Molecular Basis of Viral Persistence: a Single Amino Acid Change in the Glycoprotein of Lymphocytic Choriomeningitis Virus Is Associated with Suppression of the Antiviral Cytotoxic T-Lymphocyte Response and Establishment of Persistence†

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Isolates of lymphocytic choriomeningitis virus (LCMV) that elicit a cytotoxic T-lymphocyte response (CTL⁺) have been compared with isolates that suppress the CTL response (CTL⁻) in an effort to map this phenotype. A single amino acid change in the glycoprotein of the LCMV Armstrong (ARM) strain is consistently associated with the CTL⁻ trait and the ability of the virus to persist (P⁺). The CTL⁺ P⁻ parental strain spontaneously gives rise to CTL⁻ P⁺ variants within lymphoid tissues of mice persistently infected from birth. To map the structural basis of the phenotype, the complete RNA sequence of LCMV ARM 53b (CTL⁺) was compared with that of its variant ARM clone 13 (CTL⁻). Differences in 5 of 10,600 nucleotides were found. Three changes are noted in the large L RNA segment, and two are noted in the small S RNA segment. Only two of the changes distinguishing CTL⁺ from CTL⁻ isolates affect amino acid coding: lysine to glutamine at amino acid 1079 of the polymerase protein, and phenylalanine to leucine at amino acid 260 of the envelope glycoprotein (GP). We also analyzed two additional CTL⁻ variants and four spontaneous CTL⁺ revertants. All three CTL⁻ variants differ from the original CTL⁺ parental strain at GP amino acid 260, indicating that this amino acid change is consistently associated with the CTL⁻ phenotype. By contrast the other four mutations in LCMV are not associated with the CTL⁻ phenotype. Sequence analysis of the coding regions of four CTL⁺ revertants of ARM clone 13 did not reveal back mutations at the GP 260 locus. This finding indicates that the GP 260 mutation is necessary but not sufficient for a CTL⁻ P⁺ phenotype and that the reversion to CTL⁺ P⁻ is likely either due to secondary mutations in other regions of the viral genome or to quasispecies within the revertant population that make significant contributions to the phenotype.

Persistent virus infection can result in chronic and progressive disease. Viruses causing persistent infections in humans include the retroviruses, hepadnaviruses, adenoviruses, paramyxoviruses, and herpesviruses (for reviews, see references 1, 25, and 31). Among the molecular mechanisms used by the virus to persist are avoidance of immune surveillance and direct interference with normal immune function. The first strategy is exemplified by viruses with restricted gene expression, e.g., herpes simplex virus expressing only a latency-associated transcript when latent in neurons (46), cytomegalovirus expressing only immediate-early genes during latency (38), and arenaviruses and paramyxoviruses that express fewer viral glycoproteins during persistent infection (24, 33). Selected adenoviruses incorporate a strategy of directly interfering with the expression of major histocompatibility complex class I antigen necessary for lymphocytic recognition of infected cells. This interference also obviates immune surveillance by reducing the presentation of viral antigen (9, 35, 44). Another example of the immune interference strategy is offered by human immunodeficiency virus infection in which the virus can be selectively transmitted from monocytes to activated T lymphocytes, and the cytopathic effect of the virus eventually

exceeds the regenerative capacity of the immune system (19, 21, 30, 36).

For the arenavirus lymphocytic choriomeningitis virus (LCMV), the presence or absence of the cytotoxic T-lymphocyte (CTL) response determines whether or not an infection is cleared (10, 14, 26, 28, 50). Adoptive transfer of CD8⁺ lymphocytes from an LCMV-immune mouse to a persistently infected animal results in clearance of the virus (5, 23, 32). Correspondingly, abolition of virus-specific CTL responses during acute infection changes the outcome from clearance to persistence (10, 16, 20, 49). The commonly used LCMV strain Armstrong 53b (ARM 53b) causes an acute infection in the mouse. ARM 53b elicits a vigorous CTL response that clears virus and prevents the establishment of persistence (CTL⁺ or P⁻ virus). Spontaneous variants, CTL⁻ P⁺, can be isolated from splenic lymphoid cells of persistently infected mice (2). One such isolate, LCMV ARM clone 13, suppresses the CTL response in adult immunocompetent mice and thereby causes a persistent infection. To map the genetic basis of the immunosuppressive phenotype, we have compared ARM 53b and clone 13 by oligonucleotide-primed RNA sequencing.

