Genetic Structure, Transforming Sequence, and Gene Product of Avian Sarcoma Virus UR1

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We analyzed the genetic structure and gene products of the newly isolated avian sarcoma virus UR1, which recently has been shown to be replication defective and to contain no sequences homologous to the *src* gene of Rous sarcoma virus. The sizes of the genomic RNAs of UR1 and its associated helper virus, UR1AV, were determined to be 29S and 35S (5.9 and 8.5 kilobases), respectively, by gel electrophoresis and sucrose gradient sedimentation. RNase T₁ oligonucleotide mapping of purified viral RNAs indicated that UR1 RNA contains eight unique oligonucleotides in the middle of the genome and shares four 5'-terminal and three 3'-terminal oligonucleotides with UR1AV RNA. The unique sequences of UR1 and Fujinami sarcoma virus were found to be closely related to each other by molecular hybridization of UR1 RNA with DNA complementary to the unique sequence of Fujinami sarcoma virus RNA, but minor differences were found by oligonucleotide fingerprinting. In the regions flanking the unique sequences, UR1 and Fujinami sarcoma viral RNAs contain distinct oligonucleotides, which are shared with oligonucleotides of the respective helper viral RNAs. Cells transformed with UR1 produce a single 29S RNA species which contains a UR1 unique sequence; this species is most likely the mRNA coding for the transforming protein. In UR1-transformed cells, a phosphoprotein of 150,000 daltons (p150) was detected by immunoprecipitation with antiserum against *gag* proteins. p150 was associated with a protein kinase activity that was capable of phosphorylating p150 itself, immunoglobulin G of antiserum, and a soluble substrate, α -casein. This enzyme transferred phosphate exclusively to tyrosine residues of substrates in vitro, but p150 labeled in vivo with ³²P contained both phosphoserine and phosphotyrosine. The in vitro kinase reaction was not affected by the presence of cyclic AMP or cyclic GMP and strongly preferred Mn²⁺ over Mg²⁺. Thus, the properties of UR1 protein are almost identical to those of Fujinami sarcoma virus protein.

Two new viruses from two independently obtained solid tumors in chickens were isolated recently in the United States, and the initial biological characterizations of these viruses are presented in the accompanying paper (1). Each preparation was found to contain a virus with all of the attributes of a sarcomagenic transforming virus. After initial biological purification, these sarcoma viruses, designated UR1 and UR2, were shown to be defective in replicative functions and to be associated with helper leukosis type viruses. Molecular hybridization studies indicated that the RNAs of both of these sarcoma viruses shared no homology with sequences of the *src* gene of Rous sarcoma virus (RSV) (1). Thus, it was possible that these viruses belonged to the classes of recently characterized chicken sarcoma viruses whose transforming

genes are not related to the *src* gene of RSV. One of these classes includes Fujinami sarcoma virus (FSV) (6, 8, 13) and PRCII sarcoma virus (2, 18); in this class the transforming sequence is designated *fps*, to indicate that the sequence is shared by these two sarcoma viruses (20). Another class of non-*src*-containing sarcoma viruses contains Y73, whose transforming sequence, designated *yes*, is not related to either *src* or *fps* (12).

UR1 is a virus which was isolated from a chicken tumor originally obtained by B. Calnek, Cornell University, Ithaca, N.Y. (1). In this paper we show that the genome of UR1 contains the *fps* sequence and that the overall genetic structure of UR1 is quite similar to that of FSV. Reflecting this similarity, the UR1 genome encodes a *gag*-related polyprotein which is closely

related to the FSV polyprotein in structure and function.

MATERIALS AND METHODS

Cells and viruses. Primary chicken embryo fibroblast cultures were prepared from 11-day-old group-specific antigen-negative C/E chicken embryos, as described previously (7). Details of the origin of UR1 (University of Rochester isolate 1) sarcoma virus and its associated helper virus, UR1AV, are described in the accompanying paper (1). Briefly, a mixture of UR1(UR1AV) and UR1AV was isolated from a fibrosarcoma which spontaneously appeared in a chicken. The UR1(UR1AV) stock used in this study was derived from a single focus picked from a culture infected with the original stock of UR1 virus. Since the focus released infectious virus, our preparation contained both UR1 and UR1AV. The UR1-infected cells used in this study were prepared either by infecting chicken embryo fibroblasts with high titers of UR1 or by combining the individual foci produced after infection with a virus dilution which contained about 1,000 focus forming units. In the latter procedure, the foci were plated onto dishes preseeded with uninfected chicken embryo fibroblasts. The fully transformed cultures prepared by this method were used for labeling viral RNAs. These cultures still contained the helper virus UR1AV, but the ratio of sarcoma virus to helper virus was higher than the ratio obtained from mass cultures infected with the virus. UR1-transformed nonproducer cell cultures were prepared as described in the accompanying paper (1) from individual foci picked from cell cultures infected with high dilutions of the UR1(UR1AV) stock. FSV and its helper virus, Fujinami-associated virus (FAV), have been described previously (5, 8, 20).

