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Markedly Elevated Levels of an Endogenous *sarc* Protein in a Hemopoietic Precursor Cell Line

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The *src* gene product of Harvey murine sarcoma virus is a 21,000-dalton guanine nucleotide-binding protein. We have recently shown that a wide variety of vertebrate cell strains and cell lines express much lower levels of an endogenous p21 immunologically related to the Harvey murine sarcoma virus-coded p21. In this report, we have examined the levels of endogenous p21 in a unique hemopoietic precursor cell line, 416B, which was originally described as a continuous cell line of a hemopoietic stem cell, CFU-S. The currently available 416B cells express markedly elevated levels of endogenous p21. The level of endogenous p21 in the 416B cells is 5- to 10-fold higher than the level of p21 in Harvey murine sarcoma virus-infected cells and more than 100 times higher than the level of endogenous p21 that we have observed in a variety of other fresh or cultured cells. The results indicate that marked regulation of the levels of an endogenous *sarc* gene product can occur, and speculation about a possible role for endogenous p21 in normal hemopoietic stem cells is discussed.

Various type C retroviruses can induce malignant transformation in both cultured fibroblastic cells and certain more specialized cells in the hemopoietic system. Some examples of such retroviruses include: (i) avian erythroblastosis virus, which can transform primary chicken embryo cultures or established fibroblastic mammalian cell lines and induce erythroleukemia in chickens (7, 12-14); (ii) Kirsten sarcoma virus (Ki-SV) and Harvey sarcoma virus (Ha-SV), which can transform a wide variety of established fibroblast cell lines and induce erythroleukemia in newborn mice (24); (iii) Abelson virus, which can transform established mouse or rat cell lines but which induced B-cell or pre-B cell leukemias in susceptible mice (22, 25, 30); (iv) MC29 virus, which can transform primary chicken embryo cultures or established mammalian cell lines as well as various hemopoietic cells of the myeloid lineage (7, 13); and (v) a recently described myeloproliferative murine sarcoma virus (20). A variety of groups have identified genetic sequences that have been implicated in the oncogenic properties of these viruses, and in each case a homologous genetic sequence has been detected in normal uninfected cells (1, 2, 10, 14, 23, 28). Because of the detection of these relatively conserved homologous normal genes and the detection of messenger ribonucleic acid (RNA) products of these genes, some normal role has been postulated for these endogenous progenitors of the viral *onc* genes.

Our laboratory has been especially interested in one group of such retroviruses derived from rats: Ha-SV, Ki-SV, and a purely rat sarcoma virus (22). Ha-SV and Ki-SV were originally isolated by passage in rats of mouse type C retroviruses, and both have been shown to be recombinant viruses containing genetic sequences of the respective mouse type C parental virus and genetic sequences of endogenous rat origin (11, 15). The *src* protein of these two oncogenic retroviruses, which are homologous in their genetic structure, has been shown to be a related 21,000-dalton protein, p21 (26, 29). Recently, employing rat antiserum containing antibodies to the *src* p21 of Ha-SV, we have also demonstrated a related *sarc* p21 in a variety of vertebrate cell strains and in cell lines which have not had virus added exogenously (H. Langebeheim, T. Y. Shih, and E. M. Scolnick, Virology, in press). The levels of this endogenous p21 are extremely low in the cells examined, and infection of some of these cells with Ha-SV results in an elevation (20- to 50-fold) in levels of p21. The results of studies on the viral p21 and endogenous p21 are analogous in many ways to extensive data on the p60 *src* protein of Rous sarcoma virus and the homologous p60 *sarc* protein found in normal chicken and mammalian cells (5, 6, 19, 31).

To investigate a possible function for endogenous p21, we have examined the expression of endogenous p21 in a variety of different cells. We have concentrated on hemopoietic cells for

two main reasons: (i) because of the erythroleukemic pathology induced in the hemopoietic system in mice which have been injected with pseudotypes of Ha-SV or Ki-SV, and (ii) because of the availability of a unique series of hemopoietic precursor cell lines isolated from long-term bone marrow cultures of Friend virus-infected BDF_1 mouse bone marrow cells (8, 9). One of these cell lines, 416B, has been of special interest, since this unique cell line originally possessed at least some properties expected of a hemopoietic stem cell, CFU-S (8). We report here that currently available cultures of 416B cells express levels of endogenous p21 which are 5 to 10 times higher than the levels of p21 in cells exogenously infected with Ha-SV and at least 100 times higher than the levels of endogenous p21 that we have observed either in several fibroblast cell lines, in several other hemopoietic cell lines, or in the total population of cells derived from normal bone marrow of 6- to 8-week-old BDF_1 or NIH Swiss mice. We discuss the practical utility of the elevated level of endogenous p21 in this cell and the possible mechanisms of this dramatically elevated level of endogenous p21, and we speculate about the possibility that p21 will be a molecular marker for some hemopoietic precursor cells in normal bone marrow.

