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Proteolytic Processing of Animal Virus Proteins

BYRON E. BUTTERWORTH[1]

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Investigations of the proteins synthesized in response to animal viruses have revealed reliance to various extents on the process of proteolytic cleavage for maturation and control of function of the viral proteins. The role of protein cleavage in bacteriophage proteins has been reviewed (*Hershko* and *Fry*, 1975). The major emphasis of this critical review will be on the protein processing of the members of the picornavirus group, where proteases are used for everything from release of the nascent chains from the polysomes to maturation of the virions. An extensive literature has been accumulated in this area which has revealed interesting new insights into the details of these processes. Proteolytic processing also plays a large role in the biosynthesis of the togaviruses and the RNA tumor viruses and each is discussed in turn.

I. The Cleavage Scheme of the Picornaviruses

A. History

There are several reviews dealing with the molecular biology of picornavirus replication and the role of proteolytic cleavages in the formation of the viral proteins (*Baltimore*, 1969; *Rueckert*, 1971; *Sugiyama* et al., 1972; *Shatkin*, 1974; *Hershko* and *Fry*, 1975; *Korant*, 1975; *Butterworth* et al., 1976a; *Rekosh*, 1976).

Studies comparing the cleavage pattern of several picornaviruses show the patterns to be remarkably similar. In general, principles learned in one system seem to apply to the other members of the picornavirus group. Well-studied viruses will be used to illustrate most points. However, where there are interesting differences they will be pointed out.

Two developments facilitated the detailed study of the synthesis and cleavage of picornavirus proteins. The first was the discovery that these viruses inhibited host protein synthesis, so that under the appropriate conditions incubation of infected cells with radioactive amino acids resulted in the specific labeling of only the viral-coded proteins (Fig. 1). The second was the development of high-resolution sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) which provided a straightforward method of separating the polypeptides on the basis of molecular weight.

In 1965 *Summers* et al. published a profile of poliovirus-specific polypeptides obtained from the cytoplasm of infected cells (*Summer* et al., 1965). The pattern contained both capsid and non-capsid polypeptides and there were hints of proteolytic cleavage of some of the larger polypeptides. It was later shown using

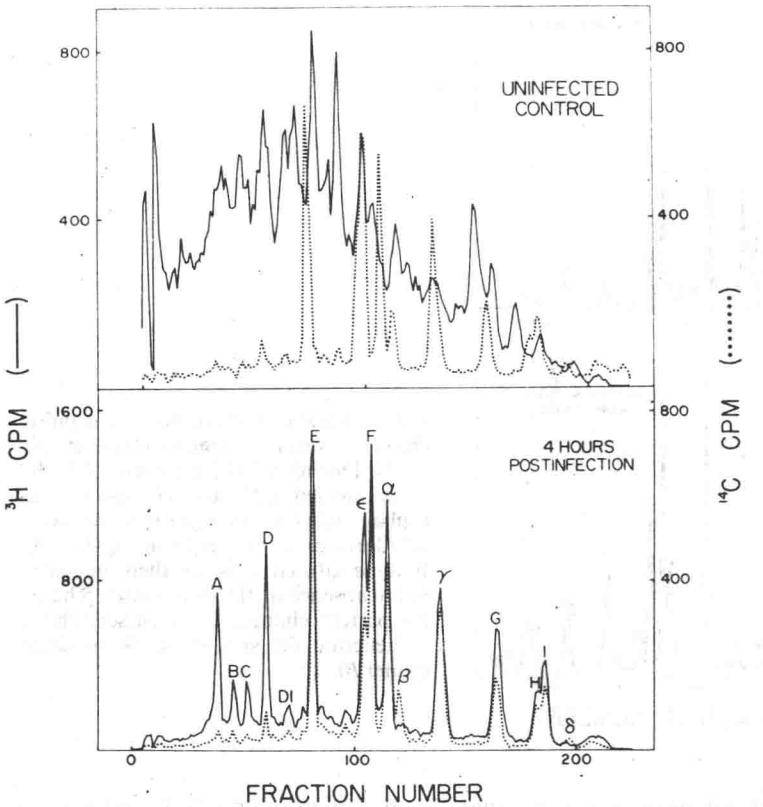


Fig. 1. SDS-PAGE profiles of proteins synthesized in uninfected HeLa cells (top panel) and 4 h after infection with EMC virus (bottom panel) (*Butterworth* et al., 1971). This dramatically illustrates the virus' ability to redirect cellular protein synthesis. Dotted line represents the known stable viral polypeptides which were coelectrophoresed as a marker. Direction of migration is from left to right

pulse-chase experiments (Fig. 2) that some of the large polioviral polypeptides were, in fact, precursor molecules that underwent a series of proteolytic cleavages to generate the smaller polypeptides, and the basic outlines of the precursor-product relationships were established (*Summers* and *Maizel*, 1968; *Maizel* and *Summers*, 1968; *Jacobson* and *Baltimore*, 1968; *Jacobson* et al., 1970; *Summers* et al., 1971).

