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Complete Protein Linkage Map of Poliovirus P3 Proteins: Interaction of Polymerase 3D^{pol} with VPg and with Genetic Variants of 3AB

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Poliovirus has evolved to maximize its genomic information by producing multifunctional viral proteins. The P3 nonstructural proteins harbor various activities when paired with different binding partners. These viral polypeptides regulate host cell macromolecular synthesis and function as proteinases, as RNA binding proteins, or as RNA-dependent RNA polymerase. A cleavage product of the P3 region is the genome-linked protein VPg that is essential in the initiation of RNA synthesis. We have used an inducible yeast two-hybrid system to analyze directly protein-protein interactions among P3 proteins. Sixteen signals of homo- or heterodimer interactions have been observed and have been divided into three groups. Of interest is the newly discovered affinity of VPg to 3D^{pol} that suggests direct interaction between these molecules in genome replication. A battery of 3AB variants (eight clustered-charge-to-alanine changes and five single-amino-acid mutations) has been used to map the binding determinants of 3AB-3AB interaction which were found to differ from the amino acids critical for the 3AB-3D^{pol} interaction. The viral proteinase 3C^{pro} was not found to interact with other 3C^{pro} molecules or with any other P3 polypeptide in yeast cells, a result confirmed by glutaraldehyde cross-linking. The weak apparent interaction between 3AB and 3CD^{pro} scored in the yeast two-hybrid system was in contrast to a strong signal by far-Western blotting. The results elucidate, in part, previous results of biochemical and genetic analyses. The role of the interactions in RNA replication is addressed.

Specific interactions between virus-encoded polypeptides are likely to play a crucial role in poliovirus replication (25, 33, 37, 43, 44, 46, 56, 57). Compared to the average size of DNA virus genomes, the genomes of RNA viruses are small, a restriction that can be related to the error-prone RNA replication of RNA virus quasispecies (55). Poliovirus is no exception. Its genome of ca. 7,500 nucleotides has coding capacity for only a limited number of proteins, all of which are derived from a single translation product, the 247-kDa polyprotein (Fig. 1A) (29, 48). The polyprotein is processed by two virus-encoded proteinases into some 27 cleavage intermediates and end products (23, 55). To maximize the information stored in the small viral genome, the cleavage end products and their precursors have, in many cases, distinct functions. This expands the menu of polypeptides useful for viral proliferation. The C-terminal region (P3) of the viral polyprotein may serve as typical example (Fig. 1B). The P3 precursor is generated through a very fast cleavage event at the amino terminus of the 3A coding region, and it undergoes further rapid processing to yield the two multifunctional, relatively stable cleavage intermediates 3AB (an RNA binding protein involved in RNA polymerization and the precursor to VPg) and 3CD^{pro} (a sequence-specific RNA binding protein that displays also proteinase activity). Subsequent slow processing of 3AB and 3CD^{pro} generates the four cleavage end products, 3A, 3B (the genome-linked protein VPg), 3C^{pro} (a proteinase), and 3D^{pol} (an RNA-dependent RNA polymerase). Table 1 summarizes the biochemical activities and known functions of these polypeptides and provides appropriate references.

Some of the viral polypeptides obtain specific functions by acquiring different binding partners. 3AB is a membrane-associated protein that serves as precursor for VPg (50). Purified detergent-solubilized 3AB is also a nonspecific RNA binding protein (44, 56) that, in association with 3CD^{pro} (37), acquires binding specificity for the 5′-terminal cloverleaf in the presence of a 1,000-fold excess of tRNA (25, 56, 57). Genetic and biochemical data have argued that this complex ([3AB/3CD^{pro}/5′-cloverleaf]) plays an essential role in the initiation of plus-strand RNA synthesis (25, 56, 57). Association with 3CD^{pro}, on the other hand, leads to another biochemical event: 3AB stimulates cleavage of the otherwise rather stable 3CD^{pro} precursor to 3C^{pro} and 3D^{pol} (37, 56). Purified preparations of 3AB have binding affinity for 3D^{pol}, and at low 3D^{pol} concentrations, the 3AB/3D^{pol} complex possesses greatly increased polymerase activity compared to that of 3D^{pol} alone (33, 44, 46). Moreover, the 3AB/3D^{pol} complex appears to have binding specificity for the 3′-terminal nontranslated region (NTR) of the poliovirus genome, a phenomenon that would be useful to guide the polymerase to the proper template site in the initiation of minus-strand synthesis (44). The activity of 3AB is also regu-

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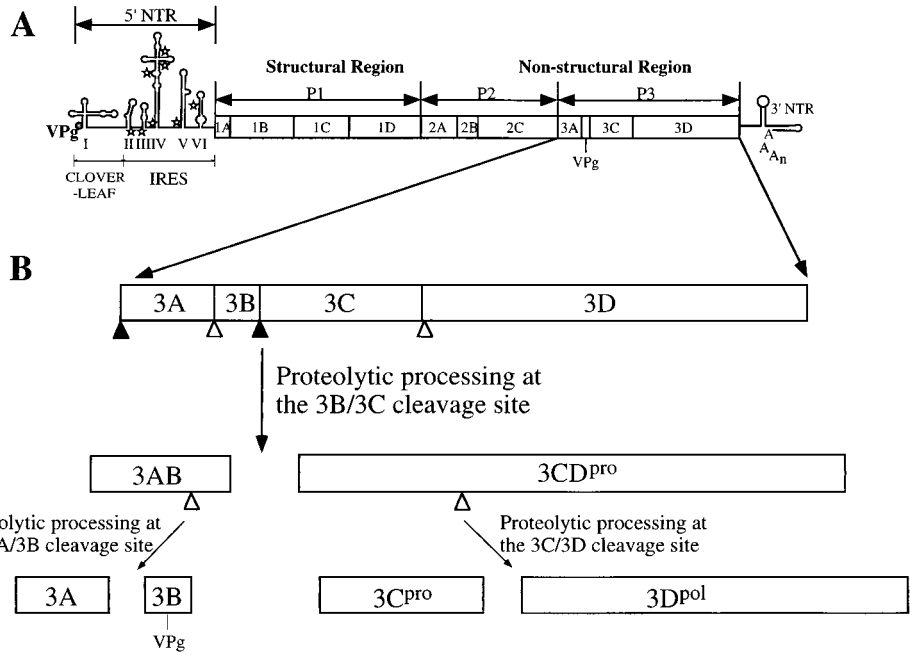

FIG. 1. Genome organization and processing of the polyprotein P3 region. (A) The poliovirus genome is divided into the 5′ NTR, polyprotein, and 3′ NTR. The 5′ NTR consists of the cloverleaf and internal ribosome entry site (IRES) elements. Open stars, unused AUG initiation codons. The polyprotein is divided into the structural and nonstructural regions, whose major cleavage products are indicated. (B) Enlargement of the P3 nonstructural precursor and its processing products analyzed in this study. Closed and open triangles indicate rapidly and slowly processed Q/G cleavage sites, respectively. For functions of the cleavage products, see Table 1.

lated by its association with $3CD^{pro}$, since membrane-bound 3AB can be cleaved by $3CD^{pro}$ into 3A and 3B (33), two cleavage products that have lost the functions of 3AB (see Fig. 5 for overview). Finally, $3D^{pro}$ can form homo-oligomers with increased RNA binding and RNA polymerization activities in vitro (43).

The molecular nature of these exceedingly complex interactions of the poliovirus P3 proteins (summarized in Fig. 5) is unknown. Where do the binding sites of the binding partners map on the corresponding polypeptide chains? Is binding sufficient for function, or can binding and function be genetically separated? Does VPg have binding affinity to $3D^{pol}$? Is the substrate for uridylylation by $3D^{pol}$ precursor 3AB or cleavage product VPg (31, 51, 52)? Have all possible interactions between P3 proteins been detected biochemically?

The yeast two-hybrid system is a powerful tool to detect protein-protein interactions (8, 16). By using this method, a protein linkage map has been established in *Escherichia coli* bacteriophage T7, allowing tabulation of special interactions of the phage gene products (3). The yeast two-hybrid system can also be used to map protein domains that are responsible for protein interactions and to identify mutations that affect these interactions. Here, we report the linkage map of poliovirus proteins mapping to the P3 domain of the polyprotein. In the course of these experiments, we have tested three versions of the two-hybrid system of which only one, the semi-inducible system (58), was adequate. Whereas the viral proteinase $3C^{pro}$ yielded no interaction with other P3 proteins, we detected interaction between VPg and the polymerase $3D^{pol}$, a result highly significant for the study of the mechanism of initiation of viral RNA synthesis. Moreover, interactions between genetic variants of 3AB with wild-type (wt) 3AB or $3D^{pol}$ revealed binding domains of 3AB to these proteins and a relationship between complex formation and function. Since the structure of $3D^{pol}$ has been elucidated at the atomic level (22), the results reported here may contribute to the elucidation of the multicomponent RNA replication complex of poliovirus.

