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Chemical determination of the ml Moloney sarcoma virus pP60^{gag} gene order: Evidence for unique peptides in the carboxy terminus of the polyprotein

(cyanogen bromide/gel electrophoresis/peptide mapping)

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ABSTRACT **The gene order of the ml Moloney sarcoma virus (mlMSV) specific pP60^{gag} (P60) was determined by direct chemical analysis of the polyprotein. P60 was cleaved with cyanogen bromide (CNBr) into eight partial and complete fragments ranging in mass from 10,000 daltons to 58,000 daltons. Peptide maps of these fragments were compared to maps of p15, p12, and three CNBr fragments of p30. The polarity of p15 and p12 in a CNBr fragment of P60 was determined by carboxypeptidase A digestion; likewise the CNBr fragments of p30 were ordered by aminopeptidase digestion. The linear arrangement of P60 CNBr fragments gave the gene order of NH_2-p15-p12-p30-COOH. The m3 isolate of MSV expresses a P70 *gag* polyprotein. Peptide maps of 48,000-dalton CNBr fragments of m3 P70 and ml P60 were similar and suggested that both polyproteins were similar through the NH_2-terminal two-thirds of p30. However, the presence of peptides unique to the 10,500-dalton COOH-terminal fragment of mlMSV p30 and not present in the p30 of either m3MSV or Moloney leukemia virus suggested that the *gag* gene deletion in the ml isolate begins in the p30 reading frame.**

By using immunological methods to detect intermediate cleavage products of cellular polyproteins expressed by defective transforming viruses and conditional mutants of Rauscher leukemia virus, Barbacid *et al.* (1) were able to determine the *gag* gene order: NH_2-p15-p12-p30-p10-COOH. Several transforming viruses have been shown to express only portions of the parental leukemia virus *gag* gene. For example, some viruses such as the ml isolate of Moloney sarcoma virus (mlMSV) lack only p10 (1, 2), whereas others lack immunological determinants for p30 and p10 (1) or for p12, p30, and p10 (3). These deleted *gag* polypeptides are believed to occur at variable distances from the 3′ end of the genome (1, 3). This has not been demonstrated directly in the polyprotein, however, and it is not known where the deletions begin, a question that can only be answered by direct chemical analyses. To begin addressing these questions, we have determined the *gag* gene order in the mlMSV polyprotein pP60^{gag} (P60). This polyprotein contains Moloney murine leukemia virus (M-MuLV) related p15, p12, and p30 and is the major component of the feline leukemia virus (FeLV) pseudotype [mlMSV(FeLV)]. By cyanogen bromide (CNBr) cleavage and peptide mapping procedures, we have determined the P60 gene order to be NH_2-p15-p12-p30-COOH.

The m3 isolate of MSV differs from mlMSV in biological reversion frequency (4) and expresses a larger *gag*-related polyprotein (P70). P70 and variable amounts of murine p30 are also detected in the FeLV pseudotype of m3[m3MSV(FeLV)] (5). The m3 P70 possesses p15, p12, and p30 MuLV-related immunological determinants, but lacks p10 (D. Bolognesi, personal communication). In these studies we show that the p30 of M-MuLV and m3MSV have comparable peptide maps and are similar to the maps of the NH_2-terminal 20,000-dalton (Dal) mlMSV p30 CNBr fragment. Moreover, ml P60 and m3 P70 have similar peptide maps in an NH_2-terminal CNBr fragment representing NH_2-p15-p12 and the 20,000-Dal NH_2-terminal portion of p30. Finally, we have identified peptides unique to a 10,500-Dal COOH-terminal fragment of mlMSV p30, which were not present in the p30s of either M-MuLV or m3MSV p30. These studies suggest that the deletion in mlMSV *gag* may begin in the COOH terminus of the p30 reading frame.

MATERIALS AND METHODS

Virus and Protein Purification. The FeLV pseudotypes of ml and m3 Moloney murine sarcoma viruses have been described (5, 6). The IC isolate of M-MuLV (5) was purified by double banding in sucrose gradients (2). mlMSV P60, p30, p15, and p12 were purified from mlMSV(FeLV) (2, 6); m3MSV P70 and p30, from m3MSV(FeLV) (5); and M-MuLV p30, from M-MuLV IC. Virus proteins were purified by guanidine agarose chromatography (6) or by polyacrylamide gel electrophoresis. For purification by the latter procedure, 1 mg of purified virus was subjected to a low-level dansylation procedure for visualization of the protein bands in the gel (7). The desired protein band was electrophoretically extracted in 10 mM ammonium bicarbonate prior to lyophilization and washing with cold acetone.