Subsequent work with encephalomyocarditis (EMC) virus showed it to be more amenable to study than poliovirus. The EMC viral protein profile was simpler, without so many secondary cleavages and there were no apparent overlapping peaks. Typical profiles for the virus-specific proteins of the three most well-studied viruses, EMC virus, human rhinovirus-1A (HRV-1A), and poliovirus are shown in Figure 3. Based on kinetics of synthesis and cleavage, CNBr mapping, and molecular weights, a cleavage scheme was obtained for EMC virus which was analogous to the one determined for poliovirus (Fig. 4) (*Butterworth* et al., 1971).

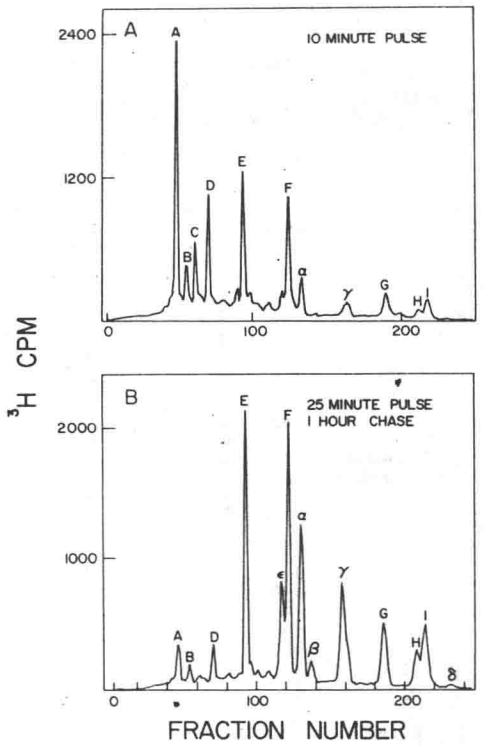


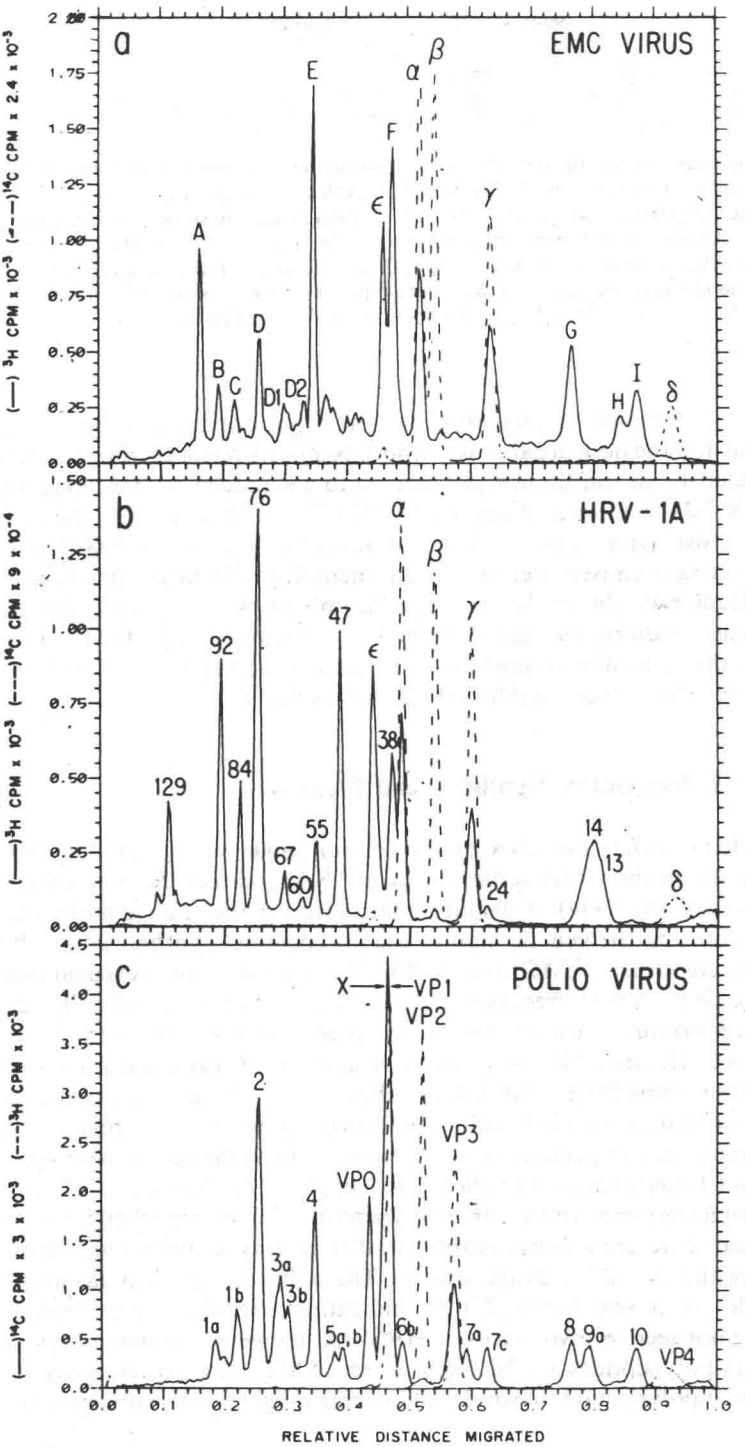
Fig. 2. Flow of radioactivity in a pulse-chase experiment (*Butterworth* et al., 1971). During a brief exposure of EMC virus-infected cells to ^{3}H-amino acids (pulse), label is incorporated primarily into large precursor molecules (panel *A*). If these labeled cells are then incubated in the absence of ^{3}H-amino acids (chase), the pattern changes as precursors cleave to generate the smaller stable products (panel *B*)