MATERIALS AND METHODS

Strains and plasmids. The *Saccharomyces cerevisiae* strains and all plasmids for two-hybrid analysis are listed in Table 2. The yeast strains and parental plasmids used in two-hybrid systems I and II are generous gifts from Stanley Fields (University of Washington) (2, 16), while those in system III are from Roger Brent (Harvard Medical School) (58). Detailed information about the yeast strains and the plasmids can be found in the cited references. The DNA fragments encoding the six P3 proteins were amplified by PCR with *Xma*I and *Sal*I sites flanking the ends. The pGBT.9, pGAD.GH, and pBTM116 vectors were digested with the same restriction enzymes and ligated with one of the above fragments. The new plasmids are named after the parental plasmids and the viral proteins (e.g., pGAD.GH3AB). Subsequently, the *Eco*RI and *Sal*I fragments were cut out from the pGBT.9 fusion plasmids and ligated to pEG202 and pJG4-5 that had been digested with *Eco*RI/*Sal*I and *Eco*RI/*Xho*I, respectively. Additionally, pRFHM1, which expresses LexA fused to a transcriptional inert fragment of the *Drosophila* Bicoid protein, served as a negative control.

The mutant 3AB genes, in which clustered charged residues were changed to alanines (see Fig. 3), were constructed previously (31, 56) except that M20 was a generous gift of Bert Semler (University of California, Irvine). In the M20 mutant, threonine 67 was replaced with lysine (19). DNA fragments that encode those mutant 3AB proteins were cloned into pJG4-5 as described above.

Two-hybrid analysis. In systems I and II (Table 2), each yeast strain was transformed with the corresponding DNA binding domain (BD)- and transcription activation domain (AD)-viral peptide fusion plasmid by the lithium acetate method (2). The transformants were plated on synthetic medium lacking tryptophan and leucine. About 10 Trp^+ Leu^+ colonies from each plate were inoculated into selective liquid medium, and β-galactosidase activity was measured in a liquid assay (2). To quantify the β-galactosidase activity in the liquid assay, Miller units were defined as 1,000 × optical density at 574 nm (OD_{574}) divided by the product of the volume (milliliters) × time (minutes) × $O.D._{600}$.

In system III (Table 2), the yeast strain was first transformed with plasmid pSH18-34, which has a *lacZ* reporter gene preceded by an upstream LexA binding site (58). The transformed cells were amplified and further transformed with pEG202- and pJG4-5–peptide fusion plasmids and plated onto synthetic medium containing 2% glucose but lacking histidine, tryptophan, and uracil. About 10 His^+ Leu^+ Ura^+ colonies from each transformation were inoculated

TABLE 1. Biochemical activities and known functions of proteins encoded in the poliovirus P3 region

Protein (size [kDa])	Activities	Reference(s)
3A (10)	Membrane association	32
	Substrate for glycosylation	13, 14
	Alteration of membrane permeability	32
	Inhibition of host protein secretion	15
	Essential for RNA replication	4, 18, 26, 54
3B (VPg) (2)	Weak stimulation of $3D^{pol}$	44
	Weak stimulation of $3CD^{pro}$ autocleavage	37
	Covalent linkage to 5′ genome RNA	29, 41
	Initiation of RNA replication	7, 30, 31, 45, 51, 52, 53
3AB (12)	Membrane association	18, 32, 50, 52, 54
	Substrate for glycosylation	13, 14
	Strong stimulation of $3D^{pol}$	33, 44, 46
	Strong stimulation of $3CD^{pro}$ autocleavage	37, 56
	Nonspecific RNA binding	44, 56
	Essential for RNA replication	18, 19, 25, 56, 57
$3C^{pro}$ (20)	Viral protein processing	20, 40
	Host protein cleavage	10, 11, 28
	Weak RNP complex formation with 5′ cloverleaf RNA and 3AB (or host factor)	25
$3D^{pol}$ (52)	Uridylylation of VPg	45
	RNA polymerase	17, 39
	Terminal adenylyltransferase	38
	Polymerization-dependent unwinding of double-stranded RNA	9
	RNA binding	43
	RNP complex with 3′ NTR RNA and 3AB	25, 44
	Binding to 3AB	27, 33, 44, 46
	Oligomerization	22, 43
	Interaction with human protein Sam68	36
$3CD^{pro}$ (72)	Viral protein processing	24, 35
	Binding to 3AB	25, 37
	Strong RNP complex with 5′ cloverleaf and 3AB (or host factor)	1, 25, 56, 57

in selective medium. When the OD_{600} of the liquid culture was around 0.6 to 1.0, the expression of the AD fusion protein was induced with 2% galactose and the incubation was continued for 4 h. The β-galactosidase activity was then assayed.

Chemical cross-linking. One microgram of purified $3C^{pro}$ or 3AB protein was incubated for 15 min at 30°C with different amounts of glutaraldehyde in a buffer containing 25 mM HEPES (pH 7.5) and 5 mM $MgCl_2$. In some reactions, 0.5% sodium dodecyl sulfate (SDS) was also included. The samples were then separated by SDS–15% polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and blotted with a specific antibody to each protein.

Far-Western blotting analysis. Two or 4 μg of purified proteins was directly dot blotted on a nitrocellulose membrane (Schleicher & Schuell). The membrane was then incubated with 56 nM purified $3CD^{pro}$ (a $3C^{pro}$-$3D^{pol}$ cleavage site mutant [20, 24]) in a buffer containing 25 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 50 mM NaCl, 1 mM dithiothreitol, and 0.075% Tween 20 overnight at 4°C (12). Bound protein was detected by enhanced chemiluminescence immunoassay (Amersham) with anti-$3C^{pro}$ serum.

RESULTS

Analysis of three versions of the yeast two-hybrid system to study interactions among poliovirus-encoded P3 proteins. There are three widely used yeast two-hybrid systems, referred to as systems I, II, and III in Table 2 (2, 58). In system I, yeast cells constitutively express the GAL4 BD and AD. The coding regions for six poliovirus P3 peptides (3A, 3B, 3AB, $3C^{pro}$, $3D^{pol}$, and $3CD^{pro}$ [Fig. 1B]) were cloned into plasmids to produce fusion proteins with both domains. The only interaction detectable was that between the BD-$3D^{pol}$ and the AD-$3D^{pol}$ fusion proteins, and the signal was unexpectedly weak (Table 3).

We then switched to system II, in which the sequence of *E. coli* repressor LexA protein replaces that of GAL4 BD. The signal for the $3D^{pol}$-$3D^{pol}$ interaction was enhanced (Table 3). Moreover, we were now able to observe interaction between BD-3D and AD-3AB. However, in both systems I and II, the growth rate of yeast transformants that expressed fusion proteins containing either 3C, 3A, or their precursors was notably low. The slow growth was pronounced in cells transformed with BD-$3C^{pro}$/AD-$3C^{pro}$, BD-3A/AD-3A, and BD-3AB/AD-3AB. Poliovirus protein $3C^{pro}$ has been reported to inhibit transcription by RNA polymerases II and III in mammalian cells (10, 11). It may also damage the microtubule network (28). 3A and 3AB, in turn, have been reported to modify membrane permeability (32) and interfere with host protein secretion (15). Thus, the constitutive expression of these viral proteins could be deleterious to the normal metabolism of the yeast cell.

To circumvent these obstacles, we chose system III, developed in the laboratory of Roger Brent (58). This system uses

TABLE 2. Three two-hybrid systems used in this work

System	Vector		Yeast strain	Expression of AD fusion	Reference
	BD	AD			
I	pGBT9 (GAL4 BD)	pGAD.GH (GAL4 AD)	Y153	Constitutive	2
II	pBTM116 (LexA BD)	pGAD.GH	CTY-10d	Constitutive	2
III	pEG202 (LexA BD)	pJG4-5 (B42)	EGY48/pSH18-34	Inducible	58

TABLE 3. Comparison of the sensitivity among the three two-hybrid systems

System	Mean β-galactosidase activity (Miller units) ± SD (BD fusion-AD fusion)			Signal/noise ratio[a]
	3D-3D	3D-3AB	3D-Vc	
I	2.3 ± 0.6	0	0	
II	51.2 ± 3.6	1.4 ± 0.2	1.1 ± 0.2	46.5 (1.27)
III	16,200 ± 1,600	20,500 ± 900	152 ± 17	107 (135)

[a] Defined as 3D-3D/3D-Vc (or 3D-3AB/3D-Vc).

the LexA DB domain as in system II. Whereas expression of the BD fusion protein is constitutive, that of the AD fusion is under the control of the inducible *GAL1* promoter and contains the bacterial peptide B42. Since the promoter is inducible, the yeast cell can proliferate before synthesis of AD fusion protein is induced. Indeed, better growth of the transformants, especially of the cells expressing AD-3C^{pro} and AD-3AB fusion proteins, was observed. This semi-inducible expression yielded the highest sensitivities and signal/noise ratios in detecting viral protein-protein interaction (Table 3). This was true especially for 3D^{pol}-3AB interactions: the β-galactosidase activity in system III was 10^4-fold higher than those in systems I and II. Such high signal in the inducible system was reported recently also in a separate study (27).

