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Acylation of Proteins with Myristic Acid Occurs Cotranslationally

CELESTE WILCOX, JING-SHAN HU, ERIC N. OLSON*

Several proteins of viral and cellular origin are acylated with myristic acid early during their biogenesis. To investigate the possibility that myristylation occurred cotranslationally, the BC_3H1 muscle cell line, which contains a broad array of myristylated proteins, was pulse-labeled with [^{3}H]myristic acid. Nascent polypeptide chains covalently associated with transfer RNA were isolated subsequently by ion-exchange chromatography. [^{3}H]Myristate was attached to nascent chains through an amide linkage and was identified by thin-layer chromatography after its release from nascent chains by acid methanolysis. Inhibition of cellular protein synthesis with puromycin resulted in cessation of [^{3}H]myristate-labeling of nascent chains, in agreement with the dependence of this modification on protein synthesis in vivo. These data represent a direct demonstration that myristylation of proteins is a cotranslational modification.

A VARIETY OF VIRAL AND CELLULAR proteins has been shown to be modified by the covalent attachment of the 14-carbon saturated fatty acid, myristate, linked through an amide bond to glycine at their amino termini (*1*). Two of the most thoroughly studied myristylated proteins are the transforming protein of Rous sarcoma virus, $p60^{v\text{-}src}$, and the proto-oncogene product, $p60^{c\text{-}src}$. These polypeptides are translated on free polysomes and myristylated before being transported to the plasma membrane (*2–4*). Deletion or modification of the first 14 NH_2-terminal amino acids of $p60^{v\text{-}src}$ does not influence intrinsic tyrosine kinase activity, but prevents myristylation and membrane association, and abolishes the transforming activity of the protein (*3–7*). Together, these observations suggest an important role for myristylation in targeting proteins to the plasma membrane and in cellular transformation.

Inhibition of protein synthesis results in immediate cessation of myristylation of cellular and viral proteins, indicating that this covalent modification is an early processing step, tightly coupled to translation (*2, 8–10*). Studies with inhibitors of protein synthesis cannot determine, however, whether myristate attachment occurs cotranslationally or within seconds after completion of polypeptide synthesis. To define precisely when myristate is attached to newly synthesized acylproteins, we have examined whether nascent polypeptide chains are modified by myristic acid.

To obtain nascent chains, BC_3H1 cells were labeled with [^{35}S]methionine for 10 minutes, cell extracts were prepared, and polyribosomes were isolated by magnesium precipitation (*11*). Ribosome pellets were solubilized and were applied to a QAE-Sephadex anion-exchange column in buffer containing 0.1*M* NaCl. This technique, which was adapted from the method described by Cioli and Lennox (*12*), allows isolation of nascent polypeptide chains because of binding of the RNA component of peptidyl transfer RNA (tRNA) to the column. At low salt concentrations, mature polypeptides are not retained by the column, whereas the negatively charged tRNA moiety attached to nascent chains binds to the column. Nascent chains covalently associated with tRNA elute when the salt concentration of the column buffer is increased to 1.0*M* (Fig. 1A).

A typical fractionation on QAE-Sephadex of a ribosomal pellet isolated from [^{35}S]-methionine-labeled cells is shown in Fig. 1, A and C. The large amount of radioactivity that passes through the column in 0.1*M* NaCl represents completed proteins associated with the ribosomal pellet. Increasing the salt concentration to 1.0*M* NaCl resulted in elution of a peak of [^{35}S]methionine-labeled material. To demonstrate that polypeptides contained in this high salt fraction were retained by QAE-Sephadex through a covalent interaction with RNA, we treated ribosomal pellets with 0.3*N* NaOH or ribonuclease (RNase) before fractionation (Fig. 1, B and D, respectively). Each treatment eliminated more than 95% of the [^{35}S]methionine-labeled material previously retained by the ion-exchange resin, demonstrating that RNA was required for its association with the column.

The specificity of the fractionation procedure for isolation of nascent chains was assessed further by diluting an aliquot of the high salt fraction to the low salt concentration of the starting buffer and reapplying the sample to a new column (Fig. 1E). Approximately 80% of the radioactivity was retained by the second column, thereby demonstrating that the high salt fraction was not contaminated with mature polypeptides. In addition, material that passed through the original column did not bind when reapplied to a new column (Fig. 1F), which

Department of Biochemistry and Molecular Biology, University of Texas, M. D. Anderson Hospital and Tumor Institute at Houston, Houston, TX 77030.

