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# Isolation and Preliminary Characterization of Temperature-Sensitive Mutants of Newcastle Disease Virus

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Temperature-sensitive (*ts*) mutants of Newcastle disease virus have been isolated and characterized genetically (complementation), biochemically (RNA synthesis), and biologically (fusion from within and hemadsorption). Fifteen of these mutants have been divided into five complementation groups. Groups A (five mutants) and E (one mutant) are *ts* for RNA synthesis ($RNA^-$) as well as for the other functions. Group B contains four $RNA^+$ mutants of which one is *ts* for fusion, one for hemadsorption and two for neither function. Group C contains one $RNA^+$ mutant which is a poor cell fuser. Group D contains two $RNA^+$ mutants which are *ts* for fusion. In addition, two noncomplementing mutants (group BC) fail to complement both group B and group C mutants while exhibiting complementation with mutants in groups A, D, and E.

Virions of paramyxoviruses and cells infected by them possess a relatively large number of easily assayable properties determined by a small number of genes and gene products (perhaps six to eight) as indicated by the number of known mRNA's and proteins specified by these genes (7, 13, 15, 33). Thus, paramyxoviruses are ideal subjects for temperature-sensitive (*ts*) mutant analysis. Indeed *ts* mutants have been isolated for a number of paramyxoviruses, including measles (1, 30), Sendai virus (24, 25), and also Newcastle disease virus (NDV) (17, 26, 27). The majority of those mutants for which RNA phenotypes have been determined have an $RNA^-$ phenotype; i.e., they synthesize little or no virus-specific RNA at the nonpermissive temperature. In this paper we report the isolation of 49 *ts* mutants of NDV and the genetic, biochemical, and biological characterization of 15 of these mutants; in contrast to other studies, the majority of these *ts* mutants have $RNA^+$ phenotypes.

(Portions of this work were presented at the Conference on Negative Strand Viruses in Cambridge, England, 1973 [31].)

## MATERIALS AND METHODS

**Cell culture.** Primary and secondary chicken embryo cell cultures were prepared and grown in a 5% $CO_2$ atmosphere at 40 C in the standard medium described by Hightower and Bratt (12). Secondary cultures were confluent after 48 h of incubation and were used for virus growth experiments and plaque assays. For fusion assays, cultures were used after 1 day of incubation, prior to confluency (11). For the isolation and characterization of *ts* mutants, incubation at 36 C was carried out in a water-jacketed incubator, whereas incubation at 41.8 C was done by submerging the plates in a water bath to ensure better temperature control.

**Virus stocks.** Wild-type virus, AV-WT, was recloned from a previously cloned stock of NDV-AV (Australia-Victoria, 1932) (3). Stocks of recloned virus were used for mutagenesis. Stocks were grown in the allantoic sac of 11-day-old embryonated eggs incubated at either 38 C for AV-WT or 36 C for *ts* mutants. Allantoic fluid was harvested after 36 to 48 h of incubation, and virus was concentrated and purified as previously described (6). Virus was resuspended in Tris saline buffer (32), pH 7.4, supplemented with 2.5% calf serum, and stored at −70 C.

**Mutagenesis.** (i) $HNO_2$. Stocks of AV-WT were treated with 1 M $NaNO_2$ at pH 4.9 in 0.25 M acetate buffer (5, 23). After incubation at room temperature (25 C) for 5 min (0.004% survival) or 7.5 min (0.00001% survival), the mixtures were diluted into cold pH 8.4 medium and frozen at −70 C.

**(ii) Nitrosoguanidine.** Nitrosoguanidine was added to a stock of AV-WT at a final concentration of 100 μg/ml (5, 21). After 60 min at 25 C (0.5% survival), the mixtures were diluted and nitrosoguanidine was removed by extensive dialysis against Tris saline buffer supplemented with 2.5% calf serum. Mutagenized virus was stored at −70 C.