Accumulated evidence suggested that the picornaviral mRNA had only a single initiation site (*Jacobson* and *Baltimore*, 1968). This provided the basis for a unique technique to map the polypeptide gene loci on the viral RNA using the drug pactamycin (*Taber* et al., 1971). Newly developed quantitative techniques allowed more detailed studies of the cleavage kinetics and of pactamycin mapping which rely on quantitation for meaningful results (*Butterworth* and *Rueckert*, 1972b).

B. Nomenclature

Unfortunately, a systematic nomenclature has not been agreed to and not only has each virus acquired its own nomenclature but newly discovered peaks have been independently named with new letters and subscripts. The preferred nomenclature and identification of analogous polypeptides for EMC virus, HRV-1A, and poliovirus, are shown in Figures 3 and 4.

Fig. 3a–c. Comparison of the SDS-PAGE profiles of ^{3}H-labeled virus-specific polypeptides of (a) EMC virus, (b) HRV-1A, and (c) Poliovirus (*Butterworth*, 1973). Dashed line represents proteins from the purified ^{14}C-labeled virions, which were coelectrophoresed to identify the virion polypeptides in each pattern


a
EMC VIRUS
A
B
C
D
D1
D2
E
F
ϵ
α
β
γ
G
H
I
δ
(——) ^{3}H CPM x 10^{-3} (- - -) ^{14}C CPM x 2.4 x 10^{-3}
b
HRV-1A
129
92
84
76
67
60
55
47
ϵ
α
β
γ
38
24
14
13
δ
(——) ^{3}H CPM x 10^{-3} (- - -) ^{14}C CPM x 9 x 10^{-4}
C
POLIO VIRUS
X
VP1
VP2
VP3
VPO
1a
1b
2
3a
3b
4
5a,b
6b
7a
7c
8
9a
10
VP4
(——) ^{14}C CPM x 3 x 10^{-3} (- - -) ^{3}H CPM x 10^{-3}
RELATIVE DISTANCE MIGRATED

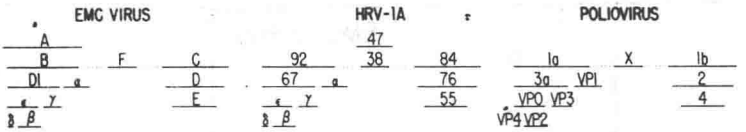


Fig. 4. Common features in the biosynthesis of virus-specific polypeptides of EMC virus, HRV-1A, and poliovirus (*Butterworth*, 1973). Based on molecular weight and position on the genetic map, above portions of the patterns of viral protein biosynthesis were the same for these three viruses. Analogous polypeptides are shown in the same relative positions. Lateral position represents relative location of the corresponding gene locus on the viral RNA (the 5' end of the RNA is to the left). Vertical position represents precursor-product relationships or alternative cleavage forms. Line lengths are proportional to molecular weight

As new viruses are studied it is suggested that the virion polypeptides be named with the prefix VP and the noncapsid polypeptides be numbered according to their apparent molecular weight (in thousands) relative to a standard, as was done for HRV-1A and HRV-2 (*McLean* and *Rueckert*, 1973). This approach was taken for the RNA tumor viruses (*August* et al., 1974). Because of differences in SDS-PAGE techniques and markers chosen there exists an uncomfortably large variation in the reported molecular weights of the virus-specific polypeptides, especially for the larger polypeptides (*Butterworth* and *Korant*, 1974; *Swaney* et al., 1974). As a point of reference the molecular weights of EMC, HRV-1A, and polioviral proteins *relative to each other* have been established (*Butterworth;* 1973).