Protein-protein interactions of P3 polypeptides. Six LexA BD-poliovirus peptide fusions (Lex-3A, -3B, -3AB, -3C^{pro}, -3D^{pol}, and -3CD^{pro} [Fig. 2]) were constructed in plasmid pEG202. For negative controls, plasmid pRFHM1, which expresses LexA-Bicoid fusion protein, was included in the analyses (58) (Materials and Methods). In addition, six B42 AD-viral peptide fusions (AD-3A, -3B, -3AB, -3C^{pro}, -3D^{pol}, and -3CD^{pro}) were constructed in vector pJG4-5. Forty-nine pairs of plasmids, including every combination of the two groups, were transformed into yeast strain EGY48. If two viral polypeptides interact with each other, transcription of the β-galactosidase gene is induced; β-galactosidase activity can then be quantified by a simple colorimetric assay. For example, BD-3A was paired with AD-3A, AD-3B, AD-3AB, etc., and the colorimetric signal is presented in Fig. 2 as bars 1, 2, 3, etc., respectively; or 3B was paired with AD-3A, AD-3B, AD-3AB, etc., and the signal was recorded as bars 8, 9, 10, etc., respectively. The β-galactosidase activities of 12 transformants were well above background (Fig. 2), an observation suggesting an affinity between corresponding poliovirus polypeptide pairs.

It should be noted that the failure to observe an interaction in the two-hybrid system does not necessarily mean that the two proteins do not interact in their normal environment.

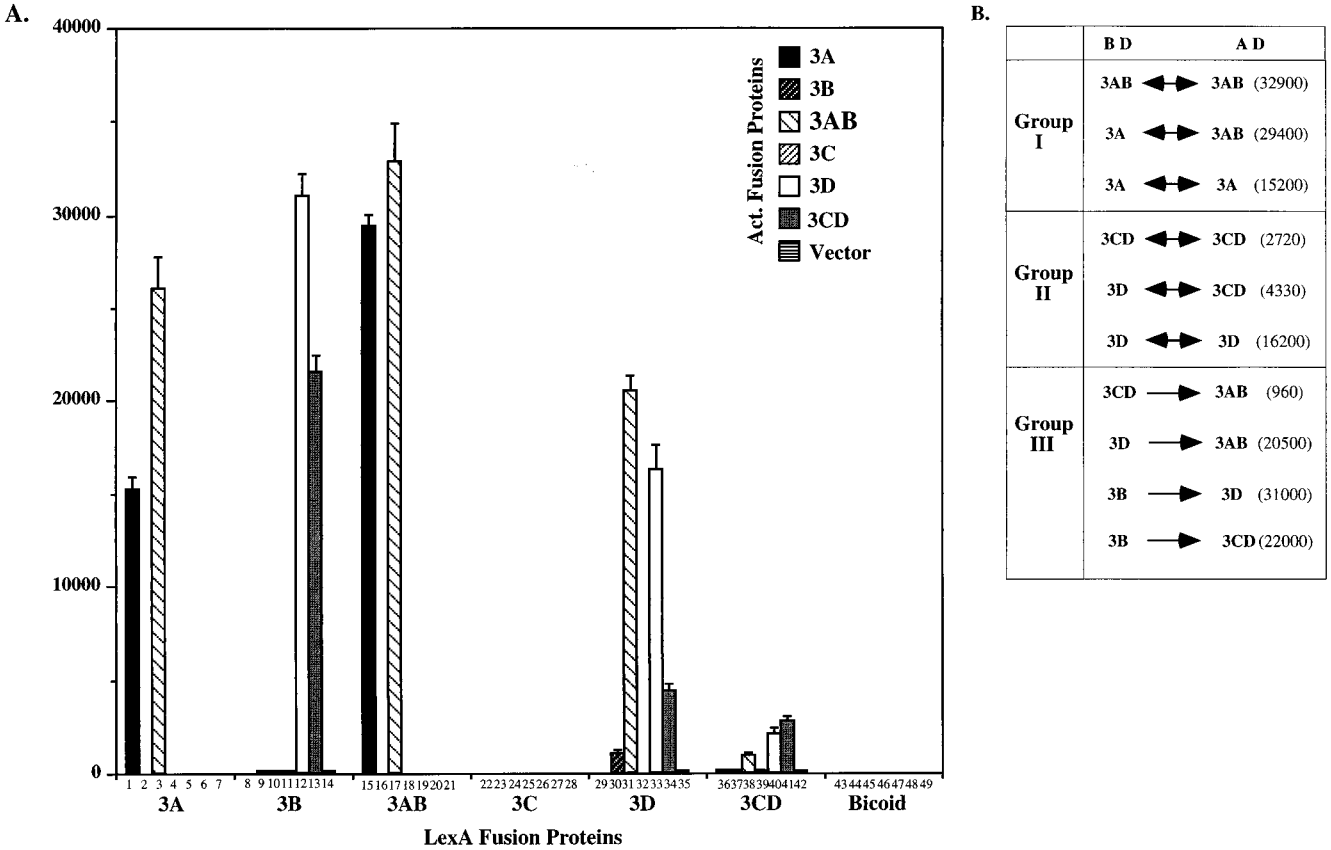

FIG. 2. Interactions of six proteins encoded in the P3 nonstructural region in the inducible yeast two-hybrid system. (A) Yeast strain EGY48, expressing the pair of indicated hybrid proteins, was analyzed for β-galactosidase activity (given in Miller units). Each bar presents the result of a pairing of a LexA fusion protein with an AD fusion protein. Thus, the interaction of LexA-3A (referred to also as BD-3A) with AD-3A, AD-3B, AD-3AB, AD-3C, etc., is given in bars 1, 2, 3, 4, etc. Lack of a bar indicates absence of a color reaction. The enzyme activity of each transformant was quantified in a liquid assay and presented as Miller units. The Bicoid segment served as negative control. (B) Detected protein interactions divided into three groups (see text). The β-galactosidase activity from each interaction is indicated in parentheses. The double-headed arrows indicate that the interaction between two different viral proteins is bidirectional; the single-headed arrows indicate unidirectional interactions.

Improper folding or poor expression of the hybrid proteins can influence whether an interaction is apparent. Often, the polarity in which two proteins are presented may play a crucial role. Likewise, in the absence of information about the relative expression levels (which can change from experiment to experiment), the relative amounts of β-galactosidase expression between unrelated hybrid pairs do not directly reflect differences in the affinities of the pairs of proteins. Nevertheless, the observation of β-galactosidase activity in the presence of a pair of hybrid proteins is a strong indication that two polypeptide chains expressed as fusion proteins do interact.

The 12 observed interactions can be divided into three groups (Fig. 2B), depending on the nature of the partners. Interactions between those polypeptides that map to the N terminus of P3 (3AB, 3A, and 3B) have been combined in group I. Homodimer pairs of 3A and 3AB strongly react with each other in this system (bars 1 and 17, respectively), the latter giving the highest value of β-galactosidase activity (~3×10^4 Miller units) scored in any of these experiments. The extraordinary homoaffinity of purified 3AB, even in the presence of strong detergent, was demonstrated also by a biochemical assay (see below). Experiments using vector pairs with a Bicoid hybrid control or AD alone failed to yield β-galactosidase activity (BD-3A/AD and BD/AD-3A [bars 7 and 43, respectively] and BD-3AB/AD and BD/AD-3AB [bars 21 and 45, respectively]), an observation showing that the interactions are due to affinities between the viral polypeptide chains. 3B, on the other hand, did not show significant binding to other 3B-containing fusion proteins in this system—3B (bar 9; BD-3B/AD in bar 14), 3A (bars 2 and 8), or 3AB (bars 10 and 16)—although the LexA-3B fusion protein was expressed, as evident from strong signals obtained with $3D^{pol}$ and $3CD^{pro}$ (bars 12 and 13) (see also below). AD-3B was also expressed since it interacted with Lex-$3D^{pol}$ (bar 30); this interaction, however, was weak and thus is not a good measure of AD-3B expression (see below).