*To whom correspondence should be addressed.

indicates that peptidyl tRNA was not contained in the low salt fraction.

To determine whether acylation with myristic acid occurred during translation, we isolated nascent chains from cells labeled with [^{3}H]myristate for 10 minutes. Because less than 1% of the [^{3}H]myristate taken up by the cells during the labeling period was incorporated into nascent polypeptide chains, it was important to develop a technique for complete removal of lipids noncovalently associated with nascent chains. Removal of lipids was achieved by placing an aliquot of each fraction from the QAE-Sephadex column onto Whatman 3 MM filter paper, which was then extracted with chloroform:methanol:acetic acid (3:6:1) followed by petroleum ether. We tested whether this method alone was sufficient to remove all noncovalently associated lipids from peptidyl tRNA. Cultures were labeled for 12 hours with [^{3}H]myristate, and total cellular lipids, obtained by extraction of cell homogenates with chloroform:methanol (2:1), were fractionated on QAE-Sephadex. More than 95% of the ^{3}H-labeled lipids was eluted from the column in 0.1*M* NaCl (Fig. 2F, closed circles). Organic extraction of the column fractions was sufficient to remove all labeled lipids (Fig. 2F, open circles). Thus, the presence of [^{3}H]myristate-labeled material on filters after organic extraction would require its covalent association with protein (see below).

