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Characterization of Y73, an avian sarcoma virus: A unique transforming gene and its product, a phosphopolyprotein with protein kinase activity

(cell transformation/defectiveness/autophosphorylation/IgG heavy chain/phosphotyrosine)

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ABSTRACT **The Y73 strain of avian sarcoma virus recently isolated in Japan is defective in replication and is associated with subgroup A leukosis virus (YAV). The virus caused sarcoma but not acute leukosis when inoculated into chickens. Studies on the viral RNA showed that a 26S RNA, estimated to be 4.8 kilobases long, was Y73 viral RNA carrying a transforming gene. The 26S RNA has sequences in common with the RNA of an avian leukosis virus but no homology with the *src* gene sequence of avian sarcoma virus (ASV). Thus, Y73 has a unique sarcoma-inducing gene. A phosphorylated polyprotein of 90,000 daltons (p90) was immunoprecipitated from extracts of Y73-transformed chicken embryo cells by a variety of antisera reacting with *gag* gene products. When a bacteria-bound immunocomplex containing the p90 protein was incubated with [γ-^{32}P]ATP, the Y73-specific p90 and the IgG heavy chain were phosphorylated by a p90-associated protein kinase. The amino acid phosphorylated *in vitro* was exclusively tyrosine in both cases, whereas p90 phosphorylated *in vivo* contained phosphoserine as a major phospho amino acid with traces of phosphotyrosine and phosphothreonine.**

The locus of the transforming gene (designated as *src*) in the avian sarcoma virus (ASV) genome has been determined, and a phosphoprotein of 60,000 daltons (pp60^{src}) has been identified as the *src* gene product (1, 2). All well-characterized ASVs have been shown to have essentially the same *src* gene, which presumably is derived from a cellular gene (*sarc*) present in uninfected vertebrate cells (3–7).

Transforming genes distinct from the *src* gene of ASV have been found in the last 3 year in avian acute leukemia viruses, which induce myeloblastosis, erythroblastosis, and myelocytomatosis in chickens. The fact that some strains of these viruses can induce transformation of chicken embryo fibroblasts (CEFs) without involvement of the *src* gene indicates that the *src* gene is not the only cause of fibroblastic transformation (8–12). Therefore, it seems to be worthwhile to characterize more ASV strains of different origins to see whether they all contain the *src* gene or another transforming gene(s) that can induce sarcomas.

In this study, we analyzed Y73, a new strain of ASV that was isolated in 1978 from a transplantable tumor originally found in 1973 in a White Leghorn hen raised on a farm in Yamaguchi Prefecture (13). Despite the pathogenic similarity of Y73 with other ASV strains, its most striking feature is its similarity with acute leukemia viruses in being defective in replication and having a small genomic RNA that lacks the *src* gene sequence but encodes a polyprotein consisting of a *gag* gene-derived part and a Y73-specific portion (14–18). This report also presents evidence for the existence of a protein kinase that phosphorylates tyrosine and is associated with a polyprotein encoded by a transforming gene other than the *src* gene of ASV.

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MATERIAL AND METHODS

Cells and Viruses. Y73 was a gift from A. Sato and colleagues (Yamaguchi University) and was grown on CEFs. The methods used (*i*) for preparation of the Prague strain of Rous sarcoma virus (RSV) of subgroup C (PR-RSV-C), the Schmidt–Ruppin strain of RSV of subgroup A (SR-RSV-A), the Rous-associated virus-2 (RAV-2), and a transformation-defective (*td*) mutant of PR-RSV-C (*td*PR-RSV-C), and (*ii*) for focus assay and soft agar cloning of viruses and transformed cells have been described (19–23). Y73-associated virus (YAV) was isolated from a Y73 stock by the end point dilution method (24).

RNA Analysis. The methods used for preparation of ^{32}P-labeled viral RNA and its analysis on 2% agarose gel have been described (19). [^{32}P]cDNAsrc and [^{32}P]cDNAcom, which were specific and complementary to *src* and total sequences in RSV, respectively, were prepared as described by Stehelin *et al.* (3), except that DNase-digested calf thymus DNA was used as a random primer to the endogenous reverse transcription reaction.

[^{32}P]cDNA·RNA hybridization was achieved by incubation at 41°C in 50% (vol/vol) formamide solution containing 50 mM Tris·HCl (pH 7.5), 1 mM EDTA, and 0.6 M NaCl, and the hybridized fraction of cDNA was monitored by digestion with nuclease S1.

