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Cleavage of four avian sarcoma virus polyproteins with virion protease p15 removes *gag* sequences and yields large fragments that function as tyrosine phosphoacceptors *in vitro*

(*gag*-linked transformation-specific proteins/protein kinase)

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ABSTRACT **The transformation-specific polyproteins of avian sarcoma viruses PRCII, PRCII-p, Fujinami sarcoma virus (FSV), and Esh sarcoma virus (ESV) consist of two domains, one derived from a partial viral *gag* gene and the other representing an apparently cell-derived insert in the defective viral genome. These *gag*-linked proteins were cleaved with retrovirion protease p15. Cleavage of PRCII-p polyprotein P170, P105 of PRCII, and P140 of FSV occurred within the *gag* domain and generated fragments of M_r 130,000, 70,000, and 115,000, respectively, containing all of the transformation-specific sequences linked to a remnant of the original *gag* sequences. ESV P80 was cleaved inside the transformation-specific domain, yielding a M_r 35,000–38,000 fragment from the NH_2-terminal half of the molecule consisting of the entire *gag* portion and some non-*gag* sequences and a M_r 48,000 fragment containing most of the transformation-specific sequences. The tyrosine phosphorylation sites of the polyproteins were found in every case in the transformation-specific fragments. The major serine phosphorylation site of ESV P80 was found to reside in the M_r 35,000–38,000 *gag*-containing fragment, probably within the transformation-specific sequences of that cleavage product. Removal of all of the *gag* domain of ESV P80 or most of the *gag* domain in PRCII-p P170, PRCII P105, and FSV P140 does not affect their ability to be phosphorylated by the polyprotein-associated tyrosine-specific protein kinase activities. This observation suggests that the *gag* sequences of the polyproteins of classes II (PRCII-p, PRCII, and FSV) and III (ESV) avian sarcoma viruses may not be required for this enzymatic function, which appears to be of importance in transformation.**

Avian sarcoma viruses have been divided into three classes on the basis of homology between their transformation-specific genetic sequences and the translation products of these sequences (1–4). Class I includes the different strains of Rous sarcoma virus, (RSV); class II encompasses PRCII sarcoma virus (PRCII) (5, 6) and Fujinami sarcoma virus (FSV) (7, 8); and Esh sarcoma virus (ESV) (9) and Yamaguchi 73 sarcoma virus (Y73) (10) represent class III. Recent studies (1, 2) have indicated that the transformation-specific genetic sequences of classes II (*fps*) and III (*yes*) sarcoma viruses, like the RSV *src* gene (11), were acquired by the retrovirus from cellular genetic sequences. The available evidence indicates that these sequences are expressed as the COOH-terminal portion of a polyprotein that obtains its NH_2-terminal domain from a partial viral *gag* gene.

Studies with conditional and nonconditional transformation mutants of RSV indicate that the *src* gene product, $pp60^{src}$, and its tyrosine-specific protein kinase (ATP:protein phosphotransferase, EC 2.7.1.37) are essential for transformation (12–15). The proteins of cells transformed by classes II and III avian sarcoma viruses have, like those transformed by class I, elevated levels of phosphotyrosine (16), and transformation-related tyrosine phosphorylation involves similar target proteins (17). Also, immunoprecipitates of the *gag*-linked polyproteins of classes II and III sarcoma viruses are associated with protein kinases specific for tyrosine residues (9, 10, 18, 19). These observations suggest that, as proposed for RSV $pp60^{src}$ (20, 21), the *gag*-linked polyproteins of classes II and III sarcoma viruses induce transformation by altering the balance of cellular protein phosphorylation at tyrosine residues.

Avian retrovirus particles have a proteolytic activity chemically indistinguishable from that of virion protein p15 and capable of cleaving the primary translation product of the viral *gag* gene, $Pr76^{gag}$, into smaller polypeptides, including virion protein p15 itself (22–24). This activity has been shown to cleave other viral proteins containing all or part of the amino acid sequence of $Pr76^{gag}$ (24). This paper reports on the susceptibility of the *gag*-linked transformation-specific polyproteins of classes II and III sarcoma viruses to cleavage with virion protease p15. The results allow further structural definition of these polyproteins and show that removal of most or all of the *gag* domains does not affect the ability of the transformation-specific domains to serve as substrate for their associated tyrosine-specific protein kinase activities.

