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Generation and Genetic Characterization of Avian Reovirus Temperature-Sensitive Mutants

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There currently is little known about the genetic and biological functions of avian reovirus (ARV), an atypical member of the family *Reoviridae* and the prototype of all nonenveloped viruses that induce syncytia formation. In this study, we created ARV temperature-sensitive (*ts*) mutants by chemical mutagenesis of ARV strain 138. We developed a novel efficiency of lysis (EOL) screening technique and used it and the classical efficiency of plating (EOP) assay to identify 17 ARV *ts* mutants. Pairwise mixed infections of these mutants and evaluation of recombinant progeny *ts* status led to their organization into seven recombination groups. This indicates that these new groups of mutants represent the majority of the ARV genome. To phenotypically characterize the *ts* mutants, progeny double-stranded RNA (dsRNA) produced at permissive and nonpermissive temperature was measured. Some mutants were capable of dsRNA synthesis at the restrictive temperature (RNA^+), which indicates the effects of their *ts* lesions occur after RNA replication. Most mutants were RNA^-, which suggests their mutations affect stages in viral replication that precede progeny genome synthesis.

INTRODUCTION

Avian reoviruses (ARV) belong to the orthoreovirus genus of the family *Reoviridae*. The orthoreoviruses have a double-layered capsid that encases 10 segments of double-stranded RNA (dsRNA) which encode 11 protein products (Nibert *et al.,* 1996; Martinez-Costas *et al.,* 2000; Shmulevitz and Duncan, 2000). Despite the agricultural and economic importance of ARV, little is known about its genetic and biological functions within the host (Barnes *et al.,* 1997; Martinez-Costas *et al.,* 2000; Sheng Yin *et al.,* 2000). Avian reoviruses are similar in many respects to the more extensively studied mammalian reoviruses (MRV). However, the ARV have a number of unique features associated with them such as a different host range, lack of hemagglutination, and ability to induce syncytia in cell culture (Martinez-Costas *et al.,* 1997; Sheng Yin *et al.,* 2000). Avian reoviruses serve as the prototype of all syncytia-inducing nonenveloped viruses (Duncan *et al.,* 1996) and a novel fusogenic protein associated with this behavior has recently been identified (Shmulevitz and Duncan, 2000). Through study of this unconventional reovirus, we may broaden our understanding of the *Reoviridae* family as well as the molecular functions of viruses as a whole, in particular, the molecular determinants of syncytia formation.

Conditionally lethal mutants have been extensively exploited to delineate many biological processes in the field of virology (Compton *et al.,* 1990; Mitraki and King, 1992; Rixon *et al.,* 1992; Schwartzberg *et al.,* 1993; Shikova *et al.,* 1993; Black *et al.,* 1994; Chen *et al.,* 1994; Millns *et al.,* 1994; Ericsson *et al.,* 1995; Nagy *et al.,* 1995; Wiskerchen and Muesing, 1995; Carleton and Brown, 1996) and availability of MRV temperature-sensitive (*ts*) mutants has allowed elucidation of many aspects of MRV replication and assembly (reviewed in Coombs, 1998). To extend similar studies to the more poorly understood ARV, we introduce here the first genetically exploited panel of chemically derived ARV *ts* mutants. An efficiency of lysis assay was developed to screen for temperature sensitivity in the group of potential *ts* mutants. Temperature-sensitive status was then confirmed by standard methods (Fields and Joklik, 1969; Ramig, 1982; Coombs, 1998). Subsequently, mutants were organized into seven groups based on having their *ts* mutations in the same or different gene segments. The ability to synthesize dsRNA at permissive and restrictive temperature was measured to localize the effects of the *ts* lesions in the viral lifecycle. These mutants will ultimately serve as useful tools to elucidate ARV protein functions and allow for comparative analyses to other reoviral proteins.

RESULTS AND DISCUSSION

Various concentrations of three chemical mutagens were applied to ARV 138 and ARV 176 infections. The percentage of progeny virus survival was calculated by comparison of mutated progeny titers to that of unmutagenized infections. Bromodeoxyuridine, a DNA mutagen (Boccadoro *et al.,* 1986), had little effect on ARV 138 survival (Fig. 1) and served as our negative control.

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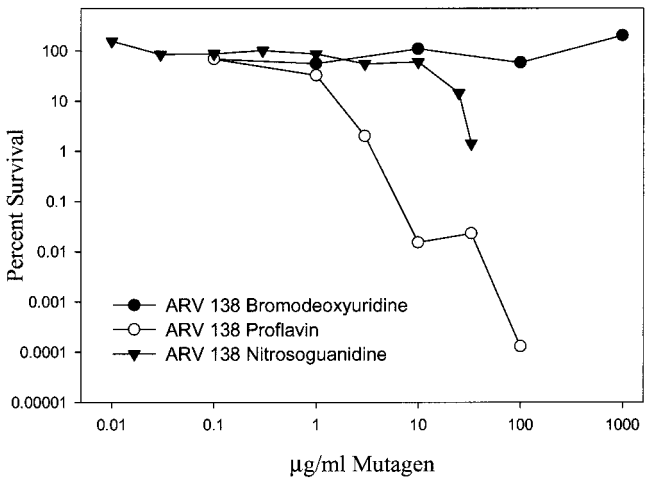

FIG. 1. Survival curves of ARV 138 after chemical mutagenesis. Various concentrations of bromodeoxyuridine, proflavin, and nitrosoguanidine were used to mutagenize ARV strain 138 infections as described under Materials and Methods and percentage survival determined.

