has supported the idea that a lower induction of interferon in Ras-transformed cells favors viral propagation and could be of major importance to the virus’ oncolytic activity (Shmulevitz et al., 2010).

To further support the idea that interferon-induced antiviral pathways are critical in the ability of reovirus to discriminate between parental and Ras-transformed cells, a mutant virus obtained by chemical mutagenesis was selected for its increased sensitivity to interferon and was actually shown to exhibit a more complete blockage than the wild-type virus in parental cells, even at extremely high multiplicity of infection (Rudd and Lemay, 2005).

Multiple reovirus genes are suspected to be involved in the control of the interferon innate immune response (Sherry, 2009; Sherry et al., 2009). The μ2 protein was shown to be the major determining factor in the control of interferon signaling induction by altering the subcellular localization of IRF-9 (Irvin et al., 2012; Zurney et al., 2009). The μ3 protein could rather affect interferon sensitivity due to its ability to sequester the dsRNA activator of the interferon-inducible protein kinase PKR; however, this property of μ3 could be modulated by μ1 since the μ3-μ1 heterohexamer does not appear to bind dsRNA (Schiff, 2008). Finally, at least in some cell types, the σ2 and λ2 protein were previously suspected to play a role in the control of either induction of interferon signaling or virus’ sensitivity to interferon (Sherry et al., 1998).

In order to clarify which genetic determinants are involved in the large difference in interferon sensitivity between the parental and previously identified P4L-12 mutant virus (Rudd and Lemay, 2005), the complete sequence of the viral genome was determined. Both viruses were then reconstructed by site-directed mutagenesis and reverse genetics, since a number of differences
were observed between the isolate used to construct the reverse genetics plasmids and the parental laboratory stock initially used to isolate the mutant virus. Each four genes exhibiting differences between the parental and mutant viruses were then individually substituted either to replace the mutant gene by its parental counterpart or replacing the parental gene by its mutant counterpart in the parental background. This clearly demonstrated that the single amino acid substitution in one of the two methyltransferase domains of λ2 is the major determinant of the difference in interferon sensitivity between these two viruses.

**Results**

**Sequencing of the interferon-sensitive P4L-12 mutant**

In order to determine the differences in sequence between the interferon-sensitive P4L-12 mutant (Rudd and Lemay, 2005) and the original wild-type type 3 Dearing laboratory stock used in the present study (referred to as T3DS) and derived mutant P4L-12.

Amino acids differences between wild-type reovirus type 3 Dearing laboratory stock (T3DS) and derived mutant P4L-12.

Table 1 Amino acids differences between different reovirus type 3 Dearing.  

| Amino acid position | T3DS | T3D^w | T3D^K | T3D^S | Reolysin^a | Amino acid position |
|---------------------|------|-------|-------|-------|------------|-----------------|
| λ2                  | M    | L     | 979   |       |            |                 |
| μ1                  | P    | S     | 315   |       |            |                 |
| μNS                 | V    | A     | 705   |       |            |                 |
| σ3                  | M    | I     | 221   |       |            |                 |

Table 2 Amino acids differences between different reovirus type 3 Dearing.

