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# Evidence that the Abelson virus protein functions *in vivo* as a protein kinase that phosphorylates tyrosine

(B-cell lymphoma/phosphotyrosine/phosphoproteins/viral transformation)

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**ABSTRACT** Both lymphocytes and fibroblasts that have been transformed by Abelson murine leukemia virus contain 6- to 12-fold increased levels of the rare modified amino acid phosphotyrosine in their proteins. This observation, coupled with the fact that the p120 protein encoded by this virus has been shown to undergo an apparent autophosphorylation to yield phosphotyrosine *in vitro*, suggests that Abelson virus encodes a protein kinase that phosphorylates tyrosine in transformed cells. These results are similar to those obtained previously with Rous sarcoma virus and suggest, by analogy, that the modification of cellular polypeptides through the phosphorylation of tyrosine may be involved in cellular transformation by Abelson virus. p120 isolated from transformed cells contains phosphoserine, phosphothreonine, and phosphotyrosine. The phosphotyrosine is found at two sites in the protein. p120 therefore may be a protein kinase that undergoes autophosphorylation *in vivo*.

Abelson murine leukemia virus (A-MuLV) transforms an unusual spectrum of murine cell types. Although the predominant targets of this virus, both *in vivo* and *in vitro*, appear to be pre-B lymphocytes (1), direct transformation of the fibroblastic 3T3 cell line has been demonstrated *in vitro* (2) and transformed macrophages have been isolated *in vivo* (3). A-MuLV was isolated (4) from a lymphosarcoma that appeared in a steroid-treated mouse after injection of Moloney murine leukemia virus (M-MuLV), a virus that transforms none of the cells listed above (5). It seems likely that A-MuLV arose by recombination between M-MuLV and cellular genetic information. The 5600-base, single-stranded RNA genome of A-MuLV consists of the two ends of the M-MuLV genome which flank 3600 bases homologous with genetic information in chromosomal DNA of normal uninfected mice (6, 7).

A-MuLV appears to encode only one protein (8, 9). The size of this protein ranges from 90,000 to 160,000 daltons in different stocks of the virus (10). A majority of the stocks of A-MuLV encode a protein of 120,000 daltons and therefore we refer here to the Abelson protein as p120. p120 is a hybrid protein encoded in part by the portion of the A-MuLV genome derived from the *gag* gene of M-MuLV and in part by the acquired cellular genetic material (8, 9). It is likely, but by no means proven, that p120 is involved in transformation by this virus. The mechanism of transformation by A-MuLV is not understood.

The biochemical basis of cellular transformation by a tumor virus is best understood in the case of Rous sarcoma virus (RSV). The transforming protein of this virus, $p60^{src}$ (11), is an unusual protein kinase (12) which phosphorylates tyrosine (13, 14). All cells transformed by RSV contain 5- to 10-fold more phosphotyrosine than do uninfected cells (15). We have demonstrated (15) that this increase in the level of this unusual modified amino acid most probably is due to the phosphorylation, by $p60^{src}$, of cellular polypeptides and that the abundance of phosphotyrosine in whole cell protein can be used to measure the activity of $p60^{src}$ *in vivo*. In contrast, cells transformed by Kirsten sarcoma virus, Moloney sarcoma virus, simian virus 40, or polyoma virus do not contain altered levels of phosphotyrosine (15). This suggests that there may be no universal mechanism of viral transformation and that the biochemistry of transformation by some tumor viruses may be different from that of transformation by RSV. It is clearly of interest to determine whether any tumor viruses other than RSV encode protein kinases that phosphorylate tyrosine *in vivo*.

Although there is essentially no structural homology (unpublished data), Abelson p120 resembles $p60^{src}$ in that it has an associated kinase activity that will phosphorylate tyrosine in an immunoprecipitate (16). p120 differs from $p60^{src}$, however, in that it is the p120 polypeptide rather than the immunoglobulin that becomes phosphorylated in this reaction (16, 17). Nevertheless, because the kinase activity associated with p120 phosphorylates the same amino acid as $p60^{src}$, it was possible that the phosphorylation of tyrosine was involved in transformation by Abelson virus. On the other hand, it was possible that the reaction observed *in vitro* was not indicative of an activity of p120 *in vivo*. Such may be the case with polyoma virus. Immunoprecipitates containing polyoma middle tumor antigen also contain a protein kinase activity that phosphorylates tyrosine *in vitro* (18). The abundance of phosphotyrosine, however, is unchanged in polyoma virus-transformed cells (15). Although this may mean simply that the protein kinase activity of polyoma virus is difficult to detect *in vivo*, it could indicate that the *in vitro* phosphorylation reaction is misleading with regard to the actual biochemistry of transformation by polyoma virus.

