

# Frame shift mutations as a novel mechanism for the generation of neutralization resistant mutants of human respiratory syncytial virus

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**The genetic characterization of four previously reported mutants of human respiratory syncytial (RS) virus resistant to monoclonal antibody 63G is described. Sequences of the G protein genes were obtained from: (i) mRNA derived cDNA recombinants, (ii) direct mRNA sequencing and (iii) amplified vRNA derived cDNAs. The results obtained indicate that the original escape mutants, recovered from individual plaques, contained heterogeneous viral populations. This heterogeneity affected the number of adenosine residues present after nucleotides 588 or 623 of the G protein gene. Mutant viruses recovered after a second plaque purification step generated homogeneous sequences but contained single adenosine insertions or deletions at those two sites compared with the Long sequence. These genetic alterations introduced frameshift changes which are reflected in both the antigenic and structural properties of the mutant G proteins. The origin and importance of frameshift mutations in the RS virus G protein gene are discussed.**

**Key words:** frameshift mutation/glycoproteins/paramyxoviruses/respiratory syncytial virus

## Introduction

The G glycoprotein of human respiratory syncytial (RS) virus is responsible for cell attachment to the membrane receptor during the initial stages of an infectious cycle (Levine *et al.*, 1987). This step is mediated in other paramyxoviruses by the hemagglutinin–neuraminidase (HN) or the hemagglutinin (H) glycoproteins (Chanock and McIntosh, 1985); however, neither sequence nor structural homology has been detected between the RS virus G protein and the HN or H protein of paramyxoviruses (Wertz *et al.*, 1985; Satake *et al.*, 1985).

The nucleotide sequence of the G protein gene predicts a polypeptide of 298 or 292 amino acids for viruses of the A and B subgroups, respectively (Johnson *et al.*, 1987), with a single hydrophobic domain between residues 38 and 66 which is postulated to serve as both signal sequence and transmembrane anchor. The C-terminal ectodomain of the G protein contains three to eight potential sites for N-linked oligosaccharide side chains in different strains, and >70 potential sites (Ser or Thr) for O-linked sugars (Johnson *et al.*, 1987). Recently, Hendricks *et al.* (1988) described two non-virion-associated (soluble) forms of the G protein

(G<sub>s</sub>), generated by spontaneous cleavage after amino acids 65 or 74.

Experiments done with glycosylation inhibitors (Lambert, 1988) and cell lines deficient in O-glycosylation (Wertz *et al.*, 1989) indicate that the G protein is synthesized as a 32 kd polypeptide precursor, and cotranslationally modified by the addition of high mannose N-linked sugars to form an intermediate of 45 kd. This step is followed by the conversion of the N-linked sugars to the complex type and the addition of O-linked side chains to achieve the mature 90 kd form.

Data from different sources indicate that the G protein is important for the induction of a neutralizing and protective immune response. Although most anti-G MAbs have only moderate *in vitro* neutralization indexes, these could be increased in some cases by the addition of complement (García-Barreno *et al.*, 1989). Neutralization enhancement was also detected with mixtures of two (Anderson *et al.*, 1988) or more anti-G MAbs (García-Barreno *et al.*, 1989). Moreover, protection against an RS virus challenge was observed in experimental animals after: (i) passive transfer of anti-G MAbs (Taylor *et al.*, 1984; Walsh *et al.*, 1984), (ii) immunization with purified antigen (Walsh *et al.*, 1987) or (iii) inoculation of vaccinia recombinants expressing the G protein (Olsmed *et al.*, 1986; Stott *et al.*, 1987).

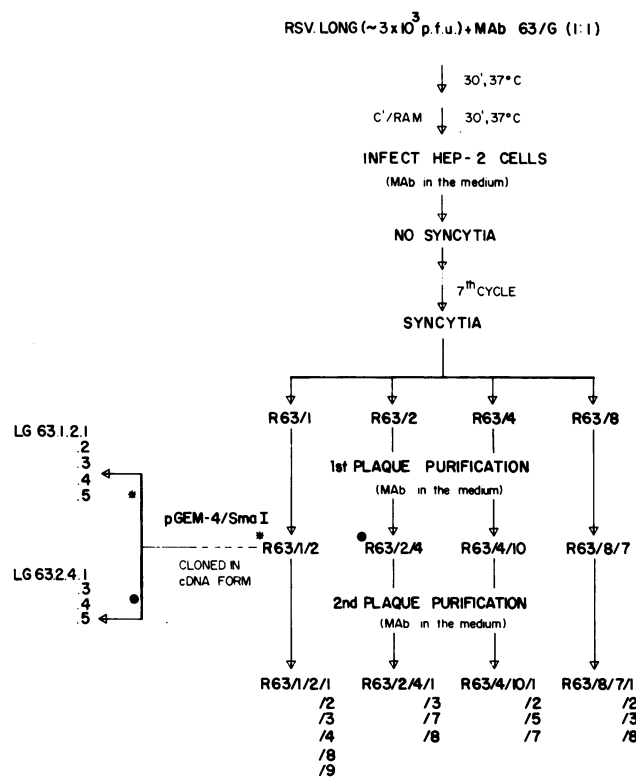
To understand the structural basis of G protein antigenicity, we have isolated and characterized RS virus mutants which escape neutralization to individual MAbs. Four such mutants were isolated after serial passage of the Long strain in the presence of MAb 63G (García-Barreno *et al.*, 1989). The 63G resistant viruses showed drastic antigenic changes reflected in the absence of antibody binding with most anti-G MAbs. The nucleotide sequence changes selected in these viruses reveal a novel mechanism, based in frameshift mutations, for the generation of neutralization resistant mutants.

