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Genome Structure of Avian Sarcoma Virus Y73 and Unique Sequence Coding for Polyprotein p90

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The genome structure of a newly isolated sarcoma virus, Y73, was studied. Y73 is a defective, potent sarcomagenic virus and contains 4.8-kilobase (kb) RNA as its genome; in contrast, helper virus associated with Y73 had 8.5-kb RNA, similar to other avian leukemia viruses. Fingerprinting analysis of these RNAs demonstrated that the 4.8-kb RNA contains a specific RNA sequence of 2.5 kb, which represents the transforming gene (*yas*) of Y73. This specific sequence was mapped in the middle of the genome and had at both ends 1- to 1.5-kb sequences in common with Y73-associated virus RNA. This structure is very similar to those of avian and mammalian leukemia viruses. In vitro translation of the 4.8-kb RNA and the immunospecificity of the products directly demonstrated that polyprotein p90, containing p19, is a product translated from capped 4.8-kb RNA and that the specific peptide portion is coded by the *yas* sequence. Protein 90, which was also found in cells transformed with Y73, was suggested to be a transforming protein.

Among avian retroviruses, Rous sarcoma virus (RSV) has been most thoroughly studied because of its competency in replication and sarcomagenic transformation. The sarcoma gene (*src*) in RSV codes a protein, $p60^{src}$ (4, 21), which has protein kinase activity phosphorylating tyrosine residues in target proteins (5, 10). Recently, we (27) reported that a newly isolated avian sarcoma virus, Y73 (11), contains a sarcomagenic transforming gene (*yas*) showing no homology with the *src* sequence of RSV. Furthermore, a specific protein, p90, was found in Y73-transformed cells and shown to have protein kinase activity, which phosphorylated tyrosine residues in p90 itself and in heavy-chain molecules of immunoglobulin G complexed with p90 (13). Fujinami sarcoma virus (7) was also recently reported to have a distinct class of transforming gene (8, 16).

Independent isolates of avian sarcoma viruses RSV and B77 are competent in replication and have virtually the same genome structure: 5′ *gag pol env src* 3′ (26). On the other hand, all avian acute leukemia viruses are defective in replication, deleting the *pol* and *env* genes and part of the *gag* gene, instead of the region containing the transforming gene in the middle of the genome (3, 6, 9, 18, 22). Since Y73 is a sarcomagenic virus, but is defective in replication (11), it seemed interesting to determine its genome structure.

In this work, we characterized the Y73 genome by RNase T_1 fingerprinting analysis and in vitro translation of the genome RNA. Results showed that the structural features of the Y73 genome are essentially the same as those of genomes of other acute leukemia viruses and that polyprotein p90 is a gene product of the specific sequence (*yas*), suggesting that p90 is a transforming protein.

MATERIALS AND METHODS

Cells and viruses. Chicken embryo fibroblasts were prepared from C/O or C/BE chicken embryos and cultured as described before (28). Y73 viral stock was grown as described previously (13), and Y73-associated virus (YAV) was isolated by two successive limiting dilutions on chicken embryo fibroblasts and confirmed to have no focus-forming ability under the standard agar layer.

Viral preparations were harvested every 12 h and purified by precipitation with ammonium sulfate and by successive discontinuous and continuous sucrose gradient centrifugations as described previously (30). Labeling of viral RNA with [^{32}P]phosphate was carried out exactly as described previously (30).

Preparation of viral RNA. Virion RNA was extracted by proteinase treatment followed by phenol extraction (30). Virions were disrupted by adding 0.1 volume of 10% sodium dodecyl sulfate in the presence of 100 μg of proteinase K per ml. After incubation for 30 min at 37°C, RNA was extracted three times with phenol-chloroform (6:4) and then precipitated with 2 volumes of ethanol. RNA was dissolved in 0.1% sodium dodecyl sulfate and heated at 100°C for 1 min and then fractionated on a gradient of 15 to 30% sucrose containing 50 mM Tris-hydrochloride (pH 7.5), 0.1 M NaCl, and 1 mM EDTA by centrifugation at 45,000 rpm for 3 h in a Beckmann SW50.1 rotor at 15°C. In

some experiments, RNA containing polyadenylic acid [poly(A)] was separated by passing the preparation through a column of oligodeoxythymidylic acid-cellulose as described before (31).