C. The Pattern of Polypeptide Synthesis and Cleavage

The single-stranded picornaviral RNA genome of molecular weight $2.6–2.8 \times 10^6$ daltons that is found in the virion serves as the mRNA to direct the synthesis of the viral proteins (*Shatkin*, 1974). A detailed comparison of the relative molecular weights and positions on the genetic map of the polypeptides synthesized by the antigenically different viruses, EMC virus, HRV-1A, and poliovirus, revealed that in each case there was a capsid precursor nearest the 5' end of the RNA followed by a stable primary product, then a family of polypeptides analogous to the EMC viral polypeptides C, D, and E (Fig. 4) (*Butterworth*, 1973; *McLean* and *Rueckert*, 1973). This suggests something fundamental about this pattern of biosynthesis because it has remained unchanged while evolutionary pressures have resulted in extensive changes in the antigenicity of the virions and the degree of homology among the RNAs (*Young* et al., 1968; *Dietzschold* et al., 1971; *Yin* et al., 1973).

It is not known if the genes for the three polypeptide families are contiguous on the viral RNA and there are a considerable number of polypeptides which have not been placed in the over-all cleavage scheme. The poliovirus pattern has many more polypeptides of a size below 27000, indicating considerable secondary cleavages that are not seen in EMC virus or HRV-1A. However, tryptic mapping does indicate that polypeptides G, H, and I in the EMC viral pattern may be mixtures of different polypeptides of similar molecular weight (*Dobos* and *Plourde*, 1973).

D. Uniformity of Protein Processing in the Picornaviruses

There have been numerous additional reports of analogous proteolytic processing for members of each of the four major picornavirus subgroups. Several coxsackievirus types, which like poliovirus are members of the enterovirus subgroup, have been studied (*Holland* and *Kiehn*, 1968; *Kiehn* and *Holland*, 1970). There is one report that up to 34 distinct polioviral polypeptides could be resolved, many of which had not been described previously (*Abraham* and *Cooper*, 1975a). However, careful comparison of this pattern with others shows the major peaks to be the same (*Butterworth*, 1973) e.g. p110 = 1a, p90 = 1b, p79 = 2, p29 = 6b. The minor polypeptides present may represent a low level of secondary cleavages or residual synthesis of host proteins. Tryptic mapping of the polypeptides confirms the relationships presented in Figure 4 (*Abraham* and *Cooper*, 1975b).

In general, additional studies characterizing the viral polypeptides of the cardiovirus subgroup, including EMC virus, mouse Elberfeld (ME) virus, and mengovirus, are consistent with the cleavage scheme presented in Figure 2 (*Ginevskaya* et al., 1972; *Dobos* and *Martin*, 1972; *Dobos* and *Plourde*, 1973; *Esteban* and *Kerr*, 1974; *Lucas-Lenard*, 1974; *Paucha* et al., 1974; *Paucha* and *Colter*, 1975).

Members of the rhinovirus subgroup (many of which are responsible for the common cold) show analogous protein processing to the other picornaviruses (*McLean* and *Rueckert*, 1973).

Studies on foot-and-mouth disease virus (FMDV) indicate that the cleavage scheme will be analogous to those of the other picornaviruses (*Vande Woude* and *Ascione*, 1974; *Black*, 1975).

II. The Translation Process

A. Single Initiation Site

The viral RNA is proposed to possess only a single initiation site so that each ribosome completes translation of the entire mRNA portion of the genome with the polypeptide products being separated by proteolytic cleavage (*Rekosh*, 1976). Primary products are released from the growing polypeptide chain by nascent cleavages. Some of these primary products are further processed by posttranslational or secondary cleavages.

Consistent with the concept of a single initiation site are the results of quantitative analysis experiments which showed that the major primary polypeptides and stable products of EMC virus were produced in equimolar amounts (Table 1) (*Butterworth* et al., 1971; *Butterworth* and *Rueckert*, 1972a). Similar values were also obtained for the polypeptides of HRV-1A and poliovirus (*Butterworth*, 1973). However, there are enough variations and minor polypeptides present in less than equimolar amounts so that multiple initiation sites or premature release of ribosomes cannot be completely ruled out.