In the process of the sequence comparison, the full extent of the LCMV coding capacity has been defined. The virus encodes a nucleocapsid protein (NP) and envelope glycoprotein (GP) in opposite orientations on its small S RNA segment and an RNA polymerase (L) and a small zinc-finger protein (Z) in opposite orientations on its large L RNA segment (4, 39-42). Initially, evidence from sequence anal-

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ysis (43) and from reassortant analysis (3) led to the interpretation that the immunosuppressive phenotype mapped to the L RNA segment. However, the use of two different strains of virus (LCMV Armstrong and LCMV Pasteur) in the reassortment analysis and the fact that sequence information was limited to the S RNA segment made the interpretation inconclusive. The studies presented here indicate that the immunosuppressive phenotype maps to the S RNA segment. Sequence analysis shows that ARM 53b (CTL⁺) differs from ARM clone 13 (CTL⁻) by 5 bases of 10,600. Additional sequence information is presented from two other CTL⁻ isolates and four CTL⁺ revertants of clone 13. One change in GP amino acid 260, located on the S RNA, is consistently associated with the CTL⁻ P⁺ phenotype. The significance of the change at GP 260 and how it could influence the CTL-inducing properties of the virus are discussed.

MATERIALS AND METHODS

Viral isolates. LCMV ARM 53b is a triple plaque-purified clone of ARM CA 1371 (17). The clone 13 variant was isolated from the spleen of an adult BALB/WEHI mouse persistently infected with ARM 53b since birth (2); it was also triple plaque purified. Preparation of stocks of these viruses for use in the experiments described here involved two further passages in BHK-21 cells.

New CTL⁻ virus isolates (ARM clone 4 and ARM clone 6) were derived from peripheral blood lymphocytes (PBLs) of adult BALB/WEHI mice persistently infected with LCMV. Persistence was initiated by inoculating newborn mice within 18 h of birth with 10⁴ PFU of ARM 53b intracerebrally. The clone 13 revertants were derived from either brain homogenates prepared 15 days postinfection (clone 13-28 and clone 13-32) or PBLs prepared more than 15 days postinfection (clone 13-11 and clone 13-14) from BALB/WEHI mice which had been persistently infected as adults by intravenous inoculation of 2 × 10⁶ PFU of clone 13. Clones 13-28 and 13-32 were isolated from homogenates prepared by R. Welsh during his sabbatical. Brain homogenates were suspended at 0.25 g of tissue per ml, PBLs were suspended at 2 × 10⁶/ml, and infectious centers from these tissues were titered on Vero cell monolayers. Plaques were picked with a sterile Pasteur pipette, ejected into 0.5 ml of medium, and allowed to elute overnight. Viral clones were derived by three subsequent rounds of plaque purification and amplified by growth in BHK-21 cells. The first BHK cell-derived stock was considered passage 1 (P1); virus used for biological characterization in mice and for sequence analysis was P3.

Characterization of the biological properties of new viral isolates. Six- to 8-week-old BALB/WEHI mice were inoculated intravenously with 2 × 10⁶ PFU of virus. At this dose the parental ARM 53b virus strain induces a CTL response and virus is cleared, whereas the clone 13 variant fails to induce an effective CTL response and establishes a persistent infection. Groups of animals were infected with each of these reference viruses and the new virus isolates. The level of LCMV-specific CTL activity mediated by spleen cells from at least two individual animals from each group was measured at 7 days after infection. Fifteen or more days after infection virus persistence was assessed by titering infectious virus remaining in the serum of two or more individual animals from each group.

CTL assays. CTL assays were performed as previously described (10, 34). ⁵¹Cr-labeled BALB clone 7 cells (*H-2^d*)

and MC57 cells (*H-2^b*), either uninfected or infected 48 h previously with ARM 53b at a multiplicity of 1 PFU/cell, were employed as target cells. Effector cells were prepared from the spleens of individual mice by mechanical disruption followed by Ficoll-Hypaque density gradient centrifugation. Lysis was measured over a 5-h period at effector-to-target ratios ranging from 50:1 to 5:1; samples were set up in triplicate. The results are expressed as percent specific ⁵¹Cr release {[(sample release - spontaneous release) / (maximum release - spontaneous release)] × 100}. Lysis of uninfected targets mediated by effector cells has been subtracted from the infected cell value to give LCMV-specific lysis.

