

# The Nuclear Matrix Protein, Numatrin (B23), Is Associated with Growth Factor-induced Mitogenesis in Swiss 3T3 Fibroblasts and with T Lymphocyte Proliferation Stimulated by Lectins and Anti-T Cell Antigen Receptor Antibody

Nili Feuerstein, Sarah Spiegel,\* and James J. Mond

Department of Medicine, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814; and

\*Laboratory of Molecular and Cellular Neurobiology, National Institute of Neurological, Communicative Disorders, and Stroke, National Institutes of Health, Bethesda, Maryland 20892

**Abstract.** Numatrin is a tightly bound nuclear matrix protein (40 kD/pI-5) whose synthesis is markedly and promptly increased in association with cellular commitment for mitogenesis in B lymphocytes. (Feuerstein, N., and J. J. Mond. 1987. *J. Biol. Chem.* 262:11389-11397). To study whether this event is exclusively associated with proliferation of B lymphocytes, we examined the synthesis of numatrin in T lymphocytes (murine and human) activated by lectins or by anti-T cell antigen receptor monoclonal antibody and in Swiss 3T3 fibroblasts stimulated by growth factors. We showed a close correlation between induction of DNA synthesis and induction of numatrin synthesis in T lymphocytes stimulated by concanavalin A, anti-T cell antigen receptor monoclonal antibody, and IL-2 in murine T cells. Similar results were observed in Swiss 3T3 fibroblasts, thus only combinations of growth factors (insulin/EGF or insulin/B subunit of cholera toxin) or serum, which induced a significant increase in DNA synthesis, were also associated with a significant increase in synthesis of numatrin. Similar to B cells, the increase in numatrin synthesis in fibroblasts was found to occur at early G1 phase. The calcium ionophores, A23187 and ionomycin, previously shown to induce an increase in c-myc and c-fos mRNA levels in fibroblasts, induced a marked increase in the synthesis of a nuclear protein at 80 kD/pI-5 but failed to induce an increase in the synthesis of numatrin indi-

cating that an increase in intracellular  $Ca^{++}$  level is not sufficient for induction of the synthesis of numatrin. This further indicates that the increase in synthesis of numatrin may be more closely correlated with cellular commitment for mitogenesis as compared with other biochemical parameters. Using a polyclonal numatrin antibody we demonstrated that mitogen stimulation is also associated with a marked increase in numatrin abundance, which reached a peak at the onset of S phase and declined at the end of S phase. Evidence is presented to show that numatrin synthesis and abundance is elevated in various lymphoma cell lines. Using indirect immunofluorescence assays we showed that numatrin is abundant in other malignant cells: KB, epidermoid carcinoma, and Hep2 human hepatoma. Immunofluorescence studies further showed that mitogen stimulation of B lymphocytes induced a marked accumulation of numatrin in the nucleoli. This observation is in accord with the recent finding of identity of numatrin with the nucleolar protein B23 (Feuerstein et al. 1988. *J. Biol. Chem.* 263:10608-10612). Taken collectively these results suggest that numatrin/B23, a nucleolar phosphoprotein associated with the nuclear matrix, is a common nuclear protein associated with receptor mediated induction of mitogenesis in normal cells and with neoplastic growth in various cell types.

SEVERAL nuclear proteins including cyclin/PCNA (6), p53 (21), dividin (7), and proteins encoded by proto-oncogenes such as c-myc, c-myb, and c-fos (review reference 2), have been implicated in processes associated with the regulation of cellular growth. Recently, we have described and characterized a nuclear protein at 40 kD/pI-5

whose synthesis was found to be rapidly induced in murine B lymphocytes after stimulation with various mitogens (12). This protein was found to be tightly associated with the nuclear matrix and was termed 'numatrin.' Kinetic studies showed that the increase in the synthesis of numatrin was detected 60-120 min after mitogen activation, reached a peak

at 16 h, and declined to almost control level during S phase of the cell cycle. The increase in the synthesis of numatrin in B lymphocytes was found to be associated exclusively with cellular commitment for mitogenesis as activation of B cells with stimuli that did not stimulate DNA synthesis failed to induce an increase in the synthesis of numatrin. Elevated synthesis of numatrin was also detected in several malignant B lymphoma cells (12) and growth arrest of one of these cell lines (WEHI 231) by anti- $\mu$  was associated with specific inhibition of numatrin synthesis (13), suggesting that numatrin might have an important role in regulation of cellular mitogenesis in normal and neoplastic B lymphocytes.

In the present study we further characterized the synthesis as well as the abundance of numatrin in several normal and malignant cellular systems. We provide evidence to suggest that numatrin is a ubiquitous protein whose synthesis and abundance are closely associated with induction of mitogenesis and with neoplastic growth in various cell types.

## Materials and Methods

### Materials

Monoclonal antibody to T cell antigen receptors (anti-TcR mAb)<sup>1</sup> was kindly provided by Dr. Jeffrey Bluestone, the National Institutes of Health, Bethesda, MD (18). The B subunit of cholera toxin was purchased from Schwarz/Mann (Spring Valley, NY). Epidermal growth factor (EGF) and insulin were obtained from Collaborative Research (Lexington, MA). Calcium ionophore A23187, 8-mercaptoguanosine, phytohemagglutinin (PHA), and concanavalin A (Con A) were purchased from Sigma Chemical Co. (St. Louis, MO). [<sup>35</sup>S]methionine (1100 Ci/mmol) and [<sup>3</sup>H]thymidine (59 Ci/mmol) were purchased from DuPont Co. (Wilmington, DE) and New England Nuclear (Boston, MA).

### Lymphoma Cell Lines

HL-60 human promyelocytic leukemia cells, U937 human histiocytic lymphoma, P388D1 murine macrophage-like lymphoma, Raji human B lymphoma, EL-4 murine T lymphoma, Molt-4 human T lymphoma, and IM-9 human B lymphoma were obtained from American Type Culture Collection (Rockville, MD) and grown in RPMI-1640 supplemented with 10% FBS.

### Purification and Culture of Murine Splenic T and B Lymphocytes

Murine splenic T cells were purified as previously described (17). Briefly, splenic cells were incubated on culture dishes that had been coated with anti-K (50  $\mu$ g/ml), and after 1 h the nonadherent cells were collected and plated on fresh anti-K-coated plates for another hour. The nonadherent cells prepared in this way contained <5% surface immunoglobulin-positive cells and were used as a source of T cells. B cells were purified from murine spleen as previously described (12). Briefly, spleens were teased apart in a petri dish and the spleen cells were treated at 4°C for 45 min with a mixture of monoclonal anti-T cell antibodies, followed by incubation for 45 min at 37°C with rabbit complement. The B cells obtained by this method contained <1% T cells. T or B lymphocytes were suspended in modified Mishell Dutton medium ( $2 \times 10^6$  cells/ml) and incubated with various reagents as specified in Results.

### Purification and Culture of Peripheral Blood T Lymphocytes

Human peripheral blood T lymphocytes were prepared from blood of human healthy donors as previously described (24) and further purified by rosetting with sheep red blood cells (24). T cells were further incubated at

$2 \times 10^6$  cells/ml in RPMI-1640 supplemented with 10% autologous serum.

### Analysis of DNA Synthesis in T Lymphocytes

For evaluation of T cell DNA synthesis,  $1 \times 10^5$  cells/well were cultured in flat-bottomed microtiter plates in a total volume of 0.2 ml in modified Mishell Dutton medium that contained 10% endotoxin-free FBS (HyClone Laboratories, Logan, UT). Experimental samples performed in triplicate were incubated in a humidified 5% CO<sub>2</sub> incubator. Cultures were pulsed with 1.0  $\mu$ Ci [<sup>3</sup>H]thymidine and incubated for 8 h. Cells were harvested onto glass fiber filter strips by a semiautomated cell harvester (PHD cell harvester; Cambridge Technology, Inc., Cambridge, MA), and the incorporation of [<sup>3</sup>H]thymidine into DNA was measured by liquid scintillation counting (12).

