Avian Sarcoma Virus UR2 Encodes a Transforming Protein Which Is Associated with a Unique Protein Kinase Activity

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UR2 is a newly characterized avian sarcoma virus whose genome contains a unique sequence that is not related to the sequences of other avian sarcoma virus transforming genes thus far identified. This unique sequence, termed ros, is fused to part of the viral gag gene. The product of the fused gag-ros gene of UR2 is a protein of 68,000 daltons (P68) immunoprecipitable by antiserum against viral gag proteins. In vitro translation of viral RNA and in vivo pulse-chase experiments showed that P68 is not synthesized as a larger precursor and that it is the only protein product encoded in the UR2 genome, suggesting that it is involved in cell transformation by UR2. In vivo, P68 was phosphorylated at both serine and tyrosine residues. Immunoprecipitates of P68 with anti-gag antiserum had a cyclic nucleotide-independent protein kinase activity that phosphorylated P68, rabbit immunoglobulin G in the immune complex, and α-casein. The phosphorylation by P68 was specific to tyrosine of the substrate proteins. P68 was phosphorylated in vitro at only one tyrosine site, and the tryptic phosphopeptide of in vitro-labeled P68 was different from those of Fujinami sarcoma virus P140 and avian sarcoma virus Y73-P90. A comparison of the protein kinases encoded by UR2, Rous sarcoma virus, Fujinami sarcoma virus, and avian sarcoma virus Y73 revealed that UR2-P68 protein kinase is distinct from the protein kinases encoded by those viruses by several criteria. Our results suggest that several different protein kinases encoded by viral transforming genes have the same functional specificity and cause essentially the same cellular alterations.

UR1 and UR2 are two newly isolated avian sarcoma viruses (ASVs) whose basic biological and biochemical properties have been recently described (1, 26, 29, 30). Both sarcoma viruses are defective in replicative functions and are associated with helper leukosis-type viruses. Biochemical studies indicated that UR1 and UR2 belong to the class of ASVs whose transforming genes are not related to the src gene of Rous sarcoma virus (RSV) (1) and whose RNA genomes consist of a unique transforming sequence linked to a viral gag sequence (3, 13, 18, 29, 30, 32).

The genome of UR1 has a unique sequence closely related to the fps transforming sequence of Fujinami sarcoma virus (FSV)-PRCII, and the overall genetic structure of UR1 is quite similar to that of FSV (29). Reflecting this similarity, the specific protein product of UR1, P150, is almost indistinguishable from FSV-P140 in structure and enzymatic properties (29).

Unlike UR1, the unique sequence of UR2 has no nucleotide sequence homology with any of the ASV transforming genes described so far, namely, src of RSV, fps of FSV-UR1-PRCII, and yes of avian sarcoma virus Y73 (Y73)-Esh sarcoma virus (26). Thus, the unique sequence present in the UR2 genome appears to represent a new type of ASV transforming gene and is designated as ros (30).

In this paper we report on the characterization of the UR2 gene product. The UR2 genome encodes a gag-containing phosphoprotein of 68,000 daltons (P68) that is immunoprecipitable by antiserum against viral gag proteins and is associated with a distinct tyrosine-specific protein kinase activity.

MATERIALS AND METHODS

Cells and viruses. Primary chicken fibroblast cultures were prepared from 11-day-old group-specific antigen-negative C/E chicken embryos as described previously (11). The origin and isolation of UR2 (University of Rochester isolate no. 2) and its helper UR2-associated virus (UR2AV) have been described (1, 26). In all the experiments described in this paper, UR2-transformed cells were also infected with UR2AV. Other avian retroviruses used were Rous-associated virus-2 (RAV-2), Schmid-Ruppin RSV of subgroup A, FSV, and Y73 (27).

Isotopic labeling of cells and preparation of cell
extracts. (i) \[^{35}\text{S}\]methionine labeling. Long-term labeling of UR2-transformed cells was as described previously for FSV-transformed cells (8).

For pulse-labeling, cells grown in 35-mm tissue culture plates were preincubated with 0.4 ml of methionine-free minimum essential medium (GIBCO Laboratories) containing 1% calf serum for 1 h. Each plate then received 120 \(\mu\)l of fresh medium containing 200 \(\mu\)Ci of l-\[^{35}\text{S}\]methionine (900 to 1,200 Ci/mmol; Amersham Corp.), and incubation was continued for either 5 min or 20 min as indicated. After incubation, cells were either harvested immediately or incubated for further times in F-10 medium supplemented with 10% tryptose phosphate broth, 5% bovine serum, and 1.5 mM unlabeled l-methionine (1,000-fold excess). At the end of each period, cells were harvested. All incubations were at 37°C.