Serum virus titers. Serum virus titers were determined by plaque assay on Vero cell monolayers. Starting with a 1:100 serum dilution, 10-fold dilutions were prepared and tested; this assay detects a minimum of 400 PFU/ml.

Sequence comparison of LCMV ARM isolates. RNA for sequence analysis was prepared from purified virus or from virus-infected BHK cells (13, 43). Virus harvested from BHK cell culture media at 48 and 72 h was concentrated by 7% polyethylene glycol precipitation and banded on a 10 to 50% renografin gradient. Banded virus was diluted threefold in TNE (10 mM Tris-HCl [pH 7.5], 1 mM EDTA, 100 mM NaCl), spun 1 h at 50,000 × *g*, and resuspended in 0.5 ml of TNE. Virus was extracted with phenol, extracted next with chloroform, ethanol precipitated, and stored in distilled water at 1 mg/ml. Infected cells were lysed in a citrate-buffered 4 M guanidine isothiocyanate solution, sonicated for 30 s, layered over a 5.7 M CsCl cushion, and spun at 25,000 rpm for 18 h in an SW28 rotor; the RNA pellet was dissolved in H₂O, phenol extracted, ethanol precipitated, and stored in H₂O at 10 to 30 mg/ml. Sequence analysis used radiolabeled oligonucleotide primers and dideoxynucleotides and was catalyzed by reverse transcriptase. In general, 2 to 7 µg of viral RNA or 40 to 100 µg of total cellular RNA was used for each sequencing reaction. The technical details have been described elsewhere (43, 45).

Sequence comparison of different strains of LCMV. The sequence of the LCMV ARM strain was compared with the Traub and Pasteur strains in the region of the coding change in the GP gene (43) and in the RNA polymerase gene (this article). The Traub strain was obtained from F. Lehmann-Grube, Heinrich-Pette Institute, Hamburg, Germany, and the Pasteur strain was obtained from Y. Riviere, Pasteur Institute, Paris, France. Virus was propagated as P3 in BHK-21 cells, and RNA was prepared as described above.

Nucleotide sequence accession numbers. The GenBank accession numbers for LCMV Armstrong sequences are M20869 (S RNA), J04331 (3' end of the L RNA), and M27693 (5' end of the L RNA).

RESULTS

Comparison of ARM 53b with clone 13. The complete RNA sequence of the LCMV ARM 53b genome and the complete genomic sequence of its variant LCMV clone 13 were analyzed in parallel. As diagrammed in Fig. 1, there were three sequence changes on the L RNA and two sequence changes on the S RNA. The S RNA changes have been described previously (43) and occur at nucleotides 856 and 1298 of the S RNA sequence. The change at 856 results in a coding change of phenylalanine to leucine at GP residue 260, a position that is close to the putative processing site between GP-1 and GP-2 (7, 8, 9a). Of the three changes on the L RNA, occurring at nucleotides 1268, 3267, and 6192,

FIG. 1. Diagram of the LCMV genome, illustrating the positions of the five nucleotides which differ between the CTL⁺ P⁻ virus ARM 53b and its CTL⁻ P⁺ variant, ARM clone 13. For each mutation, the nucleotide number, base change (ARM 53b→clone 13), and effect on coding are indicated.

only the one at 3267 effects a coding change: lysine to glutamine at amino acid 1079 of the RNA polymerase.

Comparison of ARM 53b and clone 13 with three other LCMV strains in the region of the coding change in the RNA polymerase. The coding change at polymerase amino acid 1079 occurs in a region previously designated as the most highly conserved region of RNA virus polymerases (37). It is also highly conserved among several strains of LCMV (Fig. 2). A 210-nucleotide stretch (nucleotides 4055 to 3846) was sequenced from LCMV ARM 53b, LCMV ARM clone 13, LCMV Traub, and LCMV Pasteur. Although the Traub and Pasteur strains differed from the Armstrong strain by 13% (28 of 210) and 14% (29 out of 210) of their nucleotides, respectively, their predicted amino acid sequences are identical to that of ARM 53b in that region.

