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Poliovirus replication proteins: RNA sequence encoding P3-1b and the sites of proteolytic processing

(protein sequence analysis/picornavirus/genome map/proteinase)

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ABSTRACT **A partial amino-terminal amino acid sequence of each of the major proteins encoded by the replicase region (P3) of the poliovirus genome has been determined. A comparison of this sequence information with the amino acid sequence predicted from the RNA sequence that has been determined for the 3′ region of the poliovirus genome has allowed us to locate precisely the proteolytic cleavage sites at which the initial polyprotein is processed to create the poliovirus products P3-1b (NCVP1b), P3-2 (NCVP2), P3-4b (NCVP4b), and P3-7c (NCVP7c). For each of these products, as well as for the small genome-linked protein VPg, proteolytic cleavage occurs between a glutamine and a glycine residue to create the amino terminus of each protein. This result suggests that a single proteinase may be responsible for all of these cleavages. The sequence data also allow the precise positioning of the genome-linked protein VPg within the precursor P3-1b just proximal to the amino terminus of polypeptide P3-2.**

Picornavirus genetic information is expressed as a single translation unit (1–3) that, were it not processed by proteolytic cleavage into individual proteins, would be, in the case of poliovirus, a single 240,000 M_r polyprotein corresponding to about 90% of the *ca.* 7500-nucleotide sequence of the genome. This polyprotein is organized into three domains, each of which gives rise to a different group of virus proteins (for recent reviews, see refs. 4 and 5). Initial proteolytic cleavage of the poliovirus polyprotein produces three precursor polypeptides, P1-1a (NCVP1a), P2-3b (NCVP3b), and P3-1b (NCVP1b) that correspond to these three domains. Protein P1-1a (95,000 M_r) corresponds to the 5′ portion of the genomic RNA (plus strand polarity), and it is the large precursor to the four capsid proteins (VPI, VP2, VP3, and VP4). Protein P2-3b (65,000 M_r) corresponds to the central portion of the genome (6), and it is thought to be the precursor to protein P2-X (NCVPX). Protein P2-X is reported to have proteinase activity (7). The third primary cleavage product, P3-1b, is the large precursor (84,000 M_r) to a group of noncapsid proteins and maps in the 3′ region of the genome (8), where replicase functions map. This precursor is cleaved during the poliovirus infection to yield, among other proteins, the virus-specific RNA polymerase that, as a highly purified protein, has been shown to elongate, but not to initiate, poliovirus RNA chains *in vitro* (9). We shall refer to the virus-specific RNA polymerase polypeptide protein as P3-4b (58,000 M_r). The designation 4b was given to this product by Etchison and Ehrenfeld (10). The polymerase has also been called NCVP4 and p63 (11, 9).

The mechanism of initiation of poliovirus RNA synthesis and the specific protein(s) responsible for this activity are at present unknown. Because newly initiated RNA chains isolated from infected cells all contain the small protein VPg covalently linked to their 5′ termini (12, 13), it has been postulated that VPg (or a precursor) may serve as a primer for initiation of RNA synthesis (12, 14). Although several partially purified extracts of poliovirus-infected cells supplemented with exogenous poliovirus RNA will initiate the synthesis of new RNA chains *in vitro* (15, 16), to date, none of these have been shown to attach VPg to replicase/polymerase products.

In order to determine which virus-specific proteins are involved in the initiation of RNA synthesis, we have begun to study the primary structure of the poliovirus replicase precursor-polypeptide and the cleavage products generated from it. By comparing the data obtained from a limited sequential Edman degradation of these products with the sequence data now available from the total nucleotide sequence of poliovirus RNA (unpublished; ref. 17), we have determined the cleavage sites for the replicase proteins, and we can predict their amino acid sequence. The data we present in this communication demonstrate the relationship among the P3-1b-derived products—VPg (17, 18), P3-2, P3-4b, and P3-7c—and suggest that a single proteolytic activity, presumed to be of viral origin, may function to process all of these proteins.

MATERIALS AND METHODS

Preparation of Infected-Cell Extracts and Gel Electrophoresis. Extracts were prepared from suspension cultures of S3 HeLa cells (5×10^6 cells per ml) infected with poliovirus type I (Mahoney) at a multiplicity of 50 plaque-forming units per cell. Infected cultures at 37°C were labeled with radioactive amino acids (Amersham) for 1–2 hr beginning 3 hr after infection. When labeling of large, uncleaved precursor proteins was desired, $ZnCl_2$ was added to the infected culture (final concentration 0.8 mM) 15 min prior to the addition of the radioactive amino acid (19) and was maintained at the same concentration throughout the labeling period. After the labeling period, cells were harvested immediately on ice, washed once in ice-cold phosphate-buffered saline, resuspended in a small volume (0.5–1.0 ml) of protein gel sample buffer (20), heated to 100°C for 2 min, and electrophoresed (20). Analytical gels were subjected to fluorography (21). Preparative gel bands were visualized by direct autoradiography.