Preparation of cellular and viral RNAs. Preparation of isotope-labeled cellular 18S and 28S RNAs was by previously described methods (23, 24). ^3H - and ^{32}P -labeled viral RNAs were prepared by procedures described recently (26) which were modified from the published methods (23, 24). Virus labeled with [^3H]uridine or $^{32}\text{P}_i$ was collected for 24 to 36 h at 3- to 5-h intervals after an initial 10- to 12-h labeling period. To isolate the UR1 sarcoma virus RNA component, a purified ^{32}P -labeled 60S to 70S RNA mixture of UR1 and UR1AV was fractionated on a polyacrylamide gel either before or after heat denaturation and oligodeoxythymidylic acid column selection (23, 24) of polyadenylic acid [poly(A)]-containing RNA. Similar results were obtained with both methods except that the latter procedure generally gave more distinctive peaks in gels, apparently because of the removal of degraded non-poly(A)-containing RNAs.

Gel electrophoresis and elution of RNA from gels. Separation of ^{32}P - or ^3H -labeled viral RNAs in 2.2% sodium dodecyl sulfate (SDS)-polyacrylamide gels and subsequent elution of RNA components from the gel slices were performed as described previously (3, 4). For preparative gels of [^{32}P]RNA, the RNA profile was obtained either by cutting the gel into 1-mm slices and measuring Cerenkov radiation or by direct exposure of the gel in the gel slicer to X-ray film at room temperature for 5 to 10 min when 10×10^6 to

20×10^6 cpm of RNA was used. The peak slices of each RNA component were transferred to 30-ml tubes, and the RNA was eluted in each tube by stirring with a magnetic bar at room temperature for 12 h in 5 to 7 ml of a buffer containing 0.01 M Tris-hydrochloride (pH 7.2), 0.4 M LiCl, 2 mM EDTA, 0.15% SDS, and 150 μg of yeast RNA. The polyacrylamide was removed by centrifugation at $17,000 \times g$ for 20 min and recovery of the RNA in the supernatant was usually more than 90%. The RNA was concentrated and further purified by sedimentation in a 10 to 25% sucrose gradient containing 0.01 M Tris-hydrochloride, 0.1 M NaCl, 1 mM EDTA, and 0.1% SDS at 40,000 rpm for 3 h at 22°C in an SW40 rotor to remove the contaminating polyacrylamide, which pelleted at the bottom of the centrifuge tube. More than 85% of the RNA sedimented as a single symmetric peak. This RNA was concentrated and subjected to further analysis. Denaturation of [^{32}P]RNAs in 1 M glyoxal-50% dimethyl sulfoxide and fractionation on 1% agarose gels were by the method of McMaster and Carmichael (17), except that gels were dried under a vacuum before autoradiography.

RNAse T₁ fingerprinting and oligonucleotide mapping. An analysis of the UR1-UR1AV RNA mixture was performed with gradient-purified ^{32}P -labeled 60S to 70S RNA complex. For an analysis of the sarcoma and helper viral RNA components, the individual RNA species purified by polyacrylamide gel electrophoresis and sucrose gradient sedimentation as described above were fractionated further by oligodeoxythymidylic acid cellulose binding to obtain the poly(A)-positive RNA. RNAse T₁ fingerprinting, partial sequence analysis with RNAse A, and ordering of oligonucleotides on viral RNA have been described previously (24).

Preparation of cDNA's. The preparation of ^3H - and ^{32}P -labeled DNAs complementary to FAV (cDNA_{FAV}) and to the FSV unique sequence (cDNA_{FSV}) has been described previously (20). The preparation of [^{32}P]DNA complementary to the 5'-terminal 101 nucleotides of Rous-associated virus-2 (RAV-2), designated "strong-stop cDNA" (cDNA_{ss}), was by previously described procedures (9, 11). Briefly, endogenous DNA synthesis was carried out at 41°C for 1 h in 6 ml of a reaction mixture containing 20 mM Tris-hydrochloride (pH 8.0), 17 mM NaCl, 3 mM MgCl₂, 1 mM dATP, 1 mM dGTP, 1 mM TTP, 10 mM dithiothreitol, 100 mg of actinomycin D per ml, 2 mCi of [^{32}P]dCTP, 0.025% Nonidet P-40, and RAV-2 (20 U of absorbance at 260 nm). The DNA product was freed of proteins by phenol and chloroform extractions and then concentrated by ethanol precipitation. The RNA templates were removed by incubating the concentrated DNA-RNA mixture in 0.2 N NaOH either at 100°C for 30 min or at 37°C for 12 h. After neutralization and concentration, cDNA products were fractionated on an 8% polyacrylamide gel containing 8 M urea. After autoradiography for 3 to 10 min, the gel band containing cDNA_{ss} was cut out, and the cDNA was electroeluted (16). cDNA_{ss} was freed of polyacrylamide by phenol and chloroform extractions.