## Results

### **Sequence of the G protein gene from cDNA clones of the original escape mutants to MAb 63G**

We had reported (García-Barreno *et al.*, 1989) the isolation of neutralization resistant viruses after serial passage of the Long strain in the presence of MAb 63G, as illustrated in Figure 1. Four mutants (R63/1, R63/2, R63/4 and R63/8) were isolated and plaque purified to yield the viruses: R63/1/2, R63/2/4, R63/4/10 and R63/8/7.

To identify the genetic changes selected in the escape mutants, cDNA copies of the G mRNA from cells infected with Long, R63/1/2 or R63/2/4 viruses were cloned in pGEM4 vector and sequenced by the dideoxy method with specific primers. Figure 2 shows the nucleotide (part A) and deduced amino acid (part B) sequences of the Long virus gene. Four nucleotide differences were detected when compared with the published sequence of the same strain



**Fig. 1.** Scheme for isolation of 63G escape mutants. See Materials and methods. Poly(A)<sup>+</sup> mRNAs from cells infected with mutants R63/1/2 and R63/2/4 were used for cDNA synthesis and cloning of the G protein gene in the pGEM-4 vector. Several recombinant clones (LG 63.1.2.1, .2, etc.) were isolated from each mutant cDNA.

(Johnson *et al.*, 1987). Since these differences, three of them leading to amino acid changes, were maintained in the mRNA (see later), they probably reflect different passage histories of the virus in the two laboratories.

The nucleotide sequences determined for cDNA clones from mutants R63/1/2 and R63/2/4 contained several changes with respect to the Long G sequence (Figure 3). These changes involved the addition or deletion of adenosine residues, after nucleotides 588 or 623, within short A runs. Five cDNAs from mutant R63/1/2 contained a single A deletion after nucleotide 588. One of the clones (LG63.1.2.1) had, in addition, five adenines inserted after nucleotide 623. Two other cDNAs (LG63.1.2.2 and LG3.1.2.4) had no insertions, and two (LG63.1.2.3 and LG63.1.2.5) contained a single A insertion after nucleotide 623.

Four cDNAs obtained from mutant R63/2/4 also revealed sequence heterogeneity. All four clones contained a single A deletion after nucleotide 623. Two cDNAs (LG63.2.4.1 and LG63.2.4.5) had no other changes but two (LG3.2.4.3 and LG3.2.4.4) had a single A inserted after nucleotide 588.

The adenosine insertions and deletions detected in individual cDNAs from the 63G resistant viruses introduced frameshift changes in the G protein gene, leading to drastic amino acid sequence alterations at the C-terminal end (Figure 3)

#### **mRNA sequences of the mutants resistant to MAb 63G**

The cDNA sequence heterogeneity of the escape mutants was in contrast to the situation found in the parental Long strain, where two cDNA clones from the same mRNA

preparation showed identical sequences (not shown). These results suggested that cDNA heterogeneity was not generated during the cloning process but instead was intrinsic to the mRNA preparations.

This hypothesis was tested by directly sequencing segments of the total G mRNA population which included the sites of adenosine insertions and deletions. The results obtained revealed a single nucleotide sequence for Long mRNA identical to that of cDNA (Figure 4). In contrast, the results with both R63/1/2 and R63/2/4 indicated the presence of multiple sequences in the mRNA preparations. Thus, R63/1/2 contained a single sequence up to the seven As (residues 624–630) detected in Long mRNA and from there on mixed sequences towards the mRNA 5' end. Similarly, R63/2/4 generated a single sequence up to the six As in Long mRNA (residues 589–594) and mixed sequences thereafter. The sites at which multiple sequences were detected in the mRNA preparations coincided with the sites at which sequence heterogeneity was first detected in the cDNA clones. The G mRNA sequence of mutants R63/4/10 and R63/8/7 generated identical results to those of R63/2/4 (not shown).

#### **vRNA sequences of the mutants resistant to MAb 63G**

The G mRNA heterogeneity found in the escape mutants could be explained in at least two possible ways. (i) A mixed population of infectious particles was present in each plaque purified mutant virus; or (ii) the heterogeneity at the mRNA level was generated from genetically homogenous viruses through RNA editing (Thomas *et al.*, 1988) or some other mechanism.