RESULTS

***gag*-Linked Polyproteins of PRCII, PRCII-p, FSV, and ESV Are Cleaved into Discrete Fragments by Viral Protease p15.** To test the susceptibility of various avian sarcoma virus polyproteins to cleavage with virion protease p15, chicken embryo cells transformed by PRCII, PRCII-p, FSV, or ESV were incubated for 16 hrs with L-[^{35}S]methionine and the viral-coded polyproteins were immunoprecipitated with an antiserum to virion protein p19. Incubation of such immunoprecipitates with a crude preparation of virion protease p15 generated from each protein one prominent major fragment and, from ESV P80, several additional smaller fragments (Fig. 1). From proteins P105 of PRCII, P140 of FSV, P170 of PRCII-p, and P80 of ESV, major fragments of M_r 70,000 (p70), 115,000 (p115), 130,000 (p130), and 48,000 (p48), respectively, were obtained. Cleavage of ESV P80 also produced fragments in the M_r 35,000–38,000 range. The fragment of M_r 23,000 shown in Fig. 1, lanes o and p, is not derived from P80, as it is not observed after cleavage of P80 obtained from nonproducer cells (Fig. 1, q–t). By its size and peptide composition (not shown), it is probably identical to a *gag* p19-related product described by Vogt *et al.* (24). Although

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Abbreviations: RSV, PRCII, FSV, and FSV, Rous, PRCII, Fujinami, and Yamaguchi sarcoma viruses, respectively; AMV, avian myeloblastosis virus; RAV-7, Rous-associated virus 7.

