Expression of the PRC II Avian Sarcoma Virus Genome

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We found that the genomic RNA of the replication-defective avian sarcoma virus PRC II was 4.0 kilobases long. A Northern blot analysis of the viral RNAs present in PRC II-transformed cells showed that the PRC II genome was expressed as a single 4.0 kilobase mRNA species. In vitro translation of polyadenylic acid-containing 70S virion RNA yielded two highly related proteins of 110,000 and 105,000 daltons (P110 and P105), which were synthesized from messenger activity that sedimented as expected for the 4.0 kilobase PRC II genome (at 25 to 27S). P110 and P105 were identified as in vitro translation products of the PRC II genome by immunoprecipitation and tryptic peptide mapping and were the only PRC II-specific polypeptides detected by in vitro synthesis. In addition, we found that immune complexes prepared from PRC II 70S virion RNA in vitro translation products contained a tyrosine-specific protein kinase activity. A comparison of the in vitro- and in vivo-synthesized proteins revealed that PRC II-transformed cells also contained 110,000- and 105,000-dalton proteins, which were indistinguishable from in vitro-synthesized P110 and P105 by electrophoretic mobility and tryptic peptide analysis. Both P110 and P105 were present in producer cells and in seven individual nonproducer clones. A pulse-chase analysis showed that P105 was the primary translation product of the PRC II genome and that P110 was derived from P105 by post-translational modification. Under conditions of long-term labeling with [³⁵S]methionine, P110 and P105 were present in a molar ratio of approximately 1:1. These results indicated that the transformation-specific product of the PRC II genome, previously referred to as a single component (P105), actually consists of two polypeptides related by post-translational modification.

Recently, three classes of avian sarcoma virus have been defined on the basis of biochemical and immunological differences among their transforming proteins (5, 25). PRC II and Fujinami sarcoma virus (FSV), members of one avian sarcoma virus class, are replication-defective transforming viruses which induce sarcomas in vivo and transformation of avian fibroblasts in vitro. The FSV genome has been shown to contain genetic information which was probably obtained by recombination with cellular sequences. These host-derived sequences, termed *fps*, are located in the middle of the FSV genome and are flanked by helper virus sequences (19, 22). The RNA genomes of FSV and PRC II appear to be closely related, as determined by nucleic acid hybridization, and may be the products of two independent recombinations between an avian leukosis virus genome and the same cellular gene (35).

Chicken embryo fibroblasts transformed by

PRC II contain a phosphoprotein, P105, which is similar to FSV-encoded P140 in that it is composed of helper virus-derived *gag* protein sequences which are contiguous with transformation-specific sequences from the *fps* region (5, 24). Since P105 is the only viral gene product which has been detected in transformed, non-producer cells by immunoprecipitation, it is currently the only identified transformation-specific product of the PRC II genome (8). This protein can be immunoprecipitated with antisera against p19 and p27, two virion internal structural proteins which are coded for by the 5'-proximal portion of the *gag* gene (5, 24). TBR sera from rabbits bearing tumors induced by Rous sarcoma virus (RSV), a member of a second avian sarcoma virus class, also precipitate P105 by recognition of the *gag* determinants (5, 26). Immune complexes prepared with anti-p19 or TBR serum from PRC II-transformed cells contain a tyrosine-specific phosphotransferase activity which phosphorylates either P105 alone or both P105 and the heavy chain of immunoglobulin, respectively (5, 26). Because of this protein kinase activity observed in immunoprecipitates

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which contain P105 and the elevated levels of phosphotyrosine in protein observed in PRC II-transformed cells, P105 has been implicated as a tyrosine-specific protein kinase *in vivo* (5).

Although PRC II and FSV are avian sarcoma viruses, it has been demonstrated recently that the Snyder-Theilen strain of feline sarcoma virus (ST-FeSV) and the Gardner-Arnstein strain of FSV code for proteins which are related to FSV P140 and PRC II P105 as determined by immunological criteria and tryptic peptide mapping (4, 5). In addition, hybridization studies have shown that FeSV virion RNA is partially homologous to the *fps* region of the FSV genome (35). Because the FSV and PRC II genomes have extensive homology in the *fps* region, it seems likely that the PRC II and FeSV transformation-specific regions are also partially homologous.

It was of interest to determine whether the PRC II genome expressed transformation-specific proteins other than P105. We approached this problem in two ways. First, we examined PRC II-specific intracellular RNAs in an attempt to identify species which could serve as mRNA's for proteins other than P105. Second, we examined the *in vitro* translation products of PRC II 70S virion RNA and compared these with the viral proteins synthesized in PRC II-transformed cells. In this report, we present evidence that a single PRC II-specific RNA species gives rise to two highly related forms of P105 both *in vivo* and *in vitro*. These two proteins, designated P110 and P105, appear to be related in a precursor-product fashion by post-translational modification. In addition, we show that the PRC II proteins synthesized *in vitro* display an associated protein kinase activity in immune complexes.

MATERIALS AND METHODS

Cells and viruses. PRC II virus stocks were prepared from PRC II-infected chicken cells originally provided by P. K. Vogt, University of Southern California, Los Angeles. Rous-associated virus type 2 (RAV-2) was also obtained from P. K. Vogt. FSV was obtained from H. Temin (University of Wisconsin, Madison), as described by Lee et al. (22) and Beemon (5), and from H. Hanafusa (The Rockefeller University, New York, N. Y.), as described by Feldman et al. (17) and Hanafusa et al. (19). All virus stocks were grown on *gs*⁻*chf*⁻ chicken embryo fibroblasts (CEF) that were prepared from eggs supplied by SPAFAS, Inc., Norwich, Conn., as described previously (1, 7).