MATERIALS AND METHODS

Cells. The NIH 3T3 cells, the C127 3T3 cells derived from an RIII mouse, and the Ha-SV-transformed nonproducer C127 cells have been described previously (29; Langebeheim et al., in press). NIH 3T3 cells producing either ecotropic Moloney type C virus or Ha-SV and Moloney virus have also been described previously (29). The F9 pluripotent teratocarcinoma cell, derived from a 129 mouse, was the gift of George Khoury, National Cancer Institute. WEHI-3 is a myelomonocytic leukemia cell line which was originally isolated from the bone marrow of a mineral oil-treated BALB/c mouse (17); WEHI-3 cells grow attached to the surface of plastic cell cultureware. Three suspension cell lines derived from Friend virus infection of BDF_1 long-term bone marrow cultures (8, 9) were used: (i) 427E, a myeloid leukemia cell line which can be transplanted in syngeneic mice; (ii) 458C, a continuous cell line which has many properties of a nonmalignant granulocyte precursor cell line, GM-CFC; and (iii) 416B, a cell line which originally had properties of a CFU-S or hemopoietic stem cell line. Each of these suspension cells produces the helper-independent virus, Friend murine leukemia virus (F-MuLV), but not the replication-defective virus, spleen focus-forming virus (Ruscetti et al., unpublished results). HFL/d, a spleen focus-forming virus-induced erythroleukemia cell line from BALB/c mice, was the gift of Frank Lilly, Albert Einstein College of Medicine. The three suspension cell lines from BDF_1 marrow were grown in Fisher medium with 25% horse serum. All other cells were grown in Dulbecco medium with 10 to 20% fetal calf serum.

Normal bone marrow cells were obtained by flushing the contents of a single femur from 6- to 8-week-old mice into 5.0 cm^3 of methionine-free Dulbecco medium. Bone marrow cells were also obtained from mice treated subcutaneously 3 to 4 days earlier with 80 mg of phenylhydrazine per kg of body weight. Cell suspensions were also prepared from the spleens of normal 6- to 8-week-old mice, mice pretreated with phenylhydrazine as above, or mice with F-MuLV-induced erythroleukemia (33).

Cell labeling and immunoprecipitation. Cells were washed once in methionine-free Dulbecco medium and labeled for 3.0 h in methionine-free Dulbecco medium supplemented with 2% dialyzed fetal calf serum at 37°C in an atmosphere of 10% CO_2. The serum was dialyzed against 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.5) and stored at −20°C as frozen working samples before use in metabolic labeling; serum was thawed just before use. The dialysis-freeze-thaw procedure for the serum ensured minimum proteolysis of the serum and reliable high-specific-activity labeling of cells. [^{35}S]methionine, specific activity 495 Ci/mmol, was used at a final concentration of 300 μCi/ml in all labeling studies. Suspension cells were labeled at a cell concentration of 10^6 cells per ml. Extracts of cells were prepared by lysing cells in a buffer containing 1% Triton X-100, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate, 0.02 M sodium phosphate (pH 8.0), 0.1 M sodium chloride, 1 mM ethylenediaminetetraacetic acid and 200 U of the protease inhibitor Trasylol per ml. Procedures for the immune precipitation of labeled proteins and the analysis of the precipitates on sodium dodecyl sulfate-polyacrylamide gels have been fully described previously (29). The source of the rat antiserum containing antibodies to the Ha-SV p21 *src* protein has been described previously (26, 29).

Other procedures. The guanine nucleotide binding assay for Ha-SV p21 was performed exactly as previously described (26). One-dimensional peptide mapping was carried out by the procedure of Cleveland et al. (4).

Hybridization assays. Liquid hybridization assays were performed as previously described and analyzed with S1 nuclease (27). Agarose gel electrophoresis of deoxyribonucleic acid (DNA) samples and subsequent hybridization were performed by the procedures of Southern (15). For the liquid hybridization assays, a ^{3}H-labeled complementary DNA (cDNA) was used which detects the rat-derived 30S sequences in Ha-SV and Ki-SV. Since the rat-derived 30S sequences do not code for p21 (11), but are part of the genome of Ha-SV and Ki-SV, we used this cDNA to exclude contamination of 416B cells by either virus as detailed below. The preparation of [^{3}H]cDNA was as described previously (27). For the blotting experiments, a molecularly cloned piece of Ha-SV DNA was used, designated BS9. BS9 DNA spans the 3′ 40% of the *src* gene of Ha-SV; the DNA is 460 base pairs and has 360 base pairs to the 3′ side of a single *Hin*dIII restriction site in the *src* gene and approximately 100 base pairs to the 5′ side of the *Hin*dIII site. This DNA probe has been fully detailed recently by Ellis et al.

(11) and was labeled by nick translation with [^{32}P]-cytidine triphosphate to a specific activity of approximately 5×10^7 cpm/μg.