### Radiolabeling of Cellular Proteins in Lymphocytes

Radiolabeling was done as described (10). T lymphocytes were washed in phosphate saline buffer and resuspended ( $5 \times 10^6$  cells/ml) in methionine-free RPMI supplemented with 5% dialyzed FCS (Gibco Laboratories, Grand Island, NY) and [<sup>35</sup>S]methionine (80–100  $\mu$ Ci/ml) for 4 h at 37°C.

### Culture and Radiolabeling of Swiss 3T3 Fibroblasts

Swiss 3T3 cells are from the American Type Culture Collection. Stock cultures of these cells were maintained in low glucose DME, supplemented with 2 mM glutamate, 2 mM pyruvate, penicillin (100  $\mu$ g/ml), streptomycin (100  $\mu$ g/ml), and 10% FBS in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C (23). For maintaining quiescent cell cultures, 3T3 cells were subcultured into 35-mm dishes at a density of  $1.5 \times 10^5$  cells/well with 3 ml of the above medium and refed with the same medium after 2 d. Such cultures were used at least 5 d after the last change of medium when the cells were confluent and quiescent (9). The quiescent cultures were washed twice with low glucose DME to remove residual serum, and 2 ml of DME-Waymouth's (1:1; vol/vol) supplemented with transferrin (5  $\mu$ g/ml) and BSA (20  $\mu$ g/ml) was added along with various growth factors as specified in the Results section. After various periods of time as specified, the cells were radiolabeled with [<sup>35</sup>S]methionine in methionine-free RPMI-1640 (200  $\mu$ Ci/well in 1.5 ml for 2 h) with or without various growth factors as indicated. Parallel cultures were pulsed with [<sup>3</sup>H]thymidine for measurement of DNA synthesis.

### Measurement of DNA Synthesis in Quiescent Swiss/3T3 Cells

DNA synthesis was analyzed as described (23). Quiescent cultures (prepared as described above) were pulsed with 0.5  $\mu$ Ci of [<sup>3</sup>H]thymidine for 4 or 2 h (as specified) and then washed twice with 2 ml of ice-cold PBS and twice with ice-cold 5% TCA. The insoluble material was dissolved in 0.5 ml of 0.25 M NaOH, which was transferred to glass scintillation vials containing 10 ml of Ready Solv HP (Beckman Instruments, Inc., Palo Alto, CA) and analyzed for radioactivity.

### Isolation of Nuclei

Nuclei were isolated as previously described (3, 12). Cells ( $10^7$ ) were washed in PBS (pH 7.2) and resuspended in 5 ml of buffer containing 10 mM Tris-HCl (pH 8.0), 3 mM CaCl<sub>2</sub>, 0.25 M sucrose, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF). The cells were disrupted by gentle homogenization and the lysates were centrifuged at 1,000 g for 5 min and further washed (twice) with nuclear buffer supplemented with 0.5% NP-40. Isolation of pure nuclei was confirmed by phase microscopic examination.

### Two-Dimensional Gel Electrophoresis

Whole nuclei were suspended in urea isoelectric focusing lysis solution (9.5 M urea, 2% NP-40, 5% 2-mercaptoethanol, 2% LKB ampholites pH 3.5–10.0). After vigorous vortexing the insoluble material was removed by centrifugation and the sample was subjected to two-dimensional gel electrophoresis, staining, and radiofluorography as described (11, 19).

### Measurement of [<sup>35</sup>S]Methionine in Specific Proteins in Two-Dimensional Gels

Measurement of radioactivity in proteins in the gels was done as described

1. *Abbreviations used in this paper:* anti-TcR mAb, anti-T cell antigen receptor monoclonal antibody; Con A, concanavalin A; EGF, epidermal growth factor; PHA, phytohemagglutinin.

(4). In brief, proteins were located by radioautography and were surrounded by a radiodense mask of x-ray film to exclude radioactivity from proximate gel areas. Radioactivity was measured directly with an end window Geiger-Muller tube (model 44-7; Ludlum Measurements, Inc., Sweetwater, TX) coupled to a scaler/ratemeter (model 2200). Background activity was determined by counting regions of the gel of comparable area which were free of labeled proteins.

### Production of Anti-Numatrin Antibody

Partially purified numatrin from HL-60 cells (14) or numatrin of HL-60 cells resolved by SDS-PAGE was emulsified in 1 ml of Freund Complete Adjuvant and injected subcutaneously into two rabbits. After 2 wk the rabbits were boosted with a similar amount of numatrin (~100 µg) emulsified in Freund's complete adjuvant. After four booster shots the serum tested positive in ELISA and was shown to interact specifically with numatrin in immunoblot analysis.

### Indirect Immunofluorescence Assays

Cells were cytocentrifuged onto slides and fixed for 15 min at room temperature with 2% paraformaldehyde in PBS. The cells were further permeabilized with acetone for 3 min at -20°C. After washing, the slides were incubated at 37°C (in the incubator) with anti-numatrin antibody 303 (1:150 dilution) for 60 min. The slides were extensively washed with PBS and incubated with fluorescein-conjugated affinity-purified goat anti-rabbit IgG (100 µg/ml) for 60 min at 37°C. The slides were then washed extensively (four times for 15 min) and viewed under fluorescence microscope.

### Immunoblotting Assays (Western)

Assays were done with minor modification of a previously described technique (29). Nuclear proteins were analyzed on SDS-PAGE and electrotransferred onto nitrocellulose membranes at 80 V for 90 min at room temperature. The nitrocellulose membranes were then soaked overnight in Tris saline buffer (10 mM Tris, pH 7.5, 0.150 M NaCl) containing 3% BSA and 10% FCS. The membranes were further incubated for 2 h with anti-numatrin antibody 303 (1:150 dilution) in Tris saline buffer containing 5 mM EDTA, 0.25% gelatin, and 0.05% NP-40 for 2 h at room temperature. Detection of immunoreactive bands was done by incubation with <sup>125</sup>I-labeled protein A (0.1 µCi/ml in the same medium used for antibody incubation). The membranes were washed extensively in buffer containing 50 mM Tris, pH 7.5, 5 mM EDTA, 1 M NaCl, 0.25% gelatin, and 0.8% sarcosyl for 2 h, dried, and exposed to autoradiography.

## Results

### Induction of Proliferation in T Lymphocytes by Con A, IL2, or Anti-TcR mAb Is Associated with Induction of the Synthesis of Numatrin

To examine whether induction of mitogenesis in T lymphocytes is associated with induction of the synthesis of numatrin, murine splenic T cells were incubated with Con A (2 µg/ml), IL2 (100 µ/ml), or anti-TcR mAb (10 µg/ml) and examined for DNA synthesis and the synthesis of numatrin. Table I shows that Con A and anti-TcR mAb caused a 55- and 23-fold increase in DNA synthesis, respectively. IL2 had only a minor effect on DNA synthesis, but it synergized with anti-TcR mAb to induce a 57-fold increase in DNA synthesis. Parallel cultures were set up to examine the effect of these mitogens on the synthesis of numatrin; cells were incubated with the ligands for 16 h and then radiolabeled. The nuclear proteins were extracted and analyzed by two-dimensional gel electrophoresis. Fig. 1 and Table I demonstrate the effect of various ligands on the synthesis of numatrin. Con A and anti-TcR mAb, which were potent mitogens, caused a 12.9- and 9.6-fold increase in the synthesis of numatrin, respectively, while IL2 alone which had only a small mitogenic effect, had also a relatively small effect on the synthesis of

**Table I. Effects of IL-2, Anti-TcR, and Con A on the Synthesis of Numatrin and of DNA in Murine T Lymphocytes**

Treatment	Numatrin synthesis		DNA synthesis
	cpm	E/C	cpm
None	41	—	948
Con A	530	12.9	51,895
IL-2	82	2.0	4,345
Anti-TcR	395	9.6	21,464
Anti-TcR + IL-2	544	13.3	54,470

Murine splenic T lymphocytes were purified as described and incubated with Con A (2 µg/ml), IL-2 (100 µ/ml), or anti-TcR (10 µg/ml). [<sup>3</sup>H]thymidine incorporation was measured 48 h after onset of the cultures. The results represent means of triplicate wells with SEM of <10%. Synthesis of numatrin (in cells incubated with the ligands for 16 h) was measured directly from the gels (see Fig. 1). Data are also expressed as E/C (cpm in experimental conditions/cpm in control cells).