**(iii) 5-Fluorouracil.** Confluent cultures were infected at a multiplicity of infection of 2 PFU/cell in the presence of 400 μg of 5-fluorouracil per ml (28). Unadsorbed virus was removed by extensive washing, and the infected cultures were incubated at 36 C in medium containing 400 μg of 5-fluorouracil per ml for 11 h. Supernatant fluids were removed

and frozen at −70 C. Samples of the virus were sonicated to break up possible aggregates before plating for mutant selection.

**Isolation of *ts* mutants.** Mutagenized stocks were plated at 36 C at limiting dilution. Clones were isolated after 3 days of incubation by picking single plaques with a sterile Pasteur pipette, without the addition of neutral red to enhance plaque visibility. Plaques were resuspended in 1 ml of standard medium, and appropriate portions were then replated at 36 C. Three solitary plaques were picked from each plate, and each one was screened for plaque formation at the nonpermissive temperature (41.8 C). In all cases, clones that produced either no plaques or very small plaques after 36 to 48 h of incubation at 41.8 C were designated as potential *ts* mutants, and stocks of these potential mutants were grown as described above. The cloned stock with the lowest plating efficiency, or the smallest plaque size, was then chosen from each triplicate set and used for initial characterization. Final designation as *ts* mutants was based on significantly reduced yields at 41.8 C relative to 36 C in one-step growth experiments.

**Mode of infection. (i) Plaque assays.** Infectivity titrations were performed using plaque assays as described elsewhere (3) except that incubation was at 36 C, unless otherwise specified.

**(ii) One-step growth experiments.** Confluent cell monolayers were inoculated with virus at a multiplicity of 5 to 10 PFU/cell. After 45 min of adsorption at 4 C, cultures were washed four times with medium at 4 C and once with medium prewarmed to 36 or 41.8 C. Prewarmed medium was then added to the cultures, and the cultures were incubated at 36 or 41.8 C, respectively. At various times after infection, samples of medium containing released virus were removed and frozen at −70 C for subsequent titration by plaque assays at 36 C.

**(iii) Complementation experiments.** Infection of cell monolayers was carried out as described above except that, at 4 h postinfection, duplicate infected cultures were washed again with warm medium and fresh medium was added to the plates. Incubation was then continued at 36 C (as a control) or 41.8 C until 9.5 h postinfection, at which time the medium from all samples was removed and frozen for subsequent assay. Each mutant was added at a multiplicity of 5 PFU/cell so that singly infected cultures received 5 PFU/cell and mixedly infected cultures received 10 PFU/cell. Complementation indexes were determined by calculating the ratio of the virus yield from a mixedly infected culture to the sum of the yields from singly infected cultures of each mutant, where the infected cultures were incubated at 41.8 C and released virus was assayed at 36 C: complementation index = yield ($A$ + $B$)/yield ($A$) + yield ($B$). Complementation indexes greater than 3 were usually considered to be indicative of mutants from separate complementation groups.

**Fusion and hemadsorption assays.** Fusion assays were carried out under the conditions described by Gallaher and Bratt (11). Subconfluent cultures were infected with virus at low multiplicities (multiplicity of infection, 0.5 to 1 PFU/cell). After adsorption at 4 C, infected cultures were washed and standard medium, prewarmed to either 36 or 41.8 C, was added to the cells. At 10 h postinfection, the cells were washed, fixed, and stained with Giemsa as described by Bratt and Gallaher (3, 11). Plates were scored qualitatively for the presence (+) or absence (−) of fusion. As described previously, NDV-AV (AV-WT) induces fusion from within but is unable to induce fusion from without (3).

Hemadsorption assays were carried out by infecting cells as for virus growth experiments. At 10 h postinfection, the cells were washed with iced phosphate-buffered saline (11). A suspension of washed chicken erythrocytes was then added to each plate, and adsorption was carried out for 30 min at 4 C to prevent elution (11). Plates were then washed, fixed, and stained as for fusion and scored for the presence (+) or absence (−) of hemadsorption.

