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Detection of Phosphorylated Forms of Moloney Murine Leukemia Virus Major Capsid Protein p30 by Immunoprecipitation and Two-Dimensional Gel Electrophoresis

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We detected phosphorylation of the major Moloney murine leukemia virus (M-MuLV) capsid polypeptide, p30, by using $^{32}P_i$-labeled virions. This was observed both on two-dimensional polyacrylamide gels directly or on one-dimensional gels of viral lysates that had been immunoprecipitated with monospecific goat anti-p30 serum. The phosphorylation event had been difficult to detect because pp12 the major virion phosphoprotein incorporates almost all of the ^{32}P label added to infected cells (Y. Yoshinaka and R. B. Luftig, Virology 116:181–195, 1982). When immunoprecipitates from M-MuLV lysates labeled with $^{32}P_i$ were compared with those labeled with [^{35}S]methionine, it was calculated that the degree of phosphorylation at the p30 domain of Pr65^{gag} was only 0.22 to 0.54% relative to phosphorylation at the p12 domain. Two-dimensional gel electrophoresis of the ^{32}P-labeled p30 immunoprecipitates showed that there were three phosphorylated p30 forms with isoelectric points (pIs) of 5.7, 5.8, and 6.0. These forms were generally more acidic than the [^{35}S]methionine-labeled p30 forms, which had pIs of 6.0, 6.1, 6.3 (the major constituent with >80% of the label), and 6.6. The predominant phosphoamino acid of the major phosphorylated p30 form (pI 5.8) was phosphoserine. Further, tryptic peptide analysis of this p30 form showed that only one peptide was predominantly phosphorylated. Based on a comparison of specific labeling of p30 tryptic peptides with [^{14}C]serine, [^{35}S]methionine, and $^{32}P_i$, we tentatively assigned the phosphorylation site to a 2.4-kilodalton NH_2-terminal peptide containing triple tandem serines spanning the region from amino acids 4 to 24.

Type C retroviruses are a morphologically distinct class of viruses composed of an RNA-protein inner core structure encapsulated in a lipoprotein envelope. Murine leukemia viruses (MuLVs) are structurally the most thoroughly characterized of all the mammalian retroviruses. The inner core proteins of MuLVs are synthesized via a precursor polyprotein, Pr65^{gag}, that yields the mature proteins, p15, p12, p30, and p10 (3, 16, 17, 19, 26). Pr65^{gag} itself is not glycosylated, although two covalent modifications are known to occur, viz., the protein is phosphorylated at serine residues (19) and a fatty acid, myristic acid, is joined in an amide linkage to the N-terminal glycine (8, 29).

It is widely accepted that protein phosphorylation is an important modification that occurs in the regulation of many metabolic processes. Strand and August (33) were the first to report that a MuLV contains both a protein kinase activity and substrate proteins. Thereafter, it was confirmed that all type C RNA tumor viruses contain a major structural phosphoprotein, e.g., pp12 for rodent retroviruses (23, 24).

In the present study, we show that another MuLV *gag*-encoded protein in addition to pp12, viz., p30, is also phosphorylated in virions. This phosphorylation event had gone undetected because of its relatively low level compared to the level of phosphorylation of pp12. Our study extends previous results which showed that p30, when analyzed on two-dimensional (2D) sodium dodecyl sulfate (SDS)-polyacrylamide gels, exhibits multiple forms that differ in pI (11).

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MATERIALS AND METHODS

Viruses and cells. MJD-54 cells are a line of JLS-V9 mouse fibroblasts chronically infected with Moloney MuLV (M-MuLV). Clone 2 cells are a cloned cell line of 3T3 mouse fibroblasts chronically infected with M-MuLV. Both cell lines were obtained from K. Manly, Roswell Park Memorial Institute, Buffalo, N.Y. SC-1 cells are a line of S^+L^- uninfected mouse fibroblasts (7) which were obtained from M. Dewey, University of South Carolina, Columbia. These cells were cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 5% newborn calf serum.

Radiolabeling of cells. Uninfected and chronically infected cells were grown in 60-mm-diameter dishes (Costar, Cambridge, Mass.) in DMEM supplemented with 5% fetal bovine serum. The monolayer cells were replaced with new medium containing DMEM supplemented with 2% newborn calf serum every day for 4 days. These cells were then labeled for 16 h with 2.5 mCi of $^{32}P_i$ (ICN Pharmaceuticals Inc., Irvine, Calif.) per dish in 2 ml of phosphate-free (MEM) supplemented with 4% fetal bovine serum or 300 μCi of L-[^{35}S]methionine per dish in 2 ml of MEM containing 1/10 the normal concentration of methionine and 4% fetal bovine serum. For pulse-labeling and chasing, the cells were starved for 1 h with methionine-free MEM, pulse-labeled with 200 μCi of L-[^{35}S]methionine per dish in 1 ml of methionine-free MEM, and then chased in DMEM containing 5% fetal bovine serum and 10-times excess methionine for 4 h.