Analysis of Viral Proteins. [^{35}S]Methionine- and [^{32}P]orthophosphate-labeled cell extracts of infected cells were immunoprecipitated, and the resulting immunocomplexes were subjected to electrophoresis on sodium dodecyl sulfate/polyacrylamide gels (23) essentially as described by Brugge and Erikson (2). Antiserum from a tumor-bearing rabbit induced by SR-RSV (TBR-B4) was prepared by the procedure of Brugge and Erikson (2). Rabbit antiserum prepared against disrupted RAV-2 was provided by T. Koyama. Protein kinase activity was determined in immunoprecipitates by the procedure of Rübsamen *et al.* (25).

Abbreviations: ASV, avian sarcoma virus; RSV, Rous sarcoma virus; RAV, Rous-associated virus; CEF, chicken embryo fibroblast; Y73NP, Y73-transformed non-virus-producing clones; YAV, Y73-associated virus; TBR, tumor-bearing rabbit; PR-RSV-C, Prague strain of RSV of subgroup C; SR-RSV-A, Schmidt–Ruppin strain of RSV of subgroup A; TBR-B4; antiserum from a TBR induced by SR-RSV; *td*, transformation-defective (mutant); kb, kilobase(s).

Analysis of Phosphorylated Proteins. The phosphorylated proteins extracted from bands were hydrolyzed at 110°C in 4 M HCl for 4 hr in sealed glass tubes. The hydrolysates were lyophilized and the residues were dissolved in 5 μl of electrophoresis buffer, consisting of formic acid/acetic acid/H_2O, 25:87:887 (vol/vol), and subjected to electrophoresis on cellulose thin-layer plates with marker phospho amino acids [phosphotyrosine synthesized by the method of Rothberg *et al.* (26), phosphoserine, and phosphothreonine] at pH 1.9. Ascending chromatography in the second dimension was performed in isobutyric acid/0.5 M NH_4OH, 5:3 (vol/vol). The phospho amino acid markers and ^{32}P-labeled compounds were detected as described (27).

RESULTS

Pathogenic Features of Y73 ASV. Y73 induced characteristic transformed cell foci mainly composed of fusiform cells, and infected CEFs formed rather compact colonies in soft agar.

Most transformed cell colonies isolated from soft agar cultures infected with highly diluted stocks were non-virus-producers (Y73NP). The virus rescued from Y73NP with subgroup B helper virus showed a strictly subgroup B host range, although the original Y73 belonged to subgroup A. These findings confirmed the previous conclusion that Y73 is defective in virus replication. When inoculated intramuscularly or subcutaneously into 7-day-old chickens, it induced fibro- or myxofibrosarcomas at the site of inoculation. When inoculated intravenously, it also induced sarcomas in various organs, but no leukosis of any type was observed within 1 month after inoculation.

Y73 ASV Contains 26S RNA As Viral Genome. ^{32}P-labeled viral RNA was extracted from virions semipurified from the culture fluid of Y73-producing cells and analysed by electrophoresis on 2% (wt/vol) agarose gel (Fig. 1). Most of the poly(A)-containing RNA moved as high molecular weight RNA. Heat-denaturation of the poly(A)-containing RNA gave two components: large RNA, with similar mobility to 35S RNA of *td*PR-RSV-C, and small RNA, which was smaller than 28S rRNA. The smaller RNA sedimented at 26S. Because the viral RNA analysed was extracted from a mixture of Y73 and YAV, it can be concluded that the 26S RNA, estimated to be 4.8 kilobases (kb) long, was viral RNA carrying a transforming gene of Y73 and that 35S RNA was the genome of YAV. A molecular size of 4.8 kb is one of the smallest ever observed for a genomic RNA of an RNA tumor virus.

The 26S RNA Does Not Contain the *src* Gene Sequence. Next we examined whether Y73 RNA contained a transforming gene related to *src* of ASVs. [^{32}P]cDNAsrc prepared from PR-RSV-C did not hybridize significantly with Y73-26S RNA (Fig. 2). In a parallel experiment, however, this cDNAsrc preparation hybridized efficiently with RNA of PR-RSV-C. On the other hand, cDNAcom, representing the viral replication unit, hybridized efficiently with the same preparation of Y73-26S RNA. These results clearly demonstrate that the 26S component of Y73 did not contain homologous sequences to those of *src* of ASVs. Thus, it is concluded that the transforming gene of Y73 is not related to the *src* of ASVs.

