Characterization of the Protein Apparently Responsible for the Elevated Tyrosine Protein Kinase Activity in LSTRA Cells

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The LSTRA murine thymoma cell line contains an elevated level of tyrosine protein kinase activity. When a microsomal preparation from these cells is incubated in vitro with ATP, the principal tyrosine protein kinase substrate is a 56,000-dalton protein, p56. We have found that an activity phosphorylating p56 on tyrosine can also be detected at low levels in microsomes from most, but not all, T lymphoma cell lines and from normal thymic tissue. Only 1 of 30 other lymphoma cell lines was found to contain an elevated level of such a tyrosine protein kinase. An activity that phosphorylated p56 in vitro was not detectable in the cells of other hematopoietic lineages. Anti-peptide antibodies reactive with the site of in vitro tyrosine phosphorylation of p56 allowed us to determine that the apparent abundance of the p56 polypeptide parallels closely the level of the tyrosine protein kinase activity in the cell lines examined. This suggests that p56 is the protein kinase responsible for the elevated tyrosine protein kinase activity in LSTRA cells and that the phosphorylation of p56 observed in vitro results from autophosphorylation. Two-dimensional tryptic peptide mapping revealed that p56 is distinct from the proteins encoded by the cellular genes which are the progenitors of retroviral tyrosine protein kinases, src, yes, fgr, abl, fes, and ros. Additionally, none of these proto-oncogenes was found to be transcribed at elevated levels in LSTRA or Thy19 cells. Like the catalytic subunit of the cyclic AMP-dependent protein kinase, the cellular and viral forms of p60^{src}, and the protein phosphatase calcineurin B, p56 contains covalently bound fatty acid.

Tyrosine protein kinase activity is an intrinsic property of the transforming proteins of six classes of acutely oncogenic retroviruses (39). This activity is also a property of (i) the receptors for at least four polypeptide hormones, epidermal growth factor (52), platelet-derived growth factor (13), insulin (25), and insulin-like growth factor (36), and (ii) the cellular homologs of at least two viral tyrosine protein kinases, p60^{c-src} (21, 31) and NCP98^{c-fps} (30). It is apparent that tyrosine phosphorylation is an important event in the control of cell proliferation and that malignant transformation can occur, at least in part, as a result of an imbalance of this process.

It was found recently that the LSTRA murine lymphoma cell line contains dramatically elevated levels of a tyrosine protein kinase activity (7). This activity can be detected both in vivo, by determination of endogenous phosphotyrosine levels (7), and in vitro, by measurement of tyrosine protein kinase activity associated with microsomal preparations (6–8). The major substrate of the tyrosine protein kinase activity of LSTRA cells in vitro is a protein with an apparent molecular weight of 53,000 to 56,000, p56 (Casnellie, J. E., L. E. Gentry, L. R. Rohrschneider, and E. G. Krebs, Proc. Natl. Acac. Sci. U.S.A., in press). The LSTRA cell line is unusual. The only other murine lymphoid cells which contain an abnormal and elevated level of tyrosine protein kinase activity are those transformed by Abelson murine leukemia virus (A-MuLV) (39), a virus which encodes a tyrosine protein kinase (57). The LSTRA cell line is derived from a thymoma induced by Moloney murine leukemia virus (Mo-MuLV) (16), which does not encode a transforming

protein. The tyrosine protein kinase (S) responsible for the elevated activity in LSTRA cells must therefore be cellular rather than viral.

The abundant tyrosine protein kinase activity in LSTRA cells could be due to either overexpression or activation of (i) one of the tyrosine protein kinases encoded by a cellular proto-oncogene, (ii) a growth factor receptor, or (iii) another as-yet-uncharacterized tyrosine protein kinase. The major site of in vitro tyrosine phosphorylation in p56 is identical in sequence to 10 amino acids encompassing the major site of tyrosine phosphorylation in both p60^{src} and p90^{yes} (6). This complete homology of a region of p56 with a region in two viral tyrosine protein kinases suggests that p56 may be itself a tyrosine protein kinase and that its phosphorylation in vitro results from "autophosphorylation." If so, it is possible that p56 is the tyrosine protein kinase which is responsible for the increased tyrosine protein activity in LSTRA cells. We examined whether p56 might be related to a product of one of the retroviral oncogenes by comparing two-dimensional maps of the methionine-containing tryptic peptides of the products of the v-src, v-abl, v-yes, v-fps/fes, v-ros, and v-fgr genes with that of p56.

In addition, we examined a large number of both normal and transformed murine hematopoetic cells for the presence of a tyrosine kinase activity which would phosphorylate p56 in microsomal preparations. Such an activity was found in most, but not all, T cells. It was not detectable in cells from other hematopoietic lineages.

MATERIALS AND METHODS

Cells. The LSTRA line of Mo-MuLV-induced thymoma cells (16) and the BW5147 line of spontaneously transformed mouse T lymphoma cells (23) were grown in Dulbecco-Vogt

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modified Eagle medium (DMEM) supplemented with 20% fetal calf serum and 5×10^{-5} M 2-mercaptoethanol. The Thy16, Thy19, and Thy56 lines, which were isolated from spontaneous thymomas of BALB/Mo mice (55), were a kind gift of M. Vogt. M. Haas generously provided the 136.4, 136.5, and 136.7 lines isolated from C57BL/6 murine thymomas induced by radiation leukemia virus (17); the 4SPL.C12, 4SPL.C14, 4SPLD1.C11, 4SPLD1.C15, and 4SPLD1.C19 T lymphoma lines isolated from mouse spleen; the E1.1-12 line isolated from a mouse thymoma induced by inoculation of cloned T lymphoblastoma cells (18); and the PXTL-3, PXTL-22, PXTL-4, PXTL-6, and PXTL-7 thymoma cell lines isolated from X-ray-treated C57BL/6 mice (M. Haas, A. Altman, E. Rothenberg, M. H. Bogart, and O. W. Jones, Leukemia Res., in press). The RAW309 Cr.1 line of Abelson virus-transformed macrophages (34) and the 18-48 line of Abelson virus-transformed mouse B lymphoid cells (44) were obtained from W. Raschke. The 745-6 line of Friend virus-transformed erythroid cells (24) was provided by J. Glenney. The RG17.16.ST line derived from the fusion of AD5 T-helper cells with BW5147 cells and the AJ39 T-killer cell line, identified and specified as L3C5 (15), were a kind gift of A. Glasebrook. The S194/5.XXO.BU.1 line of cloned mature plasma cells (22) was generously provided by R. Hyman. The freshly isolated cells from spontaneous thymomas of BALB/Mo mice and lymphocytes from normal thymus from 3-week-old SIM.R. mice were generously provided by M. Vogt.