| Amino acid position | T3D^w | T3D^K | T3D^K | T3D^S | Reolysin^a | Amino acid position |
|---------------------|-------|-------|-------|-------|------------|-----------------|
| λ3                  | M     | L     | 979   |       |            |                 |
| S                   | S     | R     | 1045  |       |            |                 |
| N                   | S     | S     | S     | 1048  |            |                 |
| S                   | P     |       |       | 1186  |            |                 |
| λ2                  | G     | E     | E     | E     | 504       |                 |
| G                   | R     |       |       | 509   |            |                 |
| I                   | M     | M     | 1165  |       |            |                 |
| λ1                  | A     | T     | 201   |       |            |                 |
| I                   | S     | S     | S     | 500   |            |                 |
| A                   | T     |       |       | 574   |            |                 |
| Q                   |       |       |       | 852   |            |                 |
| M                   | V     |       |       | 982   |            |                 |
| G                   | D     |       |       | 1017  |            |                 |
| μ2                  | A     | V     | 122   |       |            |                 |
| S                   | P     | P     | P     | 208   |            |                 |
| R                   | Q     | Q     | Q     | 342   |            |                 |
| A                   | S     |       |       | 528   |            |                 |
| μNS                 | K     | E     | E     | E     | 180       |                 |
| A                   | V     | V     | 705   |       |            |                 |
| G                   | D     |       |       | 707   |            |                 |
| σ1                  | V     | A     | 22    |       |            |                 |
| T                   | I     |       |       | 249   |            |                 |
| Y                   | S     |       |       | 253   |            |                 |
| T                   | A     | A     | A     | 408   |            |                 |
| σ3                  | W     | R     | 133   |       |            |                 |
| G                   | K     |       |       | 198   |            |                 |
| E                   | D     | D     | D     | 229   |            |                 |

* T3D-Kobayashi, wild-type virus from the reverse genetics system (Kobayashi et al., 2007).
* T3D-Sandekian, original wild-type virus reported in this study.
* T3D-Wollenberg, wild-type virus (R124) used in van den Wollenberg et al. (2012).
* T3D-Hosseini, wild-type unpublished virus sequence from NCBI database.
* T3D-Reolysin^a, wild-type virus used in clinical trials as described in Chakrabarty et al. (2014).

Comparative sequence analysis was also performed with the sequence of another complete T3D genome (referred to as T3D^w) that was used in a recent study by another group (van den Wollenberg et al., 2012) and the sequence of another laboratory stock deposited in the NCBI database by one further group (referred to as T3D^K); finally the sequence of the virus currently used in oncolytic virotherapy under the tradename “REOLYSIN^w” was added to the comparison (Chakrabarty et al., 2014). Only complete sequences determined by direct sequencing of the viral genome were retained for this analysis since it is difficult to assume that sequences obtained earlier from cloned viral genes are necessarily representative and that they would actually generate a replication-competent virus when combined together.

The laboratory subtype, T3D^K, did not display any more differences from T3D^w when compared to other completely sequenced genomes. While some positions are clearly more variable, all stocks showed the presence of 8 to 19 differences including 2–8
unique substitutions compared to T3D<sup>k</sup>. It thus seems that each stock, or subtype, has its own sequence “signature” and that T3D<sup>k</sup> with 12 amino acid differences including only 3 unique substitutions is not more divergent from T3D<sup>k</sup> than other virus stocks currently in use in different laboratories.

**Reverse genetics rescue of viruses harboring single-gene substitution**

In order to determine which of the amino acid differences between T3D<sup>k</sup> and P4L-12 viruses are responsible for the difference in interferon sensitivity between the two viruses, site-directed mutagenesis was used to introduce the changes in the reverse genetics plasmids encoding the different proteins; when more than one change was found on the same protein, the corresponding nucleotide changes were all introduced on the same plasmid. Since the parental T3D (T3D<sup>k</sup>) used to isolate P4L-12 differs from the plasmid-encoded T3D<sup>k</sup> sequence, a complete set of plasmids encoding “wild-type” T3D<sup>k</sup> was first constructed and then further subjected to site-directed mutagenesis to obtain P4L-12 encoding sequences.

Both a parental T3D<sup>k</sup> and P4L-12 virus were rescued by transfection of the corresponding plasmid combination. Viruses were similarly obtained using one P4L-12 gene (L2, M2, M3 and S4) in a background of nine T3D<sup>k</sup> genes. All these viruses were rescued and propagated, as described in Materials and methods. Reciprocally, viruses harboring one T3D<sup>k</sup> gene in a background of nine P4L-12 genes were also rescued and propagated.