Nitrosoguanidine and proflavin, known RNA mutagens (Singer and Fraenkel Conrat, 1967) used on the *Orthomyxoviridae* (Simpson and Hirst, 1968) and other members of the *Reoviridae* (Fields and Joklik, 1969), led to reduced ARV 138 survival with increased amounts of mutagen (Fig. 1). Similar patterns were obtained when ARV 176 was examined after mutagenesis with these three agents (data not shown). The optimal mutagen concentration to apply to maximize production of *ts* mutants results in a 1–10% survival of viral progeny (Pringle, 1996). These conditions were met when the ARV infections were treated with either 5–8 μg/ml proflavin or 34 μg/ml nitrosoguanidine (Fig. 1). We chose to continue our analyses of nitrosoguanidine-treated samples because, in the case of mammalian reovirus *ts* mutants, there are fewer proflavin-generated mutants than nitrosoguanidine-generated mutants (Fields and Joklik, 1969). In addition, the best characterized proflavin-generated MRV mutants (*ts*A201 and *ts*A279) appear to have more than a single lesion and efficiency of plating (EOP) values not significantly different from that of wild-type (Hazelton and Coombs, 1995, 1999), whereas the best characterized nitrosoguanidine-generated MRV mutants (*ts*C447 and *ts*G453) clearly have *ts* lesions in a single gene and very low EOP values (Coombs *et al.,* 1994; Shing and Coombs, 1996).

A total of 351 individual plaques, each of which failed to enlarge after transfer to 39.5°C (as detailed under Materials and Methods) and thus represent a potential temperature-sensitive mutant, were picked from a culture of ARV 138 treated with 34 μg/ml nitrosoguanidine. Each clone was double-plaque purified and then amplified through two passages as described under Materials and Methods. Eighty-one of these clones were initially screened for temperature sensitivity by the classical EOP assay. It is generally accepted for the *Reoviridae* that random variation of wild-type virus EOP values is within an order of magnitude of 1.0, whereas *ts* mutant EOP values fall below this lower limit (EOP $<$ 0.1) of random variation. Four of the 81 clones (clones 12, 37, 46, and 247) had EOP values less than 0.001, indicating they were clearly temperature sensitive, whereas the other 77 clones all had EOP values greater than 0.1. To reduce the number of clones to be screened by the standard EOP method, we developed a new technique to screen for temperature sensitivity. Avian reovirus infections are ultimately lytic to the host cell (Duncan *et al.,* 1996). Therefore, we used the four confirmed *ts* clones, and some confirmed non-*ts* clones, to create an efficiency of lysis (EOL) assay. The EOL assay compares the virus' ability to lyse cells at permissive and nonpermissive temperature. Consequently, *ts* mutations that affect any stage prior to release or cell lysis itself at restrictive temperature will be identified by the EOL assay. EOL resembles the EOP assay but is less precise and relies on general cell lysis rather than individual plaque enumeration to measure temperature sensitivity. The EOL assay allowed simultaneous analysis of 12 serial 1:3.16 (equivalent to $\sqrt{10}$) dilutions of each of eight different clones per 96-well plate. These EOL dilutions cover a dilution range similar to that of the EOP assay, where six serial 1:10 dilutions are examined. EOL incubation times were optimized such that similar amounts of cell lysis were found in comparable dilutions of the unmutagenized ARV 138 parent and the various non-*ts* clones at both 33.5 and 39.5°C (Fig. 2). In contrast, cell lysis by the confirmed *ts* clones required significantly more virus at nonpermissive temperature than the permissive temperature. Thus, clones that demonstrated decreased levels of cell lysis at the restrictive temperature, and whose EOL ratio was less than that of the unmutagenized parental ARV 138 EOL value, were identified as potentially temperature sensitive. For example, 1:316 and 1:560 dilutions of ARV 138 at restrictive and permissive temperatures, respectively, resulted in cell lysis at both temperatures and a calculated EOL ratio (the nonpermissive dilution value divided by the permissive dilution value: 560/316) of 1.8. Conversely, a 1:316 dilution of *ts* 12 was capable of cell lysis at the permissive temperature, whereas original virus stocks inoculated without dilution were not capable of cell lysis at nonpermissive temperature. Calculation of the *ts* 12 EOL ratio (1/316) resulted in a value of 0.0032. The novel EOL technique has many advantages over the standard EOP assay for initial screening of temperature sensitivity. The EOL method is not as costly since eight samples can be screened with each plate as compared to one sample per plate by EOP. The novel EOL screening approach also involves less handling and requires only 5 days to complete, in contrast to the labor-intensive efficiency of plating assay that requires a minimum of 8 days to complete. Thus, the EOL assay is a useful means to