Y73-Transformed Cells Contain a Specific Polyprotein of 90,000 Daltons. The hybridization experiment described above suggested that Y73 has a transforming gene distinct from the *src* gene of known ASVs. To identify the polypeptide(s) encoded by the Y73 gene(s), Y73-transformed and YAV-infected cells were labeled with [^{35}S]methionine and the cell extracts were immunoprecipitated with TBR-B4 serum, which can detect the *gag*, *env*, and *src* gene products of ASVs. The resulting immunocomplexes were then subjected to sodium dodecyl sulfate/polyacrylamide gel electrophoresis. Fig. 3 shows that the immunoprecipitates from Y73-transformed cell extracts contained a polypeptide (indicated by an arrow) that was not found in YAV-infected cells. Because this polypeptide migrated slightly faster than marker phosphorylase *a* (94,000 daltons), this Y73-specific protein was named "p90." Immunoprecipitation of this polypeptide was greatly reduced by preabsorption of the TBR-B4 serum with RAV-2, which suggests that the polypeptide contains viral structural protein(s) or a polypeptide sequence antigenically similar to viral structural protein(s).

The p90 was also found in [^{35}S]methionine-labeled extracts

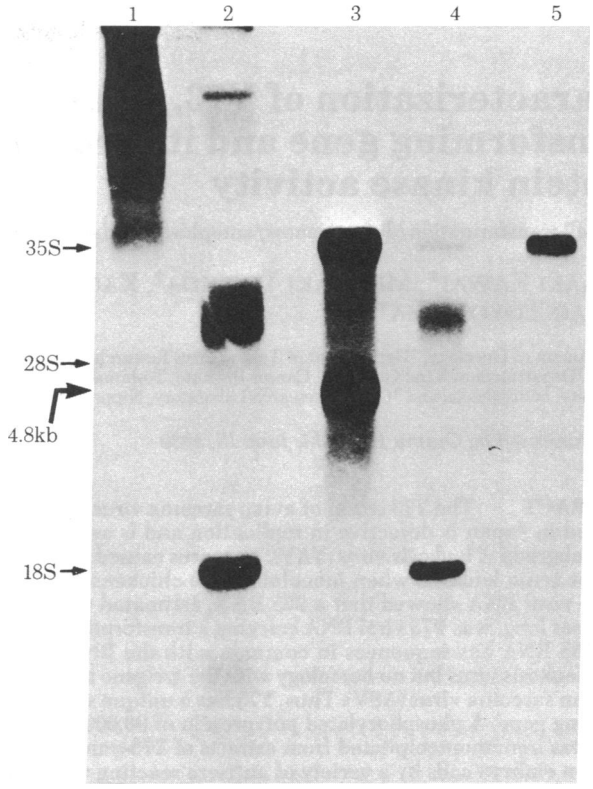

FIG. 1. Agarose gel electrophoresis of [^{32}P]RNA isolated from Y73 virions. [^{32}P]RNA from Y73 was separated into poly(A)-containing (lanes 1 and 3) and poly(A)-lacking (lanes 2 and 4) fractions on an oligo(dT)-cellulose column, and the RNAs were analyzed on 2% agarose gel before (lanes 1 and 2) and after (lanes 3 and 4) heat treatment at 100°C for 1 min. [^{32}P]RNA of *td*PR-RSV-C (lane 5) was run as a marker of 35S viral RNA.

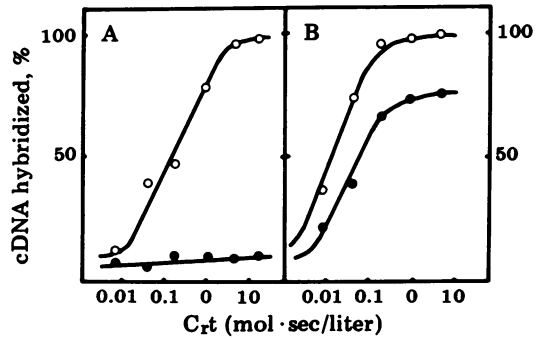

FIG. 2. Hybridization kinetics of cDNAsrc of RSV and cDNAcom (representing transformation-defective viral RNA) with the 26S RNA component of Y73. [^{32}P]cDNAsrc (600 cpm) (*A*) and [^{32}P]cDNAcom (2800 cpm) (*B*) were hybridized with various amounts of 26S RNA of Y73 virions (●) and with 35S RNA of PR-RSV-C (○).