Radioactive virion preparation. Radioactive virion fractions were prepared from the culture fluids of the labeled cells as described above. The unlabeled virions used for carrier were independently purified from large amounts (2 to

5 liters) of culture fluids by Sepharose CL-4B chromatography after precipitation of virions by polyethylene glycol 6000 in the presence of 0.4 M NaCl by the method of Aboud et al. (1). The appropriate amounts of unlabeled virions and radioactive virions were then mixed with the culture fluids to give a visible virus band and loaded onto a 20 to 50% (wt/vol) linear sucrose gradient in STE buffer (0.1 M NaCl, 0.01 M Tris hydrochloride [pH 7.4], 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride). After centrifugation at 35,000 rpm for 1 h at 4°C in an SW41 rotor (Beckman Instruments, Inc., Fullerton, Calif.), the visible virus band was removed and diluted with STE buffer. The virus was then pelleted by centrifugation at 40,000 rpm for 30 min at 4°C in a Beckman SW50.1 rotor. For the preparation of crude virus fractions, the culture fluids of the labeled cells were centrifuged through 25% (wt/vol) sucrose in STE buffer at 46,000 rpm in an SW50.1 rotor for 2 h at 4°C by the method of Ro and Ghosh (27).

Immunoprecipitation. Immunoprecipitation was carried out as follows. Labeled cell or virus pellets were solubilized by sonication for 10 to 20 s in lysis buffer (0.5% Nonidet P-40 and 0.5% sodium deoxycholate in TNE buffer [0.05 M Tris hydrochloride, pH 7.2, 0.1 M NaCl, 1 mM EDTA] containing 1 mM phenylmethylsulfonyl fluoride) (13) and centrifuged in an SW50.1 rotor at 30,000 rpm for 1 h at 4°C, and the supernatant was reacted for 16 h at 4°C with goat anti-Rauscher MuLV p30 serum (lot 78S-221), goat anti-Rauscher MuLV p12 serum (lot 77S-559), or unimmunized-goat serum (lot 81S-249), all of which were obtained through the Resources Program of the National Cancer Institute, Bethesda, Md. The immune complexes were then treated with protein A-Sepharose CL-4B beads (Pharmacia Fine Chemicals, Piscataway, N.J.) with constant shaking at room temperature for 15 min. The beads were collected in an Eppendorf tube by centrifugation and washed six times with 1% Nonidet P-40 in TNE buffer containing 1 mM phenylmethylsulfonyl fluoride and once with distilled water.

Gel electrophoresis. Samples for one-dimensional (1D) SDS-polyacrylamide gel electrophoresis PAGE were solubilized in sample buffer (1% SDS, 5% 2-mercaptoethanol, 0.0625 M Tris hydrochloride [pH 6.8], 10% glycerol, 0.001% bromophenol blue) by heating for 3 min at 95 to 100°C. If the sample had been immunoprecipitated with Sepharose CL-4B beads, the beads were removed by centrifugation. The soluble polypeptides were then analyzed by SDS-PAGE (separation gel, 6 to 12% linear-gradient polyacrylamide gel; spacer gel, 4%) by the method of Laemmli (14).

2D gel electrophoresis was carried out essentially by the method of O'Farrell and O'Farrell et al. (20, 22). Samples were suspended in 20 μl of 1% SDS containing 0.1 M DL-dithiothreitol and incubated for 1 h at 22°C before being heated for 3 min at 95 to 100°C. As above, if the sample had been immunoprecipitated, the Sepharose CL-4B beads were removed by centrifugation. Next, the solution was treated with 10 μl of 0.1 M *N*-ethylmaleimide (36) for 1 h at 4°C. Five microliters of Ampholines (13.3% Ampholine, pH 5 to 8, and 3.3% Ampholine, pH 3.5 to 10; LKB Instruments, Inc., Rockville, Md.), 10 μl of 20% Nonidet P-40, and 60 mg of solid urea were then added, and the resulting samples were separated according to charge by pH gradient electrophoresis by using 1.6% Ampholine, pH 5 to 8, and 0.4% Ampholine, pH 3.5 to 10, as described previously (9). After electrophoresis at 300 V for 15 h and 800 V for 1 h, the gels were equilibrated in SDS sample buffer (2.3% SDS, 5% 2-mercaptoethanol, 10% glycerol, 62.5 mM Tris hydrochloride [pH 6.8]) and then subjected to electrophoresis in the second dimension under the same conditions as those described above for 1D SDS-PAGE. Fluorographs were obtained by the method of Chamberlain (4).

Phosphoamino acid analysis. $^{32}P_i$-labeled polypeptides were obtained by electrophoretically eluting the protein from 2D gel slices in a dialysis bag. After being dried, the polypeptides were solubilized in 50 μl of 6 N HCl, sealed in ampoules, and hydrolyzed at 110°C for 1 h. The hydrolysates were then dried and dissolved in 10 μl of water containing an equal mixture of unlabeled phosphoserine, phosphothreonine, and phosphotyrosine. Next, the samples were electrophoresed by paper chromatography: first dimension in pH 1.9 buffer (acetic acid-formic acid-water [87:25:887, vol/vol]) at 1,500 V for 40 min; second dimension in pH 3.5 buffer (acetic acid-pyridine-water [50:5:945, vol/vol]) at 1,500 V for 30 min. The chromatography paper was reacted with ninhydrin, dried, and subjected to autoradiography.