Biological properties and sequence information from clone 13 revertants. Six LCMV isolates were obtained from the PBLs of clone 13-infected mice. Two of these, clone 13-11 and clone 13-14, proved to be CTL⁺ revertants of clone 13 by two criteria: first, they could elicit a CTL response which was assayed in vitro (Table 1), and second, they were cleared from the mouse by day 15 postinfection as assessed by the lack of infectious virus in the serum. Six additional LCMV isolates were obtained from the brains of clone 13-infected mice. Two of these, clone 13-28 and clone 13-32, proved to be CTL⁺ revertants of clone 13 by the criteria mentioned above (Table 1). Initially, the clone 13 revertants were subjected to sequence analysis only in those regions known to differ between ARM 53b and clone 13. Since none of the revertants differed from the parental clone 13 isolate in these positions (Table 2 and Fig. 3), we proceeded to look for second-site reversions in the rest of the coding regions of the viruses. A complete RNA sequence of all of the coding regions and most of the noncoding regions was determined for all four of the clone 13 revertants. Nucleotide changes were found for clone 13-32 at nucleotide 1425 in the L RNA and for clone 13-28 at nucleotide 767 in the S RNA (Table 2). The change in the clone 13-32 L RNA resulted in a coding change of methionine to isoleucine at polymerase amino acid residue number 1891, and the change in the 13-28 S RNA was a noncoding change.

Arm 53b	GGC	TTG	AAT	TTC	AAA	TTT	GAC	TGG	GAC	AAA
Clone 13	...	...	...	...	...	...	...	...	...	...
Traub	...	C.A	...	...	..G	...	...	...	...	...
Pasteur	...	...	...	...	...	...	...	...	..T	..G
Amino Acid	G	L	N	F	K	F	D	W	D	K
Arm 53b	CTG	GAA	GAA	GAC	GTG	AGA	ATC	AGT	GAG	AGG
Clone 13	...	...	...	...	...	...	...	...	...	...
Traub	...	...	..G	...	...	...	...	...	..A	...
Pasteur	...	...	...	...	..A	...	...	..C	..A	..A
Amino Acid	L	E	E	D	V	R	I	S	E	R
Arm 53b	GAA	AGT	AAT	TCT	GAG	TCC	CTT	AGT	AAA	GCT
Clone 13	...	...	...	...	...	...	...	...	...	...
Traub	...	..C	...	...	..A	..T	T.A	..C	...	..C
Pasteur	...	...	...	...	..A	..T	T.A	...	..G	..C
Amino Acid	E	S	N	S	E	S	L	S	K	A
Arm 53b	CTG	TCG	TTG	ACA	AAA	TGT	ATG	AGT	GCT	GCT
Clone 13	...	...	...	...	C..	...	...	...	..A	..C
Traub	...	..A	...	...	...	..C	...	...	..A	..C
Pasteur	T.A	..A	...	..G	...	..C	...	...	..A	..C
Amino Acid	L	S	L	T	K/Q	C	M	S	A	A
Arm 53b	TTG	AAA	AAT	CTG	TGC	TTC	TAC	TCA	GAA	GAA
Clone 13	...	...	...	...	...	...	...	...	...	...
Traub	..A	...	...	T..	..T	...	...	..G	...	...
Pasteur	..A	...	...	T..	..T	...	..T	...	..G	...
Amino Acid	L	K	N	L	C	F	Y	S	E	E
Arm 53b	TCA	CCA	ACA	TCA	TAC	ACC	TCA	GTA	GGT	CCT
Clone 13	...	...	...	...	...	...	...	...	...	...
Traub	...	...	...	...	..T	...	..C	...	...	...
Pasteur	...	...	...	...	..T	...	..T	...	...	...
Amino Acid	S	P	T	S	Y	T	S	V	G	P
Arm 53b	GAC	TCT	GGA	AGG	CTG	AAA	TTT	GCA	CTA	TCT
Clone 13	...	...	...	...	...	...	...	...	...	...
Traub	..T	..C	...	...	..G	...	...	...	..T	..A
Pasteur	..T	..G	..A	...	...	...	...	...	..T	...
Amino Acid	D	S	G	R	L	K	F	A	L	S

FIG. 2. Comparison of the sequences of the polymerase genes of three different LCMV strains in the region of the ARM→clone 13 coding change. One coding change was found on the L RNA: an A→C change at nucleotide 3953 which results in an amino acid change from lysine in ARM 53b to glutamine in clone 13. The predicted amino acid sequences of ARM 53b and two distinct LCMV strains, Traub and Pasteur, are identical within this area, illustrating the high degree of conservation in this region of the polymerase. Nucleotides identical to those of ARM 53b are designated by a dot.