Virus and viral RNA preparation. Virus, total virion RNA, and 70S virion RNA were prepared as described previously (1, 7).

Cellular RNA preparation. Whole-cell RNA was prepared from infected and uninfected CEF, as described previously (1, 20). For polysomal RNA, PRC II-infected CEF were washed and lysed, and a postnuclear supernatant was prepared essentially as de-

scribed by Lee et al. (21), except that the lysis buffer contained 0.01 M vanadyl-ribonucleoside complex (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) as an RNase inhibitor. The postnuclear supernatant contained 0.1 M KCl, 0.005 M MgCl₂, 0.025 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.5), 0.01 M vanadyl-ribonucleoside complex, 1% Nonidet P-40 (NP-40), and 0.5% sodium deoxycholate. The polysomes were then pelleted through a layer of 1.0 M sucrose and a layer of 2.0 M sucrose in the same buffer lacking detergent and vanadyl-ribonucleoside complex in a Beckman SW41 rotor at 30,000 rpm for 12 h. The RNA was extracted from the polysomal pellet by the method of Lee et al. (21).

RNA handling. The conditions used for the preparation of polyadenylic acid [poly (A)]-containing RNA, the denaturation of RNA by dimethyl sulfoxide treatment, and sucrose gradient sedimentation have been described previously (1, 7).

For gel electrophoresis, RNAs were treated at 50°C for 1 h with a solution containing 0.008 M sodium acetate (pH 5.2), 50% (vol/vol) dimethyl sulfoxide, 14% (vol/vol) glyoxal (Aldrich Chemical Co., Milwaukee, Wis.), and 400 µg of carrier tRNA (Boehringer Mannheim Corp., Indianapolis, Ind.) per ml. RNAs were then fractionated on a 1.2% agarose gel in a buffer containing 0.036 M Tris base, 0.03 M NaH₂PO₄, 0.001 M EDTA, and 0.5% sodium dodecyl sulfate (SDS) (pH 7.8) for 720 V·h. Preparation of the gel for transfer to diazobenzoyloxymethyl paper and preparation of the diazobenzoyloxymethyl paper were performed essentially as described by Alwine et al. (2), except that 0.2 M sodium acetate (pH 4.0) was used instead of borate buffer.

Hybridization to RNA coupled to diazobenzoyloxymethyl paper. After transfer of the RNA from the gel, the diazobenzoyloxymethyl paper was treated with prehybridization buffer (50% formamide [BDH], 0.75 M NaCl, 0.05 M HEPES, pH 7.5, 0.2% SDS, 0.005 M EDTA, 1% [wt/vol] glycine, 200 µg of sonicated, denatured calf thymus DNA per ml, 5× concentrated Denhardt solution [13]) for 4 h at 41°C. Hybridizations were performed with 1 × 10⁷ to 5 × 10⁷ cpm of probe in 12 ml of hybridization buffer (the same as prehybridization buffer except with 1× concentrated Denhardt solution) plus 3 ml of 50% dextran sulfate for the times and at the temperatures indicated in the legend to Fig. 2. The paper was then washed extensively with 2×, 1×, 0.5×, and 0.1× SSC containing 0.2% SDS and exposed to Kodak X-Omat R film with an intensifying screen (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate).

Probe preparations. ³²P-labeled cDNA_{rep} (containing DNA sequences complementary to the entire PRC II and helper virus genomes) was prepared by incubating heat-denatured PRC II 70S RNA in a mixture containing 0.05 M Tris-hydrochloride (pH 8.0), 0.01 M MgCl₂, 0.03 M β-mercaptoethanol, 0.12 M KCl, 0.5 mM dATP, 0.5 mM dCTP, 0.5 mM dGTP, 2 mg of calf thymus primer per ml, 4 µM [α-³²P]TTP (293 Ci/mmol; New England Nuclear Corp., Boston, Mass.), and avian myeloblastosis virus reverse transcriptase from J. W. Beard (Life Sciences, St. Petersburg, Fla.) at 37°C for 60 min. The DNA was extracted with phenol-chloroform and separated from unincorporated material by passage over a Sephadex G-75 column in a buffer

containing 0.15 M NaCl, 0.01 M Tris-hydrochloride (pH 7.4), 0.001 M EDTA, and 0.1% SDS.

To prepare a ^{32}P -labeled nick-translated probe, we used a recombinant lambda clone containing an insertion of the ST-FeSV genome, which was kindly provided by C. J. Sherr, National Institutes of Health, Bethesda, Md. (34). Nick-translation was performed on the entire recombinant clone essentially as described by Rigby et al. (29), except that the reaction buffer contained 0.01 M Tris-hydrochloride (pH 7.5), 0.01 M MgCl_2 , and 0.001 M dithiothreitol. The reaction was stopped and treated as described above for the preparation of cDNA probes.

Probe preparations were treated with 0.3 N NaOH for 10 min at room temperature and neutralized with HCl before they were added to the hybridization buffer.

In vitro translation. An mRNA-dependent reticulocyte lysate was used for in vitro translation, as previously described (1, 6). Completed reactions were incubated for 20 min at 37°C with 50 μg of RNase A per ml in the presence of 0.01 M EDTA before analysis on 12.5% SDS-polyacrylamide slab gels (6, 7).

Tryptic peptide mapping. Two-dimensional tryptic peptide analysis of [^{35}S]methionine-labeled proteins was performed as previously described (1, 7).