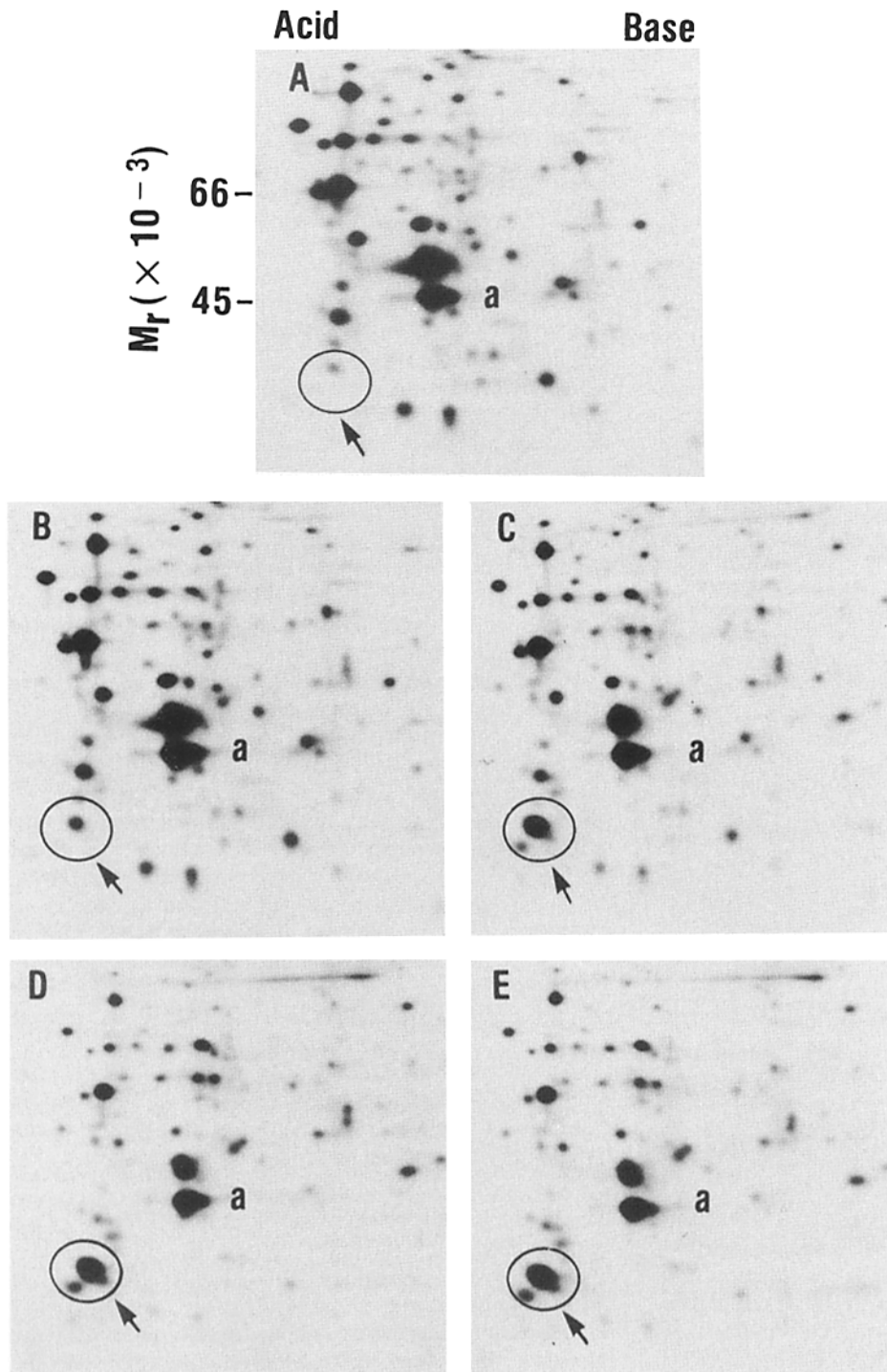
numatrin. However, similar to its effect in enhancing DNA synthesis, IL-2 synergized with anti-TcR mAb to further enhance the synthesis of numatrin, indicating a close correlation between the induction of the synthesis of numatrin and the induction of mitogenesis in murine T lymphocytes.

We further sought to examine whether a similar event could be found in human lymphocytes. PHA (a known T lymphocyte mitogen) stimulated an increase in the synthesis of numatrin in purified population of human peripheral blood T lymphocytes (Fig. 2) as well as an increase in DNA synthesis (data not shown), indicating that an increase in the synthesis of this nuclear protein is also associated with the mitogenic activation of human T cells. It should be noted that the double spots that are associated with numatrin are in accordance with the finding of its identity with B23 (14) which is composed of two major subunits; α (the more abundant and slightly higher molecular weight form) and β subunit (29). Both subunits α and β can be detected by the specific anti-numatrin antibody in two-dimensional gel electrophoresis (data not shown). (The lower molecular weight basic spot, which is occasionally detected in close proximity in other figures, might be a degradation product or posttranslational modification of one of the protein subunits.)

### Kinetics of Numatrin Abundance in Mitogen-stimulated Lymphocytes

In previous studies we have found that the incorporation of [<sup>35</sup>S]methionine into numatrin is rapidly induced at early G1 phase after mitogen stimulation of B cells but is markedly reduced during S phase of the cell cycle. To further study the kinetics of abundance of numatrin during various periods of the cell cycle, we employed immunoblotting technique using specific antibody to numatrin (antibody 303).

Murine splenic T and B cells were stimulated with mitogens for various periods of times. Nuclear proteins (equal amount of proteins) were analyzed by SDS-PAGE, electrotransferred onto nitrocellulose membranes, and immunoblotted with numatrin antibody 303. Fig. 3 demonstrates that mitogen stimulation induced an increase in numatrin abundance that was detected at early G1 phase, reached a plateau at the onset of S phase, and remained elevated during S phase. As shown in Fig. 3, A and B, the amount of numatrin declined toward the end of S phase and was completely unde-



