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Clustered Charge-to-Alanine Mutagenesis of Human Respiratory Syncytial Virus L Polymerase Generates Temperature-Sensitive Viruses

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Clustered charge-to-alanine mutagenesis was performed on the large (L) polymerase protein of human respiratory syncytial virus to identify charged residues in the L protein that are important for viral RNA synthesis and to generate temperature-sensitive viruses. Clusters of three, four, and five charged residues throughout the entire L protein were substituted with alanines. A minigenome replicon assay was used to determine the functions of the mutant L proteins and to identify mutations that caused temperature sensitivity by comparing the level of reporter gene expression at 39 and 33°C. Charge-to-alanine mutations were introduced into an antigenomic cDNA derived from RSV A2 strain to recover infectious viruses. Of the 27 charge-to-alanine mutations, 17 recombinant viruses (63%) were obtained. Seven mutants (41%) exhibited small plaque morphologies and/or temperature-sensitive growth in tissue culture. To generate mutant viruses with more temperature-sensitive and attenuated phenotypes, several clusters of charge-to-alanine substitutions were combined. Five combination mutants were recovered that exhibited shut-off temperatures ranging from 36 to 39°C in tissue culture and restricted replication in the respiratory tracts of cotton rats.

INTRODUCTION

The large (L) protein of human respiratory syncytial virus (hRSV), a pneumovirus of the Paramyxoviridae family, is the viral RNA-dependent RNA polymerase (Lamb and Kolakofsky, 2001; Collins *et al.,* 2001). The L protein is 2165 amino acids in length and is encoded by the L gene that constitutes approximately 43% of the hRSV genome (Stec *et al.,* 1991). Comparative sequence alignments of the hRSV L protein with other RNA-dependent RNA polymerases have identified conserved domains (Poch *et al.,* 1989, 1990; Sidhu *et al.,* 1993). The L protein most likely catalyzes initiation, elongation, and termination of RNA synthesis. Its large size suggests that it may also perform other functions during RSV replication. As with other negative-strand RNA viruses, the RSV genome is fully encapsidated by the N proteins to form ribonucleocapsids that serve as templates for RNA synthesis. RSV viral RNA replication and mRNA synthesis occur in the cytoplasm of the infected cells and require the phosphoprotein (P) and the L protein (Grosfeld *et al.,* 1995; Yu *et al.,* 1995). Recently, it has been shown that two other RSV proteins also play important roles in RSV RNA synthesis. The antitermination function of M2-1 is essential for processive RNA synthesis and transcriptional read-through at RSV gene junctions (Collins *et al.,* 1996; Hardy and Wertz, 1998; Fearns and Collins, 1999; Tang *et al.,* 2001). The M2-2 protein regulates the balance between RSV transcription and genome replication (Jin *et al.,* 2000; Bermingham and Collins, 1999).

hRSV is the leading viral cause of severe lower respiratory tract infection in infants, young children, the elderly, and immunocompromised individuals worldwide. Various approaches have been explored to develop RSV vaccines for the prevention of disease associated with RSV infection (reviewed by Collins *et al.,* 1999; Falsey and Walsh, 2000). Live attenuated RSV is considered the most effective approach for RSV vaccine development. Temperature-sensitive, live attenuated vaccine candidates have been generated for several viruses that cause human respiratory tract infections, such as influenza virus (Belshe *et al.,* 1998), hRSV (Wright *et al.,* 2000), and human parainfluenza virus (Hall *et al.,* 1992; Karron *et al.,* 1995). Viruses with temperature sensitivity phenotypes are able to replicate in the upper respiratory tract, stimulating anti-viral immunity, but are restricted in the lower respiratory tract, reducing the incidence of pneumonia and bronchiolitis. In the past, RSV ts mutants generated by cold adaptation and/or random chemical mutagenesis mostly mapped to the L gene, indicating that the L protein is an important ts determinant (Juhasz *et al.,* 1997, 1999; Whitehead *et al.,* 1998, 1999).

In order to identify charged residues that are important for the function of the RSV L protein and to generate RSV ts mutants, clustered charged residues in the RSV L protein were substituted with alanines. Alanine-scanning mutagenesis has been proposed to minimize disruption

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TABLE 1

Substitutions of Charged Amino Acids with Alanines in RSV L

Mutations	Amino acid positions	Sequence of charged cluster[a]	No. of substituted residues
A1	520	R[b]	1
A2	1033–1034	DDR	2
A3	135–136	KEKDK	2
A4	1187–1188	RDKRE	2
A5	1725–1726	DKK	2
A6	1957–1958	RK	2
A7	146–148	DED	3
A8	255–256	HKE	2
A9	353–355	RKR	2
A10	435–437	DER	3
A11	568–569	EHEK	2
A12	587–588	LRD[c]	2
A13	620–621	KERE	2
A14	1025–1026	KDK	2
A15	1187–1190	RDKRE	4
A16	1208–1209	RER	2
A17	157–158	KDD	2
A18	348–349	EED	2
A19	510–511	ERD	2
A20	1269–1270	EKK	2
A21	1306–1307	KDE	2
A22	1378–1379	DED	2
A23	1515–1516	EKD	2
A24	1662–1663	KHK	2
A25	2043–2044	DKE	2
A26	2102–2103	HKH	2
AA8	588–589	RD	2

[a] Residues substituted with alanines are underlined.