Tryptic peptide analysis. Slices containing specific polypeptides were cut from 2D gels and washed with 25% isopropanol and then with 10% methanol. The samples in the gel slices were then incubated at 37°C overnight with 0.5 ml of tolylsufonyl phenylalanyl chloromethyl ketone (TPCK)-trypsin (50 μg/ml; Worthington Diagnostics, Freehold, N.J.) in 0.05 M NH_4HCO_3 buffer (pH 8.0). The supernatants were removed and lyophilized. Samples were then analyzed on cellulose-coated thin-layer chromatography plates (20 by 20 cm; Eastman Kodak Co., Rochester, N.Y.), first by electrophoresis in buffer 1 acetic acid-formic acid-water [15:5:80, vol/vol]) and then by chromatography in buffer 2 (butanol-pyridine-acetic acid-water [32.5:25:5:20, vol/vol]) as described by Elder et al. (5). For determination of the molecular weights of the tryptic peptides, the peptides similarly digested with TPCK-trypsin were analyzed by 1D SDS-PAGE under the same conditions as those described above except that the size of the separation gel was substantially longer (30 cm) than usual (12 cm).

RESULTS

Initial observations of phosphorylated p30s in virions. Based on our previous observation that there was heterogeneity in the pIs of M-MuLV p30s when virions were assayed on 2D gels, we wanted to determine whether a posttranslational modification such as phosphorylation might account for formation of the more acidic p30s. Thus, we labeled chronically infected mouse cells with $^{32}P_i$ or [^{35}S]methionine, purified the virions, and analyzed the proteins on 2D gels. Figure 1 shows the pattern of heterogeneity of the pI of M-MuLV p30 when partially purified [^{35}S]methionine-labeled virions obtained from the culture fluids of two chronically infected cells lines, viz., MJD-54 (Fig. 1A) and Clone 2 (Fig. 1B), were separated by 2D gel electrophoresis. Several p30 spots can be readily identified for both MJD-54 and Clone 2 virions; one of these is a major spot, and three are minor spots. We also note that relative to p30, only a minor amount of p12 appears on the 2D gel despite the fact that it possesses one of the three *gag*-encoded methionines; p30 has the remaining two. This loss of p12 labeling may have been caused in part by the harsh urea denaturation treatment used for the 2D gel system. In support of this contention, on 1D gels of [^{35}S]methionine-labeled virus, more of p12 was labeled relative to p30 (Fig. 2A, left lane) than was labeled on 2D gels (Fig. 1A and B).

We then examined the pattern of $^{32}P_i$-labeled M-MuLV on 2D gels. We detected major labeling of pp12, as expected (Fig. 1C and D). In addition, we noted for the first time a