To examine the possibility that p120 modifies cellular proteins through the phosphorylation of tyrosine *in vivo*, we have measured the abundance of phosphotyrosine in protein in cells transformed by A-MuLV.

## RESULTS

We have used here exactly the same procedure that we used to measure the activity of $p60^{src}$ in RSV-transformed cells (15). Cellular phosphoproteins were labeled biosynthetically with $[^{32}P]$orthophosphate for 15–18 hr, extracted into phenol, precipitated with trichloroacetic acid, and washed with chloroform/methanol, 2:1 (vol/vol). The labeled phosphoamino acids were then released by partial acid hydrolysis and separated by two-dimensional electrophoresis on cellulose thin-layer plates. The terms "level of phosphotyrosine" and "abundance of phosphotyrosine" refer to the percentage of $^{32}P$ recovered in phosphotyrosine relative to that recovered in phosphotyrosine, phos-

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Abbreviations: A-MuLV, Abelson murine leukemia virus; M-MuLV, Moloney murine leukemia virus; RSV, Rous sarcoma virus.

phothreonine, and phosphoserine combined. We have reported elsewhere (15) that this procedure yields data that reflect accurately the steady-state levels of these three phosphoamino acids. There is no indication that these values are affected significantly by differences in the specific activity of precursor pools, selective extraction of phosphoproteins, or phosphorylation subsequent to cell lysis.

We first examined the abundance of phosphotyrosine in four lines of A-MuLV-transformed 3T3 cells (Fig. 1; Table 1). Each of these contained a level of phosphotyrosine that was significantly greater than the low level in uninfected 3T3 cells. This was the case both in cells productively infected with A-MuLV and a helper virus and in the transformed nonproducer cell line, ANN-1 (2). The 5.5- to 12-fold increase in the A-MuLV-transformed cells was comparable to that in 3T3 cells transformed by RSV (Table 1). Productive infection with the nontransforming Friend MuLV helper virus had no effect on the cellular level of phosphotyrosine.

We then considered the perhaps more pertinent question of whether transformation by A-MuLV affected the level of phosphotyrosine in hematopoietic cells (Fig. 1; Table 2). The three