Gel Electrophoresis. Polyacrylamide gel electrophoresis was performed in slab gels with two different buffer systems (8, 9) as previously described (2). Molecular weight standards were bovine serum albumin, ovalbumin, chymotrypsinogen, and CNBr fragments of myoglobin and cytochrome *c*.

Cyanogen Bromide Cleavage. CNBr cleaves proteins at methionine residues giving both partial and complete cleavage fragments (10). Proteins (50–200 μg) were incubated at 4° for 16–36 hr with 200 μl of 0.1 M CNBr (freshly sublimated) in 70% formic acid. The reaction was stopped by the addition of water followed by lyophilization.

NH_2-Terminal Amino Acid Analysis. NH_2-terminal amino acids were determined by the method of Weiner *et al.* (11). CNBr-cleaved p30 (60 μg) was dansylated prior to gel electrophoresis. The dansylated bands were excised and extracted as described above. The extract was hydrolyzed at 105° for 6–16 hr and chromatographed on polyamide plates (Schleicher & Schuell) as described (11).

Aminopeptidase Digestion. Enzyme solution (10 μg) was added to 30 μg of lyophilized CNBr-cleaved p30 and the mix-

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Abbreviation: mlMSV, ml isolate of Moloney sarcoma virus; P60, pP60^{gag} polyprotein; CNBr, cyanogen bromide; M-MuLV, Moloney murine leukemia virus; FeLV, feline leukemia virus; Dal, dalton.