Radiolabeling and immunoprecipitation. The procedures used for labeling cells with [^{35}S]methionine (Amersham Corp., Arlington Heights, Ill.) and $^{32}\text{P}_i$ (ICN, Irvine, Calif.) and for immunoprecipitation have been described previously (32). TBR serum was obtained by injecting the Schmidt-Ruppin strain of RSV subgroup D into newborn rabbits (9). Rabbit antiserum against avian myeloblastosis virus p19 was provided by D. P. Bolognesi, Duke University Medical Center, Durham, N.C.

Protein kinase assay. Our assay of the protein kinase activity present in immune complexes was basically as described by Collett and Erikson (12), except that the immunoprecipitation buffer contained NP-40 as the only detergent. Immunoprecipitates adsorbed onto fixed *Staphylococcus aureus* were washed with 0.15 M NaCl-0.01 M sodium phosphate (pH 7.2) and then incubated for 10 min at 30°C in 20 μl of a buffer containing 0.02 M PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid)] (pH 7.0), 0.01 M MnCl_2 and 1 to 5 μCi of [γ - ^{32}P]ATP (specific activity, >2,500 Ci/mmol; New England Nuclear Corp.). Samples were assayed by SDS-polyacrylamide gel electrophoresis, autoradiography, and scintillation counting of radioactive bands.

Analysis by partial proteolysis. [^{35}S]methionine-labeled proteins in gel slices were partially digested with *S. aureus* protease V8 and analyzed using the methods described by Cleveland et al. (11), with the modifications of Eckhart et al. (15).

RESULTS

Comparison of PRC II virion RNA and PRC II-specific intracellular RNA. The expression of the PRC II genome has been studied by utilizing antiserum which recognizes viral structural protein determinants or antiserum which recognizes undefined determinants coded for by the *fps* region (4, 5, 8, 24). In this way, a single polypeptide, P105, has been identified as a translation

product of the PRC II transforming virus genome. However, it is possible that additional polypeptides which cannot be detected with these antisera are necessary for transformation by PRC II. For this reason, we wanted to determine the size and, therefore, the coding capacity of the PRC II genome. This was accomplished by preparing ^{32}P -labeled PRC II 70S virion RNA, denaturing this RNA by glyoxal treatment, and fractionating the RNA on a neutral agarose gel. This analysis (Fig. 1, lane 3) demonstrated that the PRC II 70S virion RNA complex contained two major discrete RNA species. The larger RNA was approximately 7.5 kilobases (kb) long, which was consistent with the sizes of the RNAs previously described for avian leukosis viral genomes (19, 20, 22, 36). Therefore, it seemed likely that the larger RNA species corresponded to the helper virus genome. The smaller RNA was approximately 4.0 kb long and presumably represented the PRC

FIG. 1. Comparison of ^{32}P -labeled PRC II and FSV virion RNAs. PRC II- and FSV-transformed CEF were labeled with $^{32}\text{P}_i$ for 6 h in phosphate-free medium. The supernatant was centrifuged at 2,000 rpm for 10 min to remove the cells and then centrifuged at 45,000 rpm for 30 min in a Beckman SW50.1 rotor to pellet the virus. Virion RNA was prepared by phenol extraction, ethanol precipitation, and glyoxal treatment, as described in the text. The samples were resolved on a 1.0% neutral agarose gel. The arrows indicate the positions of chick 28S and 18S rRNA markers run on the same gel. The FSV preparation in lane 2 contained a small amount of contaminating rRNA. Lane 1, FSV virion RNA (virus obtained from H. Temin); lane 2, FSV virion RNA (virus obtained from H. Hanafusa); lane 3, PRC II virion RNA.

II genome. If this entire molecule were translated, an RNA of this size would yield a 130,000-dalton (130K) to 140K polypeptide; this is more than the observed molecular weight of 105,000 for the PRC II transformation-specific protein. However, it is unlikely that the entire RNA molecule is utilized for translation because the avian leukosis virus sequences at both ends of the genome probably include large untranslated regions. Since FSV codes for a somewhat larger but related protein, we were interested in directly comparing the PRC II and FSV genomes by the same method. Figure 1 shows the results of side-by-side electrophoresis of glyoxal-treated PRC II virion RNA and two preparations of FSV virion RNA. Although the helper virus RNAs appeared to be the same size in all cases, the FSV-specific RNA was 4.8 kb long, or 800 bases larger than PRC II RNA.

To explore the expression of the PRC II genome, we undertook an analysis of the PRC II-specific intracellular mRNAs. Since many avian and murine retroviruses express internal

coding sequences in the form of spliced, subgenomic-sized mRNA's (3, 10, 14, 16, 18, 20, 23, 30, 31, 33, 36), we were particularly interested in whether PRC II-specific mRNA's smaller than 4.0 kb could be detected. To test this possibility, total cellular poly(A)-containing RNA and polysomal poly(A)-containing RNA were prepared from PRC II-transformed CEF. These RNAs were denatured with glyoxal and fractionated on a 1.2% neutral agarose gel in parallel with RNAs prepared in a similar fashion from RAV-2 avian leukosis virus-infected CEF and uninfected CEF. The RNAs were then transferred to diazotized paper and hybridized as described above. Figure 2A shows the hybridization pattern observed when we used a 32 P-labeled cDNA probe prepared from PRC II 70S virion RNA. A comparison between the virus-specific RNAs in RAV-2-infected CEF and the virus-specific RNAs in PRC II-infected CEF showed that both contained 7.5- and 2.9-kb RNA species that were capable of hybridizing with this probe. However, an additional band at 4.0 kb was