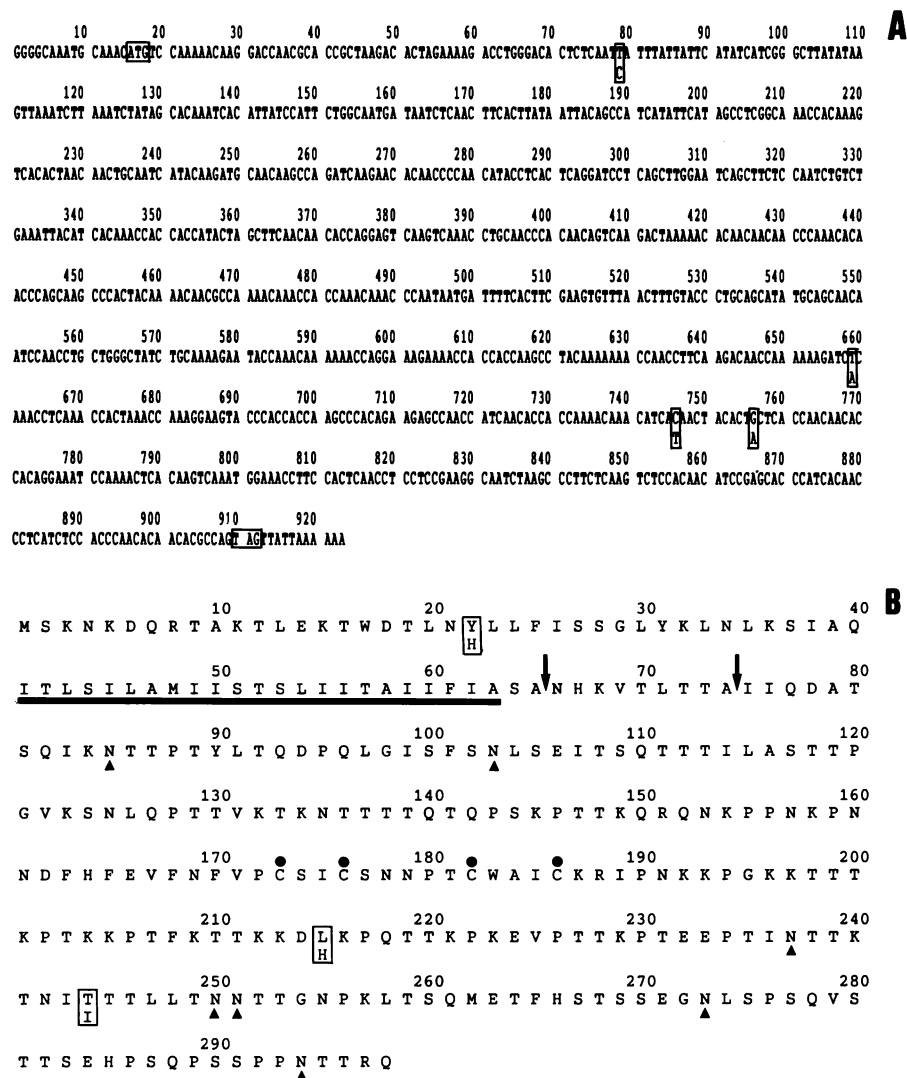
These two possibilities could be distinguished by sequencing the G protein gene from genomic RNAs (vRNAs). Since the isolation of vRNA is impractical due to poor recovery of extracellular virions, PCR technology was used for asymmetric amplification of a cDNA segment (derived from vRNA) containing the sites of sequence heterogeneity in the escape mutants. The results (Figure 5) indicated that the heterogeneity detected in the mRNA preparations (see Figure 4) was reflected in the sequences determined from amplified cDNAs of mutants R63/1/2 and R63/2/4.

#### **Second plaque purification of the 63G escape mutant viruses and sequence determination**

The above results demonstrated that the original stocks of 63G resistant mutants represented mixed virus populations. Therefore, they were plaque purified again in HEP-2 monolayers. Several plaques from each original mutant were isolated and sequences of G mRNA segments, including the sites of insertions and deletions, were determined. The nucleotide differences compared with Long virus mRNA are summarized in Table I.

In contrast to the original mutants, the G mRNAs from viruses recovered after a second plaque purification showed no sequence heterogeneity but, instead, single A insertions and deletions at three different sites. The G mRNAs from six viral plaques of mutant R63/1/2 generated identical sequences in which a single adenosine was deleted after nucleotide 588. This change implied a shift to the +1 reading frame, predicting a protein 25 amino acids shorter than wild type and with the last 81 residues changed.