Liquid hybridization. Different amounts (0.1 to 5 μg) of viral RNAs were mixed with [^3H]cDNA_{FAV} (600 cpm; specific activity, approximately 2×10^7 cpm/ μg)

or [^3H]cDNA_{fos} (500 cpm; specific activity, approximately 2×10^7 cpm/ μg) and hybridized under conditions of moderate stringency (i.e., 50°C in 30% formamide–0.45 M NaCl–0.045 M sodium citrate [pH 7.4]–5 mM EDTA–0.4% SDS) (10, 20). The extent of hybridization was measured by S1 nuclease digestion.

Isolation of unlabeled viral RNAs from infected cells. Four to six 10-cm dishes containing fully transformed cells infected with UR1 and UR1AV were used to prepare intracellular poly(A)-containing viral RNAs by the method of Hayward (10).

RNA blotting and filter hybridization. About 6 to 8 μg of poly(A)-containing RNAs prepared as described above was denatured with glyoxal and separated in a 1% agarose gel (see above). The transfer of the RNA to nitrocellulose filters and hybridization with [^{32}P]cDNA were performed by the method of Thomas (21), with some modifications. The hybridization mixture contained 0.1% SDS and 100 μg of chicken rRNA's per ml in addition to those described by Thomas. Hybridization was performed at 37°C for 2 to 3 days. Filters were washed after hybridization, as follows: three times for 5 min each at room temperature with 250 ml of 2 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 0.1% SDS; twice for 15 min each at 37°C with 250 ml of 0.1 $\times$ SSC–0.1% SDS; and twice for 30 min each at 37°C with 200 ml of 5 $\times$ SSC–50% formamide–0.1% SDS.

Protein analysis. Isotopic labeling of cells, preparation of cell extracts, immunoprecipitation, the protein kinase assay, SDS-polyacrylamide gel electrophoresis, and identification of phosphoamino acids were performed as described previously (5).

RESULTS

Size of UR1 genomic RNA. ^3H - and ^{32}P -

labeled UR1(UR1AV) 60S to 70S RNA complexes were analyzed to determine the sizes of the two genomes. Each heat-denatured RNA mixture was analyzed by using 28S rRNA and 35S RAV-2 RNA as markers in 2.2% SDS-polyacrylamide gels (Fig. 1A). Two major RNA components were observed; one comigrated with RAV-2 RNA, and the other migrated slightly slower than the 28S RNA marker. Similar results were obtained when UR1 and UR1AV RNAs were analyzed after glyoxal denaturation and electrophoresis in a 1% agarose gel (Fig. 1C). The smaller RNA component was eluted from a preparative polyacrylamide gel, and its size was determined further by velocity sedimentation in a sucrose gradient. The RNA sedimented at a position equivalent to a sedimentation coefficient of 29S (Fig. 1B). The molecular weight of the 29S RNA was estimated from its mobility in the agarose to be 1.89×10^6 , which was equivalent to about 5,900 nucleotides, assuming that the molecular weights of the 18S, 28S, and 35S RNAs were 0.7×10^6 , 1.5×10^6 to 1.7×10^6 , and 2.7×10^6 , respectively (14, 15). Since it is the 29S component and not the 35S RNA that contains sequences homologous to the transforming sequence of FSV, another independent isolate of avian sarcoma virus (ASV) characterized previously (see below), we concluded that the 29S RNA is the genome of UR1 and that the 35S RNA is the genome of the helper virus UR1AV.

Transforming sequence of UR1. The RNA sequences of UR1 and UR1AV were analyzed

FIG. 1. Analysis of the sizes of UR1 and UR1AV genomic RNAs. (A) Polyacrylamide gel electrophoresis. A ^3H -labeled UR1-UR1AV 60S to 70S RNA complex was mixed with ^{14}C -labeled 35S RAV-2 RNA and 28S chicken cellular rRNA, heat denatured, and electrophoresed in a 2.2% SDS-polyacrylamide gel. (B) Sucrose gradient sedimentation. A ^{32}P -labeled small RNA component of the UR1-UR1AV RNA mixture was eluted from a polyacrylamide gel similar to that shown in (A) and further purified by sucrose gradient sedimentation (see text). The purified small RNA component was analyzed in a 10 to 25% sucrose gradient in the presence of markers (28S, 18S, 4S) to determine its sedimentation coefficient. The sedimentation was performed in an SW40 rotor at 40,000 rpm for 4.5 h at 22°C. (C) Agarose gel electrophoresis. A ^{32}P -labeled UR1-UR1AV 60S to 70S RNA mixture (lane b) was analyzed in parallel with 35S, 28S, and 18S markers (lane a).