FIG. 2. Identification of PRC II helper-related and PRC II transformation-specific intracellular RNAs. Total cellular poly(A)-containing RNAs from PRC II-transformed CEF, RAV-2-infected CEF, and uninfected CEF and poly(A)-containing polysomal RNA from PRC II-transformed CEF were prepared as described in the text. The RNAs were denatured with glyoxal, fractionated on a 1.2% neutral agarose gel, and transferred to diazotized paper as described in the text. The blots were hybridized with a representative 32 P-labeled cDNA probe prepared from PRC II 70S virion RNA (A) and a 32 P-labeled nick-translated probe prepared from a λ ST-FeSV DNA clone (B). Hybridizations were performed in a buffer containing 10% dextran sulfate at 41°C for 4 h (A) or at 37°C for 23 h (B). The blots were washed at the hybridization temperature as described in the text and were exposed to Kodak X-Omat R film with an intensifying screen for 1.5 h (A) and 2 weeks (B). Sizes were extrapolated from RNA of the Schmidt-Ruppin strain of RSV subgroup D run on the same gels, assuming sizes of 9.5, 4.8, and 2.8 kb for genomic, *env*, and *src* mRNA's, respectively (D. Schwartz, personal communication). (A) Lane 1, 2.5 μ g of PRC II whole-cell RNA; lane 2, 2.5 μ g of RAV-2 whole-cell RNA; lane 3, 1.0 μ g of uninfected whole-cell RNA. (B) Lane 1, 0.1 μ g of PRC II 70S virion RNA; lane 2, 2.0 μ g of PRC II polysomal RNA; lane 3, 2.5 μ g of PRC II whole-cell RNA; lane 4, 1.5 μ g of RAV-2 whole-cell RNA; lane 5, 1.0 μ g of uninfected whole-cell RNA.

observed exclusively in the RNA from PRC II-transformed CEF.

A similar blot was hybridized with a ³²P-labeled probe prepared by nick-translating a recombinant DNA clone containing an insertion of the entire ST-FeSV genome. Since the PRC II and FeSV genomes share some common transformation-specific sequences but lack homology in other regions of the genome (Beemon, unpublished data), we expected that this probe would recognize only those mRNA's which were specific to the PRC II genome. As Fig. 2B shows, the FeSV probe hybridized to only the 4.0-kb RNA in polysomal and total cellular RNA preparations from PRC II-transformed CEF. The 4.0-kb RNA in preparations from PRC II-transformed CEF was identical in size to the smaller RNA in denatured PRC II 70S virion RNA. No PRC II-specific subgenomic mRNA's were detected with either the PRC II probe, the FeSV probe, or with a probe prepared from a recombinant plasmid (p 53) containing a 925-bp insert consisting of the 5'- and 3'-terminal sequences of the Prague-B RSV genome (data not shown). On the basis of this analysis, we concluded that the 7.5- and 2.9-kb RNAs observed in both RAV-2- and PRC II-infected CEF represented the helper leukemia virus genomic size and *env* mRNA's, respectively. In addition, we tentatively concluded that the PRC II genome was expressed in infected CEF as one major RNA species 4.0 kb long.

PRC II transforming protein synthesized in vivo and in vitro consists of two highly related components. We wanted to determine whether the PRC II genome contained coding information for polypeptides other than P105. To do this, we utilized the method of in vitro translation of virion RNA, which has been useful for identifying potential transforming proteins of RSV (7, 27, 28). PRC II 70S virion RNA was heat denatured, and the poly(A)-containing RNA was selected for fractionation on neutral sucrose gradients. Each gradient fraction was translated in vitro in a micrococcal nuclease-treated rabbit reticulocyte lysate, and the protein products were resolved by SDS-polyacrylamide gel electrophoresis. Figure 3 shows that the poly(A)-containing virion RNA contained two predominant messenger activities, one coding for a heterogeneous band of approximately 76K and a second coding for a similarly heterogeneous band which migrated in the 105K range. The 76K polypeptide was synthesized primarily from RNA which sedimented at 35S, whereas the 105K protein was made from 25 to 27S RNA. Tryptic peptide mapping of the [³⁵S]methionine-labeled 76K polypeptide synthesized in vitro showed a pattern similar to the patterns which we have observed previously for Pr76^{gag} en-

coded by the avian leukemia or sarcoma virus genome (1, 6). Under certain conditions of electrophoresis, it was possible to separate the heterogeneous 76K band into two components with apparent molecular weights of 80,000 and 76,000. The tryptic peptide pattern of the 80K protein was identical to that of the 76K protein (data not shown). It seemed reasonable that these two polypeptides represented translation products of different natural helper virus genomes present in the PRC II stock. In support of this hypothesis, we found that infection of CEF with limiting dilutions of the PRC II virus stock produced one transformed clone which contained only a single 76K polypeptide (see below and Fig. 6, lane 4).

The heterogeneous band in the 105K range could also be resolved into two major components with apparent molecular weights of 110,000 and 105,000. The tryptic peptide digestion pattern of the 110K protein was identical to that of the 105K protein (Fig. 4). The tryptic peptide maps of these two in vitro-synthesized proteins appeared to be highly related to the map of the P105 protein immunoprecipitated from PRC II-transformed CEF (Fig. 4A). Mixing experiments with P105 synthesized in vivo and either 110K or 105K synthesized in vitro confirmed the identity of the in vitro and in vivo products (data not shown).

The observation of a 105K doublet synthesized in vitro prompted more careful scrutiny of the in vivo-synthesized P105. Side-by-side electrophoresis of the in vivo- and in vitro-synthesized proteins demonstrated that the in vivo product could be resolved into two major components which comigrated with their in vitro counterparts (Fig. 5). However, one striking difference between the in vivo- and in vitro-generated doublets was the relative labeling intensities of the two components. The major product synthesized in vitro was the 105K polypeptide, whereas the relative amounts of the 110K and 105K proteins synthesized in vivo under steady-state labeling conditions appeared to be approximately equal.

