PB2 and PA genes control the expression of the temperature-sensitive phenotype of cold-adapted B/USSR/60/69 influenza master donor virus

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The cold-adapted (ca) and temperature-sensitive (ts) influenza master donor virus (MDV) B/USSR/60/69 was derived from its wild-type parental virus after successive passages in eggs at 32 °C and 25 °C. This strain is currently in use for preparing reassortant influenza B vaccine viruses which are used in the Russian trivalent live attenuated influenza vaccine. Vaccine viruses are obtained by classical reassortment of MDV and a currently circulating wild-type virus. The phenotypic properties cold adaptation and temperature sensitivity are inherited from the six genes encoding the internal proteins of the MDV. However, the role of the individual gene segments in temperature sensitivity and thus attenuation is not known. In this study, 35 reassortant viruses of B/USSR/60/69 MDV with current wild-type non-ts influenza B viruses were generated in eggs or MDCK cells and studied in order to identify the genes responsible for their ts phenotype. For each virus the exact genome composition was determined as well as its ts phenotype. The results demonstrated that the polymerase PB2 and PA gene segments of B/USSR/60/69 MDV independently controlled expression of the ts phenotype of B/USSR/60/69 MDV-based reassortant viruses. The other genes coding for internal proteins played no role in this respect. This suggests that mutations in the polymerase genes PB2 and PA play an essential role in attenuation of B/USSR/60/69 MDV-based reassortant influenza B vaccine viruses.

INTRODUCTION

The Russian live attenuated influenza vaccine (LAIV) is a trivalent vaccine composed of influenza A virus subtypes H1N1 and H3N2 and influenza B virus. The Russian LAIV is based on the attenuated (att) master donor viruses (MDVs) A/Leningrad/134/17/57 (H2N2) and B/USSR/60/69, which have been rendered temperature-sensitive (ts) and cold-adapted (ca) by successive passages in eggs at 32 °C and 25 °C, respectively (Alexandrova & Klimov, 1994). These MDVs are used in reassortment with current wild-type (wt) influenza A and B viruses, respectively, to generate attenuated vaccine viruses containing the appropriate surface glycoproteins.

FluMist, a LAIV that has been available since 2003 in the USA, is based on the MDVs A/Ann Arbor/6/60 and B/Ann Arbor/1/66, which were developed independently from the Russian MDVs but using the same strategy (Maassab, 1967). Both Ann Arbor-derived MDVs have been characterized, which identified the PB2, PB1 and NP genes of A/Ann Arbor/6/60, and the PA and NP genes of B/Ann Arbor/1/66, as playing a role in temperature sensitivity (Jin et al., 2003; Hoffmann et al., 2005). The MDV for the influenza A vaccine viruses, A/Leningrad/134/17/57 (H2N2), has been fully characterized, at both the genetic and phenotypic level (Klimov et al., 1992, 1995, 2001). Previously, we reported that the PB2 and PB1 gene segments play a critical role in temperature sensitivity and attenuation of A/Leningrad/134/17/57 MDV, while the PA and PBI genes are responsible for its ca phenotype (Klimov et al., 2001; Kiseleva et al., 2003). The MDV used for generating influenza B vaccine viruses, B/USSR/60/69, however, has remained uncharacterized so far. Mutations introduced in the genome of B/USSR/60/69 MDV during successive passages in eggs to render the virus attenuated have not yet been described. Also, the role of individual genes of B/USSR/60/69 MDV in the manifestation of the ts and ca phenotypes has not yet been established. Identification of mutations has been complicated by the fact that the parental wt strain of B/USSR/60/69 MDV is unknown, while studying cold adaptation has been complicated by the fact that wt influenza B viruses may...
naturally exhibit a ca phenotype. We have performed genetic and phenotypic analyses of the B/USSR/60/69 MDV. Sequences of wt influenza B viruses isolated in different years, which were obtained from databases, were compared with the sequence of B/USSR/60/69 MDV to identify amino acid substitutions in the genome of B/USSR/60/69 MDV. To more easily identify the genes that are responsible for temperature sensitivity of B/USSR/60/69 MDV, reassortants containing one, two, three or four genes derived from the MDV with the remaining genes derived from a wt influenza B virus were generated and studied.

