Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 518–522, January 1990
Biochemistry

DNA amplification-restricted transcription–translation: Rapid analysis of rhesus rotavirus neutralization sites

(polymerase chain reaction/monoclonal antibody/peptide binding/protein domain mapping)

ERICH R. MACKOW†‡§¶, MARK Y. YAMANAKA§, MINH N. DANG§, AND HARRY B. GREENBERG†§‖

Departments of ‖Medicine, †Microbiology, and ‡Immunology, Stanford University, Stanford, CA 94305; and §Palo Alto Veterans Administration Medical Center, Palo Alto, CA 94304

Communicated by Stanley Falkow, October 30, 1989

ABSTRACT **DNA amplification-restricted transcription–translation (DARTT), is based on DNA amplification by the polymerase chain reaction (PCR) and uses PCR to truncate protein-encoding DNA while adding transcriptional and translational initiation signals to the segment. The amplified DNA segments are transcribed into RNA and translated into protein *in vitro* and the synthesized proteins are used to define functional sites. DARTT was applied to rhesus rotavirus gene segment 4 cDNA in order to create a series of carboxyl-terminal truncations and new amino termini in the encoded VP4 capsid protein. The truncated VP4 polypeptides were tested for reaction with 11 VP4-specific neutralizing monoclonal antibodies to identify the minimum polypeptides required for antibody recognition. Monoclonal antibodies 2G4, M2, and M7, which neutralize a number of serologically distinct rotaviruses, required amino acids 247–474 of VP4 for binding. DARTT is potentially applicable to the identification of discontinuous epitopes and functional domains on a variety of proteins.**

Rotavirus infection is a leading cause of infant mortality in developing countries and an important cause of acute gastroenteritis in young children in the United States (1–3). Rotaviruses contain 11 double-stranded RNA segments inside a double-capsid icosahedral shell. Natural rotavirus infections result in the production of neutralizing antibodies to the outer capsid proteins, VP4 and VP7 (1–3). VP4 is an 86.5-kDa, nonglycosylated protein encoded by gene segment 4 of rhesus rotavirus (RRV) (4). VP4 is cleaved by trypsin to an amino-terminal fragment, VP8 (amino acids 1–246), and a carboxyl-terminal fragment, VP5 (amino acids 247–776), and this cleavage is associated with enhanced viral infectivity (5). Genetic and biochemical studies indicate that VP4 is associated with viral virulence (6, 7) and cell penetration (8). VP4 is responsible for binding the erythrocyte protein glycophorin (9) and is the viral hemagglutinin (10). Antibodies directed at VP4 inhibit viral hemagglutination, neutralize the virus *in vitro* (11), and passively protect mice against rotavirus challenge *in vivo* (12). VP4 also effectively induces protective immunity in animals and is immunogenic in young children (13–16).

In previous studies of monoclonal antibody (mAb) "escape" mutants, we have identified point mutations in VP4 that appeared to be involved in viral neutralization (4, 17). However, this analysis did not determine whether neutralizing antibodies actually bound to these regions or whether the identified mutations elicited conformational changes at a distant site. In order to directly define the minimal VP4 polypeptides that are bound by selected neutralizing antibodies, a broadly applicable method for synthesizing a series of truncated polypeptides has been devised. The procedure is called DNA amplification-restricted transcription–translation (DARTT) and is based on the polymerase chain reaction (PCR) (18–20). We have applied DARTT analysis to the coding region of gene segment 4 of RRV and determined the minimal VP4 polypeptide required for neutralizing mAb recognition.

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METHODS

Amplification, Transcription, and Translation. PCR, RNA transcription, and *in vitro* translation reactions were performed as described (16–18, 21, 22). Briefly, the PCR reaction scheme was as follows: denaturation at 94°C, 1 min; annealing at 42 or 50°C, depending on primer specificity, 2 min; DNA polymerization at 72°C by *Thermus aquaticus* (*Taq*) DNA polymerase, 3 min. The reaction was repeated for 30 cycles with the DNA polymerization time being extended 10 sec with each successive cycle. To complete DNA strands and remove primers competitive for RNA polymerase binding, T4 DNA polymerase was added at the conclusion of each reaction for 30 min at 37°C.