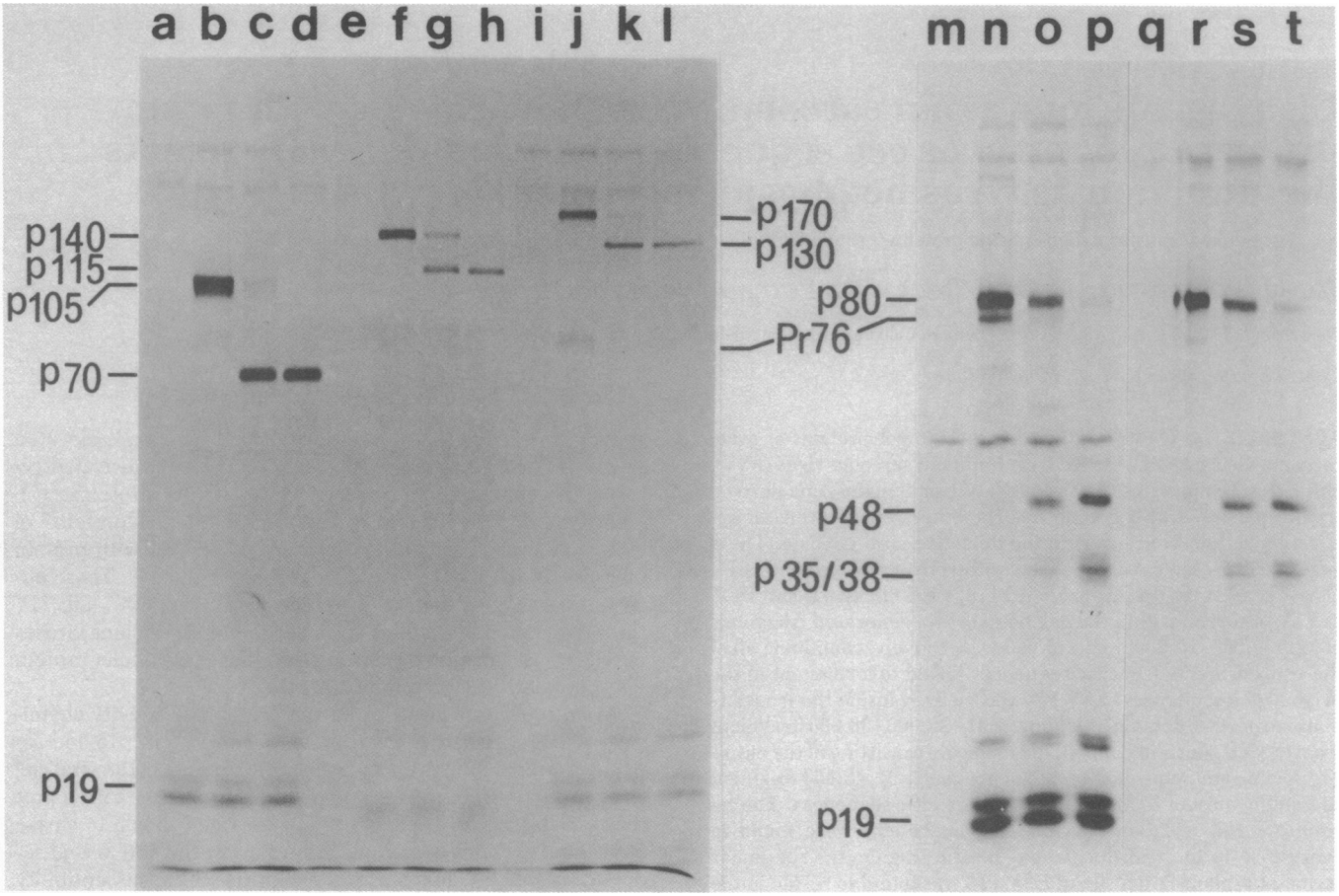

FIG. 1. Analysis of p15 cleavage fragments of polyproteins by $NaDodSO_4$/polyacrylamide gel electrophoresis. Chicken embryo cells transformed by and producing PRCII(lanes a–d), FSV(lanes e–h), PRCII-p (lanes i–l), and ESV (lanes m–p) and ESV-transformed nonproducer cells (lanes q–t) were labeled for 16 hrs with L-[^{35}S]methionine (4). Lysates were prepared, and viral proteins were precipitated with an antiserum to *gag* protein p19 (lanes b–d, f–h, j–l, n–p, and r–t) or with normal rabbit serum (lanes a, e, i, m, and q) followed by an excess of *Staphylococcus aureus* suspension. The bacterial immunoadsorbates were incubated at 37°C in 10 μl of 0.05 M Tris·HCl, pH 7.0/0.2 M NaCl/0.1% Nonidet P-40 with or without p15 for various lengths of time. Crude preparations of p15 were obtained from avian myeloblastosis virus particles that had been purified by equilibrium sedimentation, solubilized with 0.5% Nonidet P-40 and frozen and thawed three times. The 10,000 × *g* supernatant fraction of such lysates was used as the source of p15. Lanes: a, b, e, f, i, j, m, n, q, and r, no AMV; c, g, k, o, and s, 5 μg of AMV proteins for 15 min; d, h, l, p, and t, 5 μg of AMV proteins for 60 min.

structurally related to ESV P80 (4), the transformation-specific polyprotein of the other class III avian sarcoma virus, Y73, was not cleaved by virion protease p15 under the experimental conditions used here (data not shown).

Tryptic Peptide Mapping of the Cleavage Fragments. To determine the nature of the sequences in each of the cleavage fragments, we compared the two-dimensional maps of the [^{35}S]methionine-containing peptides obtained after trypsin digestion of the fragments and their parent polyproteins (Fig. 2; refs. 3 and 4). PRCII p70 and PRCII-p p130 fragments contained all of the non-*gag* peptides of PRCII P105 and PRCII-p P170, respectively, and the major peptide of *gag* protein p27 (Fig. 2 *A*, *B*, *C*, and *E*). All the other *gag* peptides common to P170 and P105 were found to be absent from p130 and p70. FSV P115 contained all of the non-*gag* peptides of FSV P140 in addition to a *gag* peptide (peptide z of Fig. 2*D*) but lacked all other *gag* peptides. These results, summarized in Fig. 5, also indicate that the non-*gag* peptides of FSV-P140 (and p115) and PRCII P105 (and p70) are a subset of those of PRCII-p P170 (and p130), with the exception of non-*gag* peptide x, which is specific for PRCII (p105 and p70). In conclusion, p15 cleaves the polyproteins of class II avian sarcoma viruses inside the *gag* domain, resulting in removal of most of the *gag* sequences and generating fragments containing residual *gag* sequences at their NH_2-terminus and all of the non-*gag* sequences.