The infectivity and replicative ability of these different viruses was then examined. L929 cells were infected at a multiplicity of infection of 2 TCID<sub>50</sub> units/cell and viral proteins in infected cells were first examined by immunoblotting (Fig. 1A); this was a first indication that all viruses were able to infect with a similar efficiency since there were no apparent differences in the amount of viral proteins produced. Production of infectious virus was next examined by infecting cells and measuring the fold-increase of infectious virus (Fig. 1B). In either the T3D<sup>k</sup> or P4L-12 background, all viruses harboring substitution of single genes from the other virus did not appear to be significantly different from their background virus. In the T3D<sup>k</sup> background a small increase (up to approximately sixfold for the S4 substitution) was observed while in the P4L-12 background a twofold increase was observed for M2 and twofold decrease for S4. Overall, there was thus no apparent significant defects when single genes were substituted in either background.

**Interferon sensitivity of rescued viruses**

Having established that the different rescued viruses can replicate to similar levels in the absence of interferon, their interferon sensitivity was then examined in comparison with their respective parental viruses.

Interferon sensitivity was first examined by measuring the titer reduction on L929 cells treated at a fixed, near saturating, β-interferon concentration (200 international units/ml), as described in Materials and methods. As expected, rescued T3D<sup>k</sup> harboring none of the P4L-12 genes exhibited resistance to interferon, its titer being almost unaffected at 200 international units/ml of interferon while rescued P4L-12 showed close to a hundred-fold decrease of its titer (Fig. 2). These values are comparable with those routinely obtained with the two parental viruses in previous experiments (data not shown).

Substitution of T3D<sup>k</sup> genes by those encoding either μNS or μ1 of P4L-12 only slightly increased sensitivity to interferon by less than tenfold while σ3 had no effect (Fig. 2). However, substitution of the L2 gene encoding λ2 significantly increased the interferon sensitivity rendering the virus as sensitive as the P4L-12 virus. The reciprocal experiment where single genes from T3D<sup>k</sup> were singly

![Fig. 1. Infectivity and replication of the different rescued viruses. L929 cells were infected at a MOI of 2 TCID<sub>50</sub> units/cell. Results for the different viruses harboring either none or single P4L-12 genes in a T3D<sup>k</sup> background or none or single T3D<sup>k</sup> genes in a P4L-12 background are presented. (A) Proteins were recovered from infected cells at 24 h and analyzed by immunoblotting using an anti-σ3 monoclonal antibody, as described in Section Material and methods. (B) Infected cells were recovered at either 24 or 48 h post-infection. Cells were disrupted by three cycles of freeze–thaw (−80 °C to room temperature) and virus titration was performed by TCID<sub>50</sub> on L929 cells in 96-well microplates. Results, on a logarithmic scale with error bars representing standard error of the mean, are presented as the average fold-increase in two distinct experiments at 24 h, 48 h, and with all four data points combined, as indicated.](image-url)
substitution of the L2 gene and presented (Fig. 3B) comparing the original T3DS and P4L-12 virus phenotype of the two viruses. A representative multiwell plate is presented on a logarithmic scale with error bars indicating the maximal value observed for each virus (n = 5 for the parental viruses, n = 3 for the two viruses harboring substitution of the L2 gene and n = 2 for the other viruses). In both the T3D5 and P4L-12 background, only L2 gene substitution statistically altered interferon sensitivity, at p = 0.002 and p = 0.05 respectively using unpaired Student’s t-test.

It appears very unlikely that differences in residual interferon in the viral inocula could affect the results, given the freeze-thaw treatment, dilutions used, and the fact that there was no differences in interferon induction between T3D5 and P4L-12 (data not shown). However, in order to further ruled out this possibility, infectious T3D5, P4L-12 and single L2 reassortant viruses were recovered from human HeLa cells before being titrated on murine L929 cells in the presence or absence of murine interferon; in these conditions any interferon from virus inoculum will be of human origins and will not act on murine cells used in the interferon assay. Identical results were obtained, correlating the presence of L2 from P4L-12 with interferon sensitivity (data not shown).