Elution of Purified Proteins. Gel strips containing individual proteins were excised from dried gels and the proteins were eluted by using an Isco electrophoretic sample concentrator (22) operated at 3 W constant power for 3–4 hr. The chamber buffer was 50 mM NH_4HCO_3/0.02% sodium dodecyl sulfate and the

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sample cup buffer was 10 mM NH_4HCO_3/0.02% sodium dodecyl sulfate. Carrier myoglobin (100 μg) was added to the sample cup just prior to protein elution.

Amino Acid Sequence Analysis. Radioactive polypeptides were applied to the spinning cup of the Beckman 890C protein sequencer directly in the electrophoretic elution buffer and the sample was dried, using the Beckman sample application subroutine. Apomyoglobin (2 mg) was applied to the sample cup in a separate application to serve as a carrier protein and as an internal standard. Sequence analysis was essentially as described (23), except that a 0.1 M Quadrol buffer program (Beckman no. 030176) was used for all samples, and phenylisothiocyanate was omitted during the first sequencing cycle (residue 0).

Nucleotide Sequence Analysis. The nucleotide sequence was determined by using a modification (17, 24) of the dideoxynucleotide method (25).

RESULTS

Analysis of Viral Proteins from Infected-Cell Extracts and Replicase. Previous studies (6, 8) have shown that the protein(s) comprising the virus-specific RNA polymerase are cleaved from a large precursor polypeptide, P3-1b. Because this protein is rapidly cleaved in infected cells, $ZnCl_2$ was added to the infected cultures to inhibit cleavage (19) and to allow the accumulation of enough radioactive P3-1b for purification and protein sequence analysis. The polypeptide composition of a typical lysate prepared with $ZnCl_2$ is shown in Fig. 1, lane A. Note the predominance of the primary cleavage products, P1-1a and P3-1b, as well as a protein we have tentatively identified as P2-3b (6) because of its apparent molecular weight (65,000) and a preliminary amino acid sequence analysis (unpublished data).

In order to obtain sufficient quantities of the radioactively labeled P3-1b cleavage products P3-2, P3-4b, and P3-7c for purification and amino acid sequence analysis, $ZnCl_2$ treatment was omitted and the labeling time was increased to 2 hr. The protein pattern resulting from this labeling regime is shown in Fig. 1, lane B. The cleavage products of P3-1b can be seen as can additional products cleaved from other precursors (e. g., VP1 and VP3 cleaved from P1-1a).

There have been a number of reports that the P3-1b cleavage products (P3-2, P3-4b, and P3-7c) are a part of the viral replicase (11, 15, 16, 26, 27). To ensure that our identification of intracellular proteins as "replicase" proteins was correct, a replicase fraction was prepared and partially purified over a phosphocellulose column according to Dasgupta *et al.* (15). The peak fractions from the column were capable of initiating RNA synthesis on exogenously added poliovirus RNA and had a low level of endogenous RNA synthesis activity (unplublished data). Fig. 1, lane C, shows the [^{35}S]methionine-labeled polypeptides found in this phosphocellulose-purified replicase fraction. The major cleavage products of P3-1b (P3-2, P3-4b, and P3-7c) are found in this replicase fraction, along with a few other virus-specific proteins (e.g., P2-X).

Amino Acid Sequence Analysis of Replicase Proteins. Our strategy for locating the genomic region that encodes each of the replicase region products was similar to that employed by Kitamura *et al.* (17). A partial amino acid sequence for each protein was obtained and then the RNA sequence of the genome was searched for regions that could encode these amino acids. This RNA sequence of 7433 nucleotides has only one open reading frame beginning within 800 nucleotides of its 5′ terminus

