JOURNAL OF VIROLOGY, Nov. 1989, p. 4850–4856
0022-538X/89/114850-07$02.00/0

Vol. 63, No. 11

# The Reovirus M1 Gene, Encoding a Viral Core Protein, Is Associated with the Myocarditic Phenotype of a Reovirus Variant

BARBARA SHERRY[1]†* AND BERNARD N. FIELDS[1,2,3]

*Department of Microbiology and Molecular Genetics, Harvard Medical School,[1] Department of Medicine (Infectious Disease), Brigham & Women's Hospital,[2] and the Shipley Institute of Medicine,[3] Boston, Massachusetts 02115*

Received 16 May 1989/Accepted 31 July 1989

**Reoviruses contain a genome composed of 10 double-stranded RNA gene segments. A reovirus reassortant, 8B, derived from type 1 Lang (T1L) and type 3 Dearing (T3D), displayed a phenotype unlike that of either of its parents in that it efficiently induced numerous macroscopic external cardiac lesions in neonatal mice (B. Sherry, F. J. Schoen, E. Wenske, and B. N. Fields, J. Virol. 63:4840–4849, 1989). A panel of T1L/T3D reassortants and a panel of reassortants derived from 8B were used to determine whether novel T1L/T3D gene associations in 8B were responsible for its myocarditic phenotype. The results eliminated the possibility that any T1L/T3D gene combination found in 8B, from 2 genes to all 10 genes, was the explanation for its induction of cardiac lesions. This suggested that a mutation(s) in an 8B gene(s) might be responsible for induction of the myocarditis. Statistical analysis of experiments with 31 reassortants derived from 8B revealed a highly significant association ($P = 0.002$) of the 8B M1 gene with induction of cardiac lesions. The reovirus M1 gene encodes a viral core protein of unknown function, although evidence suggests a potential role in core structure and/or viral RNA synthesis. This represents the first report of the association of a viral gene with induction of myocarditis.**

Reoviruses, members of the family *Reoviridae*, have a genome composed of 10 double-stranded RNA gene segments (for a review, see reference 39). The segmented nature of the genome has been used to great advantage in the past in the production of reassortant viruses which contain different combinations of gene segments derived from different parental viruses (for a review, see reference 26). When two parent viruses display distinguishable phenotypes, the progeny reassortant viruses can be used to determine which gene segment(s) cosegregates with the phenotype of interest.

Reovirus reassortants have been previously used to identify the genes associated with phenotypes in vitro, for example, inhibition of host DNA, RNA, and protein synthesis (34, 35). Reassortants have also been used to identify genes associated with aspects of viral pathogenesis in the neonatal mouse, such as growth at a primary site of entry in the animal (1), mode of spread through the animal (40), tissue tropism in the central nervous system (42), and viral shedding from the animal (14). Our overall goal is to combine the in vitro results which reveal gene function with the in vivo results to better understand the molecular basis for viral pathogenesis.

Reoviruses cause a variety of diseases in the neonatal mouse (for a review, see reference 39). The work described here stems from the fortuitous isolation of a reovirus variant which was derived as follows. Type 1 Lang (T1L) and type 3 Dearing (T3D) were coinjected perorally into neonatal mice, and a reassortant virus, called 8B, was isolated from the brain on day 13 postinjection (43). This reassortant was efficiently myocarditic when reintroduced into neonatal mice, in contrast to the parental strains T1L and T3D, which were nonmyocarditic (36).

Myocarditis is an important human disease, and viruses have been suspected for many years to be the primary agent responsible (44). Coxsackieviruses, members of the family *Picornaviridae*, have been implicated most frequently (3). Coxsackieviruses which are myocarditic or nonmyocarditic in the mouse have been studied for a number of years (for a review, see reference 15), and although there has been an extensive investigation into the role of the host immune system in the generation of the disease, there has been little insight into what viral factors are responsible for the myocarditic phenotype.

The myocarditic reovirus variant 8B offers the opportunity to use genetics to identify the viral components important in the generation of the disease. We present here the first report of the association of a viral gene with induction of myocarditis.

## MATERIALS AND METHODS

**Virus stocks and cells.** Mouse L929 (L) cells were propagated as Spinner cultures in the Joklik modification of Eagle minimal essential medium (MEM) supplemented with 2 mM glutamine, 1 U of penicillin per ml, 1 μg of streptomycin (Irvine Scientific, Santa Ana, Calif.) per ml, and either 5% fetal calf serum (Hyclone Laboratories, Logan, Utah) or 2.5% fetal calf serum and 2.5% viable serum protein (agamma VSP; Biocell, Carson, Calif.) (completed MEM). All viruses, including reassortants, were triply plaque purified and then passaged twice (to increase virus stock titer) on mouse L cells. The second-passage (P2) virus stocks were clarified by low-speed centrifugation before titration and use. Virus stocks were stored at 4°C. All gene segment derivations were confirmed by polyacrylamide gel electrophoresis of viral double-stranded RNA (see below). T1L and T3D were from standard laboratory stocks (24).

**Generation of virus reassortants.** T1L/T3D reassortants were generated previously (4, 8), using a protocol similar to the one described below. For the EW and DB series of reassortants, viruses were mixed in a 1:1 or 4:1 ratio and used to inoculate L-cell monolayers (24-well tissue culture dishes; Costar, Cambridge, Mass.) at a total multiplicity of

* Corresponding author.
† Present address: College of Veterinary Medicine, North Carolina State University, Raleigh, NC 27606.

infection of 10 PFU per cell. After 1 h of infection at 37°C, the monolayers were overlaid with 1 ml of completed MEM and incubated for 22 to 24 h at 37°C. Virus was released from cells by disruption with a microtip probe at 50% maximal setting (W225R Cell Disruptor; Heat Systems-Ultrasonics Inc., Plainview, N.Y.). The virus suspensions were then diluted in gel saline (137 mM NaCl, 0.2 mM $CaCl_2$, 0.8 mM $MgCl_2$, 19 mM $H_3BO_3$, 0.1 mM $Na_2B_4O_7$, 0.3% [wt/vol] gelatin [Fisher, Medford, Mass.]) and plated in plaque assays as described previously (41). Virus was recovered from plaques in neutral red-stained monolayers. A Pasteur pipette was used to transfer the cell debris and overlying agar to a 2-dram (~7-ml) vial containing either 1 ml of completed MEM (DB series of reassortants) or an L-cell monolayer in 1 ml of completed MEM (EW series of reassortants). Samples of the plaque suspensions of the DB series of reassortants were used to infect L-cell monolayers in 2-dram vials, and these vials as well as those containing the EW series of reassortants were incubated at 37°C until there was extensive cytopathic effect, resulting in amplified plaque suspensions.