The interferon sensitivity of the same viruses was then further examined using serial binary dilutions of interferon in 96-multiwell plates that were then stained and quantitated (Fig. 3A). When the rescued T3D5 virus and viruses harboring a single P4L-12 gene in this background were examined (left panel), the effect of L2 was again evident, especially at intermediate concentrations of interferon (approximately 10 to 50 IU/ml) while μNS and ε3 had no effect. In this assay, at lower interferon concentrations compared to Fig. 2, the μ1 protein of P4L-12 also increased interferon sensitivity.

When viruses harboring single T3D5 genes in a P4L-12 background were similarly analyzed (Fig. 3A, right panel), only L2 clearly affected interferon sensitivity, rendering the virus as resistant to interferon as T3D5. The change in interferon sensitivity conferred by M2 of P4L-12 in the T3D5 background was not reciprocal; therefore it was not further examined in the present study.

Altogether, the effect of L2 was thus most striking and this gene appears to be the main and most likely the only determinant of the difference in interferon sensitivity between T3D5 and P4L-12, its presence being clearly sufficient to reconstitute the respective phenotype of the two viruses. A representative multiwell plate is presented (Fig. 3B) comparing the original T3D5 and P4L-12 virus with their corresponding rescued virus and those where the L2 gene was singly substituted.

Expression of the L2 protein in infected cells

In order to rule out the possibility that there is a difference in the amount of L2 present in the infected cells during infection by T3D5 or P4L-12, immunoblotting was performed on the rescued viruses harboring all the genes of either viruses, as well as on the viruses harboring solely the L2 protein of the other virus (T3D5 background with L2 of P4L-12 and reciprocally). The amount of L2 protein in each case was compared with that of the major outer capsid protein μ1. There was no apparent difference between these four viruses (Fig. 3C) and a simple change in L2 expression or stability is thus unlikely.

Discussion

The present study illustrates that the state-of-the-art plasmid-based reverse genetics approach can be successfully used to study and completely reconstruct virus mutants previously identified by the use of more classical genetic techniques such as chemical mutagenesis. This has allowed to identify polymorphic amino acids residues in the mammalian orthoreovirus genome as well as clearly pinpoint an amino acid substitution that can drastically affect the interferon sensitivity of viral strains. As previously mentioned, the L2 protein was previously identified by classical reassortment studies as a possible molecular determinant of the control of interferon response in reovirus-infected cells; however, this is the first time that this has been firmly established using a completely defined genetic system. Interestingly, an immunoreceptor tyrosine-based activation motif (ITAM) that could affect interferon signaling pathways in some cell types, is observed in L2, although there is currently no evidence that it is actually functional (Stebbing et al., 2014). The amino acid substitution in P4L-12 is not part of this ITAM motif. The threonine at position 636, substituted for a methionine in P4L-12, is a conserved residue in the mammalian orthoreovirus genome as well as clearly pinpoint an amino acid substitution that can drastically affect the interferon sensitivity of viral strains. As previously mentioned, the L2 protein was previously identified by classical reassortment studies as a possible molecular determinant of the control of interferon response in reovirus-infected cells; however, this is the first time that this has been firmly established using a completely defined genetic system. Interestingly, an immunoreceptor tyrosine-based activation motif (ITAM) that could affect interferon signaling pathways in some cell types, is observed in L2, although there is currently no evidence that it is actually functional (Stebbing et al., 2014). The amino acid substitution in P4L-12 is not part of this ITAM motif.