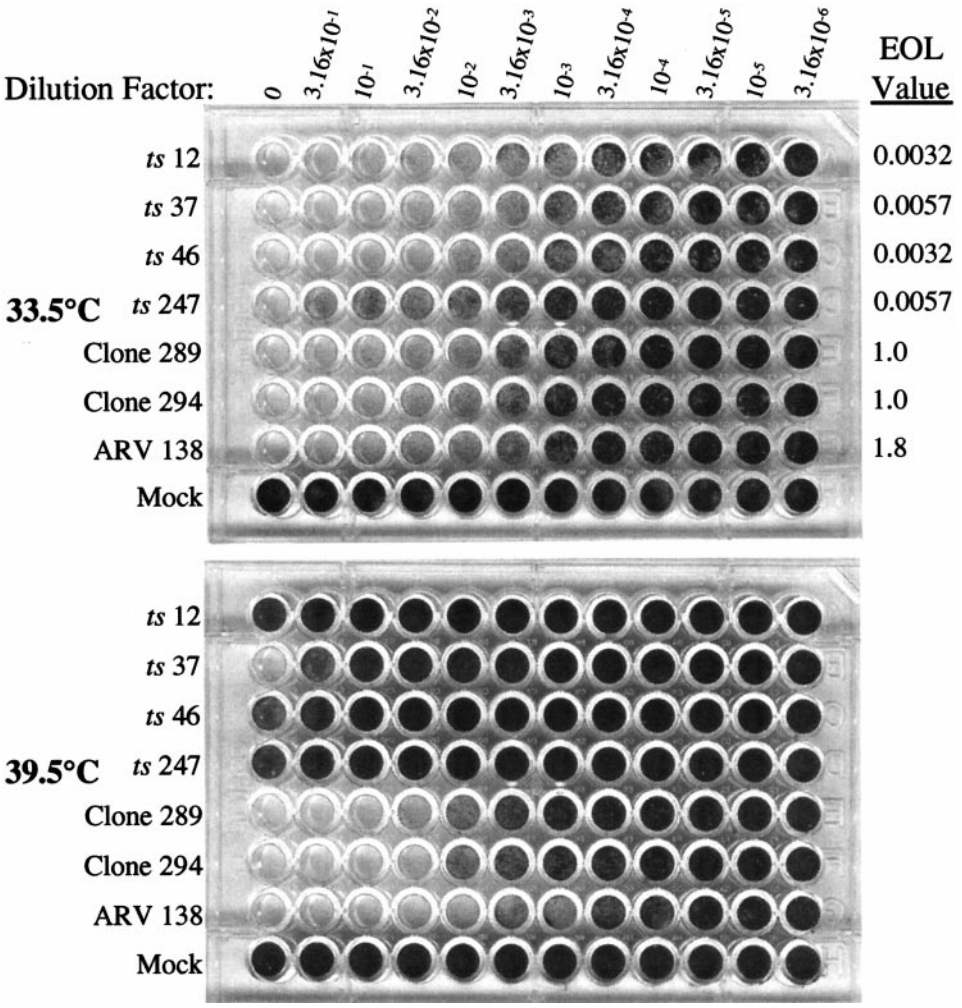

FIG. 2. Efficiency of lysis plates at permissive and restrictive temperatures. Duplicate 96-well plates of QM5 cells were infected with serial dilutions (indicated above the wells) of various virus clones, incubated at 33.5 and 39.5°C for 5 and 4 days, respectively, fixed, and stained as detailed under Materials and Methods. Lightly colored wells represent areas of cell lysis, while those with stain have intact cell monolayers. The temperature-sensitive mutants are *ts* 12, *ts* 37, *ts* 46, and *ts* 247. Clones 289 and 294 are chemically mutagenized ARV138 progeny isolated as described under Materials and Methods, but which, upon examination by EOL and EOP, were identified as non-*ts*. Mock represents uninfected cells.

screen the often hundreds of potentially *ts* clones isolated to reduce the number that need to be confirmed by the "gold standard" method of EOP.

Application of the new EOL assay to the remaining 271 picked clones indicated that 219 (81%) of the clones could be classified as non-*ts*. The other 52 clones identified as potentially *ts* by the EOL assay, as well as randomly picked clones identified as non-*ts*, were then examined by the EOP assay. All randomly chosen clones that were non-*ts* by EOL were confirmed as non-*ts* by the more accurate EOP assay, while 13 (25%) of the clones identified as *ts* by EOL were also temperature-sensitive by EOP. The efficiency of lysis screening assay consistently identified temperature-sensitive negative clones but overestimated the presence of temperature-sensitive clones according to the EOP assay. The EOL assay demonstrates high sensitivity through its negligible false negative rate but is less specific in its identification of *ts* clones compared to the EOP assay. This indicates the combined EOL/EOP approach is useful to rapidly and accurately screen many clones for temperature sensitivity, and 17 clones that represent stable ARV 138 *ts* mutants were confirmed by this combined approach. Each confirmed *ts* clone has an EOP value significantly less than that of unmutagenized ARV 138 (Table 1). Temperature-sensitive mutants occur spontaneously in the *Reoviridae* with a reported 0.1–0.3% frequency (Fields and Joklik, 1969). In contrast, the production of mammalian reovirus *ts* mutants was increased to a rate of 5% by incubation with nitrosoguanidine (Fields and Joklik, 1969). Similarly, nitrosoguanidine mutagenesis of avian reovirus resulted in a 4.8% frequency of *ts* mutant progeny generation (17 mutants isolated from 351 randomly picked plaques).

The segmented natures of the reovirus, rotavirus, and avian reovirus genomes permit recombination through

TABLE 1

Avian Reovirus Temperature-Sensitive Mutant, Efficiency of Plating Values, Recombination Values, and Their Proposed Groups

				Recombination value[c]						
Group	Clone	EOL[a]	EOP[b]	A(*ts* 12)	B(*ts* 31)	C(*ts* 37)	D(*ts* 46)	E(*ts* 158)	F(*ts* 206)	G(*ts* 247)
A	*ts* 12	0.043	0.000021	−0.001[d]	4.9	5.0	3.1	2.5	4.5	8.6
				0.5[e]	*89*	*72*	*2710*	*800*	*20*	*3621*
				1.0[f]	**263**	**453**	**2347**	**807**	**22**	**5676**
	ts 146	0.0056	0.000010	−0.002	1.0	1.8	1.7	1.3	5.0	8.1
				0.1	*14*	*13*	*880*	*406*	*15*	*3078*
				0.2	**38**	**3338**	**1018**	**445**	**25**	**4645**
B	*ts* 31	0.0056	0.0017	4.9	−0.06	2.5	2.2	2.6	6.3	8.9
				89	*0.5*	*13*	*18*	*21*	*12*	*25*
				263	**1.0**	**142**	**92**	**74**	**26**	**194**
C	*ts* 37	0.0056	0.00043	5.0	2.5	−0.1	3.1	2.6	11	8.8
				72	*13*	*0.5*	*76*	*11*	*31*	*20*
				453	**142**	**1.0**	**224**	**107**	**42**	**202**
	ts 287	0.18	0.00055	6.0	12	0.2	7.7	4.2	7.0	9.6
				72	*37*	*1.9*	*102*	*14*	*20*	*50*
				160	**147**	**4.4**	**196**	**110**	**29**	**250**
D	*ts* 46	0.0032	0.000018	3.1	2.2	3.1	−0.004	2.0	10	8.7
				2710	*18*	*76*	*0.5*	*3365*	*17*	*1038*
				2347	**92**	**224**	**1.0**	**602**	**49**	**3210**
	ts 195	0.10	0.000085	1.0	0.3	1.8	−0.001	0.9	6.0	6.0
				304	*62*	*552*	*0.3*	*34*	*1.7*	*313*
				343	**68**	**545**	**0.3**	**374**	**83**	**670**
	ts 219	0.10	0.000049	7.9	19	15	0	3.8	11	6.4
				3800	*68*	*50*	*1.0*	*703*	*21*	*1093*
				3752	**399**	**339**	**1.9**	**787**	**53**	**2026**
E	*ts* 158	0.18	0.000014	2.5	2.6	2.6	2.0	0.002	6.3	4.1
				800	*21*	*11*	*3365*	*0.5*	*9.6*	*621*
				807	**74**	**107**	**602**	**1.0**	**29**	**965**
F	*ts* 206	0.10	0.0013	4.5	6.3	11	10	6.3	−0.4	9.6
				20	*12*	*31*	*17*	*9.6*	*0.5*	*15*
				22	**26**	**42**	**49**	**29**	**1.0**	**47**
G	*ts* 247	0.10	0.000094	8.6	8.9	8.8	8.7	4.1	9.6	−0.003
				3621	*25*	*20*	*1038*	*621*	*15*	*0.5*
				5676	**194**	**202**	**3210**	**965**	**47**	**1.0**