Biosynthetic labeling. Cells were labeled with $^{32}P_i$ by incubation at a concentration of 10^6 cells per ml in phosphate-free DMEM containing 0.5 to 3.0 mCi of $^{32}P_i$ per ml (carrier free; ICN Pharmaceuticals), 5% fetal calf serum, and 15% fetal calf serum dialyzed against phosphate-free saline. Labeling was for 2 h at 37°C for immunoprecipitation and for 16 h for two-dimensional gel analysis. Several procedures were employed for labeling cells with [^{35}S]methionine. LSTRA cells were labeled with 100 to 500 μCi of [^{35}S]methionine per ml (>1,000 Ci/mmol; Amersham) by incubation for 2 h at 37°C at a concentration of 2×10^6 cells per ml in methionine-free DMEM supplemented with 20% dialyzed fetal calf serum. Chicken cells transformed by Y73 virus, rat 208F cells transformed by UR2 virus, mouse 3T3 cells transformed by Snyder-Theilen feline sarcoma virus, mouse cells transformed by the P160 strain of A-MuLV, and raccoon cells transformed by Gardner-Rasheed feline sarcoma virus, all growing on 100-mm petri dishes, were labeled for 2 to 3 h with [^{35}S]methionine at a concentration of 200 μCi/ml in 5 to 6 ml of DMEM supplemented with 4 to 10% dialyzed calf serum. Chicken cells transformed by Schmidt-Ruppin Rous sarcoma virus of subgroup A were labeled for 16 h with [^{35}S]methionine as described above. 3T6 cells were labeled for 2 h with [^{35}S]methionine at a concentration of 100 μCi/ml in 2 ml of DMEM supplemented with 10% dialyzed fetal calf serum.

Cells were labeled with radioactive fatty acids by incubation with 1.7 mCi of 9,10-[^3H]myristic acid (12.9 Ci/mmol, New England Nuclear Corp.) or 1.0 mCi of 9,10-[^3H]palmitic acid (23.5 Ci/mmol, New England Nuclear) for 2 or 18 h, respectively, at a concentration of 1.2×10^7 cells per ml in 1 ml of DMEM supplemented with 10% calf serum, 5 mM sodium pyruvate, 1% dimethyl sulfoxide, and nonessential amino acids, as described previously (42).

Preparation of anti-peptide serum. To prepare an antiserum to the site of tyrosine phosphorylation in p60^{src}, a synthetic peptide, Lys-Leu-Ile-Glu-Asp-Asn-Glu-Tyr-Thr-Ala-Arg, termed src I (20), was coupled to keyhole limpet

hemocyanin. This peptide is identical in sequence in all but its NH$_2$-terminal residue to the major site of tyrosine phosphorylation in p60^{src} (33, 45) and p90^{yes} (32). To prepare the conjugate, 5 mg of the peptide was dissolved in 1 ml of water containing 10 mg of keyhole limpet hemocyanin (Calbiochem Behring). To redissolve the precipitate which formed, the pH of the solution was adjusted to 7 with 0.1 N NaOH and 0.1 M sodium phosphate; 2 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (Calbiochem Behring) was then added and the reaction was allowed to proceed for 23 h at room temperature with constant stirring. The peptide-carrier conjugate was then dialyzed overnight against water at 4°C.

For immunization, the conjugate was emulsified with complete Freund adjuvant and injected into a rabbit at multiple subcutaneous sites. Little activity was apparent in the resultant serum. Nevertheless, immunoglobulin G was prepared by ammonium sulfate precipitation, dissolved in 0.15 M NaCl-0.01 M sodium phosphate (pH 7.2), and applied to a column containing 1 mg of the src I peptide coupled to Affi-Gel 15 (Bio-Rad Laboratories). The column was then washed sequentially with (i) 0.15 M NaCl-0.01 M sodium phosphate (pH 7.2), (ii) 0.1 M NaHCO$_3$ (pH 8.5), and (iii) 0.5 M NaCl-0.1 M NaHCO$_3$ (pH 8.5). The bound antibodies were eluted with 0.1 M glycine hydrochloride (pH 2.5), and the eluate was collected in 0.5-ml fractions in tubes containing 6 mg of dry Tris base. Fractions containing the antibodies of interest were identified by their ability to immunoprecipite p60^{src} from a lysate of chicken cells transformed by Schmidt-Ruppin Rous sarcoma virus of subgroup A. The antibody was able to immunoprecipitate p60^{src}, which was phosphorylated at the tyrosine within the site recognized by the antibodies, residue 416 (our unpublished observations).

Immunoprecipitation. To minimize background, lymphoid cells were lysed in 0.5% sodium dodecyl sulfate (SDS)-50 mM Tris hydrochloride (pH 8.0)-1 mM dithiothreitol (DTT), boiled for 60 s, and diluted with 4 volumes of 50 mM Tris hydrochloride (pH 8.0)-150 mM NaCl-1.25% Nonidet P-40-1% Trasylol-1% sodium deoxycholate-1 mM DTT. The resulting lysate was then clarified by centrifugation for 90 min at 20,000 × g. When lysates of ^{32}P-labeled cells were prepared, 2 mM EDTA was included in both solutions. The procedures for immunoprecipitation, with fixed *Staphylococcus aureus* bacteria (Pansorbin; Calbiochem Behring) used to collect immune complexes, have been described in detail (38). For all other types of cells, the labeled cells were lysed with RIPA buffer (38). p60^{src} was isolated with rabbit anti-Rous sarcoma virus tumor serum. P68^{ros} was precipitated with goat antiserum to disrupted avian myeloblastosis virus. P70^{fgr} was isolated with goat anti-Rauscher murine leukemia virus p15^{gag} serum. p53 was isolated with monoclonal antibody specific for host nonviral tumor antigen (19).

Preparation of microsomal fraction. Cells (1×10^7 to 4×10^7) were harvested and incubated in 4 ml of ice-cold 5 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) (pH 7.4)-1 mM MgCl$_2$-5 mM 2-mercaptoethanol for 10 min. Cells were broken in a Dounce homogenizer, nuclei were removed, and microsomes were separated as described in detail previously (6). The microsomal pellet was suspended in 25 mM HEPES (pH 7.4)-5 mM 2-mercaptoethanol.