The threonine at position 636, substituted for a methionine in P4L-12, is a conserved residue in the L2 protein sequence of different virus strain (Breun et al., 2001) and is located in one of the two methyltransferase domains (Reinisch et al., 2000; Breun et al., 2001; Bujnicki and Rychlewski, 2001). It is still not firmly established if this first methyltransferase domain is responsible for N-7 or the subsequent 2′-O methylation of the cap structure. However, sequence comparisons suggest the latter possibility (Bujnicki and Rychlewski, 2001) and is also consistent with the relative order of the two methyltransferase domains in Cypovirus (Zhu et al., 2014), another double-stranded RNA virus of the Reoviridae family. Methylation of the RNA cap structure, especially at the 2′-O position could be of importance to avoid recognition of viral RNA as nonself. It could thus affect either induction of interferon signaling or sensitivity to interferon-induced antiviral...
proteins, such as IFIT1 (recently reviewed by: Hyde and Diamond, 2015). This aspect has been especially well studied in members of the Flaviviridae family but has also been noticed in such different viruses as coronaviruses, vesicular stomatitis virus and metapneumovirus (Daffis et al., 2011; Zust et al., 2011; Habjan et al., 2013; Kimura et al., 2013; Dong et al., 2014; Kindler and Thiel, 2014; Ma et al., 2014; Menachery et al., 2014). An altered 2–0 methyltransferase activity of L2 could thus potentially explain the altered interferon resistance of P4L-12 compared to the parental virus. Interestingly, in rotavirus, another member of the Reoviridae family, the capping enzyme VP3 was shown to act as an antagonist of methyltransferase activity, which more specifically that of methyltransferase activity, should be examined to determine if it could affect the ability of the virus to control the innate immune reaction (Arnold et al., 2013; Ogden et al., 2014). It will be of interest to perform similar studies with the L2 protein of reovirus.

Previous work has established the μ2 protein as the major determinant of the extent of interferon signaling following reovirus infection (Zurney et al., 2009; Irvin et al., 2012; Stebbing et al., 2014). When interferon induction by T3D$^3$ and P4L-12 was compared by recovering supernatant from L929-infected cells (data not shown), both of these supernatants were able to similarly protect cells against the unrelated murine encephalomyocarditis virus (EMCV), as used in previous studies (Sandekian et al., 2011; Zust et al., 2011; Habjan et al., 2013). This is well in accordance with the fact that there is no significant differences in induction of either interferon or interferon-induced genes. Furthermore, it should be stressed that the presence of a proline at position 208 indicates that these interferon-induced antiviral effectors. In this study the L2-encoding gene, L2, appears as the major determinant of the difference between T3D$^3$ and P4L-12. The importance of multiple regulatory mechanisms directed against the interferon innate immune response has also been demonstrated in rotavirus (Arnold et al., 2013), another member of the Reoviridae, and in other unrelated viruses such as the large DNA virus vaccinia (Perdiguer and Esteban, 2009) as well as important human RNA viruses such as hepatitis C (Qashqari et al., 2013) and influenza virus (Garcia-Sastre, 2011).

The results presented herein thus further support the idea that multiple reovirus proteins are involved as determinants of reovirus’ sensitivity to interferon either by differences in the modulation of interferon signaling or differences in the sensitivity of the interferon-induced antiviral effectors. In this study the L2-encoding gene, L2, appears as the major determinant of the difference between T3D$^3$ and P4L-12. The importance of multiple regulatory mechanisms directed against the interferon innate immune response has also been demonstrated in rotavirus (Arnold et al., 2013), another member of the Reoviridae, and in other unrelated viruses such as the large DNA virus vaccinia (Perdiguer and Esteban, 2009) as well as important human RNA viruses such as hepatitis C (Qashqari et al., 2013) and influenza virus (Garcia-Sastre, 2011).

There are numerous evidence suggesting induction of interferon antiviral response or virus’ sensitivity to this response is critical in the differential ability of a virus to replicate in, and kill, different cell types. As described in the introduction, this is also likely critical in reovirus’ oncolytic ability. The possibility of modulating both the level of interferon signaling and sensitivity of reovirus strains to antiviral interferon-induced genes, by a combination of various mutations in either σ3, μ2 and L2, could certainly contribute to further optimization of viral strains for infection and killing of different cancer cell types.

Materials and methods

Cell lines and viruses

L929 mouse fibroblasts were originally obtained from the American type culture collection (ATCC® CCL-1™). The baby
hamster kidney cell line (BHK) stably expressing the T7 RNA polymerase has been described (Buchholz et al., 1999) and was a generous gift from the laboratory of Dr John Hiscott (Lady Davis Research Institute, Montréal, Canada). Both cell lines were grown in minimal Eagle medium (MEM) with 5% fetal bovine serum.