RESULTS

The expression of p21 in a variety of cultured cells and in freshly isolated bone marrow cells was investigated initially by labeling cells with [^{35}S]methionine and subsequent immunoprecipitation with rat antiserum containing antibodies to p21. Labeling was generally for 3.0 h, but similar results were obtained after labeling for either 30 min or up to 8.0 h. Immunoprecipitates were dissolved in sodium dodecyl sulfate-containing buffers and analyzed by sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis (Fig. 1 and 2). As previously reported (Langebeheim et al., in press), NIH 3T3 cells have low levels of endogenous p21, whereas the same cells infected with Ha-SV contain significantly higher levels of p21 (Fig. 1, lanes 2 and 4). Strikingly, 416B cells contain markedly elevated levels of p21 (Fig. 1, lane 8). From quantitative scans of these autoradiograms, the levels of p21 in 416B cells were calculated to be seven- to eightfold higher than the levels of p21 in Ha-SV-infected cells and more than 100 times higher than the levels of p21 in the C127 fibroblasts. We next examined other hemopoietic cell lines derived from the same Friend virus-infected BDF_1 bone marrow cultures from which the 416B cell was cloned (8, 9). The level of p21 in 416B cells was clearly higher than the level of p21 in 427E cells, from a BDF_1-derived myeloid leukemia cell line, or in 458C cells, from a BDF_1-derived hemopoietic cell line with many properties of normal GM-CFC cells. The results obtained with several other cultured cell lines and freshly isolated hemopoietic cells are shown in Fig. 2. All of these cell lines had comparably low levels of endogenous p21 and include F9, a teratocarcinoma cell in its undifferentiated state; HF1/d, an erythroleukemic cell line; WEHI-3, a myelomonocytic leukemia cell line from BALB/c mice; normal bone marrow or spleen cells from adult BDF_1 mice; bone marrow or spleen cells from phenylhydrazine-treated BDF_1 mice; and spleen cells from a Swiss mouse with F-MuLV-induced erythroleukemia (33). Two cultures of 416B were also examined: one culture cultured in Bethesda, Md., for 7 months before initial methionine labeling and the other cultured in Manchester, England, sent to Bethesda, and labeled 1 day after arrival. Only the 416B cells contained the strikingly elevated levels of p21, and the cells cultured in Manchester seemed to have somewhat higher levels than did the cells chronically cultured in Bethesda. In other studies, 416B cells were injected intravenously into BDF_1 mice which had received 800 rads of X irradiation. After 5 days the 416B cells growing in the spleen were isolated and labeled. High levels of p21 were readily detected, and the p21 level was indistinguishable from that in Fig. 2, lane 16. Since the p21 *sarc* protein was elevated in 416B cells, we also examined the levels of endogenous p60 *sarc* protein in 416B cells with antiserum given to us by Gilbert Jay, National Cancer Institute; the level of p60 *sarc* protein in 416B cells was no higher than the low levels in uninfected C127 cells.

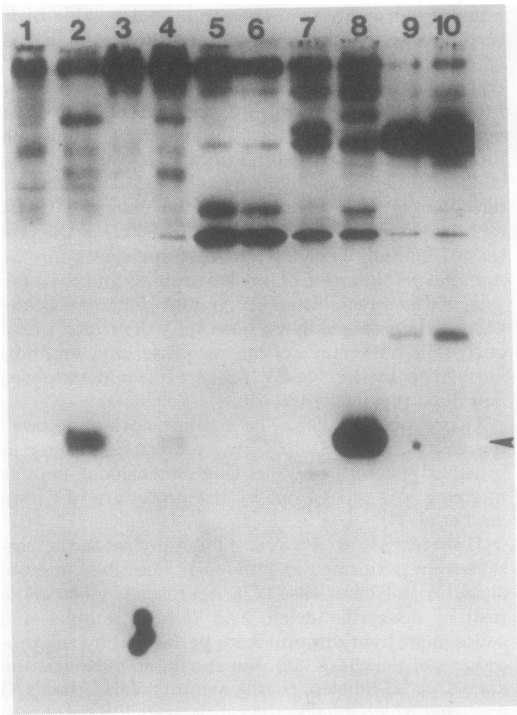

FIG. 1. *p21 in various hemopoietic cell lines. The cells were labeled and analyzed as described in the text. The gel was exposed to X-ray film for 24 h. The arrow adjacent to lane 10 indicates the p21. Lanes and extracts: 1, NIH 3T3 cells producing Ha-SV and Moloney murine leukemia virus plus control serum; 2, NIH 3T3 cells producing Ha-SV and Moloney murine leukemia virus plus antiserum; 3, NIH 3T3 cells plus control serum; 4, NIH 3T3 cells plus antiserum; 5, 458C cells plus control serum; 6, 458C cells plus antiserum; 7, 416B cells plus control serum; 8, 416B cells plus antiserum; 9, 427E cells plus control serum; and 10, 427E cells plus antiserum.*

Comparison of the endogenous 21,000-dalton band in 416B cells with Ha-SV p21. The results demonstrate that 416B cells express high levels of a methionine-labeled protein which precipitates with rat antiserum containing antibodies to Ha-SV p21. To confirm that this