**Measurement of viral RNA synthesis.** Confluent monolayers of cells were infected as for virus growth experiments. At 3 h postinfection, the cultures were washed, and medium containing 10 μg of actinomycin D (Merck, Sharpe & Dohme) per ml and dialyzed calf serum instead of the usual calf serum was added. After further incubation for 1 h at either 36 or 41.8 C, 10 to 20 μCi of [$^3$H]uridine per ml (25 Ci/mmol; New England Nuclear Corp.) was added to each culture. At 9 h postinfection, the medium was removed, the cells were washed with cold Tris saline buffer, and the cells were solubilized in 1.5 ml of sodium dodecyl sulfate-buffer (1% sodium dodecyl sulfate, 0.1 N NaCl, 0.01 M Tris, pH 8.5, 0.002 M EDTA). Aliquots of this were then assayed for trichloroacetic acid-precipitable radioactivity (2, 4), and protein determinations were performed by the method of Lowry et al. (20), using bovine serum albumin as a standard.

## RESULTS

**Mutant isolation.** Forty-nine *ts* mutants were isolated from a cloned stock of AV-WT using 36 and 41.8 C as the permissive and nonpermissive temperatures, respectively. Of these, three were isolated after growth in 5-fluorouracil, 15 were isolated after $HNO_2$ treatment, 26 were isolated after exposure to nitrosoguanidine, and five were isolated without prior mutagenesis. The frequency with which *ts* mutants were found varied from 3 to 5% for the mutagenized samples and was only slightly lower (2%) for the untreated stock of virus.

Table 1 contains a list of the 15 best-characterized mutants and the efficiency with which each mutant forms plaques at the nonpermissive temperature. The mutants fall into two distinct classes: the standard mutants, like *ts* A1, which produce plaques at 41.8 C with a relative efficiency of $5 \times 10^{-3}$ or less, and the small-plaque *ts* mutants, like *ts* B1, which plate with approximately equal efficiencies at 36 and 41.8 C but produce only very small plaques at the higher temperature. Of the mutants listed

TABLE 1. *Properties of AV-WT and ts mutants*

| Mutant | Mutagen[a] | Efficiency of plating[b] | Yield (PFU/ml) | | |
|---|---|---|---|---|---|
| | | | 36 C | 41.8 C | 41.8 C/36 C × 100 |
| AV-WT | | 1.0 | $1.2 \times 10^7$ | $4 \times 10^6$ | 33 |
| A1 | 5FU | $1 \times 10^{-4}$ | $1.4 \times 10^7$ | $2.3 \times 10^4$ | 0.16 |
| A2 | 5FU | ~1(s) | $6.3 \times 10^6$ | $1.7 \times 10^4$ | 0.27 |
| A4 | $HNO_2$ | $5 \times 10^{-4}$ | $9.5 \times 10^6$ | $3.3 \times 10^3$ | 0.35 |
| A5 | NTG | $\sim 10^{-2}$ (s) | $1.0 \times 10^7$ | $2.8 \times 10^4$ | 0.28 |
| A7 | $HNO_2$ | $5 \times 10^{-3}$ | $1.5 \times 10^7$ | $3.1 \times 10^4$ | 0.21 |
| B1 | $HNO_2$ | ~1(s) | $4.5 \times 10^6$ | $7 \times 10^4$ | 1.5 |
| B4 | NTG | $\sim 10^{-1}$ (s) | $9.5 \times 10^6$ | $5.3 \times 10^4$ | 0.56 |
| B7 | NTG | ~1(s) | $5 \times 10^6$ | $4 \times 10^4$ | 0.80 |
| B8 | $HNO_2$ | ~1(s) | $1.1 \times 10^7$ | $3.7 \times 10^4$ | 0.33 |
| BC2 | $HNO_2$ | $\sim 10^{-3}$(s) | $8.1 \times 10^6$ | $3.2 \times 10^3$ | 0.39 |
| BC3 | NTG | $1 \times 10^{-3}$ | $3 \times 10^6$ | $8.5 \times 10^3$ | 0.28 |
| C1 | NTG | $5 \times 10^{-3}$ | $4.2 \times 10^6$ | $1.5 \times 10^4$ | 0.36 |
| D1 | NTG | $5 \times 10^{-4}$ | $8.6 \times 10^6$ | $2.3 \times 10^4$ | 0.27 |
| D4 | $HNO_2$ | $2 \times 10^{-4}$ | $1.2 \times 10^7$ | $5 \times 10^4$ | 0.42 |
| E1 | NTG | $\sim 10^{-3}$(s) | $1.7 \times 10^7$ | $9.5 \times 10^3$ | 0.056 |