[b] Substitution of single charged residue at position 520, adjacent to F521L mutation in cpts530 (Juhasz *et al.,* 1997).

[c] Inadvertent change of a noncharged leucine residue. The intended changes are represented by AA8.

in protein conformation that is likely to produce a protein the function of which is only affected at a restricted temperature (Wertman *et al.,* 1992; Diamond and Kirkegaard, 1994; Hassett and Condit, 1994; Parkin *et al.,* 1996). Introduction of multiple mutations in the L protein should greatly increase the genetic stability of the virus ts phenotype. Thus, clusters of contiguous charged residues ranging from three to five residues in the L gene of RSV were selected as targets for mutagenesis. Within each of these clusters, two to four charged residues were changed to alanines. In addition, several single clustered charge-to-alanine mutations were combined in a single L protein to produce RSV that are more ts and attenuated. Here, we also demonstrated that the minigenome replicon system is very useful for screening the function of mutant L proteins *in vitro* and for identifying potential ts mutations. In general, recombinant RSV could be recovered from cDNA bearing L gene mutations that are not lethal to its function as determined by the minigenome assay. The phenotypes of recombinant RSV bearing charge-to-alanine substitutions at single or multiple charged clusters in the L gene are described.

RESULTS

Clustered charge-to-alanine mutagenesis of the L gene

Site-directed mutagenesis was carried out in the RSV L gene by changing clustered-charged amino acids to alanines. A cluster was originally defined as a window of five amino acids containing two or more charged residues (Wertman *et al.,* 1992). In order to perform alanine-scanning mutagenesis throughout the entire RSV L protein, only clusters containing three to five contiguous charged residues were targeted. Two clusters of five, 2 clusters of four, and 18 clusters of three contiguous charged residues are present in the RSV L gene. In each of these clusters two to four of the charged residues were substituted with alanines. In addition, a single Arg residue at position 521, which is adjacent to the F520L mutation in cpts530 (Juhasz *et al.,* 1997), was also substituted by alanine. The positions of the charge-to-alanine mutations in the L gene are listed in Table 1.

Functional analysis of the L protein mutants by RSV minigenome assay

The effect of single clustered charge-to-alanine mutations on RSV L protein function was determined by minigenome assays using chloramphenicol acetyltransferase (CAT) or Lac Z as a reporter gene. HEp-2 cells infected with modified vaccinia virus Ankara (MVA) at 1 pfu/cell were transfected with plasmids pN, pP, and pRSVCAT, or pRSVlacZ along with the wild-type (wt) or mutant pL. Transfected cells were incubated at a permissive temperature of 33°C or at a nonpermissive temperature of 39°C to determine whether the L protein function was temperature sensitive. The relative activities of various L mutants are summarized in Fig.1.

The effect of alanine substitutions of the charged residues on L polymerase function can be grouped into different categories based on their relative levels of CAT expression at 33 and 39°C. (1) Lethal mutations that resulted in greater than 99% reduction in CAT expression at 33°C: A9, A10, A13, A18, A20, A23, and A26. (2) Mutations that had little or no effect on protein function at both temperatures: A4, A5, A6, A7, A11, A14, A15, A21, and A24. (3) Mutations that resulted in reduced L activity at both temperatures: A1, A2, A3, A12, A22, A25, and AA8. (4) Temperature-sensitive mutations that resulted in higher levels of CAT expression at 33°C than at 39°C: A1, A12, A16, A17, A19, and AA8; these L mutations have greater potential to generate temperature-sensitive RSV mutants when introduced into the virus. In addition, several mutants, A3, A8, and A11, appeared to have a higher relative activity at 39°C compared to that at 33°C.