Fingerprinting analysis of [^{32}P]RNA. Two-dimensional gel electrophoresis was used for fingerprinting RNase T_1-resistant oligonucleotides. The procedure was essentially as described by Lee and Wimmer (17). Oligonucleotides separated on gels were extracted with 0.5 ml of water, lyophilized, and digested with RNase A (0.1 mg/ml). The digests were analyzed by electrophoresis at pH 3.5 on DEAE-cellulose paper as described by Barrell (1).

In vitro translation of viral RNA. mRNA-dependent reticulocyte lysate was prepared as described by Pelham and Jackson (20) and used to translate Y73 RNA as described previously (29). Each reaction mixture (12 μl) contained 0.2 μg of viral RNA and 20 μCi of [^{35}S]methionine in addition to the standard reagents. Two microliters of the reaction mixture was used for gel electrophoresis in the buffer described by Laemmli (15), and the gels were dried for autoradiography or fluorography. Cells were labeled with [^{35}S]-methionine as described previously (31), and immunoprecipitation was carried out according to Kessler (14).

RESULTS

RNA of Y73. Since the original Y73 stock contained a large excess of YAV of subgroup A as helper (11), the transformed cells were cloned in soft agar medium. Viruses released from the selected producer cells were labeled with [^{32}P]-phosphate, and the RNA was analyzed by agarose gel electrophoresis. As shown in Fig. 1a, most of the RNA containing poly(A) migrated as high-molecular-weight aggregates, probably 50-70S dimers. After heat treatment at 100°C for 1 min, the aggregates were dissociated into two RNA species: 4.8-kilobase (kb) and 8.5-kb RNA components (Fig. 1b). The 4.8-kb RNA extracted from the gel sedimented as 26S on sucrose gradient centrifugation (Fig. 1e), and the RNA was smaller than those of known defective acute leukemia viruses (3, 6, 12, 18). On the other hand, 8.5-kb RNA sedimented as 35S, and its mobility in the gel was the same as that of RNA of transformation-defective RSV. From these results, and from the findings that nontransforming YAV isolated by limiting dilution contained only 8.5-kb RNA and that the 4.8-kb component has a specific sequence, as shown in this paper, we concluded that the 4.8-kb RNA species is the genome of Y73 carrying the sarcomagenic transforming gene.

Analysis of Y73 RNA by fingerprinting. The relationship between 4.8-kb and 8.5-kb RNAs was analyzed by fingerprinting RNase T_1-resistant oligonucleotides. ^{32}P-labeled 4.8-kb and 8.5-kb RNAs were separated by agarose gel electrophoresis and selected for poly(A)-containing RNA on oligodeoxythymidylic acid-cellulose. The fingerprint of the 8.5-kb RNA (Fig. 2D) showed that the helper 8.5-kb RNA was a single component, and as expected, the fingerprint of 4.8-kb RNA contained the specific oligonucleotides that were missing in that of 8.5-kb RNA,