Wild-type reovirus type 3 Dearing (T3/Human/Ohio/Dearing/55) was originally obtained from the American Type Culture Collection (Reovirus 3, ATCC® VR824™). The laboratory stock was derived from a pure plaque after one passage of the original stock on L929 cells. This laboratory virus stock was thereafter used between passage 3 and 5 at a low multiplicity of infection.

Sequencing of the whole viral genomes

Viral genomic RNA was prepared and fragments for sequencing were generated by RT-PCR, as previously described (Brochu-Lafontaine and Lemay, 2012), except that Vertrel was used to prepare reovirus virions (Mendez et al., 2000). The sequences corresponding to all 10 genes of the wild-type laboratory stock were deposited under accession numbers KP208804 to KP208813 in GenBank. The sequences corresponding to all 10 genes of the reovirus interferon-sensitive P4L-12 mutant are accessible under numbers KP208814 to KP208823.

Accession numbers for sequences used for comparisons are EF494435 to EF494444 in the nucleotide database for T3D³; ADJ00316 to ADJ00325 in the protein database for T3D³; and GJ991659 to GJ991668 for T3D³ in the nucleotide database. The sequence of REOLYSIN³ was deduced from the differences noticed between each of the genes and its reference sequence as described (Chakrabarty et al., 2014).

Reverse genetics

The plasmids separately harboring each of the cDNA corresponding to the 10 genes of reovirus serotype 3 Dearing, under the transcriptional control of the T7 promoter, as previously described (Kobayashi et al., 2007), were obtained from the laboratory of Dr Terence Demdory (Vanderbilt University, Nashville, Tennessee). To obtain virus mutants in specific genes, the corresponding plasmid was directly submitted to site-directed mutagenesis and used to recover infectious virus by transfection in BHK cells expressing the T7 RNA polymerase and rescued viruses were propagated. Kinetics of viral replication and viral titers were assessed as previously described (Brochu-Lafontaine and Lemay, 2012).

Immunoblotting

Infected cells were recovered by scraping in a small volume of medium and processed for immunoblotting, as previously described (Brochu-Lafontaine and Lemay, 2012). Immunoblotting was performed using monoclonal antibodies directed against either α3 (4F2), α2 (7F4) or α1 (10F6) (Virgin et al., 1991). Images were obtained using either autoradiography on Kodak BioMax Light films or on a Typhoon Trio™ imager (GE Healthcare Life Sciences).

Determination of interferon sensitivity

Interferon sensitivity was determined by two different approaches. The reduction of titer assay was first used as previously described (Danis et al., 1997). Briefly, L929 cells were seeded in 96-well plates and were either left untreated or pre-treated with 200 international units (IU)/ml of mouse β-interferon (PBL InterferonSource) for 24 h before being used in virus titration by TCID₅₀. Following virus adsorption, interferon was added back to the pre-treated plate and the reduction in virus titer in interferon-treated cells compared to untreated cells was determined 7 days post-infection.

To measure sensitivity of viruses to different concentrations of interferon, serial twofold dilution of interferon in 96-well plates was done followed by infection with the different virus strains at the same multiplicity of infection. Plates were stained with methylene blue as previously described (Sandekian et al., 2013); images were obtained and quantitated using an Alphalager HP image analyzer.

Acknowledgments

We thank Stéphanie Côté for technical assistance in viral titrations and Dr George Szatmari for helpful suggestions to improve the manuscript.

We thank Dr. Takeshi Kobayashi and Dr. Terence Demdory (Vanderbilt University, Nashville, Tennessee) for their generous gift of the plasmids used for reverse genetics. We thank Simon Léveillé and Dr. John Hiscott (Lady Davis Research Institute, Montreal, Canada) for the original stock of BHK cells expressing the T7 RNA polymerase.

This work was supported by an operating grant number 155580 from the Natural Sciences and Engineering Research Council of Canada (NSERC) to G.L. We also thank the “Faculté des études supérieures” of “Université de Montréal” for partial financial support to V.S.

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