*Journal of General Virology* (1992), 73, 983–988. *Printed in Great Britain*



# The amino terminus of human cytomegalovirus glycoprotein B contains epitopes that vary among strains

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We mapped three antigenic domains of continuous epitopes on human cytomegalovirus (CMV) glycoprotein B (gB) by reacting a panel of independently derived monoclonal antibodies with deletion mutants expressed transiently in COS-1 cells. One of these antigenic domains, DC2, maps in the last 75 amino acids of the carboxy terminus. These epitopes are conserved in strains Towne and AD169, as well as in 19 clinical CMV isolates. ELISAs of DC2-reactive antibodies with a set of overlapping synthetic oligopeptides from the carboxy terminus showed that the epitopes of antibodies CH405-1 and CH421-5 map between amino acids 833 and 852 and that the epitope of antibody CH28-2 maps between amino acids 878 and 898. These linear epitopes were grouped into domain DC3. The third antigenic domain, $DC1_V$, maps at the amino-terminal end of CMV strain AD169 gB but is not contained in strain Towne or in 17 of 19 clinical isolates. Epitopes in this domain are likely to map between amino acids 28 and 67, an area where differences occur in the nucleotide sequence of the gB genes from AD169 and Towne. Analysis of CMV-infected cells by flow cytometry with antibodies to the amino- and carboxy-terminal domains revealed that the amino terminus of gB is extracellular and that the carboxy terminus is not exposed on the cell surface.

Human cytomegalovirus (CMV) infection is a significant cause of morbidity and mortality in transplant recipients (Meyers, 1985), congenitally infected neonates (Stagno *et al.*, 1977, 1982*a*, *b*) and immunocompromised patients. It is a danger to those with AIDS, 20 to 30 percent of whom have serious CMV disease (Drew, 1988; Jacobson & Mills, 1988). A major target of the immune response to CMV infection is glycoprotein B (gB), a constituent of the virion envelope and a homologue of herpes simplex virus 1 gB (Chee *et al.*, 1990). The CMV gB gene has been mapped in the genomes of strain AD169 (Cranage *et al.*, 1986; Mach *et al.*, 1986) and Towne (Banks *et al.*, 1989; Spaete *et al.*, 1988). This glycoprotein is produced as a full-length precursor, which is glycosylated (Britt & Auger, 1986; Pereira *et al.*, 1984; Rasmussen *et al.*, 1985*b*, 1988) and proteolytically cleaved between residues 460 and 461 after its synthesis (Spaete *et al.*, 1988, 1990). It is highly immunogenic in natural infection and elicits a strong humoral immune response in humans (Cremer *et al.*, 1985; Kniess *et al.*, 1991; Pereira *et al.*, 1982, 1983; Rasmussen *et al.*, 1991) and in animals experimentally immunized with the glycoprotein (Britt *et al.*, 1988; Gonczol *et al.* 1986; Rasmussen *et al.*, 1985*a*). A major fraction of neutralizing antibodies in sera from CMV-infected patients is directed to gB (Britt *et al.*, 1988, 1990; Kniess *et al.*, 1991; Rasmussen *et al.*, 1991) (reviewed by Rasmussen, 1990).

Certain regions of CMV gB are immunodominant, as indicated by the reactivity of monoclonal antibodies (MAbs) and convalescent-phase sera with this glycoprotein. Several laboratories have shown that antibodies to continuous (or linear) epitopes react with the carboxy-terminal half of gB (Britt *et al.*, 1988; Kniess *et al.*, 1991; Utz *et al.*, 1989). It was recently reported that an epitope recognized by a human MAb maps in the amino-terminal half of the molecule (Meyer *et al.*, 1990). In the present study, we reacted a panel of murine MAbs to CMV gB, in particular those that recognize denatured forms of this glycoprotein, with deletion mutants in the amino and carboxy termini of gB from strains Towne and AD169. We identified three antigenic domains that are composed of continuous amino acids. The first domain maps at the amino-terminal end of the molecule and contains six epitopes that were found to

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vary among CMV strains. The second domain contains seven conserved epitopes that map at the carboxy-terminal end of gB. Three antibodies in this domain reacted with specific sequences defined by a set of overlapping synthetic oligopeptides from the carboxy terminus of gB and were grouped into a third domain, DC3.