random assortment of individual gene segments during mixed infection rather than the classical means of strand breakage and rejoining (Fields and Joklik, 1969; Ramig, 1982; Coombs, 1998). Classically, mathematical recombination analyses of mammalian reovirus (Fields and Joklik, 1969) and rotavirus (Greenberg *et al.,* 1981; Ramig, 1982) *ts* mutants genetically organize the mutants into groups based on the presence of *ts* lesions in the same or different gene segments. Mixed infection crosses that involve parents whose *ts* mutations exist on different gene segments would generate a number of wild-type-like (ts^+) reassortants that have no *ts* lesion. If the parental *ts* mutations were on the same gene segments, all progeny would be *ts*. Pairwise mixed infection of the newly generated ARV *ts* mutants were performed and viral progeny titers were examined by various recombination formulae to organize the mutants into genetic recombination groups.

The standard recombination formula used previously with mammalian reovirus calculates the percentage of viral progeny capable of growth at nonpermissive temperature and accounts for background levels of replication of the two parental clones at 39.5°C. Recombination values of <0, <0.1, or <0.2% are reported to indicate the parental *ts* mutations lie on the same gene segments (same recombination group), whereas values > 1, >2, or >3% suggest *ts* mutations exist on different gene segments (different recombination groups) (Fields and Joklik, 1969; Ramig, 1982; Coombs, 1998). Use of these parameters allowed genetic organization of most of the ARV *ts* mutants. For example, when clone *ts*D46 was crossed with *ts*D195 and *ts*D219, the average recombination value obtained from three experiments was −0.001 and 0, respectively (Table 1, upper value in corresponding cells). This indicates a lack of recombination and suggests the three viruses have *ts* lesions in the same gene. Conversely, crosses between *ts*D46 and each of the other *ts* clones (except group X mutants, which failed to recombine with numerous groups of mutants; see below) generated recombination values that

TABLE 1—*Continued*

Group	Clone	EOL[a]	EOP[b]	Recombination value[c] A(*ts* 12)	B(*ts* 31)	C(*ts* 37)	D(*ts* 46)	E(*ts* 158)	F(*ts* 206)	G(*ts* 247)
X[g]	*ts* 86	0.0056	0.011	0.2	0.3	0.9	−0.001	−0.01	−17	−6.8
				16	*14*	*47*	*1.1*	*8.9*	*1.4*	*49*
				32	**41**	**130**	**0.2**	**20**	**11**	**5.1**
	*ts*108	0.018	0.0025	0.4	0.1	0.6	0.9	−0.1	nd[h]	nd
				3.7	*2.3*	*14*	*8.1*	*0.3*	*nd*	*nd*
				9.9	**4.7**	**13**	**21**	**0.9**	**nd**	**nd**
	*ts*171	0.18	0.015	−0.1	−0.8	1.1	−0.2	0	nd	nd
				0.6	*0.06*	*3.1*	*0.4*	*1.1*	*nd*	*nd*
				1.2	**0.3**	**11**	**1.0**	**3.0**	**nd**	**nd**
	*ts*188	0.10	0.046	−15	−9.7	−10	−8.0	−7.6	nd	nd
				0.009	*0.03*	*0.1*	*0.01*	*0.3*	*nd*	*nd*
				0.06	**0.1**	**0.4**	**0.04**	**0.3**	**nd**	**nd**
	*ts*205	0.18	0.0048	6.4	5.7	8.5	7.8	3.5	−1.9	−7.1
				2.8	*2.2*	*2.8*	*2.3*	*0.3*	*0.8*	*0.4*
				2.4	**2.5**	**3.2**	**3.3**	**0.8**	**1.6**	**0.9**
	*ts*207	0.056	0.0033	−0.03	−2.9	2.9	0.3	3.6	−0.7	1.0
				1.0	*0.4*	*2.3*	*1.1*	*0.6*	*0.9*	*1.2*
				0.9	**0.6**	**1.8**	**1.4**	**1.3**	**1.4**	**2.0**
	ARV138	1.8	0.52							

[a] Efficiency of lysis values determined as described under Materials and Methods, using the formula:

$$\text{EOL value} = \frac{\text{ID}_{\text{NP}}}{\text{ID}_{\text{P}}}.$$

*Ts*A12 and ARV 138 (unmutagenized control) EOL values represent averages of five trials, all others are single screening trials.

[b] Efficiency of plating values determined by the formula:

$$\text{EOP} = \frac{\text{Titer at nonpermissive temperature}}{\text{Titer at permissive temperature}}$$

[c] Identified prototype *ts* mutants of recombination groups A–G.