Interactions between those polypeptides that map to the C terminus of the P3 precursor ($3CD^{pro}$, $3C^{pro}$, and $3D^{pol}$) have been combined in group II. Only the $3D^{pol}$ homopair displayed strong interaction in this system (Fig. 2A, bar 33). In contrast, the $3D^{pol}$-$3CD^{pro}$ interactions were more difficult to detect, regardless of the polarity of the partners (bars 34 and 40). The signal for homopair interaction between $3CD^{pro}$ chains was also relatively weak (bar 41). No β-galactosidase activity was observed in attempts to pair $3C^{pro}$ with itself (bar 25), with 3AB (bars 18 and 24), or with $3CD^{pro}$ (bars 27 and 39) as a partner. The failure to obtain significant signals with the viral $3C^{pro}$ and $3CD^{pro}$ proteinases as binding partners in some of the pairings is unlikely to be the result of $3C^{pro}$-specific proteolytic activity that might interfere with the formation of, or destabilize, a protein-protein complex. This we conclude from studies with mutant polypeptides $3C^{pro}$(C147S), $3CD^{pro}$ (F83A), and $3CD^{pro}$(R87Q). Although a $3C^{pro}$(C147S) mutation constructed into the poliovirus polyprotein retained some 3C-related activity in *cis* cleavages (34), we have reported previously that the purified $3C^{pro}$(C147S), $3CD^{pro}$(F83A), and $3CD^{pro}$(R87Q) polypeptides have lost proteolytic activity below detectable levels in *trans*-cleavage assays (20, 21). When cloned into pEG202, these $3C^{pro}$(C147S), $3CD^{pro}$(F83A), and $3CD^{pro}$(R87Q) mutant polypeptides did not yield increased β-galactosidase activity (data not shown). Moreover, as mentioned, the BD-3B/AD-$3CD^{pro}$ pair showed a strong signal (bar 13).

Group III (Fig. 2B) depicts interactions between 3AB and $3CD^{pro}$ and their cleavage products. Surprisingly, all interactions in this group were found to be unidirectional. For example, $3D^{pol}$ interacted with 3AB only if presented as BD-$3D^{pol}$ (bar 31) (27). Since BD-$3D^{pol}$ does not form a detectable complex with 3A (bar 29), the binding between $3D^{pol}$ and 3AB in this system may rely predominantly on sequences of 3B. Whereas interaction between BD-$3D^{pol}$ and AD-3B was more difficult to detect (bar 30), that in the reverse direction, between BD-3B and AD-$3D^{pol}$, was strong (bar 12). As discussed later, the observation of the affinity between 3B and $3D^{pol}$ is highly significant in view of the mechanism of initiation of poliovirus minus-strand RNA synthesis (45). We have previously suggested that both the 3A and 3B moieties in 3AB are necessary to form the superactive RNA polymerase $[3AB/3D]^{super\text{-}pol}$ (44, 56). It appears, therefore, that some cooperativity of the two domains of 3AB may be important in the binding of 3AB to $3D^{pol}$.

As is the case for $3D^{pol}$-3AB and 3B-$3D^{pol}$, the $3CD^{pro}$-3AB interaction is also unidirectional (Fig. 2A; compare bar 38 with bar 20). However, the interaction between $3CD^{pro}$ and 3AB was very weak, which was a big surprise since immune precipitation experiments suggested that these polypeptides form a complex in solution (37). Therefore, the question of 3AB-$3CD^{pro}$ interaction was addressed again by another assay (see below).

The molecular basis of the unidirectional nature of some of the two-hybrid interaction is obscure. It is not due to insufficient expression of the corresponding fusion proteins because in homologous pairings, the viral polypeptides show strong signals (Fig. 1B, groups I and II). In addition, Western blotting of both 3B fusion proteins revealed adequate expression of the polypeptides (57a).

The interactions between mutants of 3AB and wt P3 proteins. Several lines of evidence argue that polypeptide 3AB plays multiple roles in the replication of poliovirus (4, 18, 19, 25, 30–33, 37, 44, 46, 50, 52, 54, 56, 57). We have provided previously biochemical evidence that the polypeptides 3AB, $3D^{pol}$, and $3CD^{pro}$ interact with each other in an as yet unknown fashion to provide crucial functions for poliovirus genome replication (25, 27, 33, 44, 56, 57). These studies included purified 3AB polypeptides that each carried clustered-charge-to-alanine mutations in the 3A portion of 3AB or point mutations in the 3B portion (Fig. 3B) (56). All of these mutations, when constructed into the poliovirus genome, were deleterious to genome replication (18, 30, 31, 56). Moreover, Giachetti et al. (18) provided evidence that mutations in a hydrophobic region of 3AB influence poliovirus RNA synthesis. It was unknown, however, to what extent the phenotypes of these mutants were related to impaired protein-protein interaction. Therefore, we tested the effects of specific 3AB mutations (18, 56) on the formation of 3AB homodimers or $3D^{pol}$/3AB heterodimers in the yeast two-hybrid system. Interaction of the mutant 3AB proteins with $3CD^{pro}$ was not analyzed because of the weak signal scored between wt 3AB and $3CD^{pro}$ fusion proteins.

Eight clustered-charge-to-alanine mutations and four point mutations, all of which cause severe defects in RNA synthesis during poliovirus proliferation in tissue culture (18, 56), were introduced into AD-3AB and tested with wt BD-3AB or with BD-$3D^{pol}$. Surprisingly, most mutant AD-3AB polypeptides gave as strong an interaction signal with wt BD-3AB as did the wt BD-3AB/AD-3AB pair (Fig. 3; compare the open bars for M6 through M19 with that for WT). There were three exceptions. M6 and M11 AD-3AB fusion proteins displayed 10- and 20-fold reductions in β-galactosidase activity, respectively. More dramatically, the M20 mutant of 3AB showed a 500-fold reduction in β-galactosidase accumulation in the presence of wt 3AB-containing AD fusion protein (Fig. 3, open bar, M20).