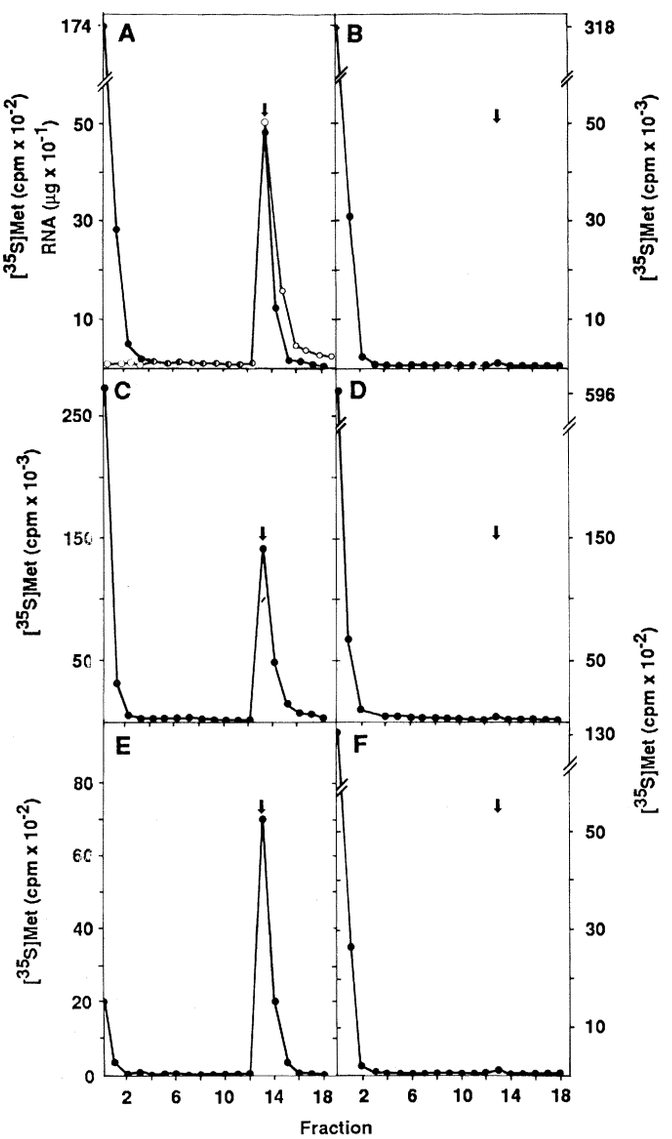

Fig. 1. QAE-Sephadex column chromatography of [^{35}S]methionine-labeled polysomes. BC_3H1 cells (*26*) were cultured as described (*27*). For pulse-labeling, cultures were incubated for 10 minutes with [^{35}S]methionine (125 μCi/ml), and ribosomes were isolated by magnesium precipitation as described by Palmiter (*11*). To isolate nascent chains, the ribosomal pellet was resuspended in 0.4 ml of column buffer [0.1*M* NaCl; 0.1*M* formate (*p*H 4.7), 0.1% Brij 35, and 6*M* urea] containing 0.5% SDS. After incubation for at least 30 minutes at room temperature, the sample was diluted to a total volume of 4 ml with column buffer and applied to a 0.4-ml QAE-Sephadex A-50 column (particle size, 40 to 120 μm; Pharmacia) equilibrated in the same buffer. The column was washed with 12 volumes of column buffer (one volume is 4 ml), and bound material containing nascent chains was eluted with column buffer containing 1.0*M* NaCl (arrow). Six 1.2-ml fractions were collected during the high salt elution. In (**A**), yeast tRNA was combined with starting buffer and applied to a QAE-Sephadex column (open circles). Binding and elution of the RNA were monitored by absorbance at 260 and 280 nm. In (A) and (B), the ribosomal pellet was resuspended and divided into equal aliquots. (A) was a control; in (**B**) the aliquot was treated with 0.3*N* NaOH at 37°C for 30 minutes and acidified with formic acid before dilution with column buffer and subsequent fractionation on QAE-Sephadex. In (C) and (D), the ribosomal pellet was resuspended and divided into equal aliquots. (**C**) was a control; in (**D**) the aliquot of resuspended ribosomal pellet was digested with RNase (200 μg/ml) for 24 hours at 37°C. In (**E**), an aliquot from the high salt fraction (A) was diluted with starting buffer to restore the original low salt concentration and was reapplied to a new QAE-Sephadex column. (**F**) An aliquot of the effluent from (A) was fractionated on a new QAE-Sephadex column. To quantitate [^{35}S]methionine-labeled material in column fractions, 150-μl aliquots from each fraction were applied to Whatman 3 MM paper (Whatman Laboratory Products Inc.). Filters were fixed with cold 10% trichloroacetic acid and rinsed with ethanol, and radioactivity was determined by liquid scintillation counting.

The elution profile of [^{3}H]myristate-labeled polysomes obtained from two separate experiments is shown (Fig. 2, A and C). As in [^{35}S]methionine-labeled polysomes, a large amount of labeled material was present in the effluent, representing completed polypeptide chains and other protein-bound lipids. However, elution with 1.0*M* NaCl resulted in a peak of [^{3}H]myristate-labeled material that was resistant to organic extraction. The peak of ^{3}H-labeled material eluted with 1.0*M* NaCl cannot be attributed to elution of negatively charged lipid species [for example, myristoyl coenzyme A (CoA) or phosphatidylserine] because, as shown above, free lipids were extractable by the organic extraction procedure. To verify that the ^{3}H-labeled material eluted with high salt was retained by the column because of its covalent association with RNA, we treated samples with NaOH or RNase as described (Fig. 2, B and D, respectively). Both treatments resulted in a reduction of more than 80% in the amount of radioactivity retained by the column, which indicated that the lipid was associated with peptidyl tRNA. When an aliquot of the unbound material was reapplied to a new column, no [^{3}H]myristate-labeled material was eluted in the high salt fraction (Fig. 2E), demonstrating that binding was specific and quantitative. Between 65 and 95% of [^{3}H]myristate-labeled material recovered in the high salt fraction from the QAE-Sephadex column also was retained when applied to a second column and was eluted with 1.0*M* NaCl.

To determine whether [^{3}H]myristate-labeling of nascent chains requires protein synthesis, as is true in vivo (*2, 8–10*), we prepared polysomes from cultures treated with 500 μ*M* puromycin for 30 minutes before addition of [^{3}H]myristate. [^{3}H]Myristate incorporation into nascent chains was inhibited by approximately 75% in the presence of puromycin; this inhibition provides indirect support for the conclusion that [^{3}H]myristate is incorporated cotranslationally into nascent polypeptide chains in vivo.