[d] Recombination values calculated using the classic formula:

$$\frac{(\text{AB})_{\text{NP}} - (\text{A} + \text{B})_{\text{NP}}}{(\text{AB})_{\text{P}}} \times 100$$

(Fields and Joklik, 1969; Ramig, 1982; 1983; Coombs, 1998), where A and B are the two *ts* parents, and AB are the resulting progeny. NP represents the titer of the virus at nonpermissive temperature and P is the titer at permissive temperature. Values represent averages of at least three trials.

[e] In italics, recombination index (RI) values calculated by the formula:

$$\text{RI} = \frac{(\text{AB})_{\text{NP}}}{(\text{A} + \text{B})_{\text{NP}}}.$$

Values represent the averages of three trials (Greenberg *et al.,* 1981).

[f] In bold, EOP recombination values calculated using a novel recombination formula:

$$\text{EOP Recombination Value} = \frac{\text{EOP}_{\text{AB}}}{0.5(\text{EOP}_{\text{A}} + \text{EOP}_{\text{B}})},$$

where the progeny (AB) EOP value is compared to that of the average EOP of the two parental *ts* mutant (A, B) EOP values. Values represent averages of at least three trials.

[g] Temperature-sensitive mutants that were found to fall into more than one recombination group-indicating that more than one gene segment had *ts* lesions.

[h] nd, not determined.

ranged from 1.7 (*ts*D46 X *ts*A146) to 10 (*ts*D46 X *ts*F206). These values indicated recombination had occurred and suggested each of these clones have their *ts* lesions in a different gene than *ts*D46. However, the organization of some ARV 138 *ts* mutants was unclear since many recombination values generated were in the range between the generally accepted limits (Table 1). For example, crosses between *ts*B31 and *ts*D195 generated an average value of 0.3 (Table 1). This value has been perceived to not represent recombination in some studies (Ramig, 1982).

To try to clarify the organization of ARV *ts* mutants, the recombination index (RI) formula (Greenberg *et al.,* 1981), originally developed to analyze bovine rotavirus *ts* mu-

tants, was applied to the ARV recombination data. The RI mathematically compares the progeny titer (AB) to the sum of the parental titers (A + B) at nonpermissive temperature. RI values greater than five suggest recombination has occurred and the mutants belong to different recombination groups, whereas values less than five suggest mutants belong to the same group (Greenberg *et al.,* 1981). In most cases, RI analysis confirmed results obtained by the classic formula. For example, in the cross that involves *ts*A12 and *ts*A146,the RI value of 0.1 (Table 1; italicized middle value in corresponding cells) indicated the mutants belong to the same recombination group. Conversely, when *ts*A12 was crossed with *ts*B31, the RI value of 89 suggested the mutants belong to different recombination groups. The RI approach clarified some of the ambiguous results obtained from the classic formula. For example, mixed infections of *ts*B31 × *ts*D195 and *ts*E158 × *ts*D195 had standard recombination values of 0.3 and 0.9, respectively; whereas RI values of 62 and 34, respectively, clearly assign these mutants to different recombination groups (Table 1). However, in some cases, the standard recombination formula and recombination index values gave opposing results. For example, crosses between *ts*D195 and *ts*F206 gave a standard recombination value of 6.0, which suggested mutations resided on different gene segments, whereas the RI value of 1.7 suggested recombination had not occurred and *ts* lesions were present within the same gene segment.

Because neither recombination analytic approach takes into account titer of the parents at permissive temperature, and because temperature-sensitive status is determined by EOP value, we developed a novel EOP-based recombination formula (K. M. Coombs, unpublished data) that compares the temperature sensitivity of the progeny, as defined by their collective EOP value, to that of the average temperature sensitivity of the two *ts* parents involved in the cross. It has been generally accepted that the random variation of wild-type virus EOP values is within $1 \pm \mathrm{Log}_{10}$. *Ts* mutants are identified by their EOP values that are at least an order of magnitude lower than the EOP value of wild-type virus. Thus, progeny EOP values that are one-half to one order of magnitude greater than parental *ts* EOP values would signify that wild-type-like (ts^+) reassortants were produced and the parental mutants belong to different recombination groups. Progeny EOP values that fall within the range of EOP value random fluctuation (<5) would indicate that all progeny are *ts*, and the *ts* parents belong to the same recombination group. For example, by this new formula, the average EOP recombination value from three experiments that involved clones *ts*A12 and *ts*A146 was 0.2 (Table 1, boldfaced lower value in each cell). This value suggests ts^+ reassortants were not produced and that the parental *ts* lesions are in the same gene. Conversely, crosses between *ts*A12 and all other *ts* clones generated EOP recombination values that ranged from 22 (*ts*A12 × *ts*F206) to 5676 (*ts*A12 × *ts*G247) (Table 1). These values indicated ts^+ reassortants were produced and that each of the other *ts* clones have *ts* lesions in a different gene segment than *ts*A12. The EOP recombination formula accentuated the differences between recombination groups and clarified the ambiguous results of the standard recombination formula. For example, in the cross that involved *ts*B31 and *ts*D195, where the classical recombination formula had failed to definitively group these mutants, the average novel EOP recombination value was 68 (Table 1) and clearly placed the mutants into separate recombination groups.

In some cases the novel EOP recombination formula, standard recombination formula, and RI formula results were inconsistent with each other. For example, in the crosses that involved *ts* 86 and *ts*D46, *ts*E158, *ts*F206, *ts*G247, the novel EOP recombination formula values of 0.2, 20, 11, and 5.1, respectively (Table 1), suggested *ts* 86 fails to recombine only with *ts*D46. The standard recombination formula values of −0.001, −0.01, −17, −6.8 (Table 1) suggest that *ts* 86 is incapable of recombination with each of these mutants, whereas the RI values suggest *ts* 86 fails to recombine only with *ts*D46 (RI value = 1.1) and *ts*F206 (RI value = 1.4) (Table 1). *ts* 86 has a mildly temperature-sensitive phenotype (EOP value of 0.011, Table 1), which may account for the discrepancy between the recombination results.