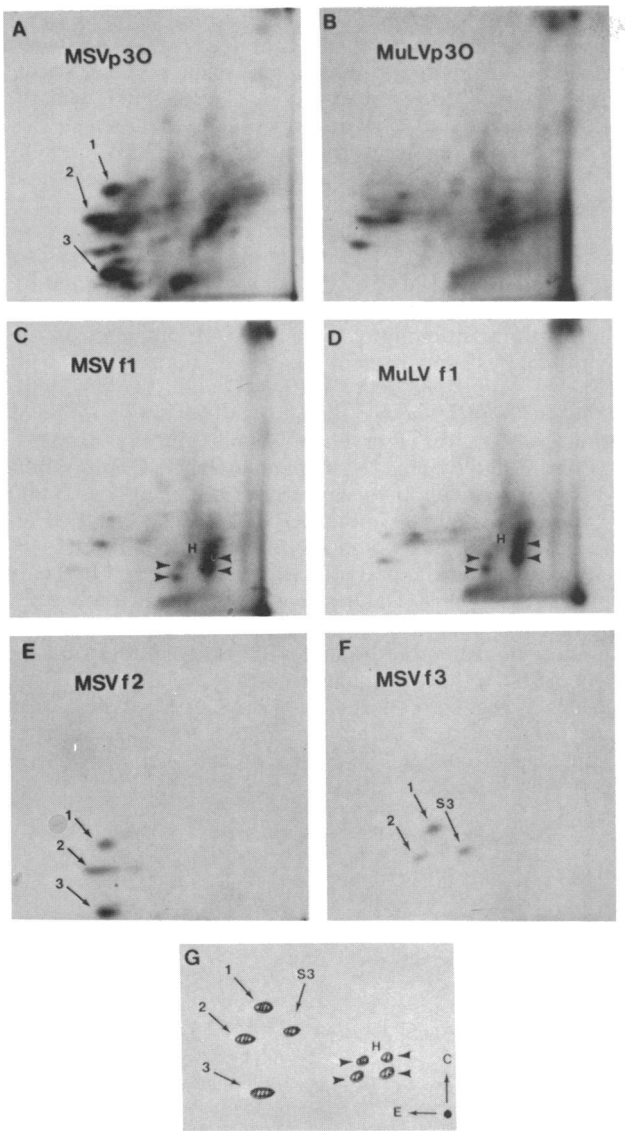

FIG. 1. ^{125}I peptide maps of CNBr fragments of m1MSV and M-MuLV p30. (*A*) m1MSV p30 showing "m1MSV" specific peptides 1, 2, and 3 not present in (*B*) M-MuLV p30; (*C*) f1 fragment (20,000-Dal) of m1MSV p30 with "horseshoe"-shaped series of peptides indicated as "H" (arrowheads) also present in (*D*) f1 of M-MuLV p30; (*E*) peptides 1, 2, and 3 of the f2 fragment (10,500-Dal) of m1MSV p30; (*F*) peptides 1, 2, and S3 of the f3 fragment; (*G*) composite drawing of important peptide features of m1MSV p30 and its CNBr fragments [the H (arrowheads) peptides of f1; peptides 1, 2, and 3 of p30 and f2 and; peptides 1, 2, and S3 of f3]. C and E in *G* indicate the direction from the origin of chromotography and electrophoresis for all peptide maps.

ture was incubated at 37° for 16 hr. Leucine aminopeptidase (118 units/mg, Worthington Biochemicals) was activated by dialysis against 0.02 M Tris·HCl, pH 8.5/1 mM manganese chloride at 40° for 4 hr, and subsequent addition of magnesium chloride to a final concentration of 5 mM. Aminopeptidase M (Boehringer Mannheim) was dialyzed against 0.06 M sodium phosphate, pH 7.5, at 4° for 4 hr before use.

Carboxypeptidase Digestion. Proteins radioiodinated as described below were digested directly in gel slices with carboxypeptidase A (Worthington Biochemicals). Carboxypeptidase (60 μg) in 100 μl of 0.05 M ammonium bicarbonate, pH 8, was added to dried gel slices containing the ^{125}I-labeled protein and the mixture was incubated at 37° overnight. These slices were treated as described below and in ref. 12.

Peptide Mapping. ^{125}I peptide maps were obtained by the method developed by Elder *et al.* (12). Proteins were labeled with iodine-125 (Amersham/Searle) directly in stained gel slices and then digested with trypsin or chymotrypsin (Worthington Biochemicals). The labeled peptides were extracted from the gel slice, lyophilized, and spotted on cellulose plates. Subsequent two-dimensional separation and autoradiography was carried out.

RESULTS

The order of p15, p12, and p30 in the m1MSV polyprotein P60 was determined by a combination of CNBr cleavage, enzyme digestion, and peptide mapping techniques as follows: (*i*) From a comparison of m1MSV and M-MuLV p30 CNBr digests, the linear arrangement of CNBr fragments in p30 was determined and specific peptide markers were identified in each m1MSV p30 fragment; (*ii*) the p30 CNBr fragments were positioned in P60 based on the presence of the p30 peptide markers and the CNBr fragment size; and, (*iii*) the p15 and p12 location and linear arrangement in P60 was determined in a CNBr fragment of the polyprotein. The p30 fragments are denoted with lower case (f) to distinguish them from the P60 (F) CNBr fragments.

Analysis of CNBr Fragments of m1MSV and M-MuLV p30. A comparison of the peptide maps of m1MSV p30 and M-MuLV p30 (Fig. 1 *A* and *B*) showed that the peptides denoted 1, 2, and 3 were present in m1MSV p30 and absent in M-MuLV p30. The location of these peptides was determined in CNBr fragments. Both m1MSV p30 and M-MuLV p30 were digested with CNBr and each produced four fragments (Fig. 2, lanes a and b). Three fragments, one 20,000 Dal (f1) and two approximately 10,500 Dal and 10,000 Dal (f2, f3) had characteristic peptide maps. Additional fragments, perhaps due to acid hydrolysis, were not identified in P60 and were not further characterized. The f1 fragments of m1MSV p30 and M-MuLV p30 were identical in both mobility (Fig. 2, lanes a and b) and peptide maps (Fig. 1 *C* and *D*). A distinguishing characteristic of peptide maps of this fragment was the "horseshoe" shaped series of peptides ("H") observed close to the origin. These peptides were not observed in uncleaved p30 and are probably at the COOH-terminus in f1 because this fragment, like p30, has NH_2-terminal proline (see below). [The H peptides were useful for identifying this cleavage site in P60 (Fig. 4).] The m1MSV p30 f2 and f3 fragments were slightly faster in mobility than the corresponding M-MuLV fragments (Fig. 2, lanes a and b). Moreover, while the M-MuLV fragments had few discernable peptides (not shown), f2 of m1MSV p30 showed the same three distinct peptides as observed in the m1MSV p30 (Fig. 1 *A* and *E*). Map positions of peptides 1 and 2 were the same in