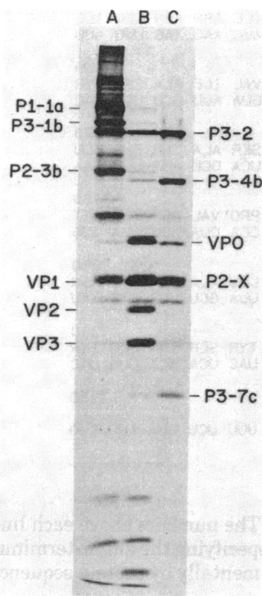

FIG. 1. Autoradiograph of a 12.5% polyacrylamide gel of intracellular poliovirus-specific proteins. Lane A, poliovirus-infected HeLa cells were labeled in the presence of 0.8 mM $ZnCl_2$ for 1 hr beginning 3 hr after infection. The concentration of the radioactive amino acid ([^{3}H]lysine) was 400 μCi/ml (1 Ci = 3.7×10^{10} becquerels). After labeling, cells were harvested and resuspended in gel sample buffer (20). Lane B, poliovirus-infected HeLa cells labeled as in A except that the $ZnCl_2$ was omitted and the labeling time was increased to 2 hr. Lane C, phosphocellulose-purified poliovirus replicase fraction. Sample was prepared and assayed as described (15). Proteins were labeled for 2 hr with [^{35}S]methionine (10 μCi/ml) beginning 2.5 hr after infection.

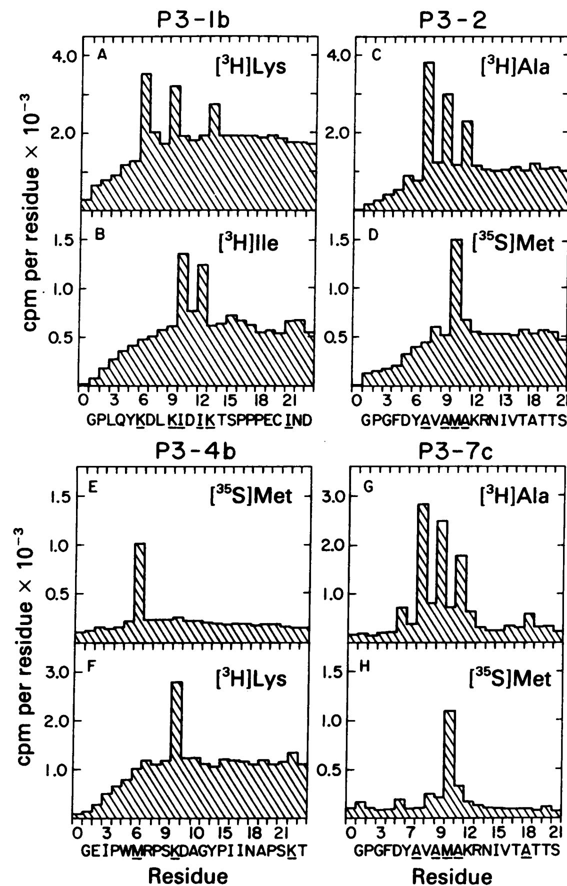

FIG. 2. Partial amino-terminal amino acid sequence determination of poliovirus polypeptides P3-1b, P3-2, P3-7c, and P3-4b. The polyacrylamide gel-purified, radioactively labeled polypeptides were subjected to automated Edman degradation. Individual panels display the radioactivity recovered in the amino acid fraction after each sequence cycle for the indicated polypeptide and radioactive amino acid. A pair of panels is shown for each of the four polypeptides. The predicted amino-terminal amino acid sequence for each of the polypeptides is given at the bottom of each panel pair in the single-letter amino acid code. Amino acids whose positions are considered proven by the presented data are underlined. The amount of radioactivity applied to the sequencer was: P3-1b (*A*) 213,600 cpm of [^{3}H]Lys; (*B*) 115,000 cpm of [^{3}H]Ile; P3-2 (*C*) 313,000 cpm of [^{3}H]Ala; (*D*) 141,000 cpm of [^{35}S]-Met; P3-4b (*E*) 39,400 cpm of [^{35}S]Met; (*F*) 185,000 cpm of [^{3}H]Lys; P3-7c (*G*) 87,000 cpm of [^{3}H]Ala; (*H*) 17,300 cpm of [^{35}S]Met.