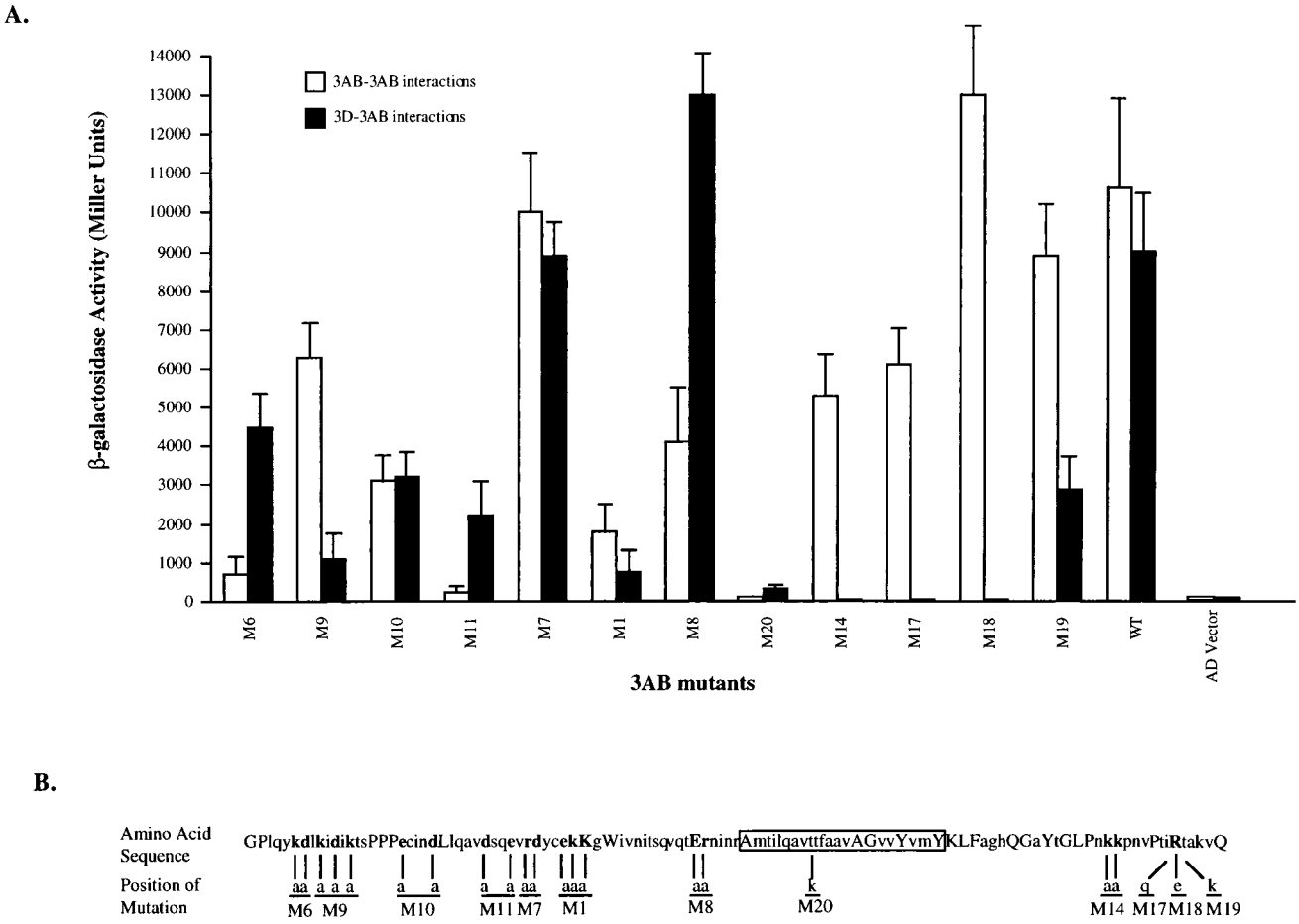

FIG. 3. Interactions of mutant 3ABs with wt 3D^{pol} or 3AB in the yeast two-hybrid system. (A) Yeast strain EGY48 was transformed with pairs of an AD-mutant 3AB and a BD-wt 3AB (or BD-3D^{pol}) expression plasmid. The β-galactosidase activity resulting from AD-mutant 3AB interaction with BD-wt 3AB or BD-3D^{pol} was quantified by the liquid assay and presented as in Fig. 2A. Open bars, 3AB-3AB interactions; shaded bars, 3D^{pol}-3AB interactions. The 3AB mutants are as designated below. (B) Polypeptide 3AB is represented as a long, open rectangle of which the hydrophobic region is shaded. Positions of the clustered-charge-to-alanine mutations (56) are indicated.

In this mutant polypeptide, a threonine residue located in the strongly hydrophobic region of 3AB (46) (boxed region in Fig. 3B) was exchanged with a positive amino acid (lysine) (T67K [18]). Finally, none of the mutations in the 3B domain affected binding between 3AB polypeptides (Fig. 3, open bars, M14 and M17 to M19). These results imply that the 3A moiety of 3AB, perhaps predominantly its hydrophobic region, is essential for homodimer formation.

It should be noted that all mutant AD-3AB fusion proteins were expressed in yeast, as judged by the strong signals scored with these hybrids either with BD-3AB (Fig. 3, open bars) or with BD-3D^{pol} (closed bars) as a partner (see below). Expression of the M20 hybrid was further analyzed by Western blotting analysis using an anti-3AB antibody, and it was found to be in the range of the other mutant hybrids (data not shown).

A different picture emerged when the AD-3AB mutant proteins were paired with wt BD-3D^{pol}. Whereas most mutations in the hydrophilic domain of 3AB failed to significantly influence interaction with BD-3D^{pol}, the M14, M17, and M18 mutations in the 3B domain of 3AB reduced 3AB/3D^{pol} binding to barely detectable levels in this system (Fig. 3, closed bars). All three mutations reduce the positive charge in the 3B domain. Interestingly, the positive charge of the lysine residue in the 3B domain of 3AB in M19 is apparently adequate for the 3AB-3D^{pol} interaction whereas glutamine or glutamic acid residues in this position are not. Only the M1 and M20 mutations in the 3A domain reduced significantly the signal of the 3AB-3D^{pol} interaction (Fig. 3). As discussed later, these data serve to explain, in part, our results previously reported for biochemical assays with 3AB mutants (56).

Binding of P3 proteins in vitro. The high score of protein-protein binding of the BD-3AB/AD-3AB fusion proteins was contrasted by a total absence of interaction of corresponding 3C^{pro} fusion proteins in the yeast two-hybrid system (Fig. 2). We tested the validity of this result by a chemical cross-linking experiment.

One microgram each of purified 3AB (33) and 3C^{pro} (40) was incubated with increasing amounts of glutaraldehyde. As is apparent in Fig. 4A, 3AB could be cross-linked, forming dimers and higher-order oligomers. Polypeptide 3AB is particularly intriguing in that it reproducibly displayed a small number of dimeric molecules even in the absence of glutaraldehyde (Fig. 4A, 3AB, lane 1), a phenomenon seen previously (33). Presence of 0.4% SDS in the chemical cross-linking reaction blocked multimerization of 3AB but, remarkably, did not completely disrupt dimerization of 3AB (lane 6). It should be noted that the method of detection of proteins in this experiment was by immune reaction with a 3AB-specific monoclonal antibody

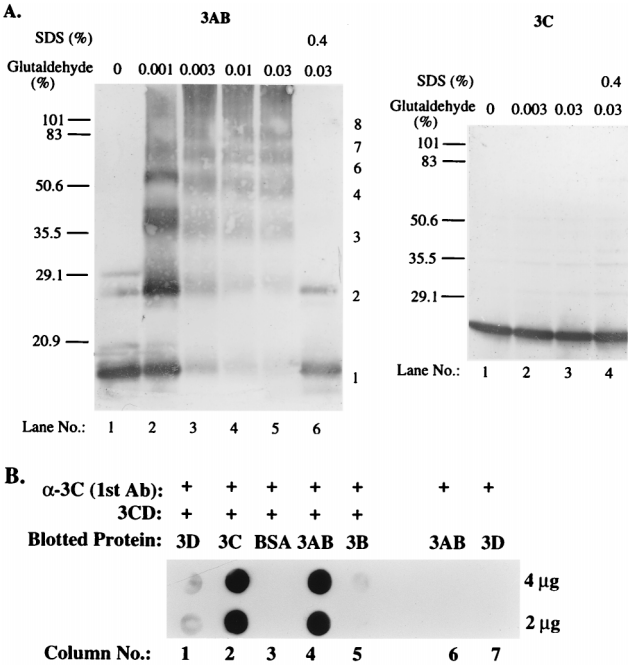

FIG. 4. Binding of poliovirus proteins in vitro. (A) Chemical cross-linking of proteins 3AB and $3C^{pro}$. As indicated, 1 ng of each protein was incubated with different concentrations of glutaraldehyde at 30°C for 30 min. In one reaction, 0.4% SDS was added together with 0.03% glutaraldehyde. The samples were loaded onto an SDS–13% polyacrylamide gel for 3AB or $3C^{pro}$ and separated by electrophoresis. The mobilities of some proteins with known molecular weights are indicated at the left in kilodaltons; numbers to the right of the 3AB panel show estimates of different forms of oligomerization. (B) Protein-protein interactions detected in a far-Western blotting assay. Two or 4 ng of different purified proteins was blotted onto a 0.45-mm-pore-size nitrocellulose membrane. The blotted membrane was incubated with or without purified $3CD^{pro}$ (24) at 4°C overnight. Bound $3CD^{pro}$ was detected by enhanced chemiluminescence (α-3C) immunoblotting with anti-$3C^{pro}$ antibody (Ab) (see Materials and Methods).

after transfer of the material to nitrocellulose membranes. This method served to ensure that the observed bands were 3AB specific.

In contrast, purified $3C^{pro}$ did not cross-link, even at the highest glutaraldehyde concentration (Fig. 4A, 3C, lane 3). This result supports the notion that the viral proteinase $3C^{pro}$ lacks the ability to form homodimers or oligomers.

Similar cross-linking experiments were carried out with purified $3D^{pol}$ and $3CD^{pro}$. In both cases, dimerization could be observed (data not shown). Indeed, we observed formation of higher-order oligomers with $3D^{pol}$, a result that agrees with previous data suggesting that $3D^{pol}$ has the propensity to form oligomers in solution (43).

The weak interaction between 3AB and $3CD^{pro}$ in the yeast two-hybrid assay (Fig. 2) prompted us to analyze heteroprotein interaction by far-Western blotting (12). In this experiment, we blotted purified 3AB, 3B, $3C^{pro}$, $3D^{pol}$, and bovine serum albumin (BSA) onto nitrocellulose membranes and probed the proteins first with purified $3CD^{pro}$. Binding of the proteinase to the blotted $3D^{pol}$, BSA, 3AB, and 3B proteins was then tested by probing with anti-$3C^{pro}$ antibodies. These antibodies are capable of detecting $3CD^{pro}$ but do not cross-react with 3AB or $3D^{pol}$ (Fig. 4B, lanes 6 and 7). $3CD^{pro}$ was able to bind strongly to immobilized 3AB (Fig. 4B, lane 4), weakly to immobilized $3D^{pol}$ (lane 1), and barely detectably to 3B (lane 5). No binding was observed to BSA (lane 3). These data support those obtained with the yeast two-hybrid assay and serve to indicate that weak binding observed in yeast cells is not necessarily a reflection of the strength of protein interaction as scored by other methods. For us, this is particularly significant with respect to the strong far-Western signal seen for the pair $3AB/3CD^{pro}$ (Fig. 4B, lane 4), in contrast to the weak apparent interaction scored in the two-hybrid system (Fig. 2A, bar 38). It must be kept in mind, however, that the biochemical assays may not necessarily reflect the binding reactions in the infected cell. This dilemma, of course, must be considered even for the results obtained in the yeast two-hybrid system.