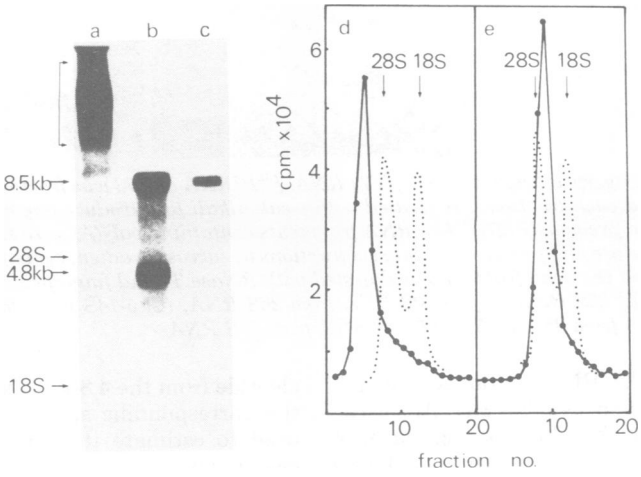

FIG. 1. *Analysis of Y73 RNA by agarose gel electrophoresis (a, b, c) and sucrose gradient centrifugation (d, e). [^{32}P]RNA of Y73 virions containing poly(A) before (a) and after (b) heat treatment at 100°C for 1 min and [^{32}P]RNA of transformation-defective Prague RSV were subjected to electrophoresis in 2% agarose gel in 40 mM Tris-hydrochloride (pH 8.0), 20 mM sodium acetate, and 1 mM EDTA. After electrophoresis, RNAs were extracted from the bands corresponding to 4.8-kb and 8.5-kb RNA and centrifuged through 15 to 30% sucrose gradients containing 20 mM Tris-hydrochloride (pH 7.5), 0.1 M NaCl, 1 mM EDTA, and 0.2% sodium dodecyl sulfate in an SW50.1 rotor at 45,000 rpm and 15°C for 180 min. (d) 8.5-kb RNA; (e) 4.8-kb RNA. Dotted lines are [^{3}H]rRNA added as internal markers..*