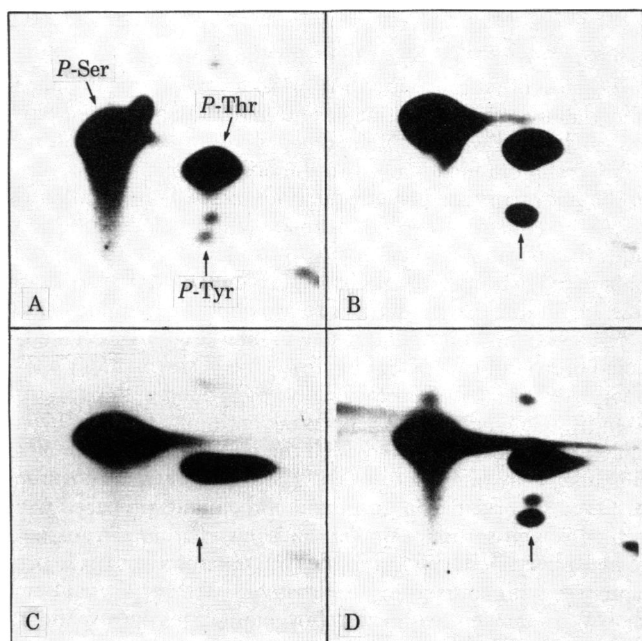


FIG. 1. Two-dimensional analysis of the acid-stable phosphoamino acids in fibroblasts and lymphocytes transformed by A-MuLV. (*A*) NIH Swiss 3T3; (*B*) A-MuLV-transformed NIH Swiss 3T3, ANN-1; (*C*) chemically transformed B-lymphoma WEHI 231.2; (*D*) A-MuLV-transformed pre-B lymphoma RAW 309.1.1. NIH Swiss 3T3 cells (19) and the ANN-1 line of A-MuLV-transformed NIH Swiss 3T3 cells (2) were labeled and extracted as described (13, 15). The B-lymphoma WEHI 231.2 (20) and the A-MuLV-transformed pre-B lymphoma RAW 309.1.1 (3) were labeled at a density of 1–2 × $10^6$ cells per ml for 18 hr at 37°C in 2 ml of phosphate-free Dulbecco's modified Eagle's medium containing 5% fetal calf serum, 5% dialyzed (against phosphate-free saline) fetal calf serum, 50 $\mu$M mercaptoethanol, and 1.2 mCi of [$^{32}$P]orthophosphate (ICN, carrier-free). The cells were concentrated by centrifugation and then extracted as described (13, 15). Separation of the phosphoamino acids released by partial acid hydrolysis (13) was by electrophoresis at pH 1.9 from right to left followed by electrophoresis from bottom to top at pH 3.5. Approximately 1–2 × $10^6$ cpm was applied to each plate. Exposure was for 15 hr with a fluorescent screen. Phosphoamino acids were identified by staining internal standards with ninhydrin. In *B*, *C*, and *D* phosphotyrosine (*P*-Tyr) is indicated simply by an arrow. Only the central portions of the electropherograms are shown here. The film response was saturated by both phosphoserine (*P*-Ser) and phosphothreonine (*P*-Thr) in all cases.

Table 1. Level of phosphotyrosine in Abelson virus-transformed fibroblasts

| Cells | Virus | Relative abundance of phosphotyrosine* |
|---|---|---|
| NIH Swiss 3T3 | None | 0.054 |
| NIH Swiss 3T3 | F-MuLV† | 0.033 |
| ANN-1 (NIH Swiss 3T3) | A-MuLV‡ | 0.391 |
| BALB 3T3/A31 | None | 0.025 |
| A31-WR19M.1 (BALB 3T3) | A-MuLV§ | 0.201 |
| A31-BR48-M-MuLV (BALB 3T3) | A-MuLV¶ | 0.308 |
| A31-RAW309Cr.1 (BALB 3T3) | A-MuLV§ | 0.137 |
| BALB 3T3/A31 | RSV | 0.200 |

Procedures for the labeling of the cells with [$^{32}$P]orthophosphate, extraction of phosphoproteins, partial acid hydrolysis, and two-dimensional analysis of the released phosphoamino acids are described in the legend to Fig. 1, in the text, and in refs. 13 and 15.

* Fraction of radioactivity recovered as phosphotyrosine relative to that recovered as phosphoserine, phosphothreonine, and phosphotyrosine combined.

† Friend MuLV.

‡ Nonproducer cells, no helper virus.

§ Producer cells, undefined helper virus.

¶ Producer cells, M-MuLV helper virus.

lines of A-MuLV-transformed lymphoid cells examined here, 18.48 (21), 18.81 (21), and RAW309.1.1 (3) contained levels of phosphotyrosine 5- to 10-fold higher than the low levels in the chemically transformed B lymphomas 70Z/3 (22), 38C13.1 (20), WEHI 231.2 (23), and WEHI 279 (23), in the spontaneous T lymphoma BW5147 (24), and in the erythroleukemias 745.6 (25) and ERT 231-4-1 (26) which were transformed by the Friend virus complex and the Rauscher virus complex, respectively. It appears that both fibroblasts and lymphocytes transformed by A-MuLV contain an increased level of phosphotyrosine.

The increase in the level of phosphotyrosine in cells transformed by A-MuLV could result from the induction or activation by the virus of $p60^{proto\text{-}src}$, the normal cellular homologue of the transforming protein of RSV (27). $p60^{proto\text{-}src}$ is present at a low level in all cells (28) and resembles the viral protein in that it is a protein kinase that phosphorylates tyrosine (13, 14). However, A-MuLV-transformed ANN-1 fibroblasts contained no more $p60^{proto\text{-}src}$ specific protein kinase activity, as measured by precipitation with anti-RSV tumor antiserum, than did uninfected cells or nontransformed cells productively infected with Friend MuLV (data not shown).