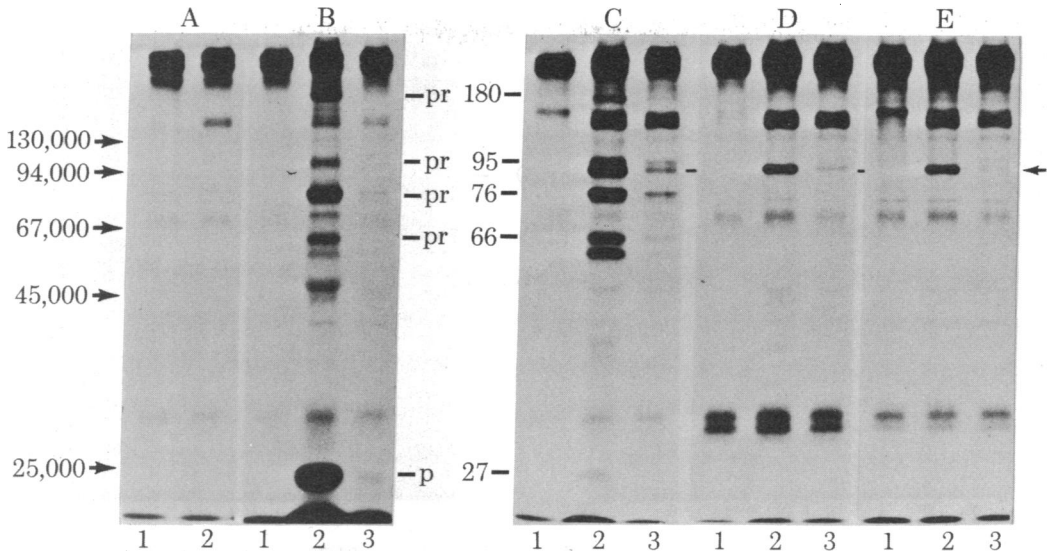

FIG. 3. Immunoprecipitates of ^{35}S-labeled extracts from uninfected, YAV-infected, and Y73-transformed chicken embryo cells. Extracts of uninfected (*A*), YAV-infected (*B*), and Y73-transformed (*C*) cells, and of Y73NP clone 1 (*D*) and clone 2 (*E*) were immunoprecipitated with rabbit nonimmune serum (lane 1), antiserum from a rabbit bearing an SR-RSV-induced sarcoma (TBR-B4) (lane 2), and TBR-B4 preabsorbed with disrupted RAV-2 virion protein (lane 3). The resulting immunoprecipitates were subjected to 10% (wt/vol) polyacrylamide gel electrophoresis. The procedures for immunoprecipitation and gel electrophoresis have been described (23). Virus-specific proteins are indicated in the center margin. The positions of molecular weight markers are indicated by arrows on the left.

of Y73NP clones (Fig. 3 *D* and *E*). No other protein was specifically immunoprecipitated with the TBR-B4 serum from the lysate of these Y73NP clones. The p90 was not immunoprecipitated with a TBR serum that could react with p27 but not with p19 (not shown). These findings suggest that the p90 contains p19 but not p27.

p90 Is a Phosphoprotein. Uninfected, YAV-infected, and Y73-transformed CEF cultures and a Y73NP clone were labeled with [^{32}P]orthophosphate and the cell extracts were immunoprecipitated with TBR-B4 serum. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis of the resulting immunoprecipitates revealed that the p90 in extracts from Y73-transformed and Y73NP cells was phosphorylated (Fig. 4). The *gag* gene products pr76 and pr66, present in YAV-infected and Y73-transformed cells, were also phosphorylated. Furthermore, pp60^{src} appeared to be immunoprecipitated from all these uninfected and infected cell cultures by this antiserum.