ESV p48 contained most of the non-*gag* peptides of P80 (Fig. 2 *F* and *G*) but lacked all of the *gag* p19-derived peptides. Analysis of the 35,000–38,000 M_r fragments (Fig. 2*H*) showed all of the p19-*gag* peptides of P80 in addition to the non-*gag* peptides missing from p48. We conclude that ESV P80 is cleaved inside the non-*gag* domain to give two major fragments: a fragment derived from the COOH-terminal half of the molecule and containing most of the non-*gag* sequences (p48) and a M_r 35,000–38,000 fragment containing the *gag* p19 sequences and part of the non-*gag* sequences. Secondary cleavage(s) inside the p48 domain generated further fragments migrating in the M_r 35,000–38,000 region. These fragments contributed p48-specific non-*gag* peptides to the peptide maps of these size class molecules.

The Major p15 Cleavage Fragments Contain the Phosphotyrosine Residues of the Polyproteins. Phosphorylation of classes II and III polyproteins at tyrosine residues by their associated protein kinases occurs at target sites specific for each class (3, 4). This fact, together with the absence of tyrosine phosphorylation of $Pr76^{gag}$ or virion *gag* proteins both *in vitro* or after [^{32}P]orthophosphate labeling *in vivo*, suggested that the ty-

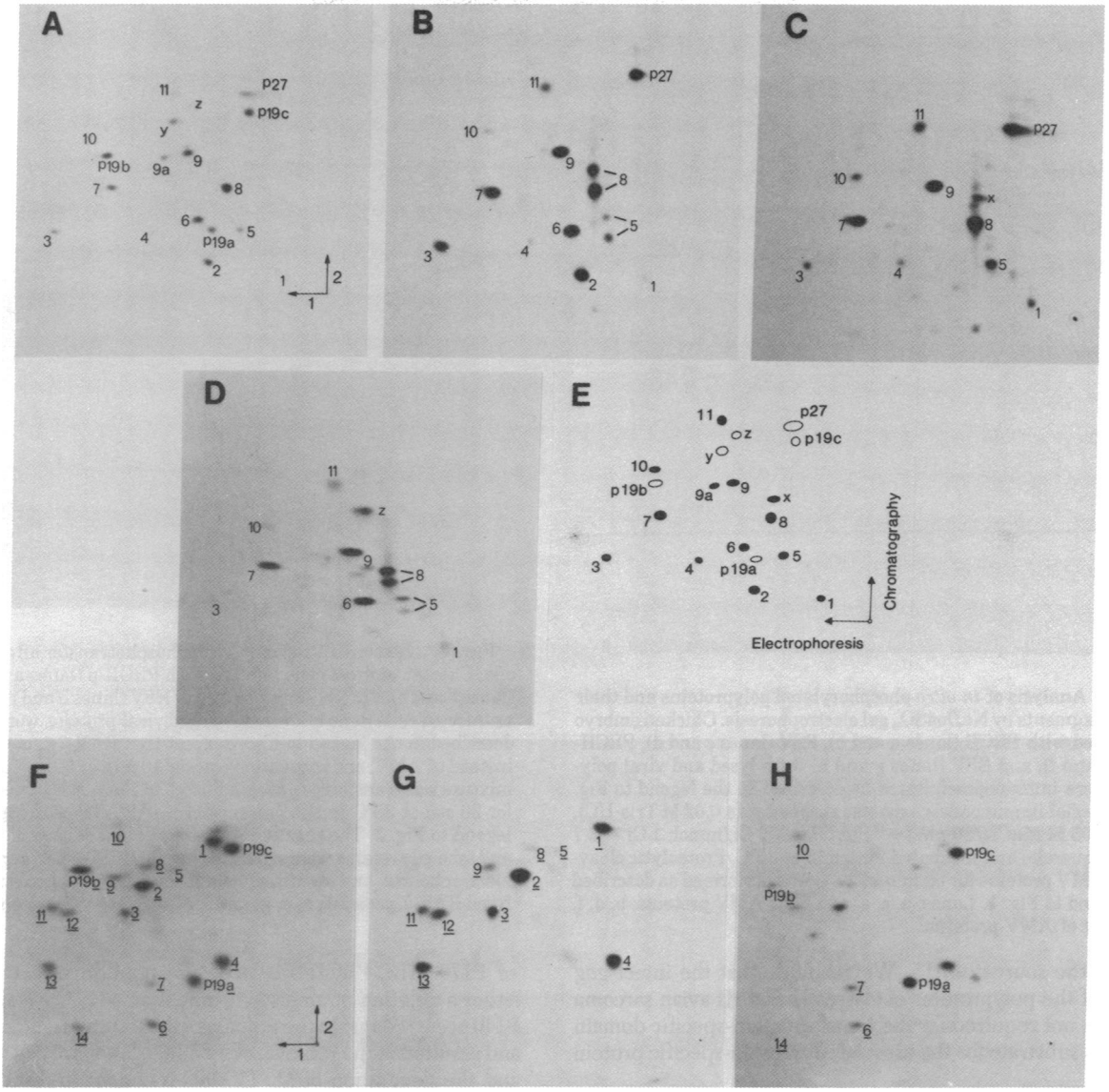

FIG. 2. Tryptic peptide maps of the polyproteins and their p15-cleavage fragments. [^{35}S]Methionine-labeled polyproteins and their fragments were eluted from gels similar to those of Fig. 1, oxidized with performic acid, and digested with *N*-tosylphenylalanine chloromethyl ketone/trypsin. The resulting peptides were separated as described (4) and visualized by fluorography. (*A*) PRCII-p P170, (*B*) PRCII-p p130, (*C*) PRCII p70, (*D*) FSV p115, (*E*) Composite diagram of class II polyproteins. Open spots, *gag* peptides; solid spots, non-*gag* peptides. Non-*gag* peptides are identified as 1–11 and x. *Gag* peptides were named after their origin where known. Occasional migration of peptides 5 and 8 as doublets is probably the result of incomplete oxidation. (*F*) ESV P80, (*G*) ESV p48, (*H*) ESV M_r 35,000–38,000 fragments. Non-*gag* peptides of ESV are numbered 1–14.