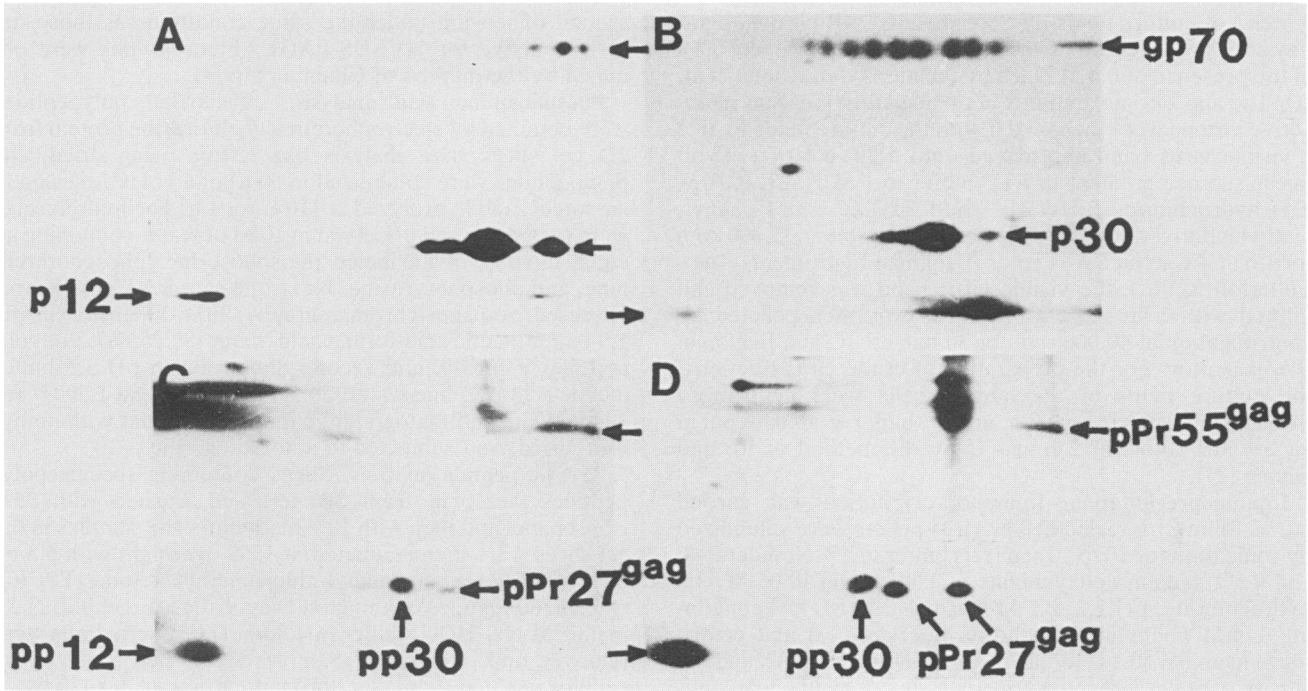

FIG. 1. 2D gel electrophoresis of virion fractions obtained by the method of Ro and Ghosh (27). Labeling of MJD-54 (A and C) and Clone 2 (B and D) cells was with [^{35}S]methionine (A and B) or $^{32}P_i$ (C and D) for 16 h. Concomitant experiments with [^{3}H] myristate labeling (12) and with MuLV p15- and p12-specific antisera identified the positions of pPr27^{gag}.

phosphorylated spot at an M_r position of p30, which we tentatively designated pp30. We also observed phosphorylation of the pp12 precursor forms, pPr27^{gag} and pPr55^{gag}, as well as phosphorylated spots at higher M_r positions that may have been due either to contaminating cellular proteins or to breakdown products from higher-molecular-weight precursors, such as Pr180$^{gag\text{-}pol}$.

Immunoprecipitation of the phosphorylated polypeptides with anti-p30 serum. We next wanted to quantitate the relative degree of phosphorylation of pp30 compared with that of pp12. This could not be done from the 2D gels shown in Fig. 1 for two reasons: (i) p12 appeared to be lost from the 2D gel, and (ii) although we had tentatively designated the newly observed phosphorylated-protein spot pp30, we had not confirmed its identity by immunological criteria and thus it could still be technically regarded as an electrophoretic variant of pPr27^{gag}. This led us to take another approach to quantitate the amount of pp30 in M-MuLV preparations. Specifically, $^{32}P_i$- or [^{35}S]methionine-labeled lysates of virions obtained from MJD-54 or Clone 2 cells were immunoprecipitated with goat anti-p30 serum and the resulting precipitates were run on 1D SDS-polyacrylamide gels. Relative to Pr65^{gag}, p30 was overwhelmingly labeled with ^{35}S (Fig. 2B and C, lanes S), whereas both were labeled to a moderate extent with ^{32}P (lanes P). Analogous precipitation of lysates with goat anti-p12 serum permitted detection of pp12 and Pr65^{gag}, albeit at much reduced levels for both compared with the corresponding amounts of p30 and Pr65^{gag} seen with anti-p30 serum (data not shown). Unimmunized goat serum did not precipitate any protein bands (Fig. 2D), nor did any appear in lysates of putative virion fractions obtained from mock-infected [^{35}S]methionine-labeled SC-1 cells (Fig. 2A, right lane).

We next performed a densitometric analysis of the protein bands shown in Fig. 2B and C and quantitated (in arbitrary densitometry units) the relative amounts of [^{35}S]methionine and $^{32}P_i$ in Pr65^{gag} and p30 (Table 1). Based on the equations presented in Table 1, footnote *b*, we were able to estimate the percentage of [^{35}S]methionine-labeled p30 domains in Pr65^{gag}, p30, and by extrapolation, p12.

Specifically, for M-MuLV obtained from MJD-54 cells, the percentage of $^{32}P_i$ in p30 was 0.22%. By subtraction, the degree of phosphorylation for pp12 was 99.78%. The corre-

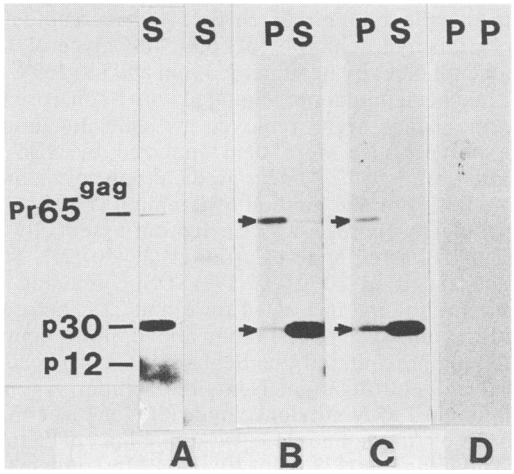

FIG. 2. 1D SDS-PAGE of detergent-treated virion lysates that had been labeled with $^{32}P_i$ or [^{35}S]methionine. MJD-54, Clone 2, or SC-1 cells were labeled with $^{32}P_i$ (lanes P) or [^{35}S]methionine (lanes S) for 16 h. (A) SDS-PAGE pattern of virion fractions obtained from MJD-54 (left lane) or mock-infected SC-1 (right lane) cells without immunoprecipitation. (B, C, and D) SDS-PAGE patterns of immunoprecipitates obtained from virion fractions of MJD-54 (B and left lane of D) or Clone 2 (C and right lane of D). Goat anti-p30 serum (B and C) or unimmunized-goat serum (D) was used for immunoprecipitation.