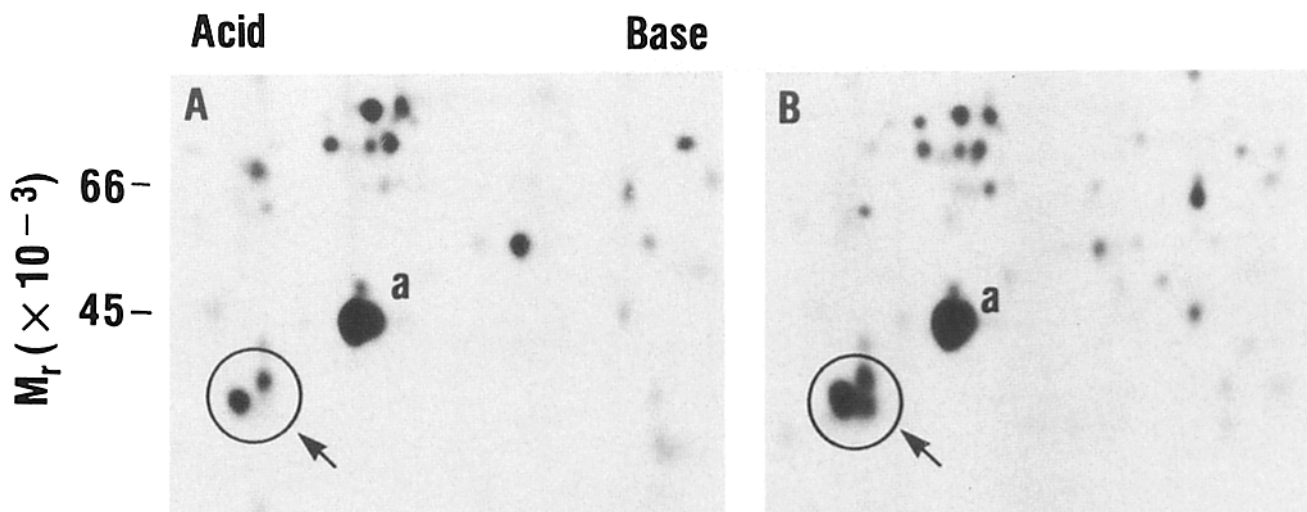
**Figure 1.** Effects of Con A, IL-2, and anti-TcR mAb on synthesis of numatrin in murine T lymphocytes. Murine splenic T lymphocytes were purified as described and incubated with Con A (2  $\mu\text{g}/\text{ml}$ ), IL-2 (100  $\mu\text{g}/\text{ml}$ ), or anti-TcR (10  $\mu\text{g}/\text{ml}$ ), singly or in combination, for 16 h. The cells were then labeled with [ $^{35}\text{S}$ ]methionine (100  $\mu\text{Ci}/\text{ml}$ ) for 4 h, nuclei were purified, and the nuclear proteins were extracted. Equal amounts of cpm of nuclear proteins were analyzed by two-dimensional gel electrophoresis. *A*, control; *B*, IL-2; *C*, anti-TcR; *D*, IL-2 + anti-TcR; and *E*, Con A. *a*, actin. Circles and arrows indicate numatrin.

tectable in B lymphocytes after 92 h incubation with the mitogen.

#### ***Evidence for Elevated Synthesis and Abundance of Numatrin in Various Lymphoma Cells***

We have previously found that numatrin synthesis is elevated in B lymphoma cells (12). The finding that numatrin synthesis is also induced in proliferating T lymphocytes led us to

examine the synthesis of numatrin in other lymphoma cell types (Fig. 4). The synthesis of numatrin was measured by the amount of radioactivity in numatrin relative to that in actin or relative to the radioactivity in a standard sample of proteins which included  $\sim 100$  nuclear proteins as shown in Table II. The synthesis of numatrin was found to be elevated in all the lymphoma cell lines (as compared to normal resting B or T lymphocytes). Notably, there was a considerable vari-



**Figure 2.** Induction of numatrin synthesis in human peripheral blood T lymphocytes stimulated by PHA. Human T cells were purified from peripheral blood as described and incubated with PHA (2  $\mu\text{g/ml}$ ) for 24 h. Thereafter, the cells were labeled with [ $^{35}\text{S}$ ]methionine, nuclei were isolated, and the nuclear proteins were analyzed by two-dimensional gel electrophoresis. *A*, control cells; and *B*, PHA-treated cells. *a*, actin. Circles and arrows indicate numatrin.

ability in numatrin synthesis among those cells which apparently is not related to the cellular origin of the tumor cells, namely whether the cells are B or T lymphoma or have a myeloid origin. Numatrin was found to be most rapidly synthesized in HL-60 promyelocytic leukemia cells as shown by quantitation of radioactivity (Table II). It was also found to be markedly abundant in HL-60 cells as demonstrated by Coomassie Blue staining of the gels (Fig. 5).

Immunoblot analysis (Fig. 6) further showed that the amount of numatrin is elevated in several leukemic cells (HL-60, Raji, and Molt-4) as compared to normal resting lymphocytes, indicating that not only the synthesis but also the amount of numatrin is enhanced in these malignant cells.

#### **Induction of DNA Synthesis in Swiss 3T3 Fibroblasts by Growth Factors is Associated with an Increase in the Synthesis of Numatrin**

Further studies were designed to examine whether induction of numatrin synthesis is also associated with mitogenesis in cell types of nonlymphoid origin. To this end we examined DNA synthesis and the synthesis of numatrin in quiescent cultures of Swiss 3T3 fibroblasts activated by growth factors.

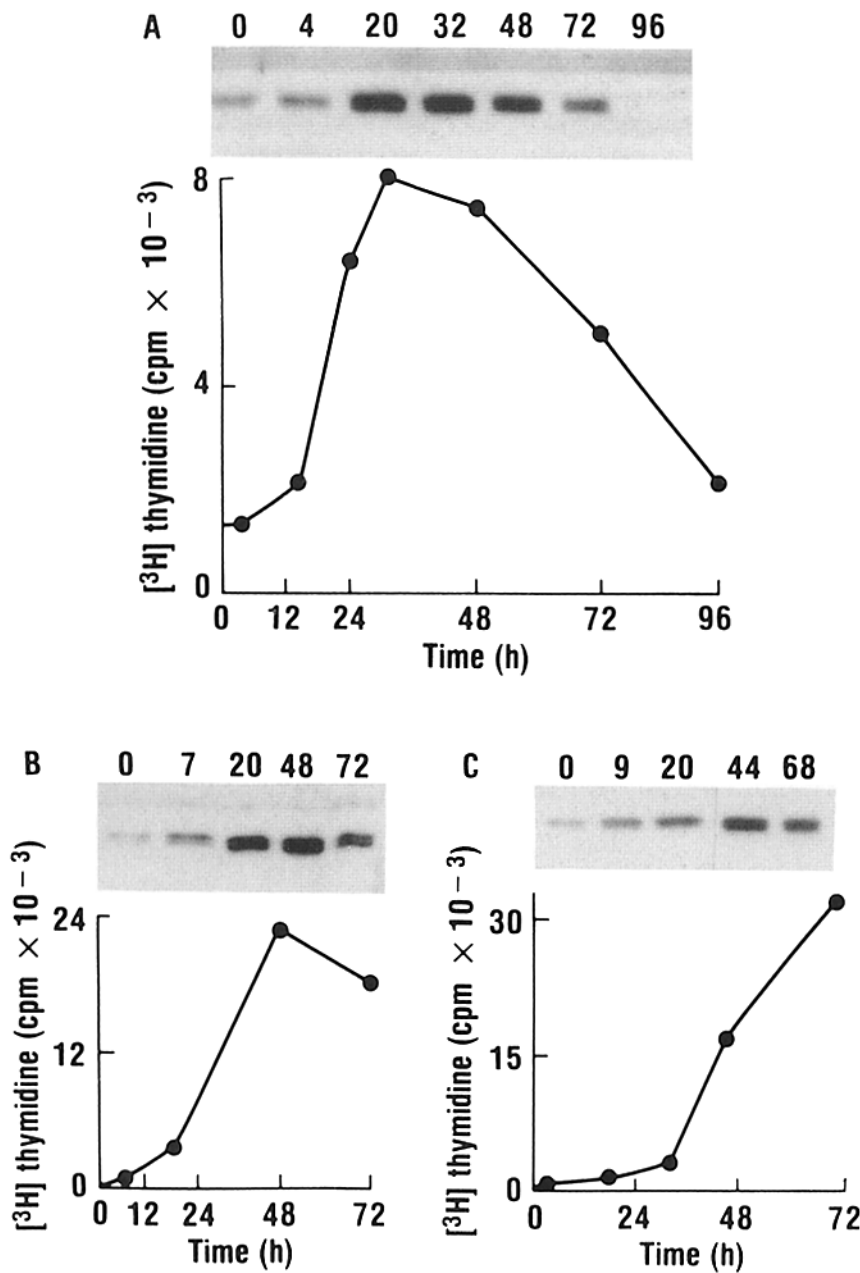
Quiescent Swiss 3T3 cells were stimulated with EGF, insulin, B subunit of cholera toxin, or serum (singly or in combination), and 4 h later the cells were labeled with [ $^{35}\text{S}$ ]methionine and the nuclear proteins were extracted and analyzed by two-dimensional gel electrophoresis. DNA synthesis was analyzed in parallel cultures 20 h after addition of the growth factors. In agreement with previous studies (review references 22, 23; Spiegel, S., and L. Panagiotopoulos, manuscript submitted for publication) EGF, insulin, and the B subunit of cholera toxin when added alone to quiescent 3T3 fibroblasts had only a small effect on DNA synthesis (Table III), but combinations of insulin and EGF or insulin and the B subunit of cholera toxin induced a 43- and 16-fold increase in DNA synthesis, respectively. Serum alone