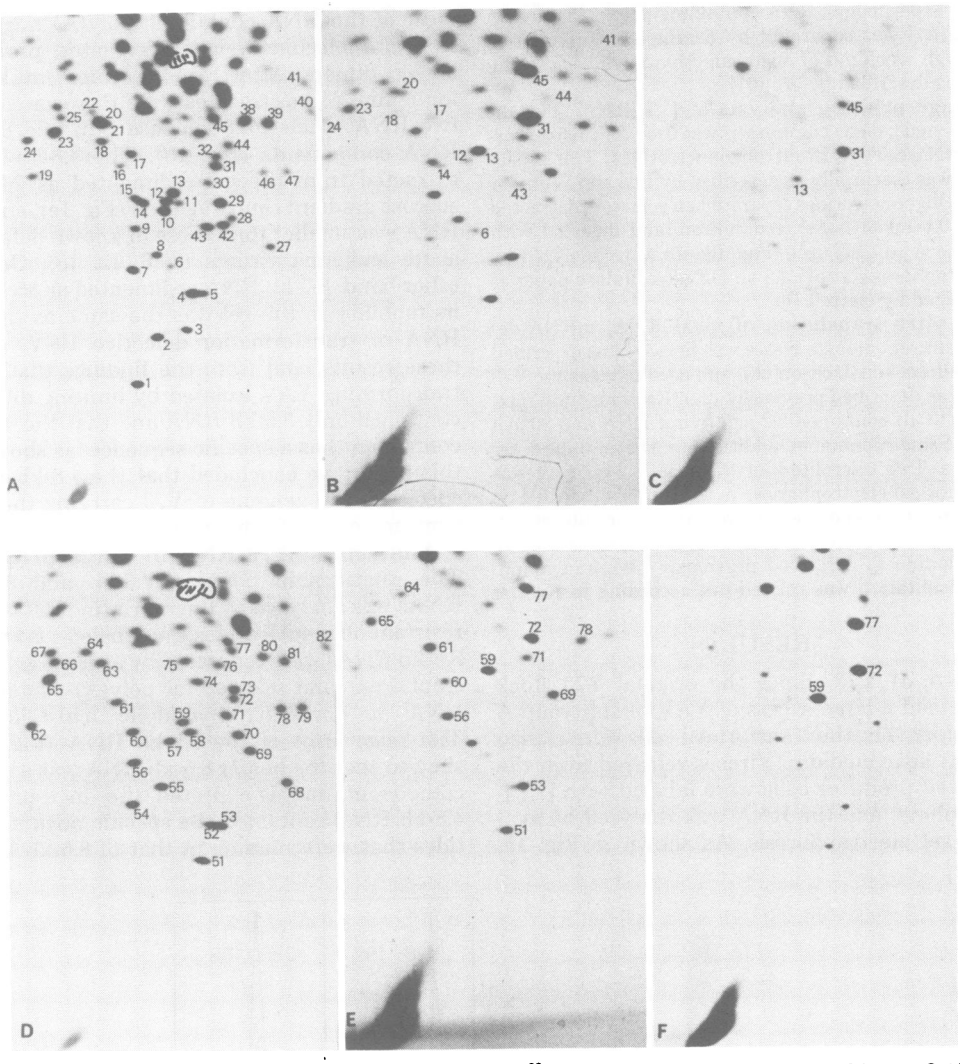

FIG. 2. *RNase T_1 fingerprinting analysis of Y73 RNA. [^{32}P]RNA of Y73 was fractionated into 26S (A) and 35S (D) fractions, and each fraction was treated with weak alkali to introduce one or two cuts in the RNA molecule, as described previously (27). Then RNA fragments containing poly(A) were separated on oligodeoxythymidylic acid-cellulose and fractionated into six fractions by sucrose gradient centrifugation. Each fraction was recentrifuged, and the peak fraction was digested with RNase T_1 and fingerprinted by two-dimensional gel electrophoresis. (A) 26S RNA; (B) 18-20S RNA from 26S RNA; (C) 5-14S RNA from 26S RNA; (D) 35S RNA; (E) 19-25S RNA from 35S RNA; (F) 5-14S RNA from 35S RNA.*

indicating that 4.8-kb RNA contained the specific nucleotide sequences (Fig. 2A). All of the oligonucleotide spots found in the fingerprint of 8.5-kb RNA were detected in that of 4.8-kb RNA, but most of them were minor components. These minor spots were probably contamination of the 4.8-kb RNA fraction with fragments of 8.5-kb RNA. To verify this explanation, each spot was cut out from the gels and quantitated by counting the radioactivity of ^{32}P. Since the accurate length of each oligonucleotide was not known, the ratio of the yield of each oligonucleotide from the 4.8-kb RNA fraction to that of the corresponding spot from 8.5-kb RNA was used to estimate the apparent molar yield of each oligonucleotide, assuming that pure 8.5-kb RNA gave each oligonucleotide in a molar ratio. In the case of spots specific to 4.8-kb RNA, the yield of oligonucleotides of 8.5-kb RNA which were similar in size was used to calculate the ratio. The results are summarized in Table 1. All spots were classified into three groups by their yield ratio, that is, less than 0.5, between 0.8 and 1.2, and more than 1.4. Oligonucleotides showing

TABLE 1. *Yields of RNase T_1-resistant oligonucleotides from 26S and 35S RNA fractions*

Spots in 26S RNA fraction		Spots in 35S RNA fraction		Ratio 26S/35S
No.	Yield (cpm)	No.	Yield (cpm)	
1	775			(1.03)[a]
2	736			(0.98)
3	295	51	753	0.39
4	985	52	643	1.50
5	291	53	779	0.37
6	126			(0.18)
7	978	54	702	1.39
8	230	55	502	0.46
9	201	56	491	0.41
10	745	57	553	1.35
11	168	58	441	0.38
12	494			(1.12)
13	779	59	562	1.38
14	556			(1.19)
15	192	60	466	0.41
16	139	61	379	0.36
17	353			(0.91)
18	320			(0.82)
19	185	62	388	0.47
23a+b	867	65	466	1.86
24	389			(1.08)
25	131	66	358	0.36
27	201	68	536	0.37
28	216	69	524	0.41
29	806	70	542	1.49
30	164	71	486	0.34
31	616	72	367	1.67
32	142	73	486	0.29
38	564	80	393	1.43
39	575	81	355	1.61
40	101	82	234	0.43
41	227			(0.97)
42	568			(1.06)
43	607			(1.13)
44	320			(0.83)
45	500	77	383	1.30
46	164	78	415	0.39
47	177	79	397	0.44