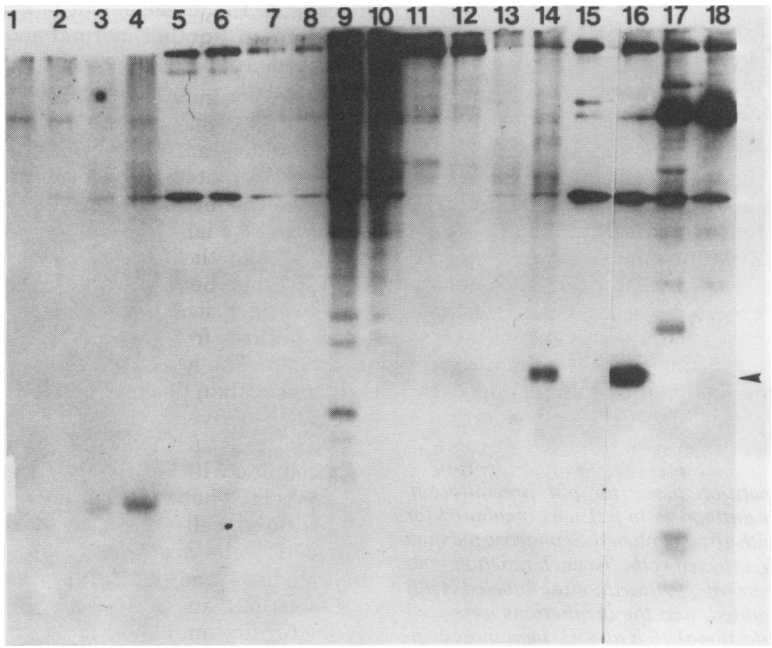

FIG. 2. *p21 in hemopoietic cells and cell lines. The indicated cells were labeled and analyzed for p21 as described in the text. All odd-numbered lanes were precipitated with control serum; all even-numbered lanes were precipitated with antiserum. The arrow adjacent to lane 18 indicates the p21. Lanes and extracts: 1 and 2, bone marrow from normal BDF$_1$ mice; 3 and 4, bone marrow from phenylhydrazine-treated BDF$_1$ mice; 5 and 6, spleen from normal BDF$_1$ mice; 7 and 8, spleen from phenylhydrazine-treated BDF$_1$ mice; 9 and 10, F9 undifferentiated teratocarcinoma cells; 11 and 12, HFL/d erythroleukemia cells; 13 and 14, 416B cells carried in Bethesda, Md.; 15 and 16, 416B cells carried in Manchester, England; and 17 and 18, WEHI-3 myeloid leukemia cells.*

21,000-dalton protein in 416B cells was in fact related to Ha-SV p21, two experiments were performed: a competition radioimmunoprecipitation and peptide mapping of the p21 in 416B cells and the p21 coded for by Ha-SV. The competition study is shown in Fig. 3. The Ha-SV p21 was purified 265-fold from Ha-SV-infected NIH 3T3 cells (Shih et al., unpublished data). The partially purified p21 was used to compete for p21 antibodies in immunoprecipitation studies on the 416B methionine-labeled p21. At each level of antiserum tested, the purified Ha-SV p21 competed effectively against the precipitation of 416B p21; at the lowest level of antibody tested (Fig. 3, lane 4 versus lane 1), virtually complete competition was achieved.

The peptide maps are shown in Fig. 4A and B. One-dimensional mapping was performed by using either *Staphylococcus* sp. V8 protease or papain to generate [^{35}S]methionine-labeled peptides. When we used V8 protease, two major peptides were produced from either the 416B p21 or the Ha-SV p21, and the relative migration of the peptides was similar. When we used papain, one major band was generated with either p21, and this band migrated similarly in both cases. These results confirm the close relationship of the 416B p21 and the Ha-SV p21. Clearly, more detailed peptide analyses are needed, and even these peptide maps show differences between the 416B p21 and the Ha-SV p21. However, the results of the competition study and the peptide map confirm a close relationship between the Ha-SV p21 and the p21 in 416B cells. In studies not shown, two other rat antisera with antibodies to Ha-SV p21 also precipitated the 416B p21; six rat antisera from rats transplanted with either Moloney sarcoma virus-transformed cells or spleen focus-forming virus-infected rat cells failed to precipitate either the Ha-SV p21 or the 416B p21.