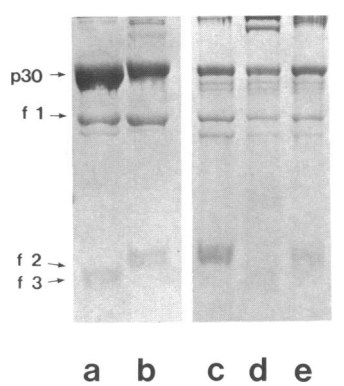

FIG. 2. Electrophoretic analyses of the CNBr fragments of m1MSV and M-MuLV p30. Proteins digested with CNBr were subjected to electrophoresis or incubated with aminopeptidase prior to electrophoresis in urea-SDS (2). Lanes: a, m1MSV p30; b, M-MuLV p30; c, m1MSV p30; d, m1MSV p30 digested with aminopeptidase M; e, m1MSV p30 digested with leucine aminopeptidase.

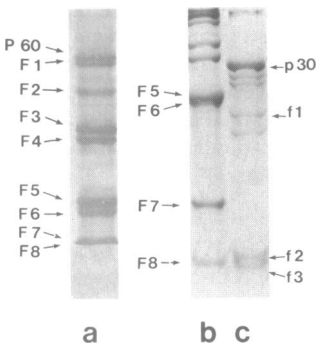

FIG. 3. Electrophoretic analyses of the CNBr fragments of P60. Procedure as in Fig. 2 except that the P60 fragments in lane a were separated by 10% polyacrylamide gel electrophoresis (8); lane b, P60; lane c, m1MSV p30. The size of the m1MSV P60 CNBr fragments were: F1 (58,000 Dal), F2 (48,000 Dal), F3 (37,000 Dal), F4 (33,000 Dal), F5 (25,000 Dal), F6 (23,000 Dal), F7 (14,000 Dal), F8 (10,000 Dal).

f2 and f3 (Fig. 1 *E* and *F*), whereas the position of peptide 3 was shifted in f3 (S3). The shift in this peptide, accompanied by the difference in size between f2 and f3 suggests that an additional CNBr site existed on f2 and cleavage at this site yielded S3. Thus the H peptides are specific for f1; peptides 1, 2, and 3 for f2; and peptides 1, 2, and S3 for f3. These peptides are schematically presented as a composite in Fig. 1*G*.

The linear arrangment of the CNBr fragments of m1MSV p30 was determined both by NH_2-terminal amino acid analysis and by digestion with aminopeptidases. Proline is resistant to hydrolysis by the aminopeptidases (13) and both p30 (as has been shown for p30 of several viruses) (14) and the f1 fragment possessed NH_2-terminal proline residues. Digestion of CNBr-cleaved p30 with either leucine aminopeptidase or aminopeptidase M resulted in an increase in mobility and a decrease in the amount of only the f2 and f3 fragments whereas p30 and f1 were unaffected (Fig. 2, lanes d and e). These results ordered the p30 fragments as NH_2-f1-f2-COOH. Clearly peptides 1, 2, and 3 of f2 were responsible for the major differences observed between the uncleaved m1MSV and M-MuLV p30 (Fig. 1 *A* and *B*) and the ordering placed these peptides in the COOH-terminus of m1MSV p30.

Analysis and Linear Arrangement of CNBr Fragments of P60. CNBr cleavage of P60 gave eight major fragments ranging in size from 10,000 Dal to 50,000 Dal (Fig. 3, lanes a and b). Three features of the peptide maps of the P60 fragments allowed for the positioning of their linear arrangement in the polyprotein. (*i*) Comparison of the peptide maps of: P60 with F1 (Fig. 4 *A* and *B*), F3 with F4 (Fig. 4 *D* and *E*), and F7 with F8 (Fig. 4 *G* and *H*) showed the same shift in map position of peptide 3 to S3 as was observed between f2 and f3 of m1MSV p30 (Fig. 1 *E* and *F*). Since S3 mapped in the COOH-terminus of p30 and was present in the largest P60 fragment (F1; 58,000 Dal), this positioned p30 in the COOH-terminus of P60 (Fig. 6). Therefore F3, F4, F7, and F8 must represent COOH-terminal fragments of the polyprotein. However, each P60 pair differed by 2000–4000 Dal in size whereas the difference between f2 and f3 was estimated to be 500 Dal. To account for this difference in size, an additional 3500-Dal peptide must be present in P60, COOH-terminal to p30 and lacking in peptides detectable by these procedures. These data further indicate that tryptic peptide 3 contains an additional CNBr cleavage site. This site is not cleaved in P60, F3, F7, and p30 f2 but is cleaved in F1, F4, F8, and f3.