rosine-containing site was located in the non-*gag* portion of the polyproteins. As shown in Fig. 3, only the major fragments are visibly labeled after p15 cleavage of the *in vitro* phosphorylated polyproteins. This result shows that the tyrosine-containing site of ESV P80 is located in the non-*gag* domain of the molecule and supports a similar conclusion for PRCII P105, FSV P140, and PRCII-p P170.

The Major Cleavage Fragments of the Polyproteins Act As Phosphoacceptors *in Vitro*. To test whether the *gag*-sequences of the polyproteins are essential for *in vitro* phosphorylation by the associated protein kinases, PRCII-p P170, FSV P140, PRCII P105, and ESV P80 were cleaved with virion protease p15 and then incubated in the presence of [γ-^{32}P]ATP/5 mM Mn^{2+} (4). Fig. 4 shows that the fragments generated by p15 cleavage of all four polyproteins can serve as *in vitro* phosphoacceptors. Analysis of the tryptic phosphopeptides obtained from the *in vitro*-labeled fragments suggested that the same site was phosphorylated in the fragments as in the intact proteins. We tested the ability of the fragments to be phosphorylated *in vitro* after only partial cleavage of the polyproteins, as longer incubation of the polyproteins at 37°C resulted in significant inactivation of the associated kinases. Under the conditions used, labeling of the fragments must be the result of phosphorylation of the fragments themselves rather than the result of phosphorylation before cleavage, as the 30-seconds duration of the kinase test is too short to allow generation of detectable cleavage fragments by p15. As source of p15 for these experiments, we used fibroblast-produced Rous-associated virus type 7 (RAV-7) rather than AMV virus produced by leukemic myeloblasts. Myeloblast-derived retroviruses contain high levels of a cell membrane-associated ATPase (25) that interferes with the phosphotransfer reaction. Preliminary control experiments had shown that the pattern of cleavage of all four polyproteins was identical whether we used Nonidet P-40-disrupted AMV or