Three out of four plaque purified viruses from R63/2/4



**Fig. 2.** Nucleotide and amino acid sequence of Long G. (A) shows the nucleotide sequence (messenger sense) of Long G determined from a full length cDNA insert in vector pGEM4. The deduced amino acid sequence of the G protein is shown in (B). Changes with respect to the published sequence (Johnson *et al.*, 1987) are shown underneath, enclosed in boxes. The protein hydrophobic domain is underlined. Also denoted are the sites of proteolytic cleavage (↓) which generate the G<sub>s</sub> forms, the cysteine residues (●) and the potential sites of N-glycosylation (▲).

generated identical sequences in which a single A was deleted after residue 623. This nucleotide change predicted a polypeptide 25 amino acids shorter than wild type with the last 69 residues changed. However, the fourth plaque (R63/2/4/1) obtained from the same stock contained the A deletion after nucleotide 623 followed, seven triplets later (after nucleotide 648), by a single A insertion which restored the initial reading frame of the wild type protein. Thus, R63/2/4/1 sequence predicted a G protein with seven amino acids changed between residues 205 and 211 but of wild type length.

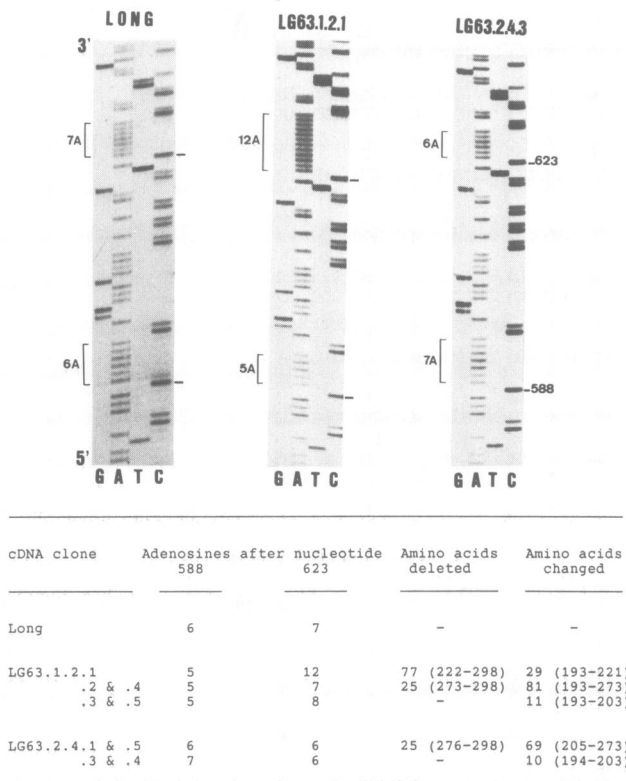
The viruses recovered after plaque purification of mutants R63/4/10 and R63/8/7 generated identical sequences to those of R63/2/4/3, /7 and /8. Again, single A deletions after nucleotide 623 predicted 25 residues shorter polypeptides with the last 69 amino acids changed with respect to the Long G protein.

Since the adenosine insertion which was detected in R63/2/4/1 had not been identified in the original mutant, the entire G mRNA sequence of the viruses denoted by an a in Table I was determined. No extra changes were found.

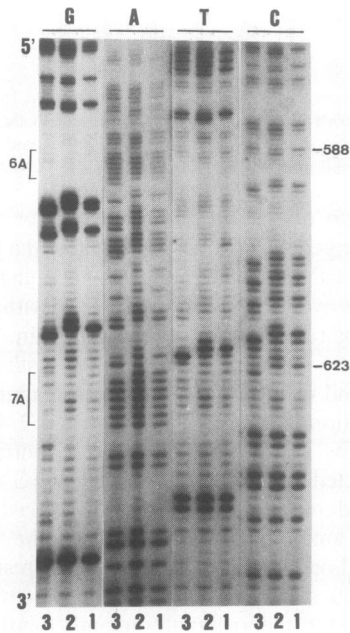
#### Characterization of the mutant proteins

To correlate the genetic changes observed in the 63G escape mutant viruses with antigenic changes in the mutant proteins, purified virions were tested in an ELISA with a panel of 19 anti-G MABs raised against the Long strain. The results shown in Figure 6 include the data obtained with the original mutant stocks and with the viruses recovered after the second plaque purification. All mutants, except R63/2/4/1, reacted only with MABs 71G, 77G and 9G. In contrast, mutant R63/2/4/1 reacted with all MABs except 63G used in the selection procedure (see Figure 1). Since the last mutant has changed only amino acids 205–211 of the G protein, compared with Long (see Table I), the ELISA results indicate that those residues are essential for epitope 63G integrity.