Fig. 1 illustrates CMV gB and deletion mutants in this gene. Details of the procedures used to construct the gB mutants and their electrophoretic properties will be described elsewhere (I. Qadri, D. Navarro, P. Paz & L. Pereira, unpublished results). Mutant gB-(1-832), constructed in strain Towne, lacked the carboxy-terminal 75 residues but retained the transmembrane region and all of the amino terminus; mutant gB-(1-258), constructed in strain AD169, lacked the carboxy-terminal 648 residues of the molecule; and mutant gB-(716-906), also in AD169, lacked all of the amino-terminal extracellular domain of the molecule.

In the first set of experiments, we analysed the antigenic properties of these mutants by immunofluorescence assays on COS-1 cells transfected with plasmid DNAs and compared these properties with those of wild-type gB. Properties of the panel of MAbs used were published elsewhere (Pereira & Hoffman, 1986; Pereira *et al.*, 1984), but the following properties of antibodies in groups 2 and 4 are relevant to this study. Group 2 antibodies reacted by immunoblot analysis with intact gB and other high $M_r$ forms but failed to react with the carboxy-terminal cleavage fragment, which has an apparent $M_r$ of approximately 58·5K. Protease V8 protection experiments showed that these antibodies protected two fragments of gB, a broad band of 85K to 70K and another band 50K in size, from V8 cleavage. One of the group 2 antibodies, CH408-1, had complement-dependent neutralizing activity. Group 4 antibodies reacted in immunoblot assays with uncleaved, glycosylated forms of wild-type gB and also with the carboxy-terminal half of the cleaved gB molecule. These antibodies failed to protect any portion of gB from protease V8 cleavage and had no detectable neutralizing activity.

Results of immunofluorescence assays of the deletion mutants in CMV gB showed that the molecule contained two antigenic domains composed of continuous epitopes (Table 1). The first antigenic domain, which we designated as $DC1_V$, was located in the amino terminus of the molecule and contained continuous epitopes recognized by group 2 antibodies. $DC1_V$ was expressed on strain AD169, as indicated by the reactivity of antibodies with wild-type gB and the deletion mutant gB-(1-258), but was not expressed by strain Towne gB or any of the deletion mutants in this gene (Banks *et al.*, 1989). In a collaborative study (Meyer & Mach, 1991; H. Meyer, M. Mach & L. Pereira, unpublished results), epitopes in the amino terminus were fine-mapped to residues 27 to 84 of the amino terminus of strain AD169 by reactions with a β-galactosidase fusion protein of gB expressed in *Escherichia coli*.

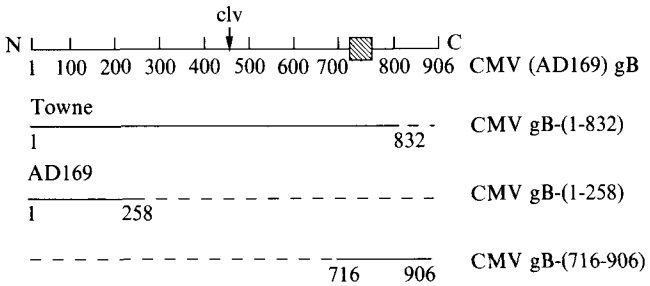


Fig. 1. Schematic representation of CMV gB and deletion mutants. Stick models of the structure of wild-type gB AD169 and of deletion mutants in Towne and in AD169 are shown. Construction of deletion mutants in AD169 gB was as follows. *Bam*HI fragment T containing the gB gene of CMV (AD169) was excised from cosmid 7091 (Fleckenstein *et al.*, 1982) and cloned into the *Bam*HI site of pUC18. The *Eag*I fragment (3125 bp) encoding gB, was cloned into the *Sma*I site of the eukaryotic expression vector pMT2 (Wong *et al.*, 1985) and designated as pMTgB. A derivative, pMT3, was constructed by inserting a synthetic *Spe*I linker that encodes stop codons in all three reading frames, at the blunt-ended *Eco*RI site. Plasmid p258, which encodes gB-(1-258), was obtained by cutting pMTgB with *Bss*HII, then blunt-ending this site with mung bean nuclease, and cleaving the plasmid with *Bgl*II. This fragment was cloned into the *Bgl*II and *Sma*I sites of pMT3. Plasmid p716-907, which encodes gB-(716-907), was obtained by cloning an 865 bp *Sal*I–*Eco*RI fragment into these sites in pMT2. The first ATG at residue 716 was utilized as an initiation codon for this 191 amino acid sequence, which includes the hydrophobic transmembrane sequence and the carboxy terminus of the molecule. To construct gB-(1-832) the *Eag*I fragment containing the Towne gB gene was excised and digested with *Nsi*I and *Ban*II. The ends were blunted with mung bean nuclease and religated, resulting in the loss of 461 bp (or 75 amino acids from gB) at the carboxy terminus. The hydrophobic transmembrane region is depicted as a hatched box. The first and last residues of the molecules are indicated by numbers. Names used to refer to the mutant glycoproteins are indicated to the right. Dashed lines indicate deleted portion of the glycoprotein. Arrow shows cleavage site (clv) (Spaete *et al.*, 1988).