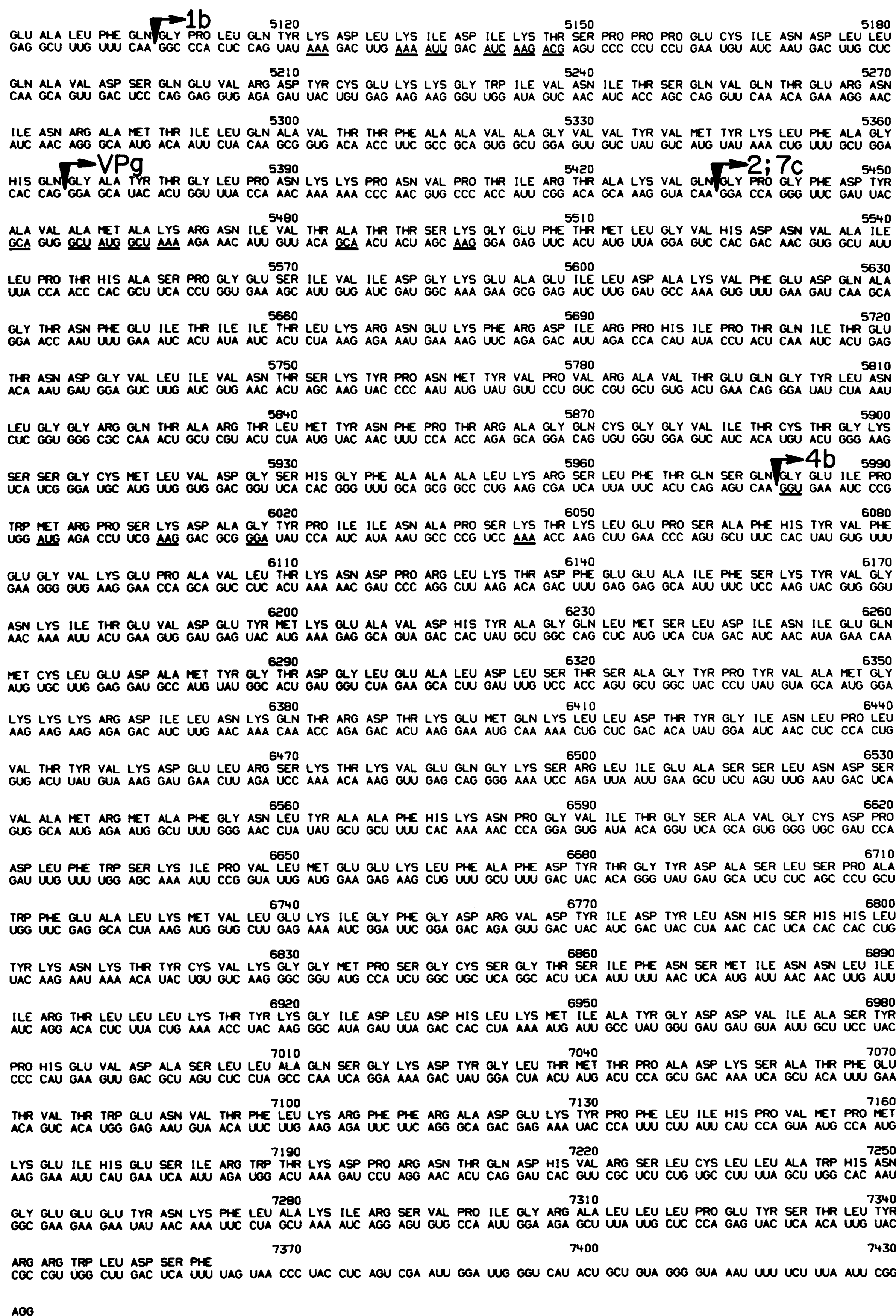

FIG. 3 Nucleotide sequence of the 3′ region of the poliovirus RNA genome and the amino acids encoded therein. The numbers above each line refer to the nucleotide number, starting from the 5′ end of the RNA. The arrows indicate the sites of protein cleavage, specifying the amino terminus of the newly cleaved protein. The underlined codons are those whose amino acid residues have been confirmed experimentally by protein sequence analysis. The 3′-terminal poly(A) has been omitted.

and ending within 200 nucleotides of the 3′ terminus (unpublished data). In the following discussion, we have found it convenient to refer to specific nucleotide locations, as numbered from the 5′ end of the genome. Nucleotide uncertainties in isolated regions of the RNA may necessitate minor changes in nucleotide numbering.