DISCUSSION

We have obtained a protein linkage map of poliovirus P3 proteins, using all possible combinations of protein-protein pairing of those P3 cleavage products known to be relevant in poliovirus RNA replication. The study has led to the discovery of hitherto unknown protein-protein interactions. Ultimately, our studies are aimed at solving the nature of the replication complex functioning in poliovirus genome replication in vivo, a task that will be greatly aided by the known crystal structure of $3D^{pol}$ (22). Our experiments with a battery of mutants mapping to 3AB have allowed us, for the first time, to map binding domains on 3AB and provide an explanation of phenotypes of polioviruses carrying such mutations in vivo (56). These studies are useful to explain the outcome of biochemical reactions performed with purified poliovirus P3 proteins (25, 44, 46, 56, 57).

Both the power and the limitations of the yeast two-hybrid system have been demonstrated here. We note that only one of three commonly used strategies of the yeast two-hybrid system was successful: the method in which one of the binding partners is inducibly expressed (58) (Table 3). The restriction is most likely related to the toxicity of some viral polypeptides that may impair cell growth when expressed constitutively as fusion proteins in yeast cells.

The uridylylation of VPg (=3B) by purified $3D^{pol}$ in the presence of poly(A) template is the most unusual (45) reaction in which proteins of the P3 region of the poliovirus polyprotein are engaged. The reaction performed in vitro requires that VPg interact very specifically with the polymerase in the absence of the 3A moiety of its precursor 3AB (45). The data presented here provide for the first time evidence of direct binding of VPg to $3D^{pol}$, a result consistent with a model in which VPg is the substrate for uridylylation in vivo.

Polypeptide 3AB is a poliovirus protein for which several different functions in replication have been assigned (Table 1; Fig. 5). During purification of the protein, we noticed previously that 3AB has the propensity to dimerize (33). We were surprised to observe, however, that 3AB dimerizes also in the presence of SDS; moreover, we now report that in the absence of the strong detergent, 3AB will form oligomers in solution (Fig. 4A). An analysis of 3AB interaction in the yeast two-hybrid system confirmed the high affinity between two 3AB polypeptide chains (Fig. 2). Making use of 3AB mutants (56), we have mapped the region that is mostly responsible for 3AB homointeractions to the hydrophobic domain (Fig. 3). It was surprising to us that clustered-charge-to-alanine mutations in the hydrophilic portion of 3A or in 3B exerted little influence on the 3AB interaction, with the possible exception of mutants M6 and M11. Lama and Carrasco have found that 3AB molecules can permeabilize membranes of yeast and bacterial cells (32). Perhaps several 3AB molecules form a pore by penetrating through, and aligning their hydrophobic segment within, intracellular membranes. It is known that mutations engineered into the hydrophobic segment influence membrane association of 3AB (54) and that mutations in this region display

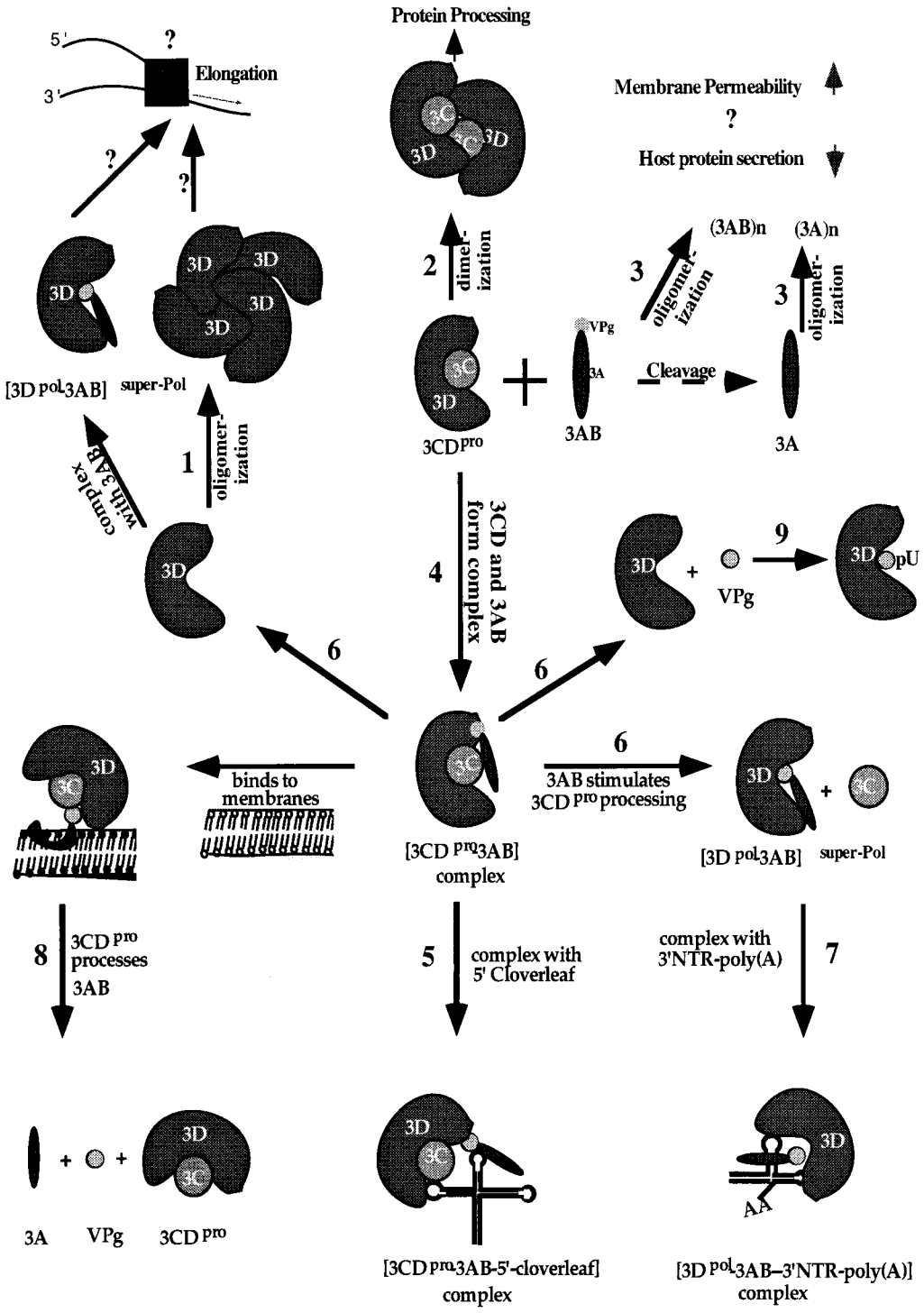

FIG. 5. Working model for interactions and diverse functions of the P3 polypeptides. (1) $3D^{pol}$ is depicted as a tetramer although its precise status in solution is not known. $3D^{pol}$ oligomers are more active than monomers in RNA chain elongation, and the same may be true for the $3AB/3D^{pol}$ complex. (2) The function of $3CD^{pro}/3CD^{pro}$ dimers is not known, but they may be required for autocleavage and/or viral protein processing. (3) 3AB and its cleavage product 3A alter host membrane permeability and inhibit protein secretion. Whether these proteins must assemble to dimers or multimers (through interactions of their hydrophobic domain) to produce these effects is unknown. (4) 3AB and $3CD^{pro}$ can form a complex in solution that (5) can bind with high specificity to the 5′ cloverleaf, a process required for the initiation of positive-strand RNA synthesis. (6) When 3AB and $3CD^{pro}$ are bound to each other, 3AB can stimulate the autocleavage of $3CD^{pro}$ into 3C and $3D^{pol}$. $3D^{pol}$ and 3AB may remain in a tight complex with increased RNA polymerization activity. (7) A $3AB/3D^{pol}$ complex is likely to recognize the 3′ NTR of the genome for the initiation of negative-strand RNA synthesis. (8) The half-life of 3AB is regulated by $3CD^{pro}$, since membrane-bound 3AB can be cleaved by $3CD^{pro}$ into 3A and 3B (VPg), two cleavage products whose functions are distinct from those of the 3AB precursor. (9) VPg, in turn, is uridylylated by $3D^{pol}$ to VPgpU(pU), the primer for $3D^{pol}$. For references, see Table 1. 3′NTR, 3′ nontranslated region.

a strong defect in poliovirus RNA replication (4, 18, 19). It is possible, therefore, that oligomerization is an important determinant of 3AB in RNA replication.