[a] The ratio of oligonucleotides specific to the 26S RNA fraction was calculated by using the yield (counts per minute; cpm) of oligonucleotides of 35S RNA, which are similar in size to the specific one; this ratio is shown in parentheses.

a ratio of 0.8 to 1.2 were all specific to 4.8-kb RNA, and all other oligonucleotides showing a ratio of less than 0.5 or more than 1.4 were confirmed by RNase A digestion to have the same nucleotide composition as the corresponding spots obtained from 8.5-kb RNA (data not shown). From these findings it was concluded that 22 oligonucleotides produced at a ratio of more than 1.4 (10 spots) and between 0.8 and 1.2 (12 spots) originated from 4.8-kb RNA and that the rest of the minor spots produced at a ratio of less than 0.5 represented 8.5-kb RNA fragments contaminating the 4.8-kb RNA fraction.

Spot no. 6 showed a very low yield although it was specific to the 4.8-kb RNA fraction. This spot might be explained by a microheterogeneity in the sequence of 4.8-kb RNA, but the reason is unknown. Thus, spot no. 6 was removed from the following considerations.

Analysis of the nucleotide composition of 22 oligonucleotides originating from 4.8-kb RNA suggested that 11 oligonucleotides were specific and produced in a ratio of 0.8 to 1.2 and that 10 oligonucleotides were shared with YAV 8.5-kb RNA and thus were produced at a ratio of more than 1.4 (Table 2). Assuming a random distribution of the oligonucleotides along the genome, the results suggest that about half the 4.8-kb sequence is specific to 4.8-kb RNA.

For determination of the location of the oligonucleotides specific to 4.8-kb RNA within the genome, RNA fragments containing poly(A) were fractionated and purified by sucrose gradient centrifugation, followed by recentrifugation of each fraction and fingerprinting (Fig. 2B and C). Although the 4.8-kb RNA fraction was contaminated with fragments of 8.5-kb RNA, only 22 spots, which were concluded to be produced from 4.8-kb RNA (Table 2), were followed, and the other spots were ignored. Thus, a tentative oligonucleotide map was constructed (Fig. 3). YAV 8.5-kb RNA was analyzed similarly (Fig. 2E and F), and the two oligonucleotide maps were compared. Ten oligonucleotides were common to both RNAs, and 4 of these 10 were

TABLE 2. *Qualitative analysis of RNase T_1 oligonucleotides of Y73 26S RNA and YAV 35S RNA*

Spot no. of Y73 26S RNA	Spot no. of YAV 35S RNA	RNase A digestion products
1		U, C, G
2		U, C, AC, G
4	52	U, C, AC, AU, AAG
6		U, C, AC, AU, AAAU, G
7	54	U, C, AC, AU, AAC, AAG
10	57	U, C, AAG, AAAU
12		U, C, AC, AG, AAAU
13	59	U, C, AU, AG, AAC, AAAC
14		U, C, AC, AU, AAC, G
17		U, C, AC, AU, AAC, AAU, G
18		C, AC, AU, AAC, AAU, AAAC, G
23 a + b	65?	U, C, AC, AU, AG, AAC, AAAG
24		C, AC, AG, AAAC
29	70	U, C, AC, AU, G
31	72	U, C, AC, AU, AAC, G
38	80	U, C, AU, AAU, G
39	81	U, C, AU, G
41		U, C, AU, AG
42		U, C, AC, AU, AAU, AAAN,[a] G
43		U, C, AC, AU, AG, AAC, AAU
44		U, C, AC, AU, AG
45	77	C, AU, AAU, AAAC, G

[a] N, Not identified.

mapped within about 1 kb adjacent to poly(A) at the 3′ end; the other 6 were detected only in the poly(A)-containing RNA fragments larger than 20S for the Y73 and 30S for YAV, and were thus mapped within about 1 to 1.5 kb in the 5′ regions of the genomes. The remaining oligonucleotides specific to 4.8-kb RNA were mapped in the middle portion of the 4.8-kb genome RNA. These results suggest that the 4.8-kb specific RNA sequence is composed of a continuous sequence of about 2.5 kb of RNA and is flanked at the two ends with 1- to 1.5-kb RNA sequences common with those of helper YAV RNA.