(ii) \[^{32}\text{P}\] labeling. Labeling of UR2-transformed cells was as described previously for FSV-transformed cells (8).

Cell-free protein synthesis. The preparation of 24S UR2 and 35S UR2AV genomic RNAs for in vitro translation was as described previously (30). In vitro translation was carried out in the mRNA-dependent rabbit reticulocyte lysate system described by Pelham and Jackson (24). The translation mixtures, containing either 20 \(\mu\)g of 24S RNA or 25 \(\mu\)g of 35S RNA per ml, were incubated at 28°C for 1 h, after which amino acid incorporation was terminated by the addition of 1 \(\mu\)M of pancreatic RNase per ml. Translation products were then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) either directly or after immunoprecipitation with antisera against structural proteins of RAV-2.

Protein analysis. Preparation of cell extracts, immunoprecipitation, the protein kinase assay, SDS-PAGE, quantitation of radioactivity in gel bands, and identification of phosphoamino acids were performed as described previously (8). Tryptic peptide analysis was carried out essentially as described (8), except that electrophoresis in the first dimension was at pH 1.9 in a buffer containing acetic acid, formic acid, and water (78:25:87, vol/vol) and ascending thin-layer chromatography in the second dimension was in butanol, pyridine, acetic acid, and water (15:10:3:12, vol/vol). Antiserum against p19 and against RAV-2 viral proteins, and sera of rabbits bearing tumors induced by Schmidt-Ruppin RSV (TBR sera) were used for immunoprecipitation; their preparations were as described previously (8).

RESULTS

Products of the UR2 transforming gene. The presence of gag sequences linked to the unique sequence in the genome of UR2 (30) suggested that UR2 might code for a fusion protein containing gag antigenic determinants. Therefore, to identify this protein we first analyzed immunoprecipitates of \[^{35}\text{S}\]methionine- or \[^{32}\text{P}\]phosphate-labeled proteins from extracts of UR2-transformed cells with antisera against structural proteins of RAV-2. Figure 1 shows that antiserum against total virion proteins precipitated the viral proteins p15, p19, p27, Pr76, P180, and, in addition, a 68,000-dalton protein (P68) from extracts of \[^{35}\text{S}\]methionine-labeled cells. P68 was not precipitated with preimmune serum (Fig. 1), and it was absent from uninfected or UR2AV-infected cells (data not shown). As shown in Fig. 1, the antivirion antibody was also able to precipitate P68 from extracts of \[^{32}\text{P}\]labeled cells, indicating that P68 is a phosphoprotein in vivo.

Fate of newly synthesized P68. The detection of P68 described above was carried out in UR2-infected cells that had been labeled with \[^{35}\text{S}\]methionine for 8 h. To determine whether a short-lived precursor of P68 could be detected in UR2-infected cells and to see whether P68 underwent any proteolytic cleavage during its lifetime, UR2-transformed cells were pulse-labeled with \[^{35}\text{S}\]methionine for 5 min and chased for up to 90 min, or they were pulse-labeled for 20 min and chased for up to 22 h. Labeled proteins were analyzed by SDS-PAGE after immunoprecipitation of cell extracts with limiting amounts of antivirion antisera. As shown in Fig. 2A, no larger precursors of P68 were detectable after a 5-min pulse. In addition, Fig. 2A and B show that P68 did not undergo any size modifications.
either soon after chain termination or during a period comprising several half-lives of P68. It should be noted, however, that a proteolytic cleavage removing the gag determinants in P68 would have escaped detection by this assay. The band observed above P68 in Fig. 2 corresponds to a host cell polypeptide that is non-specifically immunoprecipitated even after preabsorption of the antivirion antiserum with disrupted RAV-2 virus particles (data not shown).