In addition to the 76K and 105K doublets, a number of smaller polypeptides were synthesized primarily from RNA in the 25 to 27S region of the gradient (for example, the 42K protein doublet synthesized from messenger activity which cosedimented with the 110K and 105K messenger activities). It was possible to assign these proteins to one of two distinct classes, each containing helper virus structural protein tryptic peptides (data not shown). Members of one class contained only *gag*-related tryptic peptides and presumably resulted from premature termination of translation within the *gag* portions of the PRC II genome. The second class

FIG. 3. In vitro translation of PRC II virion RNA. The poly(A)-containing fraction of heat-denatured PRC II 70S virion RNA was denatured with dimethyl sulfoxide as described in the text, loaded onto a 10 to 30% sucrose gradient, and spun in a Beckman SW41 rotor at 25,000 rpm for 16 h at 22°C. The RNA in each gradient fraction was then translated in a messenger-dependent rabbit reticulocyte lysate in the presence of [35 S]methionine, and the products were resolved on a 12.5% acrylamide slab gel. The position of 35S RNA was extrapolated from 28 and 18S rRNA's run in a parallel gradient.

of proteins contained *pol* peptides in addition to some *gag* peptides. We did not detect any *fps*-specific tryptic peptides in proteins from either of these classes.

Another polypeptide of approximately 34K was synthesized from RNA in the 18S region of the gradient. Tryptic peptide mapping of this protein demonstrated that it was identical to the 34K protein which has been described previously as the translation product of a cellular mRNA packaged into the virions of most avian sarcoma and leukosis viruses (1).

We expected that any internal coding sequences within the PRC II genome would be

expressed as polypeptides translated from fragmented RNAs with sedimentation values less than 25S. However, aside from the smaller polypeptides described above, no additional translation products were observed. From these results, it seemed probable that the P105-related proteins were the major, if not the only, translation products of the PRC II genome. In addition, it appeared that the transforming protein, which has been referred to previously as a single species (P105), actually consists of two major electrophoretically distinct components of 110K and 105K.

P110 and P105 are both present in PRC II-

FIG. 4. Tryptic peptide mapping of in vivo- and in vitro-synthesized P110 and P105. PRC II-transformed CEF were labeled with [^{35}S]methionine and immunoprecipitated with anti-p19 serum. Heat-denatured PRC II 70S virion RNA was translated in a messenger-dependent lysate as described in the legend to Fig. 3. The P110 and P105 proteins were identified by preparative gel electrophoresis, removed from the gel, and digested with trypsin. The products were resolved on cellulose thin-layer plates by electrophoresis at pH 4.7 toward the cathode, with the origin on the left, followed by ascending chromatography from bottom to top. (A) In vivo-synthesized P110 and P105. (B) In vitro-synthesized P110. (C) In vitro-synthesized P105.

transformed, nonproducer clones. It was important to determine whether P110 and P105 were synthesized independently from distinct RNA genomes in transformed cells or whether one protein was produced by modification or processing of the other. If P110 and P105 were synthesized from different genomic RNAs, infection of CEF with limiting dilutions of a PRC II virus stock should have resulted in the segregation of two separate genomes. To test this possibility, we prepared nonproducer clones by infecting CEF with limiting dilutions of the PRC II virus stock. These cells were then plated in soft agar, and individual foci were isolated after 10 days and after 2 weeks. Each clone was grown up separately, labeled with [^{35}S]methionine and $^{32}\text{P}_i$, and immunoprecipitated with anti-p19 serum. From a total of nine foci picked, one was completely negative for the expression of either helper or transformation-specific viral proteins. This focus presumably represented a clump of cells none of which had been infected and conveniently acted as an uninfected control (Fig. 6, lane 8). Among the remaining eight clones, one had clearly received both a helper virus genome and a transforming genome since both the P105 complex (P110 and P105) and Pr76^{gag} were present (Fig. 6, lane 4). However, seven of the foci picked consisted of clones of nonproducer cells since Pr76^{gag} was absent from these preparations. In each of these seven clones, it was evident that P110 and P105 were present in approximately equimolar quantities. Therefore, it seemed unlikely that P110 and P105 were synthesized from different RNAs present

in our PRC II virus stock. From these experiments, we tentatively concluded that P110 and P105 shared a common origin in that a single species of the PRC II transforming genome in nonproducer cells appeared to express both proteins.

P105, the primary translation product of the PRC II genome, is a precursor to P110. We were interested in determining whether a precursor-product relationship existed between the P110 and P105 proteins observed in vivo. To address this question, PRC II-transformed CEF were pulse-labeled for 5 min with [^{35}S]methionine and then chased with medium containing an excess of cold methionine for varying time intervals. The cells were then lysed, immunoprecipitated with anti-p19 serum, and analyzed by SDS-polyacrylamide gel electrophoresis. As Fig. 7 shows, the major translation product observed with a 5-min pulse was the lower member of the doublet, P105. The upper component, P110, began to appear after a 10-min chase, and after 30 min of chase time P110 and P105 were present in essentially equimolar amounts. This ratio was also observed under conditions of long-term labeling, as shown in Fig. 7, lane S. In addition, pulse-chase experiments in which the chase was performed for periods as long as 20 h revealed that P110 and P105 had roughly equal half-lives (data not shown). These results suggested that approximately 50% of the newly synthesized P105 in transformed cells was modified post-translationally to give rise to P110.