caused a 45-fold increase in DNA synthesis, and this effect was further potentiated by the B subunit of cholera toxin. Fig. 7 (and quantitation of these results in Table II) show the effect of these growth factors on the synthesis of numatrin. EGF, insulin or the B subunit of cholera toxin alone, which had only a small effect on DNA synthesis, caused only 43, 36, and 26% increases in the synthesis of numatrin, respectively. However, serum or a combination of insulin and EGF or insulin and the B subunit of cholera toxin, which had a large effect on DNA synthesis, induced a 220, 150, or 180% increase in the synthesis of numatrin, respectively. The combination of serum and the B subunit of cholera toxin caused the largest increase in the synthesis of numatrin, 330% (4.3-fold).

It should be noted that in 3T3 cells we did not detect the two subunits  $\alpha$  and  $\beta$  of numatrin/B23 (29). Conceivably, only the more abundant subunit ( $\alpha$ ) can be detected. Since lower amounts of cpm were analyzed on the gels, the amounts of the  $\beta$  subunit probably were below the level of detectability. The possibility that  $\beta$  subunit is not found in these cells is very unlikely, but cannot be excluded by this experiment.

#### **Kinetics of Numatrin Synthesis in Quiescent 3T3 Fibroblast Stimulated by Mitogens**

To study the kinetics of the change in the synthesis of numatrin, quiescent 3T3 cells were incubated with serum and the B subunit of cholera toxin (a combination which resulted in the largest increase in DNA synthesis and numatrin synthesis as shown in Table III) for 2, 4, 8, 16, 24, and 35 h. 2 h before the end of the incubation period the cells were labeled with [ $^{35}\text{S}$ ]methionine (for 2 h) and equal cpm of nuclear proteins were further analyzed by two-dimensional gel electrophoresis. Parallel cell cultures were set up to determine the kinetics of the change in [ $^3\text{H}$ ]thymidine incorporation. The increase in synthesis of numatrin was detected 2 h after ex-



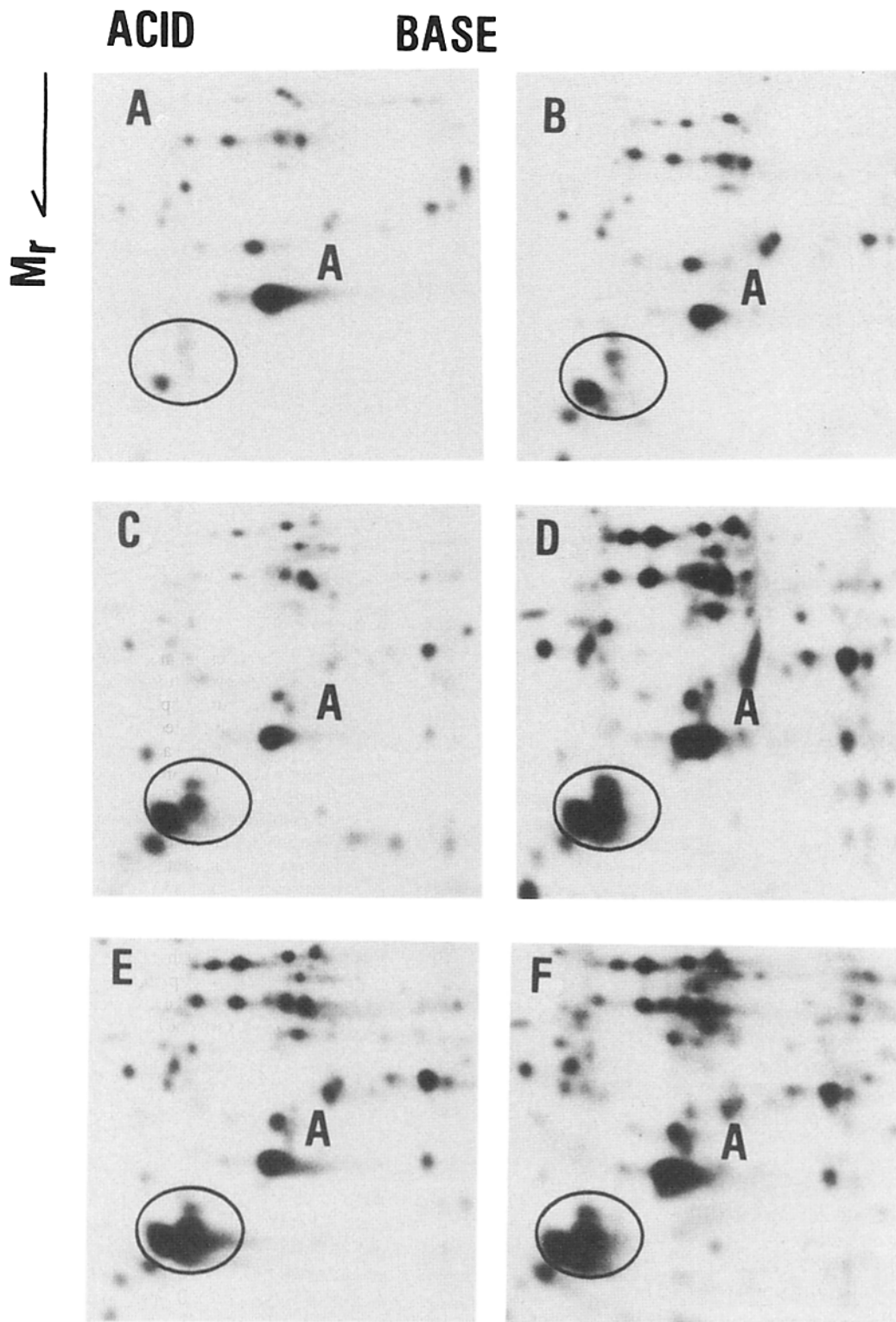
**Figure 3.** Kinetics of numatrin abundance during the cell cycle in mitogen-stimulated lymphocytes. Murine splenic T and B lymphocytes were stimulated with mitogens for various periods of time and then nuclei were purified, and nuclear proteins (20 µg) were analyzed by SDS-PAGE. The proteins were electrotransferred onto nitrocellulose membranes and immunoblotted with antibody 303 as described in Materials and Methods. Immunoreactive bands were detected by incubation with <sup>125</sup>I-protein A followed by autoradiography. Parallel cultures were set up for assay of [<sup>3</sup>H]thymidine incorporation (in A, cells were pulsed for 4 h and in B and C for 8 h). (A) B cells stimulated with anti-Ig (10 µg/ml) and 8-mercaptopguanosine (0.5 mM). (B) B cells stimulated with anti-Ig (conjugated to dextran). (C) T cells stimulated with Con A (2 µg/ml).

posure of the cells to the mitogens and reached a peak at 8 h (Fig. 8). The increase in [<sup>3</sup>H]thymidine incorporation was detected at 16 h and reached a peak at 24 h. These results indicate that the increase in numatrin synthesis in quiescent fibroblasts that are stimulated to proliferate is an early event that precedes the entry into S phase of the cell cycle. This conclusion is in accord with previous kinetic studies in B lymphocytes (12).