**RESULTS**

Contribution of the genes encoding internal proteins of B/USSR/60/69 MDV to the ts phenotype

In this study we focused on identification of gene segments of influenza B virus reassortants, obtained from B/USSR/60/69 MDV and wt influenza B viruses, that contribute to the ts phenotype. Therefore, the phenotype of reassortant viruses with different genome constellations was studied in order to identify which gene or genes are responsible for the ts phenotype and, thereby probably confer attenuation.

All the influenza B viruses chosen as wt parents were non-ts at elevated temperature. In contrast, the B/USSR/60/69 MDV appeared to exhibit a clear ts phenotype (Table 1). The effect of each gene segment of B/USSR/60/69 MDV was assessed by phenotypic analysis of single and multiple (two, three, four or six) gene reassortants of B/USSR/60/69 MDV with wt influenza B viruses (Table 1).

The results show that all reassortant viruses that inherited the PB2 or PA gene of B/USSR/60/69 MDV have a ts phenotype. Exchange of the PB2 (reassortants R1–R3) or the PA (R7–R11) gene of the wt virus with the corresponding gene of B/USSR/60/69 MDV led to a dramatic decrease in the replication properties of these single-gene reassortants at elevated temperature. In contrast, exchange of the PB1 (R4–R6), NP (R12–R17), M (R18) or NS (R19) genes of the wt virus with those of B/USSR/60/69 MDV did not significantly decrease the ability of single-gene reassortants to replicate at elevated temperature (Table 1).

Replication at elevated temperature of multiple-gene (two, three and four) reassortant viruses that inherited at least the PB2 (R24–R26, R28–R29) and/or PA (R20, R24, R27) gene from B/USSR/60/69 MDV was reduced compared to reassortants that inherited these genes from wt parental viruses (R21–R23). So-called 6:2 vaccine reassortants (R30–R35), containing the gene segments encoding the surface glycoproteins HA and NA of the wt virus and all other genes from B/USSR/60/69 MDV, always displayed a ts phenotype (Table 1).

The results of this study demonstrate that the PB2 and PA polymerase genes play a critical role in temperature sensitivity.

**Identification of unique amino acid substitutions in the genome of B/USSR/60/69 MDV**

Wt influenza B viruses isolated in the 1960s and 1970s demonstrated a non-ts phenotype at elevated temperature (Rudenko et al., 2003). Although the exact parent of B/USSR/60/69 is unknown, this virus was isolated in the same time frame and therefore it is likely that its parental strain was also able to replicate at elevated temperature. A virus designated B/USSR/17/69 that was passaged 17 times at an optimum temperature of 32 °C in embryonated eggs was available at the Department of Virology of the Institute of Experimental Medicine (St Petersburg, Russia). This virus is an intermediate strain between B/USSR/60/69 and its parental strain and has been used in the past as a live attenuated influenza B vaccine for adults (Alexandrova, 1971). We found this intermediate strain to be ts but non-ca (Table 2). By comparison with several recent non-ca/non-ts wt influenza B viruses, B/USSR/17/69 demonstrated a clear ts phenotype but a non-ca phenotype. Successive passages (60) at 25 °C of this intermediate non-ca/ts B/USSR/17/69 resulted in ca/ts B/USSR/60/69 MDV (Alexandrova, 1996), which replicates to relatively high titre at a temperature of 25 °C (Table 2).