Myristate is highly specific for amide linkages to proteins and can be released only by acid hydrolysis (*8–10, 13*). In contrast, palmitate is preferentially attached to proteins through highly labile thiol ester bonds that are sensitive to hydrolysis with NH_2OH (*8,*

9, 13). To determine the nature of the linkage through which [^{3}H]myristate was attached to nascent polypeptide chains, we treated material contained within the QAE-Sephadex high salt fraction from [^{3}H]myristate-labeled polysomes with 1*M* NH_2OH, *p*H 7, for 4 hours at room temperature. Samples were then applied to filter paper, which was extracted to remove noncovalently associated lipids. To ensure that this method was sufficient to distinguish between amide and ester linkages of fatty acids to proteins, we labeled proteins with [^{3}H]palmitate and treated them in an identical manner. NH_2OH treatment released 84% of palmitic acid from proteins (control, 18,853 cpm; NH_2OH-treated proteins, 3108 cpm), but only 8% of myristic acid from nascent chains (control, 453 cpm; NH_2OH-treated nascent chains, 416 cpm). These results suggest that the lipid moeity is attached to nascent chains through an amide bond, consistent with the previously observed specificity of myristylation.

To ensure that the radioactivity associated with ^{3}H-labeled nascent chains was not due to the conversion of myristate into amino acids or other lipid species, we subjected nascent chains contained in the high salt fraction from QAE-Sephadex to acid methanolysis. Extraction of the hydrolysate with petroleum ether and analysis of the organic phase by thin-layer chromatography revealed a single labeled species that comigrated with methyl myristate (Fig. 3).

Together, our results show that myristic acid can be attached to acylproteins through an amide linkage during translation in vivo. The possibility remains, however, that some myristate-containing proteins also may be acylated very soon after completion of polypeptide chain biosynthesis. That myristylation may occur with subtly different kinetics is suggested by the previous observation that labeling of BC_3H1 cells with [^{3}H]myristate for short periods results in visualization of three major myristylated proteins (*10*), whereas a labeling period of 4 hours reveals 20 or more myristylated proteins (*10, 13, 14*).

Metabolic labeling of tissue culture cells with [^{3}H]myristate yielded the first indications of the remarkable specificity of *N*-myristoyl acyltransferase (NMT) with respect to fatty acid donor and protein acceptors (*2, 8–10, 13, 14*). Using synthetic octapeptides as substrates for NMT in vitro, Towler *et al.* demonstrated subsequently that myristoyl CoA was the preferred lipid donor for the modification and that the enzyme was specific for NH_2-terminal Gly residues (*15–17*). Chemical identification of the NH_2-terminal residue of several myristylated polypeptides also has revealed the presence of Gly in all cases (*18–22*).

Because myristylation can be reconstituted in vitro with artificial acceptor peptides (*15–17*), this modification is not coupled obligatorily to polypeptide elongation. The lack of dependence of myristylation on polypeptide elongation in vitro resembles *N*-linked glycosylation and acetylation, both of which occur cotranslationally and have been reconstituted in vitro with artificial acceptor peptides containing the appropriate enzyme-recognition sequences (*23, 24*).

In the future, it will be important to establish the precise intracellular distribution of NMT and to determine the exact time during polypeptide elongation when myristate is attached to nascent polypeptide chains. It is tempting to speculate that NMT might associate with ribosomes as has been shown for some *N*-acetyltransferases (*25*), thereby facilitating interaction with the NH_2-termini of appropriate polypeptide chains.