[a] 5FU, 5-Fluorouracil; NTG, nitrosoguanidine.

[b] Efficiency of plating is defined as the ratio of plaques formed at 41.8 to 36 C. An (s) indicates small-plaque phenotype.

in Table 1, nine are standard mutants and six are small-plaque mutants. The remaining mutants are distributed in a similar manner into these two classes (data not shown). When a number of these small plaques from 41.8 C were harvested, cloned at 36 C, and then tested for temperature sensitivity, it was found that 31 of the 48 plaques tested yielded only virus that was still temperature sensitive (indicating that these small-plaque *ts* mutants were slightly "leaky" for virus production under multiple cycle conditions), 15 plaques yielded no measurable PFU, and only 2 of the 48 plaques tested yielded both *ts* virus and apparent revertants.

**Virus growth.** AV-WT grows well at temperatures ranging from 36 to 41.8 C. By 11 h post-infection at either 36 or 41.8 C, approximately equal numbers of infectious virus are found in the supernatant fluid, in spite of a longer lag in virus production at the lower temperature (Fig. 1A). Increasing the temperature much above 41.8 C or below 35.5 C, however, causes a decrease in the production of virus, often resulting in as much as a 10-fold reduction in yield.

The growth of each mutant was measured at the permissive and nonpermissive temperatures, and examples of growth curves for AV-WT and several *ts* mutants are presented in Fig. 1. As can be seen, these mutants grow well at 36 C and very little, or not at all, at 41.8 C. The yield of each *ts* mutant at both 36 and 41.8 C is given in Table 1. Although all of the mutants are defective for growth at the nonpermissive temperature (under single cycle conditions), there are variations in the 41.8 C yield of different mutants. For most of the mutants these variations can be attributed to two independent phenomena: one is the continued presence of a variable amount of input virus in the culture fluids, which can only partially be eliminated by extensive washing; the second is variations in the leakiness of individual mutants.

**Virus-specific RNA synthesis.** Determinations of the ability of each mutant to induce virus-specific RNA synthesis at 41.8 C were carried out by labeling infected cultures with [$^3$H]uridine, in the presence of actinomycin D, as described in Materials and Methods. The cultures were labeled for a relatively long period of time (5 h) to allow detection of RNA synthesized at any time during the viral growth cycle. The results, expressed as the ratio of [$^3$H]uridine incorporated at 41.8 C to that at 36 C, are presented in Table 2. Under these labeling conditions, AV-WT virus-infected cultures accumulate 2.6 times as much label in RNA at 41.8 C as at 36 C, whereas mutant-infected cells accumulate from 0.008 to 12.4 times as much at 41.8 C as at 36 C. The mu-