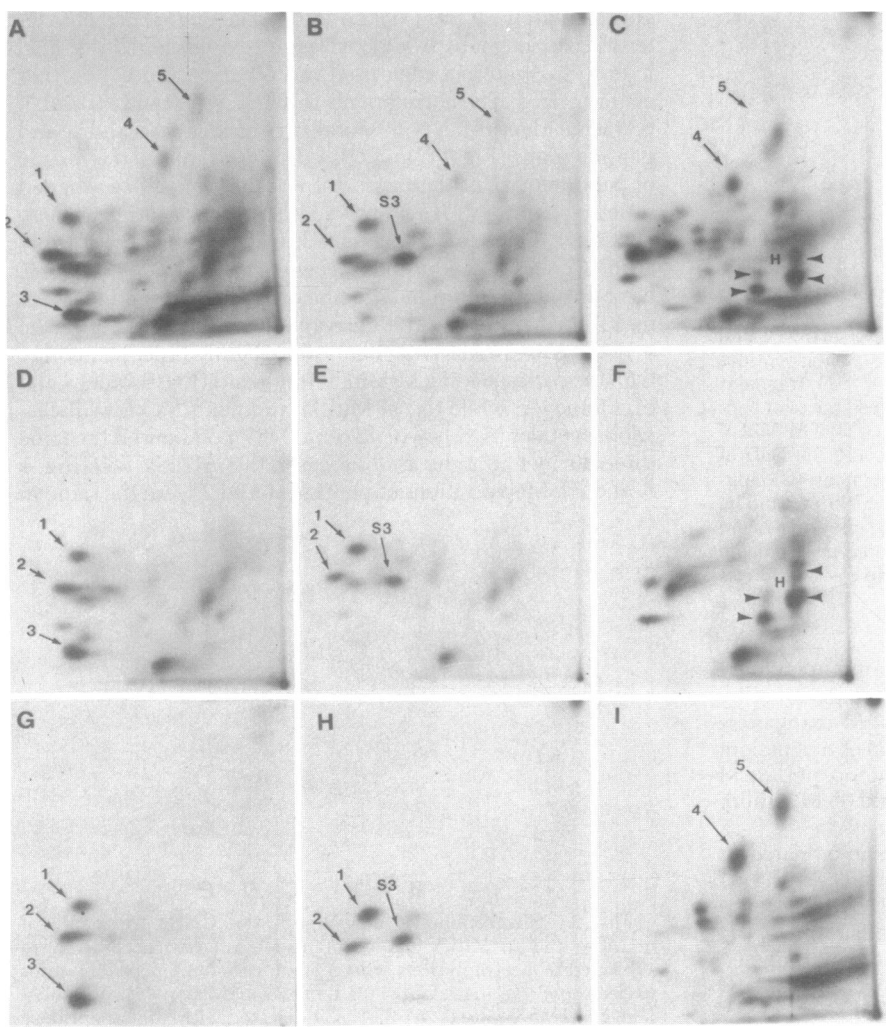

FIG. 4. ^{125}I peptide maps of CNBr fragments of P60. (*A*) P60; (*B*) F1; (*C*) F2; (*D*) F3; (*E*) F4; (*F*) F6; (*G*) F7; (*H*) F8; (*I*) F5. The "m1MSV"-specific peptides 1, 2, and 3 of f2 are denoted in (*A*) P60, (*D*) F3, and (*G*) F7. Peptides 1 and 2, and the shift in map position of peptide 3 to S3 are denoted in (*B*) F1, (*E*) F4 and (*H*) F8. The H peptides (arrowheads), specific for f1 of p30 are indicated in F2 and F6. F5, containing p15 and p12 (Fig. 5), displays two characteristic peptides denoted 4 and 5 that are also indicated in P60, F1 and F2.