Guanine nucleotide-binding activity. Recently, we described a biochemical activity of the p21 *src* protein of Ha-SV and Ki-SV. The p21 was shown to have a high affinity for either guanosine diphosphate (GDP) or guanosine triphosphate, and a binding assay was devised which allows the purification and characterization of p21 (26). Since the [^{3}H]GDP binding activity could be used as an assay for p21 in crude extracts of cells, extracts of NIH 3T3 cells, Ha-SV-infected NIH 3T3 cells, 416B cells, and

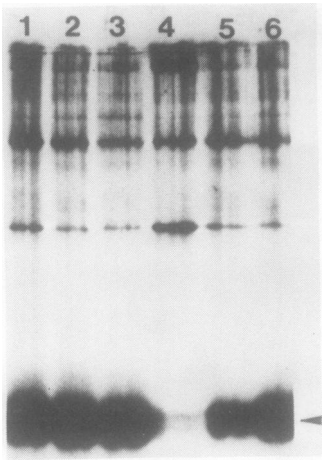

FIG. 3. *Competition assay for p21 precipitation. Antiserum with antibodies to p21 was incubated for 60 min at 4°C with 10 μg of phenyl-Sepharose purified p21 from Ha-SV-infected cells. To each reaction mixture 2 × 10^6 cpm of [^{35}S]methionine-labeled 416B cells was then added, and the incubations were continued for an additional 16 h at 4°C. Immunoprecipitates were then analyzed by sodium dodecyl sulfate-gel electrophoresis. The arrow adjacent to lane 6 indicates the p21. Lanes: 1, 0.002 ml of antiserum; 2, 0.005 ml of antiserum; 3, 0.010 ml of antiserum; 4, 0.002 ml of antiserum preincubated with p21; 5, 0.005 ml of antiserum preincubated with p21; 6, 0.010 ml of antiserum preincubated with p21.*

427E cells were prepared and assayed for [^{3}H]-GDP binding in this assay (Fig. 5). Uninfected NIH 3T3 cells or 427E cells had essentially no detectable GDP-binding activity since the levels of endogenous p21 are so low in these cells. As previously reported, Ha-SV-infected cells had elevated amounts of GDP-binding activity. 416B cells showed an even higher level of GDP-binding activity; the level of p21 in 416B cells, as quantitated from the linear portion of the binding curve, was approximately 10-fold higher than the level of p21 in the Ha-SV-infected cells and at least 100-fold higher than the p21 level thus far observed in the other uninfected cells which express low levels of p21. The results confirm the elevated levels of p21 in 416B cells by an assay that does not rely on metabolic labeling of viable cells.

Is the p21 in 416B cells endogenous? The level of p21 in 416B cells was so much higher than that in other cells not deliberately infected with Ha-SV or Ki-SV that we next performed a series of experiments (i) to study whether 416B cells had been either inadvertently infected with Ha-SV or Ki-SV or even infected with a novel recombinant virus coding for p21, and (ii) to investigate the arrangement of gene(s) in these cells and in another normal mouse cell that could code for p21-like proteins. With the use of a molecularly cloned piece of DNA from the 3′

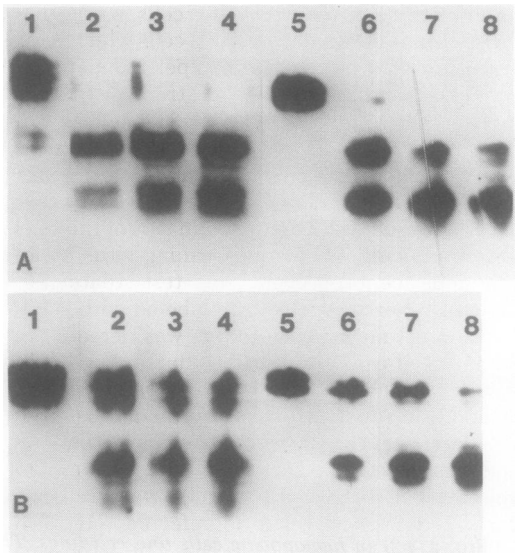

FIG. 4. *Peptide map of Ha-SV p21 and 416B p21. One-dimensional peptide analysis of the respective p21 proteins was performed as described previously (4). In A, Ha-SV p21 and 416B p21 were cleaved with V-8 protease. In B, Ha-SV p21 and 416B p21 were cleaved with papain. Lanes: 1, uncleaved Ha-SV p21; 2, Ha-SV p21 plus 10 μg of enzyme; 3, Ha-SV p21 plus 25 μg of enzyme; 4, Ha-SV p21 plus 50 μg of enzyme; 5, uncleaved 416B p21; 6, 416B p21 plus 10 μg of enzyme; 7, 416B p21 plus 25 μg of enzyme; and 8, 416B p21 plus 50 μg of enzyme.*

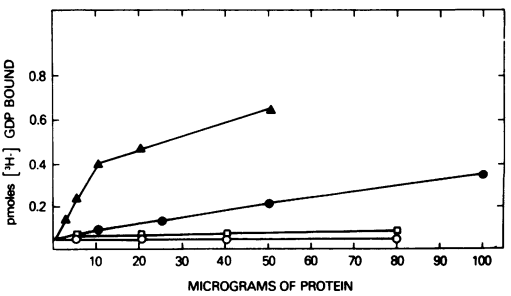

FIG. 5. *GDP-binding activity of 416B cells. Extracts of the indicated cells were assayed for [^{3}H]-GDP binding at 4°C as previously described (26); 1 pmol of [^{3}H]GDP represents approximately 7,500 cpm. All assays were in the presence of 0.007 ml of antiserum. All extracts had less than 0.05 pmol with control serum. Symbols: (▲) 416B cells; (●) NIH 3T3 cells producing Ha-SV and Moloney murine leukemia virus; (□) NIH 3T3 cells producing Moloney murine leukemia virus; and (○) 427E cells.*

portion of the *src* gene of Ha-SV, Ellis et al. have recently demonstrated the presence of conserved p21-coding gene(s) in the DNA from a wide variety of vertebrate species (11).

