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A Putative Nucleoside Triphosphate-Binding Domain in the Nonstructural Protein of B19 Parvovirus Is Required for Cytotoxicity

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Cytotoxicity secondary to B19 parvovirus infection is due to expression of the viral nonstructural protein. Nonstructural proteins of many parvoviruses contain a well-conserved nucleoside triphosphate (NTP)-binding motif, which has been shown to be essential for a variety of protein functions. We show here that cytotoxicity of the B19 parvovirus nonstructural protein was abolished by single mutations of amino acids within the NTP-binding domain, especially within the A motif, implicating NTP-binding in virus-induced cell death.

B19 parvovirus causes a variety of human diseases (29), including fifth disease in children and a polyarthralgia and/or arthritis syndrome in adults. Transient aplastic crisis follows acute parvovirus infection in persons with underlying hemolytic anemia, and chronic anemia due to persistent infection occurs in the immunocompromised host. In utero infection can produce hydrops fetalis or congenital anemia.

The target of B19 parvovirus responsible for its hematologic syndromes is the human erythroid progenitor cell. Only hematopoietic cells of bone marrow, the liver, and blood support viral replication; in a few cell lines with erythroid characteristics, there may be semipermissive or abortive viral propagation (22, 26). The very limited tropism of the virus for erythroid cells is due to its receptor on an erythrocyte globoside or P antigen (4), as well as intracellular factors present in permissive cells. However, B19 parvovirus infection may kill cells in which viral replication cannot be demonstrated. Platelet counts commonly fall in experimental and natural infection, and colony formation by the megakaryocytic progenitor cell is inhibited by virus. Neonatal myocarditis may also be the result of nonproductive viral infection of developing heart cells (21).

Cytotoxicity secondary to B19 parvovirus is due to expression of the viral nonstructural protein (23). Infected cells have ultrastructural features typical of apoptosis (20). A nonstructural protein is encoded from the left side of the genome, and its apparent molecular mass is 77 kDa (23). In nonpermissive cells transfected with viral DNA, mRNA from the left side of the genome, encoding this protein, is preferentially expressed because of a functional block in full-length transcription, and transfected nonpermissive cells fail to survive when the nonstructural protein is expressed (18). Nonstructural protein genes are highly homologous among the parvoviruses (10), and the cytotoxic effect of nonstructural protein gene expression has been demonstrated for other members of this family (3, 15, 24).

By computer analysis, the B19 parvovirus nonstructural protein shows a nucleoside triphosphate (NTP)-binding motif in the middle of the protein (12). This motif is well conserved among the nonstructural proteins of parvoviruses as well as in various viral and cellular proteins, including ATP synthase, myosin heavy chains, guanylate kinase, thymidine kinase, ATP-dependent helicases, GTP-binding elongation factors, the Ras family of proteins, bacterial RecA and RecF proteins, and G protein alpha-subunits (27). Among these, the nonstructural proteins of parvoviruses are most closely related to the family of ATP-dependent helicases or DNA-dependent ATPases. The DNA- and RNA-dependent nucleoside triphosphatases are key enzymes in biochemical pathways of replication, repair, recombination, transcription, and translation. However, the molecular mechanism of B19 nonstructural protein cytotoxicity is not known. In this study, we examined whether the NTP-binding domain of the B19 parvovirus nonstructural protein was required for its cytotoxic effect, using a stable transfection assay with plasmids carrying mutant nonstructural protein genes.

The NTP-binding motif is composed of two sites, A and B with the consensus sequences GxxxxGK[T/S] and [D/E][D/E], respectively (27). Figure 1A compares the consensus sequences of several parvoviruses with those of other viruses. Figure 1B shows the putative topography of the B19 parvovirus nonstructural protein NTP-binding domain, based on homology with simian virus 40 T antigen (1) and cH-ras p21 (2), and the secondary structure as predicted by the Chou-Fasman method (7). This domain is likely to form a supersecondary structure, termed beta-alpha-beta motif, or Rossmann fold (25). The A and B sites each may form a flexible loop that constitutes the binding site of phosphate groups in NTP. Two glycine residues in the A site help to form a turn from the beta-sheet to the alpha-helix. The positively charged lysine residue and the overall dipole moment of the alpha-helix are predicted to facilitate capture of the negatively charged phosphate groups.

We designed a series of mutants in which one or two residues within the NTP-binding domain were substituted as shown in Fig. 2. The name of each mutated gene is derived from the substituted amino acid, its location in the nonstructural protein, and the new, mutant amino acid, in order. Each amino acid in the A site was replaced with valine, a neutral and hydrophobic amino acid. In addition, a serine or threonine residue was replaced by a similar hydroxyl amino acid, threonine or serine, respectively, to create a conservative substitution. To alter the charge of amino acid, threonine at position 332 was changed to the acidic amino acid glutamate or the basic amino acid lysine. Also, the lysine at 334 was replaced with an acidic amino acid, glutamate, or a polar but uncharged amino acid, glutamine.