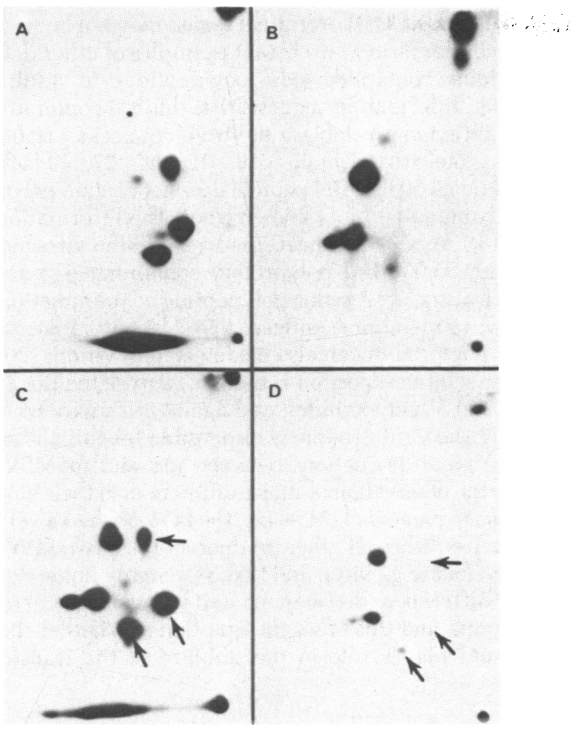

FIG. 5. ^{125}I-labeled chymotrypsin peptide maps of F5, p12, and p15. (*A*) p12; (*B*) p15; (*C*) F5; (*D*) F5 digested with carboxypeptidase A showing the disappearance of p12 peptides (arrows).

(*ii*) The H peptides detected in p30 f1 were present in the F2 and F6 fragments of P60 (cf. Fig. 1 *C* and *D* and Fig. 4 *C* and *F*). F2 was larger than F6 by 25,000 Dal and also contained peptides 4 and 5. This was consistent with F2 containing the COOH-terminal f1 portion of p30 with p15 plus p12 at its NH_2 terminus (Fig. 6). (*iii*) Peptides 4 and 5, identified in P60, F1, and F2, were markedly enriched for in F5, but absent in all other fragments. The F5 fragment contained both p15 and p12. For these polypeptides, chymotrypsin peptide maps were more illustrative and indeed show that F5 contained both p15 and p12 peptides (Fig. 5). To order p15 and p12 in this fragment, F5 was digested with carboxypeptidase A (Fig. 5*D*). This treatment resulted in the disappearance of p12 peptides only, and thereby placed p12 COOH-terminal in F5. The size of F5

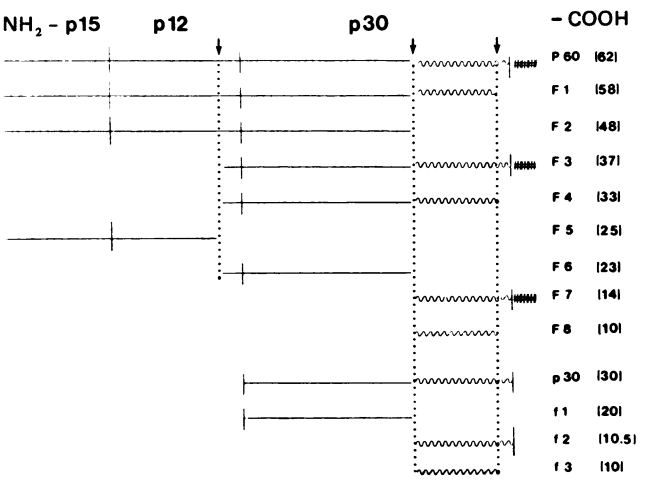

FIG. 6. CNBr cleavage maps of P60 and p30. ↓, Methionine cleavage sites: ∿∿, unique portion of m1MSV p30; ##########, 3500 Dal peptide present in P60 and absent in p30. The molecular weights were estimated from numerous gel analyses and their sum suggests that the mass of P60 is 62,000 Dal.