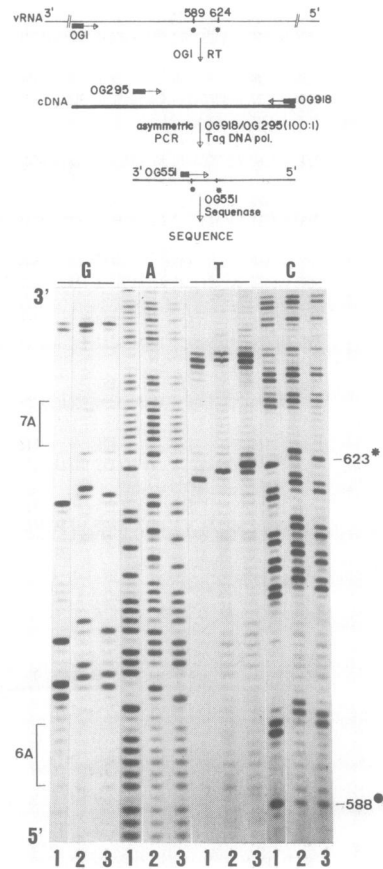
As reported for the original mutants (García-Barreno *et al.*, 1989), none of the G proteins from the twice purified viruses, except R63/2/4/1, were recognized by a rabbit antiserum raised against the soluble form of the Long G protein (not shown). These results emphasize the drastic effects that the genetic changes selected in the mutant viruses have upon the G protein antigenicity.



**Fig. 3.** Sequence analysis of cDNA clones. Partial sequences of cDNA recombinants from Long, LG63.1.2.1 and LG63.2.4.1 are shown in the upper part. The sites after nucleotides 588 and 623 which contained a different number of adenosines in the three clones are indicated in the left hand margin. 5' and 3' denote the mRNA orientation. The sequence changes found in different recombinants compared with Long cDNA are summarized in the lower part with the predicted amino acid changes.



**Fig. 4.** G mRNA sequences of Long and 63G escape mutants. Partial sequences of the mRNAs from mutants R63/2/4 (3), R63/1/2 (2) and the Long wild type (1) are shown. The G, A, T and C lanes, indicated at the top, are contiguous for the three viruses. The position of the adenosine runs, with differences between mutants and wild type, are indicated in the left hand margin. 5' and 3' denote mRNA orientation.



**Fig. 5.** Sequences of amplified vRNA derived cDNAs. A 623 nucleotide segment of vRNA from R63/1/2 (1), R63/2/4 (2) and Long (3) viruses, including most part of the G protein ectodomain, was amplified as illustrated at the top. The lower part shows partial sequences of the amplified cDNAs obtained with the oligonucleotide OG551 (see Materials and methods). The sites of sequence heterogeneity after nucleotides 588 (●) and 623 (\*) are indicated in the left margin.

The genetic changes detected in the mutants resistant to MAb 63G predicted other structural differences, illustrated in Figure 7. The Long G sequence contains seven potential sites for the addition of N-linked sugars which are conserved in the mutant R63/2/4/1; however, five of those sites are lost in the other 63G escape mutants. The Long G protein contains, in addition, 74 serine and threonine residues susceptible of O-glycosylation which in the mutant viruses, except R63/2/4/1, are considerably reduced. These changes, together with the 25 amino acid shortening at the C-terminal end, should lead to marked reductions of the protein apparent molecular weights.

Although the escape viruses reacted in part with MAbs 71G, 77G and 9G in the ELISA of Figure 6, these antibodies recognized the mutant proteins inefficiently in both immunoprecipitation and Western blot assays. Thus, the visualization of mutant proteins was done by SDS-PAGE and autoradiography of purified virions and soluble proteins from [<sup>3</sup>H]-glucosamine-labelled infected cultures. The results obtained (Figure 8A) revealed the presence of broad G bands (80–90 kd) in the lanes corresponding to Long and R63/2/4/1 viruses whereas the other mutants contained a new band of 60–65 kd. Similarly, analysis of the proteins present in culture fluids (Figure 8B) revealed the presence of an 80 kd band corresponding to the soluble form (Gs) of the

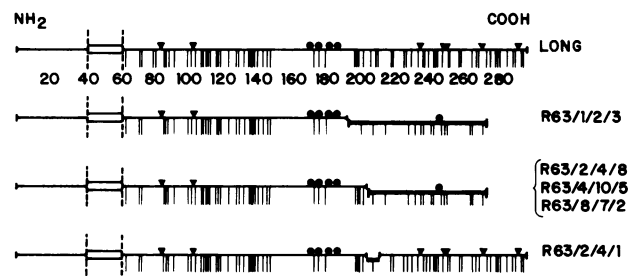
**Table I.** G mRNA sequence changes in the mutants resistant to MAb 63G recovered after a second plaque purification

Virus	Adenosines after nucleotide			Amino acids deleted	Amino acids changed
	588	623	648		
Long	6	7	6	—	—
R63/1/2/1	5	7	6	25 (274–298)	81 (193–273)
/2	5	7	6	25 (274–298)	81 (193–273)
/3 <sup>a</sup>	5	7	6	25 (274–298)	81 (193–273)
/4	5	7	6	25 (274–298)	81 (193–273)
/8	5	7	6	25 (274–298)	81 (193–273)
/9	5	7	6	25 (274–298)	81 (193–273)
R63/2/4/1 <sup>a</sup>	6	6	7	—	7 (205–211)
/3	6	6	6	25 (274–298)	69 (205–273)
/7	6	6	6	25 (274–298)	69 (205–273)
/8 <sup>a</sup>	6	6	6	25 (274–298)	69 (205–273)
R63/4/10/1	6	6	6	25 (274–298)	69 (205–273)
/2	6	6	6	25 (274–298)	69 (205–273)
/5 <sup>a</sup>	6	6	6	25 (274–298)	69 (205–273)
/7	6	6	6	25 (274–298)	69 (205–273)
R63/8/7/1	6	6	6	25 (274–298)	69 (205–273)
/2 <sup>a</sup>	6	6	6	25 (274–298)	69 (205–273)
/3	6	6	6	25 (274–298)	69 (205–273)
/8	6	6	6	25 (274–298)	69 (205–273)