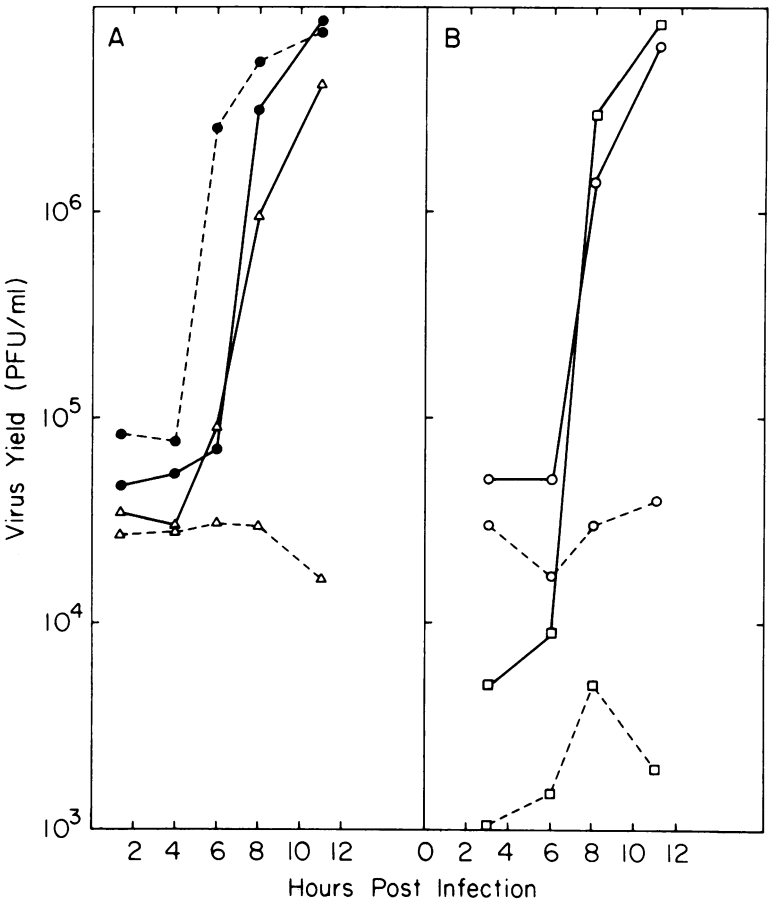


FIG. 1. *Growth of AV-WT and ts mutants at 36 and 41.8 C. (A) (●) AV-WT; (▽) ts A1. (B) (○) ts B1; (□) ts D4. Solid lines represent growth at 36 C and dashed lines represent growth at 41.8 C.*

tants can be divided into two groups on the basis of their ability to synthesize virus-specific RNA: $RNA^+$ mutants, which accumulate as much as, or more, label in RNA than AV-WT at 41.8 C; and $RNA^-$ mutants, which at 41.8 C incorporate little, if any, label above the uninfected culture background level but which clearly synthesize RNA at 36 C. A summary of the RNA phenotypes of each mutant is also included in Table 2.

**Fusion from within and hemadsorption.** Fusion from within and hemadsorption are two phenomena that occur in NDV-infected cultures late in the infectious cycle. Fusion from within occurs by 6 h postinfection, in cells infected with low multiplicities of infectious virus. It requires viral protein synthesis and occurs optimally when cells are maintained at pH 8.4 (3, 11). Mutants were tested for their ability to fuse cells into polykaryocytes at 36 and 41.8 C under these optimal conditions, and the results are shown in Table 3.

Hemadsorption refers to the ability of NDV-infected cells to adsorb erythrocytes due to the presence of viral hemagglutinin on the infected cell's outer membrane and, like fusion, first appears by 6 h postinfection and requires new viral protein synthesis. However, unlike fusion, it occurs at high and low multiplicities and occurs best when cells are maintained at pH 7.2, the optimal pH for virus production (3, 11). Mutants were tested for the production of hemadsorbing material on the surface of the infected cell, and the results are summarized in Table 3.