In addition to the phenotypic study, B/USSR/60/69 MDV and the intermediate strain, B/USSR/17/69, were also genetically characterized. To identify unique amino acid substitutions in the genome of B/USSR/60/69 MDV the sequences of the genes encoding the internal proteins of both B/USSR/60/69 MDV and B/USSR/17/69 were compared to sequences of wt influenza B viruses isolated from 1940 to 2006 which were deposited in the Influenza Sequence Database. In total, 45 sequences of the PB2 gene, 69 sequences of the PB1 gene, 57 sequences of the PA gene, 108 sequences of the NP gene, 31 sequences of the M1 gene, 31 sequences of the BM2 gene, 87 sequences of the NS1 gene and 90 sequences of the NS2 gene (Table 3) were aligned with the corresponding genome segments of B/USSR/60/69 MDV and B/USSR/17/69. In addition, influenza B viruses (B/Ann Arbor/1/66, B/Hong Kong/5/72, B/Singapore/222/79, B/Panama/45/90, B/Johannesburg/5/99) for which complete genome sequences were available were used for comparison. The sequence alignment results of B/USSR/60/69 MDV, B/USSR/17/69 and wt influenza B viruses are summarized in Table 3.
Table 1. Correlation between genome constellation of reassortant viruses and their ts phenotype

<table>
<thead>
<tr>
<th>Wt parental virus</th>
<th>Code</th>
<th>Gene segment</th>
<th>RCT&lt;sub&gt;37(38)&lt;/sub&gt;</th>
<th>Phenotype</th>
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<td></td>
<td></td>
<td>PB2</td>
<td>PB1</td>
<td>PA</td>
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<td>wt</td>
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<td>wt</td>
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<td>60</td>
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</tr>
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<tr>
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<td><strong>Parental viruses</strong></td>
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<td>B/USSR/60/69 MDV</td>
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<tr>
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<tr>
<td>B/Florida/4/06†</td>
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*RCT<sub>37(38)</sub>= log<sub>10</sub>[EID(TCID)<sub>50</sub> ml<sup>-1</sup>] at 32 °C− log<sub>10</sub>[EID(TCID)<sub>50</sub> ml<sup>-1</sup>] at 37(38) °C.
†Virus belongs to the B/Yamagata/16/88 lineage.
‡Gene belongs to the B/USSR/60/69 MDV.
§Gene belongs to the wt parental virus.
‖Virus belongs to the B/Victoria/2/87 lineage.
in the PB2 gene and one in the BM2 gene) appeared to be non-unique; these amino acid changes were also found in the genome of 15 out of 45 and 3 out of 31 wt influenza strains, respectively. In total, one non-unique amino acid mutation and four unique amino acid mutations were found in the PB2 and PA genes. Interestingly, six of these mutations were already observed in the genes encoding internal proteins of the intermediate strain B/USSR/17/69, indicating that these were most likely introduced during the 17 passages of the parental strain of B/USSR/60/69 at 32 °C. Only two extra coding mutations (one in the PB2 gene and one in the BM2 gene) were observed in the ca/ts B/USSR/60/69 MDV when compared to the non-ca/ts B/USSR/17/69. It appears that these two substitutions were introduced during passages of B/USSR/17/69 at 25 °C (Table 3).

No amino acid changes in the PB1 and NP genes were found. Importantly, neither of these genes is responsible for manifestation of the ts phenotype (reassortants R4–R6