Samples of amplified plaque suspensions were used to infect L-cell monolayers in six-well tissue culture dishes, overlaid with 3 ml of completed MEM, and incubated at 37°C. These infected monolayers were used to isolate viral double-stranded RNA and to determine the gene segment derivations as described below. Suspensions containing reassortant viruses were identified, and the original plaque suspension (DB series of reassortants) or the amplified plaque suspension (EW series of reassortants) was used as the inoculum for a second round of plaque purification. Viruses were plaque purified a third time and amplified, characterized, and stored as P2 virus stocks (see above).

**Determination of gene segment derivations.** The 10 double-stranded RNA gene segments of T1L and T3D display characteristic and distiguishable mobilities on polyacrylamide gels (24), and this technique was used to determine the gene segment derivations in the reassortant viruses. Specifically, monolayers in six-well tissue culture dishes were infected, incubated until all cells were dead, and then frozen (−20°C) and thawed (37°C) once. A Pasteur pipette was used to disperse the cells and transfer them to a 15-ml tube. Nonidet P-40 was added to a final concentration of 0.5%, and after incubation for 30 min on ice the suspension was centrifuged for 10 min at 2,000 rpm (IEC centrifuge [850 × *g*]) to pellet the nuclei. Supernatants were decanted to fresh 15-ml tubes, sodium dodecyl sulfate was added to 1%, and the RNA was extracted by vigorous mixing with 1 volume of phenol (Mallinckrodt, Inc., St. Louis, Mo.) freshly equilibrated with 100 mM NaCl–50 mM Tris (pH 7.4)–1 mM EDTA followed by several rounds of incubation for 5 min at 37°C and centrifugation for 5 min at 3,000 rpm (1,600 × *g*) on a centrifuge obtained from Ivan Sorvall, Inc., Norwalk, Conn. The aqueous layer was mixed with 2.5 volumes of cold 100% ethanol, incubated at −20°C overnight, and centrifuged for 10 min at 10,000 rpm (Sorvall centrifuge [12,100 × *g*]). Supernatants were decanted, and the RNA was lyophilized. The RNA was suspended in 200 μl of Laemmli sample buffer, and 20 to 50 μl was used for electrophoresis on 10% polyacrylamide gels as previously described (16).

**Mice, inoculations, and specimen removal.** Pregnant NIH Swiss (NIH/Sw) mice were purchased from the National Cancer Institute (Frederick, Md.), housed in individual filter-topped cages, and checked daily for births. Neonates (2 days old) were inoculated in the left hindlimb with 20 μl of virus diluted in gel saline with a 1-ml tuberculin syringe and a 30-gauge needle. On the indicated day postinjection, mice died or were sacrificed by cervical dislocation, and the hearts were removed and examined for the presence of macroscopic external cardiac lesions. The hearts were disposed of or treated as described below for plaque assays.

| VIRUS | GENE SEGMENT (8B OR WILD TYPE) S1 | S2 | S3 | S4 | M1 | M2 | M3 | L1 | L2 | L3 | % MICE WITH EXTERNAL CARDIAC LESIONS |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 8B | ● | ● | ● | ● | ● | ● | ● | ● | ● | ● | 100% |
| WT | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | 0% |
| EW29 | ● | ○ | ● | ● | ○ | ○ | ● | ○ | ○ | | 0% |
| EW43 | ● | ● | ○ | ● | ○ | ○ | ● | ○ | ○ | | 0% |
| EW27 | ○ | ● | ○ | ● | ○ | ● | ● | ○ | ● | | 0% |
| DB93A | ○ | ○ | ● | ● | ○ | ● | ○ | ● | ○ | ○ | 0% |
| DB93B | ○ | ○ | ○ | ● | ○ | ● | ● | ● | ○ | ○ | 0% |
| DB188 | ○ | ● | ○ | ○ | ○ | ● | ● | ● | ○ | ● | 0% |
| EW50 | ○ | ○ | ○ | ○ | ○ | ● | ○ | ● | ● | | 0% |
| DB95 | ● | ○ | ● | ○ | ○ | ● | ○ | ● | ○ | | 0% |
| EW116 | ○ | ● | ○ | ● | ○ | ○ | ○ | ● | ○ | | 0% |
| EW46 | ○ | ○ | ○ | ● | ○ | ● | ○ | ● | ○ | | 0% |
| EW10 | ○ | ● | ○ | ● | ● | ● | ● | ○ | ○ | | 0% |
| EW26 | ○ | ○ | ● | ● | ● | ● | ● | ○ | ○ | | 0% |
| EW102 | ● | ● | ● | ● | ● | ○ | ● | ○ | ○ | | 0% |
| EW38 | ● | ● | ● | ○ | ● | ○ | ○ | ○ | ○ | | 0% |
| DB76 | ○ | ● | ● | ○ | ● | ● | ○ | ● | ○ | ○ | 0% |
| EW40 | ○ | ○ | ● | ○ | ● | ● | ○ | ● | ○ | | 8% |
| EW47 | ● | ● | ● | ● | ● | ● | ○ | ● | ○ | | 12% |
| DB62 | ○ | ● | ● | ○ | ● | ● | ● | ● | ○ | ● | 18% |
| DB68 | ○ | ○ | ● | ● | ○ | ● | ● | ● | ● | ● | 18% |
| EW90 | ● | ● | ● | ● | ○ | ● | ● | ● | ○ | | 19% |
| EW67 | ● | ● | ● | ● | ● | ○ | ● | ● | ● | | 33% |
| EW93 | ● | ● | ● | ● | ● | ○ | ● | ○ | ● | | 43% |
| EW100 | ● | ● | ○ | ● | ● | ● | ○ | ○ | ○ | | 44% |
| EW25 | ● | ● | ● | ● | ● | ● | ● | ● | ○ | | 53% |
| DB69A | ○ | ○ | ● | ● | ● | ● | ● | ○ | ● | ○ | 56% |
| EW96 | ○ | ● | ○ | ● | ● | ● | ○ | ● | ● | | 80% |
| EW112 | ● | ● | ○ | ● | ● | ● | ● | ● | ○ | | 82% |
| EW89 | ● | ○ | ● | ● | ● | ● | ○ | ● | ○ | | 82% |
| DB181 | ○ | ● | ● | ○ | ● | ● | ● | ● | ● | ● | 89% |
| EW60 | ○ | ● | ● | ● | ● | ● | ● | ● | ● | | 94% |
| DB88 | ○ | ● | ○ | ● | ● | ● | ○ | ● | ○ | ● | 100% |