These observations, plus the fact that partially purified p120 becomes phosphorylated on tyrosine *in vitro*, led us to favor the idea that p120 functions as a protein kinase that phosphorylates tyrosine *in vivo*. Because phosphotyrosine has not been detected in p120 labeled biosynthetically with [$^{32}$P]orthophosphate (16), it has been argued that the phosphotyrosine that is generated *in vitro* during incubation of p120 with ATP is not a true end-state protein modification but is rather a normally short-lived reaction intermediate that is trapped *in vitro* because of the absence of the appropriate acceptor. Because of the significant increase in the abundance of phosphotyrosine in cells transformed by A-MuLV, we thought that reexamination of whether p120 isolated from transformed cells contained phosphotyrosine was in order.

p120 labeled by incubation of ANN-1 fibroblasts with [$^{32}$P]orthophosphate for 2.5 hr contained phosphoserine, phosphothreonine, and a significant amount of phosphotyrosine (Fig. 2). The steady-state level of phosphotyrosine was one-quarter that of phosphoserine. Tryptic peptide analysis dem-

Table 2. Level of phosphotyrosine in tumor cells

| Hematopoietic cells | Tumor cell type | Mode of induction | Relative abundance of phosphotyrosine* |
|---|---|---|---|
| 70Z/3 | Pre-B lymphoma | Nitrosourea | 0.011 |
| 38C13.1 | B lymphoma | Dimethylbenz[a]-anthracene | 0.020 |
| WEHI 231.2 | B lymphoma | Mineral oil | 0.017 |
| WEHI 279 | B lymphoma | Mineral oil | 0.047 |
| BW 5147 | T lymphoma | Spontaneous | 0.015 |
| 745.6 | Erythroleukemia | Friend virus complex | 0.013 |
| ERT 231-4-1 | Erythroleukemia | Rauscher virus complex | 0.019 |
| 18.48 | Pre-B lymphoma | A-MuLV† | 0.170 |
| 18.81 | Pre-B lymphoma | A-MuLV† | 0.109 |
| RAW309.1.1 | Pre-B lymphoma | A-MuLV‡ | 0.158 |

See Table 1 for procedures.

* Fraction of radioactivity recovered as phosphotyrosine relative to that recovered as phosphoserine, phosphothreonine, and phosphotyrosine combined.

† Producer cells, M-MuLV helper virus.

‡ Producer cells, undefined helper virus.

onstrated that p120 contained at least eight sites that were phosphorylated *in vivo* (Fig. 3A). Peptides 4 and 8 contained phosphotyrosine (Fig. 3B). Peptides 1 and 2 are also present in the $Pr65^{gag}$ encoded by M-MuLV (unpublished data) and thus are not derived from the portion of p120 encoded by the acquired sequences. These two peptides and peptides 6 and 7 contained phosphoserine; peptides 3 and 5 contained phosphothreonine. It is not yet clear whether peptides 3, 4, 5, 6, 7, and 8 are derived from the viral or the acquired portion of p120.

It seemed possible that p120 was a protein kinase that phosphorylated tyrosine and could undergo autophosphorylation both *in vivo* and *in vitro*. To test this hypothesis, we compared the phosphorylated tryptic peptides generated *in vitro* during