The second antigenic domain, designated as DC2, was located in the carboxy terminus of the molecule and contained the continuous epitopes of group 4 antibodies (Table 1). All of the group 4 antibodies reacted with intact gB from both strains AD169 and Towne and with a deletion mutant in AD169, gB-(716-906), which contained 190 residues of the carboxy-terminal portion of gB, including the transmembrane anchor sequence, but lacked the entire amino-terminal extracellular domain. In contrast, these antibodies failed to recognize the carboxy-terminal deletion mutants in strain Towne, gB-(1-832) in this study, and gB-(1-619) and gB-(1-680) in an earlier study (Banks *et al.*, 1989). Insofar as mutant gB-(1-832) lacks only 75 amino acids of the carboxy

Table 1. *Immunofluorescence reactions of MAbs with CMV gB from wild-type strains Towne and AD169, and deletion mutants lacking the amino or the carboxy terminus*

| Group* | MAb | AD169 | Towne | gB-(1-832)† | gB-(1-258)‡ | gB-(716-906)‡ |
|---|---|---|---|---|---|---|
| 2 | CH45-1 | + | − | − | + | − |
| | CH86-3 | + | − | − | + | − |
| | CH386-3 | + | − | − | + | − |
| | CH404-4 | + | − | − | + | − |
| | CH408-1§ | + | − | − | + | − |
| | CH412-2 | + | − | − | + | − |
| 4 | CH28-2 | + | + | − | − | + |
| | CH158-2 | + | + | − | − | + |
| | CH216-2 | + | + | − | − | + |
| | CH340-4 | + | + | − | − | + |
| | CH381-1 | + | + | − | − | + |
| | CH385-3 | + | + | − | − | + |
| | CH402-5 | + | + | − | − | + |
| | CH405-1 | + | + | − | − | + |
| | CH410-3 | + | + | − | − | + |
| | CH421-5 | + | + | − | − | + |

* Antibodies reacted by immunoblot assay with intact gB and the 58·5K carboxy-terminal cleavage fragment (Group 2) or with intact gB and the high $M_r$ forms (Group 4) (Pereira & Hoffman, 1986; Pereira *et al.*, 1984). Procedures used for immunofluorescence tests were published (Banks *et al.*, 1989).

† Constructs made in gB gene of strain Towne (I. Qadri *et al.*, unpublished results).

‡ Constructs made in gB gene of strain AD169 (I. Qadri *et al.*, unpublished results).

§ Complement-dependent neutralizing antibody.

Table 2. *Reactivity of MAbs in ELISA assays with overlapping synthetic oligopeptides from the carboxy terminus of CMV (Towne) gB*

| Amino acid number | Peptide sequence* | Reactive antibody | Titre† |
|---|---|---|---|
| 773–792 | TRQRRLCMQPLQNLFPYLVS | | |
| 788–807 | PYLVSADGTTVTSGNTKDTS | | |
| 803–822 | TKDTSLQAPPSYEESVYNSG | | |
| 818–837 | VYNSGRKGPGPPSSDASTAA | | |
| 833–852 | ASTAAPPYTNEQAYQMLLAL | CH405-1, CH421-5 | >3200, 400 |
| 848–867 | MLLALVRLDAEQRAQQNGTD | | |
| 863–882 | QNGTDSLDGQTGTQDKGQKP | | |
| 878–898 | KGQKPNLLDRLRHRKNGYRH | CH28-2 | >3200 |
| 894–907 | NGYRHLKDSDEENV | | |

* Oligopeptides were synthesized by the method of Houghten (1985).