24S viral RNA directs the synthesis of P68 in vitro. Since there is apparently no subgenomic mRNA in UR2-infected cells (30), it seems likely that the gene product of UR2 is translated directly from 24S RNA. The fact that P68 accounted for only 80% of the coding capacity of the 24S UR2 RNA left open the possibility that, in addition to P68, 24S RNA coded for a non-gag protein which would not be immunoprecipitable by antiserum against gag-coded proteins or that P68 was synthesized as a very short-lived precursor which escaped detection in pulse-chase experiments. To address these questions directly, we carried out in vitro translation of 24S UR2 and 35S UR2AV viral RNAs in the cell-free reticulocyte lysate system described by Pelham and Jackson (24). As shown in Fig. 3, 35S UR2AV RNA directed the synthesis of Pr76 only. On the other hand, 24S UR2 RNA directed the synthesis of P68 as a doublet that was immunoprecipitable by antiserum against virus structural proteins. The reasons for the synthesis of P68 but not Pr76 as a doublet are not clear to us. No translation products of 24S viral RNA other than P68 were observed with the exception of a small amount of Pr76, which was probably due to contaminating helper virus RNA fragments present in the 24S UR2 RNA preparation.

From these results and those obtained by in vivo labeling of UR2-transformed cells, we conclude that UR2 codes for a gag-containing phosphoprotein, P68, which is the transforming protein of UR2 sarcoma virus.

Phosphorylation of acceptor proteins by UR2-P68 kinase activity. The transforming proteins of all the ASVs described so far, namely, RSV-p60, FSV-P140, UR1-P150, PRCI-P105, Y73-P90, and Esh sarcoma virus-P80, are phosphoproteins associated with a protein kinase activity in immunoprecipitates (5, 8, 10, 16, 19, 21, 23, 29). Since UR2-P68 is a phosphoprotein, we investigated whether immunoprecipitates of P68 were also associated with a protein kinase activity. As shown in Fig. 4, when antisera against p19 or against total RAV-2 virion proteins were used, immunoprecipitates from extracts of UR2-transformed cells catalyzed the transfer of [γ-32P]ATP to P68 and to a very small extent to the heavy chain of immunoglobulin G (IgG). Preimmune serum was unable to

![Fig. 2. Fate of newly synthesized P68. UR2-transformed cell cultures were pulse-labeled with [35S]methionine for 5 min (panel A) or 20 min (panel B) and chased for the periods indicated. Cells were then lysed, and the resulting cell extracts were immunoprecipitated with limiting amounts of antivirion antiserum. Immunoprecipitates were analyzed by SDS-PAGE in 5 to 15% gradient gels followed by autoradiography. Panels A and B correspond to two separate acrylamide gels.](http://jvi.asm.org/)

FIG. 2. Fate of newly synthesized P68. UR2-transformed cell cultures were pulse-labeled with [35S]methionine for 5 min (panel A) or 20 min (panel B) and chased for the periods indicated. Cells were then lysed, and the resulting cell extracts were immunoprecipitated with limiting amounts of antivirion antiserum. Immunoprecipitates were analyzed by SDS-PAGE in 5 to 15% gradient gels followed by autoradiography. Panels A and B correspond to two separate acrylamide gels.
UR2-transformed cells by TBR serum shares antigenic determinants with virus structural proteins but not with RSV-p60. As shown in lane E of Fig. 4, when α-casein was added to the kinase reaction mixture, both P68 and α-casein became phosphorylated. However, as in the case of IgG phosphorylation, the phosphorylation of α-casein was less efficient than we have observed with FSV-P140/UR1-P150 and Y73-P90 (Table 1; 8, 29). Calf thymus histone (H1) or phosvitin were not phosphorylated by P68 immunoprecipitates (data not shown).

**Characteristics of the P68 kinase reaction.** Several variables of the P68 kinase reaction were examined. The kinase reaction was monitored by measuring $^{32}P$ radioactivity in the P68 and α-casein bands after the reaction products were separated by SDS-PAGE. The presence of cyclic AMP or cyclic GMP had no effect on the P68 kinase reaction. The temperature optimum of the reaction was $24^\circ C$. At this temperature the reaction was complete in 10 min; at $0^\circ C$ the reaction proceeded at one-half of the rate at $24^\circ C$.