by RNase T₁ oligonucleotide mapping and nucleic acid hybridization. The ³²P-labeled UR1-UR1AV 60S to 70S RNA mixture was heat denatured and separated in a polyacrylamide gel similar to that shown in Fig. 1A. Both the 35S and 29S RNA components were eluted from the gel and further purified by sucrose gradient sedimentation and oligodeoxythymidylic acid column chromatography. Figure 2 shows the fingerprints of the 60S to 70S RNA mixtures and the purified 29S and 35S RNA components. The oligonucleotides present in the 29S and 35S RNAs each represented a subset of those present in the 60S to 70S RNA mixture, which contained all oligonucleotides of both components. Each oligonucleotide from the fingerprints of the 60S

to 70S RNA mixture and of the purified 29S and 35S RNAs was analyzed for RNase A-resistant fragments (Table 1). In addition to visual identifications by chromatographic locations, the compositions of the RNase A-resistant fragments provided the basis for identifying the corresponding oligonucleotides in different fingerprints. Eight oligonucleotides (oligonucleotides 51 to 57) present in the 60S to 70S RNA mixture and in the purified 29S RNA were absent in the purified 35S RNA (Fig. 2). Therefore, these oligonucleotides must have been derived from the specific sequence of UR1 29S RNA. This conclusion was supported by the finding that five of the eight oligonucleotides were either identical or homologous to certain FSV-specific

FIG. 2. Fingerprint patterns of RNase T₁-resistant oligonucleotides of UR1 and UR1AV RNAs. (A) 5S to 16S poly(A)-containing RNA prepared from gel-purified 29S UR1 RNA. (B) 29S UR1 RNA eluted from a polyacrylamide gel and further purified by sucrose gradient sedimentation and oligodeoxythymidylic acid cellulose binding. (C) 35S UR1AV RNA purified as in (B). (D) 60S to 70S RNA mixture of UR1 and UR1AV. (E) 5S to 11S poly(A)-containing RNA prepared from gel-purified 35S UR1AV RNA. (F) 12S to 20S poly(A)-containing RNA prepared from gel-purified 35S UR1AV RNA. (G) 21S to 32S poly(A)-containing RNA prepared from gel-purified 35S UR1AV RNA. (H) Tracing of the fingerprint in (D). The spots indicated by the arrows in (D) and shaded in (H) are UR1-specific oligonucleotides.

oligonucleotides identified previously (8). The FSV-specific oligonucleotides homologous to the oligonucleotides of UR1 RNA are shown in Fig. 3. The homology between the specific sequences of UR1 and FSV was also demonstrated by hybridization studies (see below). Besides the specific oligonucleotides, the 29S RNA shared seven oligonucleotides with its 35S helper virus RNA (Fig. 2 and 3). No *src*-specific oligonucleotides present in the RNAs of RSV and its related sarcoma viruses (26) were found in the UR1 29S RNA. The fingerprint pattern of UR1 29S RNA was distinct from that of FSV RNA analyzed previously (8), despite the homology in

TABLE 1. RNase T₁-resistant oligonucleotides of UR1 and UR1AV RNAs

Spot	RNase A digestion products
UR1AV	
Cap	m ⁷ G ^{ppp} GmpCp(3U, 5C, 2.3AC, 3AU)G
37	5U, 6.6C, AAG, A ₄ N
36	5U, 10C, G, AU
35	3U, 3C, G, AC, AU, AAU
34	3U, 5C, G, 3AC
33	2U, 6C, G, 2AC, AU
32	U, 2C, G, AU, AAU, 2A ₃ U
31	U, 4.5C, 3AC, AU, AG, A ₃ C
30	3U, C, AC, 2AU, AG, A ₃ U
29	9C, 4AC, 2AG
28	3U, 2C, AC, AU, AG, A ₃ C
27b	4U, 4C, G, AC, AU
26	4U, 3C, 2AC, 3AU, AG
25	U, 6C, 3AC, AAG
24	4U, 2C, G, AC, AU
23	6U, 6C, G, AU
22	5U, C, AU, AG
21	4U, 3C, G, AU
20	4U, 7C, 2AC, AU, AAC, AAG
19	2U, C, G, 3AU, AAU
18	5C, AU, AG, A ₄ N
17	2U, 2C, G, AC, 2AU, A ₆ N
16	4.5C, G, 4AC
15	U, 1.5C, G, 2AC, A ₃ U, A ₆ N
14	U, 7C, G, 3AC, A ₃ N
13	5U, 2C, G, 3AU, AAU
12	5U, 8C, G, AC, AU, A ₃ C
11	4U, 7C, G, 2AU
10	5U, 5C, AC, 2AU, AG, AAU, A ₃ C
9	4U, 10C, G, 2AC, AAU
8	2U, 2C, G, AC, 2AU, AAU
7	4U, 3C, G, AU, AAU
6abc	7U, 10C, 2G, 3AC, AU, AG, 3AAU
5	4U, 2C, G, AC, 2AU, 2AG, A ₄ N
4abc	U, 13C, G, 7AC, 2AU, 2AG, AAC, AAU, A ₃ N, A ₄ N
3	3U, 4C, AC, AAU, A ₃ C, A ₃ G
2	2U, 3C, G, AC, AU
1ab	4U, 4C, G, 2AC, AU, AG, AAU
C	G, AC, AU, AAU, A ₃ C
UR1 specific	
51	U, 4C, G, AC, 2AU
52	U, 4C, G, AC, 2AU, 2AAC
53	3U, 5C, G, 2.5AC, 2.5AU
54	U, 4C, G, 2AC, AAC
55ab	U, 8C, 3AC, AU, 2AG, A ₃ U
56	U, 6C, G, AC, AU
57	2U, 6C, 4AC, AU, AG