To determine whether a p21-encoding virus could be transmitted from 416B cells, we filtered the supernatant fluid from 416B cells which have been shown to produce the helper-independent type C retrovirus F-MuLV and transmitted this supernatant to uninfected NIH 3T3 cells, a cell line highly permissive for F-MuLV replication. One week later the cells were analyzed for p21 by [^{35}S]methionine labeling and for F-MuLV by the XC plaque test. Greater than 99% of the cells were producing XC plaque-forming virus by an infectious center-XC plaque test (data not shown). When the cells were labeled with [^{35}S]-methionine, no increase in p21 was detected in the infected cells compared with the low levels observed in uninfected NIH 3T3 cells (Fig. 6). In addition, no foci of transformed cells were noted in the infected cultures even after subculturing the infected NIH 3T3 cells for 2 additional weeks. Next, total cellular RNA was prepared from NIH 3T3 cells, Ha-SV-infected NIH 3T3 cells, 416B cells, and 427E cells. The RNA was hybridized to a [^{3}H]cDNA which detects the rat-derived 30S virus-like sequences of Ki-SV or Ha-SV (27), sequences which are part of Ha-SV and Ki-SV but which do not code for p21 (11). The results of the hybridization are shown in Table 1. The RNA from 416B cells contained no rat-derived 30S sequences, whereas the RNA from Ha-SV-infected cells readily hybridized. As a positive control for the quality of the RNA prepared from 416B cells, this RNA was also hybridized to a cDNA from F-MuLV. High levels of F-MuLV RNA were readily detected ($C_rt_{1/2}$, less than 50 mol·s·liter^{-1}). The results of the RNA hybridization study and the lack of transmissibility of any p21 or focus-forming virus exclude the possibility that the 416B cells had been inadvertently infected with Ha-SV or Ki-SV. As further corroboration of this conclusion, we examined a culture of 416B cells obtained directly from Manchester, England. This culture had never previously been in our laboratory. The cells showed the same markedly elevated levels of p21 when labeled just 1 day after arriving in our laboratory (Fig. 2, lane 16).

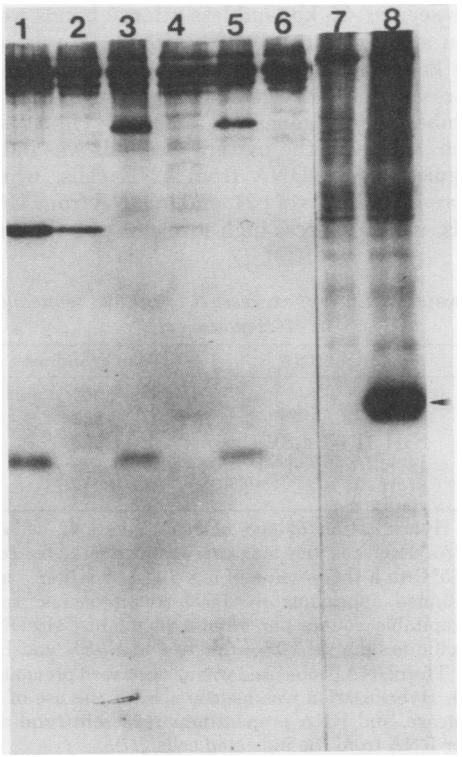

FIG. 6. *Lack of transmissibility of p21 from 416B cells. 416B cells were seeded at 10^6 cells per ml in Fisher medium containing 25% horse serum and incubated for 24 h at 37°C. The supernatant fluid was filtered through a 0.45-μm membrane filter (Millipore Corp.) and used to infect NIH 3T3 cells with the aid of 8 μg of polybrene per ml. The multiplicity of infection was approximately 1.0. When the cells reached confluence in about 6 days, some of the cells were analyzed by an infectious center test for F-MuLV, and the rest were analyzed by metabolic labeling for p21. All odd-numbered lanes were precipitated with control serum; all even-numbered lanes were precipitated with antiserum. The arrow adjacent to lane 8 indicates the p21. Lanes and extracts: 1 and 2, NIH 3T3 cells; 3 and 4, NIH 3T3 cells infected with F-MuLV 57 (3); 5 and 6, NIH 3T3 cells infected with 416B-derived F-MuLV; 7 and 8, 416B cells.*