#### **Effect of Calcium Ionophores on Synthesis of Numatrin in 3T3 Fibroblasts**

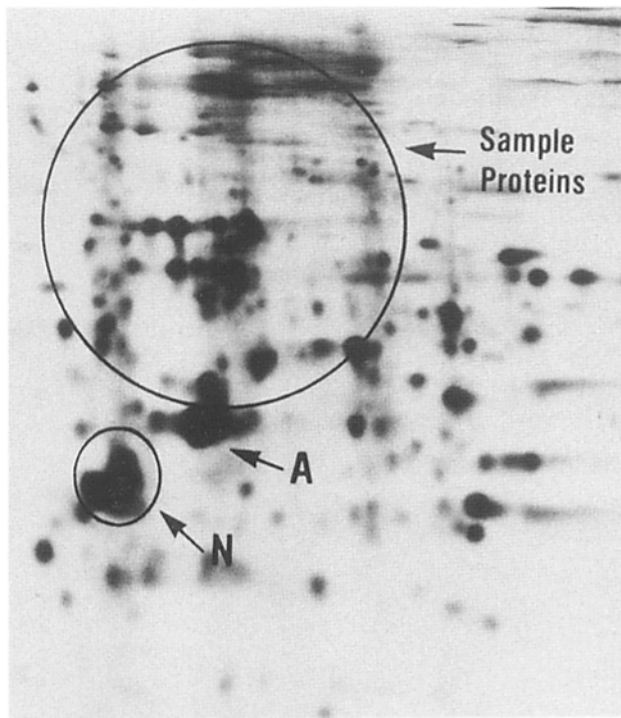
The calcium ionophores A23187 and ionomycin have been previously shown to stimulate an increase in expression of c-fos and c-myc in Swiss 3T3 fibroblasts (25-27). It was therefore important to examine whether activation of these

cells by calcium ionophores also induces an increase in the synthesis of numatrin. To this end we incubated quiescent Swiss 3T3 cells with A23187 or with ionomycin for 4 and 8 h at concentrations previously shown to induce an increase in c-myc and c-fos (A23187, 0.5 and 1.0 µM; ionomycin 1 µM) and then labeled the cells with [<sup>35</sup>S]methionine for 90 min. (Viability as measured by exclusion of trypan blue was >95%, except for cells incubated with ionomycin for 8 h which had 90% viability.) While both calcium ionophores failed to induce an increase in expression of numatrin, they induced a marked increase in expression of one nuclear protein at 80 kD/pI-5 (Fig. 9). The synthesis of this protein was increased in a dose dependent manner by A23187 after 4 h incubation and was further enhanced after 8 h, indicating that the synthesis of certain discrete nuclear proteins such as



**Figure 4.** Synthesis of numatrin in various lymphoma cells. Lymphoma cell lines and murine splenic B lymphocytes were labeled with [<sup>35</sup>S]methionine (100 μCi/ml for 4 h). Nuclear proteins were extracted and analyzed (~500,000 cpm) by two-dimensional gel electrophoresis. *A*, resting B lymphocytes; *B*, LPS-stimulated (50 μg/ml, 16 h) B lymphocytes; *C*, Raji cells; *D*, Molt-4 cells; *E*, HL-60 cells; and *F*, MI-9 cells.

Table II. Comparison of the Relative Synthesis of Numatrin in Various Lymphoma Cell Lines



Cells	Origin	N/A	N/Sample proteins
HL-60	human, promyelocytic leukemia	3.08	0.52
Raji	human, B lymphoma	1.90	0.49
MOLT-4	human, T lymphoma	1.54	0.40
IM-9	human, B lymphoma	0.95	0.29
EL-4	murine, T lymphoma	1.34	0.20
U-937	human, histiocytic lymphoma	0.40	0.18
P388D1	murine, macrophage-like lymphoma	1.20	n.t.
B lymphocytes (resting):	murine, normal cells	0.07	0.05
B lymphocytes (LPS-activated):	murine, normal cells	0.83	0.23

Numatrin was identified by autoradiography of gels that were obtained as described in Fig. 3 (for T lymphocytes as described in Fig. 1). In each gel the radioactivity of numatrin as well as that of actin and a standard sample of proteins (as shown above) was measured directly from the gels. The level of numatrin in each cell type is presented as the ratio of cpm in numatrin relative to actin (*N/A*) or relative to the sample proteins shown above (*N/sample proteins*).

p80/pI-5 is specifically regulated by an increase in the intracellular level of calcium, but these conditions are not sufficient for induction in the synthesis of numatrin.

#### ***Intranuclear Localization of Numatrin by Indirect Immunofluorescence in Various Cell Types***

Indirect immunofluorescence assays using anti-numatrin antibody 303 (Fig. 10) showed that stimulation of B lymphocytes with mitogens for 16 h (late G1 phase) was associated with a marked accumulation of numatrin in the nucleoli. Distinct nucleolar staining was also obtained in HL-60 cells (not shown) and in nonlymphoid malignant cells, such as KB human epidermoid carcinoma and Hep-2 human hepatoma cell lines (Fig. 10), indicating the ubiquitous nature of numatrin. These results are in accord with the recent discovery that numatrin is identical to the nucleolar protein B23 (14).

#### ***Discussion***

We have previously shown that induction of the synthesis of the nuclear matrix protein, numatrin, is an event that occurs at early G1 phase of the cell cycle, and that it is associated with cellular commitment for mitogenesis in B lymphocytes (12, 13). In the present study we show that the induction of numatrin is also associated with induction of mitogenesis in human and murine T lymphocytes activated by lectins or by anti-TcR mAb, as well as in nonlymphoid cells, quiescent Swiss 3T3 fibroblasts stimulated by growth factors. These data suggest that the involvement of numatrin in mitogenesis is not unique to B cells but is rather a common event that may be associated with induction of cellular mitogenesis.