An expression plasmid containing the B19 nonstructural

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A

viruses	protein	A site	B site
parvoviruses			
B19	NS	TLWFYGPPSTGKTNLAMAIAK	SLVVWDEGIIKS
AAV	Rep	TIWLFGPATTGKTNIAEAIAH	MVIWWEEGKMTA
BPV	NS1	STLFYGPASTGKTNLAQAICH	MILWWEECIMTT
ADV	NS1	CIWFYGPGGTGKTLLASLICK	NIIWAEECGNFG
CPV	NS1	TVLFHGPASTGKSIIAQAIAQ	NLIWIEEAGNFG
FPV	NS1	TVLFHGPASTGKSIIAQAIAQ	NLIWIEEAGNFG
H1	NS1	TVLFHGPASTGKSIIAQAIAQ	NLIWVEEAGNFG
MVM	NS1	TVLFHGPASTGKSIIAQAIAQ	NLIWVEEAGNFG
PPV	NS1	TILFHGPASTGKSIIAQHIAN	NLIWIEEAGNFS
other viruses			
SV40	T	YWLFKGPIDSGKTTLAAALLE	FLVVFEDVKGTG
HPV1a	E1	CLLIFGPPNTGKSMFCTSLLK	KIGLLDDATKPC
HSV1	UL5	VYLITGNAGSGKSICVQTINE	NVIVIDEAGLLG
CONSENSUS		.....G....GKT.....	.....DD.....
		S	EE

B

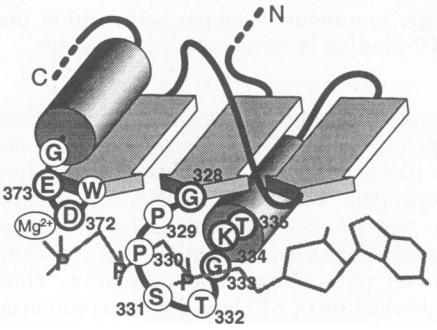

FIG. 1. (A) Conserved sequences of NTP-binding-site motif (sites A and B) in the parvoviruses and the other viruses. BPV, bovine parvovirus; ADV, Aleutian disease virus; CPV, canine parvovirus; FPV, feline parvovirus; H1, parvovirus H-1; MVM, minute virus of mice; PPV, porcine parvovirus. (B) Putative tertiary structure of NTP-binding domain in B19 nonstructural (NS) proteins (1, 2, 7). The consensus amino acids for NTP-binding are indicated by bold circles. The numbers beside the amino acids represent the location from amino-terminal methionine.

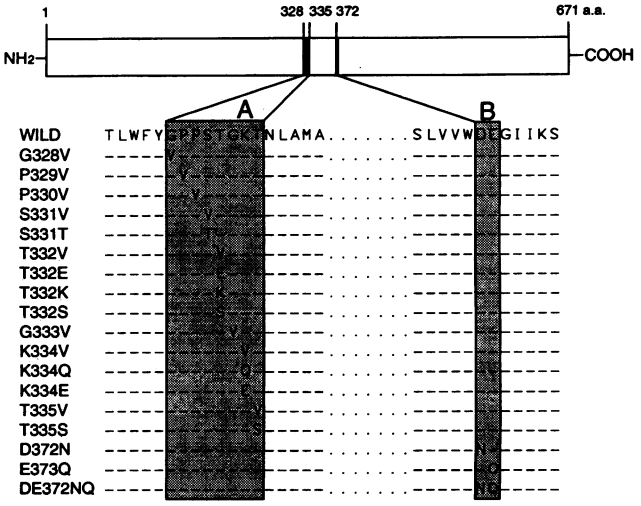

FIG. 2. Schematic diagram of mutants in nonstructural protein NTP-binding site (based on previously published data [1, 2, 7], and see the text). The name of mutants were designated by the mutated amino acid(s) (a.a.), the location from the amino-terminal methionine, and the substituted amino acid(s) from left to right. WILD, wild type.

protein, pREP9/NS, was constructed. The nonstructural protein gene from pYT103c, a nearly full-length B19 parvovirus clone (a kind gift from P. Tattersall [9]), was subcloned into the eukaryotic episomal expression vector, pREP9 (Invitrogen, San Diego, Calif.), which contains the neomycin phosphotransferase gene. A linker sequence (CCTCGAGG; New England BioLabs, Beverly, Mass.) was inserted between two *Pvu*II sites in pYT103c to produce an *Xho*I site downstream of nonstructural protein-coding region. The plasmid was subjected to digestion with *Eco*RI and blunt-ended with mung bean nuclease, followed by *Xho*I digestion. The excised fragment contained the P6 promoter and nonstructural protein-coding region and was inserted between *Xba*I (blunt-ended with mung bean nuclease) and *Xho*I sites in pREP9. Since the original Rous sarcoma virus (RSV) promoter in pREP9 was replaced by B19 P6 promoter, expression of nonstructural protein would be controlled by the P6 promoter.