Protein P3-1b samples labeled (in the presence of $ZnCl_2$) separately with [^{3}H]lysine, [^{3}H]isoleucine, [^{3}H]threonine, [^{3}H]alanine, or [^{35}S]methionine were purified by preparative gel electrophoresis and subjected to radiochemical sequence analysis on a Beckman sequencer. Substantial yields of radioactivity (above a background) were obtained for lysine-labeled P3-1b in the amino acid residue from cycles 6, 9, and 13, and from the isoleucine-labeled protein in residues 10 and 12 (Fig. 2 *A* and 2 *B*). In addition, a sequencer run of threonine-labeled P3-1b indicates that residue 14 is a threonine (data not shown). Samples labeled with [^{3}H]alanine and [^{35}S]methionine gave no significant recovery of radioactivity above a background in any of the first 23 sequencer cycles (data not shown). A computer search of the poliovirus genomic sequence found only one region that could code for the peptide -Lys-Xaa-Xaa-Lys-Ile-Xaa-Ile-Lys-Thr- (nucleotides 5121–5147). We therefore conclude that the amino terminus of protein P3-1b is encoded beginning at nucleotide 5106 (Fig. 3).

Radioactively labeled samples of proteins P3-2, P3-4b, and P3-7c were prepared (in the absence of $ZnCl_2$) and analyzed to determine their partial amino-terminal sequence in a manner analogous to that used for P3-1b. Radioactivity from [^{3}H]alanine-labeled P3-2 was found in residues 7, 9, and 11, and [^{35}S]-methionine-labeled P3-2 yielded significant radioactivity only in residue 10 (Fig. 2 *C* and *D*). An analysis of [^{3}H]alanine-labeled P3-7c and [^{35}S]methionine-labeled P3-7c gave a pattern of radioactivity that is almost identical to that obtained for P3-2 (Fig. 2 *G* and *H*, respectively). A computer search of the poliovirus genomic sequence indicates that this pattern is consistent with only one encoding location (Fig. 3). We conclude that P3-2 and P3-7c have the same amino-terminal sequence and that both are encoded beginning at nucleotide 5433. In confirmation of this conclusion, we have found that the 12th residue of P3-2 is lysine (data not shown), and the result shown in Fig. 2G is compatible with the assignment of alanine as the 18th residue of P3-7c. These data also define the size of VPg, the coding sequence of which begins at nucleotide 5360 (17).

The analysis of radioactively labeled protein P3-4b indicates that methionine is the 6th residue (Fig. 2*E*), lysine is the 10th residue (Fig. 2*F*), and glycine is the 1st and 13th residues (data not shown). A computer search of the genome sequence indicates that P3-4b can be encoded only beginning at nucleotide 5979.

DISCUSSION

The nucleotide sequence of the 3′ region of the poliovirus genome is presented in Fig. 3 along with the amino acid sequence predicted by the single open reading frame found in this sequence. The exact locations of the amino terminus of each of the replicase proteins P3-1b (nucleotide 5106), P3-2 and P3-7c (nucleotide 5433), and P3-4b (nucleotide 5979) are indicated in Fig. 3 by arrows. Data reported here and previously (17) allow us to also position the genome-linked protein VPg within this region at a site immediately proximal to the amino-terminal location of P3-2 and P3-7c (Figs. 3 and 4). These data thus confirm and extend the data obtained by tryptic peptide analysis and demonstrate further the similarities in the processing patterns of poliovirus and encephalomyocarditis virus, another picornavirus (18). Note also that while the amino termini of P3-2 and P3-7c are encoded by the same sequence, P3-2 has an apparent molecular weight of 74,000, whereas P3-7c has an apparent molecular weight of only 20,000 (ref. 5; Fig. 1). A consideration of the required coding capacity for P3-7c suggests that the carboxy terminus of P3-7c may be adjacent to the amino terminus of P3-4b. These relationships are schematically illustrated in Fig. 4.

In an earlier report (17) it was noted that the amino terminus of VPg was generated by cleavage of the precursor polypeptide between glutamine and glycine residues (encoded by nucleotides 5364–5369 in Fig. 3). In addition, the possibility was raised that the carboxy terminus of VPg may be generated by another Gln-Gly cleavage (encoded by nucleotides 5429–5435). Note that each of the replicase proteins studied in this report is also predicted to have glycine at its amino terminus, and the predicted preceding amino acid in each case is glutamine. We have shown that the amino terminus of P3-4b is in fact glycine. These findings suggest that there is a common element, cleavage between a glutamine and a glycine residue, to the processing of each of the replicase proteins, including the primary cleavage producing P3-1b itself. The observed cleavage of these replicase proteins at a Gln-Gly pair strengthens the previous suggestion (17) that the glutamine residue encoded by nucleotides 5430–5432 (Fig. 3) is the carboxy terminus of VPg. Such a cleavage would generate a VPg that is 22 amino acids in length and has a molecular weight of 2354.