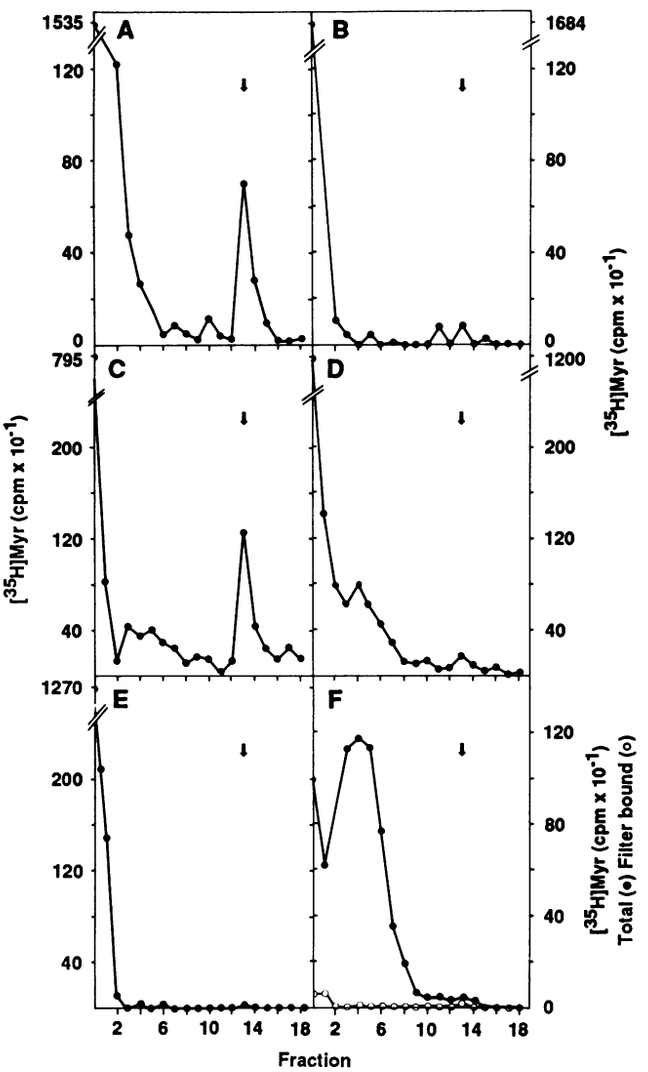

Fig. 2. QAE-Sephadex column chromatography of [^{3}H]myristate-labeled polysomes. BC_3H1 cells were labeled for 10 minutes with [^{3}H]myristate (0.5 mCi/ml), and a ribosomal pellet was isolated. Conditions for (**A**) to (**D**) are exactly as described in Fig. 1. Elution with column buffer containing 1.0*M* NaCl is shown by arrow. In (**E**), an aliquot of the effluent from the fractionation shown in (A) was rerun on a second QAE-Sephadex column. To remove residual lipid and determine the amount of lipid covalently bound to nascent chains, aliquots from each column fraction were placed on Whatman 3 MM filter paper and extracted extensively with chloroform:methanol:acetic acid (3:6:1) followed by petroleum ether. Filters were then dried, and radioactivity was determined by liquid scintillation counting. Values represent filter-bound radioactivity (counts per minute) after extraction with chloroform:methanol:acetic acid and petroleum ether. In (**F**), lipids obtained by extraction of [^{3}H]myristate-labeled cells with chloroform:methanol (2:1) were combined with column buffer and applied to a QAE-Sephadex column. Closed circles indicate total radioactivity (counts per minute) per fraction, and open circles indicate filter-bound radioactivity (counts per minute) per fraction after organic extraction.

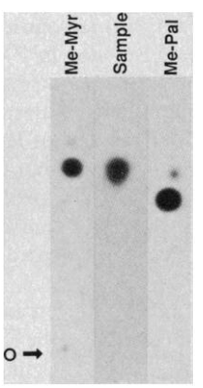

Fig. 3. Thin-layer chromatography of the organic extract of [^{3}H]myristate-labeled nascent chains hydrolyzed in methanolic HCl. [^{3}H]Myristate-labeled polysomes were applied to a QAE-Sephadex column, and material eluting in the presence of 1.0*M* NaCl was applied to Whatman 3 MM filters as described in Fig. 2. Filters were treated with chloroform:methanol:acetic acid (3:6:1) and petroleum ether to remove noncovalently associated lipids and then heated for 18 hours at 100°C in vacuo in 2*N* HCl, 83% methanol. The hydrolysate was extracted with petroleum ether, and the organic phase was analyzed by thin-layer chromatography with acetonitrile:acetic acid (1:1) as the mobile phase. After separation, the plate was treated with EnHance spray (New England Nuclear Corporation) and exposed to film for 10 days. ^{3}H-labeled methylmyristate (Me-Myr) and methylpalmitate (Me-Pal) standards were run in adjacent lanes. O, origin.