† Procedures used for ELISA were published previously (Hubenthal-Voss *et al.*, 1988; Pereira *et al.*, 1989).

terminus, epitopes of the group 4 antibodies appear to map within the missing residues. These antibodies also failed to react with comparable deletion mutants lacking the carboxy terminus of strain AD169 gB (I. Qadri, D. Navarro, P. Paz & L. Pereira, unpublished results).

To fine-map the group 4 epitopes, we tested the antibodies in ELISAs with a set of overlapping synthetic oligopeptides that spanned the last 134 residues of the carboxy terminus of gB (Table 2). Antibodies CH405-1 and CH421-5 reacted with a peptide containing amino acids 833 to 852 and antibody CH28-2 with a peptide containing amino acids 878 to 898. The finding that three of 10 antibodies to continuous epitopes which mapped in the carboxy terminus of gB recognized synthetic peptides from this region of the molecule indicated that these epitopes are linear. The lack of reactivity of the other seven antibodies suggests that although the remaining epitopes in this region of gB are largely sequential, they depend in part on local folding of the polypeptide. On the basis of the reactions with these synthetic peptides, we subdivided the continuous epitopes, grouping the linear ones into domain DC3.

Next, we analysed the antigenic properties of 19 CMV clinical isolates to determine whether strains propagated minimally in cell culture share epitopes expressed by laboratory strains with long passage histories. For these experiments, group 2 antibodies to domain $DC1_V$ in the amino terminus and group 4 antibodies to the carboxy-terminal domains DC2 and DC3 were reacted by immunofluorescence with patient isolates propagated for one passage in cell culture. We found that all of the strains expressed the DC2 and DC3 epitopes, whereas only two strains expressed the $DC1_V$ epitopes of the amino terminus. This analysis of a small number of isolates indicates that CMV strains cluster into at least two groups, based on the reactivity of group 2 antibodies; strains that express the amino-terminal epitopes shared by strain AD169, and those strains, like Towne, that do not. Additional analysis of the antigenic properties of CMV isolates indicates that the midregion of gB contains conformation-dependent epitopes that vary among strains (D. Navarro, E. Lennette, S. Frank, I. Qadri & L. Pereira, unpublished results). Differences in the nucleotide sequence of the midregion of gB from different CMV strains have also been identified; but their antigenic properties have not been analysed (Chou & Dennison, 1991).

Secondary structure analysis of the CMV gB gene indicates the presence of a hydrophobic region, which serves as a potential transmembrane anchor sequence, near the carboxy terminus of the molecule (Chee *et al.*, 1990; Spaete *et al.*, 1988). Because the hydrophobic domain contained two broad adjacent hydrophobic peaks, it was predicted that gB could assume two possible orientations in the membrane of CMV-infected cells and the virion envelope (Spaete *et al.*, 1988). If the hydrophobic domain crossed the membrane once, then the amino terminus would be outside and the carboxy terminus inside the cell. Conversely, if the two hydrophobic subdomains each traversed the membrane once, then both the amino and the carboxy terminus would be