FIG. 3. Oligonucleotide maps of UR1 and UR1AV RNAs. The scale on the left represents the UR1AV genome and is measured in kilobases (kb). The gene order, including the *c* region (22), and the approximate range of each gene are shown. The circled oligonucleotides are the oligonucleotides that are highly conserved among avian retroviruses (22). The order of the oligonucleotides within each set of parentheses is not certain. The oligonucleotides within the brackets are UR1 specific, and their homologous counterparts in FSV RNA are shown. Double lines connect the oligonucleotide pairs which have identical RNase A-resistant fragments, and single lines connect the pairs with similar but not identical RNase A digestion products.

the specific sequences of these RNAs. For example, FSV RNA did not contain oligonucleotide C; instead, it contained three distinct oli-

gonucleotides in the 3' region (8) which were absent in UR1 RNA. Similarly, oligonucleotides characteristic of UR1 or FSV were also found in the 5' region; for example, UR1 RNA oligonucleotide 36 was absent in FSV RNA. Oligonucleotide 37 and the cap-containing oligonucleotide were common in UR1 and FSV RNAs. Differences in oligonucleotide were also found between UR1AV and FAV (Fig. 2) (8). These results were consistent with the hypothesis that two different helper viruses were involved in the generation of these two independently isolated sarcoma viruses (1, 6).

To locate the UR1 unique and shared sequences on the UR1 genome, we constructed oligonucleotide maps of UR1 and UR1AV RNAs (Fig. 3) by a previously described method (24). Figure 2 shows fingerprints of different sizes of poly(A)-containing RNA fragments of UR1 and UR1AV. Both RNA species contained the highly conserved oligonucleotide C of avian retroviruses (22) at the 3' end; in addition, they shared two oligonucleotides (oligonucleotides 1a and 2) within the 3'-terminal 1,000 nucleotides. The homology in the 3' regions of the two RNA species was shown clearly by the fingerprints of the small poly(A)-tagged RNA fragments (Fig. 2A and E). Figure 2A shows that UR1-specific oligonucleotides 51 and 52 appeared immediately after the shared oligonucleotides C, 1a, and 2. In contrast, Fig. 2F shows that in the case of UR1AV RNA, oligonucleotides C, 1a, and 2 are followed by oligonucleotides 3 and 4, which presumably were derived from the 3' region of *env*, based on their map locations and homologies to the highly conserved *env*-specific oligonucleotides of other avian retroviruses analyzed previously (22, 24). Therefore, oligonucleotides 2 and 51 or 52 define the 3' junction of shared and unique sequences in UR1 RNA. Besides the homology at the 3' region, UR1 RNA and its helper virus RNA shared four oligonucleotides at the 5' end, including the terminal cap oligonucleotide, which was similar to the oligonucleotide present in Prague RSV and B77 ASV RNAs (25). We concluded that the UR1 genome shares 3'- and 5'-terminal sequences with its helper virus RNA and has a unique sequence in the middle. The UR1 unique sequence contains RNase T₁ oligonucleotides which are also present in the FSV unique sequence. We did not determine whether the RNase T₁ oligonucleotides unique to FSV and UR1 are localized at any specific regions within their respective unique sequences.