TABLE 1. Degrees of phosphorylation in M-MuLV $Pr65^{gag}$ and p30

Cell line used for M-MuLV	Radio-isotope	Intensity[a]		Phosphorylation (%)[b]	
		$Pr65^{gag}$	p30	pp12	pp30
MJD-54	^{35}S	3.35	305	99.78	0.22
	^{32}P	3.35	1		
Clone 2	^{35}S	3.35	1,030	99.46	0.54
	^{32}P	3.35	8		

[a] The intensities of $Pr65^{gag}$ and p30 polypeptide bands are shown in arbitrary units obtained by tracing of densitometric scans.

[b] The degree of phosphorylation of $Pr65^{gag}$ at its p12 and p30 domains was calculated by the following steps, taking into account that the relative methionine contents in the p15, p12, p30, and p10 regions of $Pr65^{gag}$ are 0, 1, 2, and 0, respectively. (i) The total [^{35}S]methionine counts on all p30 domains = 2/3(^{35}S counts on uncleaved $Pr65^{gag}$) + ^{35}S counts on p30 = 2/3(3.35) + 305.00 = 307.25 (2/3 represents the ratio of methionine in p30 domains of $Pr65^{gag}$ to the total number of methionine residues in $Pr65^{gag}$). (ii) The percentage of p30 domains in $Pr65^{gag}$ remaining uncleaved = 2/3(residual [^{35}S]methionine counts on $Pr65^{gag}$)/(total [^{35}S]methionine counts on all p30 domains) = 2/3(3.35)/307.25 = 2.25/307.25 = 0.74%. (iii) By using the $^{32}P_i$ counts for MJD-54 cells obtained from the table and letting x equal the total amount of ^{32}P counts in arbitrary units originally present in uncleaved $Pr65^{gag}$, we find that 0.74%(x) = 3.35, in terms of ^{32}P units. Thus, 0.0074(x) = 3.35 units and x = 452.70 ^{32}P units originally present in uncleaved $Pr65^{gag}$. (iv) By using the p30 intensity data, we find that the percentage of $^{32}P_i$ in p30 = 1 U/452.70 U = 0.22%.

sponding values for pp30 and pp12 for virus isolated from Clone 2 cells were 0.54 and 99.46%.

Thus, in confirmation of previous results (38), it appears that the predominant phosphorylation of $Pr65^{gag}$ is at the p12 domain and that only trace amounts (0.22 and 0.54% in MJD-54 and Clone 2 virions, respectively) of phosphorylation occur at the p30 domain.

Identification of the pI of the phosphorylated M-MuLV p30 spots. To accurately identify the pI of pp30 (Fig. 1C and D), we immunoprecipitated $^{32}P_i$-labeled p30 from detergent lysates of virions and compared it with [^{35}S]methionine-labeled p30 by using 2D gel electrophoresis. There were approximately four [^{35}S]methionine-labeled p30 spots, with pIs of 6.0, 6.1, 6.3, and 6.6, that could be identified in immunoprecipitates from MJD-54 virion lysates (Fig. 3A); a similar, albeit decreased, minor-spot profile can be seen for Clone 2 virion lysates (Fig. 3C). When virion lysates were labeled with $^{32}P_i$ and immunoprecipitated with M-MuLV p30 antiserum, we could identify a major ^{32}P-labeled p30 spot with a pI of 5.8 and two minor spots with pIs of 5.7 and 6.0 (Fig. 3B and D). The [^{35}S]methionine- or $^{32}P_i$-labeled p30s were then sliced out of the gel, and the radioactivity was counted. This provided quantitation of the relative amount of label in each p30 spot (Table 2). Based on these results, the amount of p30 with a pI of 5.8 is <1% of the total [^{35}S]methionine-labeled p30. To confirm that the phosphorylated p30s actually differed from the [^{35}S]methionine-labeled p30 forms, we performed a mixing experiment. When $^{32}P_i$-labeled p30 (Fig. 4C) was mixed with [^{35}S]methionine-labeled p30 (Fig. 4B), an increased density appeared in the acidic region of p30 on 2D gels of the mixed sample (Fig. 4A). This additivity (Fig. 4A) supports the contention that the major phosphorylated spot was distinct from the previously identified [^{35}S]methionine-labeled spots.