FIG. 1. Frequency of macroscopic external cardiac lesions. WT (wild-type) indicates virus EB121 for the EW series of reassortants and E3 for the DB series of reassortants (see Table 2). Hearts were examined when animals died or at 14 days postinjection, and a heart was scored as positive if any external lesions were detected. For each virus tested, between 9 and 30 mice were injected with $2 \times 10^5$ to $2 \times 10^6$ PFU. In the case of viruses which induced lesions in <50% of the mice, additional mice were injected with $4 \times 10^6$ to $5 \times 10^7$ PFU, for a total of 16 to 45 mice each (data not shown). Viruses which failed to induce lesions behaved the same regardless of dose; viruses which induced lesions in a low-to-intermediate fraction of mice behaved the same or were more myocarditic at the higher dose. ●, Genes derived from 8B (either directly or indirectly through EW60); ○, genes derived from nonmyocarditic (wild-type) parents.

**Plaque assays.** Hearts were minced into approximately 1-mm pieces by using a razor blade and placed in 2 ml of gel saline. Samples were frozen (−70°C) and thawed (37°C) three times and then were sonicated for 15 to 30 s with a microtip probe as described above. The virus suspensions were serially diluted in 10-fold steps in gel saline and plated in duplicate on L-cell monolayers for plaque assays as described previously (41). Viral titers were expressed as PFU per heart.

**Statistical analyses.** (i) Chi-square analysis. Reassortants in Fig. 1 were considered to be nonmyocarditic (0% frequency) or myocarditic (>0% frequency), and a chi-square analysis was applied. (ii) Wilcoxon rank sum analysis. Reassortants in Fig. 1 were ordered by the frequency with which they induced lesions and were then assigned a rank consistent with their order. Viruses inducing lesions at equal frequencies were assigned identical ranks, equal to the sum of the

TABLE 1. Frequency of macroscopic external cardiac lesions

| Virus | Source[a] of gene segment: | | | | | | | | | | No. of mice with cardiac lesions[b]/no. tested on days postinjection: | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | S1 | S2 | S3 | S4 | M1 | M2 | M3 | L1 | L2 | L3 | 5 to 8 | 9 to 14 |
| T3D | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 0/31 | 0/27 |
| T1L | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1/32 | 3/28 |
| 8B | 3 | 1 | 1 | 1 | 1 | 3 | 1 | 1 | 1 | 1 | 51/55 | 8/8 |
| G2 | 3 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 3 | 1 | 0/4 | 2/24 |
| EB143 | 3 | 1 | 1 | 1 | 1 | 1 | 1 | 3 | 1 | 1 | 0/4 | 2/33 |
| EB138 | 3 | 3 | 1 | 1 | 1 | 3 | 1 | 3 | 1 | 1 | | 1/19 |
| H27 | 1 | 1 | 1 | 1 | 1 | 3 | 1 | 1 | 3 | 3 | | 0/19 |
| H15 | 3 | 3 | 3 | 1 | 1 | 3 | 3 | 1 | 3 | 3 | 0/4 | 0/28 |
| EB86 | 3 | 3 | 3 | 1 | 3 | 3 | 1 | 1 | 3 | 3 | | 0/16 |
| EB129 | 3 | 1 | 1 | 3 | 3 | 3 | 1 | 3 | 3 | 1 | | 0/19 |

[a] 3, T3D; 1, T1L.

[b] Mice were injected with $1 \times 10^5$ to $4 \times 10^6$ PFU and died or were sacrificed on the days indicated. A heart was scored as positive if it displayed any external lesions.

appropriate set of ranks divided by the number of viruses at that rank position (e.g., the first 15 viruses, each inducing 0% myocarditis, were each assigned the rank 8 [=(1 + 2 + 3 + . . . + 15)/15]). Wilcoxon rank sum analysis was then applied (18).

## RESULTS

**Evidence that the 8B myocarditic phenotype is due to a viral mutation(s).** Reassortant virus 8B was efficiently myocarditic, whereas its parent viruses, T1L and T3D, were not. This was evident macroscopically; 8B induced large, white, calcified lesions covering the surface of the heart, while T1L induced only rare lesions and T3D induced none at all (Table 1) (36). In addition, 8B induced at least 10-fold more myocardial necrosis than did either T1L or T3D (36). One explanation for the new phenotype was that 8B contained particular genes derived from each parent which, when expressed together in the same virus, generated the myocarditic phenotype. This phenomenon has been described previously for, for example, reovirus (12), influenza virus (31, 32), and lymphocytic choriomeningitis virus (30). Another (not mutually exclusive) explanation was that 8B contained a mutation(s) critical for the phenotype.

To address the hypothesis that novel T1L/T3D gene associations in 8B were responsible for the phenotype, we tested a panel of T1L/T3D reassortants for their induction of macroscopic lesions in neonatal mice (Table 1). Three reassortants (G2, EB143, and EB138) were mildly myocarditic, analogous to T1L (5 to 11% of the hearts displayed lesions between 9 and 14 days postinjection), while the remaining four reassortants induced no macroscopic external lesions. Thus, none of the seven reassortants behaved like 8B.