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Tumor Cell Rejection Through Terminal Cell Differentiation

JOAQUIN J. JIMENEZ AND ADEL A. YUNIS

Leukemic cells cultured in the presence of various conditioned media differentiate into macrophages. This finding suggested that the maintenance of undifferentiated state and self-renewal in vivo may be related to the inability of the host to generate an appropriate level of differentiation factor (DF). Evidence for this hypothesis was derived from experiments in vitro and in vivo with myeloid leukemia of rat. The following results were obtained: (i) in vitro, the percentage of cell differentiation at a fixed concentration of DF was inversely related to the concentration of cells; (ii) leukemic cell inoculates that were lethal to 7-day-old rats were rejected by 21-day-old rats; (iii) leukemic cells in diffusion chambers underwent differentiation in 21-day-old rats but not in 7-day-old rats; (iv) organs from 21-day-old rats contained more DF activity than those of 7-day-old rats; (v) treatment of rats with DF in diffusion chambers resulted in leukemic cell differentiation inside the chamber; and (vi) the development of leukemia in 7-day-old rats was aborted by treatment with DF. These results show that the differentiation of rat leukemia cells requires the appropriate level of DF. The proliferation of transplanted leukemia cells in 7-day-old rats goes unchecked because of inadequate generation of DF. Conversely, in the 21-day-old rats, rejection is accomplished by differentiation of the transplanted cells.

INTENSE INTEREST IS CURRENTLY FOCUSED on the role of colony-stimulating factor in leukemic cell differentiation and its potential applicability to the therapy of human myelogenous leukemia. In both the murine and human systems, factors derived from various conditioned media have been identified that induce the terminal differentiation of leukemia cells in vitro (*1–3*). Preparations containing granulocyte colony-stimulating factor (G-CSF) have been shown to suppress self-renewal of myeloid leukemia stem cells in vitro and the leukemogenicity of treated myeloid leukemic cells in vivo (*4–10*). Although the responsiveness of the leukemic cell to differentiation factors (DFs) relative to normal stem cells is uncertain, the studies cited above have clearly established that the leukemic cell can be induced to undergo terminal differentiation in response to physiological factors. This raises a number of questions, perhaps most important among them: What maintains the leukemic cell in the undifferentiated state in vivo? In vitro and in vivo observations reported herein on the myelogenous leukemia of rat suggest that the maintenance of the undifferentiated state and self-renewal may be related to the host's inability to generate the appropriate level of DF. Rejection of a leukemic cell transplant in 21-day-old recipient rats is accomplished by terminal cell differentiation of the transplanted cells.

The experimental model we have used in these studies is the chloroleukemia of rat (*11, 12*). This is a myelogenous leukemia with many similarities to the human disorder. It was originally produced by gastric instillation of 20-methylcholanthrene and later successfully transferred by the injection of chloroleukemia cells into newborn rats (*12, 13*). In 1975, the establishment of a permanent cell line, MIA C51 (*14*), from this tumor was reported. MIA C51 is maintained in suspension culture in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (DMEM-FCS). It has a doubling time of 12 hours and maintains many of the characteristics of the parent tumor, including transferability to newborn rats.

It has long been known from the work of Shay *et al.* (*12*) and Moloney *et al.* (*13*) that the successful transfer of rat chloroleukemia depends on the age of the recipient animal (best in newborn) and the number of leukemic cells injected. This is generally interpreted as a tendency to immune rejection as the rat matures. An alternative explanation was initially suggested to us by our in vitro observations. When C51 cells were cultured at increasing cell density (from 1×10^4 to 8×10^4 cells per milliliter) at a fixed level of

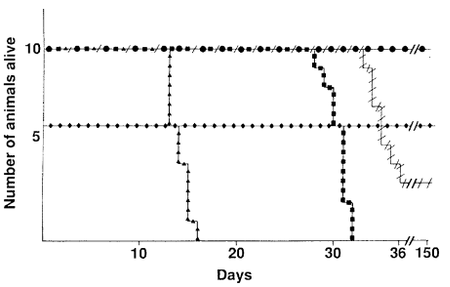

Fig. 1. Relation of survival to the age of recipient animal and to the number of transplanted leukemia cells. C51 cells in log phase were suspended in serum-free DMEM at 5×10^5 cells per milliliter. Appropriate dilutions were made and the indicated numbers of cells were injected intraperitoneally in 0.1 ml volume: (▲) 7-day-old rat with 1×10^5 cells injected; (■) 7-day-old rats, with 2.5×10^4 cells injected: (+) 7-day-old rats with 2×10^4 cells injected; (●) 7-day-old rats with 10^4 cells injected; and (◆) 21-day-old rats with 2.5×10^4 cells injected. At death, the abdomen of each animal was opened through an incision and inspected for leukemic ascites and for macroscopic leukemic infiltrates.

Departments of Medicine, Biochemistry, and Oncology, University of Miami School of Medicine, Miami, FL 33101.