<table>
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<tr>
<th>Virus</th>
<th>Virus titre [log10(EID50 ml−1)]</th>
<th>RCT37</th>
<th>RCT25</th>
<th>Phenotype</th>
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<td></td>
<td>32 °C</td>
<td>37 °C</td>
<td>25 °C</td>
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<td>8.3 ± 0.2</td>
<td>1.3 ± 0.1</td>
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<tr>
<td>B/Harbin/07/94*</td>
<td>9.2 ± 0.1</td>
<td>9.1 ± 0.2</td>
<td>1.5 ± 0.2</td>
<td>0.1</td>
</tr>
<tr>
<td>B/Shandong/7/9788†</td>
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<td>7.7 ± 0.2</td>
<td>3.1 ± 0.1</td>
<td>0.5</td>
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<td>7.8 ± 0.1</td>
<td>2.8 ± 0.1</td>
<td>0.4</td>
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<td>8.5 ± 0.2</td>
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<td>9.0 ± 0.2</td>
<td>3.4 ± 0.2</td>
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<tr>
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<td>9.5 ± 0.2</td>
<td>7.6 ± 0.2</td>
<td>3.0 ± 0.2</td>
<td>1.9</td>
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<tr>
<td>B/Malaysia/2506/04†</td>
<td>8.8 ± 0.1</td>
<td>7.9 ± 0.2</td>
<td>6.4 ± 0.2</td>
<td>0.9</td>
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<tr>
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<td>3.4 ± 0.1</td>
<td>2.5 ± 0.2</td>
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<td>8.9 ± 0.2</td>
<td>3.6 ± 0.1</td>
<td>6.4 ± 0.2</td>
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*Virus belongs to the B/Yamagata/16/88 lineage.
†Virus belongs to the B/Victoria/2/87 lineage.

| Table 3. Amino acid mutations in the internal proteins of ca/ts B/USSR/60/69 compared to FluMist MDV and B/Ann Arbor/1/66, and the role of these proteins in the virus phenotype |
|--------------------------------------------------|-----------------|-----------------|-----------------|
| Gene segment                                      | No. of sequences | No. of coding mutations | Temp. at which coding mutation appeared | Coding mutation not found in wt virus sequence | Phenotype | No. of amino acid substitutions | Phenotype |
|                                                  |                 |                          |                               |                                             |           |                                |           |
| PB2                                              | 45              | 1                         | 32 °C                         | 45/45                                       | ts        | 1†§                           | –          |
|                                                  |                 | 1                         | 32 °C                         | 45/45                                       |           |                                |           |
|                                                  |                 | 1                         | 25 °C                         | 30/45                                       |           |                                |           |
| PB1                                              | 69              | 0                         | –                             | –                                           | –         | –                             | –          |
|                                                  |                 | 1                         | 32 °C                         | 45/45                                       |           |                                |           |
| PA                                               | 57              | 1                         | 32 °C                         | 57/57                                       | ts        | 2                             | ts         |
|                                                  |                 | 1                         | 32 °C                         | 57/57                                       |           |                                |           |
| NP                                               | 108             | 0                         | –                             | –                                           | –         | 4                             | ts         |
| M1                                               | 31              | 1                         | 32 °C                         | 31/31                                       | non-ts    | 2                             | possibly ts |
| BM2                                              | 31†§            | 1                         | 25 °C                         | 28/31                                       | –         |                                |            |
| NS1                                              | 87              | 1                         | 32 °C                         | 87/87                                       | non-ts    | –                             | –          |
| NS2                                              | 90              | 0                         | –                             | –                                           | –         | 6 + 1†§                        | –          |
| RNP complex (PB2, PA, NP)                        | 4 + 1†§         |                            |                               |                                             |           |                                |           |

*Compared to wt reference sequences.
†Adapted from Hoffmann et al. (2005).
‡Number of sequences of wt influenza viruses aligned with sequences of B/USSR/60/69.
§Mutation is non-unique (is present in one wt influenza strain, B/Harbin/7/94).
‖Mutation is non-unique (is present in 3 out of 31 sequences of wt viruses).
and R21–R23, and reassortants R12–R17 and R23, respectively) (Table 1). The mutations that were found in the M1, BM2 and NS1 genes also appear not to play a role in the ts phenotype. While it cannot be excluded that the mutation in BM2 plays a role in the ca phenotype, this is not likely for the mutations in M1 and NS1 as they were introduced during passaging at 32 °C. Therefore, the biological relevance of these mutations remains unknown.