New immunosuppressive isolates. The first CTL⁻ isolate, LCMV ARM clone 13, had been obtained from the spleen mononuclear cells of a mouse persistently infected with LCMV ARM. Eleven new LCMV isolates were obtained from the PBLs of LCMV ARM-infected carrier mice after two different attempts at isolation. Two of the isolates (ARM clone 4 and ARM clone 6) proved to be CTL⁻ by two criteria (Table 1); they were found to persist after more than 15 days postinfection, and they failed to elicit a good CTL response as assessed by the chromium release assay. They were studied further. Limited sequence analysis, across the five loci differing between ARM 53b and clone 13, revealed that both isolates resembled the parental isolate ARM 53b at four loci and the CTL⁻ variant at the locus encoding GP amino acid 260 (Table 2 and Fig. 3).

DISCUSSION

Comparison of the entire genomic sequence of an LCMV isolate that elicits a CTL response (ARM 53b, CTL⁺) and one that suppresses the CTL response (ARM clone 13, CTL⁻) revealed changes in 5 of 10,600 nucleotides. One nucleotide change in the center of the polymerase gene and one in the center of the GP gene affect the amino acid sequences of the proteins encoded. Three of the nucleotide changes do not alter the sequence of any of the four defined LCMV gene products. These data and sequence information from two additional CTL⁻ isolates and four CTL⁺ revertants identify the mutation, or combination of mutations,

TABLE 1. Characterization of the biological properties of new virus isolates

Virus isolate ^a	CTL activity ^b	Persistence ^c	Phenotype
ARM 53b	58.4, 57.0	<400, <400	CTL ⁺ P ⁻
Clone 13	12.3, 11.3	8.0×10^4 , 1.6×10^5	CTL ⁻ P ⁺
Clone 13-11 ^d	36.6, 33.2	<400, <400, 1.6×10^3	CTL ⁺ P ⁻
Clone 13-14 ^d	37.4, 22.3	<400, <400, <400	CTL ⁺ P ⁻
ARM 53b	64.0, 72.0	<400, <400	CTL ⁺ P ⁻
Clone 13-28 ^e	54.3, 56.7	<400, <400	CTL ⁺ P ⁻
Clone 13-32 ^e	70.0, 74.3	<400, <400	CTL ⁺ P ⁻
	70.5, 66.7	<400, <400	CTL ⁺ P ⁻
ARM clone 13	3.4, 5.6	1.0×10^5 , 1.3×10^5	CTL ⁻ P ⁺
ARM clone 4 ^f	12.9, 22.4	8.0×10^2 , 3.6×10^3	CTL ⁻ P ⁺
ARM clone 6 ^f	24.2, 29.8	3.6×10^3 , <400	CTL ⁻ P ⁺

^a Groups of four or five mice were infected intravenously with 2×10^6 PFU of ARM 53b (CTL⁺ P⁻ control), ARM clone 13 (CTL⁻ P⁺ control), or a new virus isolate of unknown properties. Sources of the new viruses are listed in footnotes d, e, and f.

^b The levels of LCMV-specific CTL activity of spleen cells from two animals were determined 7 days postinfection by ⁵¹Cr release assays. Results are expressed as the percentage of LCMV-specific lysis mediated by spleen cells and are shown for each animal. Effector-to-target ratios in the lysis were 10:1, 25:1, and 50:1 from isolates from PBL (ARM clone 13), brain (ARM clone 13), and PBL (ARM 53b).

^c The remaining animals (two or three) in each group were bled on day 15 postinfection, and serum virus titers were determined by plaque assay on Vero cell monolayers. The level of sensitivity of the assay was 400 PFU/ml. Values shown are for individual animals.

^d Isolated from PBLs of mice originally infected with ARM clone 13.

^e Isolated from brains of mice originally infected with ARM clone 13.

^f Isolated from PBLs of mice originally infected with ARM 53b.

associated with the immunosuppressive CTL⁻ P⁺ phenotype.