After phenol extraction and ethanol precipitation, amplified DNA (0.1–1 μg) was added to a 100-μl transcription reaction mixture containing 50 mM NaCl; 40 mM Tris·HCl (pH 8.0), 8 mM $MgCl_2$; 2 mM spermidine; 15 mM dithiothreitol; 200 μM each rATP, rGTP, rCTP, and rUTP; 80 units of RNasin (Promega), and 50 units of phage T3 RNA polymerase. After 1 hr at 37°C the RNA was phenol/chloroform-extracted and ethanol-precipitated. Decreasing the amount of RNA polymerase in the reaction was found to decrease the amount of complete 2.4-kilobase (kb) RNA transcripts. Approximately 20 μg of plus-stranded RNA was obtained from this reaction and was ready to use in *in vitro* translation reactions.

Translations were performed with 1–5 μg of the synthesized run-off transcript RNA in addition to 15–100 μCi (1 μCi = 37 kBq) of [^{35}S]methionine, 10–30 μl of rabbit reticulocyte lysate, and 1 unit of RNasin per μl for 30–60 min at 30°C. Translation mixtures were diluted directly in 0.1–1.0 ml of RIPA buffer [0.3 M NaCl/0.1 M Tris·HCl, pH 7.5/1% (wt/vol) sodium deoxycholate/1% (vol/vol) Triton X-100/1 mM phenylmethylsulfonyl fluoride] in the presence or absence of 0.1% sodium dodecyl sulfate (SDS). Immunoprecipitations were performed using previously characterized neutralizing mAbs (23) and *Staphylococcus aureus* protein A-agarose beads.

Oligonucleotide Primers. Primers were synthesized on an Applied Biosystems 380A DNA synthesizer. The designation, location of sequence in RRV gene 4 (2), and orientation

Abbreviations: DARTT, DNA amplification-restricted transcription–translation; mAb, monoclonal antibody; PCR, polymerase chain reaction; RRV, rhesus rotavirus.

¶To whom reprint requests should be addressed at: Palo Alto VA Medical Center, Building 1, (111) GI, 3801 Miranda Avenue, Palo Alto, CA 94304.

(P, positive or coding strand; N, negative or noncoding strand) are listed here: 4R33, 172–189, N; 4R37, 349–366, N; 4R7, 508–525, N; 4R570N, 553–570, N; 4R621N, 604–621, N; 4R672N, 655–672, N; 4R40, 673–690, N; 4R2, 794–811, N; 4R38, 1022–1042, N; 4R32, 1221–1238, N; 4R1299N, 1282–1299, N; 4R1359N, 1342–1359, N; 4R1398N, 1381–1398, N; 4R1431N, 1414–1431, N; 4R27, 1440–1457, N; 4R28, 1564–1585, N; 4R24, 1759–1776, N; 4R22, 1945–1962, N; 4R19, 2134–2151, N; 4R30, 2342–2362, N; 4RT3163*, 163–177, P; 4RT3211*, 211–225, P; 4RT3367*, 367–381, P; 4RT3526*, 526–540, P; 4RT3VP5*, 751–765, P; 4RT3853*, 853–867, P; 4RT3997*, 997–1011, P; 4RT31222*, 1222–1236, P; 4RT31300*, 1300–1314, P; an asterisk indicates that the T3 RNA polymerase transcription initiation signal and an in-frame ATG translation initiation site have been synthesized at the 5′ end of the oligonucleotide. The added T3–ATG signals used were GGTACCGAATTAACCCTCACTAAAGGGATG or ATTAACCCTCACTAAAGATG. Additional bases at the 5′ end of the first T3 transcriptional site were added to avoid potential digestion of the T3 RNA polymerase recognition site by the 5′ → 3′ exonuclease activity associated with *Taq* DNA polymerase. However, no noticeable translational product differences were attributable to using the shorter sequence in the PCR.

RESULTS

DARTT requires adding transcriptional and, in some cases, translational signals to the DNA being amplified. Initiation signals can be included in amplified DNA products if a plasmid, or an oligonucleotide contains a T3, T7, or SP6 DNA-dependent RNA polymerase initiation site adjacent to a cloned DNA's translation initiation codon, ATG (Fig. 1*A*). PCR amplification with a vector-derived primer and an opposing cDNA insert-derived primer results in the synthesis of a truncated DNA that can subsequently be transcribed and translated. Applying this strategy to a number of negative-sense insert primers results in the synthesis of discrete polypeptides containing a native amino terminus and a series of new carboxyl termini (Fig. 1*A*).