To investigate the possibility that P110 resulted from the modification of a specific region of

FIG. 5. Comparison of in vivo- and in vitro-synthesized P110 and P105 by polyacrylamide gel electrophoresis. PRC II-transformed CEF were labeled for 16 h with [35 S]methionine as described in the text. The cells were washed, lysed, and immunoprecipitated with anti-p19 serum. Heat-denatured PRC II 70S virion RNA was translated in a messenger-dependent rabbit reticulocyte lysate at a final concentration of 25 μ g/ml in the presence of [35 S]methionine. The proteins were resolved on a 12.5% polyacrylamide gel. Lane 1, [35 S]-labeled PRC II CEF immunoprecipitated with anti-p19 serum; lane 2, [35 S]methionine-labeled in vitro translation products of PRC II 70S viral RNA; lane 3, [35 S]methionine-labeled in vitro translation products with no added RNA.

P105, we subjected 35 S-labeled P110 and P105 to partial digestion with *S. aureus* protease V8. Figure 8 shows that most of the smaller digestion products were identical for P110 and P105. However, the largest partial digestion products of these proteins did not comigrate and had apparent molecular weights of 83,000 and 77,000 for P110 and P105, respectively. This result suggested that the modification of P105 which generated P110 probably occurred within a 77K portion of the protein. Preliminary evidence from the V8

digestion patterns of P110, P105, and Pr76^{gag} labeled at the amino terminus by in vitro synthesis with [35 S]formyl methionyl-tRNA^{met} suggested that the modified region does not include the extreme amino terminus.

We attempted to test whether this modification was the result of glycosylation by using (i) treatment of immunoprecipitates with endoglycosidase H and (ii) treatment of PRC II-transformed CEF with tunicamycin. In neither case did we detect a change in the relative representation of P110 and P105 (data not shown). This was in good agreement with the results obtained by Neil et al. (24), whose attempts to label P105 with [3 H]glucosamine or [3 H]mannose were unsuccessful. Although we could not exclude the possibility that some sugars are present in linkages not affected by endoglycosidase H or tunicamycin treatment, it seemed unlikely that the observed change in electrophoretic mobility was due to glycosylation.

It has been reported that P105 is phosphorylated in vivo at several sites and contains both phosphoserine and phosphotyrosine (5, 26). This suggested the possibility that additional phosphorylation of a portion of the P105 molecules resulted in an increase in apparent molecular weight, producing the protein which we called P110. In general, we observed that P110 from 32 P-labeled PRC II-infected CEF was more highly phosphorylated than P105 on a molar basis. A preliminary analysis of the phosphoamino acid contents of separated P110 and P105 indicated that each protein contained both phosphoserine and phosphotyrosine. However, the ratios of phosphoserine to phosphotyrosine appeared to be different in that P110 was more highly phosphorylated at tyrosine residues than P105. We are presently continuing these investigations by examining the phosphotryptic peptides of P105 and P110.

Although the precise nature of the modification is still unclear, we concluded that P110 arises by a post-translational modification of P105 and that this modification occurs within a 77K portion of P105.

Kinase activity associated with the in vitro translation products of PRC II viral RNA. PRC II P105 synthesized in vivo and immunoprecipitated with either anti-p19 serum or RSV TBR serum has been shown to be associated with protein kinase activity, which results in phosphorylation of P105 or the heavy chain of immunoglobulin G or both (5, 26). We were interested in determining whether the P105 complex (that is, P110 and P105) synthesized in vitro would likewise demonstrate phosphotransferase activity in the immune complex. To explore this possibility, PRC II denatured 70S virion RNA was translated in vitro in the rabbit reticulocyte

FIG. 6. P110 and P105 immunoprecipitated from chick nonproducer cells infected with PRC II. Subconfluent cultures of CEF were infected with serial dilutions of the PRC II virus stock for 1 h at 37°C. The cells were then trypsinized, replated in 1.2% agar, and placed at 37°C for 1 to 2 weeks. Individual foci were picked with a drawn-out Pasteur pipette and transferred to 17-mm dishes containing 5×10^4 uninfected CEF. Subsequently, the cultures were transferred to duplicate plates; one of these plates was labeled for 16 h with [^{35}S]methionine, and the other was labeled with $^{32}\text{P}_i$ for 16 h as described in the text. Lanes 2 through 10 contain anti-p19 serum immunoprecipitates from cultures of nine individual picked foci. Lane 1 contains an anti-p19 serum immunoprecipitate of a control culture of uninfected cells.

lysate. When TBR serum was used to immunoprecipitate the *in vitro* products in a buffer containing NP-40 as the only detergent, phosphorylation of the heavy chain of immunoglobulin was observed (Fig. 9, lane 1). The control reaction, in which no RNA was added to the translation mixture, showed a small degree of phosphorylation of the heavy chain. This residual activity was probably due to the fact that the TBR serum used cross-reacted with endogenous pp60^{c-src} in the rabbit reticulocyte lysate. When immune serum was preabsorbed with detergent-disrupted virus, immunoprecipitation of the P105 complex synthesized *in vitro* was prevented. Under these conditions, immunoprecipitation of the endogenous pp60^{c-src} was not affected. The level of incorporation into the

immunoglobulin heavy chain was the same in this case (Fig. 9, lane 2) as in the precipitations where no RNA was added (Fig. 9, lane 3). Quantitation of the amount of radioactivity in the heavy chain in each case revealed a five- to sevenfold enhancement of protein kinase activity in the sample in which P105 was present in the precipitate compared with the controls. A phosphoamino acid analysis of the heavy chain phosphorylated in immunoprecipitates of *in vitro*-synthesized P110 and P105 revealed that tyrosine was the only acceptor amino acid in this reaction (data not shown).