Earlier observations based on limited sequence and reassortant data initially suggested that the mutations affecting the CTL phenotype did not map to the S RNA. Comparison of the S RNA sequence of ARM 53b (CTL⁺) and ARM clone 13 (CTL⁻) and three other LCMV strains, Traub, Pasteur, and WE (all CTL⁺), demonstrated that the CTL⁺ strains

carried the same amino acid as clone 13 at GP 260 (43). In the light of present data that the amino acid sequences of Traub, Pasteur, and WE each differ from the ARM strain by roughly 10% (41a) and that CTL⁺ revertants can also retain the GP 260 mutation (this paper), it is likely that several different regions of the GP sequence can determine the CTL⁻ phenotype. Earlier studies reassorting the ARM CTL⁻ variant and the CTL⁺ Pasteur strain indicated that the immunosuppressive phenotype mapped to the L segment (3). However, the interpretation of this experiment is difficult because of the use of the Pasteur strain and not the ARM strain as the donor of the CTL⁺ RNA segment.

Sequence information presented here from new CTL⁻ isolates, ARM clone 4 and ARM clone 6, indicates the importance of the S segment mutation at GP 260 in production of the CTL⁻ phenotype (Table 2). Among this group of isolates, the four variant loci other than GP 260 that were examined were not changed. Clone 13, clone 4, and clone 6 were isolated independently, and yet they all show the same sequence change at GP amino acid 260. Recent experiments using reassortants between a CTL⁺ virus (LCMV ARM 53b) and its CTL⁻ variant (ARM clone 13) also led to the conclusion that the immunosuppressive phenotype reassorts with the S RNA segment (27). Hence, the results of two independent studies support the view that the GP 260 amino acid change on the S RNA segment is essential to the CTL⁻ P⁺ immunosuppressive phenotype. Unquestionable evidence will require an infectious cDNA for LCMV that can be altered at the five mutant loci by site-specific mutagenesis. Unfortunately this reagent does not yet exist.

Despite complete sequence analysis of the entire open reading frames of four clone 13 revertants that did not show a back mutation at the GP 260 position, no coding changes were observed in the viral glycoprotein. One revertant, clone 13-32, has a coding change in the L polymerase that might account for reversion to the CTL⁺ phenotype. Additionally, clone 13-32 has a noncoding change in the GP gene.

It is also possible that the CTL⁺ phenotype of the revertants is due to a quasispecies or a subpopulation of virus in the stock that may not be represented in the consensus RNA sequence obtained via direct RNA sequence analysis. The

TABLE 2. Sequence analysis^a of CTL⁻ P⁺ virus isolates and revertants to the CTL⁺ P⁻ phenotype

Phenotype and isolate	Nucleotide					Other deviations from ARM 53b coding sequence ^b
	L RNA 5952	L RNA 3953	L RNA 1028	S RNA 856	S RNA 1298	
CTL ⁺ P ⁻ ARM 53b	G	A	C	U	U	
CTL ⁻ P ⁺						
ARM clone 4 ^c	G	A	C	C	U	ND
ARM clone 6 ^c	G	A	C	C	U	ND
ARM clone 13 ^c	A	C	U	C	C	None
CTL ⁺ P ⁻						
Clone 13-11 ^d	A	C	U	C	C	None
Clone 13-14 ^d	A	C	U	C	C	None
Clone 13-28 ^d	A	C	U	C	C	S RNA 767 A→C, noncoding
Clone 13-32 ^d	A	C	U	C	C	L RNA 1425 G→C, Met→Ile

^a RNA from the two new CTL⁻ P⁺ virus isolates (clone 4 and clone 6) and three revertants to the CTL⁺ P⁻ phenotype (clone 13-11, clone 13-14, clone 13-28, and clone 13-32) was sequenced by oligonucleotide primer extension across the five areas where the ARM 53b and ARM clone 13 sequences were known to differ; the bases found at the relevant positions are listed.

^b No further sequence analysis was performed on clone 4 and clone 6, but the whole of the coding sequence and parts of the intergenic regions of the four revertant viruses were determined and compared with that of ARM 53b; the changes so uncovered are listed. ND, Not done.

^c Isolate derived from mice initially infected with ARM 53b.

^d Isolate derived from mice initially infected with ARM clone 13.