A complementary second method for truncating the polypeptides entails adding internal transcriptional and translational start signals to the DNA segment (Fig. 1*B*). This is accomplished by synthesizing a 5′-end primer that contains the T3 RNA polymerase transcriptional start signal and adds a new ATG initiation codon in-frame to the protein coding regions of the DNA (Fig. 1*B*). Amplifying between this T3–ATG primer and a negative-sense downstream primer (18, 19) results in 5′-end-restricted fragments that can be transcribed into RNA and translated into protein. Since the latter procedure truncates the protein product from the amino terminus and the former technique produces truncations from the carboxyl terminus, virtually any polypeptide can be synthesized by combining the two PCR protocols.

Fig. 2 illustrates a series of amplified DNAs produced by using primers that create 3′-end truncations of the RRV gene 4 cDNA. A 2355-base-pair clone of the RRV gene 4 in pBluescript KS (Stratagene), 4EKS35, was used in the amplifications. 4EKS35 (0.1–1 μg) and 100 ng of each 5′-end and 3′-end primer were included in 30 cycles of DNA amplifica-

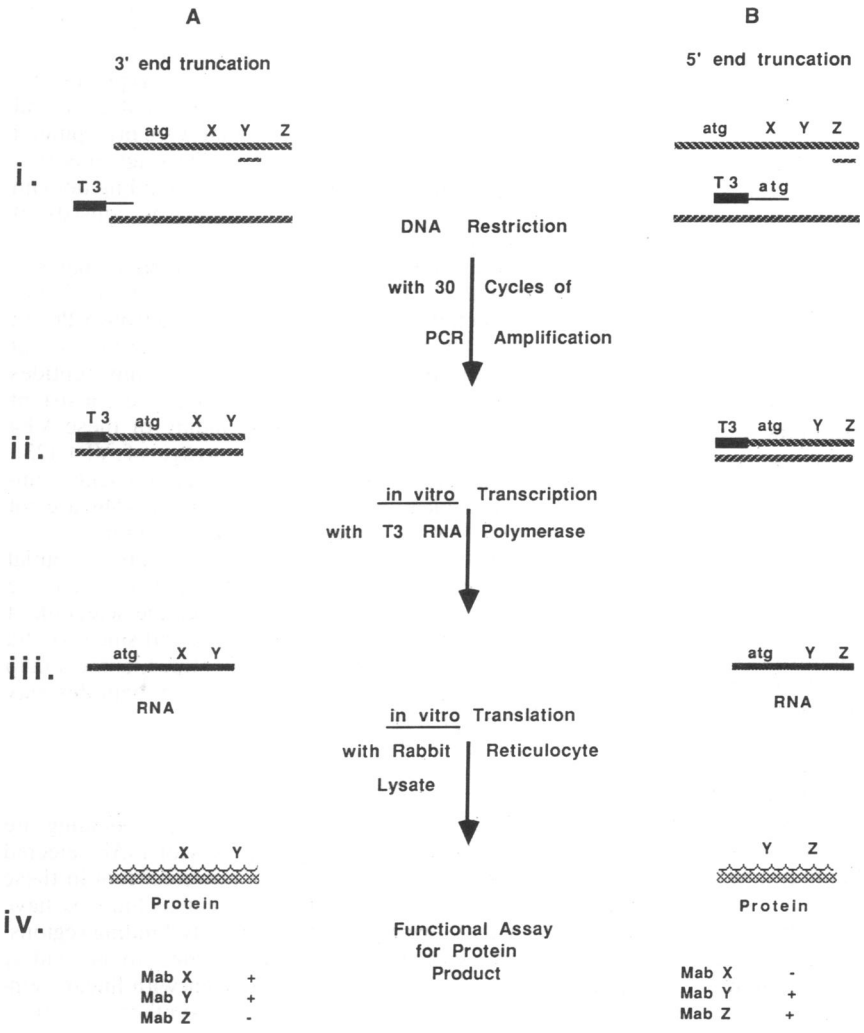

FIG. 1. DARTT strategies for protein structure–function analysis. (*i*) DNA encoding a single protein is depicted by two bars with 5′ → 3′ orientation specified by diagonals. X, Y, and Z represent functional regions of the encoded polypeptide and are preceded by a translational initiation codon (atg). Primers that delimit the region to be amplified are shown to anneal to their complementary DNA strands. In *A*, the 3′-end limiting primer anneals at an internal position in the gene, and the 5′-end primer contains a noncomplementary T3 polymerase transcription initiation site (solid bar) upstream from a complementary sequence (thin line) and utilizes the native translational start site of the protein. In *B*, the primer pair includes the 3′ end of the gene but the upstream primer contains a noncomplementary T3 polymerase transcription initiation site and an ATG translational initiation site, the latter being