The interaction of 3AB with the viral polymerase $3D^{pol}$ may play a central role in the enzyme's template recognition and polymerase activity (27, 33, 44, 46, 56). Particularly intriguing is 3AB's ability to bind to $3D^{pol}$ and stimulate $3D^{pol}$ polymerase activity at low enzyme concentrations (33, 44, 46, 56). 3AB and $3D^{pol}$ may form a complex that we have described as $[3AB/3D]^{super\text{-}pol}$ (44). We have previously shown that certain clustered-charge-to-alanine mutations in 3AB diminish this stimulating activity (56). We now correlate the lack of $[3AB/3D]^{super\text{-}pol}$ formation with the apparent loss of binding of 3AB to $3D^{pol}$ (Fig. 3A). The 3AB mutations abrogating enzyme stimulation as well as $3AB/3D^{pol}$ binding map to the same basic amino acids in the 3B moiety (Fig. 3B). Thus, specific residues in the 3B moiety of 3AB form contact points between this protein and the polymerase, an interaction that we speculate is true also for the complex formation between VPg and $3D^{pol}$. It should be noted, however, that the M20 mutation in the hydrophobic domain of 3AB and the M1 mutation in the 3A moiety reduced binding to $3D^{pol}$ in the two-hybrid system (Fig. 3). This may explain why both 3A and 3B portions of 3AB are required to yield a maximally functioning $[3AB/3D]^{super\text{-}pol}$ complex (44). At least one residue in $3D^{pol}$, Val391, has been identified as being required for binding of $3D^{pol}$ to 3AB in the two-hybrid system and in biochemical assays (27).

The interaction in the two-hybrid system between 3AB and $3CD^{pro}$ was relatively weak (Fig. 2A, bar 38). On the other hand, we have reported that $3AB/3CD^{pro}$ coprecipitated in immune reactions, an observation suggesting interaction in solution (37). Indeed, 3AB has been reported to stimulate proteolysis of $3CD^{pro}$, and $3AB/3CD^{pro}$ exhibits a strong and very specific affinity to the 5′-terminal cloverleaf of the viral genome (25, 56, 57). When tested by far-Western blotting, a strong interaction between 3AB and $3CD^{pro}$ was observed (Fig. 4B). This result underscores the contention that weak signals in the two-hybrid assay are not necessarily indicative of the strength of specific protein-protein interaction.

The complexity of the different interactions of the P3 polypeptides of poliovirus is summarized in Fig. 5. It is apparent that the polypeptide chains produced during the cascade of proteolytic cleavages engage in a variety of very different biochemical reactions. Several of these reactions have been deduced by genetic analyses only (55). In addition to the viral polypeptides, there may also be cellular proteins that specifically interact with P3 cleavage products of the poliovirus polyprotein (1, 6, 42). The recently described poly(C) binding protein 2 may be such a host factor (6, 42).

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REFERENCES

1. **Andino, R., G. E. Rieckhof, P. L. Achacoso, and D. Baltimore.** 1993. Poliovirus RNA synthesis utilizes an RNP complex formed around the 5′-end of viral RNA. EMBO J. **12:**3587–3598.
2. **Bartel, P. L., C.-T. Chien, R. Sternglanz, and S. Fields.** 1993. Using the two-hybrid system to detect protein-protein interactions, p. 153–179. *In* D. A. Hartley (ed.), Cellular interactions in development: a practical approach. Oxford University Press, Oxford, England.
3. **Bartel, P. L., J. A. Roecklein, D. SenGupta, and S. Fields.** 1996. A protein linkage map of Escherichia coli bacteriophage T7. Nat. Genet. **12:**72–77.
4. **Bernstein, H. D., and D. Baltimore.** 1988. Poliovirus mutant that contains a cold-sensitive defect in viral RNA synthesis. J. Virol. **62:**2922–2928.
5. **Bernstein, H. D., P. Sarnow, and D. Baltimore.** 1986. Genetic complementation among poliovirus mutants derived from an infectious cDNA clone. J. Virol. **60:**1040–1049.
6. **Blyn, L. B., K. M. Swiderek, O. Richards, D. C. Stahl, B. L. Semler, and E. Ehrenfeld.** 1996. Poly(rC) binding protein 2 binds to stem-loop IV of the poliovirus RNA 5′ noncoding region: identification by automated liquid chromatography-tandem mass spectrometry. Proc. Natl. Acad. Sci. USA **93:**11115–11120.
7. **Cao, X. M., R. J. Kuhn, and E. Wimmer.** 1993. Replication of poliovirus RNA containing two VPg genes leads to a specific deletion event. J. Virol. **5572–5578.**
8. **Chien, C.-T., P. L. Bartel, R. Sternglanz, and S. Fields.** 1991. The two-hybrid system: a method to identify and clone genes for proteins that interact with a protein of interest. Proc. Natl. Acad. Sci. USA **88:**9578–9582.
9. **Cho, M. W., O. C. Richards, T. M. Dmitrieva, V. Agol, and E. Ehrenfeld.** 1993. RNA duplex unwinding activity of poliovirus RNA-dependent RNA polymerase $3D^{pol}$. J. Virol. **67:**3010–3018.
10. **Clark, M. E., T. Hammerle, E. Wimmer, and A. Dasgupta.** 1991. Poliovirus proteinase 3C converts an active form of transcription factor IIIC to an inactive form: a mechanism for inhibition of host cell polymerase III transcription by poliovirus. EMBO J. **10:**2941–2947.
11. **Clark, M. E., P. M. Lieberman, A. J. Berk, and A. Dasgupta.** 1993. Direct cleavage of human TATA-binding protein by poliovirus protease 3C in vivo and in vitro. Mol. Cell. Biol. **13:**1232–1237.
12. **Cuervo, A. M., and J. F. Dice.** 1996. A receptor for the selective uptake and degradation of proteins by lysosomes. Science **273:**501–503.
13. **Datta, U., R. Banerjee, X. Liu, and A. Dasgupta.** 1996. A point mutation in the glycosylation site of poliovirus polypeptide 3AB produces a dominant lethal phenotype. Presented at the ASV Meeting, Ontario, Canada.
14. **Datta, U., and A. Dasgupta.** 1994. Expression and subcellular localization of poliovirus Vpg-precursor protein 3AB in eukaryotic cells: evidence for glycosylation in vitro. J. Virol. **68:**4468–4477.
15. **Doedens, J. R., and K. Kirkegaard.** 1995. Inhibition of cellular protein secretion by poliovirus proteins 2B and 3A. EMBO J. **14:**894–907.
16. **Fields, S., and O.-K. Song.** 1989. A novel genetic system to detect protein-protein interactions. Nature **340:**245–246.
17. **Flanegan, J. B., and D. Baltimore.** 1977. Poliovirus-specific primer-dependent RNA polymerase able to copy poly(A). Proc. Natl. Acad. Sci. USA **74:**3677–3680.
18. **Giachetti, C., S.-S. Hwang, and B. L. Semler.** 1992. *cis*-acting lesions targeted to the hydrophobic domain of a poliovirus membrane protein involved in RNA replication. J. Virol. **66:**6045–6057.
19. **Giachetti, C., and B. L. Semler.** 1991. Role of a viral membrane polypeptide in strand-specific initiation of poliovirus RNA synthesis. J. Virol. **65:**2647–2654.
20. **Hammerle, T., C. U. Hellen, and E. Wimmer.** 1991. Site-directed mutagenesis of the putative catalytic triad of poliovirus 3C proteinase. J. Biol. Chem. **266:**5412–5416.
21. **Hammerle, T., A. Molla, and E. Wimmer.** 1992. Mutational analysis of the proposed FG loop of poliovirus proteinase 3C identifies amino acids that are necessary for 3CD cleavage and might be determinants of a function distinct from proteolytic activity. J. Virol. **66:**6028–6034.
22. **Hansen, J. L., A. M. Long, and S. C. Schultz.** 1997. Structure of the RNA-dependent RNA polymerase of poliovirus. Structure **5:**1109–1122.
23. **Harris, K. S., C. U. T. Hellen, and E. Wimmer.** 1990. Proteolytic processing in the replication of picornaviruses. Semin. Virol. **1:**323–333.
24. **Harris, K. S., S. R. Reddigari, M. J. H. Nicklin, T. Hämmerle, and E. Wimmer.** 1992. Purification and characterization of poliovirus polypeptide 3CD, a proteinase and a precursor for RNA polymerase. J. Virol. **66:**7481–7489.
25. **Harris, K. S., W. Xiang, L. Alexander, A. V. Paul, W. S. Lane, and E. Wimmer.** 1994. Interaction of the poliovirus polypeptide $3CD^{pro}$ with the 5′ and 3′ termini of the poliovirus genome: identification of viral and cellular cofactors necessary for efficient binding. J. Biol. Chem. **269:**27004–27014.
26. **Heinz, B. A., and L. M. Vance.** 1995. The antiviral compound enviroxime targets the 3A coding region of rhinovirus and poliovirus. J. Virol. **69:**4189–4197.
27. **Hope, D. A., S. E. Diamond, and K. Kirkegaard.** 1997. Genetic dissection of interaction between poliovirus 3D polymerase and viral protein 3AB. J. Virol. **71:**9490–9498.
28. **Joachims, M., K. S. Harris, and D. Etchison.** 1995. Poliovirus protease 3C mediates cleavage of microtubule-associated protein 4. Virology **211:**451–461.
29. **Kitamura, N., B. L. Semler, P. G. Rothberg, G. R. Larsen, C. J. Adler, A. J.**