How completely did the seven reassortants address the novel T1L/T3D gene associations found in 8B? These reassortants tested all 16 of the novel T1L/T3D gene pairs found in 8B (e.g., T3D-S1 with T1L-S2, T3D-S1 with T1L-S3, etc.), eliminating the possibility that a novel gene pair was responsible for the phenotype. These reassortants tested 60 of the 64 novel T1L/T3D gene trios and 109 of the 140 novel T1L/T3D gene quartets found in 8B, suggesting that a novel combination of three or four genes was not responsible for the phenotype. The data therefore suggested that 8B contained a mutation(s) critical to the myocarditic phenotype. In fact, the remaining four novel trios and 31 novel quartets, as well as all larger novel combinations, were tested with another reassortant set (Fig. 1; see Discussion), verifying that the new myocarditic phenotype of 8B was not due to novel T1L/T3D gene associations.

**Generation of reassortants between 8B and nonmyocarditic viruses.** The data above suggested that 8B contained a mutation(s) critical for the myocarditic phenotype. In order to identify the gene(s) containing the critical mutation(s), we generated reassortants between 8B and nonmyocarditic (wild-type) viruses (Table 2). Note that neither T1L nor T3D could be used as the nonmyocarditic parent in this reassortment experiment, since the origin of certain genes in the resulting progeny reassortants would be unclear (e.g., in a reassortment experiment between T3D and 8B the origin of the S1 and M2 genes in the progeny would be unclear). A reassortant virus with a gene segment pattern exactly opposite that of 8B did not exist in the laboratory virus stocks, and it was therefore necessary to perform two reassortment experiments (Table 2). First, we derived reassortants between 8B and a nonmyocarditic T1L/T3D reassortant (EB121), chosen because 9 of its 10 segments were distinguishable from those of 8B. We resolved the tenth segment (L3) in a second reassortment experiment, in which the parents were a myocarditic progeny reassortant of 8B (EW60) and a second nonmyocarditic T1L/T3D reassortant (E3). For simplification, genes derived from 8B (either directly or indirectly through EW60) are indicated as filled

TABLE 2. Strategy for reassortments

| Parent viruses | Phenotype[a] | Source[b] of gene segment: | | | | | | | | | | Progeny isolated[c] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | S1 | S2 | S3 | S4 | M1 | M2 | M3 | L1 | L2 | L3 | |
| T1L | Nonmyocarditic | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 8B |
| T3D | Nonmyocarditic | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | |
| 8B | Myocarditic | 3 | 1 | 1 | 1 | 1 | 3 | 1 | 1 | 1 | 1 | EW series |
| EB121 | Nonmyocarditic | 1 | 3 | 3 | 3 | 3 | 1 | 3 | 3 | 3 | 1 | |
| EW60 | Myocarditic | 1 | 1 | 1 | 1 | 1 | 3 | 1 | 1 | 1 | 1 | DB series |
| E3 | Nonmyocarditic | 3 | 3 | 3 | 3 | 3 | 1 | 3 | 3 | 3 | 3 | |

[a] Phenotypes are described as myocarditic or nonmyocarditic on the basis of the data in Table 1 (T1L, T3D, and 8B) or Fig. 1 (EW60). When EB121 and E3 were used in studies as described for Fig. 1, they induced heart lesions in 0 of 19 and 0 of 17 mice, respectively.

[b] 3, T3D; 1, T1L.

[c] 8B was derived by reassortment in vivo in a neonatal mouse (43). The EW and DB series of reassortants were derived in vitro as described in Materials and Methods.

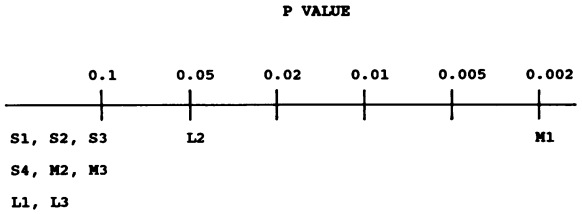


FIG. 2. Results of chi-square and Wilcoxon rank sum statistical analyses performed on the data shown in Fig. 1.

circles, and those derived from the nonmyocarditic (wild-type) parents are indicated as open circles (Fig. 1).

**The 8B-M1 gene is associated with the myocarditic phenotype.** We tested the 8B-derived reassortants for their induction of macroscopic lesions in neonatal mice (Fig. 1). The viruses fell into two groups: those that never induced external cardiac lesions, and those that induced them in a fraction or all of the mice. Viruses which induced lesions in a fraction of the mice did so reproducibly in separate experiments. No single 8B gene or combination of 8B genes segregated perfectly with the induction of external cardiac lesions, suggesting that a statistical analysis might be useful.

We applied a chi-square analysis, in which the viruses were considered to be myocarditic (inducing any detectable lesions) or nonmyocarditic. However, T1L characteristically induced a low frequency of lesions (Table 1), suggesting that it might be inappropriate to group reassortants that induced a low frequency of lesions (T1L-like) with those that induced a high frequency (8B-like). Therefore, in addition we applied a Wilcoxon rank sum analysis, in which the data were treated as a continuum spanning from 0 to 100% myocarditis. In this way, viruses which induced lesions in a greater percentage of the mice were ranked as more myocarditic (more 8B-like). The results of the two analyses were the same (Fig. 2): 8 of the 10 gene segments were not significantly associated with the induction of lesions (i.e., $P > 0.05$), while the L2 gene segment was implicated at only a low significance ($P = 0.05$). In contrast, the M1 gene was associated with this phenotype at a highly significant level ($P = 0.002$), indicating it was the predominant determinant of the myocarditis. Note, however, that certain reassortants did not behave as would be predicted by their M1 gene derivation (see Discussion).

It is worth noting that we verified the suitability of this data set for statistical analysis as follows. The results were randomized so that each of the 31 reassortants was arbitrarily assigned one of the 31 observed frequencies. This randomization was invoked 10 times and was followed by analysis by using the Wilcoxon rank sum method. Of the 100 opportunities for association of a gene with the myocarditic phenotype (10 randomizations involving 10 genes each), there were 94 cases with $P > 0.05$, 4 cases with $P = 0.05$, and 2 cases with $P = 0.02$. This was the distribution predicted to occur by chance, confirming the validity of a statistical analysis of this data set. This range of $P$ values also emphasizes the significance of the $P$ value (0.002) which associated the 8B-M1 gene and myocarditis.