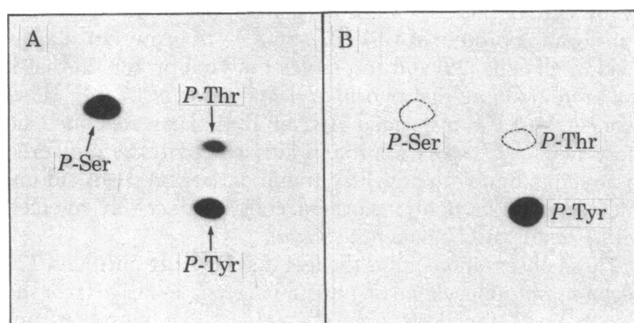


FIG. 2. Comparison of the phosphoamino acids of p120 labeled *in vivo* (A) and *in vitro* (B). ANN-1 cells growing on a 50-mm plastic tissue culture dish were labeled with 1 mCi of [$^{32}$P]orthophosphate in 2.5 ml of phosphate-free Dulbecco's modified Eagle's medium containing 10% dialyzed (against phosphate-free saline) fetal calf serum and 50 $\mu$M mercaptoethanol for 2.5 hr. Labeled p120 was isolated by immunoprecipitation with goat anti-Rauscher MuLV $p15^{gag}$ essentially as described (29). p120 for *in vitro* labeling was prepared similarly from unlabeled cells and the immunoprecipitate was incubated with 25 $\mu$Ci of [$\gamma$-$^{32}$P]ATP in 100 $\mu$l of 0.01 M sodium 1,4-piperazinediethanesulfonate, pH 7.0/0.01 M $CoCl_2$ for 10 min at 30°C. Labeled p120 was released from the immunoprecipitates and subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis as described (30). The polypeptide was located by autoradiography and electroeluted. Partial acid hydrolysis was for 60 min at 110°C in 6 M HCl. Phosphoamino acids were analyzed as described in the legend to Fig. 1 and in ref. 13 and identified by comparison with internal standards stained with ninhydrin. Only the central portions of the electropherograms are shown here.

incubation with ATP (Fig. 3C) with those present in p120 labeled biosynthetically (Fig. 3A). p120 was reproducibly phosphorylated at at least six major and many minor sites *in vitro* (Fig. 3C), all of which contained phosphotyrosine (Fig. 2B; ref. 16). Careful examination of two-dimensional maps of mixtures of the phosphorylated tryptic peptides of p120 phosphorylated *in vivo* and *in vitro* revealed that none of the major tryptic peptides that became labeled *in vitro* was identical to those phosphorylated in transformed cells (e.g., Fig. 3D). More surprising was the finding that neither of the two peptides (peptides 4 and 8) that contained phosphotyrosine *in vivo* (Fig. 3B) became labeled to a significant extent *in vitro*. One of the peptides phosphorylated *in vitro* (E) occasionally comigrated with peptide 7 from p120 labeled *in vivo*. This is adventitious. Peptide 7 contained phosphoserine whereas all the peptides labeled *in vitro* contained only phosphotyrosine. The map shown here is from p120 phosphorylated in an immunoprecipitate prepared with anti-$p15^{gag}$ antiserum. A similar, but somewhat simpler, pattern of phosphorylated tryptic peptides was obtained from p120 precipitated with antiserum to disrupted M-MuLV (data not shown). It showed two major phosphopeptides corresponding to peptides B and E.

Because p120 itself contained phosphotyrosine, it was important to determine to what extent the increase in the abundance of phosphotyrosine in cells transformed by A-MuLV was due to the presence in them of the p120 polypeptide. We therefore immunoprecipitated p120 with an excess of antibody from a lysate of A-MuLV-transformed ANN-1 fibroblasts and then measured the level of phosphotyrosine remaining in the lysate. Precipitation of p120 had no measurable effect on the abundance of phosphotyrosine in the cell lysate (data not shown). Although we do not know whether we precipitated all of the p120 in the cell lysate, it appears likely that there are phosphorylated polypeptides other than p120 that contribute to the increased level of phosphotyrosine in cells transformed by A-MuLV.

## DISCUSSION

Both fibroblastic tissue culture cells and hematopoietic cells transformed by Abelson virus contain a phosphotyrosine level 6 to 12 times higher than normal. This increase in the abundance