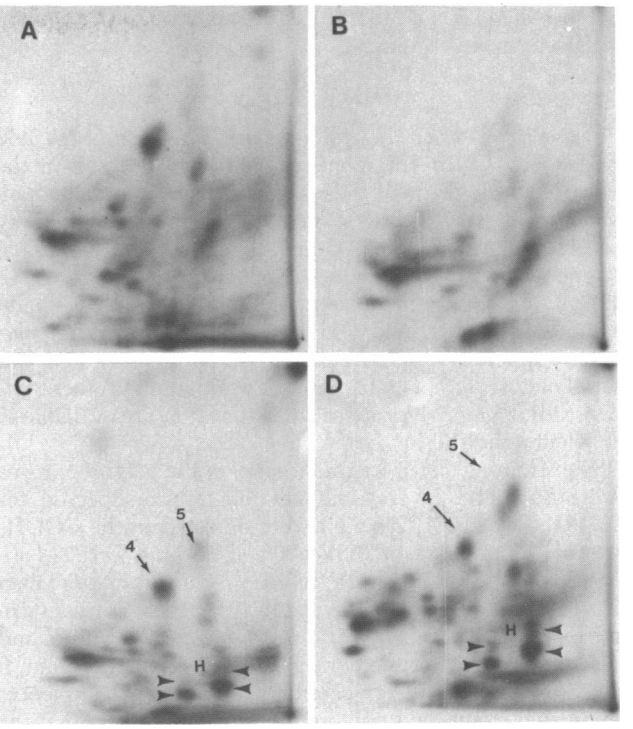

FIG. 7. ^{125}I-labeled peptide maps of proteins from the m3 strain of MSV(FeLV). (*A*) m3 P70; (*B*) m3 p30; (*C*) 48,000-Dal CNBr fragment from P70; (*D*) F2 48,000-Dal fragment of m1MSV P60. Peptides 4 and 5, representing p12 and p15 peptides, are denoted in *C* and *D*, as are the H peptides of the f1 fragment (arrowheads) of p30 (Fig. 1 *C* and *D*).

indicated that it was smaller than the combined size of both polypeptides [p12 has a molecular weight of 14,500 Dal in this gel system (2)]. Thus, CNBr must remove a small portion of either p12 or p15. Since p15 lacks methionine (2) and therefore CNBr cleavage sites, a segment of p12 must be present in another fragment. In limited CNBr digestions P60 was cleaved into two major fragments: F5 and F3 (37,000 Dal). F3 contained a full complement of the m1MSV p30 peptides (compare Fig. 1*A* with Fig. 4*D*). This suggests that these two fragments represented the entire polyprotein and that F3 should contain a small portion of the COOH-terminus of p12. Similarly both F4 and F6 are 3000 Dal larger than their p30 peptide map counterpart—i.e., F4 (33,000 Dal) should equal f1 plus f3 (30,000 Dal) and F6 (23,000 Dal) should equal f1 (20,000 Dal) (Fig. 6). These data provide evidence for a CNBr site in the COOH-terminus of p12 and again places p15 NH_2-terminal in P60.

Analysis of CNBr Fragments of m3MSV P70. The m3 isolate of MSV expresses a P70 gag that is present in m3MSV(FeLV) (5). Because the CNBr fragments of m1MSV P60 were arranged, it was of interest to see whether fragments with similar peptide maps could be identified in m3MSV P70. Comparison of the peptide maps of both MSV polyproteins and their specific p30 show that the m3MSV proteins did not possess the m1MSV-specific peptides 1, 2, and 3 but were similar to M-MuLV p30 (Fig. 7: compare Fig. 7*B* to Fig. 1*B*). However, CNBr cleavage of m3MSV P70 yielded a fragment that was equal in size to F2 of P60, and comparison of the peptide maps of these fragments showed great similarities (Fig. 7 *C* and *D*). This suggested that these polyproteins were similar from the NH_2-terminus through the p30 f1 fragment but differed in the remaining COOH-terminal region in both size and peptide maps. Although the size of P70 suggested the presence of p10, the polyprotein was negative for p10 peptides (not shown). Thus

both m1MSV P60 and m3MSV P70 appear to have abberant COOH-terminal regions.