Recently Hunter and Sefton (28) showed that pp60^{src} of ASVs contains two phosphorylated amino acids (i.e., phosphoserine and phosphotyrosine). Although phosphotyrosine is very rare in normal cells, specific phosphorylation of tyrosine has been observed with several tumor viruses (29, 30). Therefore, the phosphorylated amino acid residue(s) in the Y73-specific p90 protein was determined. ^{32}P-Labeled p90 and pr76 were eluted from the gel and hydrolysed in 4 M HCl at 110°C, and the products were analysed by electrophoresis and chromatography on thin-layer cellulose. The major phospho amino acid of p90 was phosphoserine, with small amounts of both phosphothreonine and phosphotyrosine, whereas pr76 contained exclusively phosphoserine (see Fig. 6). Since the p90 appears to be a fused protein, consisting of a *gag*-related portion and a Y73-unique portion, most of the phosphoserine may have been derived from the *gag*-related portion, while phosphotyrosine was derived from the other part, which presumably bears the transforming function.

Protein Kinase Activity Is Associated with p90. Collett and Erikson (31) and others (25, 32) found that when the bacterially bound immunocomplex of pp60^{src} was incubated with [γ-^{32}P]ATP, the heavy chain of IgG was phosphorylated. But protein kinase activity has not been found to be associated with polyproteins of avian acute leukemia viruses (25, 32). Therefore, we investigated the capability of the p90 protein to phosphorylate the IgG heavy chain. Extracts of SR-RSV-A-transformed and Y73-transformed CEFs were immunoprecipitated with TBR-B4 serum, and the immunoprecipitates bound to *Staphylococcus aureus* were incubated with [γ-^{32}P]ATP. The heavy chain of IgG was phosphorylated to the same extent in both case (Fig. 5). This result suggests that a protein kinase, possibly associated with p90, was immunoprecipitated from the Y73-transformed cell extract. In addition to the phos-

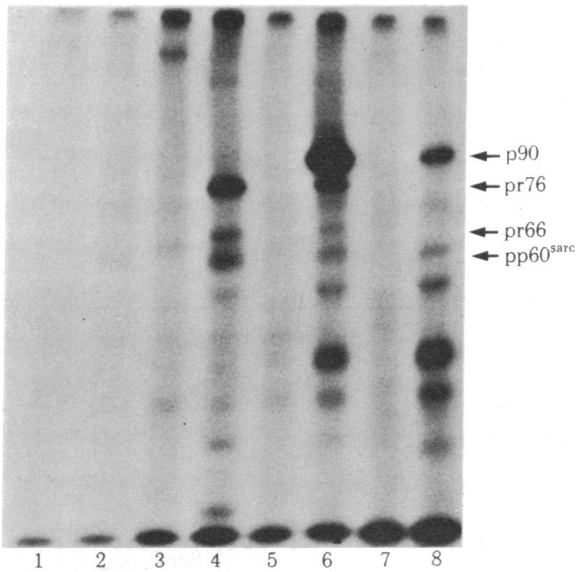

FIG. 4. Autoradiogram of immunoprecipitates of ^{32}P-labeled cell extracts from Y73-transformed cells. Lanes: 1 and 2, uninfected; 3 and 4, YAV-infected; 5 and 6, Y73-transformed; 7 and 8, Y73NP clone 3 cells, incubated in phosphate-free medium containing 5 mCi (1 Ci = 3.7×10^{10} becquerels) of [^{32}P]orthophosphate for 4 hr. Immunoprecipitation of the cell extracts with nonimmune serum (lanes 1, 3, 5, and 7) and TBR-B4 serum (lanes 2, 4, 6, and 8) and gel electrophoresis were carried out as described (23).