**The S1 gene and potentially other genes can modify the 8B phenotype.** The observed continuum in the frequency of myocarditis induced by progeny reassortants of 8B (Fig. 1) suggested that genes other than M1 could modify the phenotype. However, the statistical analysis had not revealed any likely candidate genes (the L2 gene was implicated at a significance level very similar to those of the other genes).

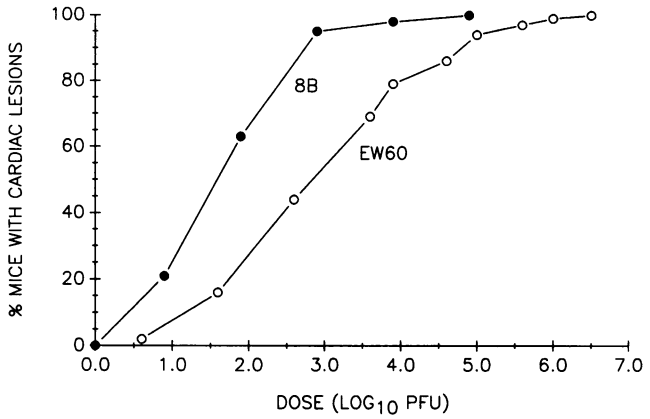


FIG. 3. Frequency of macroscopic external cardiac lesions in 8B- and EW60-infected mice. A minimum of 8 and a maximum of 35 mice were injected for each datum point indicated, and hearts were examined for macroscopic external lesions as described for Fig. 1 except that animals were not sacrificed until 21 days postinjection. Reed and Muench (27) calculations were performed, and the results were plotted.

We therefore chose to examine the role of the S1 gene, because it encodes the viral attachment protein and is the determinant of tissue tropism in the mouse central nervous system (for a review, see reference 39). We wished to examine its role in myocarditis in experiments that were more quantitative than those shown in Fig. 1, and we selected reassortant EW60 for our analysis (Fig. 1) because it had (potentially) only the S1 gene substituted with respect to 8B (the derivation of its L3 gene was unclear [Table 2]). When 8B and EW60 were injected into mice at a range of doses, EW60 was clearly less efficiently myocarditic than 8B at doses lower than $10^5$ PFU (Fig. 3). The calculated dose required to induce lesions in 50% of the mice was $7 \times 10^2$ PFU for EW60 but only $4 \times 10^1$ PFU for 8B, an 18-fold difference. Thus, although the 8B-M1 gene was the predominant determinant of myocarditis, the S1 gene could affect the phenotype. These quantitative studies, as illustrated for the S1 gene, can be used in future studies to assess the role of other genes in myocarditis.

T3D induces a lethal encephalitis in neonatal mice, and this neural tropism and disease is determined by the S1 gene (for a review, see reference 39). Since the 8B-S1 gene segment was derived from T3D and 8B was lethal even at low doses (see below), it was possible that the lethality of 8B reflected an S1 gene-specified encephalitis. Indeed, brain sections from 8B-infected animals displayed the typical encephalitis characteristics of T3D (data not shown). To examine the role of the S1 gene segment in lethality, we injected mice with a range of doses of either 8B or EW60 and recorded the number of survivors (Fig. 4). EW60 was lethal (although less lethal than 8B), demonstrating that a T3D-derived S1 gene (and therefore an encephalitis) was not a prerequisite for death. Note, however, that while 8B killed virtually every mouse showing cardiac lesions (superimposable curves in Fig. 3 and 4), this was not the case for EW60. One possible explanation for the reduced lethality of EW60 is that neonatal mice undergo significant developmental changes during the time of the experiment. If the EW60-induced lesions progressed more slowly, the developing mouse might have a change to respond to the infection. Survival of these mice could be analogous to survival of

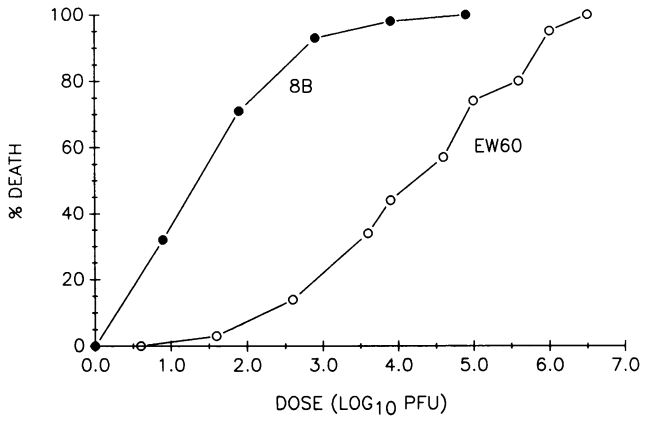


FIG. 4. Lethality of 8B and EW60. Mice were injected, Reed and Muench calculations were performed, and the data were plotted as described for Fig. 3.

T1L-infected mice, in which cardiac lesions, when present, resolve as focal scarred areas (data not shown).

**Viral replication in the heart is not correlated with myocarditic potential.** T1L grows nearly as well as 8B in the hearts of infected mice (36), suggesting that growth in the heart is not the predominant determinant of the 8B-induced myocarditis. In order to determine whether a threshold titer in the heart (achieved by 8B, but just missed by T1L) was necessary for induction of macroscopic external lesions, we assayed several efficiently myocarditic reassortants for their growth in the heart (Fig. 5). On each of the days examined, one, two, or all three of the myocarditic reassortants achieved titers only less than or equal to the T1L titer. This confirmed that the high titers of virus found in 8B-infected hearts were not a prerequisite for the myocarditic phenotype.