It can be seen that all of the $RNA^-$ mutants are, as expected, temperature sensitive for both fusion from within and the production of hemadsorbing cell surfaces. The $RNA^+$ mutants, however, fall into several classes: those that behave like AV-WT and fuse and form hemadsorbing cell surfaces at 36 and 41.8 C; those that are temperature sensitive for either fusion or hemadsorption but not both; and those that

TABLE 2. *Virus-specific RNA synthesis*

| Virus | [$^3$H]uridine incorporation (counts/min × $10^{-3}$)[a] | | 41.8 C/36 C[b] | % AV-WT[c] | RNA phenotype |
|---|---|---|---|---|---|
| | 36 C | 41.8 C | | | |
| AV-WT | 113.0 | 295.0 | 2.61 | 100 | + |
| A1 | 116.0 | 11.4 | 0.098 | 3.7 | − |
| A2 | 21.2 | 3.8 | 0.18 | 6.9 | − |
| A4 | 116.0 | 10.1 | 0.08 | 3.1 | − |
| A5 | 54.9 | 8.3 | 0.15 | 5.8 | − |
| A7 | 52.9 | 6.8 | 0.13 | 4.9 | − |
| B1 | 93.2 | 270.5 | 2.90 | 111.1 | + |
| B4 | 72.6 | 217.0 | 2.99 | 114.5 | + |
| B7 | 8.4 | 66.9 | 7.94 | 304.2 | + |
| B8 | 127.8 | 381.5 | 2.99 | 114.5 | + |
| BC2 | 39.4 | 146.2 | 3.71 | 142.1 | + |
| BC3 | 115.7 | 265.5 | 2.29 | 87.7 | + |
| C1 | 86.7 | 273.9 | 3.16 | 121.0 | + |
| D1 | 76.1 | 204.3 | 2.68 | 102.6 | + |
| D4 | 26.0 | 322.3 | 12.38 | 474.3 | + |
| E1 | 138.1 | 1.1 | 0.008 | 0.3 | − |

[a] Radioactivity incorporated between 4 and 9 h postinfection per culture of $1.5 \times 10^6$ cells. Data have been corrected to reflect only virus-induced RNA synthesis by subtracting radioactivity incorporated into uninfected control cultures (5,000 counts/min per sample).

[b] Ratio of radioactivity incorporated after 41.8 C incubation to that at 36 C incubation for each virus-infected culture.

[d] Ratio of radioactivity incorporated after 41.8 C incubation to that at 36 C incubation as percentage of AV-WT-infected cultures.

TABLE 3. *Fusion from within (FFWI) and hemadsorption*

| Virus | RNA phenotype | FFWI | | Hemadsorption | |
|---|---|---|---|---|---|
| | | 36 C | 41.8 C | 36 C | 41.8 C |
| AV-WT | + | + | + | + | + |
| *ts* A1, A2, A4, A5, A7 | − | + | − | + | − |
| *ts* E1 | − | + | − | + | − |
| *ts* B7 | + | + | + | + | − |
| *ts* B1, B4 | + | + | + | + | + |
| *ts* B8 | + | + | − | + | + |
| *ts* BC3 | + | + | − | + | + |
| *ts* D1, D4 | + | + | − | + | + |
| *ts* C1 | + | − | − | + | + |

induce very little fusion but normal hemadsorption even when the infected cells are incubated at the permissive temperature.

**Thermal inactivation of mutants.** The results of tests on thermal stability at 55 C of the infectivity of AV-WT and several *ts* mutants are presented in Fig. 2. The two RNA$^-$ mutants shown, *ts* A1 and *ts* E1, were inactivated with the same kinetics as AV-WT; this was also true for *ts* A4 and *ts* A7, the other RNA$^-$ mutants tested (unpublished data). The RNA$^+$ mutants, however, fell into two classes: those like AV-WT (*ts* B1) and those that were inactivated more rapidly than AV-WT (*ts* BC3, *ts* B4, *ts* C1).

**Complementation between *ts* mutants.** Complementation experiments have been carried out on these 15 mutants by the procedure described in Materials and Methods, and the complementation indexes obtained for each cross are presented in Fig. 3. Complementation groups have been defined by complementation indexes of 3 or less. With the exception of the low level of complementation between groups B and C, complementation between mutants in different groups was generally good, and the yield of virus from mixedly infected cultures was often as high as the yield from AV-WT-infected cultures.