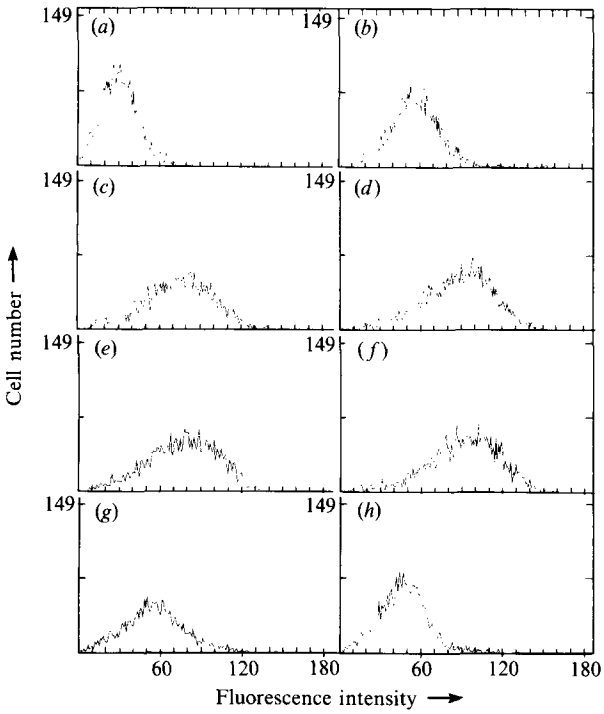


Fig. 2. Fluorescence-activated cell sorting of HFF cells infected with CMV (AD169) at 96 h post-infection. Cells were suspended in PBS with 2% BSA and incubated with a 1:100 dilution of the MAbs for 1 h on ice, then with fluorescein-conjugated anti-mouse immunoglobulin for 1 h. Cells were washed twice with PBS, and propidium iodide was added to each sample at a 1:100 dilution. Antibodies used were (*a*) FITC-conjugated anti-mouse IgG, (*b*) CH160, (*c*) CH177-3, (*d*) CH382-2, (*e*) CH45-1, (*f*) CH408-1, (*g*) CH28-2, (*h*) CH158-2. Mean fluorescence: (*a*) 31·31, (*b*) 57·54, (*c*) 74·58, (*d*) 87·62, (*e*) 76·66, (*f*) 91·22, (*g*) 55·57, (*h*) 47·38.

on the extracellular side of the plasma membrane after translocation to the cell surface. In the next series of experiments, we performed flow cytometry on CMV-infected cells to determine whether the carboxy terminus of gB was inside or outside the plasma membrane. Reactivities of antibodies in groups 2 and 4 were compared with those of selected antibodies from group 3, which recognize epitopes that map in the extracellular domain of gB (Banks *et al.*, 1989). Human foreskin fibroblast (HFF) cells were infected with AD169 at 1 p.f.u. per cell, collected after 96 h, reacted with the test antibodies for 1 h on ice, and then reacted with fluorescein-conjugated antisera to murine antibodies for 1 h. Results of these experiments are shown in Fig. 2.

Control reactions, which were negative for surface fluorescence, consisted of fluorescein-conjugated antisera (Fig. 2*a*) and MAb CH160 to the CMV immediate-early protein IE72 (C. Gimeno & L. Pereira, unpublished data), which is not expressed on the cell surface (Fig. 2*b*). Positive controls for surface reactivity were neutralizing antibodies CH177-3 (Fig. 2*c*) and CH382-2 (Fig. 2*d*), which were reported as recognizing epitopes in

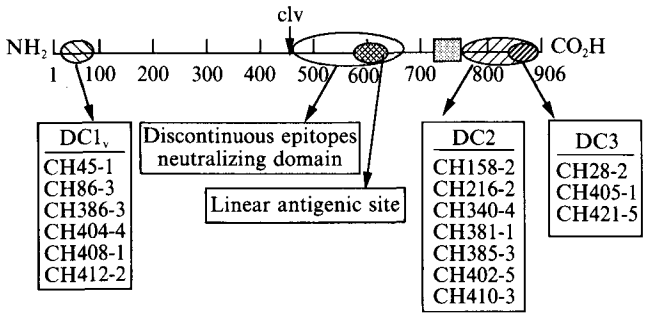


Fig. 3. Schematic representation of a topographical map of MAb epitopes on CMV (AD169) gB. Antigenic domains are indicated by ellipses. Amino acids are designated by numbers inclusive of the signal sequence. The cleavage site is marked by an arrow at amino acid 460 (Spaete *et al.*, 1988) and the transmembrane anchor domain is boxed. Continuous epitopes in the amino and the carboxy termini mapped in the present study are listed. Crosshatched ellipse indicates region containing a linear antigenic site mapped by Kneiss *et al.* (1991) and Utz *et al.* (1989). Mapping of domain containing discontinuous neutralizing epitopes has been published (Banks *et al.*, 1989).

the extracellular domain of gB (Banks *et al.*, 1989). Analysis of antibodies CH45-1 (Fig. 2*e*) and CH408-1 (Fig. 2*f*) to the amino-terminal $DC1_V$ domain showed that these antibodies reacted with the cell surface, showing a strong surface fluorescence intensity equivalent to that found with the surface-reactive antibodies CH177-3 and CH382-2. In contrast, antibodies CH28-2 (Fig. 2*g*) and CH158-2 (Fig. 2*h*) to carboxy-terminal domains DC3 and DC2, respectively, failed to react with the surface of CMV-infected cells. Our results indicate that the carboxy terminus of gB is not exposed on the cell surface and is most likely intracellular. The finding that none of the antibodies to domains DC2 and DC3 neutralize CMV infectivity (Pereira & Hoffman, 1986) supports this conclusion. Assuming that the orientation of gB is retained during virion envelopment and egress from infected cells, we conclude that the carboxy terminus is not exposed on the virion surface.