There are reports that the major polypeptides of mengovirus may be produced in less than equimolar amounts (*Lucas-Lenard*, 1974; *Paucha* et al., 1974; *Paucha*

Table 1. Size and molar ratios of the major EMC viral polypeptides

Polypeptide	Apparent molecular weight	Molar ratio following short pulse	Molar ratio following chase
A	100000	0.80	0.01
B	90000	0.09	0.01
C	84000	0.29	0.01
D	75000	0.29	0.04
E	56000	0.47	0.72
F	38000	1.00	1.00
α	34000	0.20	1.00
G	16000	0.79	1.06
A + B + α		1.09	1.02
F		1.00	1.00
C + D + E		1.05	0.77

Data are from *Butterworth* et al., 1971 and *Butterworth* and *Rueckert*, 1972a. There are several other reports that indicate that the molecular weight of A may be closer to 110000 (*Esteban* and *Kerr*, 1974; *Lucas-Lenard*, 1974; *Paucha* et al., 1974)

and *Colter*, 1975). This could result from premature termination, selective degradation, or preferential loss from the cells (in each case the cells were washed before lysing). The differences observed are small, so it is doubtful that this represents the operation of a major control mechanism in the relative amounts of the polypeptides synthesized. Indeed, this lack of regulation is puzzling, as it appears that the capsid polypeptides should be needed in substantially larger amounts than the other viral polypeptides.

B. Pactamycin Mapping

The single initiation site scheme with each ribosome translating one copy of each gene provided the basis for a unique mapping system in which quantitative changes in the viral protein profile were measured following inhibition of initiation of protein synthesis by pactamycin. During that brief period when the already initiated ribosomes completed translation, those genes nearer the 5' end of the RNA were translated with less frequency than those nearer the 3' end (Fig. 5). The relative amount of each polypeptide formed provided a criterion for ordering the viral polypeptide genes on the RNA (*Taber* et al., 1971; *Summers* and *Maizel*, 1971; *Butterworth* and *Rueckert*, 1972a, *Rekosh*, 1972; *Butterworth*, 1973). The results confirmed previously established relationships and allowed the placement of several unassigned polypeptides in the cleavage scheme. Furthermore, this technique would not have worked if there had not been a single initiation site, supplying further support for the single initiation site hypothesis.

Other means of ordering the genes such as observing the kinetics of appearance of the polypeptides following a short pulse (*Rekosh*, 1972; *Butterworth* and *Rueckert*, 1972b) or following reversal of inhibition of initiation (*Saborio* et al., 1974) have confirmed the relative gene orders in Figure 4.