In vitro translation of Y73 RNA. To study the gene products coded by the specific sequence, poly(A)-containing RNA isolated from Y73 virions was fractionated by size (Fig. 4A) and translated in an mRNA-dependent reticulocyte lysate (20). Polyacrylamide gel analysis (Fig. 4B) of the translation products showed that Pr76 was the main product from RNA fractions larger than 28S, as found in previous experiments on translation of avian erythroblastosis virus RNA (29). This result was also expected, because the higher-molecular-weight fraction of RNA contained mainly helper YAV RNA in which the *gag* gene is located near the 5′ terminus and the protein-synthesizing system from

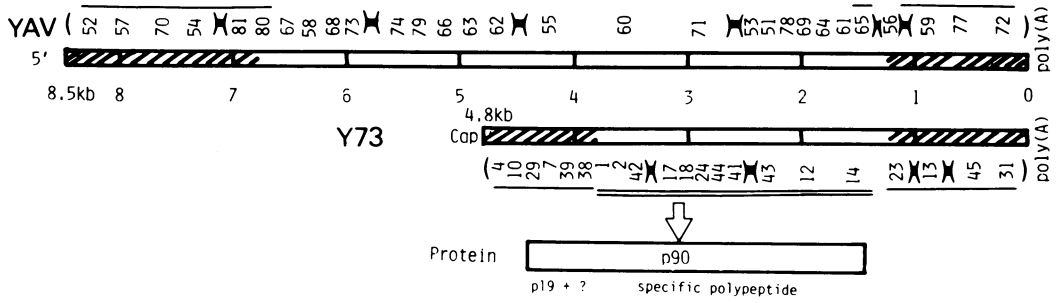

FIG. 3. *Physical maps of oligonucleotides and polypeptide coded by Y73. Spot numbers with a single line are oligonucleotides shared with 4.8-kb and 8.5-kb RNAs, and those with a double line are specific to 4.8-kb RNA. The orders of the oligonucleotides within the respective parentheses are uncertain.*

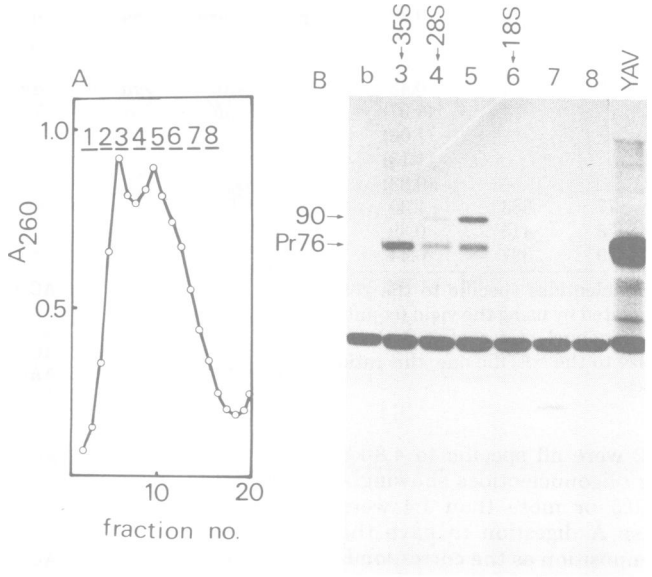

FIG. 4. *Polyacrylamide gel electrophoresis of the translation products of Y73 RNA and helper YAV RNA. Y73 RNA containing poly(A) was fractionated on a sucrose gradient (A) as described in Fig. 1, and about 0.2 μg of RNA in each fraction was translated in an mRNA-dependent rabbit reticulocyte lysate, containing 20 μCi of [^{35}S]methionine, and analyzed on a 7 to 15% polyacrylamide gradient gel (B). Track b had no added RNA; other tracks are labeled with numbers corresponding to the fractions of RNA from the sucrose gradient in (A). Track YAV is 0.5 μg of heat-treated 70S RNA of helper YAV.*

eucaryotic cells translates only one gene near the 5′ end of polycistronic viral mRNA (19, 21, 25).