Assay of tyrosine protein kinase activity in microsomes. The reactions contained either 14 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol; Amersham) or 140 μCi of [γ-^{32}P]ATP (30 Ci/mmol) in 25 μl of 10 mM PIPES (piperazine-N-N'-bis(2-ethanesulfonic acid) (pH 7.0)-10 mM MnCl$_2$. A 25-μl volume of resuspended microsomes (derived from 10^6 cells) were add-
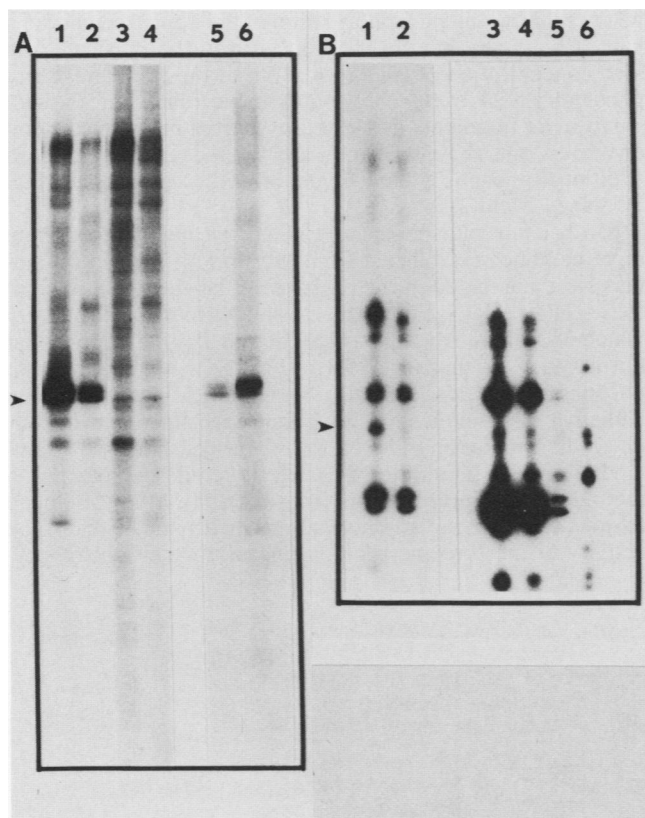
FIG. 1. Analysis of phosphorylated proteins from microsomes. Microsomal fractions were prepared from various cell lines, incubated with 140 μCi (50 μM) of [γ-^{32}P]ATP for the specified time at 4°C, and analyzed by SDS-polyacrylamide gel electrophoresis as described in the text. (A) Lane 1, LSTRA cells, 10 min; lane 2, Thy19 cells, 10 min; lane 3, Thy56 cells, 10 min; lane 4, BW 5147 cells, 10 min; lane 5, LSTRA cells, 2 min; lane 6, LSTRA cells, 60 min. The arrow indicates the position of p56. (B) Analysis by partial proteolysis of p56 phosphorylated in microsomes. p56 phosphorylated by incubation of microsomes for 5 min with [γ-^{32}P]ATP in vitro was excised from a gel, digested with 50 ng of S. aureus V8 protease, and analyzed by electrophoresis on a 15% polyacrylamide gel as described in the text. The digestion products were detected by autoradiography. Unless indicated the proteins analyzed were the lower of the two bands phosphorylated in vitro. Arrow indicates the position of the additional proteolytic fragment. Lane 1, upper band from phosphorylated doublet from LSTRA cell microsomes; lane 2, lower band from phosphorylated doublet from LSTRA cell microsomes; lane 3, p56 from LSTRA cell microsomes; lane 4, p56 from Thy19 cell microsomes; lane 5, p56 from Thy56 cell microsomes; lane 6, 56,000-dalton protein from BW5147 cell microsomes.

ed to start the reaction. The mixtures were incubated at 4°C for 10 min. The reactions were stopped by addition of an equal volume of 4% sodium dodecyl sulfate–10 mM sodium phosphate (pH 7.0)–0.2 M dithiothreitol–10% 2-mercaptoethanol–20% glycerol.

Angiotensin phosphorylation. Each reaction contained 10 μl of microsome suspension and 1 μl of 50 mM [Val^5]-Angiotensin II (Sigma) dissolved in water and 10 μl of a solution containing 140 μCi of [γ-^{32}P]ATP (30 Ci/mmol) in 10 mM PIPES (pH 7.0)–10 mM MnCl$_2$. The reactions were carried out at 30°C for 10 min, stopped by placing on dry ice and analyzed by electrophoresis on a cellulose thin-layer plate as described previously (20).

TABLE 1. Presence of p56 in cell lines and tissues[a]

Cell type	No. containing p56
Tissues	
Normal thymus	1/1
Mo-MuLV-induced thymoma	2/2
Cell lines	
Mo-MuLV-induced T lymphoma	4/4
Radiation-MuLV-induced T lymphoma	21/21
Radiation-induced T lymphoma	4/5
Spontaneous T lymphoma	0/1
A-MuLV-transformed macrophages	0/1
Friend MuLV-transformed erythroid leukemia cells	0/1
A-MuLV-transformed pre-B cells	0/1
Mature plasma cells	0/1
Normal T-killer cells	0/1
Normal t-helper cells	0/1

[a] The presence of p56 was determined by incubation of the microsomal fractions with [γ-^{32}P]ATP in vitro. Phosphorylated proteins were separated on SDS-polyacrylamide gels, and proteins comigrating with p56 were subjected to partial proteolysis with S. aureus V8 protease to confirm the identity of p56.

SDS-polyacrylamide gel electrophoresis. Kinase reaction mixtures were heated to 100°C for 60 s and then analyzed by electrophoresis on gels containing 15% acrylamide and 0.09% bisacrylamide as described previously (38). Immunoprecipitates were analyzed as described previously (38).

Analysis by partial proteolysis. Gel slices containing p56 were inserted into the wells of a 15% polyacrylamide gel. Digestion with 10 or 50 ng of S. aureus protease V8 and analysis of digestion products was performed as described (9, 41). Detection of the proteolytic fragments was by autoradiography or fluorography.