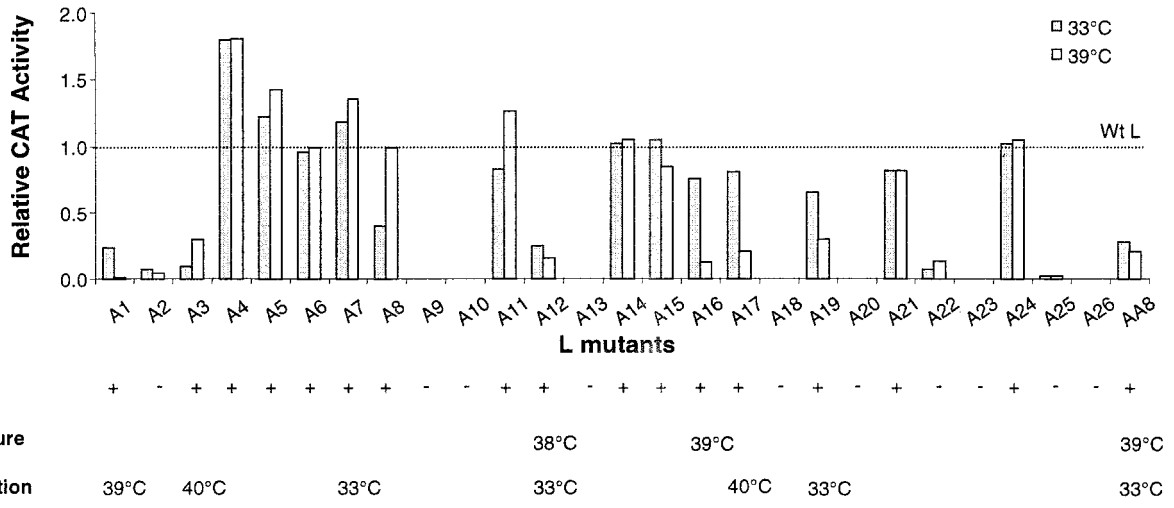

FIG. 1. Functional analysis of the L protein mutants by minigenome assay. HEp-2 cells were transfected with plasmids pN, pP, and pRSVCAT together with wt or mutant pL in triplicates and incubated at 33 or 39°C for 36 h, and CAT protein expression was analyzed by ELISA. The levels of CAT expression in each L-mutant-transfected cell are reported as relative to wt L (1.00). The relative activity of each mutant is an average from three independent experiments. Infectious RSV recovered from L mutants is indicated as (+) and the converse by (−). The plaque-forming efficiency of each recovered virus was evaluated at 33, 39, and 40°C, and the temperatures at which different mutants exhibited altered plaque phenotypes are indicated.

L mutants that had no activity or altered activity in the RSVCAT minigenome assay were further examined with the RSVLacZ minigenome to determine whether their activity could be enhanced by M2-1, since expression of Lac Z was dependent on the processivity function of M2-1. The RSVLacZ minigenome assay was performed only at 33°C because the enzymatic activity of β-galactosidase is rapidly lost at 39°C. The A15 mutant that showed a wild-type level of CAT expression at both 33 and 39°C was included for comparison. As shown in Fig. 2, the relative level of β-galactosidase expressed by each of the L mutants closely resembled that observed in the RSVCAT minigenome assay. However, the levels of β-galactosidase expression were generally higher than that observed for CAT expression, with the exception of A19. L mutants that were nonfunctional by RSVCAT minigenome assay were also inactive in the RSVLacZ minigenome assay. Mutants A3, A22, and A25 had less than 10% of wt L activity in the CAT minigenome assay, but their relative activity was much higher in the presence of M2-1 as shown by the level of β-galactosidase expression, suggesting that these L mutations might have affected the processivity of the L protein.

Recovery of recombinant RSV with mutations in L

All the L mutations that did not completely abolish CAT expression at 33°C were introduced into the L gene of a full-length RSV antigenomic cDNA derived from the A2 strain to recover recombinant RSV mutants. Except for mutants A2, A22, and A25, which had very low activity in the RSVCAT minigenome assay, viruses were recovered from the antigenomic cDNA bearing mutations that were not lethal to the protein function as determined *in vitro*. A total of 17 viruses was obtained, as indicated in Fig. 1. Multiple attempts were also made to recover A18 and A23, two representative lethal L mutations. As expected, no viruses were recovered from cDNA bearing the A18 or A23 mutations.

The recombinant viruses were plaque-purified and amplified in Vero cells. Most of the mutants had a titer of 10^6–10^7 pfu/ml. The introduced mutations in the recombinant viruses were confirmed by sequence analysis of the viral cDNA fragments obtained by RT–PCR using pairs of primers spanning the mutated sites in the L gene.

Temperature sensitivity of recovered RSV L mutants

The plaque-forming efficiency of the recombinant RSV L mutants was initially evaluated at 33, 39, and 40°C in HEp-2 cells. As indicated in Fig. 1, three mutants exhibited the ts phenotype. rA2-A12 was ts at 38°C, while rA2-A16 and rA2-AA8 had a shut-off temperature of 39°C. In addition, several L mutants formed small plaques at various temperatures. rA2-A1 formed smaller plaques at 39°C, but the small-plaque phenotype of rA2-A3 and rA2-17 was detected only at 40°C. The small-plaque phenotype of rA2-A7 and rA2-A19 was not dependent on temperature; both had small-plaque morphology at 33°C. rA2-AA8 also exhibited a small-plaque morphology at 33°C, in addition to a reduction in virus titer at 39°C. rA2-12 had the lowest shut-off temperature among the single clustered charge-to-alanine mutations showing a virus titer reduction of 5.9 $\log_{10}$ pfu/ml at 38°C.