DISCUSSION

The MDV for influenza A vaccine viruses used in the Russian live attenuated influenza vaccine, A/Leningrad/134/17/57 (H2N2), has been genetically characterized in detail (Klimov et al. 1992, 1995). However, little is known about the genetic make-up of B/USSR/60/69, which is in use as a MDV for influenza B vaccine viruses (Rudenko et al., 1993). This MDV has evolved from the propagation of the wt parental B/USSR/69 strain in chicken embryos for 17 passages at optimum temperature (32 °C) to yield B/USSR/17/69 (Alexandrova, 1971), followed by an additional 60 passages at 25 °C (Alexandrova, 1996). The resulting B/USSR/60/69 strain was characterized in eggs using two phenotypic markers, cold adaptation (ca phenotype), which is characterized by higher reproductive yield at 25 °C compared to wt virus, and temperature sensitivity (ts phenotype), which is characterized by a comparatively lower reproductive yield at elevated temperature. With respect to ts, both B/USSR/60/69 and B/USSR/17/69 show a substantial reduction in their replication at elevated temperature. The B/USSR/60/69 MDV, in contrast to B/USSR/17/69, replicates at higher titre at low temperature and thus also exhibits a ca phenotype.

A relation between attenuation and the ts phenotype of cold-adapted viruses has been demonstrated previously for influenza A virus (Klimov et al., 2001). There appears to be no relation between mutations which are responsible for manifestation of the ca phenotype and attenuation. A direct relation between temperature sensitivity and attenuation makes sense, as replication of ts viruses is limited to the nasopharynx of the upper respiratory tract, where lower temperatures prevail. Conversely, non-ts viruses may replicate in the relatively warmer lower respiratory tract, where they could potentially cause viral pneumonia or predispose the host to other viral or bacterial infections.

The PB2 gene of A/Leningrad/134/17/57 (H2N2) MDV (Klimov et al., 2001; Kiseleva et al., 2003), ca A/Ann Arbor/6/60 (H2N2) (Jin et al., 2003), and even some avian influenza viruses (McCauley & Penn, 1990), has been shown to be related to the temperature sensitivity of reassortant viruses. Other studies have shown that the PA gene is responsible for the ts phenotype conferring attenuation to reassortant viruses obtained from ca B/Ann Arbor/1/66 MDV and wt B/Hong Kong/1732/76 (Donabedian et al., 1987, 1988). In the present study we focused on finding a relation between the genome constellation of B reassortant viruses and the ts phenotype. The results of our study demonstrate that the PB2 gene and the PA gene of B/USSR/60/69 MDV are independently responsible for the ts phenotype and are likely to be the determinants of attenuation. This does not necessarily mean, however, that these gene segments are solely responsible for the att phenotype. Other genes that do not seem to play a role in temperature sensitivity may still contribute to the att phenotype. Indeed, it has been demonstrated that in addition to the PA and NP genes of B/Ann Arbor/1/66, the M gene also plays a role in attenuation without contributing to the ts phenotype (Hoffmann et al., 2005). Animal studies with selected reassortants will probably reveal the role of each of the genes in attenuation. Our RCT results showed that reassortant viruses containing a combination of the PB2 and PA genes, including 6:2 vaccine viruses, tend to be the most temperature sensitive, followed by those that inherited the PA gene only. Single-gene reassortants containing only the PB2 gene of MDV were less temperature sensitive than those that inherited the PA gene only.

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Sequence comparisons of B/USSR/60/69 MDV with wt influenza B virus sequences from the databases have identified eight coding mutations in the genes coding for the internal proteins (six unique and two non-unique mutations) that have probably been introduced during successive passages of the parental virus in eggs at optimal and low temperature. These mutations are present in PB2 (3), PA (2), M1 (1), BM2 (1) and NS1 (1). Of these eight coding mutations, five are in genes coding for the RNP complex (PB2 and PA). Compared to B/USSR/60/69 MDV, the MDV used in FluMist B/Ann Arbor/1/66 contains nine mutations in the genes coding for the internal proteins (one non-unique and eight unique mutations), seven of which are in RNP-encoding genes (PB2, PA and NP) (Hoffmann et al., 2005). In our study we did not find any substitutions at the amino acid level in the PB1 and NP genes in B/USSR/60/69 MDV and we showed that neither of these genes is responsible for manifestation of the ts phenotype of B/USSR/60/69 MDV. Compared to other MDVs it is interesting to note that neither in the A/Leningrad/134/17/57 (H2N2) MDV (Klimov et al., 1992) nor in the A/Singapore/1/57/ca MDV (Romanova et al., 2004) were mutations found in the NP gene. As in B/Ann Arbor/1/66, there were no changes in the PB1 gene of B/USSR/60/69 (Hoffmann et al., 2005).