FIG. 3. Analysis of the in vitro translation products of 24S UR2 and 35S UR2AV virion RNA. 24S UR2 (C, E, and F) and 35S UR2AV (B) virion RNAs were translated in the reticulocyte lysate system. Total translation mixtures (A, B, C, and E) or immunoprecipitates by antiserum against viral structural proteins (F) were analyzed by SDS-PAGE in a 5 to 15% gradient gel followed by autoradiography. Lane A contained no added RNA. Lane D corresponds to an immunoprecipitate of UR2-transformed cells labeled in vivo with $^{35}$S methionine. Lanes A–D and E and F correspond to two different acrylamide gels.

precipitate any kinase activity (Fig. 4, lane F). No kinase activity was detectable in uninfected (Fig. 4, lane A) or UR2AV-infected cells (Fig. 4, lane B). P68 was also precipitated by TBR sera, because these sera were reactive with the products of the gag gene of avian retroviruses. The immunoprecipitates of P68 by TBR sera were able to transfer $^{32}$P phosphate to P68 and to a lesser extent to the heavy chain of IgG (Fig. 4, lane G). As shown in lane H of Fig. 4, TBR serum preabsorbed with disrupted virus precipitated very little protein kinase activity from extracts of UR2-transformed cells. However, the absorption did not affect the capacity of the serum to precipitate the RSV-p60 protein kinase activity from RSV-infected cells (Fig. 4, lanes I and J). The residual kinase activity observed in lane H was probably due to the presence of endogenous p60 in the immune complexes or to incomplete absorption of the TBR serum, or to both. These results, therefore, indicate that the kinase activity precipitated from extracts of

FIG. 4. Protein kinase activity in immunoprecipitates containing P68. Unlabeled cell extracts prepared from uninfected (A), UR2AV-infected (B), UR2-infected (C–H), and RSV-infected (I and J) cell cultures were immunoprecipitated with the indicated antisera, and immunoprecipitates were assayed for protein kinase activity, as described in the text, in the absence (A–D and G–J) or in the presence (E and F) of 1 mg of α-casein per ml. Kinase reaction products were analyzed by SDS-PAGE in 8.5% gels followed by autoradiography. (A, B, D, E) Avitivirus antiserum; (F) preimmune serum; (C) anti-p19 antiserum; (G, I) TBR serum; (H, J) absorbed TBR serum. A–F and G–J correspond to two separate gels.
TABLE 1. Effect of cations on phosphorylation of UR2-P68, FSV-P140, Y73-P90, and α-casein by P68, P140, and P90 immunoprecipitates

<table>
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<tr>
<td></td>
<td>UR2-P68 kinase</td>
<td>FSV-P140 kinase</td>
<td>Y73-P90 kinase</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>P68</td>
<td>Casein</td>
<td>P140</td>
<td>Casein</td>
<td>P90</td>
</tr>
<tr>
<td>None</td>
<td>&lt;0.2</td>
<td>&lt;0.2</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
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<td>13.4</td>
<td>218.6</td>
<td>235.1</td>
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<tr>
<td>MgCl₂</td>
<td>12.4</td>
<td>2.8</td>
<td>34.8</td>
<td>75.3</td>
<td>39.3</td>
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<tr>
<td>CaCl₂</td>
<td>&lt;0.2</td>
<td>&lt;0.2</td>
<td>3.0</td>
<td>&lt;0.5</td>
<td>&lt;0.4</td>
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* Unlabeled cell extracts from UR2-, FSV-, and Y73-transformed cells were immunoprecipitated with antivirion antiserum, and immunoprecipitates were assayed for protein kinase activity as described in the text, except that the kinase buffer was supplemented with 1 mg of α-casein per ml. The cation concentration in the buffer was 10 mM in all cases. Kinase reaction products were analyzed by SDS-PAGE in 8.5% gels, and the radioactivity in the P68, P140, P90, and α-casein gel bands was determined.

b Data for each kinase were obtained from the results of independent experiments. Results with FSV-P140 kinase are reproduced from reference 8.

The enzyme had an absolute requirement for an appropriate divalent cation (Table 1). Mn²⁺ ions were found to be four times more effective than Mg²⁺ ions. Ca²⁺ ions did not support any detectable level of ³²P incorporation. The effect of divalent cations on the extent of phosphorylation was found to be similar for both α-casein and P68 (Table 1). Table 1 also shows that Mn²⁺ had a marked stimulatory effect on FSV-P140 and Y73-P90 kinases and that the effect of the different cations was in all cases similar for both autophosphorylation and phosphorylation of α-casein. (Our results on the stimulation of Y73-P90 kinase by Mn²⁺ are not consistent with those reported by Ghysdael et al. [9]. The reason for the discrepancy is not clear.)