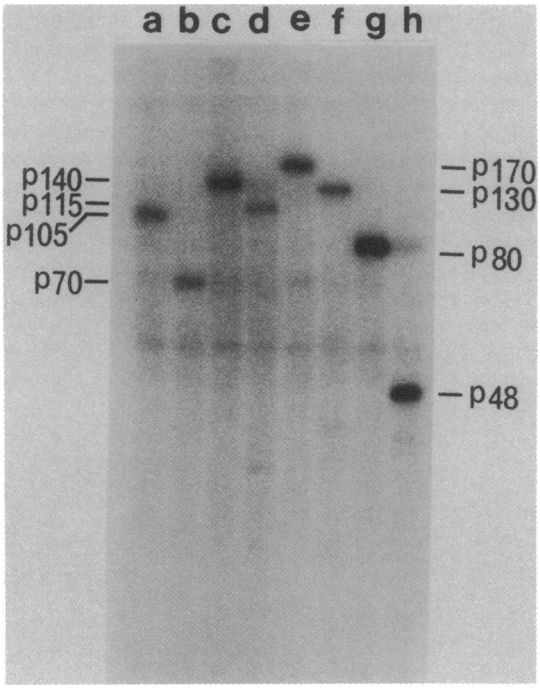

FIG. 3. Analysis of *in vitro* phosphorylated polyproteins and their cleavage fragments by $NaDodSO_4$ gel electrophoresis. Chicken embryo cells infected with PRCII (lanes a and b), FSV (lanes c and d), PRCII-p (lanes e and f), and ESV (lanes g and h) were lysed and viral polyproteins were immunoprecipitated as described in the legend to Fig. 1. The bacterial immunoadsorbant was suspended in 0.02 M Tris·HCl, pH 7.0/0.005 M $MnCl_2$/30 nM [γ-^{32}P]ATP (4000 Ci/mmol; 1 Ci = 3.7 × 10^{10} becquerels) and incubated for 5 min at 30°C. Proteolytic cleavage with AMV proteins for 60 min at 37°C was performed as described in the legend to Fig. 1. Lanes: a, c, e, and g, no AMV proteins; b, d, f, and h, 5 μg of AMV proteins.