Ts mutant groups were assigned if conclusions were the same from at least two of the applied formulae. This allowed us to identify a total of seven recombination groups where each group represents *ts* mutations in different genes. Thus, 7 of the 10 ARV 138 dsRNA gene segments have representative temperature-sensitive mutations. Prototype *ts* mutants were chosen (arbitrarily assigned for those recombination groups that contain more than one clone): *ts*A12, *ts*B31, *ts*C37, *ts*D46, *ts*E158, *ts*F206, *ts*G247 (Table 1). Six *ts* mutants (*ts* 86, *ts* 108, *ts* 171, *ts* 188, *ts* 205, *ts* 207) could not be assigned to groups because they failed to recombine with numerous mutants. Some of these mutants (for example, *ts* 188) exhibit a mild temperature-sensitive phenotype (EOP = 0.046; Table 1) and are capable of marginal growth at nonpermissive temperature. It has similarly been difficult to group other reovirus *ts* mutants that are associated with moderate *ts* phenotypes.

The capacity to produce progeny dsRNA genome at the nonpermissive temperature has been used to characterize mammalian reovirus (Cross and Fields, 1972) and rotavirus *ts* mutants (Chen *et al.,* 1990). Mutants are placed into one of two groups: those capable of dsRNA synthesis (RNA^+), and those that are not (RNA^-). This localizes the effects of the *ts* lesion to a certain stage within the viral lifecycle: either prior to or during genome replication (RNA^-), or at the later stages of capsid assembly and virus release (RNA^+). Thus, characterization

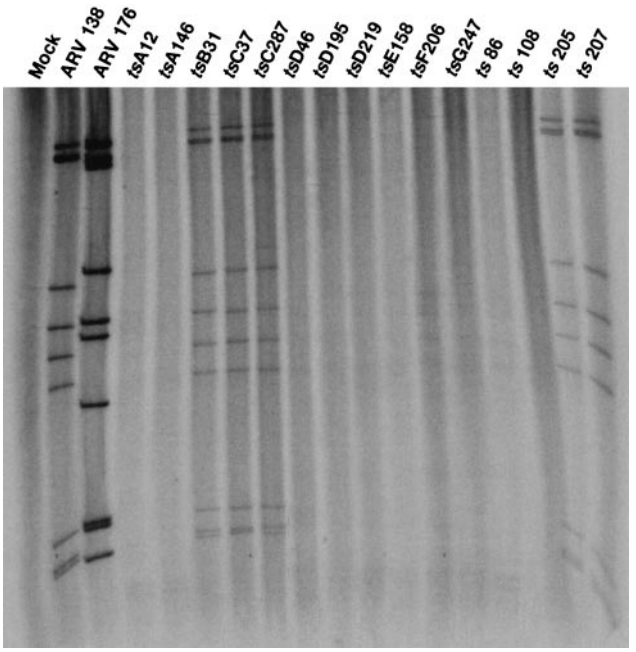

FIG. 3. Total dsRNA production at nonpermissive temperature. Cells infected at an m.o.i. of 10 PFU/cell with wild-type ARV 138, wild-type ARV 176, ARV 138 *ts* mutants, or mock infected were labeled with ^{32}P-orthophosphate and incubated at nonpermissive temperature (39.5°C) for 18 h. After incubation, dsRNA was purified as detailed under Materials and Methods and resolved in 12.5% SDS–polyacrylamide gel. The gel was dried and labeled progeny dsRNA visualized by phosphorimaging. The *ts* mutant dsRNA profiles at permissive temperature (33.5°C) were similar to wild-type virus (data not shown).

of genome synthesis in the ARV *ts* mutants serves as a preliminary analysis of the biological implications of the *ts* lesions. To phenotypically characterize the isolated ARV *ts* mutants, the total dsRNA produced from one cycle of replication was measured at both permissive and nonpermissive temperatures. The dsRNA profiles of each mutant at 33.5°C were similar to that of the wild-type controls (ARV 138 and ARV 176) (data not shown). In contrast, few mutants (*ts*B31, *ts*C37, *ts*C287) were capable of dsRNA synthesis at 39.5°C (Fig. 3). Hence, these mutants are designated RNA$^+$, whereas all other mutants failed to synthesize dsRNA at 39.5°C and were identified as RNA$^-$. Interestingly, all mutants that belong to the same recombination group were phenotypically similar with respect to genome synthesis (recombination group A and D members: RNA$^-$; group C members: RNA$^+$). This suggests similar protein functions may be targeted by the individual *ts* lesions within each recombination group. In addition, some of the nongroupable *ts* mutants were examined for total dsRNA production. *Ts* 86 and *ts* 108 were RNA$^-$ and unable to synthesize or package their viral genomes, while *ts* 205 and *ts* 207 were capable of dsRNA synthesis (RNA$^+$), as their *ts* lesions may affect later stages of the viral lifecyle. Due to complications in interpretation of gene and protein function relationships, these ungrouped *ts* mutants were not examined further in our study.

To characterize the *ts* mutants in recombination groups A–G further, an efficiency of plating profile for each clone was examined over a series of nonpermissive temperatures (Fig. 4). Clones *ts*A12, *ts*D195, and *ts*D219 expressed temperature sensitivity at a nonpermissive temperature of 37°C, while clones *ts*D46 and *ts*D158 required a temperature of 38°C to show temperature sensitivity. Clones *ts*A146, *ts*B31, *ts*C37, *ts*C287, *ts*F206, and *ts*G247 demonstrated the *ts* phenotype only at restrictive temperatures in excess of 38°C. The EOP profiles of most individual mutants in recombination groups that contain more than one member (groups A, C, and D) were unique. This suggests that the different mutants within each group represent different viral isolates.