Phosphoamino acid analysis. To learn more about p30 phosphorylation, we sliced the pI 5.8 form from a 2D gel obtained with an anti-p30 immunoprecipitate of a detergent lysate of Clone 2 virions and performed a phosphoamino acid determination on the sample. As a control we used the phosphoprotein pp12 which had been sliced from a 2D gel of similarly labeled Clone 2 virions. Both the pp30 with a pI of 5.8 (Fig. 5A) and pp12 (Fig. 5B) contained phosphoserine as the predominant phosphorylated amino acid.

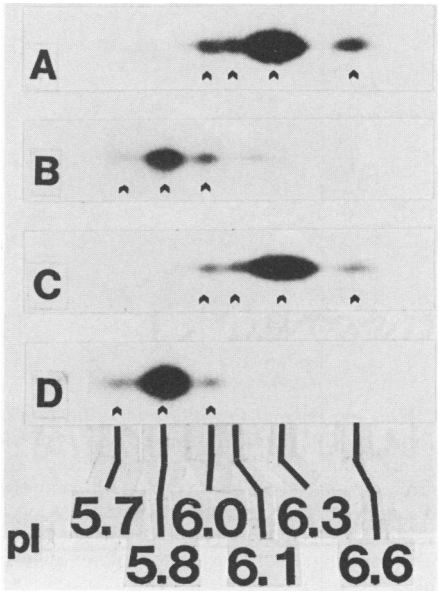

FIG. 3. 2D gel electrophoresis of immunoprecipitates obtained with anti-p30 serum from detergent-treated virion lysates labeled with $^{32}P_i$ or [^{35}S]methionine. MJD-54 (A and B) and Clone 2 (C and D) cells were labeled with [^{35}S]methionine (A and C) or $^{32}P_i$ (B and D) for 16 h.

Phosphorylated tryptic peptide analysis. The $^{32}P_i$-labeled p30 with a pI of 5.8 obtained from Clone 2 virions was next subjected to digestion with trypsin, and a tryptic peptide map was prepared (Fig. 6B). This map was compared with that obtained with [^{14}C]serine (Fig. 6A), and only one of about seven putative serine-containing tryptic peptides of p30 overlapped the phosphorylated p30 peptide (Fig. 6B). We also note that when tryptic peptide maps of [^{35}S]methionine-labeled p30 with a pI of 6.0 or 6.3 were prepared, only two spots, closely juxtaposed, were observed, one of which corresponded to the lower-right [^{14}C]serine-labeled tryptic peptide (Fig. 6A).

Site of phosphorylation. We next tried to locate the site of phosphorylation on pp30. This was done by using the 2D-gel tryptic peptide data shown in Fig. 6, as well as data obtained by using 30-cm-long 1D SDS-polyacrylamide gels with differentially labelled p30 tryptic peptides. The size of the $^{32}P_i$-labeled p30 tryptic peptide can be said initially to be consistent with a molecular mass in the range of 3 kilodal-

TABLE 2. Relative amount of [^{35}S]methionine or $^{32}P_i$ label in virion p30s separated by 2D SDS-PAGE

Source of virion[a]	Radio-isotope	Amt (%) of p30 with pI[b]:					
		5.7	5.8	6.0	6.1	6.3	6.6
MJD-54	^{35}S	0.3	0.9	3.6	2.9	83.4	8.9
Clone 2	^{35}S	0.2	0.9	2.5	1.9	91.5	3.0
	^{32}P	15.2	71.7	13.1			

[a] The virion fraction was used for all determinations.

[b] The amounts of the p30s were calculated as percentages after the radioactivity in each p30 in the slice was determined in a cocktail (Beckman Ready-Solv MP) by liquid scintillation counting.

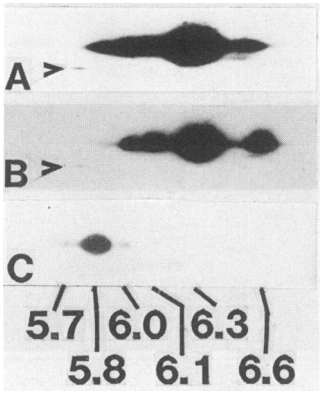

FIG. 4. Mixing experiment to confirm identification of the phosphorylated forms of p30. Detergent-treated lysates of Clone 2 virus were immunoprecipitated with goat anti-p30 serum. The immunoprecipitates from virions labeled with [^{35}S]methionine (B) or $^{32}P_i$ (C) or the mixed samples of both immunoprecipitates (A) were analyzed similarly by 2D gel electrophoresis. >, Marker for purposes of comparison. A similar result was observed for detergent-treated Clone 2 virus.