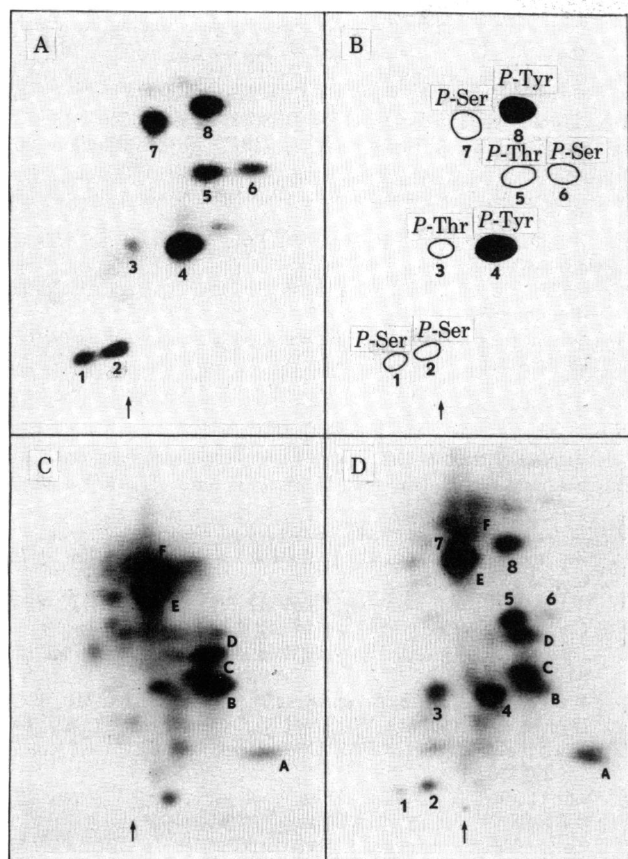


FIG. 3. Comparison of the phosphorylated tryptic peptides of p120 labeled *in vivo* and *in vitro*. Preparations of p120 labeled *in vivo* and *in vitro* were obtained as described in the legend to Fig. 2. Tryptic digestion was as described (13). Tryptic peptides were subjected to electrophoresis at pH 8.9 and ascending chromatography as described (13). The origins are indicated by vertical arrows; the anode is on the left. The amounts of radioactivity applied to each plate and the exposure times with a fluorescent screen were as follows: (*A*) p120 labeled *in vivo*, 10,000 cpm, 7 hr; (*C*) p120 labeled *in vitro*, 20,000 cpm, 10 hr; (*D*) mixture of p120 labeled *in vivo*, 7000 cpm, and p120 labeled *in vitro*, 7000 cpm, 10 hr. The tryptic digests used for the mixture were different from the ones used for the maps shown in *A* and *C*. The phosphoamino acid composition of individual peptides was determined by elution of the peptides followed by partial acid hydrolysis in 6 M HCl for 1 hr at 110°C and electrophoresis in one dimension at pH 3.5. These are shown in the schematic of the digest of p120 labeled *in vivo* (*B*); the phosphotyrosine-containing peptides are shown filled in.

of phosphotyrosine could be due to a viral protein kinase, to the induction or activation of a cellular protein kinase, or, conceivably, to a viral inhibitor of a cellular protein phosphatase. Because we found no evidence for an increased activity of the only cellular protein kinase identified to date that will phosphorylate tyrosine, $p60^{proto\text{-}src}$, and because partially purified p120 is known to phosphorylate tyrosine *in vitro* (16), it is not unreasonable to conclude that p120 is a protein kinase that modifies cellular proteins through the phosphorylation of tyrosine in both lymphoid cells and fibroblasts.

Is this activity of the protein essential for cellular transformation? Recent results indicate that it is. Blomberg *et al.* (31) have isolated nontransformed Abelson virus-infected cell lines that express a p120 that lacks *in vitro* kinase activity. Witte *et al.* (32) have isolated a deletion mutant of A-MuLV that has lost both its ability to transform cells and its *in vitro* kinase activity. Further, Rosenberg *et al.* (33) have found that variants of A-MuLV which encode truncated forms of p120 have both a decreased ability to transform lymphoid cells and a decreased *in vitro* protein kinase activity. Thus, although the activities of the proteins encoded by these mutants have not been measured *in vivo*, the correlation between *in vitro* protein kinase activity and the ability to transform is good.

Witte *et al.* (16) first demonstrated that p120 became phosphorylated on tyrosine *in vitro*. Because phosphotyrosine was not detected in p120 *in vivo*, it was reasoned that what occurred *in vitro* was not autophosphorylation by a protein kinase but rather the generation and trapping of a reaction intermediate due to the lack of the appropriate substrate. It was suggested that p120 functioned *in vivo* in a transphosphorylation reaction of unknown nature and that the phosphotyrosine that was detected only *in vitro* normally served as the proximal donor of the phosphate moiety.