p21-related DNA sequences in 416B cells. To begin to investigate the mechanism by which the p21 elevation came about in 416B cells, we examined, by the Southern blotting procedure, DNA sequences in normal liver cells from an adult (6- to 8-week-old) BDF_1 mouse, in 427E myeloid leukemia cells, and in 416B cells by using a cloned DNA fragment from the *src* gene of Ha-SV. The DNA from BDF_1 liver cells, 427E cells, and 416B cells was digested with either *Eco*RI or *Hin*dIII endonuclease and then analyzed by the Southern blotting procedure with a ^{32}P-labeled *src* DNA (see above). *Eco*RI endonuclease does not cut within the p21 *src* gene, and *Hin*dIII cuts a single time within the p21 *src* gene in Ha-SV (11) (Fig. 7). In each mouse cell DNA digested with *Eco*RI, three high-molecular-weight bands were seen, one at approximately 23 kilobases (kb), one at approximately 10 kb, and one at approximately 7 kb; in each DNA digested with *Hin*dIII, an intense band

was seen at 4.5 kb, and less intense bands were seen at approximately 10 and 3 kb. We do not yet know whether the total number of bands represents one *sarc* gene with introns or a small number of *sarc* genes related to the Ha-SV *src* gene. However, the pattern of bands was indistinguishable in DNA from 427E cells, which express low levels of p21, and in DNA from 416B cells, which express high levels of p21. The results indicate that reiteration of the Ha-SV-related p21 gene has not occurred in the high p21 expressor 416B cells.

TABLE 1. *Lack of expression of rat 30S sequences in 416B mouse cells*[a]

Source of RNA	cpm hybridized
None	212
NIH 3T3	232
NIH 3T3/Ha-SV	958
427E	225
416B	238

[a] Hybridization of 30S cDNA to various cellular RNAs. Each reaction mixture was incubated for 36 h at 65°C to a $C_rt_{1/2}$ value of 5×10^3 mol·s·liter^{-1} and contained approximately 1,500 trichloroacetic acid-precipitable counts per minute (cpm) of [^{3}H]cDNA specific for the rat 30S sequences in Ki-SV and Ha-SV. The cDNA probe used was as described previously (27). Hybridization was measured with the use of S1 nuclease, and RNA preparations represent total cellular RNA from the indicated cells (27).

DISCUSSION

The current studies have identified a continuous cell line, 416B, which expresses markedly elevated levels of a 21,000-dalton protein related to the *src* protein of Ha-SV. The p21 protein expressed in 416B cells is endogenous, and the levels of expression of endogenous p21 in this cell line are far higher than the levels we have observed thus far in any other cultured cells or fresh cells, including cells deliberately infected with Ha-SV or the related virus, Ki-SV.

Before discussing either the possible mechanism by which the p21 has become elevated in 416B cells or the possible physiological significance of the p21 elevation, it is necessary to review the history of 416B cells and the current biological properties of 416B cells. This continuous cell line was isolated from a long-term culture of BDF_1 mouse bone marrow cells which had been infected approximately 4 to 6 months earlier with Friend virus complex (8, 9). 416B cells produce the helper-independent ecotropic virus F-MuLV, but does not produce the replication-defective virus, spleen focus-forming vi-

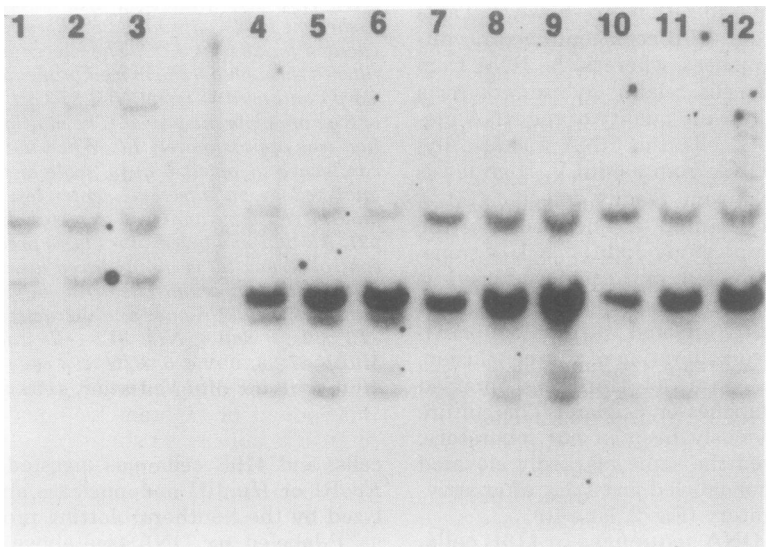

FIG. 7. *Southern blot analysis of Ha-SV-related sarc DNA in BDF_1 liver cells, 427E cells, and 416B cells. DNAs were digested with either EcoRI endonuclease (lanes 1, 2 and 3) or HindIII endonuclease (lanes 4 through 12) at 1 enzyme unit per μg of high-molecular-weight DNA for 24 h at 37°C. The DNAs were electrophoresed on 1.0% agarose gels and analyzed by the Southern transfer method with ^{32}P-labeled BS9 DNA from the src gene of molecularly cloned Ha-SV (11). Lanes: 1, EcoRI-digested 416B DNA (20 μg); 2, EcoRI-digested 427E DNA (20 μg); 3, EcoRI-digested BDF_1 mouse liver cells (20 μg); 4, 5, and 6, 10, 20, and 50 μg, respectively, of HindIII-digested BDF_1 mouse liver DNA; 7, 8, and 9, 10, 20, and 50 μg, respectively, of HindIII-digested 427E DNA; and 10, 11, and 12, 10, 20, and 50 μg, respectively, of HindIII-digested 416B DNA.*