In contrast to Pr76, a product with a molecular weight of about 90,000 (p90) was synthesized most efficiently from slightly smaller RNA than 28S (Fig. 4b). Since fractions of similar sized RNA of YAV did not support detectable synthesis of p90, the results indicate that p90 was coded by the 4.8-kb RNA of Y73. The effect of 7-methyl-GTP, a cap analog, on the synthesis of p90 was tested to know whether p90 was synthesized by intact capped RNA or fragmented RNA (2). As seen in Fig. 5, the syntheses of p90 and Pr76 were both strongly inhibited by the addition of 7-methyl-GTP. On the other hand, the amount of smaller minor products was not reduced by addition of 7-methyl-GTP. These findings strongly support the idea that p90 is synthesized by intact 4.8-kb RNA which has a cap structure at the 5′ terminus and also that smaller products are synthesized by fragmented RNA.

The in vitro product p90 was found to be immunoprecipitated with serum against disrupted virions of B77 (Fig. 5). Since this antiserum mainly contained antibody to *gag* proteins, p90 was suggested to contain amino acid sequences related to *gag* proteins. This anti-B77 antiserum precipitated p90 from an extract of cells transformed with Y73, and, as seen in Fig. 5, no detectable difference was observed in the molecular sizes of the in vivo and in vitro products. This strongly suggests that these two p90 proteins are identical. When another antiserum that had no antibody activity against p19 was used, Pr76 and p27 were precipitated from transformed cells, but p90 was not (Fig. 5). This result demonstrated that p90 contained the amino acid sequence of p19, but not that of p27. However, this experiment does not exclude the possible presence of a partial sequence of p27 in the p90 molecule.

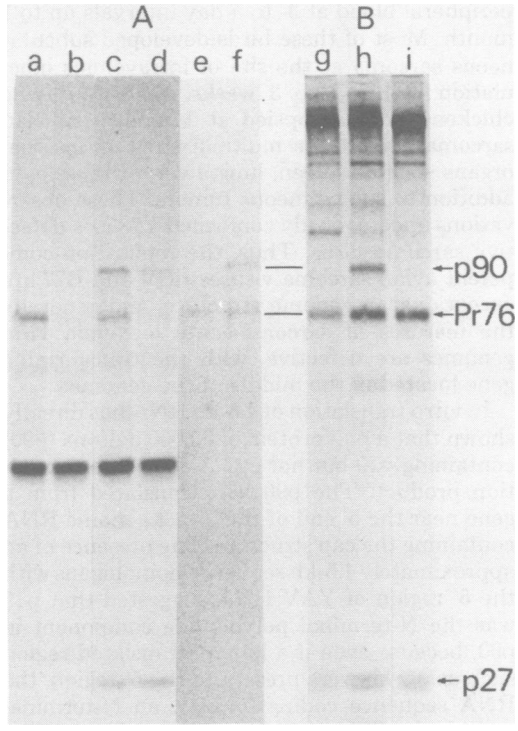

FIG. 5. *Comparison of in vitro and in vivo translation product p90. (A) Effect of 7-methyl-GTP on in vitro synthesis of p90; 35S RNA (a, b) and 26S RNA (c, d) were translated as described in Fig. 4 in the absence (a, c) or presence (b, d) of 200 mM 7-methyl-GTP. The translation products in the absence of 7-methyl-GTP were immunoprecipitated with rabbit antiserum against disrupted virions, and precipitates were analyzed on a 9% gel; (e) and (f) are immunoprecipitated translation products from 35S RNA and 26S RNA, respectively. (B) Extracts of cells infected with YAV (g) or with Y73 (h, i) were mixed with anti-gag serum, which had anti-p19 activity (g, h) or no anti-p19 activity (i), and the precipitates were analyzed on the gel.*