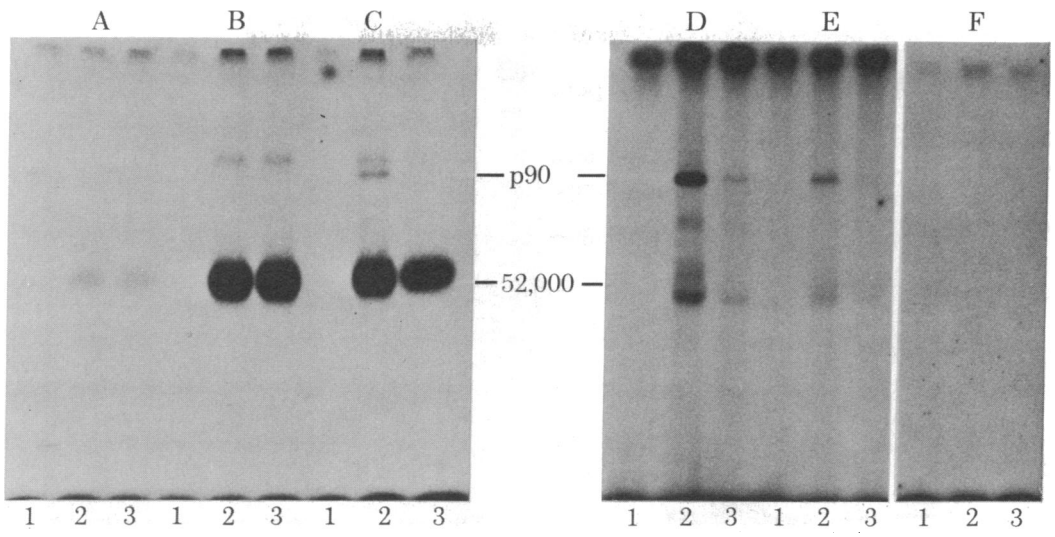

FIG. 5. Detection of p90-associated protein kinase activity in immunoprecipitates of Y73-transformed cells. Cell extracts of uninfected (*A*), SR-RSV-A-infected (*B* and *F*) and Y73-transformed (*C* and *D*) and Y73NP clone 3 (*E*) cells were prepared and immunoprecipitated with various antisera. Lanes in *A*, *B*, and *C*: 1, rabbit nonimmune serum; 2, TBR-B4; 3, TBR-B4 preabsorbed with RAV-2 virion protein. Lanes in *D*, *E*, and *F*: 1, rabbit nonimmune serum; 2, rabbit antiserum prepared against disrupted RAV-2 virion protein, unabsorbed; 3, same rabbit antiserum preabsorbed with RAV-2 virion protein. Immunoprecipitates were incubated with 0.2 μM [γ-^{32}P]ATP (New England Nuclear, 2000 Ci/mmol) as described by Rübsamen *et al.* (25).

phorylated protein of about 100,000 daltons present in both extracts, another protein of 90,000 daltons was specifically phosphorylated in the immunoprecipitate of the Y73-transformed cell extract. The fact that the amount of this phosphorylated protein was greatly reduced when RAV-2-preabsorbed TBR-B4 serum was used for immunoprecipitation strongly suggested that the protein phosphorylated *in vitro* was p90.

However, because the TBR-B4 serum also precipitated pp60^{src} from uninfected CEF extracts (Fig. 5), we used rabbit antiserum against RAV-2 whole virions instead of TBR-B4 serum to eliminate the effect of phosphorylation by pp60^{src}. The IgG heavy chain of the serum and Y73-specific p90 were likewise phosphorylated in the immunoprecipitates of extracts of Y73-infected CEFs or in a Y73NP clone (Fig. 5 *D* and *E*) but not in those of SR-RSV-A-transformed CEFs (Fig. 5*F*), uninfected CEFs, or AEV-transformed CEFs (not shown); this demonstrates that protein kinase activity was associated with the Y73-specific p90 and was capable of phosphorylating itself.

Phospho amino acids in the p90 and the IgG heavy chain of rabbit antiserum against whole virions were determined after