In conclusion, we have presented the isolation of 17 avian reovirus temperature-sensitive mutants using a novel efficient screening process and standard techniques. Our genetic analysis of these mutants is the first reported for these atypical reoviruses and resulted in the identification of seven recombination groups. Currently, we are in the process of identifying the mutated protein represented by each recombination group by reassortant mapping as well as characterizing each mutant morphologically and biologically. These ARV *ts* mutants will ultimately serve as useful genetic tools to further characterize protein function in avian reoviruses and allow for comparison to other members of the family *Reoviridae.*

MATERIALS AND METHODS

Viral stocks and cell culture

ARV strains 138 and 176 are prototypic lab stocks (Hieronymus *et al*., 1983; Duncan and Sullivan, 1998) that were passaged in a QM5 continuous quail cell line as previously described (Duncan and Sullivan, 1998). Cells were maintained in Medium199 supplemented to contain 10% fetal calf serum, 10% tryptose phosphate broth, and 2 mM L-glutamine. Viral stocks were routinely passaged at a multiplicity of infection (m.o.i.) less than 1 plaque forming unit (PFU)/cell, harvested by freeze thawing three times, and stored at either 4 or −80°C.

Plaque assay

Serial dilutions of virus stocks were made in gel/saline [137 mM NaCl, 0.2 mM $CaCl_2$, 0.8 mM $MgCl_2$, 19 mM HBO_3, 0.1 mM $Na_2B_4O_7$, 0.3% (wt/vol) gelatin]. Subconfluent QM5 monolayers in six-well tissue culture dishes were infected with virus and overlaid with 1× Media199/1% agar. Overlaid plates were incubated at the desired temperature and monolayers were fed and stained according to the following schedule. Plates incubated at 36°C or greater were fed with additional

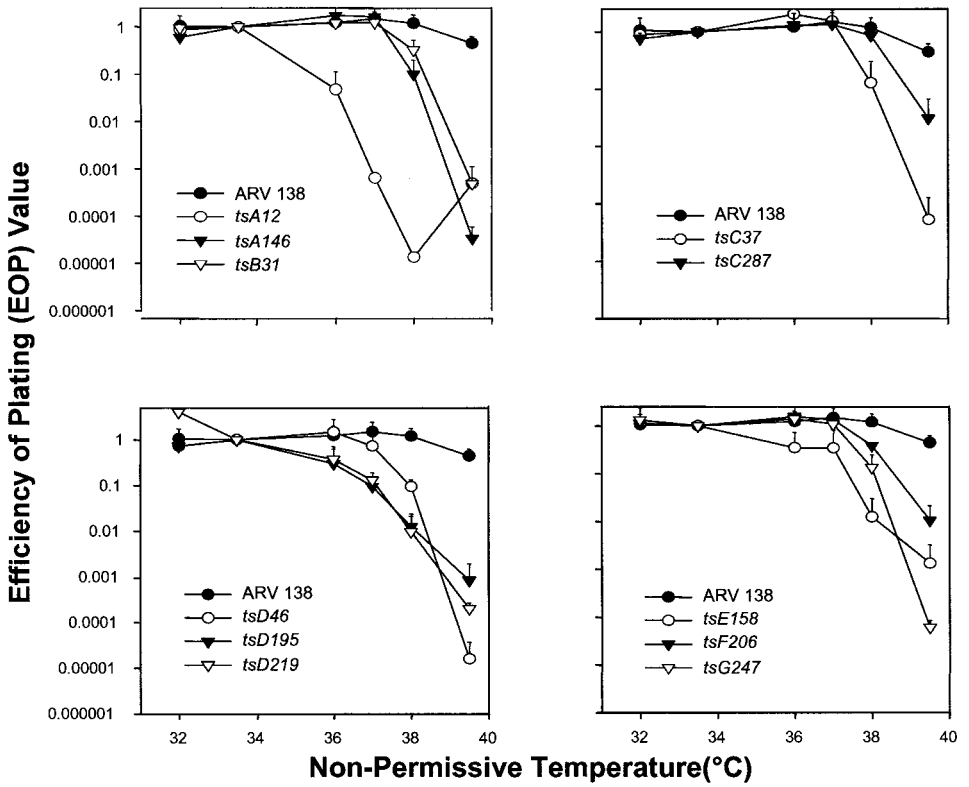

FIG. 4. Efficiency of plating profiles for *ts* mutants that belong to recombination groups A–G. In each graph ARV 138 represents unmutagenized wild-type virus. Efficiency of plating values were calculated as described under Materials and Methods with a permissive temperature of 33.5°C and indicated nonpermissive temperatures. Recombination group A: *ts*A12 (prototype), *ts*A146. Recombination group B: *ts*B31 (prototype). Recombination group C: *ts*C37 (prototype), *ts*C287. Recombination group D: *ts*D46 (prototype), *ts*D195, *ts*D219. Recombination groups E, F, and G: *ts*E158 (prototype), *ts*F206 (prototype), *ts*G247 (prototype).

Medium199/agar overlay on day 3 postinfection (p.i.) and stained with 0.02% neutral red 5 days p.i. Plates incubated at less than 36°C were fed on day 4 p.i. and stained 6 days p.i. Plaques were counted 18 h after staining.

Chemical mutagenesis and plaque purification of *ts* clones

To generate *ts* mutants, sets of QM5 cells were infected with ARV 138 and ARV 176 at an m.o.i. of 0.5–3 PFU/cell. After 1 h adsorption, the infections were overlaid with media that contained 0.1–1000 μg/ml of nitrosoguanidine, bromodeoxyuridine, or proflavin and infected cells were incubated at 37°C for 24 h. Progeny virions were harvested by freeze thawing three times and titered to determine effects of mutagen on virus replication and survival. The cell lysate from cultures that had been treated with 34 μg/ml nitrosoguanidine showed about 4% survival compared to unmutagenized controls. Subconfluent QM5 monolayers in P100 dishes were inoculated with this sample, overlaid with Media199/agar, and incubated at 33.5°C (chosen as the permissive temperature for this study) for 8 days. Plates were then lightly stained with 0.005% neutral red (Fields and Joklik, 1969) and returned to 33.5°C incubation. On day 9 the plaques were circled and the plates were placed at 39.5°C (chosen as the nonpermissive temperature for this study) for 2 days. Plaques that were identified at 33.5°C but failed to enlarge when placed at 39.5°C for 2 days were classified as potentially temperature sensitive and were picked. Picked virus clones were then plaque purified an additional time and amplified twice for subsequent study.