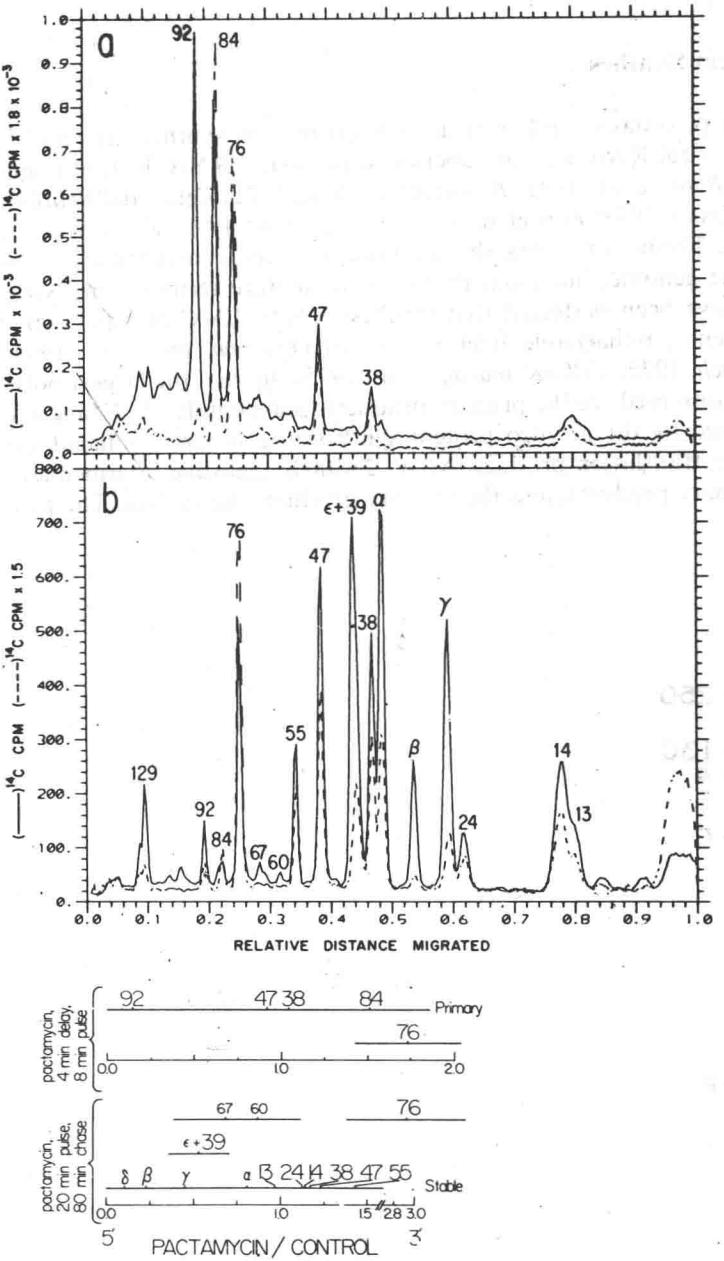


Fig. 5. Effect of pactamycin on the distribution of radioactivity incorporated into virus-specific polypeptides of HRV-1A (*Butterworth*, 1973). Solid line in panel (a), shows normal distribution of label found by pulse labeling HRV-1A-infected HeLa cells. If cells are labeled during that brief period of time following inhibition of initiation of protein synthesis by pactamycin, in which previously initiated ribosomes are running off the RNA, the pattern is changed *(dashed line)*. Those polypeptides translated from nearer the 5' end of the RNA are labeled with less frequency than those translated from nearer the 3' end. Chasing the label into the stable polypeptides allows them also to be ordered [panel (b)]. Pactamycin map derived from these figures is shown below

C. In Vitro Protein Synthesis

Initial work using picornaviral RNA in an in vitro protein synthesizing system showed that EMC viral RNA was far superior to polioviral RNA in stimulating protein synthesis (*Boime* et al., 1971; *Roumiantzeff* et al., 1971; *Egger* and *Shatkin*, 1972; *Boime* and *Leder*, 1972; *Kerr* et al., 1972; *Laskey* et al., 1972; *Kalinina* et al., 1974; *Hunt*, 1976). The in vitro work showed initiation and translation of a substantial part of the genome, but often there was premature termination. Now, various systems have been perfected that synthesize both EMC and polioviral RNA with apparently remarkable fidelity (Fig. 6) (*Esteban* and *Kerr*, 1974; *Lawrence* and *Thach*, 1975; *Villa-Komaroff* et al., 1975). In vivo the largest polypeptides normally observed are the primary products (polypeptides A, F, and C). Products synthesized by the in vitro protein synthesizing systems included the presumed complete translation product (M.W. 250000), cleavage intermediates leading to the primary products, and the primary products themselves. The pro-

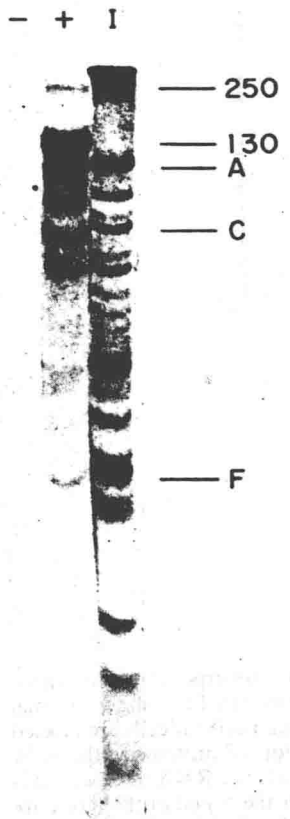


Fig. 6. ^{14}C-polypeptides synthesized in an in vitro protein synthesizing system programmed with EMC viral RNA (*Esteban* and *Kerr*, 1974). SDS-PAGE profiles are from an incubation period of 120 min in the presence (+) or absence (—) of EMC viral RNA. Gel (*I*) shows authentic EMC virus-specific polypeptides labeled in infected cells

duction of primary products in the in vitro protein synthesizing system indicates that those enzymes responsible for performing the nascent cleavages are present and functioning. The extensive secondary and maturation cleavages that occur in vivo are not observed in the in vitro systems.