FIG. 3. Analyses of the RNA sequences from three CTL⁻ virus isolates (clone 13, clone 4, and clone 6) and four revertants to the CTL⁺ phenotype (clone 13-32, clone 13-28, clone 13-14, and clone 13-11) was performed by oligonucleotide primer extension catalyzed by reverse transcriptase in a mixture of nucleotides and dideoxynucleotides. From left to right, the lanes were loaded in the order A G C T. The position of the nucleotide which differs between ARM 53b (A) and clone 13 (G) causing a coding change from phenylalanine to leucine at GP residue 260 is indicated by an arrow. All three CTL⁻ virus isolates show an A→G change at this position, but the four phenotypic revertants retain the G, rather than reverting genotypically to A.

concept of quasispecies predicts that even in a stock of thrice plaque-purified virus, given the high frequency of random RNA mutations (10^{-3} to 10^{-4} per base pair [22]), spontaneous revertants to any CTL phenotype are expected and may be isolated. Assuming that two mutations are necessary to produce the CTL⁻ phenotype (both must be knocked out to get a revertant), and knowing that each virion has 10^4 bases of genome, the probability of getting two mutations in the same event is $(10^{-3} \times 10)^2 = 10^4$, i.e., 1 in 10^4 virions should be a revertant. In a rapidly changing population which is under little selective pressure, it is possible that 10% of the pool could account for its overall phenotype (15). It is therefore reasonable to expect that a CTL⁻ virus stock, when inoculated in vivo for phenotype testing, could exhibit a CTL⁺ phenotype, especially if only a minor portion of the virus population is needed to induce the CTL response.

The mechanism by which the mutation at GP amino acid 260 in the S RNA contributes to the immunosuppressive phenotype of the virus is not clear. The amino acid change does not affect CTL recognition of LCMV, because (i) CTL epitopes have been mapped in several haplotypes and the variant locus does not fall within any of them (47, 48); (ii) the immunosuppressive phenotype is haplotype independent, since clone 13 causes persistent infection in adult mice of *H-2^b*, *H-2^d*, *H-2^q*, and *H-2^k* haplotypes; and (iii) target cells

infected with CTL⁻ virus are lysed as efficiently as CTL⁺ virus-infected target cells in a CTL assay. It is more likely that the GP 260 mutation influences CTL induction by affecting early stages of viral replication, i.e., by altering virus entry or GP processing and stability. The portions of the envelope glycoprotein which are involved in the attachment and penetration of LCMV are not yet identified. There is a precedent in other viral systems for a single missense mutation affecting the pH of endosome fusion of an enveloped virus and thus altering its tropism (6). The in vivo tropism of CTL⁺ and CTL⁻ LCMV ARM isolates is discussed elsewhere (6a). An effect on GP processing is also a possible route to the immunosuppressive phenotype. The GP 260 mutation is close to the putative cleavage site where GP-1 and GP-2 are generated from the precursor GP-C (7, 8, 9a). Endoproteolytic processing is often followed by carboxypeptidase trimming, the extent of which may be influenced by the carboxy-terminal amino acid sequence. Differences in envelope glycoprotein processing might affect how this material is handled by antigen-presenting cells, which would determine whether or not a CTL response is induced (11, 29).

Although we present evidence to support the idea that the coding change at GP amino acid 260 is necessary to produce the CTL⁻ phenotype, changes at the other four loci known to differ between ARM 53b and clone 13 could also contribute to this phenotype. One of the sites which differs between ARM 53b and clone 13 is in the middle of the polymerase gene and produces an amino acid change of lysine to glutamine. The change occurs within the region of the polymerase that is most highly conserved among the RNA virus polymerases (37) (Fig. 2). It is not known whether this mutation has an effect on polymerase function. Another mutation in the L RNA in the region between the polymerase gene and the gene for the small zinc-binding protein was noted. The region contains what is potentially a small open reading frame, although there is as yet no evidence that a viral protein is produced from it (41). The latter mutation lies at the 3' end of the Z gene mRNA and may affect the level of RNA expression rather than protein function. Either of the L RNA mutations could act in synergy with the S RNA mutations to generate a CTL⁻ phenotype; for example, a proteolytic processing function encoded on the L RNA could affect the outcome of an amino acid change near a processing site encoded by the S RNA. Mutations in the coding regions of measles virus (12) and the noncoding regions of poliovirus (18) have both been shown to affect viral tropism and thereby alter the pathogenicity of the virus.

In conclusion, the genetic basis for the interaction between CTL and LCMV has been mapped to a limited number of loci. One of these loci, a single amino acid change of phenylalanine to leucine at position 260 of LCMV GP, is associated with suppression of the CTL response and virus persistence. Knowing how this mutation might affect viral tropism and the kinetics of penetration and/or processing will lead to a better understanding of the molecular basis of virus-mediated immunosuppression.

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