Tryptic peptide mapping. Protein bands were excised from the gel, precipitated, oxidized with performic acid, digested with trypsin, and analyzed as described previously (2). Fluorography of the [^{35}S]methionine-containing tryptic peptides was enhanced by coating the thin-layer plates with 0.4% diphenyloxazole in 2-methylnaphthalene (4).

RESULTS

Distribution of p56. An activity that phosphorylates p56 has been detected at low levels in normal mouse lymphocytes and in a Mo-MuLV-transformed lymphoma cell line, YAC-1 (7). To determine what other cells contained this tyrosine protein kinase activity and whether an elevated

TABLE 2. Measurement of tyrosine protein kinase activity in microsomal fractions from thymoma cell lines[a]

Cell line	Phosphorylation of p56 (fmol/5 min)	Phosphorylation of angiotensin (fmol/5 min)
LSTRA	582	1,233
Thy19	141	383
Thy16	35	109
Thy56	32	59

[a] Microsomal fractions from 10^6 cells were incubated with 50 μM [γ-^{32}P]ATP for 5 min at 4°C as described in the text. Phosphorylated p56 was excised from SDS-polyacrylamide gels and radioactivity was determined by scintillation spectrometry, using toluene-PPO (2,5-diphenyloxazole) as scintillation fluid. Angiotensin phosphorylation reactions were carried out at 30°C for 10 min in the presence of 50 μM [γ-^{32}P]ATP as described in the text and analyzed by electrophoresis on cellulose thin-layer plates at pH 3.5. The phosphorylated peptide was aspirated from the plate and eluted with pH 4.72 buffer, and radioactivity was determined by scintillation counting, using 3a70B (RPI) as scintillation fluid.

tyrosine protein kinase activity was unique to LSTRA cells, we prepared microsomal fractions from a large variety of cells, incubated them with [γ-^{32}P]ATP, and analyzed phosphorylated proteins by SDS-polyacrylamide gel electrophoresis. In every case, any phosphoprotein which comigrated with p56 from LSTRA cells was excised and subjected to partial proteolysis with *S. aureus* V8 protease (Fig. 1). This was essential because an apparently unrelated protein with a size identical to that of p56 becomes phosphorylated in vitro in microsomes from some cell lines.

The apparent size of p56 phosphorylated in microsomes was found to vary as a function of both the length of the reaction and the concentration of ATP. When the microsomes from LSTRA cells were incubated with a low concentration of ATP (40 nM), only a single phosphorylated 56,000-dalton protein was detected. In contrast, when the microsomes were incubated with 100 μM ATP, two labeled bands of this approximate size appeared. The lower band was identical in size to that seen at the low ATP concentrations (data not shown). This phenomenon is somewhat similar to what is seen with the p75 kinase from rat liver,

where preferential phosphorylation of the upper member of a pair of bands is also observed in vitro in the presence of a high concentration of ATP (60). Both of the p56 bands were phosphorylated exclusively on tyrosine (data not shown). Analysis of fragments generated by partial proteolysis demonstrated that the two bands are related and revealed an additional phosphorylated fragment in the upper band (Fig. 1B, lanes 1 and 2).

p56 became phosphorylated when the microsomes from three cell lines established from Mo-MuLV-induced thymomas were incubated in the presence of labeled ATP. One of these cell lines, Thy19, had a tyrosine protein kinase activity which was obviously higher than that in Thy16 or Thy56 but not as great as that in LSTRA (Fig. 1A). An activity that phosphorylated p56 was also found in 21 of 21 radiation leukemia virus-induced T lymphoma cell lines and in four out of five radiation-induced thymoma cell lines we examined (Table 1). In addition this activity could be detected in the microsomal preparations from both Mo-MuLV-induced thymus tumor tissue and normal murine thymus tissue. The activity of the tyrosine protein kinase(s) which phosphory-
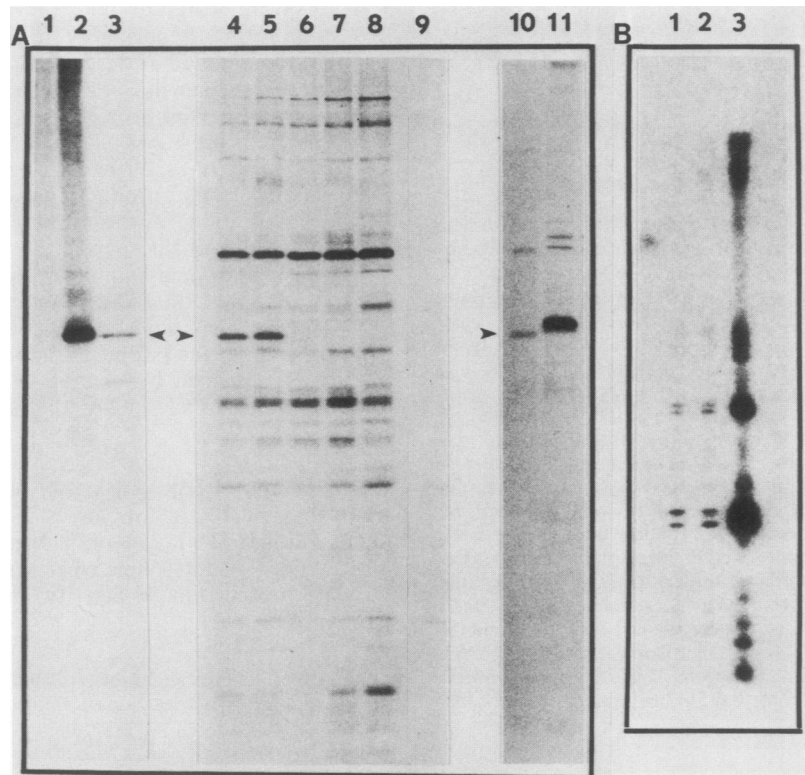