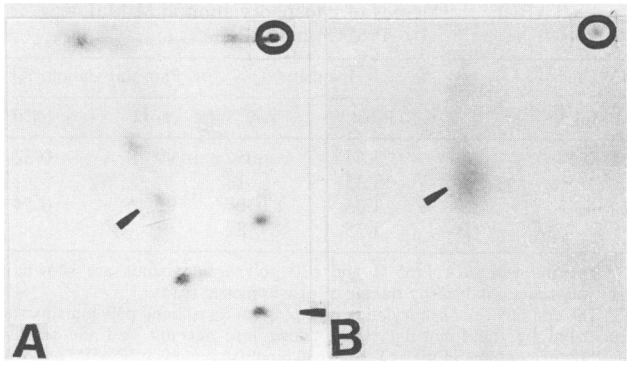

FIG. 6. Tryptic peptide maps of Clone 2 p30s with pIs of 6.3 and 5.8. Clone 2 virions labeled with [^{14}C]serine (pI 6.3) (A) or $^{32}P_i$ (pI 5.8) (B) were immunoprecipitated with anti-p30 serum, and the immunoprecipitates were separated by 2D gel electrophoresis. The p30s were sliced from the 2D gel, trypsinized, and analyzed as described in the text. The central arrowhead locates the major phosphorylated peptide, and the arrowhead at the lower right of panel A denotes the jointly labeled [^{14}C]serine-[^{35}S]methionine tryptic peptide. Circles represent spots at which material was loaded onto the thin-layer plates.

tons (kDa) (Fig. 7, lane 2), relative to a known series of low-molecular-mass markers (Fig. 7, rightmost lane). Since this is a nonlinear portion of the SDS-polyacrylamide gel, this molecular mass estimate is only approximate. We next tried to compare the position of the phosphorylated tryptic peptide with those of peptides labeled by other radioisotopes. The phosphorylated peptide (Fig. 7, lane 2) was slightly smaller than the smallest [^{35}S]methionine-labeled tryptic peptide (lane 4) and was the same size as a [^{14}C]serine-labeled tryptic peptide (lane 1). Based on this size relationship, the molecular masses of expected tryptic peptides, about 1 kDa or larger (Table 3), the M-MuLV p30 sequence obtained from GenBank, and the data shown in Fig. 6, it appears that the phosphorylation site on pp30 is located within the domain of tryptic peptide 3 (Table 3), which extends from NH_2-terminal amino acid residues 4 to 24. Specifically, this determination was based on the following assumptions: (i) the smallest [^{35}S]methionine-labeled band (Fig. 7, lane 4) corresponds to tryptic peptide 2, since it was the only methionine-labeled peptide in the 3-kDa size range (Table 3); (ii) the major phosphorylated tryptic peptide (Fig. 7, lane 2) was slightly smaller than this methionine peptide; (iii) the large [^{14}C]serine-labeled tryptic peptide (Fig. 7, lane 1) appears to be close in size to both the small [^{35}S]methionine-labeled peptide (Fig. 7, lane 4) and the ^{32}P-labeled tryptic peptide (Fig. 7, lane 2), suggesting that it may be a mixture of the two; (iv) the $^{32}P_i$-labeled tryptic peptide shown in Fig. 6 overlaps only one [^{14}C]serine-labeled peptide, whose location is distinct from that of the [^{35}S]methionine-labeled tryptic peptide; and (v) it appears that tryptic peptide 4 has no serine.

If our assumptions are correct, then phosphorylation of one or more of the triple tandem serines spanning the NH_2-terminal region from amino acids 4 to 24 represents the major site of p30 phosphorylation.

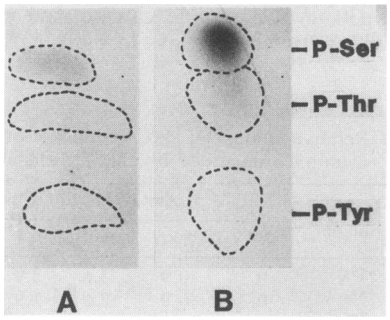

FIG. 5. Identification of the phosphoamino acid in Clone 2 pp30 with a pI of 5.8. Clone 2 pp30 with a pI of 5.8 (A) and pp12 (B) were obtained by cutting the slices from 2D gels in which the virions labeled with ^{32}Pi were separated. P-Ser, Phosphoserine; P-Thr, phosphothreonine; P-Tyr, phosphotyrosine.

DISCUSSION

The presence of a major phosphorylated form of p30 with a pI of 5.8 and two minor forms with pIs of 5.7 and 6.0 was clearly demonstrated in M-MuLV by using 2D gel electrophoresis and immunoprecipitation followed by 1D or 2D

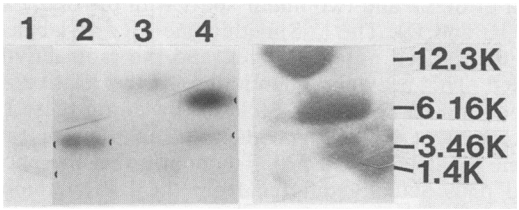

FIG. 7. Tryptic peptides of partially purified p30. Clone 2 cells were labeled with [^{14}C]serine (lane 1), $^{32}P_i$ (lane 2), a ^{14}C-labeled amino acid mixture (lane 3), or [^{35}S]methionine (lane 4). Initially, the detergent-treated virion fractions obtained from culture fluids of appropriately labeled cells were immunoprecipitated with goat anti-p30 serum, and the precipitates were separated by 2D gel electrophoresis. The p30 with a pI of 5.8 was sliced from the 2D gel of the precipitate that had been labeled with $^{32}P_i$. Correspondingly, the p30s with a pI of 6.3 were sliced from the 2D gel of the other labeled precipitates. The p30s in the gel slices were then trypsinized and subjected to SDS-PAGE on long, 30-cm separating gels. The marker peptides used for the determination of molecular weight were cytochrome *c* (12,300), bovine trypsin inhibitor (6,160), glucagon (3,460), and bacitracin (1,400). K, Molecular weight in thousands.