in-frame to the adjacent complementary primer sequence. (*ii*) PCR amplification between the primer pairs results in DNA that is restricted at the 3′ end (*A*) or the 5′ end (*B*) and that includes both transcriptional and translational elements. (*iii*) T3 RNA polymerase is used to synthesize plus-stranded RNA *in vitro* that is specific to the DNA fragment. The transcribed DNA contains native (*A*) or new (*B*) translational initiation signals. (*iv*) RNA is translated into the encoded polypeptide in rabbit reticulocyte lysates *in vitro*. The polypeptide product is then assayed for the presence of retained functions (antigenicity, binding, catalysis, etc.) that exist in the full-length protein.

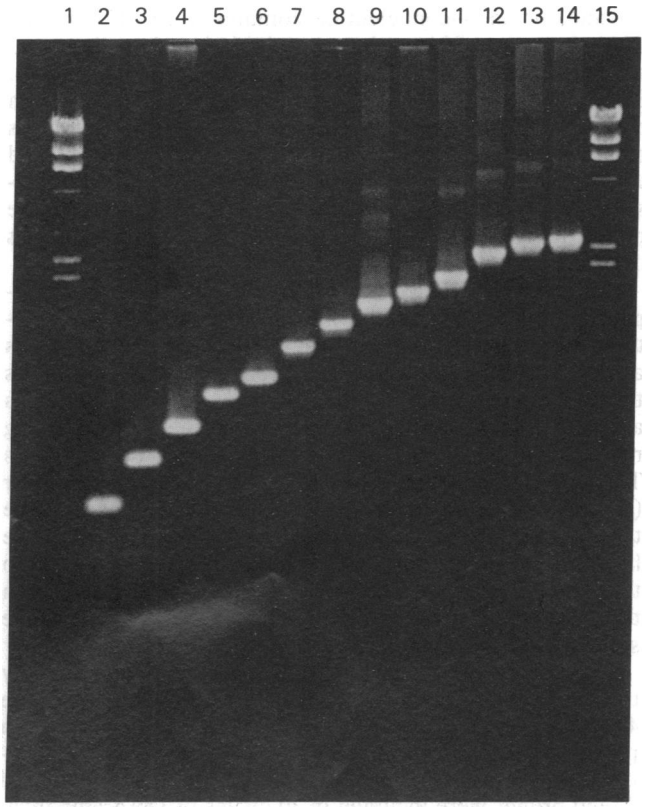

FIG. 2. Representative amplified DNAs from oligonucleotide pairs resulting in a series of 3′-truncated PCR products. DNAs were amplified for 30 cycles (18, 20) and 5% of the total DNA was electrophoresed in a 1% agarose gel, stained with ethidium bromide, and visualized by ultraviolet light. A pBR322-derived primer, PC18M, and the indicated gene 4-specific primer were used for amplifying DNAs. Amplified DNAs were sized as indicated. For lanes 2–14, primer designation (see *Methods*) and base *x–y* of RRV gene 4 sequence (2) were as follows: lane 2, 4R33, 1–189; lane 3, 4R37, 1–366; lane 4, 4R7, 1–525; lane 5, 4R40, 1–690; lane 6, 4R2, 1–811; lane 7, 4R38, 1–1042; lane 8, 4R32, 1–1238; lane 9, 4R27, 1–1457; lane 10, 4R28, 1–1585; lane 11, 4R24, 1–1776; lane 12, 4R22, 1–1962; lane 13, 4R19, 1–2151; lane 14, 4R30, 1–2362. Lanes 1 and 15, λ DNA digested with *Hin*dIII as markers. Each amplified product contains an extra 130 base pairs of vector-derived DNA at the 5′ end resulting from amplification with the PC18M primer (CACACAGGAAACAGCTAT).

tion (16–19). Amplified DNAs representing 1/20th of the total were electrophoresed in a 1% agarose gel and stained with ethidium bromide to assay the PCR reaction (Fig. 2). Each amplification-restricted DNA contains transcriptional and translational initiation signals that were used in subsequent DARTT steps.