FIG. 2. Analysis of p56 by immunoprecipitation. (A) Cells were labeled biosynthetically with either ^{32}P$_i$ or with [^{35}S]methionine as described in the text. p56 was isolated by immunoprecipitation with anti-src I antibody. p53 was isolated by immunoprecipitation with anti-p53 monoclonal antibody. Immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis, and the precipitated proteins were detected by fluorography. Lane 1, LSTRA cells, [^{35}S]methionine, normal rabbit serum; lane 2, LSTRA cells, ^{32}P$_i$, anti-src I antibody; lane 3, LSTRA cells, [^{35}S]methionine, anti-src I antibody; lane 4, LSTRA cells, [^{35}S]methionine, anti-src I antibody; lane 5, Thy19 cells, [^{35}S]methionine, anti-src I antibody; lane 6, Thy16 cells, [^{35}S]methionine, anti-src I antibody; lane 7, Thy56 cells, [^{35}S]methionine, anti-src I antibody; lane 8, BW5147 cells, [^{35}S]methionine, anti-src I antibody; lane 9, LSTRA cells, [^{35}S]methionine, normal rabbit serum; lane 10, LSTRA cells, [^{35}S]methionine, anti-src I antibody; lane 11, 3T6 cells, [^{35}S]methionine, anti-p53 antibody. The arrow indicates the position of p56. (B) Comparison of phosphorylated p56 from LSTRA microsomes and [^{35}S]methionine-labeled p56 from immunoprecipitates by partial proteolysis. p56 was labeled biosynthetically with [^{35}S]methionine and isolated by immunoprecipitation. Phosphorylated p56 was isolated by SDS-polyacrylamide gel electrophoresis of LSTRA cell microsomes which were incubated with [γ-^{32}P]ATP in vitro. The proteins were digested with 50 ng of *S. aureus* protease V8 and analyzed by electrophoresis on a 15% polyacrylamide gel as described in the text. The digestion products were detected by fluorography. Lane 1, LSTRA cells [^{35}S]methionine; lane 2, Thy19 cells [^{35}S]methionine; lane 3, LSTRA cell microsomes phosphorylated in vitro.

lated p56 in microsomes from all of these cells was much lower than that in LSTRA cells and resembled the level detected in the Mo-MuLV-induced thymomas Thy16 and Thy56 (Table 2).

No phosphorylated p56 was detected after incubation with ATP of microsomes from the BW5147 line of murine T lymphoma cells derived from a tumor induced by mineral oil. Similarly, microsomes from a line of normal T-killer cells, AJ39, and a line of T-helper hybridoma cells also did not exibit a similar tyrosine protein kinase activity in vitro. Furthermore, the microsomal preparations from a number of hematopoietic cell lines derived from other lineages, including A-MuLV-transformed macrophages, A-MuLV-transformed pre-B cells, Friend virus-induced erythroid leukemia cells, and mature plasma cells contained no activity that phosphorylated p56.

We compared more quantitatively the phosphorylation of p56 in the three Mo-MuLV-induced T lymphomas and in LSTRA cells. When assayed under the conditions in which phosphorylation of the p56 was linear with time and protein concentration, microsomes from LSTRA cells and Thy19 cells had 20-fold and 4-fold more activity that phosphorylated p56, respectively, than did microsomes from Thy16 and Thy56 cells. We also utilized [Val^5]-Angiotensin II, a good in vitro substrate for a number of tyrosine protein kinases (59), to measure tyrosine protein kinase activity. Microsomes from LSTRA cells and Thy19 cells phosphorylated 20-fold and 6-fold more [Val^5]-Angiotensin II, respectively, than microsomes from Thy16 cells and Thy56 cells (Table 2).

Analysis by immunoprecipitation. The amino acid sequence encompassing the site of in vitro tyrosine phosphorylation in the 56,000-dalton protein is identical with that surrounding the major site of tyrosine phosphorylation in p60^{src} (6). We isolated p56 from LSTRA cells by using affinity-purified antibodies to a synthetic peptide, src I, analogous to the site of tyrosine phosphorylation in p60^{src} (20).

When LSTRA cells were lysed in RIPA buffer (38), immunoprecipitates formed with any serum were contaminated with p56 (data not shown). This nonspecific binding could be avoided by lysing cells in 0.5% SDS–1mM DTT, boiling the lysate, and then diluting it into RIPA buffer lacking SDS but containing DTT. A 56,000-dalton protein was specifically precipitated with antibodies to the src I peptide from boiled lysates of the LSTRA cells labeled biosynthetically with [^{35}S]methionine. This protein comigrated with the lower band of the p56 phosphorylated in vitro (Fig. 2A). Partial proteolysis of the [^{35}S]methionine-labeled 56,000-dalton protein demonstrated that it was identical to the p56 phosphorylated in vitro (Fig. 2B). The anti-src I peptide antibody could therefore be used to measure the abundance of the p56 in cells.

p56 was also readily detectable by immunoprecipitation of lysates of [^{35}S]methionine-labeled Thy19 cells. The amounts of labeled p56 protein in LSTRA and Thy19 cells were very similar when immunoprecipitates were prepared with an excess of the antibody. p56 was almost undetectable in the immunoprecipitates from [^{35}S]methionine-labeled Thy16 and Thy56 cells, and not detectable in BW5147 cells. The amount of p56 in these cells thus correlated fairly well with both the activity of the tyrosine protein kinase that phosphorylated [Val^5]-Angiotensin II in vitro (Table 2) and the abundance of phosphotyrosine in cellular protein (data not shown).

Phosphorylation of p56 in vivo. ^{32}P-labeled p56 could also be immunoprecipitated from LSTRA cells labeled biosynthetically with ^{32}P$_i$ (Fig. 2A, lane 2). Phosphoamino acid

analysis revealed that it contained both phosphotyrosine and phosphoserine (data not shown).

Fatty acid modification of p56. The cellular tyrosine protein kinase, p60^{c-src}, is modified by the attachment of the fatty acid myristic acid to its amino terminus (42; J. E. Buss and B. M. Sefton, J. Virol., in press). We examined whether p56 shared this property with p60^{c-src}. LSTRA cells were labeled wtih [^{3}H]myristic acid or [^{3}H]palmitic acid, and p56 was precipitated with anti-src I antibodies. Labeled p56 was detected in both [^{3}H]myristic acid- and [^{3}H]palmitic acid-labeled cells (Fig. 3). No [^{3}H]myristic acid-labeled p56 protein was precipitated with the anti-p15^{gag} sera, although the Mo-MuLV gag protein precursor Pr65^{gag}, which is known to contain myristic acid (37), was readily detected. The number of cellular proteins which contain myristic acid is small (Buss and Sefton, submitted), and Pr65^{gag} could also be identified among the several [^{3}H]myristic acid-containing proteins present in the unfractionated lysate from LSTRA cells (Fig. 3, lane 1). Examination of the lysate of [^{3}H]myristic acid-labeled LSTRA cells also revealed a [^{3}H]myristic acid-labeled protein which comigrated with the p56 protein from LSTRA cells. A prominent protein of 56,000 daltons was also detected in the lysates from [^{3}H]palmitic acid-labeled cells. Both [^{3}H]palmitic acid and [^{3}H]myristic acid that incorporated into p56 were stable to hydroxylamine (39). Acid hydrolysis of the fatty acids attached to p56 revealed that both labeled palmitic acid and myristic acid were present when p56 was isolated from cells labeled with either radioactive precursor (data not shown). The five labeled proteolytic fragments generated from either the [^{3}H]palmitic acid- or [^{3}H]myristic acid-labeled p56 were identical. Four of these fatty acid-containing fragments were also identical to the fragments generated from p56 phosphorylated in vitro (data not shown).