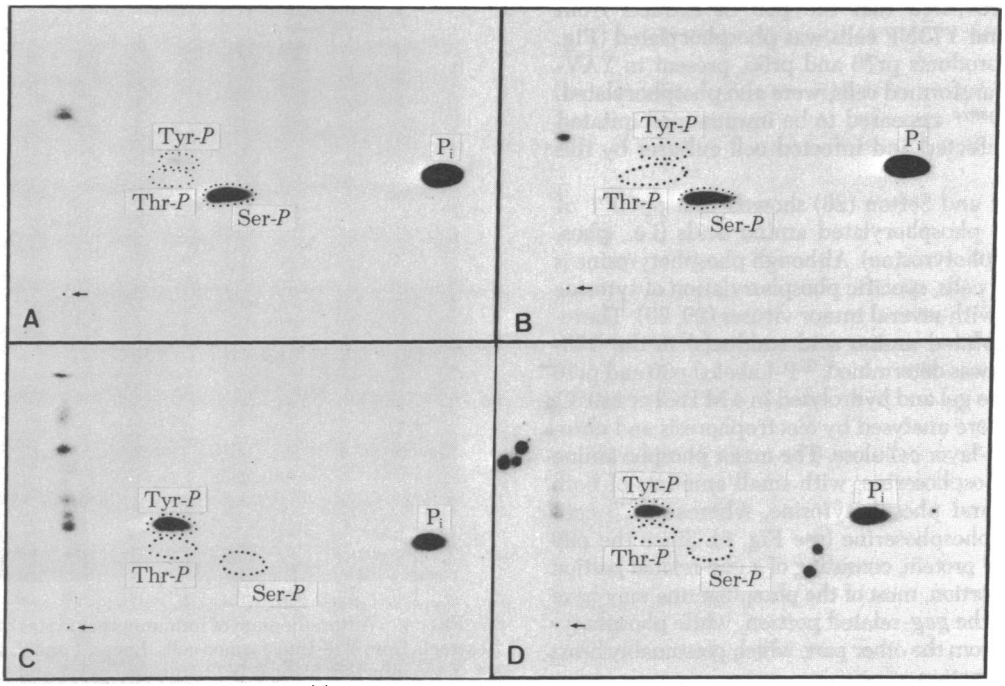

FIG. 6. Identification of phospho amino acids in phosphorylated proteins. Y73-specific p90 (*A*) and pr76 (*B*) of YAV phosphorylated *in vivo* were eluted from the gels shown in Fig. 4; p90 (*C*) and IgG heavy chain (*D*) phosphorylated in immunoprecipitates were eluted from the gels shown in Fig. 5 *D* and *E* and subjected to acid hydrolysis. The hydrolysates were then subjected to electrophoresis and chromatography. The origin is indicated by an arrow and the positions of the stained marker phospho amino acids (phosphoserine, Ser-*P*; phosphothreonine, Thr-*P*; phosphotyrosine, Tyr-*P*) are shown by broken lines.

phosphorylation *in vitro* by p90-associated protein kinase. In contrast to the phosphorylation of p90 *in vivo*, where phosphoserine was observed as a major product, the phosphorylations of both proteins yielded only phosphotyrosine (Fig. 6 *C* and *D*).

DISCUSSION

The Y73 strain of ASV was recently isolated from a transplantable chicken tumor of spontaneous origin. The virus induced sarcomas *in vivo* and transformation of fibroblasts *in vitro*, but it did not induce acute leukosis. The present study clearly demonstrates that Y73 contains a new type of sarcoma-inducing gene distinct from the *src* gene of other well-characterized ASVs.

Y73 was defective in replicating capacity and had 26S RNA as a transforming agent. The 26S RNA encodes a 90,000 polyprotein consisting of a *gag*-gene derived part and a Y73-specific portion. The fact that a protein kinase that specifically phosphorylated tyrosine, as observed with pp60^{src}, was associated with p90 strongly suggests that the p90 is a product of the transforming gene of Y73.

In the mechanism of cell transformation by tumor viruses, products of both the *src* gene of known ASVs and the Y73-transforming gene have protein kinase activity that specifically phosphorylates tyrosine residues, although these genes and their products are not related, according to molecular hybridization and immunological tests. The p90 resembles the transforming gene products of polyoma virus (29) and Abelson leukemia virus (30) more closely than that of the pp60^{src} of ASVs, because the two former gene products phosphorylate themselves through tyrosine residues *in vitro*, although phosphorylated tyrosine is scarcely observed in these products phosphorylated *in vivo*. In contrast, the pp60^{src}-associated protein kinase phosphorylates tyrosine in the IgG heavy chain, although it does not phosphorylate itself *in vitro*; but phosphorylated tyrosine is observed in pp60^{src} phosphorylated *in vivo*.

PRC II (33) and Fujinami sarcoma virus (34) are similar to Y73 in that they were isolated from spontaneous chicken tumors, are defective in replication, have much smaller genomic RNA than other nondefective strains of ASV, and produce specific polyproteins in infected cells. In addition, their genomes lack the *src* sequence (35–38). Therefore, it is interesting to examine whether these viruses share the second sarcoma-inducing gene and compose a second group of ASV or whether they contain different transforming genes.

By analogy with other ASVs and acute leukemia viruses (4, 5, 39, 40), it is very likely that normal cells contain a gene(s) related to the transforming gene of Y73. Indeed, this was shown to be the case in studies by the Southern transfer technique (unpublished data). This cellular gene(s) may play an important role in growth regulation of the cells. However, since it is unlikely that products of the cellular gene(s) also contain a *gag*-related portion like the viral counterparts, specific antiserum against the polypeptide specific to the respective transforming genes would be necessary for analysis of the nature and function of these cellular gene products and their relation with their viral counterparts.

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