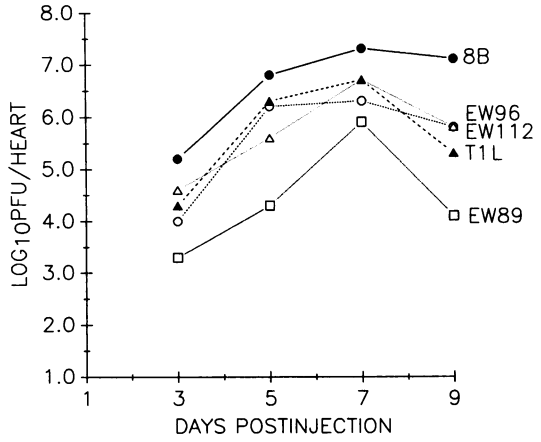


FIG. 5. Growth of virus in the hearts of infected mice. Mice were injected with $10^4$ PFU of virus. Hearts were removed on the day indicated, and viral titers were determined by plaque assay as described in Materials and Methods. Datum points on day 3 represent an average of 2 mice for the reassortant viruses and 6 mice for T1L and 8B; all other datum points represent an average of at least 6 mice for the reassortant viruses and 12 mice for T1L and 8B (except on day 9 [7 mice for T1L and 3 mice for 8B]). The standard error of the mean was always ≤0.6 for the reassortant viruses (except 1.5 for EW112 on day 3) and ≤0.25 for T1L and 8B.

## DISCUSSION

**Roles of mutation and reassortment in the 8B myocarditic phenotype.** The reassortant virus 8B displayed a myocarditic phenotype unlike that of either of its parents, T1L and T3D. We addressed the possibility that the 8B phenotype was due to novel T1L/T3D gene associations and found that none of the T1L/T3D reassortants we tested was efficiently myocarditic (Table 1). This small panel of reassortants addressed all T1L/T3D gene pairs and most T1L/T3D gene trios and quartets characteristic of 8B. Another panel of reassortants (Table 2, Fig. 1) addressed the remaining T1L/T3D gene trios and quartets as well as all higher combinations of T1L and T3D genes. That is, the T1L/T3D gene associations in 8B all include the T3D-S1 and/or the T3D-M2 gene (in association with one or several T1L-derived genes). However, many myocarditic reassortants in Fig. 1 possess a T1L-S1 gene, and several possess a T1L-M2 gene, indicating that neither the T3D-S1 nor the T3D-M2 gene is required for the myocarditic phenotype. This eliminates the possibility that any T1L/T3D gene combination found in 8B, from 2 genes to all 10 genes, is the explanation for its myocarditic phenotype.

RNA viruses mutate at a frequency of approximately $10^{-4}$ (11, 37), suggesting a simple alternative explanation for the novel phenotype. We used a panel of reassortants derived from 8B to identify any 8B gene(s) required for the myocarditic phenotype (Fig. 1). Statistical analyses of the results indicated that the 8B-M1 gene is the predominant determinant of the myocarditis (see below), suggesting that a mutation(s) in that gene is responsible for the phenotype. This mutation(s) may have been selected for in vivo during the generation of 8B (see Introduction) and/or may have been selected for because of the particular combination of T1L/T3D genes found in 8B.

**The 8B-M1 gene is associated with the myocarditic phenotype.** Statistical analyses of the myocarditic potential of 31 8B-derived reassortants (Fig. 1 and 2) indicated that the 8B M1 gene is the predominant determinant of the disease ($P$ = 0.002). Previous studies suggest that 8B is directly cytopathic to cells and that virus-mediated injury to the cardiac myocytes may be the mechanism underlying the disease (36). Together, the data suggest that alterations in the M1 gene have resulted in a highly cytopathic virus and that this is the basis for the myocarditic phenotype of 8B. Note that 8B is pantropic, as are many reoviruses, and the damage caused to other tissues may reflect the same M1-determined mechanism.

What is the function of the M1-encoded protein, and how might it be altered? Reoviruses have an outer protein capsid and an inner core, composed of proteins surrounding the viral RNA (for a review, see reference 39). Viral cores are capable of synthesizing RNA in vitro and therefore contain all the necessary enzymes for RNA synthesis. The M1 gene encodes the μ2 protein, which is present in small quantities (perhaps 12 copies per virion) in the viral core. Its low copy number suggests two possible roles (not mutually exclusive): (i) enzyme activity involved in RNA synthesis, or (ii) location at the icosahedral vertices of the core and therefore a structural function. If μ2 is involved in RNA synthesis, then it is possible that altered timing of synthesis of viral RNA or its overproduction could be responsible for the phenotype of 8B. For example, increased or altered synthesis of S1 and/or S4 RNA, which are genetically linked to inhibition of host DNA, RNA, and protein synthesis (34, 35), could lead to a more rapid shutoff of host functions and therefore cell death.

Viral genes encoding outer capsid or envelope proteins

have been the most frequently implicated determinants of pathogenicity (e.g., reovirus S1 gene [38, 42], Sindbis virus E1 and/or E2 proteins [20, 22], lymphocytic choriomeningitis virus S gene [29], mouse hepatitis virus type 4 E2 protein [5], JHM murine coronavirus E2 protein [10], rabies virus glycoprotein [6, 7, 23, 33], mumps virus hemagglutinin/neuraminidase protein [19], influenza virus hemagglutinin protein [2], and poliovirus VP1 protein [21]). However, genes encoding viral proteins with polymerase function (e.g., lymphocytic choriomeningitis L gene [28] and influenza virus PB1, PB2, PA, and NP proteins [31]) as well as noncoding regions of the genome (poliovirus 5′ noncoding region [9, 17]) have also been indicated as determinants of virulence. Since the reovirus M1 gene encodes a viral core protein, studies on the 8B-M1 gene will provide further insight into the role of noncapsid viral proteins in pathogenesis.

**Role of other reovirus genes in myocarditis.** Although the 8B-M1 gene was the predominant determinant of the myocarditic phenotype, five 8B-derived reassortants possessing the 8B-M1 gene were nonmyocarditic, and two possessing the wild-type M1 gene were (inefficiently) myocarditic (Fig. 1). Statistical analysis proved to be a powerful tool, in that it demonstrated that these exceptions were still within the limits acceptable for a significant association of the 8B-M1 gene and the disease. Nonetheless, these exceptions occurred at a fairly high frequency. One possible explanation is that these viruses contain second mutations which have suppressed the myocarditic phenotype. These mutations could be in the M1 gene (intragenic) or on another gene (extragenic). When Ramig and Fields (25) examined revertants of temperature-sensitive reovirus mutants to determine what class of suppressing mutation was most common, they found that 25 of 28 (89%) of the independently derived revertants contained extragenic suppressing mutations. Preliminary experiments with EW38 (a nonmyocarditic reassortant with an 8B-derived M1 gene [Fig. 1]) suggest that it may contain an extragenic suppressing mutation(s). Future studies will address this possibility as well as the role of intragenic and extragenic suppressing mutations in the other exceptional viruses.