Comparison of the effects of IL-2, lectin, and anti-TcR mAb on the synthesis of numatrin and that of DNA showed



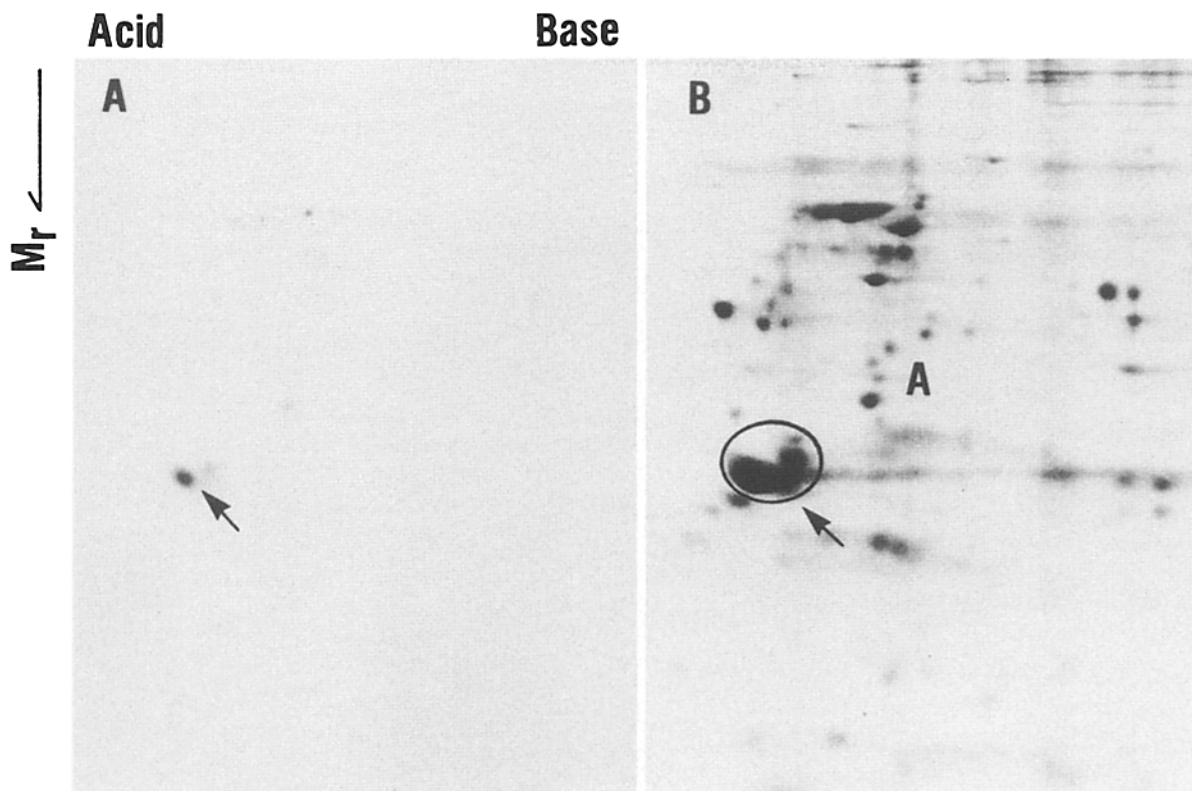


Figure 5. Abundance of numatrin in HL-60 promyelocytic leukemic cells (Coomassie Blue). Nuclear proteins were isolated from HL-60 cells at their logarithmic growth and analyzed by two-dimensional gel electrophoresis. Shown are Coomassie Blue-stained gels. A,  $\sim 15 \mu\text{g}$  nuclear proteins; and B,  $\sim 100 \mu\text{g}$  nuclear proteins of HL-60 cells.

that there is a close correlation between these two parameters. Thus, Con A and anti-TcR mAb caused a marked increase in the synthesis of numatrin which was followed by a prominent increase in DNA synthesis after 48 h. Conversely, IL-2 which by itself had only a small effect on the synthesis of numatrin, also failed to induce a significant effect on DNA synthesis. However, when combined with anti-TcR mAb, it caused a synergistic effect on the synthesis

of numatrin which was followed by a synergistic effect on DNA synthesis.

Similar results were observed in quiescent Swiss 3T3 fibroblasts activated by growth factors. Thus, combinations of growth factors (insulin/EGF and insulin/B subunit of cholera toxin) or serum which induced a significant increase

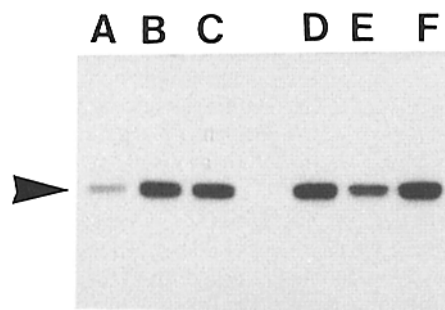
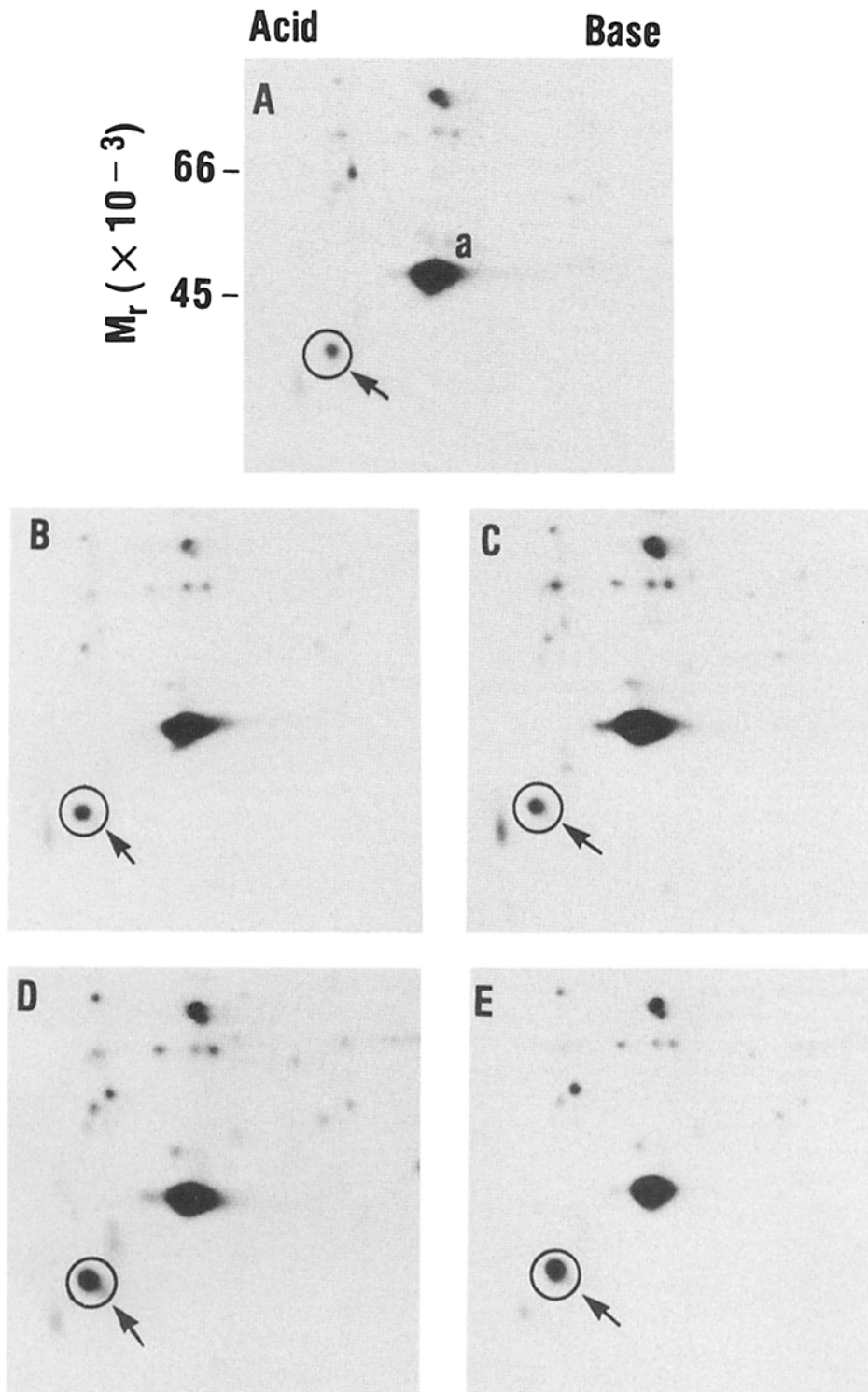


Figure 6. Elevated amounts of numatrin in leukemic cells. Nuclear cell extracts ( $20 \mu\text{g}$ ) were analyzed by SDS-PAGE. After electrotransfer, the nitrocellulose membrane was immunoblotted with antibody 339 as described in Materials and Methods. A, resting B lymphocytes; B, anti-Ig/dextran-stimulated murine B lymphocytes (24 h); C, Con A-stimulated murine T lymphocytes (48 h); D, Raji cells; E, MOLT-4; and F, HL-60 cells.