It is possible that our conditions for *in vitro* translation were not optimal for the synthesis of an enzymatically active P105 complex. We were not able to detect appreciable kinase activity

FIG. 7. Pulse-chase analysis of P110 and P105 in PRC II-transformed CEF. Five identical 35-mm plates of PRC II-transformed CEF were pulse-labeled for 5 min with 400 μ Ci of [35 S]methionine per ml in medium lacking methionine. The labeling medium was then replaced with the chase medium, which contained an excess of cold methionine. The chase was carried out for 0, 10, 30, 60, or 120 min as indicated above the respective lanes. At the end of each chase period, cell extracts were prepared and immunoprecipitated with anti-p19 serum as described in the text. Lanes S contains immunoprecipitates from PRC II-transformed CEF labeled for 16 h with [35 S]methionine. Lane P contains an immunoprecipitate from PRC II-transformed CEF labeled for 16 h with 32 P_i.

when immunoprecipitations of in vitro-synthesized proteins were performed with anti-p19 serum or with immunoprecipitation buffer which

contained SDS and deoxycholate or when the kinase buffer contained magnesium instead of manganese as the source of divalent cations, although these conditions worked well with in vivo-synthesized P105.

This demonstration of phosphotransferase ac-

FIG. 8. Partial proteolytic digestion of P110 and P105 with *S. aureus* protease V8. [35 S]methionine-labeled PRC II-transformed CEF were immunoprecipitated with anti-p19 serum, and P110 and P105 were identified on a 12.5% preparative acrylamide gel. The samples were prepared as described in the text and were subjected to proteolysis with 0, 1, 10, and 300 ng of *S. aureus* protease V8 as indicated. The partial proteolytic digestion products were resolved on a 15% acrylamide gel. Lanes A, P110; lanes B, P105.

FIG. 9. Kinase activity associated with the in vitro translation products of PRC II 70S virion RNA. Heat-denatured PRC II 70S virion RNA was translated in a messenger-dependent rabbit reticulocyte lysate, and the sample was diluted with NP-40 buffer and immunoprecipitated in the presence (lane 2) or absence (lane 1) of detergent-disrupted RSV virions. Protein kinase activity was assayed in immune complexes as described in the text. Lane 1, PRC II 70S virion RNA, unblocked TBR serum; lane 2, PRC II 70S virion RNA, blocked TBR serum; lane 3, no RNA, unblocked TBR serum. HC, indicates the immunoglobulin heavy chain, which was visualized by staining.

tivity associated with in vitro-synthesized P110 and P105 supported the hypothesis that the tyrosine-specific kinase activity observed in immune complexes containing in vivo-synthesized proteins was an intrinsic enzymatic property of P110 or P105 or both.

DISCUSSION

PRC II and FSV contain very closely related transformation-specific sequences and may have been generated by independent recombinations with the same or related cellular genes (35). However, these viruses are distinct from one another in that the FSV putative transforming protein (P140) is approximately 35,000 daltons larger than PRC II P105 and contains two or

three *fps*-specific [35 S]methionine-labeled tryptic peptides that are not found in P105 (5, 25). These observations may be explained in several ways. One possibility is that both PRC II and FSV contain *fps*-related sequences of similar or identical lengths but that in the case of FSV, more of this region is translated. Another possibility is that the amount of *fps*-related sequence obtained during the recombinational event or maintained thereafter is different for FSV and PRC II. In this paper, we have shown by a direct comparison of PRC II and FSV virion RNAs that the FSV genome is approximately 800 bases larger than the PRC II genome. This difference in size between the PRC II and FSV RNA genomes is consistent with the molecular weight differences previously observed between P140 and P105. It would be interesting to determine whether the FSV information that is not found in PRC II is of viral or cellular origin and where these additional sequences map relative to the 5'- and 3'-ends of the acquired cellular sequences.

It has been reported that the unique region of FSV RNA is partially homologous to the genomes of ST-FeSV and Gardner-Arnstein FeSV (35). Since PRC II and FSV are related, we were able to use a molecular clone of ST-FeSV DNA as a probe for PRC II-related RNA sequences in transformed cells. The fact that hybridization was detected directly demonstrated that PRC II is at least partially homologous to ST-FeSV and enabled us to determine that the PRC II genome is expressed in transformed CEF as a single full-length RNA species of 4.0 kb. It is important to point out the possibility that additional species of PRC II-specific RNA might be present in transformed cells, but such species were not detected with any of the probes that we used. Therefore, it seems very likely that transformed cells do not contain PRC II-specific subgenomic mRNA's. The 4.0-kb RNA in PRC II-transformed CEF comigrates with the smaller of the two RNAs found in PRC II virion RNA. In addition, the 4.0-kb PRC II intracellular RNA is approximately the size expected for an RNA of 25 to 27S, which we have shown to be the peak sedimentation value in virion RNA preparations for the messenger activity for P110 and P105. Therefore, it is probable that a single PRC II-specific RNA or two slightly different RNAs having similar molecular weights are translated in vivo to yield P110 and P105. The absence of any smaller, subgenomic RNAs in transformed cells makes it unlikely that transformation-specific proteins other than P110 and P105 are expressed as primary translation products of the PRC II genome. Indeed, in vitro translation of poly(A)-containing RNAs prepared from denatured 70S PRC II virion RNA failed to reveal

any PRC II-specific polypeptides aside from P110 and P105. Since this technique has been used successfully to identify candidates for the transforming protein of RSV (7, 27, 28), the lack of *in vitro* messenger activity for PRC II-specific polypeptides other than P110 and P105 supports the hypothesis that these proteins are the only transformation-specific proteins synthesized *in vivo*.