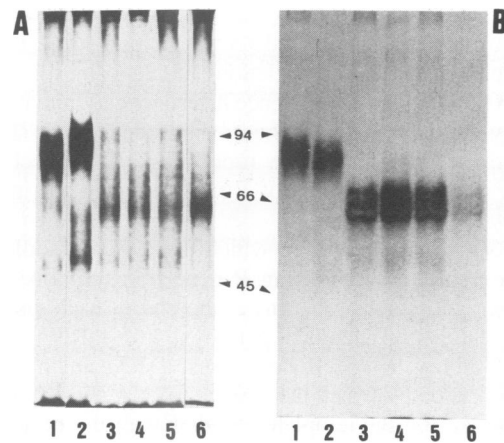
<sup>a</sup>indicates the viruses in which the entire G mRNA sequence was determined.

Antigenic Area	Mab	Antibody used for selection										
		Long	63 G					63 G				
			R63/1/2	R63/2/4	R63/4/10	R63/8/7	R63/1/2/3	R63/2/4/1	R63/2/4/8	R63/4/10/5	R63/8/7/2	
I	25	■										
	63	■										
	68	■										
	78	■										
	71	■	■	■	■	■	■	■	■	■	■	
	61	■										
	VII	62	■									
		26	■									
		28	■									
		59	■									
64		■										
65		■										
72		■										
VIII	74	■										
	77	■	■	■	■	■	■	■	■	■	■	
	27	■										
	8	■										
	58	■										
IX	9	■										
	9	■										

**Fig. 6.** Binding of anti-G monoclonal antibodies to Long and 63G resistant viruses. The binding of antibodies was tested by ELISA using purified viruses to coat microtitre plates. Symbols: <25% (□), 25–50% (▨) and >50% (■) of the absorbance values obtained with the Long strain.



**Fig. 7.** Diagram of the G protein primary structure and changes in the 63G escape mutants. The Long G protein amino acid sequence deduced in Figure 2 is represented, denoting the transmembrane domain (□), the potential acceptor sites for O-(|) and N-glycosylations (▼) and the cysteine residues (●). The structure of the mutant G proteins indicated at right are represented, denoting the sites of frameshift changes by the displaced thick lines.



**Fig. 8.** Analysis of glycosylated proteins from virions and culture supernatants. The extracellular virus (A) and soluble proteins (B) were purified from culture supernatants of [<sup>3</sup>H]-glucosamine-labelled cells infected with Long (1), R63/2/4/1 (2), R63/2/4/8 (3), R63/4/10/5 (4), R63/8/7/2 (5) or R63/1/2/3 (6) viruses. The same amount of radioactivity was applied to each lane for SDS-PAGE (8% polyacrylamide gels). The position of mol. wt markers is denoted.

Long and R63/2/4/1 G proteins and bands of 50–60 kd in the supernatants of cells infected with the other mutants. These results are compatible with the predicted molecular size reductions.

## Discussion

The results reported here represent the first genetic characterization of RSV mutants selected for resistance to anti-G monoclonal antibodies. The sequence data indicate that the escape viruses originated by single adenosine insertions and/or deletions in the C-terminal third of the G protein gene, generating frameshift mutations responsible for the resistant phenotype. These results are in sharp contrast with the situation found in the majority of other RNA viruses, where single nucleotide substitutions account for the generation of neutralization resistant mutants (review by Domingo and Holland, 1989). The other exception to this rule was found in the coronavirus MHV-4 (mouse hepatitis virus type 4), where some escape mutants contain large sequence deletions in the S glycoprotein gene (Gallagher *et al.*, 1990). Nevertheless, the RSV mutants described here represent, to our knowledge, the first example of frameshift changes responsible for the neutralization resistant phenotype. Even RSV

escape mutants selected with an anti-F monoclonal contained single nucleotide substitutions (López *et al.*, 1990).

The original 63G escape mutants represented mixed viral populations, as determined by sequencing mRNAs preparations, cDNA clones and vRNA derived cDNAs. Although the artefactual mixing of two or more plaques during the recovery from agar plates is difficult to rule out, several findings support the idea of truly heterogeneous populations being recovered from individual plaques: (i) the heterogeneity was found in all four independently isolated viruses, (ii) the heterogeneity was only found at one position of the G gene for each mutant (for instance, position 623 in mutant R63/1/2) and (iii) the heterogeneity was lost after the second plaque purification. Thus, it is possible that the original mutants were generated by errors made by the viral polymerase during RNA replication and that the best adapted viruses were selected after the second plaque purification step.