At the three concentrations that we tested (0.01 to 1.0 μM), [γ-³²P]GTP did not serve as a [³²P]phosphate donor in the P68 kinase reaction, whereas the incorporation of [³²P]phosphate into P68 increased with increasing concentrations of [γ-³²P]ATP added (Table 2). Table 2 also shows that, unlike UR2-P68 and FSV-P140, RSV-p60 and to a lesser extent Y73-P90 were capable of using [γ-³²P]GTP as the donor of [³²P]phosphate.

P68 protein kinase was active over a wide range of pH. As shown in Fig. 5, the reaction proceeded at near maximal rates between pH 6.5 and 8.5, and at pH 10.0 P68 still retained 60% of its kinase activity. However, evaluation of the reaction properties at alkaline pH is hampered by the increasing instability of ATP to alkaline pH. The reaction rate fell off rapidly below pH 6.5, probably as a consequence of irreversible inactivation of P68 kinase at low pH (see below). As shown in Fig. 5, Y73-P90 kinase was also active over a wide range of pH. By contrast, FSV-P140 kinase, which had a pH optimum of about 7.0, was active only over a narrow range of pH.

To study the effect of pH on the inactivation of P68 kinase, we washed immunoprecipitates of P68, first several times in a buffer of the desired pH and then several times in a buffer of pH 7.5. As shown in Fig. 6, P68 kinase was stable at neutral and alkaline pH, but it was rapidly and irreversibly inactivated below pH 6.5. Interestingly, the temporary exposure of P68 immunoprecipitates to pH 9.5 to 10.0 resulted in enhanced enzymatic activity. This might result from conformational changes in the immunocomplexes favoring the reaction rate or from the removal of an inhibitor of P68 kinase from the immune complexes. It is worth noting that the

TABLE 2. Phosphate donors for the UR2-P68, Y73-P90, and RSV-p60 kinase reactions

<table>
<thead>
<tr>
<th>³²P donor</th>
<th>Conc (nM)</th>
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<tr>
<td></td>
<td>UR2-P68 kinase</td>
<td>FSV-P140 kinase</td>
<td>Y73-P90 kinase</td>
</tr>
<tr>
<td>[γ-³²P]ATP</td>
<td>10</td>
<td>30.4</td>
<td>40.7</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>74.6</td>
<td>92.6</td>
</tr>
<tr>
<td></td>
<td>1,000</td>
<td>201.3</td>
<td>437.8</td>
</tr>
<tr>
<td>[γ-³²P]GTP</td>
<td>10</td>
<td>&lt;0.2</td>
<td>18.3</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>&lt;0.2</td>
<td>40.5</td>
</tr>
<tr>
<td></td>
<td>1,000</td>
<td>&lt;0.2</td>
<td>175.1</td>
</tr>
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</table>

* Unlabeled cell extracts from UR2- and Y73-transformed cells were immunoprecipitated with antivirion antiserum. Unlabeled cell extracts from Schmidt-Ruppin RSV-transformed cells were immunoprecipitated with TBR serum and immunoprecipitates were assayed for protein kinase activity as described in Materials and Methods, except that [γ-³²P]ATP and [γ-³²P]GTP (2,000 Ci/mmole; New England Nuclear) were present at the indicated concentrations. Kinase reaction products were analyzed by SDS-PAGE in 8.5% gels, and the radioactivity in the P68, P90, and IgG gel bands was determined.

b Data for each kinase were obtained from the results of independent experiments.
P68 phosphorylates tyrosine of acceptor proteins. The identities of the amino acids serving as phosphate acceptors in the P68 kinase reaction were determined. As shown in Fig. 7B, the amino acid acceptor of phosphate in P68 in vitro is tyrosine. IgG and α-casein are also phosphorylated at tyrosine in the P68 kinase reaction (data not shown). Similar analysis of the phosphoamino acid composition of in vivo $^{32}$P-labeled P68 showed that it contained both phosphoserine and phosphotyrosine (Fig. 7A).

P68 is phosphorylated in vitro at a unique tyrosine site. The tyrosine sites that are phosphorylated in the in vitro kinase reaction were examined by tryptic fingerprint analysis of in vitro $^{32}$P-labeled P68. As shown in Fig. 8C, only one labeled phosphopeptide was present in tryptic digests. This indicated that P68 was phosphorylated in vitro at only one tyrosine site. It was also observed that the P68 phosphopeptide migrated with a different mobility compared with the phosphopeptides derived from in vitro-labeled FSV-P140 (Fig. 8A) and Y73-P90 (Fig. 8B). This difference in mobility, which was confirmed by mixing experiments (data not shown), suggests that P68 has a unique tyrosine phosphorylation site in vitro that is different from those of FSV-P140 and Y73-P90. It can also be seen in Fig. 8 that FSV-P140 and Y73-P90 do not share any phosphopeptide.