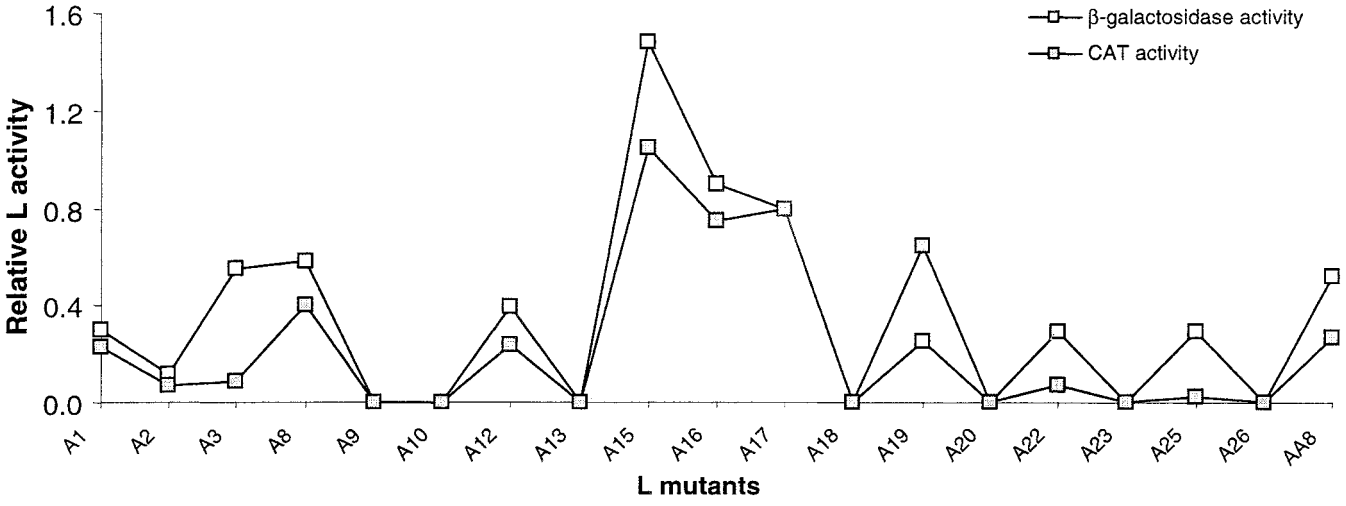

FIG. 2. Comparison of relative activities of L mutants by RSVCAT and RSVLacZ minigenome assays. L mutants with reduced or no activity by RSVCAT minigenome assay were compared to the relative activity obtained by the RSVLacZ minigenome assay in the presence of pM2–1. The assays were performed at 33°C.

Generation of RSV with multiple charged cluster-to-alanine mutations

To generate hRSV with lower shut-off temperatures, multiple mutations were introduced into the L gene by combining single charged cluster mutations that gave rise to altered virus phenotypes in tissue culture. A3, A7, A15, and A16 were selected for combination to test the functional importance of the two highly charged patches (135–146 aa and 1187–1209 aa) in L. AA8 was also incorporated into one of the combination mutants. Several combination mutations were lethal to the L protein function and did not result in virus recovery. Only five recombinant viruses were obtained, and mutations introduced into these combination mutants are listed in Table 2. The polymerase functions of the combination mutants were analyzed by RSV minigenome assays for expression of CAT or Lac Z reporter gene (Fig. 3). A3 or A7 alone did not show temperature sensitivity in the minigenome assay, but a combination of these two mutations (C7) resulted in an L protein that was ts for CAT expression at 39°C. Combining the AA8 mutation with C7 produced C27 with greater reduced L function. A15 and A16 individually displayed wt-like L activity at 33°C, but a combination of these two mutations (C9) resulted in a protein that was ts at 39°C, as shown by the RSVCAT minigenome assay. C22, a combination of A3 and A16 mutations, had a level of activity similar to that of A3 at 33°C but showed reduced activity at 39°C, as observed for A16. C25 bearing C7 and C9 mutations at four different charged clusters was still functional but exhibited a level of activity lower than that of C7 or C9. All the L combination mutants also had a higher relative activity in the RSVLacZ minigenome compared to that of the RSVCAT minigenome at 33°C, indicating enhanced polymerase activity in the presence of the M2-1 protein (Fig. 3).

TABLE 2

Combined Clustered Charge-to-Alanine Mutations

Mutations	Combined charged clusters	No. of residues changed	Single charge-to-alanine clusters
C7	135–136, 146–148	5	A3, A7
C9	1187–1190, 1208–1209	6	A15, A16
C22	135–136, 1208–1209	4	A3, A16
C25	135–136, 146–148, 1187–1190, 1208–1209	11	A3, A7, A15, A16
C27	135–136, 146–148, 588–589	7	A3, A7, AA8

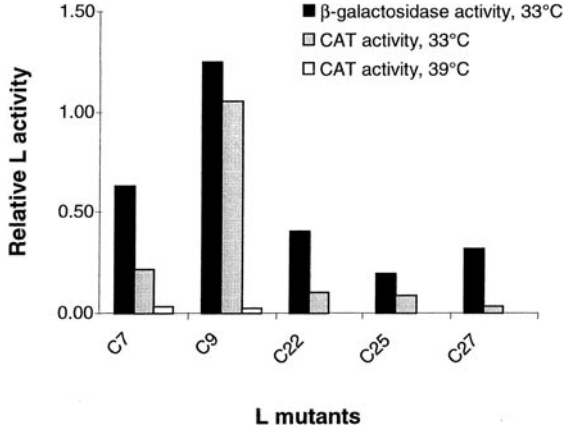

FIG. 3. Functional analysis of combination L mutants by RSVCAT and RSVLacZ minigenome assays. The RSVCAT minigenome assay was performed at 33 and 39°C, whereas the pRSVLacZ minigenome assay was performed only at 33°C. The relative activity of each L mutant is shown.