All the genetic changes detected in the escape mutants involved the insertion or deletions of adenosines in A runs of the G protein gene. Sequence heterogeneity in that region of the viral genome might, then, be introduced by a slippery polymerase during genome replication. This mechanism would resemble the form of RNA editing found in some paramyxoviruses (Thomas *et al.*, 1988; Cattaneo *et al.*, 1989; Vidal *et al.*, 1990a), which involves the addition of one or two extra Gs during P gene transcription, or the reiterative copying of U runs at the end of each cistron to generate the poly(A) tails of paramyxovirus mRNAs (Gupta and Kingsbury, 1986). In both cases, a stuttering model has been proposed by which the nascent mRNA slips backwards or upstream on the template together with the polymerase before the next base is incorporated (Vidal *et al.*, 1990b). For slippage to be viable the downstream open reading frames must be exchangeable, as it seems is the case in the RS virus G gene (see below). When this situation applies, slippage mutants may even predominate; i.e. the polymerase could slip more frequently than misincorporate. This might apply to all RNA viruses but can only be detected in situations like the RS virus G because of the exchangeable downstream sequences.

The nucleotide sequence changes detected in the 63G escape mutants bear important implications for the structural and antigenic properties of the RS virus G protein.

(i) The amino acid changes deduced for mutant R63/2/4/1 (Table I) indicate that some or all of the G protein residues 205–211 are essential for the integrity of epitope 63G. Since this antibody reacts with the unglycosylated G protein precursor in Western blots (C. Palomo, B. García-Barreno, C. Peñas and J. A. Melero, in preparation), it is likely that the antibody binding site is determined by a sequence of consecutive amino acids including some or all of the changed residues.

(ii) The amino acid changes predicted for the other mutants recovered after the second plaque purification (Table I) introduced drastic changes in the G protein C-terminal third. Since these viruses have lost the capacity to bind most anti-G MAbs, this region seems to play an essential role in determining the antigenic structure of the mature G molecule. This effect could be direct, if the antibody epitopes were located in the C-terminal end of the G protein, or indirect if the mutant proteins were altered in the maturation or modification pathways. For instance, it is feasible that

truncated G proteins were not folded in the same manner as wild type during intracellular traffic, resulting in different modifications which affected the antigenicity of the mature protein. In any case, the results obtained indicate extensive epitope overlapping in the G molecule which explains, at least in part, the high degree of antigenic variation detected with anti-G MAbs between strains of the same (García-Barreno *et al.*, 1989) or different subgroups (Anderson *et al.*, 1985; Mufson *et al.*, 1985).

Since the mutant proteins were 25 amino acids shorter than wild type (except in R63/2/4/1) and their amino acids were changed after residue 193 or 205, it seems that the G protein C-terminal end can accommodate many sequence changes without losing its function. These results are in agreement with previous observations made by Olmsted *et al.* (1989) on the expression of truncated RS virus G proteins using SV40 recombinants. Proteins containing only the N-terminal 71, 180 or 230 amino acids were transported to the cell surface, indicating that the C-terminal end was dispensable for efficient membrane insertion and cell surface expression.

Although the amino acid sequence predicted for the last 81 residues of the mutant R63/1/2/3 G protein has only marginal identity to the sequence predicted for the same region of the Long protein, their amino acid compositions and hydrophobicity profiles were strikingly similar (not shown). This might reflect possible restrictions to the amino acid changes tolerated in the G protein C-terminus. It remains to be seen whether or not viruses isolated from clinical specimens exhibit changes similar to those detected in the 63G escape mutants, correlating with the high degree of antigenic variation observed among the G proteins of natural isolates.

## Materials and methods

### Viruses

The Long strain (subgroup A) of human RS virus was used as the prototype throughout. The virus was grown in monolayers of actively growing HEp-2 cells and purified from culture supernatants as previously described (García-Barreno *et al.*, 1988).

The isolation of the original escape mutant viruses resistant to MAb 63G has been described (García-Barreno *et al.*, 1989) and is illustrated in Figure 1. Several samples of plaque purified Long virus ( $3 \times 10^3$  p.f.u.) were independently incubated with MAb 63G, in the presence of complement (C') and a rabbit anti-mouse serum (RAM), before being used to infect HEp-2 cells growing in 96 well microtitre plates. After viral adsorption, growth medium was added with 1/5 dilution of 63G antibody containing supernatant. Resistant viruses emerged after seven identical selective cycles and were cloned by plaque isolation. Their resistance to 63G neutralization in the presence of C' was confirmed. One plaque recovered from each culture supernatant was selected for further analysis. Since these original mutants were genetically heterogeneous (see Results), they were cloned again and several plaques selected for further analysis.