To construct a frameshift mutant that blocked nonstructural protein expression (pREP9/NSfs), pREP9/NS was digested with *Xba*I, blunt-ended by a filling reaction with the Klenow fragment of DNA polymerase I, and recircularized. Eighteen plasmids carrying mutant nonstructural protein genes were constructed by PCR-mediated site-directed mutagenesis with pREP9/NS as a template (13). For this purpose, eighteen pairs of complementary mutagenic primers were designed. Primary PCR was performed with an antisense mutagenic primer and a sense outside primer (TCCTAACATGGAGCTATTTAG, corresponding to nucleotides (nt) 430 to 450 of pYT103c) or with a sense mutagenic primer and an antisense outside primer (GTGGTTTGTCCAAACTCATC, corresponding to nucleotides 736 to 717 of pREP9). The two amplified fragments were mixed, denatured, and reannealed. After extension of the annealed products in the absence of primers, secondary PCR was carried out with the sense and the antisense outside primers described above. The amplified products were digested with *Xba*I and *Xho*I and inserted between the *Xba*I and *Xho*I sites of pREP9/NS, followed by confirmatory sequencing, to obtain the desired base-substituted mutants.

For the stable transfection assay, these plasmids were used. HeLa cells were maintained in monolayer in Improved Minimal Essential Medium Zinc Option (Gibco BRL, Grand Island, N.Y.) supplemented with 10% fetal bovine serum, 50 U of penicillin per ml, and 50 μg of streptomycin per ml. Plasmid DNA (20 μg per dish) was transfected into HeLa cells (1.5×10^5 per 100-mm-diameter dish) by calcium phosphate coprecipitation (6). After overnight incubation, dishes were washed with phosphate-buffered saline (pH 7.2), and fresh medium was added and at 2 days after transfection was replaced with fresh medium supplemented with G418 (450 μg/ml) to select antibiotic-resistant colonies. G418-containing medium was replaced every 3 days, and at 14 days after transfection resistant colonies were counted. All experiments were repeated five times under similar conditions, with at least two clones for each plasmid.

Figure 3 shows the cytotoxic effect of wild-type or mutant nonstructural protein gene expression on colony formation, expressed as the mean percentage of the number of colonies transfected with pREP9/NS. The number of G418-resistant colonies transfected with pREP9/NS was 11% of the control, similar to our previous published data (23). Mutations of glycine 328, glycine 333, lysine 334, and threonine 335, all consensus amino acids in the A site, greatly reduced the cytotoxicity of the nonstructural protein. G328V or G333V, in which glycine was replaced with valine, allowed colony formation at 70 or 85% of the control, respectively, suggesting that

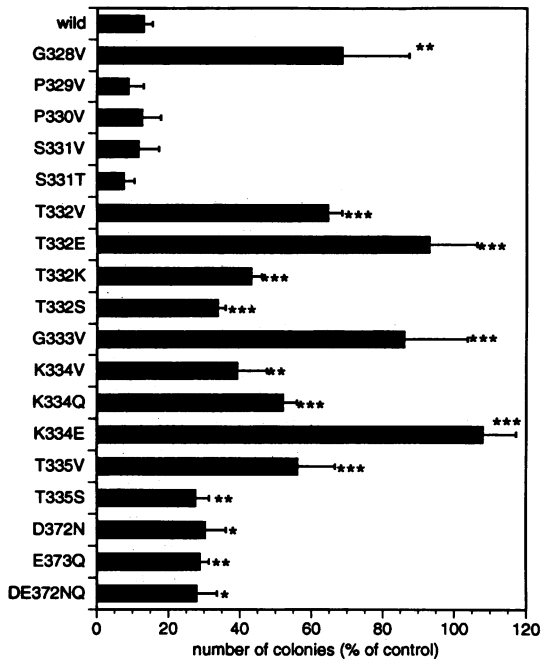

FIG. 3. The numbers of colonies transformed by pREP/NS or its mutants. The results were standardized to the number of colonies transformed by pREP9/NSfs. Data are the means ± standard errors of the means (error bars) for five experiments. The statistical significance of difference between each mutant and the wild type (wild) was determined by *t* test: *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