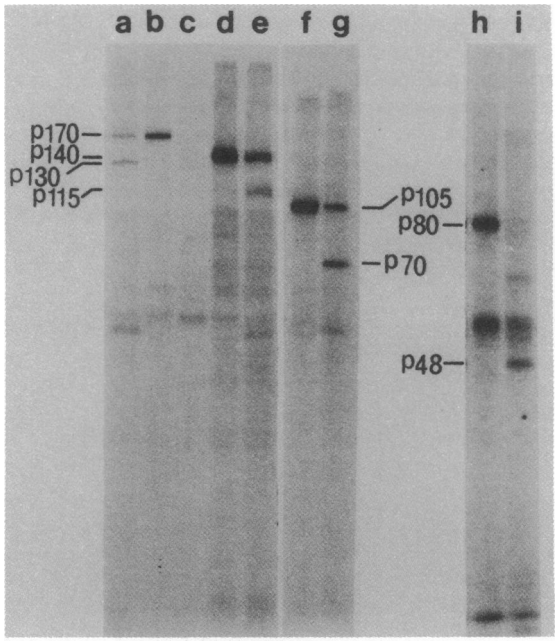

FIG. 4. Demonstration of *in vitro* phosphotransfer after p15 cleavage. Chicken embryo cells infected with PRCII-p (lanes a and b), FSV (lanes d and e), PRCII (lanes f and g), or ESV (lanes h and i) and control uninfected cells (lane c) were lysed, and viral proteins were cleaved as described in the legend to Fig. 1 except that RAV-7 protein was used instead of AMV, and incubation was for 10 min at 37°C. Each reaction mixture was transferred on ice, adjusted to 5 mM $MnCl_2$ and incubated for 30 sec at 30°C in the presence of [γ-^{32}P]ATP as described in the legend to Fig. 3. The reaction was stopped by addition of $NaDodSO_4$, and labeled proteins were separated by $NaDodSO_4$/polyacrylamide gel electrophoresis and identified by autoradiography. Lanes: b, d, f, and h, no RAV-7 proteins; a, e, g, and i, 5 μg of RAV-7 proteins.

RAV-7 as the source of p15. We conclude that the intact *gag* domains of the polyproteins of classes II and III avian sarcoma viruses are not required for the transformation-specific domain to serve as substrate for the associated tyrosine-specific protein kinases.

DISCUSSION

The *gag*-linked transformation-specific polyproteins of avian sarcoma viruses PRCII-p, PRCII, FSV and ESV, are cleaved by viral protease p15. We have used that property to analyze the structure of these polyproteins. The results of this analysis are summarized in Fig. 5. Cleavage of PRCII P105 and PRCII-p P170 occurred probably at the NH_2 terminus of the p27 sequences and resulted in both cases in the removal of ≈35,000 daltons of *gag* sequences and in the generation of fragments of M_r 70,000 (p70) and 130,000 (p130) that contained information derived from the class II transformation-specific sequences linked to part of *gag* protein p27. We propose that only part the p27 sequences are present, as (*i*) certain anti-p27 sera do not precipitate P105 and P170 although they precipitate p27 (6) and (*ii*) the COOH-terminal end of p27 contains a p15 cleavage site (24) that, if present, would have led to the removal of all p27 sequences from the major fragments. The M_r 35,000 *gag* fragment is not detected, probably because of further cleavage. Several lines of evidence described here and elsewhere (27) are consistent with the idea that the coding sequences of PRCII P105 were generated from those of PRCII-p P170 by either deletion or early termination. First, P105 and P170 seem to contain the same partial *gag* sequences and, second, the non-*gag* peptides of P105 are, with one exception, a subset of those of P170. The PRCII P105-specific peptide x may represent either a deletion or an early termination site. Cleavage of FSV P140 occurred inside the domain of the putative *gag* protein p10 and resulted in the removal of ≈25,000 daltons of *gag* sequences and the generation of M_r 115,000 fragment (p115) containing the non-*gag* peptides and one *gag* peptide. The different *gag* content of FSV P140 on the one hand and of PRCII-p P170/PRCII P105 on the other are consistent with the independent acquisition of the cellular *fps* sequences by these viruses. The transformation-specific polyproteins of class III avian sarcoma viruses ESV and Y73 contain part of *gag* p19 sequences covalently linked to transformation-specific non-*gag* sequences (3, 4, 9). As there is no p15 cleavage site within the amino acid sequence of p19, we expected P90 of Y73 and P80 of ESV to remain uncleaved. Although this was the case for Y73 P90, ESV P80 was efficiently cleaved inside the transformation-specific domain to give a M_r 35,000–38,000 fragment from the NH_2-terminal half of P80 and a M_r 48,000 (p48) fragment containing most of the non-*gag* peptides.