The relationship between UR1 and FSV unique sequences was studied further by DNA-RNA hybridization. [³H]cDNA_{fsv} was prepared

and hybridized to unlabeled UR1 and UR1AV RNAs under conditions of moderate stringency. As Fig. 4A shows, cDNA_{fsv} hybridized with the UR1-UR1AV RNA mixture to the same extent (90%) as the hybridization between cDNA_{fsv} and the FSV-FAV RNA mixture. The lower value of $C_{t1/2}$ in the hybridization between cDNA_{fsv} and the UR1 RNA mixture compared with the hybridization between cDNA_{fsv} and the FSV RNA mixture was most likely due to a higher ratio of sarcoma virus RNA to helper virus RNA in the former virus pair. The specificity of cDNA_{fsv} was demonstrated by the lack of hybridization with FAV RNA. This result clearly showed that there is extensive homology between the unique sequences of UR1 and FSV. The FSV-related transforming sequence must be present in UR1 RNA but not in UR1AV RNA, since, as shown above, the former RNA contains oligonucleotides homologous to the FSV-specific oligonucleotides (8). The relationship between UR1AV and FAV was examined by using cDNA_{FAV}. The results obtained demonstrated that these two viruses were not completely homologous; they shared about 70% of their genomic sequences. Such homology is typical among different isolates of avian leukosis viruses, as shown by the relationship between FAV and RAV-2 (Fig. 4B).

FIG. 4. Relationship of FSV unique sequence and UR1 RNA. The details of the hybridization conditions are described in the text. (A) [³H]cDNA_{fsv} was hybridized with a mixture of UR1 and UR1AV RNAs (●), a mixture of FSV and FAV RNAs (○), and FAV RNA (Δ). (B) [³H]cDNA_{fav} was hybridized with a mixture of UR1 and UR1AV RNAs (●), FAV RNA (○), and RAV-2 RNA (Δ).

Again, these data are consistent with independent origins of FSV and UR1. The lack of homology between the UR1 unique sequence and the *src* gene of RSV is described in the accompanying paper (1). We concluded from this study that UR1 is another isolate of *fps*-containing ASV.

mRNA of UR1. To check for the presence of UR1 subgenomic mRNA, we analyzed RNAs isolated from cells infected with UR1 (UR1AV) and UR1AV by using RNA blotting and hybridization techniques. Since all of the retroviral mRNA's analyzed so far contain the leader sequence derived from the 5' end of the viral genomic RNA, we first used ^{32}P -labeled cDNA_{ss} from a typical leukosis virus, RAV-2, to detect the mRNA's present in UR1- and UR1AV-infected cells. As Fig. 5 shows, bands corresponding to 35S genomic RNA and, presumably, UR1AV 22S *env* mRNA were present, whereas the 29S UR1 RNA band was only barely visible. The relative intensities of the UR1 RNA band and the UR1AV bands reflected both the abundance of each mRNA species in the infected cells and the number of cells in the cultures

infected by each virus. The higher intensity of the 22S UR1AV RNA band than of the 35S RNA band (Fig. 5, lane C) did not necessarily reflect the relative abundance of each RNA in the cells, but may have been due to the higher efficiency of transfer of the smaller RNA from the gel to the nitrocellulose paper. In the same RNA blot, mRNA's of other ASVs were detected readily by the RAV-2 cDNA_{ss} probe, and the bands of these mRNA's were about 10-fold more intense than the bands of UR1 and UR1AV RNAs (data not shown). In addition, UR1 29S RNA in the same RNA blot could be detected easily with cDNA_{fps} (see below); this indicated that the low intensities of the RNA bands detected with RAV-2 cDNA_{ss} were unlikely due to an insufficient amount of RNA present in the blot. These results indicate that the 5'-terminal sequences of UR1 and UR1AV are substantially different from those of RAV-2. Since our data indicated that UR1 and FSV are very homologous in their unique sequences, we next used ^{32}P -labeled FSV-specific cDNA (cDNA_{fps}) to detect UR1 mRNA after removal of the RAV-2 cDNA_{ss} from the same RNA blot. Figure 5, lane D, shows that cDNA_{fps} detected one major RNA species (corresponding in size to the 29S UR1 genomic RNA) and some smearing of possibly degraded RNAs in the lower-molecular-weight region of the gel. The same UR1 and UR1AV RNA preparation was reanalyzed with cDNA_{fps}. Figure 5, lane E, shows that only the 29S UR1 RNA could be hybridized with cDNA_{fps}. This is consistent with our assumption that RNAs corresponding to the sizes of 35S and 22S RNAs are UR1AV genomic RNA and UR1AV *env* mRNA, respectively. We conclude that the 29S mRNA species, which is probably identical to the UR1 genomic RNA, is the only mRNA derived from the UR1 genome and must be the mRNA coding for the protein product p150 described below.