Comparison of p56 with nonviral tumor antigen p53. We examined the possibility that p56 might be related to the nonviral tumor antigen p53, which is found in a great variety of transformed cells (27, 29). The electrophoretic mobilities of the two proteins were clearly different, with p53 migrating more slowly than p56 (Fig. 2A, lanes 10 and 11). Moreover, p53 and p56 appeared completely unrelated when compared by partial proteolysis (data not shown).

Tryptic peptide map analysis. To examine whether the p56 protein was closely related to any of the products of the viral oncogenes, we compared two-dimensional maps of [^{35}S]methionine-labeled tryptic peptides of p56, p60^{src} of Rous sarcoma virus, P90$^{gag-yes}$ of Y73 virus, P70$^{gag-fgr}$ of Gardner-Rasheed feline sarcoma virus, P140$^{gag-fps}$ of Fujinami sarcoma virus, P85$^{gag-fes}$ of Snyder-Theilen feline sarcoma virus, P160$^{gag-abl}$ of A-MuLV, and P68$^{gag-ros}$ of UR2 virus.

Five major tryptic peptides could be resolved from p56 (Fig. 4). One of these peptides, peptide 1, comigrated with 1 of the 10 tryptic peptides from p60^{v-src}, peptide 14. The other tryptic peptides of p56 and p60^{v-src} were different. Three peptides of p56, peptides 1, 4, and 5, migrated very similarly to peptides 14, 15, and 16 of P70$^{gag-fgr}$ of Gardner-Rasheed feline sarcoma virus (Fig. 4), but they were found to be different when a mixture of both proteins was analyzed. In addition, comparison [^{35}S]cysteine-labeled tryptic peptides and [^{35}S]methionine-labeled chymotryptic peptides revealed that p56 and P70$^{gag-fgr}$ shared no peptides (data not shown). Two peptides of p56, peptides 1 and 5, migrated very similarly to peptides 5 and 9 of P68$^{gag-ros}$, but they were found to be dissimilar when a mixture of both proteins was analyzed. p56 and P90$^{gag-yes}$ had no [^{35}S]methionine-labeled

peptides in common. No similarity was detected between the peptide maps of p56 and P140$^{gag-fps}$, p85$^{gag-fes}$, or P160$^{gag-abl}$ (data not shown).

DISCUSSION

We characterized the cellular protein apparently responsible for the elevated tyrosine protein kinase activity in the LSTRA murine lymphoma cell line. The principal substrate of this tyrosine protein kinase in vitro is a 56,000-dalton protein, p56 (6; Casnellie et al., in press). There is a good reason to think that p56 is itself responsible for the elevated tyrosine protein kinase activity in these cells. First, it contains a tryptic peptide identical in sequence to a peptide found in two other tyrosine protein kinases, p60^{src} and P90^{yes} (6). More significantly, this common peptide is the major site of tyrosine phosphorylation in p56 as well as in both retroviral protein kinases. Second, the apparent abundance of the p56 in four Mo-MuLV induced thymoma cell lines parallels closely to the level of the tyrosine protein kinase activity, as assayed by phosphorylation of either endogeneous polypeptides or an exogenous synthetic peptide. Thus, the variations in phosphorylation of p56 apparently do not reflect variation in the ability of an unrecognized kinase to phosphorylate a common abundant substrate, p56. Rather, such correlation over a 20-fold range suggests that p56 is the enzyme responsible for the elevated tyrosine protein kinase activity in these cells and that, like all known tyrosine protein kinases, it can undergo autophosphorylation in vitro.

Is p56 closely related to a tyrosine protein kinase which has been described before? Because the major site of the tyrosine phosphorylation in the 56,000-dalton protein is identical to that in the retroviral tyrosine protein kinases p60^{src} and P90$^{gag-yes}$, it is possible that p56 is encoded by either the c-src or the c-yes gene. This is almost certainly not the case. First, p56 is clearly smaller than p60^{src} and is not recognized by antitumor sera which recognize p60^{c-src} in mouse cells (unpublished data). Second, comparison of the two-dimensional tryptic maps of [^{35}S]methionine-labeled tryptic peptides revealed no homology between the 56,000-dalton protein and P90$^{gag-yes}$ from Y73 virus and very little similarity between the p56 protein and p60^{src} of Schmidt-Ruppin Rous sarcoma virus of subgroup A. The lack of homologous methionine-containing tryptic peptides is not inconsistent with the identity of the sites of tyrosine phosphorylation in these proteins. The phosphotyrosine-containing tryptic peptide common to all three proteins does not contain methionine. Despite the fact that we compared here the peptide maps of a murine cellular protein with those of viral proteins of avian origin, it is likely that identity of p56 with the proteins encoded by the c-src or c-yes genes would have been apparent. The maps of [^{35}S]methionine-labeled p60^{v-src} and p60^{c-src} from mouse cells are extremely similar (40). Further, peptide mapping reveals clearly the homology of the c-fps and v-fps proteins and of the c-myb and v-myb proteins (26, 30). Additionally, inspection of the deduced sequences of the proteins encoded by the fos, ras, myc, and erb-B oncogenes (10, 48, 51, 53, 54, 56) reveals that the products of the cellular forms of these oncogenes all share a number of methionine-containing tryptic peptides with the products of the cognate v-onc genes.