rus (Ruscetti et al., unpublished data). Originally, 416B cells formed CFU-S-like colonies in the spleens of irradiated syngeneic mice and differentiated to mature granulocytes and megacaryocytes in such spleens. Later cultures of 416B seemed to be able to differentiate into granulocytes, megacaryocytes, and erythroid cells in such spleen colony assays. For the past 3 to 5 months the 416B cells have proliferated diffusely in the spleens of irradiated mice and have matured to cells that resemble erythroblasts in such mice (Dexter et al., unpublished data). The reason for the changing biology of 416B cells is not known, and chromosomal changes have occurred during the continuous passage of the cells. Unfortunately, samples of the 416B cells frozen early after isolation of the cell line can be cultured only for a few passages. We have found equally high levels of p21 in such cells frozen 2 years ago. However, we have not yet been able to study the expression of p21 in relation to the hemopoietic biology of the cell. It is worth noting that the early 416B cells did not produce tumors when the cells were injected intravenously into syngeneic irradiated BDF_1 mice (8). Studies are in progress to assess, in normal and immunosuppressed mice, the tumorigenicity of the current 416B cells. Clearly, the exact relationship between the physiology of 416B cells and the expression of the endogenous p21 in 416B cells remains to be determined. Nevertheless, 416B cells are of obvious practical utility since they are a rich source of high levels of endogenous p21; purification of endogenous p21 and detailed comparison with the Ha-SV *src* p21 are now possible. We have no data yet on the messenger RNA for endogenous p21 in these cells, but these studies also should now be readily possible.

What is the mechanism by which the p21 has become elevated in 416B cells? The DNA blotting data indicate that the Ha-SV-related p21 gene(s) is not reiterated in 416B cells. DNA blotting data (Scolnick et al., unpublished data) have not yet indicated that the F-MuLV proviruses in 416B cells are integrated in close proximity to the p21 gene. Thus, F-MuLV proviral gene integration does not appear to have led to increased transcription of an adjacent p21 gene. Thus, there seem to be two broad possibilities to explain the enhanced expression of p21 in 416B cells. Either the p21 gene or the genes that control its expression are mutated in a way that has rendered the p21 gene more active, or the stage of hemopoietic development that 416B cells occupy favors at some molecular step elevated levels of p21. To test these hypotheses, it will be necessary to compare the methylation patterns of the p21 gene or to molecularly clone the p21 gene and its promotor sequences from 416B cells versus 427E cells and to compare them structurally and functionally. Whatever the mechanism which explains the elevated p21 expression, the results clearly indicate that the expression of an endogenous *sarc* gene can be markedly regulated. There have been no previous reports of the regulation of any endogenous viral *onc* gene product that is this marked. However, our results are somewhat reminiscent of the findings of Witte et al. (34), who detected a 150,000-dalton normal cellular protein (NCP 150) related to the Abelson virus p120 in mouse thymocytes but not in mouse fibroblasts. However, the level of the NCP 150 in the total population of thymocytes that they examined was 1/40 to 1/100 the level of the Abelson viral p120 in Abelson virus-infected cells. In our results, the levels of p21 in 416B cells were even higher than those in Ha-SV-infected cells.

The last issue that needs to be addressed is whether the p21 *sarc* is normally elevated in CFU-S stem cells at some stage in their normal biological cycle or hematological development. Unfortunately, 416B is the only cell line available to us with any CFU-S properties, and thus far a limited number of other kinds of hemopoietic cells have not expressed elevated levels of p21. In normal bone marrow, CFU-S cells represent a heterogeneous population which comprises approximately 0.1% of the total marrow cells (17, 18, 32, 35). Furthermore, pluripotent stem cells and precursor cells of more restricted potential can exist in a quiescent or dividing state (3, 16–18). Thus, analysis of the expression of p21 in normal bone marrow will require assays which detect p21 in single cells rather than in populations of cells. Because we have no data on p21 in single normal bone marrow cells, the current results clearly do not allow us to assign a role to the p21 in the normal physiology of early hemopoietic cells. However, we do feel justified in speculating that p21 expression may be regulated at some stage in the complex differentiation program of normal pluripotent or bipotent hemopoietic precursor cells. It is hoped that studies on the p21, the p21 gene(s) and its messenger RNA, and the cellular proteins that interact with p21 will be helpful in defining steps within the complex differentiation program of the CFU-S cells and in developing new assays for early pluripotent or bipotent hemopoietic cells.

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