Not surprisingly, only the genes coding for the RNP complex proteins are responsible for manifestation of the ts phenotype in both B/USSR/60/69 (PA and PB2 genes) and

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the MDV used in FluMist, B/Ann Arbor/1/66 (PA and NP genes). The same holds true for the influenza A MDV A/ Leningrad/134/17/57 (PB2 and PB1 genes) and the MDV used in FluMist, A/Ann Arbor/6/60 (PB2, PB1 and NP genes). While the mutations in the influenza A and B MDVs used in the Russian vaccine and FluMist are not identical, it is obvious that the molecular basis for the expression of the ts phenotype of both influenza A and B MDVs is close and lies within the genes that control virus replication.

**METHODS**

**Viruses.** Influenza B wt viruses and ca B/USSR/60/69 MDV were obtained from CDC (Atlanta, GA, USA) and the Institute of Experimental Medicine (St Petersburg, Russia), respectively.

**Preparation of influenza B reassortant viruses.** Thirty-five influenza B reassortant viruses were prepared by classical reassortment of B/USSR/60/69 MDV and current non-ts wt influenza B viruses belonging to the B/Victoria/2/87 lineage (B/Shandong/7/97, B/Hong Kong/330/01 and B/Malaysia/2506/04) and the B/Yamagata/16/88 lineage (B/Harbin/7/94 and B/Johannesburg/5/99) in eggs or in MDCK cells as described previously (Klimov et al., 2001; Kiseleva et al., 2004).

**RNA isolation.** RNA was isolated from influenza-virus-infected allantoic fluid or cell culture supernatant by using the QIAamp Viral RNA minikit (Qiagen).

**Genome composition of reassortants.** Genome composition of reassortant influenza B viruses was monitored by standard haemagglutination inhibition assay (for HA) and RT-PCR followed by RFLP analysis (for NA and the other six genes). The segment-specific PCR primers for screening the genome composition of reassortants were designed on the basis of sequences of 12 influenza B viruses deposited in sequence databases (http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html and http://www.flu.lanl.gov): B/Geneva/5079/03, B/Moscow/3/03, B/Barcelona/215/03, B/Israel/95/03, B/Trieste/28/02, B/Hong Kong/330/01 and B/Shangdong/7/97 (all belonging to the Victoria lineage) and B/Hong Kong/293/02, B/Ulan Ude/4/02, B/Hong Kong/692/01, B/Sichuan/579/99 and B/Youamashi/166/98 (all belonging to the Yamagata lineage). Multiple sequence alignment analysis was performed using Clone Manager 9 in order to identify regions containing nucleotides coinciding with restriction enzyme recognition sequences such that a PCR product of one parental strain is cut by a particular restriction enzyme while the corresponding PCR product of the other parental strain is not.

For cDNA synthesis universal primers were used so that in a single reaction cDNAs were generated for all gene segments. These cDNAs were used as templates in the PCRs. The universal forward primer used was BUNIVERSALF (5'-ACGTTGCAAGACGAGAACG) and the universal reverse complementary primer used was BUNIVERSALR (5'-ACGTTGCAATGGAAGA) (W=A/T).