The observed continuum in the frequency of myocarditis induced by progeny reassortants of 8B (Fig. 1), as well as the increased 50% myocarditis-inducing dose of a virus with a substituted S1 gene (Fig. 3), suggests that additional genes may modify the 8B-M1 gene-mediated phenotype. It is worth noting that while reovirus neurotropism is determined by one gene (S1 [42], encoding the viral attachment protein), neurovirulence is modified by a second gene (M2 [12], encoding another outer capsid protein). Virulence in La Crosse virus is also primarily determined by one gene segment, although other gene segments can affect the phenotype (13).

We have used a panel of 8B-derived reassortants to demonstrate the association of the 8B-M1 gene and induction of myocarditis. We can now used this same panel of reassortants to investigate the role of other genes in inhibiting or modifying 8B-M1 gene function and in this way further investigate the genetic basis of viral pathogenesis.

## ACKNOWLEDGMENTS

Research support came from Public Health Service grant 5 R37 AI13178 from the National Institute of Allergy and Infectious Diseases. B. Sherry is a DuPont Fellow of the Life Sciences Research Foundation.

We thank Elizabeth Wenske, David Beatty, and Sam Newell for help in the generation of virus reassortants and Elaine Freimont for general technical assistance. We also thank Dinah Bodkin, Kevin Coombs, Terence Dermody, Leslie Schiff, Kenneth Tyler, and Herbert Virgin, as well as the Fields laboratory in general, for interesting discussions of this work.

## LITERATURE CITED

1. **Bodkin, D. K., and B. N. Fields.** 1989. Growth and survival of reovirus in intestinal tissue: role of the L2 and S1 genes. J. Virol. **63**:1188–1193.
2. **Bosch, F. X., M. Orlich, H.-D. Klenk, and R. Rott.** 1979. The structure of the hemagglutinin, a determinant for the pathogenicity of influenza viruses. Virology **95**:197–207.
3. **Bowles, N. E., P. J. Richardson, E. G. J. Olsen, and L. C. Archard.** 1986. Detection of coxsackie B virus-specific RNA sequences in myocardial biopsies from cases of myocarditis and dilated cardiomyopathy. Lancet **i**:1120–1122.
4. **Brown, E. G., M. L. Nibert, and B. N. Fields.** 1983. The L2 gene of reovirus serotype 3 controls the capacity to interfere, accumulate deletions, and establish persistent infection, p. 275–287. *In* R. W. Compans and D. H. L. Bishop (ed.), Double-stranded RNA viruses. Elsevier Science Publishing, Inc., New York.
5. **Dalziel, R. G., P. W. Lampert, P. J. Talbot, and M. J. Buchmaier.** 1986. Site-specific alteration of murine hepatitis virus type 4 peplomer glycoprotein E2 results in reduced neurovirulence. J. Virol. **59**:463–471.
6. **Dietzschold, B., T. J. Wiktor, J. Q. Trojanowski, R. I. Macfarlan, W. H. Wunner, M. J. Torres-Anjel, and H. Koprowski.** 1985. Differences in cell-to-cell spread of pathogenic and apathogenic rabies virus in vivo and in vitro. J. Virol. **56**:12–18.
7. **Dietzschold, B., W. H. Wunner, T. J. Wiktor, A. D. Lopes, M. Lafon, C. L. Smith, and H. Koprowski.** 1983. Characterization of an antigenic determinant of the glycoprotein that correlates with pathogenicity of rabies virus. Proc. Natl. Acad. Sci. USA **80**:70–74.
8. **Drayna, D., and B. N. Fields.** 1982. Genetic studies on the mechanism of chemical and physical inactivation of reovirus. J. Gen. Virol. **63**:149–159.
9. **Evans, D. M., G. Dunn, P. D. Minor, G. C. Schild, A. J. Cann, G. Stanway, J. W. Almond, and J. V. Maizel.** 1985. Increased neurovirulence associated with a single nucleotide change in a noncoding region of the Sabin type 3 poliovaccine genome. Nature (London) **314**:548–550.
10. **Fleming, J. O., M. D. Trousdale, F. A. K. El-Zaatari, S. A. Stohlman, and L. P. Weiner.** 1986. Pathogenicity of antigenic variants of murine coronavirus JHM selected with monoclonal antibodies. J. Virol. **58**:869–875.
11. **Holland, J. J.** 1984. Continuum of change in RNA virus genomes, p. 137–143. *In* A. L. Notkins and M. B. A. Oldstone (ed.), Concepts in viral pathogenesis. Springer-Verlag, New York.
12. **Hrdy, D. B., D. H. Rubin, and B. N. Fields.** 1982. Molecular basis of reovirus neurovirulence: role of the M2 gene in avirulence. Proc. Natl. Acad. Sci. USA **79**:1298–1302.
13. **Janssen, R. S., N. Nathanson, M. J. Endres, and F. Gonzalez-Scarano.** 1986. Virulence of La Crosse virus is under polygenic control. J. Virol. **59**:1–7.
14. **Kerouac, M., and B. N. Fields.** 1986. Viral shedding and transmission between hosts determined by reovirus L2 gene. Science **232**:1635–1638.
15. **Khatib, R., A. Probert, M. P. Reyes, G. Khatib, and J. L. Chason.** 1987. Short review: mouse strain-related variation as a factor in the pathogenesis of coxsackievirus B3 murine myocarditis. J. Gen. Virol. **68**:2981–2988.
16. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
17. **La Monica, N., J. W. Almond, and V. R. Racaniello.** 1987. Mouse model for poliovirus neurovirulence identifies mutations that attenuate the virus for humans. J. Virol. **61**:2917–2920.
18. **Lentner, C. (ed).** 1982. GEIGY scientific tables, vol. 2. Introduction to statistics, p. 156–162. CIBA-GEIGY Corp., West Caldwell, N.J.
19. **Love, A., R. Rydbeck, K. Kristensson, C. Orvell, and E. Norrby.**

1985. Hemagglutinin-neuraminidase glycoprotein as a determinant of pathogenicity in mumps virus hamster encephalitis: analysis of mutants selected with monoclonal antibodies. J. Virol. **53**:67–74.