Prior to utilizing DARTT, deletions can be made without PCR by using unique restriction sites in the coding sequence that create 3′-end truncations. Run-off RNA transcripts can then be synthesized directly from plasmid-derived RNA polymerase signals and these transcripts can be translated *in vitro*.

Fig. 3*A* illustrates the total translation products derived by DARTT from the same series of amplified DNAs that appear in Fig. 2. Each translated polypeptide was immunoprecipitated by hyperimmune anti-RRV guinea pig serum and screened for the presence of full-length translation products (Fig. 3*B*). All of the DARTT-synthesized peptides were recognized by the hyperimmune serum, except for the shortest peptide (amino acids 1–60). Incomplete translation products seen in Figs. 3 and 4 presumably resulted from premature transcriptional or translational termination, or from RNA or protein degradation, and migrated faster than the full-length VP4 or the VP4 truncations.

The series of nested polypeptides were also studied by immunoprecipitation using a series of 11 previously characterized neutralizing mAbs (Table 1). An example of this form of analysis is seen in Fig. 4. mAbs M2, M7, and 2G4 were previously shown by serologic and variant competition studies to be directed at a single neutralization domain on VP4 (23). This epitope was classified as a crossreactive or heterotypic neutralization domain, since each of these mAbs neutralizes a group of serotypically diverse rotaviruses. Sequence analysis of neutralization-escape mutants produced with the M2, M7, and 2G4 mAbs localized the heterotypic neutralization epitope to the region of amino acid 392 in VP5 (4). We attempted to locate the functional peptide comprising the heterotypic neutralizing epitope on VP4 by determining the minimal essential polypeptide that could be immunoprecipitated by the neutralizing mAbs M2, M7, and 2G4. Immunoprecipitation revealed that M2, M7, and 2G4 reacted with the full-length VP4 protein and with truncated polypeptides including VP4 amino acids 1–474 but not with VP4-(1–450), a peptide extending from amino acid 1 to 450 (Table 1). Further analysis demonstrated that VP4-(247–474) was efficiently precipitated (Fig. 4, lanes 6–8) but that VP4-(247–450) (lanes 1–3) and VP4-(282–474) did not react with the mAbs (Table 1). Hence it appears that these heterotypic, VP4-specific mAbs all require a minimum polypeptide of ≈228 amino acids (amino acids 247–474) of VP5 for binding and that deletions at either terminus abolish recognition by these neutralizing mAbs.

Table 1 summarizes the results of immunoprecipitation studies using a series of DARTT-generated proteins to localize the remaining neutralizing mAb-binding domains of VP4. In addition to the heterotypic neutralizing mAbs, we analyzed the binding domain of eight largely serotype-specific neutralizing mAbs (M11, A1, A15, 1A9, 5D9, M14, 5C4, and 7A12). Table 1 lists the VP4 fragments that were precipitated by these VP8-specific mAbs as well as the fragments that were not recognized. The minimal essential binding domain for all of the VP8-specific neutralizing mAbs consists of amino acids 55–222 of VP4.

As might have been expected from mAb variant analysis, the eight mAbs that selected mutations in the VP8 region of VP4 immunoprecipitated polypeptides derived from VP8 but not ones derived from VP5 (Table 1). The three mAbs that selected mutations in VP5 immunoprecipitated only peptides that were derived from VP5. These findings are consistent with previous observations that the binding of these VP4 mAbs is unaffected by proteolytic cleavage of VP4 (23). Current findings further establish that the amino acids comprising the trypsin cleavage site (amino acids 231–246) are not present in the minimum peptide required for binding.

Although our studies have characterized minimal essential peptides required for binding of neutralizing mAbs, these findings must be taken as provisional. Since the selection of the primers used in the PCR was arbitrary and since we did not exhaustively generate nested polypeptides, some amino acids at the boundaries of the listed minimum peptides may not be required for mAb binding (Table 1).