On the basis of these results, the mutants have been divided into five complementation groups: group A includes five mutants that are all RNA$^-$; group B includes four RNA$^+$ mu-

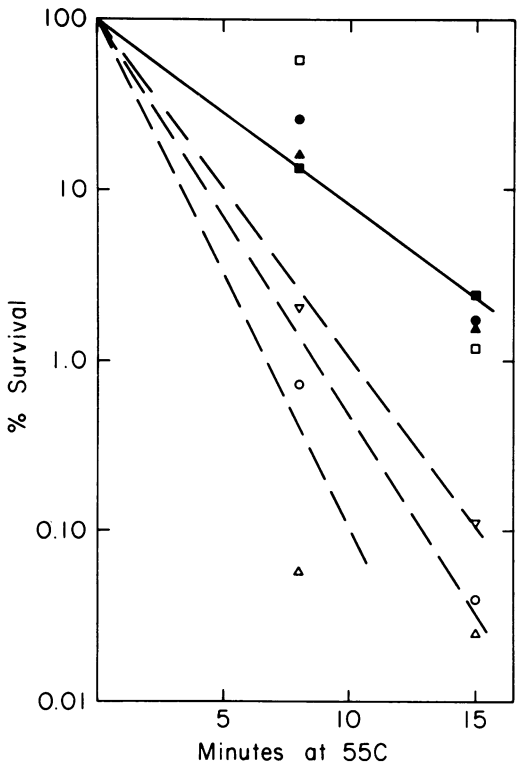


FIG. 2. *Thermal inactivation of AV-WT and ts mutants. Replicate samples of each virus tested were diluted to $10^8$ PFU/ml in Tris saline buffer and incubated at 55 C. At 0, 8, and 15 min a sample of each virus was removed and immediately diluted 10-fold with iced Tris saline buffer. Samples were kept on ice and then plated for infectious virus at 36 C. Symbols: (●) AV-WT; (▲) E1; (■) A1; (○) C1; (□) B1; (△) B4; (▽) BC3.*

tants; group C contains one RNA$^+$ mutant that is a poor cell fuser; group D contains two mutants, both of which are RNA$^+$ and *ts* for cell fusion; group E contains only one mutant that is RNA$^-$. In addition, two mutants, *ts* BC2 and *ts* BC3, fail to complement with each other or with mutants in either group B or C but exhibit good complementation with mutants in groups A, D, and E. These mutants have been designated group BC.

## DISCUSSION

NDV virions contain a single-stranded RNA genome of molecular weight $4.5 \times 10^6$ to $6 \times 10^6$ (8, 16, 18). This RNA serves as a template for five to seven 18–22S RNAs (7, 15, 33) and a 35S single-stranded RNA (B. B. Spanier and M. A. Bratt, manuscript in preparation). The 35S RNA (B. B. Spanier, C. W. Clinkscales, T. G. Morrison, and M. A. Bratt, manuscript in preparation) and at least some of the 18–22S RNAs (22) serve as templates for virus-specific proteins. Cells infected by the virus contain the six virus-specified polypeptides, which are incorporated into virions, and an additional polypeptide, which is the precursor of one of these virion proteins (13). The six polypeptides that appear to be primary translation products range in molecular weight from 41,000 to 220,000 and have a combined molecular weight of approximately $5 \times 10^5$. These proteins therefore represent most, if not all, of the coding capacity of the viral genome.

The data presented here suggest that the *ts* mutants characterized to date represent five or possibly six complementation groups; thus it would appear that mutants have been isolated in most of the genes of NDV. However, the existence of group BC (which fails to complement with either group B or C) and the low level of complementation between groups B and C suggest the possibility that these three groups might represent mutations affecting the same polypeptide; the low level of complementation between groups B and C might then reflect intracistronic complementation. Alternatively, group BC might represent either double mutants or single mutants defective in a protein that interacts with both the B and C polypeptides independently.