TABLE 3

Replication of L Mutants in HEp-2 Cells and in Cotton Rats

RSV L mutants	33°C	36°C	37°C	38°C	39°C	Replication in lungs of cotton rats (mean $\log_{10}$ pfu/g ± SE)[a]
rA2-A3	7.26	ND	ND	ND	6.88	ND
rA2-A7	7.00[b]	ND	ND	ND	6.30	4.90 ± 0.10
rA2-A15	6.00	ND	ND	ND	6.47	ND
rA2-A16	6.78	6.74	6.30	6.30	—	4.90 ± 0.10
rA2-AA8	6.08[b]	6.08[b]	6.08[b]	5.78[b]	—	1.53 ± 1.23
rA2-C7	5.48[b]	5.74[b]	5.53[b]	5.30[b]	5.51[b]	ND
rA2-C9	5.80	5.57	5.49	—	—	2.29 ± 0.91
rA2-C22	7.04	7.11[b]	7.11[b]	—	—	<1.7
rA2-C25	6.45[b]	—	—	—	—	<1.7
rA2-C27	6.90	5.96[b]	—	—	—	<1.7
wt rA2	7.00	6.94	6.94	6.70	6.70	4.80 ± 0.27

Note. —, Plaques not visible to the naked eye after immunostaining.

[a] Cotton rats were infected with 10^6 pfu of RSV intranasally. Four days postinfection, lung tissues were homogenized and titrated on Vero cells.

[b] Plaque size reduction of 50–80% relative to wt rA2.

Phenotypes of combination L mutants

Recombinant viruses containing mutations at multiple charged clusters were obtained from RSV antigenomic cDNA bearing the following mutations: C7, C9, C22, C25, and C27. These mutants exhibited altered plaque-forming efficiencies in HEp-2 cells, and most of them showed shut-off temperatures ranging from 36 to 38°C (Table 3). With the exception of rA2-C7, which is not temperature sensitive, the other four mutants were much more temperature sensitive than viruses with individual charged cluster mutations. Further combination of C7 with AA8 generated rA2-C27, which was ts at 37°C with reduced plaque-size formation at 36°C. rA2-C25 exhibited the cumulative effects of both rA2-C7 and rA2-C9. It was the most ts combination mutant, with a shut-off temperature at 36°C and reduced plaque size at 33°C in both HEp-2 and Vero cells.

Multiple-cycle growth analysis of the three combination mutants with shut-off temperatures between 36 and 38°C were examined in HEp-2 and Vero cells at 33 and 37°C (Fig 4). Consistent with their ts phenotypes, the level of replication for rA2-C25 was more restricted than those for rA2-C27 and rA2-C22 at 37°C. At 33°C, the peak titers of these three mutants were similar to that of wt rA2 in HEp-2 cells, although rA2-C25 showed a slower growth rate. In Vero cells, rA2-C22 and rA2-C27 had growth kinetics similar to that of wt rA2. However, rA2-C25 had a slower rate of growth throughout the entire 5-day period. At 37°C, rA2-C25 did not replicate well in both HEp-2 and Vero cells, showing a peak titer reduction of approximately 5.2 $\log_{10}$ in Vero cells. The peak titer of rA2-C27 was reduced by approximately 1.5 $\log_{10}$ in both HEp-2 and Vero cells. rA2-C22 had a growth rate similar to that of the wt rA2 in both cell lines.

Replication of the combination ts mutants was evaluated in respiratory tracts of cotton rats (Table 3). Consistent with the phenotypes observed in tissue culture, the four combination L mutants had restricted replication in the lower respiratory tract of cotton rats. Several individual, clustered charge-to-alanine mutants were also tested in cotton rats. As shown in Table 3, rA2-A16 was ts at 39°C in HEp-2 cells, but it replicated as efficiently as wt rA2 in cotton rats. However, rA2-C9 containing A15 and A16 mutations had a reduction of 2.5 $\log_{10}$ compared to rA2 in the lungs of cotton rats.

DISCUSSION

In this report clustered charge-to-alanine mutagenesis of the RSV L polymerase protein was used to generate temperature-sensitive viruses. Substitutions at 27 individual charged clusters by alanines in the RSV L protein generated 17 recombinant viruses (63%). Seven mutants (41%) exhibited ts and/or small plaque phenotypes in tissue culture. Several mutants with more ts and attenuated phenotypes were produced by the introduction of multiple clustered charge-to-alanine mutations in the L protein. To our knowledge, this is the first report to describe the generation of RSV ts mutants by clustered charge-to-alanine mutagenesis of the RSV L protein. A similar approach has been used to generate ts mutants for polioviruses (Diamond and Kirkegaard, 1994), influenza A viruses (Parkin *et al.,* 1996), vaccinia viruses (Hassett and Condit, 1994), and Dengue virus (Hanley *et al.,* 2002). Alanine substitutions of charged amino acids have also yielded temperature-sensitive L polymerases of Sendai virus with defects in viral RNA transcription and replication *in vitro* (Feller *et al.,* 2000a).