The specific forward primers used were BPB2F1743 (5'-CAATGGGATGTCATGTTAAGGTTG), BPB1F906 (5'-AGAGGGATCATCAGGATG), BPAFL1539 (5'-CAATCTCATGAGGAGCA), BPNI148 (5'-AAATGGCAGAACTCAGG), BNA225 (5'-AAATTCTCTGGAATGCTCACC), BMF319 (5'-GCCTGAGAGAAAAATGGAAG) and BNSF142 (5'-CTTCTGACGAAGGCTTCTT). The specific reverse complementary primers used were BPB2R2029 (5'-GTTGAGACTTTCTGTTTGG), BPB1R1284 (5'-TCCCAACACGCTTAGACTAG) and BPA1R1882 (5'-CAATACTGAAATTTTGG), BNPRI548 (5'-TTGACATGTGACATCACG) and BMF655 (5'-TCATGGTGCATGTTTCAAC), BMR655 (5'-CTCAATGGTGCTTGCAG) and BNSR449 (5'-GGTTCTCTCCTATGTCTAC). The PCR programme used was: 94 °C, 15 min; 45 × 94 °C (1 min); 50 °C, 2 min; 72 °C, 1 min; 72 °C, 10 min.

The RFLP assay was performed as described by Klimov & Cox (1995). In brief, restriction enzymes MseI or HindIII were used for RFLP analysis of PCR products of the PB2 gene (nucleotides 1743–2029) when comparing B/USSR/60/69 MDV with B/Victoria/2/87 or B/Yamagata/16/88-like viruses, respectively. BspHI was used for the PB1 gene (nucleotides 906–1284). BclI for the PA gene (nucleotides 1539–1882), AvrII for the NP gene (nucleotides 1148–1548), Msel for the NA gene (nucleotides 196–1487), BfaI for the NS gene (nucleotides 142–449) and BgII for the M gene (nucleotides 319–655).

**Determination of ts and ca phenotype.** Ts phenotype was determined by titration of reassortant viruses in eggs and/or MDCK cells (ATCC catalogue no. CCL-34) as described previously (Klimov et al., 2001; Kiseleva et al., 2004) at permissive (32 °C) and restrictive (37 °C or 38 °C) temperatures and expressed as reproduction capacity at restrictive temperature (RCT) at restrictive temperature: RCT<sub>37</sub> [log<sub>10</sub>(EID<sub>50</sub> ml<sup>−1</sup>)] or RCT<sub>38</sub> [log<sub>10</sub>(EID<sub>50</sub> ml<sup>−1</sup>)] or (log<sub>10</sub>(EID<sub>50</sub> ml<sup>−1</sup>)] at 32 °C–log<sub>10</sub>(EID<sub>50</sub> ml<sup>−1</sup>)] or (log<sub>10</sub>(EID<sub>50</sub> ml<sup>−1</sup>)] at 37 °C (or 38 °C). Viruses were classified as ts when their RCT<sub>37</sub> was ≥2 logs; viruses with RCT<sub>37</sub> < 2 logs were considered as non-ts.

Ca phenotype was determined by titration of the reassortants in eggs at 32 °C and 25 °C and expressed as reproduction capacity at low temperature: RCT<sub>25</sub> [log<sub>10</sub>(EID<sub>50</sub> ml<sup>−1</sup>)] [log<sub>10</sub>(EID<sub>50</sub> ml<sup>−1</sup>)] at 32 °C–log<sub>10</sub>(EID<sub>50</sub> ml<sup>−1</sup>)] at 25 °C. Viruses were classified as ca when their RCT<sub>25</sub> was ≤3 logs; viruses with RCT<sub>25</sub> > 3 logs were considered as non-ca.

Each assay was performed a minimum of three times. The median infectivity (TCID<sub>50</sub> ml<sup>−1</sup>, EID<sub>50</sub> ml<sup>−1</sup>) was determined according to Reed & Muench (1938) and expressed as the mean log<sub>10</sub>(EID<sub>50</sub> ml<sup>−1</sup> (EID<sub>50</sub> ml<sup>−1</sup>) ± SD.

**REFERENCES**