Of the five complementation groups, only groups A and E contain mutants defective in virus-specific RNA synthesis, whereas all the mutants in the other three groups induce the synthesis of as much viral RNA as AV-WT. This distribution of complementation groups and the overall distribution of RNA$^+$ and RNA$^-$ mutants ($^9/_{15}$ of the mutants described here and $^{27}/_{35}$ of all the mutants tested thus far [unpublished data] are RNA$^+$) is similar to that found with *ts* mutants of vesicular stomatitis virus, a rhabdovirus, which has a similar pattern of replication (9, 10, 14, 19, 21, 28, 29), but quite different from that found by other workers with *ts* mutants of Sendai virus, another paramyxovirus (24, 25), and NDV itself (26, 27). The *ts* mutants of vesicular stomatitis virus, which Pringle and others isolated, fall into five complementation groups: mutants in groups I and IV are all defective in viral RNA synthesis; group II contains both RNA$^+$ and RNA$^-$ mutants; and mutants in groups III and V are all RNA$^+$ (10, 28). In contrast, the *ts* mutants of Sendai virus fall into seven complementation groups, and mutants in six of these are defective in viral RNA synthesis (24). Only the single mutant in group G is capable of synthesizing virus-specific RNA under nonpermissive conditions. Preble and Youngner (27), working with RNA$^-$ mutants isolated from cells persistently infected with NDV, were unable to obtain

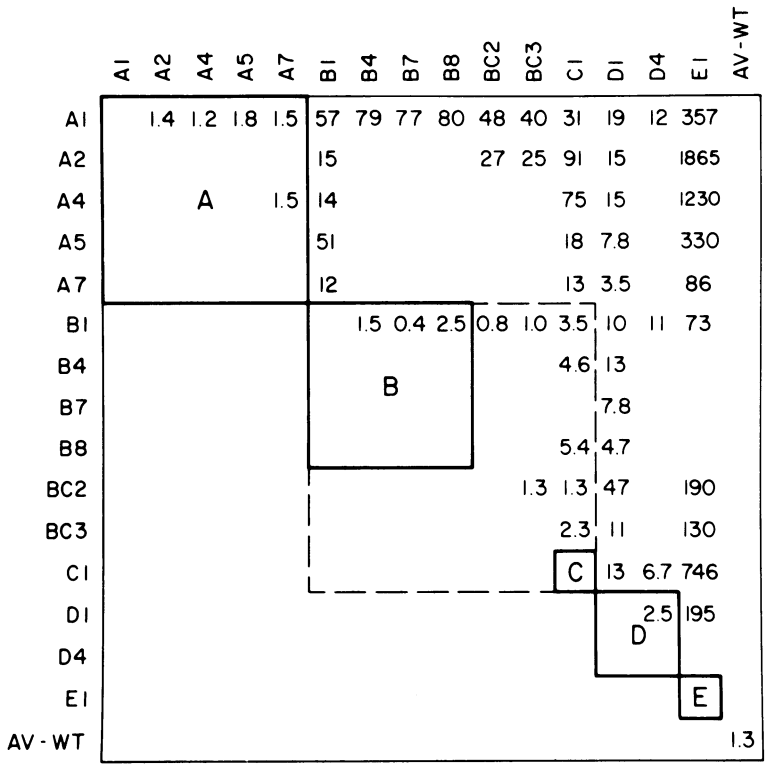


FIG. 3. *Complementation indexes for ts mutants.*

complementation between any of their *ts* mutants. This must be due to an interference phenomenon, to a genetic similarity between their mutants, or to multiple mutations.

One explanation for some of the differences observed between the earlier paramyxovirus studies and the work reported here is that different mutant isolation and selection procedures were used. The isolation of small-plaque *ts* mutants in addition to standard *ts* mutants enabled us to obtain $RNA^+$ mutants (and group B mutants in particular) with higher frequency.

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