Minigenome replicon assays have been demonstrated to be very useful in analyzing the functions of viral proteins involved in RNA synthesis and in identifying potential ts mutations (Marriott *et al.,* 1999; Feller *et al.,* 2000b; Lu *et al.,* 2002). Seven of the 27 clustered charge-to-alanine L proteins that were not functional by the minigenome assay also failed to produce infectious RSV. It was also difficult to recover infectious viruses that exhibited a very low level of activity *in vitro,* such as the A2, A22, and A25 mutations. Potential ts mutations can be identified by comparing L protein activity at 33°C with that at 39°C. For example, A12 and A16 showed greatly reduced activity at 39°C compared to 33°C *in vitro,* and the recovered mutants bearing these mutations were also ts at 38 and 39°C, respectively. Mutant L protein with reduced *in vitro* activities correlated well with the altered virus phenotypes. However, the minigenome assay is not completely predictive of virus phenotypes.

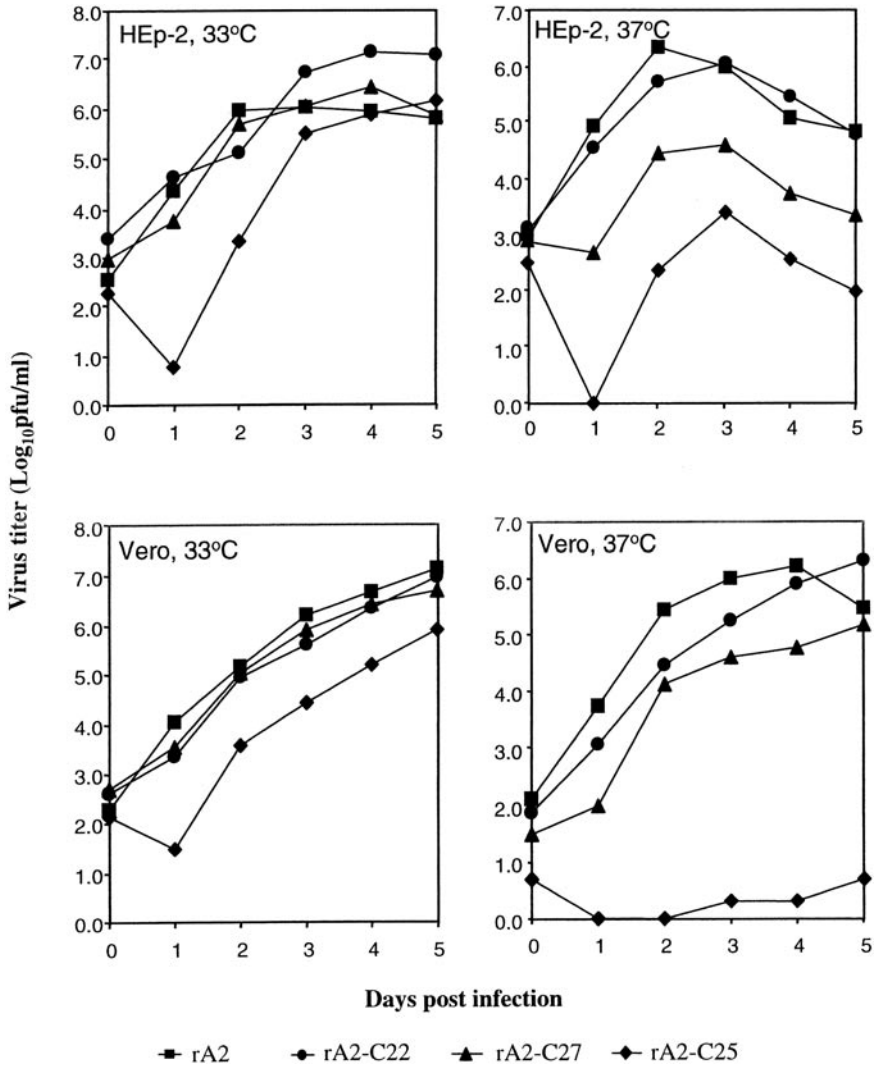

FIG. 4. Multiple-cycle growth curves of combination L mutants analyzed in Vero and HEp-2 cells. Cells were infected at a m.o.i. of 0.1 and incubated at 33 or 37°C. Aliquots of culture medium were collected at 24-h intervals and virus titers were determined by plaque assay on Vero cells.

Discrepancies between the *in vitro* activity of the mutant L protein and the virus phenotype were observed for several charge-to-alanine mutations. For example, A7 did not show reduced activity *in vitro,* but rA2-A7 formed small plaques even at the permissive temperature. The A19 L mutant was ts *in vitro,* but rA2-A19 was not ts; instead it exhibited small-plaque morphology at 33°C. Interestingly, several L mutants showed higher activity at 39°C than at 33°C. With the exception of rA2-A3, which exhibited reduced plaque size at 40°C, these mutations did not result in any observable changes in plaque-forming efficiency at the permissive and nonpermissive temperatures.