Efficiency of plating assay

Serial 1:10 dilutions of virus clones were added to subconfluent monolayers of QM5 cells in duplicate six-well tissue culture plates and incubated at 33.5 and 39.5°C, as described earlier. Plaques were counted to obtain viral titer at the two temperatures and the EOP ratio calculated to determine *ts* status by the formula:

$$\text{EOP} = \frac{\text{Titer at nonpermissive temperature}}{\text{Titer at permissive temperature}}.$$

Classically, *ts* mutants are defined as having EOP values at least 10-fold lower than that of wild-type unmutagenized parental virus EOP values (Coombs, 1998).

Efficiency of lysis assay

Duplicate 96-well flat-bottom plates of subconfluent QM5 monolayers were infected with aliquots of 12 serial 1:3.16 ($\sim\sqrt{10}$) viral dilutions made in gel/saline (eight different samples could be plated in each 96-well plate). After 1 h adsorption, the wells were overlaid with Medium199. One 96-well plate was placed at 33.5°C and the other at 39.5°C. On days 4 and 5 postinfection, the 39.5 and 33.5°C plates, respectively, were removed, washed with 1× phosphate buffered saline (PBS: 137mM NaCl, 0.3mM KCl, 0.8mM Na_2HPO_4, 0.1mM KH_2PO_4), fixed with 2% formaldehyde in PBS, and stained with 1% crystal violet. Permissive and restrictive temperature efficiency of lysis incubation times were determined such that at each dilution the extent of cell lysis was similar for both unmutagenized ARV 138 and various known non-*ts* clones. The ability of each virus clone to lyse cells at both temperatures was compared using an EOL value,

$$\text{EOL value} = \frac{\text{ID}_{\text{NP}}}{\text{ID}_{\text{P}}},$$

where ID = infectious dose required to lyse cells in monolayer; NP = nonpermissive temperature; and P = permissive temperature. Clones that exhibited reduced levels of cell lysis at the nonpermissive temperature compared to the permissive temperature, and whose EOL ratio was less than the EOL ratio of unmutagenized parental ARV 138 in the same assay, were considered potentially *ts*. These potential *ts* clones were then examined by the EOP assay.

Recombination analysis

Ts mutants were subjected to pairwise mixed infections in 24-well tissue culture plates. Each mutant was added at an m.o.i. of 5 PFU/cell. Self-crosses with each clone were also performed, using an m.o.i. of 10 PFU/cell. After adsorption, the wells were overlaid with media and incubated at 33.5°C for 32 h. After incubation, the plates were frozen and thawed twice and viral aggregates were disrupted by sonication. Samples were then immediately titered at 33.5 and 39.5°C as described earlier. Mutant cross results were analyzed by each of three recombination formulae.

The standard *Reoviridae* recombination formula,

$$\frac{(\text{AB})_{\text{NP}} - (\text{A} + \text{B})_{\text{NP}}}{(\text{AB})_{\text{P}}} \times 100$$

where AB = progeny from a mixed infection of parental viruses A and B; NP = nonpermissive temperature (39.5°C); and P = permissive temperature (33.5°C) (Fields and Joklik, 1969).

The recombination index formula (used to assess recombination of bovine rotavirus *ts* mutants) was also applied to ARV *ts* mutant mixed infection data:

$$\text{Recombination Index (RI)} = \frac{(\text{AB})_{\text{NP}}}{(\text{A} + \text{B})_{\text{NP}}}.$$

RI values > 5 indicate recombination between the *ts* mutants has occurred, whereas values < 5 suggest recombination has not taken place (Greenberg *et al.,* 1981).

Data were also analyzed by a novel formula that examines changes in progeny EOP values compared to parental EOP values:

$$\text{EOP Recombination Value} = \frac{\text{EOP}_{\text{AB}}}{0.5\,(\text{EOP}_{\text{A}} + \text{EOP}_{\text{B}})}.$$

Analysis of $^{32}PO_4^{-3}$-labeled viral dsRNA synthesis

Viral clones were used to infect both P60 and six-well dishes of subconfluent QM5 monolayers at an m.o.i. of 10 PFU/cell. Dishes were incubated at 39.5°C (P60 dish) or 33.5°C (six-well dish) and, at 30 min and 45 min PI, respectively, ^{32}P-orthophosphate (Mandel Scientific, Boston, MA) was added to a final concentration of 24 μCi/ml. The 39.5 and 33.5°C infections were incubated for a total of 18 and 24 h p.i., respectively (slightly less than one complete replication cycle at each temperature). Immediately after incubation, infections were placed on ice and cell monolayers were washed with ice-cold PBS to remove the unincorporated label. Cells were then scraped from the plastic dishes, harvested, and pelleted. Cell membranes were lysed in NP-40 buffer (140 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris–HCl pH 7.4, 0.5% NP-40), and the nuclei were pelleted. Double-stranded RNA was isolated from the cell lysates by phenol/chloroform extraction and precipitated overnight at −20°C in 0.108 mM sodium acetate and 2.5 vol of ice-cold ethanol. Viral dsRNA pellets were lyophilized and resuspended in 30 μl 1× electrophoresis sample buffer (0.24 M Tris–HCl pH 6.8, 1.5% dithiothreitol, 1% sodium dodecyl sulfate (SDS)). The radiolabeled samples were heated to 65°C for 5 min, and RNA was resolved in a 12.5% SDS–polyacrylamide gel (16 × 16 × 0.1 cm) for a total of 819 mAmp h (typically per gel 1.5 h at 18 mAmps followed by 66 h at 12 mAmps). The gel was dried at 80°C for 2 h, then exposed to a Molecular Imager Fx Imaging screen (BioRad) for various periods of time at room temperature. Phosphor images of the dried gel were analyzed by a Personal Molecular Imager Fx (Bio-Rad).

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