Fig. 3 is a schematic diagram showing the location of the three domains of continuous epitopes on CMV gB of strain AD169 identified in this study. The amino-terminal domain contains epitopes that vary among laboratory strains, as well as among freshly isolated strains from patients. Comparison of the nucleotide sequence of gB encoded by strains AD169 and Towne indicates that differences in the amino acid sequences of these molecules cluster between residues 28 and 67 of their coding sequences (Spaete *et al.*, 1988). Thus, it is likely that residues in this region of the amino terminus encode the epitopes of antibodies in group 2, and that these amino acid differences affect the antigenic properties of the amino terminus of CMV gB. In a recent report on the structure of the amino terminus of CMV gB, residues 27 to 84 of strain AD169 gB produced in a

prokaryotic expression vector were shown to contain an epitope recognized by a human MAb (Meyer *et al.*, 1990). It was later shown that this epitope was conserved among CMV strains and mapped to amino acids 68 to 77, which are adjacent to an antigenic site between amino acids 50 and 54 that varies among laboratory strains (Meyer & Mach, 1991). The variable but not the conserved site is contained within domain $DC1_V$ mapped in the present study. It is notable that antibody CH408-1, which maps in domain $DC1_V$, has complement-dependent neutralizing activity for strain AD169 (Pereira & Hoffman, 1986). We are currently testing this antibody against a number of CMV isolates to determine whether it has neutralizing activity for other strains.

The carboxy terminus of gB contains two domains of continuous epitopes, DC2 and DC3, which are expressed by all of the CMV strains we have tested thus far. Kneiss and coworkers (Kneiss *et al.*, 1991) also identified a region containing continuous epitopes at the carboxy terminus; mapping between residues 703 and 906, it overlaps domains DC2 and DC3 mapped in the present study. The carboxy terminus of gB contains still another linear antigenic region, which is located in the carboxy-terminal half of the extracellular domain between amino acids 589 to 645 (Kneiss *et al.*, 1991) and 608 to 625 (Utz *et al.*, 1989).

In mice immunized with extracts of CMV-infected cells, the carboxy-terminal intracellular domain of gB is immunogenic, as evidenced by the number of murine MAbs that recognize this region of the molecule. It is notable that epitope CH28-2, mapped in this study, has been particularly useful as a serological marker for uncharacterized gene products encoded by open reading frames identified by nucleotide sequence analysis. This strategy was recently demonstrated by inserting a synthetic oligonucleotide encoding epitope CH28-2 at the carboxy terminus of a gene predicted to encode the herpes simplex virus type 1 family 35 proteins (Liu & Roizman, 1991). Analysis of the epitope-tagged gene products revealed that two transcriptional units are contained within this gene. We have initiated immunofluorescence assays of CMV-immune patient sera on transfected cells expressing the CMV deletion mutant gB-(716-906) that encodes the intracellular carboxy terminus (D. Navarro, S. Frank, E. Lennette & L. Pereira, unpublished results). Results of these studies indicate that the intracellular domain of gB is recognized by a subset of high-titred patient sera and suggest that the intracellular domain of gB is not highly immunogenic in CMV infection, when compared with the extracellular portion of the molecule. Studies are in progress to analyse the immune response of CMV-infected patients to antigenic domains on gB identified with murine MAbs.

We thank Pedro Paz for assistance in the epitope analysis and Paul Dazin for the FACS analysis. These studies were supported in part by Public Health Service grants from the National Institute of Allergy and Infectious Diseases (AI23592 and AI30873) to the University of California San Francisco, (AI24009) to the University of Chicago, a grant (HL33713) from the National Heart and Lung Institute to Stanford University, and by a grant from the Thrasher Research Fund to the University of California San Francisco. D.N. was supported by a grant from the Spanish Ministry of Education and Science.

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*(Received 17 September 1991; Accepted 9 December 1991)*