The RSVLacZ minigenome was also used to test the functions of the L mutants. Overall, the relative polymerase activity was higher using the RSVLacZ minigenome that is dependent on the processivity function of M2-1 (Tang *et al.,* 2001). None of the L mutants showed specific defects in M2-1-mediated processive RNA synthesis. The activity of A3 was relatively more enhanced by M2-1 compared to the other L mutations tested, suggesting that the A3 mutation may have affected the processivity of the L protein.

Charged residues that are important for L polymerase function can be identified by the clustered charge-to-alanine mutagenesis. Sequence alignment showed that six regions, designated domain I to VI, are highly conserved among 10 different paramyxovirus L proteins (Sidhu *et al.,* 1993). Although specific functions have yet to be assigned to each of these domains, mutagenesis of conserved residues in domains I to VI of Sendai virus L protein revealed that many of these residues are important for polymerase function (Chandrika *et al.,* 1995; Smallwood *et al.,* 1999; Feller *et al.,* 2000a; Cortese *et al.,* 2000). The highly conserved GDNQ motif (Poch *et al.,* 1989), the putative site of phosphodiester bond forma-

tion, is located in domain III (Schnell and Conzelmann, 1995; Sleat and Banerjee, 1993). Alignment of hRSV L with other paramyxovirus L protein sequences indicates that the A13 mutation is located in the putative RNA binding domain (Poch *et al.,* 1990). The A13 mutation is coincidentally located at the same position as mutant 2-2 of Sendai virus L protein, which was also inactive for RNA synthesis (Smallwood *et al.,* 1999). A20 is located in a highly conserved charged region in domain V of paramyxovirus L protein. An analogous mutation in Sendai virus L (SS21) was also completely inactive for RNA replication *in vitro* (Cortese *et al.,* 2000). This suggests that functionally important residues identified in one paramyxovirus L protein may also be important in a different paramyxovirus polymerase even when the viruses are distantly related (Feller *et al.,* 2000b). This was also demonstrated by the importation of the F521L mutation present in the L protein of RSV strain cpts530 into human parainfluenza virus type 3 strain cp45, which resulted in increased attenuation of cp45 (Skiadopoulos *et al.,* 1999). cpts530 has a shut-off temperature at 39°C in tissue culture (Juhasz *et al.,* 1997). Mutations of the charged residues in linear proximity to F521, such as E511AR512A (A19) and R520 (A1), also rendered the L protein sensitive to temperature *in vitro,* and the cognate viruses formed small plaques at 33 and 39°C, respectively. This result further indicates the importance of the regions adjacent to the 521 residue for L protein function and virus replication.

Only two clusters of five contiguous charged residues are found in the L protein at residues 134–138 and 1186–1190. Alanine substitutions of two charged residues in the first cluster reduced L protein activity *in vitro,* and the recombinant virus rA2-A3 exhibited small-plaque morphology at 40°C. Substitution of two (A4) or four amino acids (A15) in the second cluster had no effect on L protein function *in vitro.* When additional mutations were engineered around these charged clusters, the resulting viruses rA2-C7 and rA2-C9 showed altered virus replication in tissue culture. Combination of the C7 and C9 mutations produced a very attenuated virus, rA2-C25, which had a very low shut-off temperature at 36°C. Thus, incrementally more attenuated and temperature-sensitive RSV could be generated by successive introduction of mutations at multiple charged clusters in the L protein.

Recombinant RSV ts mutants with clustered charge-to-alanine mutations possess characteristics that are particularly attractive for the development of live attenuated vaccines. In the past, RSV ts mutants could only be obtained by cold passage and/or chemical mutagenesis making it difficult to control the level of virus attenuation. Using reverse genetics, it is now feasible to introduce mutations into the RSV genome to fine-tune and obtain the desired level of attenuation. The ts mutations identified in the L polymerase protein can also be utilized to attenuate other RSV strains such as subgroup B chimeric RSV (Cheng *et al.,* 2001). Since the ts phenotype is usually specified by three or more nucleotide changes in the L protein, the recombinant virus will be less prone to phenotypic reversions. The usefulness of L ts mutants for RSV vaccine development is currently being evaluated.

MATERIALS AND METHODS

Cells and viruses

Monolayer cultures of HEp-2 and Vero cells were maintained in DMEM supplemented with 10% fetal bovine serum. Modified vaccinia virus Ankara expressing bacteriophage T7 RNA polymerase (MVA-T7) was provided by Dr. Bernard Moss (Sutter *et al.,* 1995; Wyatt *et al.,* 1995) and propagated in CEK cells (SPAFAS). Recombinant A2 RSV was grown in Vero cells as described previously (Jin *et al.,* 1998).

Mutagenesis of the RSV L gene and functional analysis of the mutant L proteins

The L gene expression plasmid (pL) was engineered under the control of the T7 promoter as described by Jin *et al.* (1998). Mutations were introduced into pL by pairs of mutagenic primers using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). The introduced mutations were verified by DNA sequence analysis. The entire L gene cDNA of two select mutants was sequenced and no unintended mutations were identified, suggesting that the pfu polymerase used for mutagenesis had very high fidelity and was unlikely to introduce spurious mutations during the mutagenesis reaction. The positions of the charged residues that were changed to alanines are listed in Table 1.