20. **Lustig, S., A. C. Jackson, C. S. Hahn, D. E. Griffin, E. G. Strauss, and J. H. Strauss.** 1988. Molecular basis of Sindbis virus neurovirulence in mice. J. Virol. **62**:2329–2336.
21. **Murray, M. G., J. Bradley, X. F.-Yang, E. Wimmer, E. G. Moss, and V. R. Racaniello.** 1988. Poliovirus host range is determined by a short amino acid sequence in neutralization antigenic site I. Science **241**:213–215.
22. **Polo, J. M., N. L. Davis, C. M. Rice, H. V. Huang, and R. E. Johnston.** 1988. Molecular analysis of Sindbis virus pathogenesis in neonatal mice by using virus recombinants constructed in vitro. J. Virol. **62**:2124–2133.
23. **Prehaud, C., P. Coulon, F. Lafay, C. Thiers, and A. Flamand.** 1988. Antigenic site II of the rabies virus glycoprotein: structure and role in viral virulence. J. Virol. **62**:1–7.
24. **Ramig, R., R. K. Cross, and B. N. Fields.** 1977. Genome RNAs and polypeptides of reovirus serotypes 1, 2, and 3. J. Virol. **22**:726–733.
25. **Ramig, R., and B. N. Fields.** 1979. Revertants of temperature-sensitive mutants of reovirus: evidence for frequent extragenic suppression. Virology **92**:155–167.
26. **Ramig, R., and B. N. Fields.** 1983. Genetics of reovirus, p. 197–228. *In* W. Joklik (ed.), The Reoviridae. Plenum Publishing Corp., New York.
27. **Reed, L. J., and H. A. Muench.** 1938. A simple method of estimating fifty percent endpoints. Am. J. Hyg. **27**:493–497.
28. **Riviere, Y., R. Ahmed, P. J. Southern, M. J. Buchmaier, and M. B. A. Oldstone.** 1985. Genetic mapping of lymphocytic choriomeningitis virus pathogenicity: virulence in guinea pigs is associated with the L RNA segment. J. Virol. **55**:704–709.
29. **Riviere, Y., R. Ahmed, P. Southern, and M. B. A. Oldstone.** 1985. Perturbation of differentiated functions during viral infection in vivo. II. Viral reassortants map growth hormone defect to the S RNA of the lymphocytic choriomeningitis virus genome. Virology **142**:175–182.
30. **Riviere, Y., and M. B. A. Oldstone.** 1986. Genetic reassortants of lymphocytic choriomeningitis virus: unexpected disease and mechanism of pathogenesis. J. Virol. **59**:363–368.
31. **Rott, R., M. Orlich, and C. Scholtissek.** 1979. Correlation of pathogenicity and gene constellation of influenza A viruses. III. Non-pathogenic recombinants derived from highly pathogenic parent strains. J. Gen. Virol. **44**:471–477.
32. **Scholtissek, C., A. Vallbracht, B. Flehmig, and R. Rott.** 1979. Correlation of pathogenicity and gene constellation of influenza A viruses. II. Highly neurovirulent recombinants derived from nonneurovirulent or weakly neurovirulent parent virus strains. Virology **95**:492–500.
33. **Seif, I., P. Coulon, P. E. Rollin, and A. Flamand.** 1985. Rabies virulence: effect on pathogenicity and sequence characterization of rabies virus mutations affecting antigenic site III of the glycoprotein. J. Virol. **53**:926–934.
34. **Sharpe, A. H., and B. N. Fields.** 1981. Reovirus inhibition of cellular DNA synthesis: role of the S1 gene. J. Virol. **38**: 389–392.
35. **Sharpe, A. H., and B. N. Fields.** 1982. Reovirus inhibition of cellular RNA and protein synthesis: role of the S4 gene. Virology **122**:381–391.
36. **Sherry, B., F. J. Schoen, E. Wenske, and B. N. Fields.** 1989. Derivation and characterization of an efficiently myocarditic reovirus variant. J. Virol. **63**:4840–4849.
37. **Smith, D. B., and S. C. Inglis.** 1987. Review article: the mutation rate and variability of eukaryotic viruses: an analytical review. J. Gen. Virol. **68**:2729–2740.
38. **Spriggs, D. R., R. T. Bronson, and B. N. Fields.** 1983. Hemagglutinin variants of reovirus type 3 have altered central nervous system tropism. Science **220**:505–507.
39. **Tyler, K. L., and B. N. Fields.** 1985. Reovirus and its replication, p. 823–862. *In* B. N. Fields, D. M. Knipe, R. M. Chanock, J. L. Melnick, B. Roizman, and R. E. Shope (ed.), Virology. Raven Press, New York.
40. **Tyler, K. L., D. A. McPhee, and B. N. Fields.** 1986. Distinct pathways of viral spread in the host determined by reovirus S1 gene segment. Science **233**:770–774.
41. **Virgin, H. W., IV, R. Bassel-Duby, B. N. Fields, and K. L. Tyler.** 1988. Antibody protects against lethal infection with the neurally spreading reovirus type 3 (Dearing). J. Virol. **62**:4594–4604.
42. **Weiner, H. L., D. Drayna, D. R. Averill, Jr., and B. N. Fields.** 1977. Molecular basis of reovirus virulence: role of the S1 gene. Proc. Natl. Acad. Sci. USA **74**:5744–5748.
43. **Wenske, E. A., S. J. Chanock, L. Krata, and B. N. Fields.** 1985. Genetic reassortment of mammalian reoviruses in mice. J. Virol. **56**:613–616.
44. **Woodruff, J. F.** 1980. Viral myocarditis, a review. Am. J. Pathol. **101**:427–479.