A 10% dissimilarity in amino acid sequences could, however, make the peptide maps of two proteins completely different. Therefore, the alternative approach of nucleic acid hybridization was also used. Analysis of the transcription of the c-src and c-yes genes by dot blot hybridization detected no elevated levels of transcripts of either gene in LSTRA or

Thy19 cells (data not shown). The homology of the v-src and v-yes genes, which are of avian origin, with the murine c-src and c-yes genes is not complete (43, 46). Nevertheless, we feel that we would have detected noticeably elevated transcription of either cellular gene because the hybridization
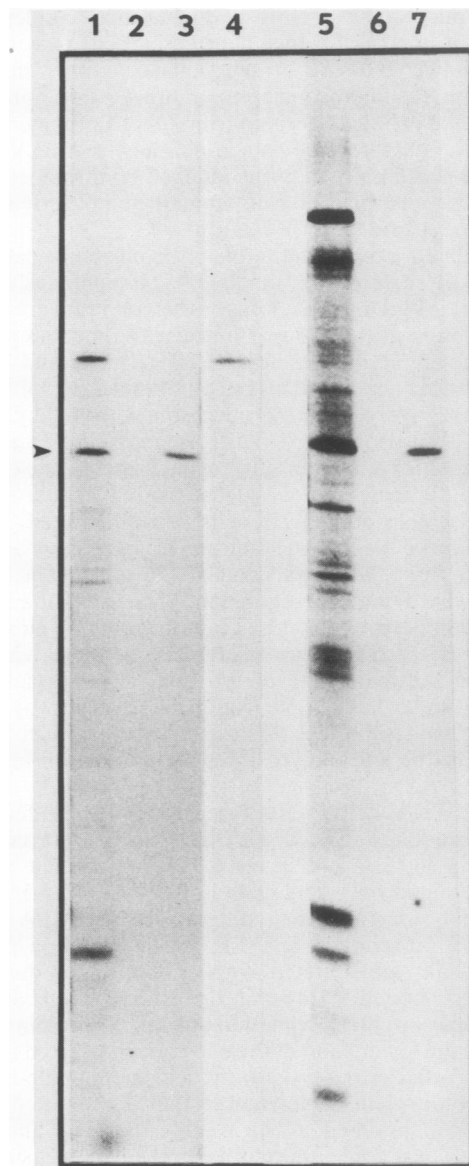

FIG. 3. Incorporation of [^{3}H]myristic acid and [^{3}H]palmitic acid into p56. Rapidly growing LSTRA cells were labeled with either [^{3}H]myristic acid or [^{3}H]palmitic acid for 2 or 18 h, respectively. Immunoprecipitates were prepared from cell lysates with anti-src I peptide antibody, anti-p15^{gag} serum, or normal rabbit serum, as described in the text. Immunoprecipitates and 1% of the total cell lysate were analyzed by SDS-polyacrylamide gel electrophoresis. [^{3}H]myristic acid-labeled proteins in lanes 1 through 3 were detected by fluorographic exposure for 60 days or for 52 days for lane 4. Exposure time for the [^{3}H]palmitic acid-labeled proteins was 15 days. The arrow indicates the position of p56. Lane 1, [^{3}H]myristic acid, total cellular proteins; lane 2, [^{3}H]myristic acid, normal rabbit serum; lane 3, [^{3}H]myristic acid, anti-src I antibody; lane 4, [^{3}H]myristic acid, anti-p15^{gag} serum; lane 5, [^{3}H]palmitic acid, total cellular proteins; lane 6, [^{3}H]palmitic acid, normal rabbit serum; lane 7, [^{3}H]palmitic acid, anti-src I antibody.
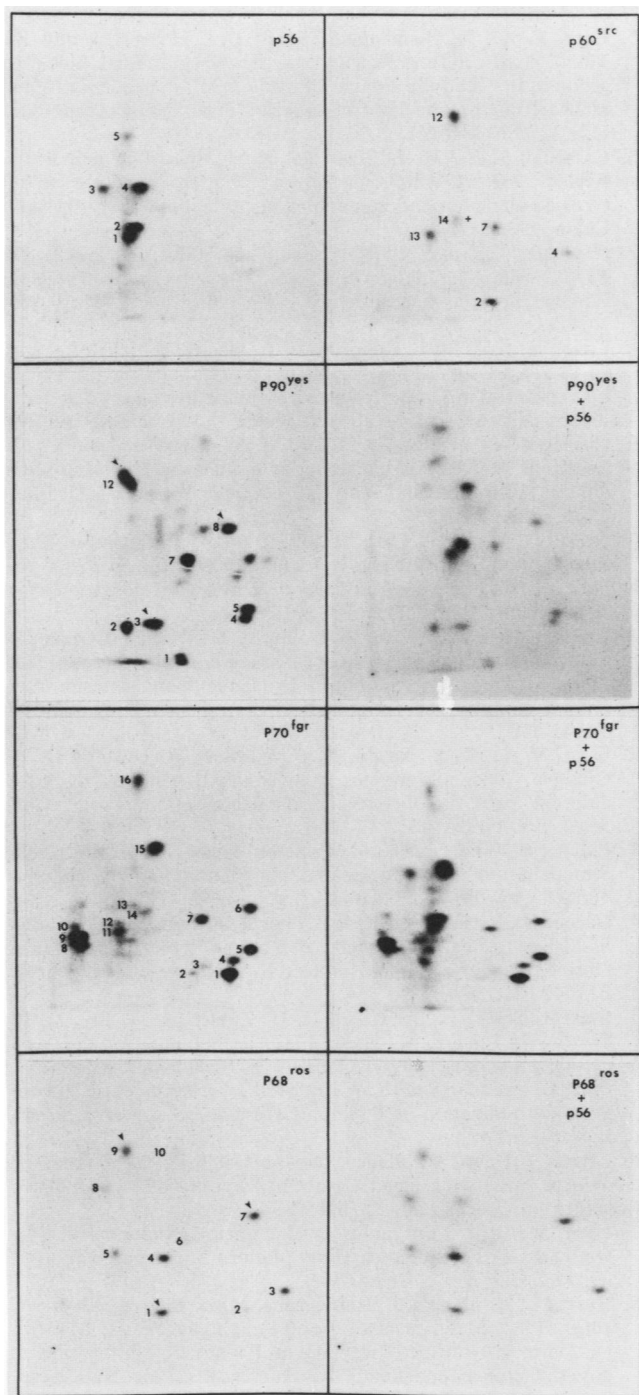
FIG. 4. Comparison of methionine-containing tryptic peptides of p56 from LSTRA cells, p60^{src} of Schmidt-Ruppin Rous sarcoma virus of subgroup A, p90^{yes} of Y73 virus, p68^{ros} of UR2 virus, and p70^{fgr} of Gardner-Rasheed feline sarcoma virus. The proteins were labeled biosynthetically with [^{35}S]methionine, isolated by immuno-precipitation, and digested with trypsin as described in the text. The digests were separated on cellulose thin-layer plates by electrophoresis at pH 4.72 in the first dimension and by ascending chromatography in the second. The origin is at lower left, and the cathode is at the right. Arrows indicate gag-related peptides in p90^{yes} and p68^{ros}. Peptides which were found to comigrate in a mixture of digests are indicated by +. The numbers for the p60^{src} peptides were adapted from Beemon et al. (3) and for p90^{yes} from Patschinsky and Sefton (33).

conditions used here permitted annealing of the viral DNA to mouse cellular DNA.