D. The Initiation Sequence

Met-tRNA$_f^{Met}$ appears to be the unique initiator-RNA in eukaryotic protein synthesis, while Met-tRNA$_M^{Met}$ donates all internal methionyl residues. The fMet residue from (^{35}S)-fMet-tRNA$_f^{Met}$ is incorporated as the amino-terminal peptide into protein coded for by EMC viral RNA in in vitro protein synthesizing systems. Tryptic digests of this product yield only one major ^{35}S-labeled tryptic peptide (*Oberg* and *Shatkin*, 1972) of N-terminal sequence fMet-Ala-Thr (*Smith*, 1973), once again confirming a single initiation site on the picornaviral RNA. Similarly, only one initiation peptide could be identified from in vitro protein synthesizing systems programmed with mengovirus RNA, mouse Elberfeld (ME) viral RNA (*Oberg* and *Shatkin*, 1972; *Oberg* and *Shatkin*, 1974) and polioviral RNA (*Villa-Komaroff* et al., 1975). In contrast, there is a report that two distinct polioviral initiation peptides exist (*Celma* and *Ehrenfeld*, 1975). In fact, careful examination of the tryptic digests of ^{35}S-fMet in vitro labeled EMC viral and mengoviral polypeptides shows small amounts of a second peptide. However, the rigorous characterization of the EMC products shows minor peptides to be overdigestion products and strongly supports the presence of only on initiation peptide (*Smith*, 1973).

The corresponding ^{35}S-fMet initiation peptide could not be located in tryptic digests of EMC virus or mengovirus. Since the capsid polypeptides are translated from the 5' end of the mRNA portion of the genome, this suggests that there is a lead-in peptide sequence, which is removed by a cleavage process in the infected cell (*Oberg* and *Shatkin*, 1972; *Smith*, 1973; *Oberg* and *Shatkin*, 1974). The in vitro protein synthesizing system programmed by poliovirus RNA did produce a protein of the same electrophoretic mobility as polypeptide 1a, which was labeled with ^{35}S-fMet-tRNA$_f^{Met}$, suggesting that 1a does contain the initiation sequence (*Villa-Komaroff* et al., 1975). A major product of the in vitro protein synthesizing system programmed with EMC viral RNA is a polypeptide of apparent molecular weight 130000 that appears to be a precursor of polypeptide A. The species labeled with ^{35}S-fMet-tRNA$_f^{Met}$ had an apparent molecular weight of 130000 indicating that this polypeptide contains the initiation sequence (*Esteban* and *Kerr*, 1974).

III. The Major Viral Polypeptides

A. The Capsid Precursor

Based on size, cleavage rate in pulse-chase experiments, and tryptic mapping, polioviral polypeptide 1a was shown to be the precursor of the capsid proteins VP0, VP1, and VP3 (*Summers* and *Maizel*, 1968; *Jacobson* and *Baltimore*, 1968; *Jacobson* et al., 1970; *Abraham* and *Cooper*, 1975b). The same criterion, with the

addition of cyanogen bromide mapping, were used to establish that the EMC polypeptide A was the capsid precursor (*Butterworth* et al., 1971; *Dobos* and *Plourde*, 1973). The largest nascent EMC viral and polioviral polypeptides observed are the size of the capsid precursor, indicating that the capsid precursor is released by a nascent cleavage very soon after synthesis (*Jacobson* et al., 1970; *Butterworth* and *Rueckert*, 1972b).

Detailed kinetic studies show that the EMC viral polypeptide A is cleaved further only after it is released from the polyribosome (*Butterworth* and *Rueckert*, 1972b). This agrees with evidence that polypeptide A actually begins to assemble and form subviral structures before further cleavage occurs (*McGregor* et al., 1975). In fact, the cleavage of A is probably intimately associated with the assembly process. The cleavage proceeds through the probable intermediates D1 and possibly D2 (3a and 3b in the case of poliovirus) to generate ε, α, and γ (VP0, VP1, and VP3 in the case of poliovirus). At this point EMC has formed the 13s immature promoter $(\varepsilon, \gamma, \alpha)_5$ and poliovirus has formed an entire empty shell consisting of $(\text{VP0, VP3, VP1})_{60}$ (*McGregor* et al., 1975; *Rekosh*, 1976). ε (VP0) is then cleaved to generate the virion polypeptides δ and β (VP2 and VP1). This is termed the maturation cleavage and occurs simultaneously with the addition of the viral RNA to the capsid precursor structures to form the complete virion (*Rueckert*, 1971). This cleavage may be imperative in picornaviral architecture because it occurs in all picornaviruses examined thus far and may play a role such as altering the protein configuration to lock the RNA in the virus structure. There are some reports that the cleavage process is not always complete and small amounts of polypeptide intermediates, ε and D2, are found in the virions (*Rueckert* et al., 1969; *Ziola* and *Scraba*, 1974).