DISCUSSION

In this paper, we characterized the genome structure of Y73, a newly isolated avian sarcoma virus which, like RSV, induces fibrosarcoma in chickens (11). The transforming gene (*yas*) of Y73 was previously characterized as a new type of sarcoma gene with no homology with *src* of RSV or *fps* of Fujinami sarcoma virus (27). In the present study, the *yas* sequence, which was characterized by specific oligonucleotides, was mapped in the middle portion of the genome, with portions of 1 to 1.5 kb common to the 3′ and 5′ ends of helper YAV RNA at the two ends. This structural feature of the genome makes it very similar to the genomes of avian acute leukemia viruses such as avian erythroblastosis virus (3) or MC29 (18), to those of Fujinami sarcoma virus (8, 16), and to those of mammalian sarcoma-acute leukemia viruses (23, 24). Because of the close similarity of the genome structure of Y73 to that of avian acute leukemia viruses, we have tested its leukemogenic capacity in chickens. One-week-old chickens were inoculated intravenously with Y73 (1.5×10^4 focus-forming units) or with avian erythroblastosis virus pseudotyped with Rous-associated virus type 1 (10^4 focus-forming units) as control. All chickens inoculated with avian erythroblastosis virus developed erythroblastosis within 8 days; however, no sign of leukosis was detected in chickens inoculated with Y73 by examination of

peripheral blood at 3- to 5-day intervals up to 1 month. Most of these birds developed subcutaneous sarcoma at the site of intravenous inoculation within 2 to 3 weeks. Eight surviving chickens were autopsied at 1 month; six had sarcomas in one or multiple sites or internal organs such as spleen, lung, liver, or muscle, in addition to subcutaneous tumors. These observations unequivocally confirmed Y73 as a defective sarcoma virus. Thus, the replication-competent avian sarcoma viruses RSV and B77 are exceptional in genome structure, and generally the features of sarcoma-acute leukemia viral genomes are defective, with the transforming gene located in the middle of the genome.

In vitro translation of 4.8-kb RNA has directly shown that a polyprotein of 90,000 daltons (p90) containing p19 but not p27 is a specific translation product. The p90 was translated from a gene near the 5′ end of the intact genome RNA containing the cap structure. The presence of an approximately 1.5-kb sequence homologous with the 5′ region of YAV RNA suggested that p19 was the N-terminal polypeptide component in p90, because even if a long untranslated region such as 0.5 kb were present in the 5′ region, the RNA sequence coding for p19, an N-terminal peptide in Pr76, could be included in the 5′ region homologous with YAV RNA. Thus, a polypeptide of about 71,000 daltons should be coded by a specific nucleotide sequence of about 2.5 kb and therefore would be a specific polypeptide. This conclusion was supported by the findings that anti-*gag* serum that had no anti-p19 activity did not precipitate p90 and that rabbit antiserum against $p60^{src}$ coded by Schmidt-Ruppin strain RSV did not cross-react with p90 (13).

Since most of the specific sequence in the 4.8-kb genome would be used for p90, p90 seems to be the only polypeptide coded by the Y73 genome. This possibility is consistent with the finding that 4.8-kb RNA is the only species detectable with specific complementary DNA ($cDNA_{yas}$) in cells transformed with Y73. These considerations strongly suggest that p90 is involved in the mechanism of sarcomagenic transformation of infected cells. This prediction is supported by the finding that p90 detected in cells transformed with Y73 had protein kinase activity for phosphorylation of tyrosine residues in p90 itself and in heavy-chain immunoglobulin G complexed with p90 (13). However, we did not exclude the possible formation of smaller products coded by the Y73 and undetectable by our methods.

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