the glycine residue also was important for nonstructural protein function, perhaps by conferring conformational flexibility on its structure. Among the mutations of the consensus amino acid lysine 334, alteration to glutamate abolished cytotoxicity completely, whereas other mutations (to valine or glutamine) showed modest cytotoxicity in the assay (50 to 60% colony formation compared with that of the control), a result consistent with an important role for lysine's negative charge in this position. Alterations of proline 329, proline 330, or serine 331, none of which is a consensus amino acid within the A site, did not significantly affect cytotoxicity. However, mutation of the nonconsensus amino acid threonine 332 suppressed cytotoxicity equivalent to that of mutations of consensus amino acids: mutation to a negatively charged glutamate abolished the cytotoxic function of the nonstructural protein, substitution with the neutral amino acid valine suppressed cytotoxicity to a lesser degree, and replacement with a positively charged lysine or hydroxyl amino acid serine modestly affected cytotoxicity. Although the threonine or serine residues corresponding to threonine 332 in the B19 nonstructural protein is not always located in the A site, they are well conserved among NTP-binding domains of viral proteins, suggesting some importance for those residues in the function of viral nonstructural proteins.

In contrast to alterations within the A site, mutations in the B site (D372N, E373Q, and DE372NQ) did not suppress the cytotoxic activity of the nonstructural protein as strongly, suggesting that these two acidic amino acids in the B site in the B19 nonstructural protein are not crucial for cytotoxicity.

Cytotoxicity of the nonstructural protein was abolished by single mutations of amino acids within the consensus sequence of the NTP-binding domain but not by mutations in nonconsensus amino acids in the domain. These results from systematic mutations in the domain implicate NTP-binding in this function of the nonstructural protein. For the homologous nonstructural protein 1 of parvovirus H-1, also responsible for cytotoxicity of rat host cells, substitution of serine for lysine within the NTP-binding domain diminished the cytotoxicity (17). Our analogous experimental result was the complete suppression of cytotoxicity by mutation of lysine 334 to glutamate. For another rodent parvovirus, minute virus of mice, the effects of nonstructural protein 1 on cellular transformation, as an indirect measure of cytotoxicity, were localized within the amino- and carboxyl-terminal domains; mutations in the middle of the gene affected not only cytotoxicity but also other protein functions and may have been indirect in their action (3, 16). Rep, the nonstructural protein of the adeno-associated virus, also inhibits cellular transformation (15). By mutational analysis, the transformation suppression domains were demonstrated not to be limited to the amino-terminal region, as deletions throughout the protein altered its suppression capabilities (28). To some extent, differences in the location of the domains for cytotoxicity might be due to the mode of mutation, since mutations by deletion or insertion are more likely to cause a gross conformational change that might affect a physically remote domain than would point mutations. In our experiments, we could not formally exclude the possibility that some mutations affected nonstructural protein expression and its effects on the B19 promoter, because of the small numbers of cells that can be obtained from individual colonies. Other possible sites in the B19 nonstructural protein which may affect cytotoxicity are now being examined in our laboratory.

The biochemical functions of the B19 nonstructural protein have not been reported except for *trans* activation of the P6 promoter (11), in contrast to pleiotropic functions reported for nonstructural proteins of other parvoviruses. The lack of permissive cell lines, convenient propagation systems, or infectious clones has prevented the extensive study of the B19 nonstructural protein. For H-1 parvovirus, the lysine residue in the NTP-binding domain is crucial for viral DNA replication and *trans* activation of the P38 promoter as well as for cytotoxicity (17). For minute virus of mice, mutations in the NTP-binding motif of nonstructural protein 1 affected multiple functions: ATP-binding, ATPase and helicase enzymatic activities, *trans* activation, and DNA replication (14). In the work of Jindal et al., some of the mutants appeared to retain ATP-binding and ATPase activities but not helicase activities and some mutations in the NTP-binding site decreased viral replication but did not affect *trans*-activating activity (14). For adeno-associated virus, mutation of a consensus purine nucleotide-binding-site motif, leading to a lysine conversion to histidine in the protein sequence, caused overproduction of Rep; the mutation inhibited viral DNA replication both of the mutant and wild-type adeno-associated virus in *trans* (5). Other mutations within the domain also cause defects in replication and *trans* activation in vivo as well as in the ATP-dependent endonuclease activity in vitro (19). ATP binding and/or hydrolysis functions of the simian virus 40 T antigen have been also reported to be strongly linked to viral replication (8). NTP-binding domains of viral nonstructural proteins uniformly influence DNA replication, likely through their helicase activity. The cytotoxicity of the B19 nonstructural protein also might be associated with helicase activity, an association under active study in our laboratory.

The B19 parvovirus nonstructural protein mutants defective in cytotoxicity might be useful in the further characterization of this protein. For example, analysis of the biochemical functions of those mutants should help to indicate which cellular events

are required for viral cytotoxicity as well as the mechanism of cell killing by the nonstructural protein. Study of the interactions of the nonstructural protein with cellular factors or *cis* elements should be possible in transfected cells, as target cells will now survive expression of this otherwise toxic gene.

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