FIG. 5. pH dependence of UR2-P68, FSV-P140, and Y73-P90 kinase reactions. Unlabeled cell extracts prepared from UR2- (○), FSV- (◇), and Y73- (■) transformed cells were immunoprecipitated with antivirion antiserum and immunoprecipitates were assayed for protein kinase activity as described in the text, except that the pH of the kinase buffer was as indicated. Kinase reaction products were analyzed by SDS-PAGE in 8.5% gels, and the radioactivity in the P68, P140, and P90 gel bands was determined. The concentration of buffer in the kinase reaction mixtures was 25 mM in all cases. Buffers used were as follows: pH 5.0 and 5.5, sodium acetate; pH 6.0 to 7.5, sodium phosphate; pH 8.0 to 9.0, Tris-hydrochloride; pH 9.5 and 10.0, glycine-sodium hydroxide.

pH inactivation pattern of P68 kinase was similarly reflected in the phosphorylation of both P68 and α-casein. This strongly suggests that the phosphorylation of α-casein and P68 was catalyzed by one rather than two kinase molecules since it would be very unlikely that two different enzymes would exhibit the same pattern of pH inactivation.

Y73-P90 kinase but not FSV-P140 kinase was also found to be very stable at alkaline pH (data not shown).

FIG. 6. pH inactivation curve of P68 kinase. Unlabeled cell extracts prepared from UR2-transformed cells were immunoprecipitated with antivirion antiserum. After five washes in RIPA (8) buffer, immunoprecipitates were equilibrated to the desired pH by three washes in the indicated buffer (50 mM) over a period of 30 min. After this, immunoprecipitates were re-equilibrated to pH 7.5 by three washes in 50 mM N-2-hydroxyethylpiperazine- N'-2-ethanesulfonic acid (HEPES)-KOH, pH 7.5, over a period of 30 min. All washes were carried out at 4°C. Immunoprecipitates were then assayed for protein kinase activity as described in the text, except that the kinase buffer was supplemented with 1 mg of α-casein per ml. Kinase reaction products were analyzed by SDS-PAGE in an 8.5% gel, and the radioactivity in the P68 (○) and α-casein (◇) bands was determined. Buffers used for pH equilibration were the same as given in the legend to Fig. 5.
FIG. 7. Analysis of the phosphoamino acid composition of P68 phosphorylated in vivo and in vitro. Partial acid hydrolysates of 32P-labeled P68 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 the lower left corner, is marked X. The positions of the internal phosphoamino acid standards are indicated. The other 32P-labeled spots observed in the chromatogram are probably partially hydrolyzed phosphopeptides. (A) P68 phosphorylated in vivo; (B) P68 autophosphorylated in vitro. S, Phosphoserine; T, phosphothreonine; Y, phosphotyrosine.

DISCUSSION

In this paper we present evidence suggesting that P68 is the transforming gene product of UR2 and that it is associated with a tyrosine-specific protein kinase activity. We also compare this enzymatic activity with that of other ASV transforming proteins.

The product of the fused gag-ros gene of UR2 (30) was identified as a 68,000-dalton protein (P68). This protein was detected in extracts of UR2-transformed cells by immunoprecipitation with antiserum against gag-coded proteins. A TBR serum absorbed with virion proteins, which is considered to be specific to the src gene product, failed to precipitate any P68, suggesting that P68 does not share antigenic determinants with RSV-p60. This is in agreement with the fact that src, fps, and yes sequences are not present in the UR2 genome (26, 30). Although P68 accounts for only 80% of the coding capacity of the 24S viral genome of UR2 (30), no other gene products of UR2 were detected and P68 was not derived from a larger precursor: P68 was the only protein synthesized in vitro by 24S genomic RNA, and it was the only protein detectable in vivo after very short pulses. Our data also indicate that P68 is a final product; i.e., it does not undergo any detectable proteolytic cleavage during its entire lifetime. Thus, P68 is the only defined product of the viral genome of UR2, both in vitro and in vivo, and it is associated only with UR2-transformed cells, strongly suggesting that P68 is responsible for cell transformation.