TABLE 3. Tryptic peptides of M-MuLV p30[a]

Peptide	Amino acid no.[b]	Mol wt	Total no. of residues	pI	No. of serines	No. of methionines
1	37–68	3,566.850	32	4.72	1	0
2	164–192	3,301.390	29	4.17	3	1
3	4–24	2,418.480	21	7.00	3	0
4	86–101	1,796.900	16	4.72	0	0
5	141–156	1,702.750	16	5.32	2	0
6	102–112	1,309.310	11	5.32	0	0
7	25–36	1,305.220	12	5.32	2	0
8	120–131	1,253.400	12	11.20	0	0
9	113–119	938.030	7	11.20	0	0

[a] Original sequence obtained from GenBank database.
[b] Range of amino acids, starting from the NH_2 terminus.

SDS-PAGE. Previously, there have been reports of the presence in vivo or in vitro or both of phosphorylated forms of M-MuLV $Pr65^{gag}$ (p15, p12, p30, and p10), $Pr40^{gag}$ (p30 and p10), $Pr27^{gag}$ (p15 and p12), and p12 or p10, (19, 30, 37, 38). Of these forms, pp12 was the predominantly phosphorylated species.

Analogous retroviruses of primate and feline origin also have a predominant phosphoprotein, pp15. In avian viruses the predominant phosphorylation is of pp19. Avian pp19 is unusual in that it also has properties similar to those of the membrane-binding protein, p15, of MuLVs. All of the predominant phosphoproteins listed above occur in the virion as variously charged molecular species corresponding to various degrees of phosphorylation, except for pp12 of wild-mouse ecotropic and amphotropic viruses. In these viruses, a large subpopulation of molecules remain in a nonphosphorylated form. It has also been shown that the number and relative proportion of the variously charged species of MuLV phosphoprotein pp12 are virus specific and independent of the cell lines used for propagation of the virus (23). This suggests the possibility that the MuLV-associated protein kinase is involved in phosphorylation of MuLV p12, which is supported by results of in vivo and in vitro experiments (19, 24, 30, 37, 39). The functional significance of such phosphorylation by putative endogenous viral kinases may reflect some regulatory role of p12 in viral replication, since the degree of phosphorylation of p12 appears to determine the extent of p12 binding to genomic RNA (31).

As noted above, all retroviruses share the presence of a major phosphoprotein. However, only M-MuLV appears thus far to have in addition to pp12, another phosphoprotein, viz., pp30, which is present as a minor subset of M-MuLV p30. We believe that our use of immunoprecipitation together with 2D gel electrophoresis enabled this relatively minor phosphorylation of p30 (pp30) to be clearly identified. The predominant phosphorylation (>99%) was at the p12 domain of $Pr65^{gag}$, with only <1% of labeling at the p30 domain. It is possible that other retroviruses, when analyzed for $^{32}P_i$ incorporation into protein, as we have done for M-MuLV, will also exhibit labeling of a minor subset of the major capsid protein.

We additionally observed that the ^{32}P-labeled phosphoamino acid on M-MuLV p30 with a pI of 5.8 was phosphoserine. Further, it appears likely that the phosphorylation site is located on a 2.4-kDa peptide containing triple tandem repeated serines spanning the NH_2-terminal region from amino acids 4 to 24 (32). However, the location of this site is best determined by radiosequencing of the ^{32}P-labeled peptide.

Our results for M-MuLV capsid protein phosphorylation are consistent with the finding of phosphorylation sites on the capsid proteins of papovaviruses, viz., simian virus 40 VP_1 (21, 34, 35) and polyomavirus VP_1 (2, 6). Ponder et al. (25) estimated that 12 to 15% of VP_1 monomers in the polyomavirus virion are phosphorylated, which is consistent with a structural role for them at the vertices of icosahedral capsids. As an aside, in a recent study with purified polyomavirus VP_1 molecules obtained from an *Escherichia coli* expression system, the unmodified VP_1 molecules could be assembled into capsidlike structures (28). This might suggest that VP_1 phosphorylation is important for distinguishing how different polyomavirus populations arising from infection of primary mouse kidney or embryo cell cultures differ in their binding to specific cell receptors. This alternative, as well as the potential effect VP_1 phosphorylation may have on the efficiency of capsid assembly and/or the ability of VP_1 to assemble around minichromosomes, still leaves open the role of VP_1 phosphorylation in virus capsid assembly.

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