The results presented here make it seem likely that the transphosphorylation reaction in which p120 is involved is the phosphorylation of proteins. However, the apparent autophosphorylation of p120 *in vitro* remains difficult to interpret. It seems unlikely that this represents trapping of an enzyme reaction intermediate; as many as 12 sites are phosphorylated on tyrosine *in vitro*. One would expect an enzyme intermediate to have a single phosphorylation site. Furthermore, in contrast to the apparent autophosphorylation reaction observed with $p60^{src}$ *in vitro* (34), the tyrosine sites in p120 that are phosphorylated *in vivo* are only weakly labeled *in vitro*. Perhaps the physiological tyrosine phosphorylation sites are already fully occupied. The efficient phosphorylation *in vitro* of tyrosines that are not detectably modified *in vivo* may be a consequence of the fact that the membrane localization of p120 (35) is lacking *in vitro*. The discrepancy between the phosphorylation patterns observed *in vivo* and *in vitro* stresses the need to search for substrates of p120 in whole cells rather than *in vitro*.

We have previously shown (15) that, although cells transformed by RSV contain noticeably increased phosphotyrosine in cellular proteins, cells transformed by simian virus 40, Moloney sarcoma virus, Kirsten sarcoma virus, polyoma virus, benzo[*a*]pyrene, or 20-methylcholanthrene do not. This suggested two points. First, transformation does not necessarily cause a detectable increase in the level of phosphotyrosine. Second, there may be no universal biochemical basis of transformation by tumor viruses or by chemicals. We have found here that various hematopoietic cells transformed spontaneously, by chemicals, by M-MuLV, or by the Friend and Rauscher virus complexes contain the low level of phosphotyrosine characteristic of normal cells. This suggests that the transforming events that led to these tumor lines did not involve either the introduction or the induction of a protein kinase whose activity leads to a noticeable increase in the level of phosphotyrosine in protein.

These results therefore extend the list of tumor viruses and tumor cell lines for which there is evidence that the initiating event in transformation is different from that which occurs during transformation by RSV or A-MuLV. The increase in the level of phosphotyrosine in cells transformed by RSV or A-MuLV is useful because it allows one to detect the activity of the viral transforming protein. There is no direct evidence, however, as to what fraction of the new protein modifications that result from the activity of p120 or $p60^{src}$ contributes to the transformed state. It is possible that many of these modifications are completely incidental to transformation. This leaves open the possibility that these other viruses encode or induce a protein kinase that phosphorylates tyrosine in only a few crucial substrates and these are present in a cell at such a low level that the activity of these kinases is undetectable by the assay we have used here.

p120 and p60$^{src}$ modify the same amino acid yet are not obviously structurally related (unpublished data). Do they modify the same cellular polypeptides? Initial indications are that at least some of the cellular proteins that are phosphorylated on tyrosine in RSV-transformed fibroblasts are also phosphorylated on tyrosine in A-MuLV-transformed fibroblasts. The cytoskeletal protein vinculin is an apparent substrate of p60$^{src}$ in both avian and mammalian fibroblasts (36). Vinculin from the ANN-1 line of Abelson virus-transformed mouse fibroblasts contains the same greatly increased level of phosphotyrosine as it does in RSV-transformed cells (36). We do not yet know, however, whether the same tyrosine residues are phosphorylated in the two cases. It is also clear that p60$^{src}$ phosphorylates a cellular protein of 36,000 daltons (29, 37, 38). This 36,000-dalton protein is also phosphorylated on tyrosine in A-MuLV-transformed fibroblasts (K. Radke and S. Martin, personal communication; J. Cooper, personal communication). The presence of phosphotyrosine in vinculin and the 36,000-dalton protein in A-MuLV-transformed fibroblasts demonstrates directly that the increased abundance of phosphotyrosine that we detect in these cells is due at least in part to the modification of cellular proteins. RSV and Abelson virus cause quite different diseases *in vivo* (sarcomas and lymphomas, respectively) yet appear to encode protein kinases that have similar activities in fibroblastic tissue cells. It seems possible therefore that the *in vivo* tissue specificity of each virus results not from the fact that these viruses encode transforming proteins that can participate in the transformation of only specific cells but rather from factors that affect the ability of the two viruses to express their transforming proteins in specific cells.

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