DISCUSSION

Various strategies have been used to localize the binding site of neutralizing mAbs. Sequence analyses of mAb-selected escape mutants have proven to be a powerful tool in these studies (4, 17, 24–27). Other biochemical techniques have also been used to directly identify antibody-binding regions, but these have not yet been demonstrated to be widely applicable (28, 29). When antibodies react with linear, nonconformationally determined epitopes, synthetic peptides

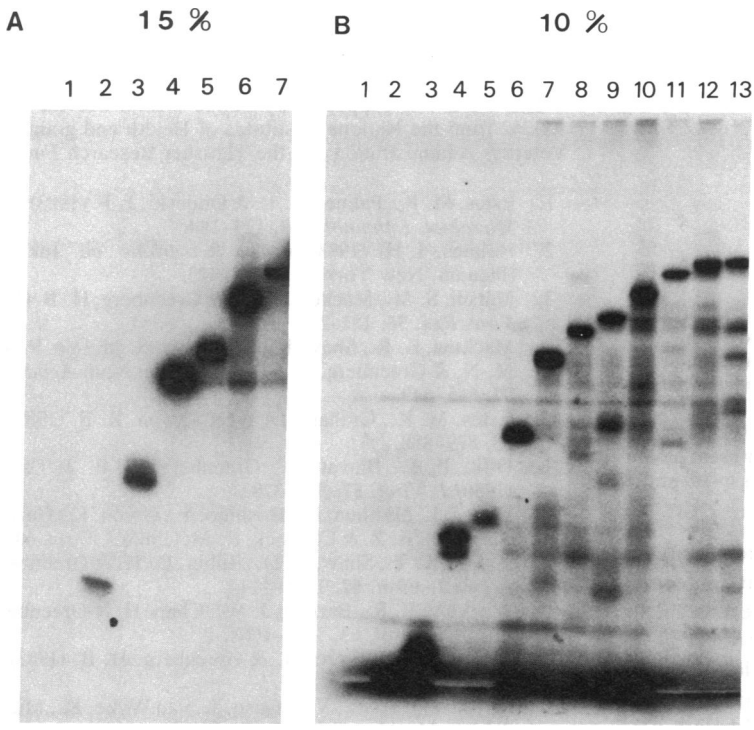

FIG. 3. DARTT-generated translation products. (*A*) Amplified DNA products (assayed in Fig. 2) were transcribed into RNA by T3 RNA polymerase and the plus-stranded RNAs were translated into protein by rabbit reticulocyte lysate with [^{35}S]methionine (21, 22) and directly assayed by SDS/15% PAGE followed by autoradiography. (*B*) Protein products were immunoprecipitated with hyperimmune anti-RRV antiserum and analyzed by SDS/10% PAGE to size the resulting polypeptides and to assay for incomplete or degraded protein products. The VP4 amino acids contained in each polypeptide (as predicted from the primer pair used in DARTT) were as follows: lanes 1, 1–60 (no product was immunoprecipitated); lanes 2, 1–119; lanes 3, 1–172; lanes 4, 1–227; lanes 5, 1–267; lanes 6, 1–344; lanes 7, 1–409; lane 8, 1–482; lane 9, 1–525; lane 10, 1–589; lane 11, 1–651; lane 12, 1–714; lane 13, 1–776.

are useful in localizing binding regions (30). If synthetic peptide analysis is feasible, it is possible to initially apply DARTT to identify and localize appropriate regions for later peptide synthesis. However, in many cases, neutralizing antibody-binding domains appear to be produced by complex protein structures, an in these instances short synthetic peptides may not be able to mimic the binding site. DARTT, on the other hand, provides a means of creating a broad array of protein products in order to localize an antibody-binding region of any potential size.

In the present study we have used DARTT to rapidly and efficiently analyze a library of nested polypeptides for direct antibody binding. The essential binding structures for antibodies directed toward both VP8 and VP5 are relatively large. This implies that some secondary or tertiary structure is necessary for binding. There are two cysteine residues present in each minimal peptide that potentially are involved in the formation of secondary structures through intramolecular disulfide bonds. However, the mAb–polypeptide interaction is unaffected by reduction with 10 mM dithiothreitol. In all cases, the mutations identified in variants selected by the neutralizing mAbs occurred within the polypeptide identified by DARTT analysis of VP4 (Table 1). The position of selected mutations did vary widely (amino acid 87 for M11 to amino acid 188 for 7A12) (Table 1), but these mAbs still require the same large polypeptide for binding (amino acids 55–222). However, Shaw *et al.* (16) demonstrated that each of these VP8 neutralizing mAbs competes with the others for VP4 binding, suggesting that these mAbs bind to adjacent domains on the VP4 protein. When the mAb competition, variant selection analysis, and the minimum polypeptide binding domains are considered together, it can be further