Inspection of the translated sequence shown in Fig. 3 indicates that the dipeptide sequence Gln-Gly occurs six times. We have provided direct evidence that four of these Gln-Gly pairs are sites for protein processing. Processing may also occur at the remaining Gln-Gly sequences encoded by nucleotides 5796–5801 and 6486–6491. Etchison and Ehrenfeld (10) have recently reported finding an unstable precursor to P3-4b, which they have designated 4a. This precursor accumulates in the presence of the proteinase inhibitor iodoacetamide. A consideration of the molecular weight reported for protein 4a (65,000) suggests that it could be produced by cleavage from a larger precursor at the Gln-Gly sequence encoded by nucleotides 5796–5801. Likewise Rueckert *et al.* (6) and K. Wiegers and R. Dernick (personal communication) have described alternative

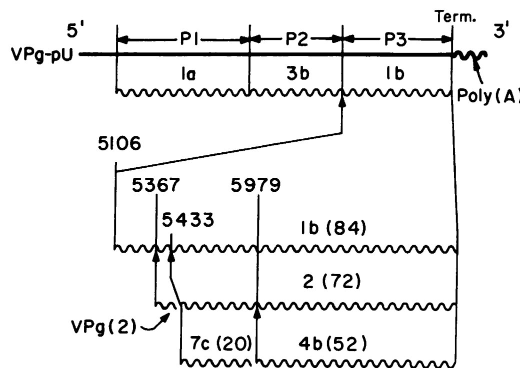

FIG. 4. Processing scheme for poliovirus protein P3-1b and its cleavage products. The numbers (starting from the 5′ end of the RNA) refer to the first nucleotide of the codon specifying the amino-terminal glycine residue for the proteolytically processed proteins described in the text. P1, P2, and P3 are the three regions of the genome represented by the three primary cleavage products of the polyprotein (1a, 3b, and 1b, respectively). Polypeptide molecules are denoted by wavy lines and are not drawn to scale. Cleavage sites in the precursor molecules are indicated by ▲. The polio virus genome RNA is represented by the thick horizontal line. Term. refers to the proposed site for termination of translation. This scheme is based upon the data presented in this paper as well as that of previous reports (6, 18). The numbers in parentheses are the molecular weights ($\times 10^{-3}$) of the proteins as determined from the data presented in this paper and unpublished data.

replicase cleavage products having molecular weights of 41,000 (P3-6a) and 34,000 (P3-6b).

Our finding that all of the replicase proteins and VPg appear to be generated by proteolytic cleavage between a common amino acid pair was unexpected and suggests that they may be processed by the same proteinase. Previously it has been suggested that primary cleavage of the polyprotein (yielding P1-1a, P2-3b, P3-1b) resulted from processing by a cellular proteinase (28, 29), but the evidence in support of this hypothesis is indirect (for a discussion, see ref. 4). Secondary cleavages that produce the remaining products (e. g., P3-2, P3-4b, and P3-7c from P3-1b) are thought to be due to the action of a virus-coded proteinase (7, 27). In support of the hypothesis that both primary and secondary cleavages may be due to the action of a single virus-coded proteinase with a primary specificity for the dipeptide sequence -Gln-Gly-, our prelimiary analysis of several other polio products from regions P1 and P2 suggests that other proteins may also be created by cleavage between a glutamine and a glycine (unpublished data). Inspection of the sequence around the limited number of Gln-Gly pairs we now know are processing sites has not revealed to us other constant sequence elements that are likely to contribute to processing specificity, although we presume that other processing determinants besides the Gln-Gly sequence exist (4).

Recently an encephalomyocarditis viral protein, identified as p22 (30–32) and corresponding in map position to poliovirus protein P3-7c (33), has been isolated from infected cell extracts as well as from *in vitro* translation products and shown to possess proteolytic activity. In encephalomyocarditis virus, the precursor of this proteinase (analogous to P3-2 in poliovirus) can undergo autocatalytic cleavage to yield the proteinase (p22) and the putative RNA polymerase (34). An analogous situation may exist for poliovirus and primary cleavages may result from autocatalytic events or the *trans* action of one polyprotein on another. As the infection proceeds, the remaining primary cleavages and all of the secondary replicase cleavages could be carried out by the mature protease (33). However, the requirement for a cellular proteolytic activity that acts on poliovirus-specific proteins or the existence of a second poliovirus-encoded protease cannot at present be ruled out, and the data we have presented do not necessarily imply that all poliovirus processing is by a single activity.

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