Based on comparative SDS-PAGE studies, the EMC capsid precursor, A, is disproportionately large compared to 92 of HRV-1A and 1a of poliovirus and may go through the intermediate B (*Butterworth*, 1973).

It has been suggested that there can be ambiguity or multiple cleavage sites in the capsid precursor polypeptides, resulting in multiple forms of the polypeptides (*Cooper* et al., 1970; *Vanden Berghe* and *Boeye*, 1972; *Phillips* and *Fennell*, 1973; *Fennell* and *Philips*, 1974). It is difficult to reconcile these observations with other detailed studies which consistently show only single peaks for each of the virion polypeptides (*McGregor* et al., 1975; *Lonberg-Holm* and *Butterworth*, 1976).

A class of defective interfering (DI) poliovirus particles has been identified in which approximately 15% of the length of the normal RNA has been deleted from the capsid coding region of the genome (*Cole* et al., 1971; *Cole* and *Baltimore*, 1973a, 1973b). Even with this defect DI particles can still inhibit cellular macromolecular synthesis, direct the synthesis of viral RNA and proteins, and serve as a mRNA in an in vitro protein synthesizing system (*Cole* and *Baltimore*, 1973a; *Villa-Komaroff* et al., 1975). Of course, no progeny virus is produced. The profile of viral polypeptides produced by DI particles shows a normal complement of noncapsid proteins. In place of the capsid-related polypeptides (1a, VP1, VP2, VP3) there is only synthesis of a new polypeptide termed DI(1)-P, which migrates in the position of 3a and maps with pactamycin in the capsid coding region of the viral RNA (*Cole* and *Baltimore*, 1973a). This protein is rapidly digested and is either the residual fragment of 1a encoded by the DI genome, or may actually be

the capsid intermediate 3a (Fig. 4) which is transiently formed as part of the degradation pathway of the aberrant capsid precursor. These experiments would suggest that the capsid polypeptides are neither responsible for inhibition of cellular RNA and protein synthesis, nor facilitate the synthesis of the viral macromolecules.

B. Polypeptides Analogous to F

EMC viral polypeptide F which is analogous to polioviral polypeptide X and HRV-1A polypeptide 38 is a stable primary product with an apparent M.W. of 38000 that is translated from the center of the genome. Little is known about the function of this polypeptide. However, X has been shown to have an affinity for phospholipid membranes and may play a role in the association of the viral RNA polymerase with cellular membranes (*Butterworth* et al., 1976b). The HRV-1A polypeptide 38 appears to have an alternate cleavage form (polypeptide 47) (*Butterworth*, 1973) which may also be the case for HRV-2 (*McLean* and *Rueckert*, 1973).

Based on molecular weight measurements and peptide mapping it has been suggested that X may be translated independently from the other viral polypeptides (*Abraham* and *Cooper*, 1975a). This is inconsistent with results of pactamycin mapping, molar ratio data, and patterns obtained during inhibition of cleavage; all of which suggest that X is under the same translational controls as the other polypeptides.

C. Polypeptides 1b, 2, and 4 and the Viral Polymerase

The EMC viral polypeptides C, D, and E are analogous to the polioviral polypeptides 1b, 2, and 4 and the HRV-1A polypeptides 84, 76, and 55 (Fig. 4). Relative to the other viral polypeptides C maps nearest the 3′ end of the viral RNA. Polypeptide C cleaves to produce D which in turn cleaves to produce E (*Butterworth* et al., 1971). Kinetic studies show that although C can be translated in the intact form, most of the time the natural cleavages occur in the growing chain, generating D and E as primary products (*Butterworth* and *Rueckert*, 1972b). There are large differences among the picornaviruses in the rates that these proteins are processed. In the profile of EMC viral polypeptides E is always present in large amounts relative to the other polypeptides, whereas in the HRV-1A profile 55 is present in small amounts. In all cases the cleavage analogous to C $\rightarrow$ D appears to be rapid (the half-life of C is 10 min (*Butterworth* and *Rueckert*, 1972b)).

The observation that the polymerase functions are on the opposite end of the genetic map relative to the capsid functions (*Cooper*, 1969) suggests that C, D, and E may be components of the viral RNA polymerase. Partial purification of the polioviral RNA polymerase complex did show an enrichment in polypeptide 4 and similar experiments with the EMC viral polymerase showed an enrichment in polypeptides D and E (*Lundquist* et al., 1974; *Traub* et al., 1976). However, these experiments are extremely difficult and it still has not been rigorously shown that these polypeptides are constituents of the polymerase (*Butterworth* et al., 1976).