DISCUSSION

We have determined the *gag* gene order in the m1MSV P60 polyprotein. CNBr fragments of P60 were ordered in the polyprotein by virtue of their size and specific peptide map markers. Partial CNBr digest fragments of P60 were especially useful for identifying the p30 peptides in the COOH-terminal fragment and for identifying the p30 NH_2-terminal overlap into p12. This linear arrangement of CNBr fragments should facilitate primary amino acid sequence analysis of the polyprotein. In addition, as demonstrated with m3MSV P70, this map should serve as a useful basis for comparison to other MSV or M-MuLV *gag* polyproteins when the latter are available in sufficient quantities.

By examining CNBr fragment maps of the polyprotein, we have also found several features that appear specific for m1MSV. Peptides 1, 2, and 3 were detected in the COOH-terminal fragment of m1MSV p30 and P60 and were not observed in the p30 of M-MuLV, m3MSV, or in any of more than 50 isolates of MuLV (15). It is possible that these peptides can be explained by strain-specific differences in M-MuLV and therefore account for the normal size p30 product present in m1MSV(FeLV). However, considering that the p10 sequences are deleted in this *gag* polyprotein, it is more likely that the unique peptides in the COOH-terminus of p30 occur as a consequence of the *gag* deletion. These peptides and the 3500-Dal additional P60 peptide could then represent either the normal *gag* COOH-terminus or peptides from some other region of the M-MuLV genome. Peptide map analyses (2) and immunological studies (1) appear to exclude the possibility that these peptides and the 3500-Dal P60-specific segment represent any portion of the p10 molecule. The m3MSV p30 and P70 lacked the m1MSV-specific peptides and the m3MSV p30 peptide maps were very similar to M-MuLV p30. This polyprotein also lacks p10 determinants (D. Bolognesi, personal communication) and thereby raises the question of what the additional 10,000-Dal COOH-terminal sequences in P70 represent. Heteroduplex mapping between the 124 strain of MSV and M-MuLV indicate that a deletion of 1.81 kbases begins at 2.25 kbases from the 5′ end of this MSV genome (16), and although the m1 and m3MSV strains have not been studied in this manner, variable sized deletions in the same region could account for loss of p10 information, the differences in the sizes of the defective *gag* gene products, and the presence of unique COOH-terminal peptides coded for by other regions of the genome.

Additional evidence for unusual sequences in the COOH-terminus of the *gag* transcript exists for at least two strains of MSV. Results from *in vivo* pulse-chase studies demonstrated that P60 is the largest viral polyprotein detectable in m1MSV-transformed cells (5). Philipson *et al.* (17) have demonstrated *in vitro* that translation products of the RNA of the 124 strain of MSV cannot be enhanced with suppressor tRNA to yield products larger than 72,000 Dal, whereas M-MuLV RNA can be enhanced to yield a 180,000-Dal *gag-pol* product. Thus, both isolates indicate that there is some block which prevents readthrough into the *pol* gene. It will be very important to compare the COOH-terminal peptides of the 124 and other MSV *gag* polyproteins to those of m1MSV to determine whether they have similar peptides indicating that they are terminating in the same region. The present analysis of m1 and m3MSV polyproteins indicate these regions are dissimilar.

Although this COOH-terminal region may not be worthy of further consideration, two recent examples of other defective polyproteins containing *gag* polypeptides in addition to "non-*gag*" information suggests that this is a common event. The MC29 avian myeloblastosis virus expresses a 110,000-Dal *gag* polyprotein that contains p19, p12, and p27, but lacks p15. The additional 50,000-Dal peptide does not contain polymerase determinants and it is not known where this information originates (18). Also, cells transformed with feline sarcoma virus express an 85,000-Dal polyprotein containing p15 and p12 covalently associated with a polypeptide containing the feline oncornavirus membrane antigen (FOCMA) (19). Like m1MSV P60 this polyprotein can also be detected in virions (20). The membrane antigen portion is neither *gag* related nor related to other FeLV polypeptides, and again, it is unknown which portion of the viral genome is responsible for this antigen.

The original distinctions between m1 and m3MSV were based on the observation of major differences in their biological reversion frequences (4, 21)—i.e., the m1MSV had a very high reversion frequency. If other products of these two MSV genes are equivalent (e.g., sarc), and that is presently unknown, then the only differences between m1 and m3 would be in the *gag* polyproteins, and this raises the question of whether these regions could play a role in the stability of the transformed state.

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