Products of the UR1 transforming gene. Because of the similarities in the genetic structures of UR1 and FSV, the protein products of these two viruses probably have similar properties. Therefore, we used the same approach to study the gene products of UR1 that was used for studies of the FSV transforming protein (5). First, [^{35}S]methionine- or ^{32}P -labeled proteins of UR1-transformed producer cells were immunoprecipitated with preimmune serum or with antisera against disrupted RAV-2 virions. The immunoprecipitates were collected as a complex with *Staphylococcus* protein A-Sepharose and were analyzed by SDS-polyacrylamide gel electrophoresis (5). Figure 6 shows that antiserum directed against total virion protein, but not preimmune serum, precipitated the viral proteins p15, p27, Pr76, and P180 and, in addition,

FIG. 5. Viral RNAs present in UR1- and UR1AV-infected cells. About 8 μg of poly(A)-containing RNA isolated from uninfected cells (lane B) or from cells infected with UR1 and UR1AV (lane C) was separated in an agarose gel, transferred to nitrocellulose paper, and hybridized with RAV-2 [^{32}P]cDNA_{ss}. The cDNA_{ss} was then removed from the filter, and, after autoradiography confirmed the efficient removal of cDNA_{ss}, the filter was rehybridized with [^{32}P]cDNA_{fps}. Lane D is the result of rehybridization of lane C. Lane E shows a repeated analysis of the same UR1 and UR1AV RNAs using cDNA_{fps}. Lane A shows ^{32}P -labeled RNA markers analyzed in parallel.

a 150,000-dalton protein (p150) from extracts of [35 S]methionine-labeled cells. p150 was the only major protein immunoprecipitated from UR1-transformed nonproducer cells (Fig. 6), and this protein was not present in uninfected or UR1AV-infected cells (data not shown). Antiserum against viral proteins also precipitated p150 from extracts of 32 P-labeled cells (Fig. 6), indicating that p150 is a phosphoprotein *in vivo*.

Phosphorylation of acceptor proteins by UR1 p150 kinase activity. Immunoprecipitates of p150 appeared to have a protein kinase activity associated with them. As Fig. 7 shows, when antiserum against RAV-2 virion proteins was used, immunoprecipitates from extracts of UR1-transformed nonproducer cells catalyzed the transfer of 32 P_i from [32 P]ATP to p150. Preimmune serum did not precipitate any kinase activity (Fig. 7). p150 was precipitated by antisera from rabbits bearing tumors induced by Schmidt-Ruppin RSV (TBR serum), because these sera were reactive with the products of the

FIG. 6. *In vivo* labeling of UR1 p150 by [35 S]methionine and 32 P_i. UR1-transformed producer (lanes A and B) and nonproducer (lanes C and D) cell cultures were labeled with [35 S]methionine (lanes A, B, and C) or with 32 P_i (lane D) and immunoprecipitated with preimmune serum (lane A) or antiserum against total virion protein (lanes B, C, and D). Immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography. Lanes A and B, 5 to 15% acrylamide gradient gel; lanes C and D, 8.5% acrylamide gel.

FIG. 7. *Protein kinase activity in immunoprecipitates containing UR1 p150.* Unlabeled cell extracts prepared from uninfected (lane A), FSV-infected (lane B), UR1-infected (lanes C through G), and Schmidt-Ruppin RSV-infected (lanes H and I) cell cultures were immunoprecipitated with preimmune serum (lane E), antiserum against total virion proteins (lanes A through D), TBR serum (lanes F and H), or RAV-2-absorbed TBR serum (lanes G and I). Immunoprecipitates were assayed for protein kinase activity and analyzed on a 8.5% SDS-polyacrylamide gel. In lanes D and E the kinase buffer contained 1 mg of α -casein per ml in addition to the kinase assay components. IgG, Immunoglobulin G.

gag gene of standard RSV strains (5). Immunoprecipitates of p150 by TBR serum were able to transfer 32 P_i to the heavy chain of immunoglobulin G, as well as to p150 (Fig. 7). As Fig. 7 shows, TBR serum preabsorbed with disrupted virus precipitated very little protein kinase activity from extracts of UR1-transformed cells. However, the same absorbed serum could precipitate the p60^{src} protein kinase activity from extracts of RSV-transformed cells to the same extent of activity that was precipitated by control unabsorbed TBR serum. Therefore, these results indicate that the kinase activity precipitated from extracts of UR1-transformed cells by TBR serum shares antigenic determinants with virus structural proteins but not with p60^{src}. As Fig. 7 shows, when α -casein was added to the kinase reaction mixture, both α -casein and p150 were phosphorylated.

The presence of cyclic AMP or cyclic GMP (1 to 100 μ M) had no effect on the p150 kinase reaction. p150 kinase required an appropriate divalent cation for its activity; at a concentration of 10 mM, Mn²⁺ was three to four times more effective than Mg²⁺, and Ca²⁺ was totally inef-

fective. At the three concentrations tested (0.01 to 1 μ M), [γ - 32 P]GTP did not serve as a 32 P donor, whereas the incorporation of 32 P into p150 was proportional to the concentration of [γ - 32 P]ATP added (data not shown).

The identities of the amino acids serving as phosphate acceptors in the p150 kinase reaction were determined. As Fig. 8 shows, the amino acid acceptor of phosphate in the p150 molecule was identified as tyrosine. The amino acid residue phosphorylated in immunoglobulin G and α -casein in the *in vitro* kinase reaction was also tyrosine (data not shown). A similar analysis of the phosphoamino acid composition of p150 labeled *in vivo* with 32 P showed that this protein contained both phosphoserine and phosphotyrosine at similar levels (Fig. 8).