Dorner, E. A. Emini, R. Hanecak, J. Lee, S. van der Werf, C. W. Anderson, and E. Wimmer. 1981. Primary structure, gene organization and polypeptide expression of poliovirus RNA. Nature **291:**547–553.
30. **Kuhn, R. J., H. Tada, W. M. Ypma, J. J. Dunn, B. L. Semler, and E. Wimmer.** 1988. Construction of a "mutagenesis cartridge" for poliovirus genome-linked viral protein: isolation and characterization of viable and nonviable mutants. Proc. Natl. Acad. Sci. USA **85:**519–523.
31. **Kuhn, R. J., H. Tada, M. F. Ypma-Wong, B. L. Semler, and E. Wimmer.** 1988. Mutational analysis of the genome-linked protein VPg of poliovirus. J. Virol. **62:**4207–4215.
32. **Lama, J., and L. Carrasco.** 1995. Mutations in the hydrophobic domain of poliovirus protein 3AB abrogate its permeabilizing activity. FEBS Lett. **367:** 5–11.
33. **Lama, J., A. V. Paul, K. S. Harris, and E. Wimmer.** 1994. Properties of purified recombinant poliovirus protein 3AB as substrate for viral proteinases and as co-factor for viral polymerase 3Dpol. J. Biol. Chem. **269:**66–70.
34. **Lawson, M. A., and B. L. Semler.** 1991. Poliovirus thiol proteinase 3C can utilize a serine nucleophile within the putative catalytic triad. Proc. Natl. Acad. Sci. USA **88:**9919–9923.
35. **Lawson, M. A., and B. L. Semler.** 1992. Alternate poliovirus nonstructural protein processing cascades generated by primary sites of 3C proteinase cleavage. Virology **191:**309–320.
36. **McBride, A. E., A. Schlegel, and K. Kirkegaard.** 1996. Human protein Sam68 relocalization and interaction with poliovirus RNA polymerase in infected cells. Proc. Natl. Acad. Sci. USA **93:**2296–2301.
37. **Molla, A., K. S. Harris, A. V. Paul, S. H. Shin, J. Mugavero, and E. Wimmer.** 1994. Stimulation of $3C^{pro}$-related proteolysis by the genome-linked protein, VPg, and its precursor, 3AB. J. Biol. Chem. **269:**27015–27020.
38. **Neufeld, K. L., J. M. Galarza, O. C. Richards, D. F. Summers, and E. Ehrenfeld.** 1994. Identification of terminal adenylyl transferase activity of the poliovirus polymerase $3D^{pol}$. J. Virol. **68:**5811–5818.
39. **Neufeld, K. L., O. C. Richards, and E. Ehrenfeld.** 1991. Purification, characterization, and comparison of poliovirus RNA polymerase from native and recombinant sources. J. Biol. Chem. **266:**24212–24219.
40. **Nicklin, M. J. H., K. S. Harris, P. V. Pallai, and E. Wimmer.** 1988. Poliovirus proteinase 3C: large-scale expression, purification, and specific cleavage activity on natural and synthetic substrates in vitro. J. Virol. **62:**4586–4593.
41. **Nomoto, A., B. Morgan-Detjen, R. Pozzatti, and E. Wimmer.** 1977. The location of the polio genome protein in viral RNAs and its implication for RNA synthesis. Nature **268:**208–213.
42. **Parsley, T. B., J. S. Towner, L. B. Blyn, E. Ehrenfeld, and B. L. Semler.** 1997. Poly (rC) binding protein 2 forms a ternary complex with the 5′-terminal sequences of poliovirus RNA and the viral 3CD proteinase. RNA **3:**1124–1134.
43. **Pata, J. D., S. C. Schultz, and K. Kirkegaard.** 1995. Functional oligomerization of poliovirus RNA-dependent RNA polymerase. RNA **1:**466–477.
44. **Paul, A., X. Cao, K. S. Harris, J. Lama, and E. Wimmer.** 1994. Studies with poliovirus polymerase 3Dpol: stimulation of poly(U) synthesis in vitro by purified poliovirus protein 3AB. J. Biol. Chem. **269:**29173–29181.
45. **Paul, A. V., J. V. Boom, D. Filipov, and E. Wimmer.** 1998. Protein-primed RNA synthesis by purified poliovirus RNA polymerase. Nature, **393:**280–284.
46. **Plotch, S. J., and O. Palant.** 1995. Poliovirus protein 3AB forms a complex with and stimulates the activity of the viral RNA polymerase, $3D^{pol}$. J. Virol. **69:**7169–7179.
47. **Reuer, Q., R. J. Kuhn, and E. Wimmer.** 1990. Characterization of poliovirus clones containing lethal and nonlethal mutations in the genome-linked protein VPg. J. Virol. **64:**2967–2975.
48. **Rueckert, R. R.** 1996. Picornaviridae and their replication, p. 507–548. *In* B. Fields (ed.), Virology. Raven Press, New York, N.Y.
49. **Schultz, S. C., J. L. Hansen, and S. Hobson.** 1996. Three-dimensional structure of the RNA-dependent RNA polymerase of poliovirus. Presented at the Keystone Symposia on Viral Genome Replication, Tamarron, Colo.
50. **Semler, B., C. Anderson, R. Hanecak, L. Dorner, and E. Wimmer.** 1982. A membrane-associated precursor to poliovirus VPg identified by immunoprecipitation with antibodies directed against a synthetic heptapeptide. Cell **28:**405–412.
51. **Takeda, N., R. J. Kuhn, C. F. Yang, T. Takegami, and E. Wimmer.** 1986. Initiation of poliovirus plus-strand RNA synthesis in a membrane complex of infected HeLa cells. J. Virol. **60:**43–53.
52. **Takegami, T., R. J. Kuhn, C. W. Anderson, and E. Wimmer.** 1983. Membrane-dependent uridylylation of the genome-linked protein VPg of poliovirus. Proc. Natl. Acad. Sci. USA **80:**7447–7451.
53. **Takegami, T., B. L. Semler, C. W. Anderson, and E. Wimmer.** 1983. Membrane fractions active in poliovirus RNA replication contain VPg precursor polypeptides. Virology **128:**33–47.
54. **Towner, J. S., T. V. Ho, and B. Semler.** 1996. Determinants of membrane association for poliovirus protein 3AB. J. Biol. Chem. **271:**26810–26818.
55. **Wimmer, E., C. U. T. Hellen, and X. M. Cao.** 1993. Genetics of poliovirus. Annu. Rev. Genet. **27:**353–436.
56. **Xiang, W., A. Cuconati, A. Paul, X. Cao, and E. Wimmer.** 1995. Molecular dissection of the multifunctional poliovirus RNA binding protein 3AB. RNA **1:**892–904.
57. **Xiang, W., K. S. Harris, L. Alexander, and E. Wimmer.** 1995. Interaction between the 5′-terminal cloverleaf and $3AB/3CD^{pro}$ of poliovirus is essential for RNA replication. J. Virol. **69:**3658–3667.
57a. **Yin, J., and E. Wimmer.** Unpublished data.
58. **Zervos, A. S., J. Gyuris, and R. Brent.** 1993. Mxi1, a protein that specifically interacts with Max to bind Myc-Max recognition sites. Cell **72:**223–232.