We attempted to identify both the differences and the similarities between P110 and P105. The tryptic peptide maps of [³⁵S]methionine-labeled P110 and P105 synthesized *in vitro* were indistinguishable from one another and were identical to the maps of the *in vivo*-synthesized products. A side-by-side comparison by one-dimensional polyacrylamide gel electrophoresis demonstrated the comigration of the *in vivo*- and *in vitro*-synthesized P110 and P105 proteins. However, the ratio of [³⁵S]methionine label incorporated into P110 and P105 was not the same *in vivo* and *in vitro*. The products synthesized *in vitro* reproducibly showed a preferential labeling of P105. In contrast, when PRC II-transformed cells were labeled to steady state with [³⁵S]methionine and immunoprecipitated with anti-p19 serum, P110 and P105 were labeled to approximately the same extent, suggesting a 1:1 molar ratio between the two proteins. We considered two possible explanations which could account for the synthesis both *in vivo* and *in vitro* of the two very closely related proteins, P110 and P105. The first possibility was that P110 and P105 were synthesized from different RNA species present in our uncloned stock of PRC II. If present, these different RNA species could represent either independent recombination events between the helper virus genome and the same cellular sequences or, alternatively, a divergence of the parental transforming PRC II genome after the recombinational event. Whatever the potential origin, we reasoned that it should be possible to separate different genomes by isolating transformed clones made by infecting CEF with limiting dilutions of our PRC II stock. Clones prepared in this manner were examined for the presence of P110 and P105 in the absence of the other. All of the nonproducer clones analyzed contained both P110 and P105. This result suggested that the two proteins were probably not synthesized from different RNA species. The alternative explanation was that P110 and P105 were related to each other in a precursor-product fashion (e.g., by post-translational modification or proteolytic cleavage). This possibility was tested by analyzing the accumulation of P110 and P105 in transformed cells which had been subjected to a brief pulse with [³⁵S]methionine and then chased for increasing periods of time. This experiment dem-

onstrated that P105 is the primary translation product of the PRC II genome and that within 10 to 15 min after synthesis of P105, some portion of the P105 molecules is converted to P110 by post-translational modification. From these experiments, we concluded that the PRC II genome is expressed in transformed CEF in the form of two very closely related proteins, P110 and P105, which are present at steady state in roughly equimolar amounts, and that P110 is derived by post-translational modification of P105.

The fact that the tryptic peptide maps of [³⁵S]methionine-labeled P110 and P105 were identical can be accounted for by any one of three possible explanations. The modification may be on a tryptic peptide which lacks methionine. Alternatively, the P110 population may be heterogeneous with respect to the sites of modification so that only a small percentage of the P110 molecules are modified at an individual tryptic peptide. In this case, the modified peptide would make a minor contribution to the total map. It is also possible that the modification might be destroyed during preparation of the samples for peptide mapping or that the modification might not result in a change of migration in this system. Although we have not been able to distinguish among these possibilities, we have shown that the partial proteolytic digestion patterns of [³⁵S]methionine-labeled P110 and P105, generated with *S. aureus* protease V8, are different. This analysis allowed us to assign at least one region of modification to a 77K dalton portion of the protein.

We have not been successful in identifying the type of modification which produces P110. The available evidence argues against glycosylation, but it is still possible that sugar residues are present in a form which has not been detected yet. The most likely possibility is that phosphorylation of P105 produces P110, perhaps at specific tyrosine residues. We are currently examining the phosphotryptic peptide maps of P110 and P105 to test this idea.

We have observed that the *gag*-related presumptive transforming proteins encoded by FSV (P140), ST-FeSV (P85), and Y73 (P90) are also present in transformed cells as two electrophoretically distinct forms (Beemon, unpublished data). It is not clear whether a functional significance can be assigned to the generation and maintenance of two very closely related proteins. However, it is conceivable that only one of the two forms has an associated enzymatic activity or that each of the forms can be localized to a different subcellular compartment. Using cell fractionation techniques, we are currently investigating the latter possibility.

Several laboratories have reported the exist-

tence of a tyrosine-specific protein kinase activity in immunoprecipitates containing in vivo-synthesized PRC II P105 (5, 26). The substrates for this kinase activity include P105 or the heavy chain of immunoglobulin or both, depending on the antiserum used for immunoprecipitation. Although these data suggest that PRC II P105 is the protein kinase responsible for phosphotransferase activity in the immune complex, the possibility that the activity is due to an associated cellular protein kinase which immunoprecipitates with P105 has not been ruled out. We have demonstrated in this study that immunoprecipitates of the in vitro translation products of PRC II 70S virion RNA likewise contain a protein kinase activity capable of phosphorylating the heavy chain of immunoglobulin at tyrosine residues. This activity could be detected only if NP-40 buffer and TBR serum were used for immunoprecipitation. Little if any phosphorylation of P105 or P110 was observed, suggesting that the in vitro-synthesized proteins are poor substrates for phosphorylation. Although less likely in this case, it is still possible that the protein kinase activity that we have observed is due to coprecipitation of in vitro-synthesized P110 and P105 with an endogenous kinase from the rabbit reticulocyte lysate used for translation. It will be necessary to demonstrate kinase activity in purified preparations of P110 and P105 to assign definitively an enzymatic function to one or both of these proteins. Nonetheless, our data are consistent with the proposal that the PRC II genome encodes a tyrosine-specific protein kinase.

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