DISCUSSION

The overall structure of the UR1 genome described here is similar to that of FSV, which has been analyzed recently (8). In accordance with its defectiveness in replicative functions (1), the genome of UR1 appears to lack the sequences of a part of the *gag* gene and most or all of the *pol* and *env* genes. In place of these genes in the middle of the genome is the UR1 unique sequence.

UR1 RNA contains fewer large RNase T₁-resistant oligonucleotides than expected from a genome containing approximately 5,900 nucleotides. Judging from the molarity of the oligonucleotides generated from the RNA of this virus, we found no evidence for duplication of a portion of the UR1 genome. We can only attribute this phenomenon to the intrinsic property of the UR1 RNA sequence. Assuming that the large oligonucleotides are distributed evenly throughout UR1 RNA, we estimate that UR1 RNA shares about 1,600 nucleotides at the 5' end and 1,000 nucleotides at the 3' end with UR1AV helper virus RNA. Thus, the remaining 3,300 nucleotides represent the unique sequence in the middle. The combination of *gag* and unique sequences is sufficient to code for the transformation-specific polypeptide of 150,000 daltons that we observed. The genome of UR1 is slightly larger than that of FSV (8), and this is also reflected in the large protein product encoded by UR1 RNA. We do not know whether the sequences which contribute to the large size of the genome are located within the *gag* gene or within the unique region of the UR1 genome.

The close relationship between the unique sequences of UR1 and FSV has been shown clearly by both RNase T₁ oligonucleotide analysis and cDNA-RNA hybridization. cDNA_{fps} hybridized completely with UR1 RNA. Since we have shown previously that cDNA prepared un-

FIG. 8. Phosphoamino acid composition of UR1 p150 phosphorylated *in vivo* and *in vitro*. Partial acid hydrolysates of *in vivo* and *in vitro* 32 P-labeled p150 were separated in two dimensions; electrophoresis at pH 1.9 was carried out from left to right, and electrophoresis at pH 3.5 was carried out from bottom to top. The origin in each plate is marked (x). The positions of the internal phosphoamino acid standards are indicated (Y, phosphotyrosine; T, phosphothreonine; S, phosphoserine). The 32 P-labeled spots on the left are probably partially hydrolyzed phosphopeptides. (A) p150 phosphorylated *in vivo*. (B) p150 autophosphorylated *in vitro*.

der the conditions which we used is fairly representative (20), our results indicate that the homology between the unique sequences of FSV and UR1 is quite extensive. RNase T₁ fingerprinting, which is more sensitive in detecting minor differences between RNA species, shows that five of eight UR1-specific oligonucleotides are also present in FSV RNA. Three UR1-specific oligonucleotides were absent in FSV RNA; conversely, some of the FSV-specific oligonucleotides were missing in UR1 RNA.

Consistent with the homology of the unique sequences of FSV and UR1, the protein products of the FSV and UR1 transforming genes are almost identical in structure and function. UR1 p150 is a phosphoprotein with protein kinase activity which phosphorylates tyrosine residues of substrate proteins, as is FSV p140 (5). The ion requirements and phosphate donors (5) are similar in UR1 p150 and FSV p140 protein kinases.

Our previous studies have shown that the transforming sequence of FSV is about 50 to 60% homologous to the transforming sequence of PRCII (20). It has also been demonstrated that PRCII codes for a polypeptide of 105,000 daltons which has a tyrosine-phosphorylating protein kinase activity (18, 19). Thus, it appears very likely that the sarcoma viruses FSV, PRCII, and UR1 have transforming sequences with a common origin. As Table 2 shows, the isolations of the three avian sarcoma viruses containing *fps* sequences were widely separated in time and place. The observation that the sequences flanking the transforming sequence are common between UR1 and UR1AV and between FSV and

TABLE 2. Isolation of three ASVs which contain the *fps* transforming sequence

Virus	Place	Year	Reference
FSV	Kyoto, Japan	1909	6
PRCII	Edinburgh, Scotland	1958	2
URI	Ithaca, N.Y.	1969	1

FAV supports the hypothesis that these sarcoma viruses were generated independently via recombination between their respective avian leukosis viruses and a cellular *c-fps* sequence. This mechanism has been postulated for many transforming retroviruses. It is intriguing that these sarcoma viruses have genetic structures of similar sizes with respect to both unique and shared sequences. This could mean that in addition to having similar transforming insertions, viruses of this group may have additional constraints on their genetic structures. A precise determination of the junctions between the *fps* sequence and the viral replicative sequences in each sarcoma virus would provide useful information concerning the process of recombination involved.

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