At face value, the 56,000-dalton protein is too small to be the c-abl or the c-fes product. It is formally possible, however, that p56 is a fragment of one of the proteins encoded by these two c-onc genes. This is argued against by the fact that a large number of independently isolated lymphomas contain p56, and it is unlikely that the same fragmentation process occurred in each of them. The [^{35}S]methionine tryptic peptide mapping showed no obvious identity between p56 from LSTRA cells and P160$^{gag-abl}$ of A-MuLV of P85$^{gag-fes}$ of Snyder-Theilen feline sarcoma virus. In addition there was no obvious elevation of the expression of the c-fes or c-abl genes in LSTRA or Thy19 cells, which contain elevated tyrosine protein kinase activity (data not shown).

Finally, it was possible that the protein was encoded by the c-ros or c-fgr gene, because their products have not yet been identified. The fact that the maps of [^{35}S]methionine-labeled tryptic peptides of p56, P68$^{gag-ros}$, and P70$^{gag-fgr}$ were different suggests that this is not the case. Our observations thus indicate that the tyrosine protein kinase which phosphorylates p56 may be unique, for it is not encoded by any of these six retroviral oncogene homologs which encode tyrosine protein kinases.

The 56,000-dalton protein is also clearly not a known growth factor receptor, since it is significantly smaller in size than the receptors for epidermal growth factor, platelet-derived growth factor, insulin-like growth factor, and insulin, which are all larger than 95,000 daltons. An interesting possibility is that the 56,000-dalton protein is a subunit of an as-yet-uncharacterized T cell-specific growth factor receptor.

In addition to these characterized tyrosine protein kinases, there are two other kinases of unknown function, p75 kinase found in rat liver (58, 60) and a 55,000-dalton protein found in rat spleen and to a lesser extent in other rat tissues (47). The size of the p75 tyrosine protein kinase makes a relationship to p56 from LSTRA cells unlikely. On the other hand, the 53,000-55,000-dalton tyrosine protein kinase detected by Swarup et al. (47) in the microsomal fraction of rat spleen cells may well be the same as that expressed at a high level in LSTRA cells. It is likely that p58, a phosphorylated protein detected in T-cell membranes by Earp et al. (12) is also the same protein.

The size of the 56,000-dalton LSTRA protein is similar to that of nonviral tumor antigen p53, which has been detected in a large number of transformed cells (35). Direct comparison of the two proteins rules out the possibility that they are related. The p53 nonviral tumor antigen is larger than the LSTRA protein and appears completely different as determined partial proteolytic analysis.

p56 appears to undergo at least two forms of protein modification in LSTRA cells. It is phosphorylated on both tyrosine and serine and appears as an abundant alkali-resistant phosphoprotein with a pI of 5.2 to 5.7 when LSTRA cells labeled biosyntheticaly with ^{32}P$_i$ are analyzed by two-dimensional gel electrophoresis (data not shown).

In addition, like p60^{src}, p56 contains lipid. Unlike either p60^{src} or Pr65^{gag}, however, which preferentially incorporate [^{3}H]myristic acid, p56 can be efficiently labeled with either [^{3}H]myristic acid or [^{3}H]palmitic acid. Our results indicate that both fatty acids are present in p56 in hydroxylamine-stable (presumably amide) linkages. We have not yet determined whether the presence of both palmitic and myristic acids indicates that there are two potential sites of acylation

in p56 or whether the acyltransferases of LSTRA cells exhibit less fatty acid specificity than the enzymes of chicken cells (Buss and Sefton, in press). p56 is thus the fourth enzyme involved in protein phosphorylation or dephosphorylation which has been found to be covalently modified by a fatty acid. The catalytic subunit of the cyclic AMP-dependent protein kinase (5), the viral and cellular forms of p60^{src} (Buss and Sefton, in press), and the protein phosphatase calcineurin B (1) each contain a myristyl moiety. It will be important to determine whether fatty acid plays a role in the enzymatic activity of these proteins.

p56 appears to be expressed at low levels in many but not all cells of the T-cells lineage. It was not found in cells of other hematopoietic lineages. Such a distribution suggests that p56 may have some normal cellular function in T cells. Only 2 of 30 virally transformed T cell lines, LSTRA and Thy19, contained relatively high levels of tyrosine protein kinase activity. It is clear therefore that the activation of the transcription of the gene encoding p56 is not a frequent event.

It is not yet known why this tyrosine protein kinase is expressed at high levels in these two Mo-MuLV-induced tumors. It was possible that recombination between the Mo-MuLV genome and a cellular gene in LSTRA cells produced a new oncogenic virus, much in the manner that A-MuLV was created. Gacon et al. (14) were, however, unable to detect any virus produced by LSTRA cells except Mo-MuLV. In addition, we found that neither p56 nor any novel gag-onc protein can be precipitated by anti-p15^{gag} sera from LSTRA cells. Thus, there is no reason to suspect that these cells harbor a cryptic acutely transforming virus. Another possibility is that the presence of the Mo-MuLV provirus in the cellular genome causes the transcriptional activation of the gene encoding this protein kinase. A Mo-MuLV provirus is found repeatedly at a small number of specific chromosomal loci in Mo-MuLV-induced rat and murine thymomas (11, 28, 49, 50). Two of these loci, pMo-1C and Pim-1, have been studied in some detail (11, 28). No Mo-MuLV provirus is found near either of these sites in either Thy19 or LSTRA cells (P. Jolicoeur and A. Berns, personal communications). Molecular cloning and DNA sequence analysis of the gene encoding p56 may reveal why the gene is overexpressed in LSTRA cells.

ACKNOWLEDGMENTS

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