Table 1. Summary of immunoprecipitation of VP4 polypeptides with neutralizing mAbs

VP4-specific neutralizing mAbs	Variant selected amino acid change	Minimum VP4 peptide required for immunoprecipitation	VP4 peptides immunoprecipitated		VP4 peptides not immunoprecipitated	
Serotype-specific						
M11	87	55–222	55–222	1–525		
A1	88		1–222	1–589	67–222	1–187
A15	89		1–227	1–645	119–222	1–172
1A9	100		1–267	1–651	172–222	1–119
5D9	114		1–344	1–661	247–776	67–776
M14	148		1–394	1–709	247–474	119–776
5C4	150		1–451	1–714	247–394	172–776
7A12	188		1–474	1–776		
			1–482			
						247–451
				1–474	1–451	247–409
Heterotypic			247–474	1–482	1–409	247–344
M2	388	247–474	247–525	1–525	1–394	282–474
M7	393		247–589	1–589	1–344	329–474
2G4	393		247–651	1–645	1–267	404–474
			247–714	1–651	1–253	282–776
			247–776	1–661	1–222	329–776
				1–709		404–776
				1–776		430–776

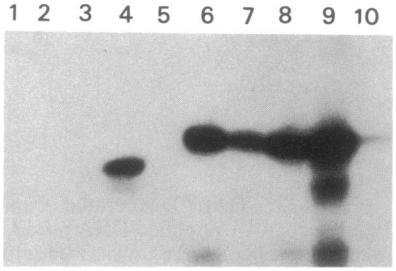

FIG. 4. Recognition of DARTT-generated proteins by neutralizing antibodies. Shown is an autoradiogram of immunoprecipitated DARTT proteins separated by SDS/15% PAGE. Immunoprecipitations were performed using VP4-specific neutralizing mAbs M2 (lanes 1 and 6), M7 (lanes 2 and 7), and 2G4 (lanes 3 and 8), or hyperimmune anti-RRV serum (lanes 4 and 9), or VP7-specific mAb 60 (lanes 5 and 10). The polypeptides precipitated represent amino acids 247–450 (lanes 1–5) or amino acids 247–474 (lanes 6–10) of the VP4 protein.

suggested that the amino acids defined by selected mutations (amino acids 88–188) could actually be present at conformationally adjacent positions of the VP8 polypeptide. Our observations suggest that synthetic peptides or short polypeptides of VP4 do not contain these neutralization epitopes and, therefore, may not be effective in stimulating neutralizing antibody. Conversely, expression of the full-length VP4 (9), the VP5 polypeptide 247–474, or the VP8 polypeptide 55–222 has been demonstrated to maintain these epitopes and the immunogenicity of these peptides should be investigated. It is likely that DARTT technology can also be used to investigate structure–function relationships for additional VP4 roles (hemagglutination and glycophorin binding, viral activation, and potential functions in fusion and cell attachment).

The major limitations of DARTT are the rather small quantities of protein produced by *in vitro* translation and the absence of posttranslational protein modifications. In addition, the truncated peptides studied here could potentially mask some epitopes through context-dependent secondary and tertiary protein conformations. However, we detected no evidence of masked binding sites, since more substantial polypeptide truncations from either end of VP4 failed to reveal additional reactive peptides (Table 1).

It is likely that DARTT can be used to investigate structure–function relationships for a variety of proteins other than VP4. The utility of DARTT for a particular protein product can be determined rapidly, with or without sequence information, by using plasmid-derived primer pairs, a cDNA clone, and a functional assay for the protein. Essentially any of the established PCR techniques can be extended to DARTT in order to study encoded proteins. This includes starting from RNA and reverse-transcribing the first cDNA strand or amplifying directly from cDNA libraries or genomic DNA. DARTT may also be valuable in screening for mAbs to selected polypeptide regions, or for detecting ligand-binding or catalytic domains, or in studies of folding or oligomerization of proteins. DARTT could potentially be used to analyze protein polymorphism and diversity among individual genes and individuals. If a number of DARTT-synthesized proteins were screened immunologically with a battery of well-characterized mAbs, DARTT could provide an antigenic fingerprint of specific protein determinants directly from amplified DNA precursors.

This work was supported by Public Health Service Grant R22 AI1362 from the National Institutes of Health and grants from the Veterans Administration and the Thrasher Research Fund.

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