The major fragments generated from PRCII-p, PRCII, FSV, and ESV polyproteins by p15 cleavage contain the tyrosine phosphorylation sites. ESV P80 and Y73 P90 are also specifically phosphorylated *in vivo* at a serine residue (4, 9). This phosphorylation site is absent from the p48 fragment of P80 but is part of the M_r 35,000–38,000 fragment and is probably contained in the non-*gag*-derived portion of that fragment (9). This distribution of the phosphorylation sites of P80 (and presumably P90) in which the phosphoserine site is located in the NH_2-terminal half of the molecule and the phosphotyrosine site is in its COOH-terminal half is similar to that of $pp60^{src}$ (28). Together

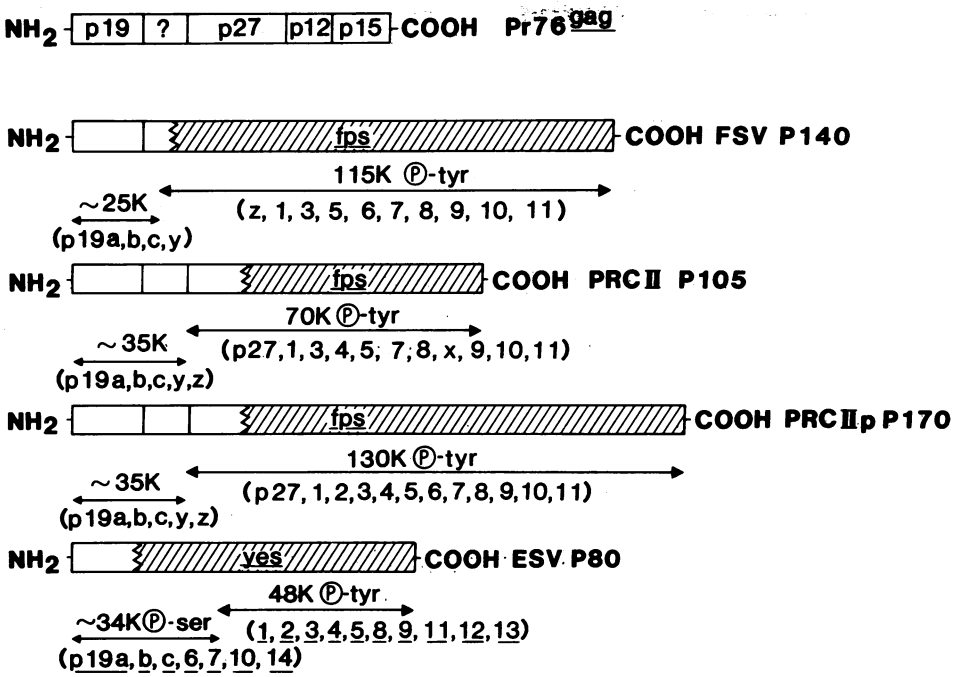

FIG. 5. Proposed structures of transformation-specific P140 of FSV, P105 of PRCII, P170 of PRCII-p, and P80 of ESV. The order of the individual *gag* virion proteins on $Pr76^{gag}$ is derived from recent pactamycin mapping data (23) and DNA and protein sequence data on the RSV *gag* gene (D. Schwartz and E. Hunter, personal communication). In accordance with the sequence data, we indicate the presence of a low molecular weight polypeptide (p10) between the coding sequences of p19 and p27. Hatched areas, transformation-specific sequences translated from *fps* or *yes*; open areas, *gag* sequences; jagged line, junction of *gag* and non-*gag* sequences; ↔, sequence covered by p15 cleavage fragments. The peptide composition of each fragment is indicated in parentheses below the segments, with the exception of peptide 9a (see Fig. 2), which is not reproducibly detected. Fragment sizes are given in kilodaltons.

with the close resemblance of the tyrosine phosphorylation sites of $pp60^{src}$ and P80 and P90 at the primary structure level (29), these observations strengthen the evidence for the functional similarities of classes I and III transformation-specific proteins.

The *gag* portions of the transformation-specific polyproteins of classes II and III avian sarcoma viruses do not appear to be required for phosphorylation of the tyrosine target site by their respective associated protein kinases. As polyprotein phosphorylation at tyrosine residues seems to be related to transformation (refs. 30 and 31; unpublished results), possibly by activation of protein kinases intrinsic to the polyproteins, these results suggest that the *gag* sequences may not be essential to the transforming function of these avian sarcoma viruses. In the provirus, the significance of the retroviral sequences linked to the transformation-specific sequences may be primarily to provide an efficient promoter for expression of the acquired cellular sequences.

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