P68 immunoprecipitates were found to be associated with a protein kinase activity that phosphorylates acceptor proteins at tyrosine residues. The P68 protein kinase exhibited a marked preference for autophosphorylation over the phosphorylation of other acceptor proteins. Nevertheless, as was observed with some of the other gag-containing polypeptides (8, 10, 16, 21, 29), the P68 enzyme was found to be capable of transferring phosphate to IgG or α-casein in addition to P68 itself. Several lines of evidence support the idea that α-casein phosphorylation is catalyzed by P68 protein kinase rather than by a contaminating cellular protein coprecipitating with P68. The phosphorylation of α-casein was observed only with the immunoprecipitates from UR2-transformed cells, and not from uninfected or UR2AV-infected cells; various parameters affecting the phosphorylation of P68 itself, such as salts, temperature, and pH, have the same effect on the phosphorylation of α-casein; and finally, the α-casein molecules were phosphorylated almost exclusively at tyrosine residues.

Analysis of the tryptic tyrosine phosphopep-
tides of UR2-P68, FSV-P140, and Y73-P90 labeled in vitro showed that they had different chromatographic properties, suggesting that the primary structure in the vicinity of the tyrosine phosphorylation sites in P68, P140, and P90 is also different. The difference between in vitro tyrosine phosphorylation sites in FSV-P140 and Y73-P90 confirms a previous report by Neil et al. (20).

The properties of the protein kinases associated with the src, gag-fps, gag-yes, and gag-ros gene products are summarized in Table 3. RSV-p60 kinase is the only enzyme of the group that does not exhibit any preference for Mn2+ over Mg2+ ions; Y73-P90 shares with RSV-p60 the ability to utilize GTP as well as ATP as a donor of γ-phosphate; UR2-P68 and Y73-P90 differ markedly from FSV-P140 in that they are very stable to alkaline pH and they have a different and wider range of optimal pH. It should be noted, however, that these comparisons were made with enzyme-antibody complexes, and a more definitive analysis should be made with purified proteins. Nevertheless, the differences recognized thus far strongly suggest that the protein kinase activity associated with each ASV transforming protein is distinct.

The fact that the four types of ASV transforming protein discussed above are all associated with tyrosine-specific protein kinase activities (6, 8, 10, 12, 14, 16, 21, 23, 29) is of great interest. The elevated expression of these enzyme activities in infected cells appears to have the same effect of cellular alteration to the transformed state. Similar involvement of protein kinase specific to tyrosine phosphorylation has been implicated in cell transformation with some mammalian retroviruses (2, 28, 31). Whether these different enzymes act on common substrates and affect similar metabolic pathways is an interesting question that remains to be answered.

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LITERATURE CITED


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<th>P donor</th>
<th>P transfer</th>
<th>Substrate for immune complex</th>
<th>Cation</th>
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<td>RSV-p60</td>
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<td>Independent (6, 14)</td>
<td>ATP, GTP (17, 19, 25)</td>
<td>Tyrosine (6, 14)</td>
<td>IgG, p60 (6, 15, 19)</td>
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<td>7.0–8.0</td>
</tr>
<tr>
<td>FSV-P140</td>
<td>Serine, tyrosine</td>
<td>Independent (8)</td>
<td>ATP (8)</td>
<td>Tyrosine (8, 12, 23)</td>
<td>IgG, P140, casein (8, 12)</td>
<td>Mn &gt; Mg (25)</td>
<td>6.5–7.5</td>
</tr>
<tr>
<td>Y73-P90</td>
<td>Serine, tyrosine</td>
<td>Independent (9)</td>
<td>ATP, GTP (9)</td>
<td>Tyrosine (16)</td>
<td>IgG, P90, casein (9, 16)</td>
<td>Mn &gt; Mg (25)</td>
<td>6.5–8.5</td>
</tr>
<tr>
<td>UR2-P68</td>
<td>Serine, tyrosine</td>
<td>Independent</td>
<td>ATP</td>
<td>Tyrosine</td>
<td>IgG, P68, casein</td>
<td>Mn &gt; Mg (25)</td>
<td>6.5–8.5</td>
</tr>
</tbody>
</table>

^a For results obtained in this study, no reference is given. Other data were from previously published reports as indicated by the numbers in parentheses.

^b R. A. Feldman, unpublished data.

TABLE 3. Comparison of the src, gag-fps, gag-yes, and gag-ros gene products of the transforming protein of new ASV.