The functions of the mutant L proteins were analyzed for their ability to support transcription and replication of a RSV minigenome, pRSVCAT or pRSVLacZ, that encoded the chloramphenicol acetyltransferase or β-galactosidase (*lacZ*) gene in the negative-sense under the control of the T7 promoter as described previously (Tang *et al.,* 2001). The protein expression plasmids encoding the RSV N or P proteins under the control of the T7 promoter have been described previously (Jin *et al.,* 1998). Subconfluent HEp-2 cells were infected with MVA-T7 at a multiplicity of infection (m.o.i.) of 1 pfu/cell, followed by transfection with 0.2 μg of pN, 0.2 μg of pP, and 0.1 μg of wt or mutant pL, together with 0.1 μg of pRSVCAT or 0.2 μg of pRSVLacZ. pM2-1 (0.3 μg) also was included in the pRSVLacZ transfections. Each transfection reaction was performed in duplicate using LipofecTACE (Invitrogen) according to manufacturer's protocols. The transfected cells were incubated at 33 or 39°C for 36 h, and the level of reporter gene expression was

measured. The levels of CAT protein expression were determined using an ELISA kit (Roche Molecular Biochemicals). The β-galactosidase protein expression was measured by monitoring the change in optical density at 550 nm (OD550) following incubation of the cell extract with chlorophenol red-β-D-galactopyranosidase (Roche Molecular Biochemicals) as described by Tang *et al.* (2001). The levels of the reporter gene expressed by the L mutants are expressed relative to that of wt L.

Recovery of recombinant RSV bearing mutations in the L gene

The L gene with the engineered mutations was excised from pL through the unique Bam*HI* and Not*I* restriction sites and introduced into pRSVC4G that contained a C to G change at the fourth position of the RSV leader in the antigenomic sense (Jin *et al.,* 1998). All the L mutations introduced into the full-length antigenomic cDNA clones were confirmed by DNA sequencing.

Recovery of recombinant RSV was performed as described previously (Collins *et al.,* 1995; Jin *et al.,* 1998). Subconfluent HEp-2 cells were infected with MVA-T7 at 1 pfu/cell and transfected with 0.4 μg pN, 0.4 μg pP, 0.2 μg mutant pL, and 0.4 μg of antigenomic cDNA containing the corresponding mutations engineered in the L gene. Following transfection, cells were incubated at 33°C for 3 days and the cell culture supernatants were used to infect fresh Vero cells to amplify rescued virus. Transfections that did not result in virus recovery were repeated in the presence of wt pL, and the mutants were plaque isolated. Individual plaques were subsequently screened for the introduced mutations in the L gene. All of the mutations introduced into the recombinant RSV mutants were confirmed by sequence analysis of the RT–PCR cDNA fragments spanning the introduced mutation sites in the L gene. Recombinant viruses were grown in Vero cells and titrated by plaque assay on Vero cells.

Determination of temperature sensitivity of recombinant mutants

Temperature sensitivity of the recovered recombinant viruses was examined by plaque assay on Vero or HEp-2 cells at temperatures ranging from 33 to 40°C. Monolayers of HEp-2 or Vero cells were infected with 10-fold serially diluted virus and overlaid with L15 medium containing 1% methylcellulose and 2% fetal bovine serum. The infected cells were incubated for 6 days at 33°C in a CO_2 incubator or in water-tight containers containing 5% CO_2 submerged in circulating water baths. The circulating thermoregulators were calibrated to 38, 39, or 40°C and maintained the set temperatures to within 0.1°C. After 6 days of incubation, plaques were enumerated following immunostaining. The shut-off temperature was defined as the lowest temperature that had a titer reduction of 100-fold or greater compared to that at 33°C.

Growth kinetics of RSV L mutants in cell culture

Subconfluent HEp-2 and Vero cells were used for multiple-cycle growth analyses of the RSV L mutants. Cell monolayers in 6-cm dishes were infected with virus at a m.o.i. of 0.1. After adsorption for 1 h at room temperature, the infected cells were washed twice with PBS, overlaid with 2 ml of OptiMEM, and incubated at 33 or 37°C. At 24-h intervals, 180 μl of culture supernatant was removed, stabilized with 0.2 M sucrose, 3.8 mM KH_2PO_4, 7.2 mM K_2PO_4, and 5.4 mM monosodium glutamate (SPG), and stored at −80°C prior to titration. Virus titers were determined by plaque assay on Vero cells.

Replication of RSV L mutants in cotton rats

The replication of RSV L mutants was compared with that of wt rA2 in 4- to 6-week-old cotton rats (*Sigmodon hispidus*) that were free of respiratory pathogens. Groups of five cotton rats were inoculated with 10^6 pfu of virus in 0.1 ml intranasally under light methoxyflurane anesthesia. Four days after infection, cotton rats were sacrificed by CO_2 asphyxiation and lungs were homogenized in 2 ml of OptiMEM I. Virus titers were determined by plaque assay on Vero cells and expressed as pfu per gram of tissue (Prince *et al.,* 1978; Crowe *et al.,* 1994).

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