The plaque purification steps are indicated in the mutant nomenclature. For instance, the original mutant R63/1 was cloned in agar plates and a single plaque (R63/1/2) was selected, which was cloned again in agar to generate several plaques (R63/1/2/1, R63/1/2/2, etc.).

### Enzyme-linked immunosorbent assay (ELISA)

It was done in 96 well microtitre plates coated with purified virus as described (García-Barreno *et al.*, 1989). The bound antibodies were developed using the biotin–streptavidin system and OPD–H<sub>2</sub>O<sub>2</sub> as recommended by the manufacturer (Amersham Corp.)

### Radiolabelling of viral proteins

HEp-2 cells were infected as described (García-Barreno *et al.*, 1988). Eighteen hours post-infection, medium was replaced by glucose depleted Dulbecco's modified Eagle's (DMEM) medium containing 2.5% dialysed fetal calf serum, 2 mM uridine and 20  $\mu$ Ci/ml of [<sup>3</sup>H]-glucosamine. When

cytopathic effect was complete, medium was collected and cleared of cell debris by low speed centrifugation.

Virus particles were precipitated from clarified supernatants with 6% polyethyleneglycol 6000, 4 h at 4°C. The virus was resuspended in TNE buffer (50 mM Tris-HCl pH 7.5, 0.1 M NaCl and 10 mM EDTA), pelleted through a 33% sucrose cushion at 230 000 g for 60 min and resuspended in TNE.

The G soluble ( $G_s$ ) forms were obtained from parallel culture supernatants as follows. After clarification at low speed, viral particles were sedimented at 65 000 g for 120 min. Then,  $G_s$  were precipitated from the virus free supernatants with 45–65% ammonium sulphate, resuspended and dialysed against PBS.

The radiolabelled proteins present in either purified virions or  $(NH_4)_2SO_4$  precipitated material were analysed by SDS-PAGE (Studier, 1972) and fluorography.

#### cDNA cloning

The procedure used has been described (Cristina *et al.*, 1990). Briefly, poly(A)<sup>+</sup> RNA from Hep-2 cells infected with either the Long strain or escape mutant viruses was used for the synthesis of the first DNA strand using reverse transcriptase (RT) primed with oligo(dT). The second strand was synthesized with Klenow polymerase primed with the oligonucleotide 5'-GGGGCAAATGCA-3', identical to the 5' end of the A2 strain G mRNA (Johnson *et al.*, 1987). These cDNAs were blunt end ligated to *Sma*I linearized pGEN4 DNA and used to transform *Escherichia coli* DH5. Ampicillin resistant colonies were screened by hybridization with the oligonucleotide 5'-ATTGCCAGAATGGATAATGTGA-3' (nucleotides 137–158 in the anti-messenger sense of G from the A2 strain) (Johnson *et al.*, 1987). Plasmid DNAs from positive colonies were isolated and characterized by both restriction mapping and sequencing of the insert ends by the dideoxy method (Sanger *et al.*, 1977). This method was also used for sequencing the entire inserts of some recombinants with primers synthesized according to the reported sequence of the A2 G protein gene (Satake *et al.*, 1985; Wertz *et al.*, 1985).

#### Polymerase chain reaction

Asymmetric amplification of a 623 nucleotide segment of the G protein gene was initiated with cDNAs synthesized using either mRNA or vRNA as templates. The first strand of mRNA derived cDNA was synthesized as above. The vRNA derived single stranded cDNA was synthesized with 10 µg of RNA extracted from purified virus and RT primed with the oligonucleotide 5'-GGGGCAAATGCA-3' (residues 1–12 of G mRNA).

The single stranded cDNAs were amplified (Saiki *et al.*, 1988) using two oligonucleotides specific for the G protein gene (see Figure 5): 5'-GATCTCAGCTTGGAAATCAGC-3' (nucleotides 295–315 messenger sense) and 5'-AAGTAACTACTGGCGTGTGTGGTGG-3' (nucleotides 889–918 anti-messenger sense). Each reaction contained in 100 µl of PCR buffer (10 mM Tris-HCl pH 8.0; 1.5 mM MgCl<sub>2</sub>, 100 mM KCl, 0.05% Tween 20 and 0.05% NP-40): 0.2 pmol of the first primer and 20 pmol of the second, 10 ng of cDNA and 2.5 units of *Thermus aquaticus* DNA polymerase. The thermal profile involved 35 cycles of denaturation at 93°C for 30 s, primer annealing at 50°C for 1 min and extension at 72°C for another minute. The amplified DNAs were used for sequencing by the dideoxy method (Sanger *et al.*, 1977) using the oligonucleotide 5'-ATCCAACCTGCTGGGCTATCT-3' (nucleotides 551–571 messenger sense).

#### mRNA sequencing

This was carried out by the dideoxy method using 5'-<sup>32</sup>P-labelled oligonucleotides, reverse transcriptase and poly(A)<sup>+</sup> RNA extracted from HEp-2 infected cells, followed by a chase with terminal deoxynucleotidyl transferase (DeBorde *et al.*, 1986).

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