Table III. Effects of Growth Factors and Serum on the Synthesis of Numatrin and of DNA in Swiss 3T3 Fibroblasts

Treatment	Numatrin synthesis		DNA synthesis
	cpm	% increase	cpm
None	300	—	14,350
EGF	430	43	51,592
Insulin	410	36	26,383
B subunit	380	26	30,736
Insulin + EGF	750	150	613,785
Insulin + B subunit	850	180	230,140
FCS	960	220	414,982
FCS + B subunit	1,290	330	643,173

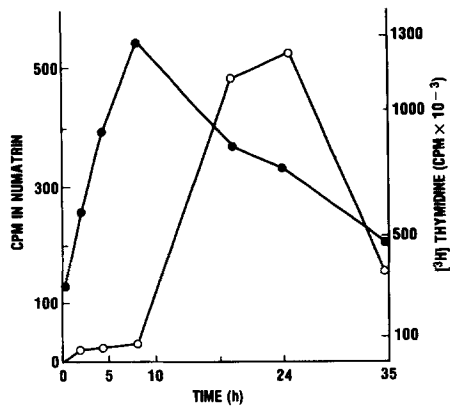
Quiescent Swiss 3T3 cells were incubated with EGF (5 ng/ml), insulin (2  $\mu\text{g}/\text{ml}$ ), B subunit of cholera toxin (1  $\mu\text{g}/\text{ml}$ ), and FCS (10%) for 4 h and then labeled with [ $^{35}\text{S}$ ]methionine (150  $\mu\text{Ci}/\text{ml}$ ) for 2 h. Thereafter, nuclei were isolated and equal amounts of cpm of nuclei proteins were analyzed by two-dimensional gel electrophoresis (see Fig. 5). Numatrin was identified and its radioactivity was measured directly in the gels as described. DNA synthesis was analyzed in parallel cultures by incorporation of [ $^3\text{H}$ ]thymidine (0.5  $\mu\text{Ci}/\text{ml}$  for 4 h) 20 h after addition of the growth factors. Results represent means of triplicate cultures with SEM  $<10\%$ .



**Figure 7.** The effect of growth factors (insulin and EGF) on the synthesis of numatrin in murine Swiss 3T3 fibroblasts. Quiescent Swiss 3T3 fibroblasts were stimulated with serum (10%), EGF (5 ng/ml), or insulin (2  $\mu$ g/ml) singly or in combination for 4 h and then labeled with [ $^{35}$ S]methionine (150  $\mu$ Ci/ml for 2 h). Thereafter, nuclei were isolated and equal amounts of cpm of the nuclear proteins were analyzed by two-dimensional gel electrophoresis. *A*, nontreated cells; *B*, EGF; *C*, insulin; *D*, EGF + insulin; and *E*, FCS.

in DNA synthesis were also associated with a larger increase in numatrin synthesis. However, it should be noted that the magnitude of the increase in the synthesis of numatrin by growth factors in fibroblasts was reproducibly lower (2.5–4.3-fold) when compared to T or B lymphocytes (5–20-fold increase). This difference might be due to a higher basal level of numatrin in the nonactivated Swiss 3T3 cells, which might result from the fact that Swiss 3T3 cells are an immortalized cell line while the lymphocytes are normal resting cells.

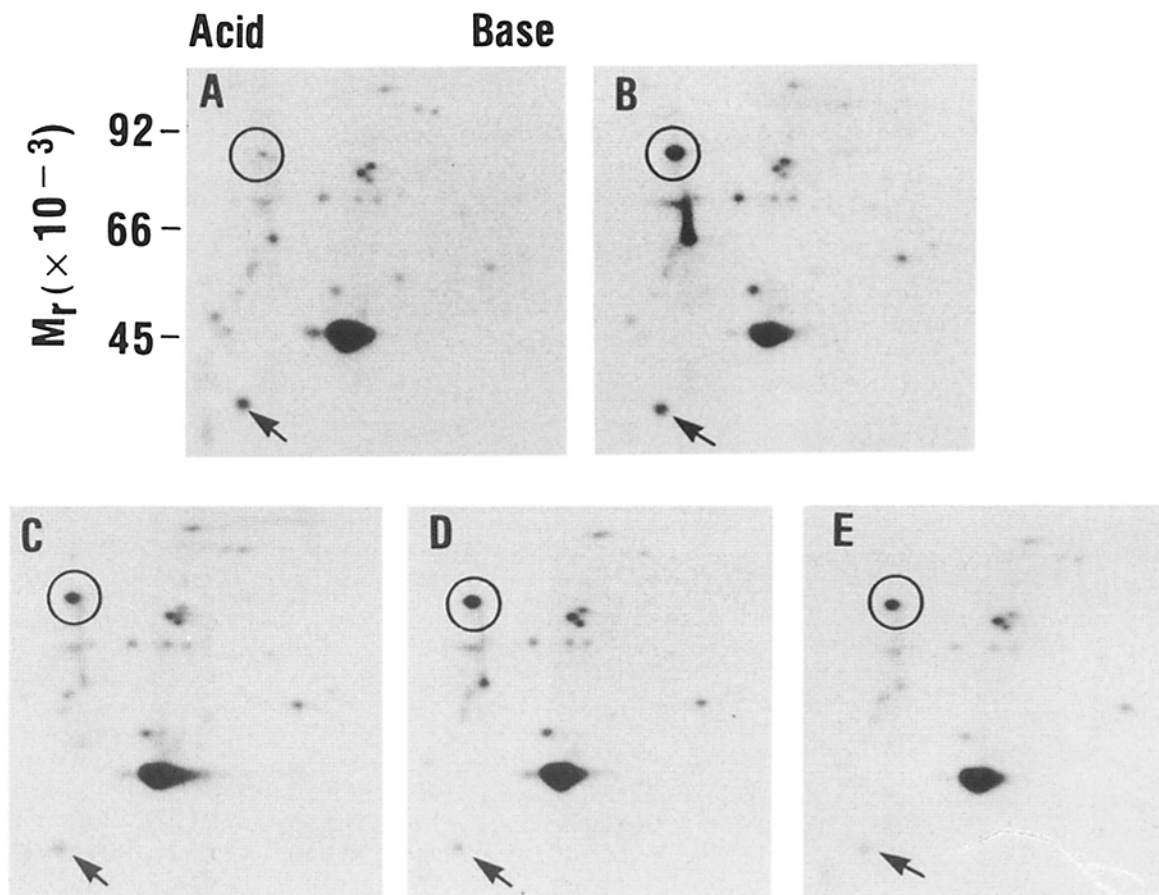
An important finding was the observation that the calcium ionophores A23187 and ionomycin, which have been previously shown to induce an increase in *c-myc* and *c-fos* mRNA level in Swiss 3T3 cells (25–27), failed to induce the synthesis of numatrin. These experiments further demonstrated that A23187 and ionomycin induced a marked and specific increase in the synthesis of a discrete nuclear protein at 80 kD/pI-5. Hence, these 3T3 cells activated by calcium ionophores are specifically stimulated to increase the expression



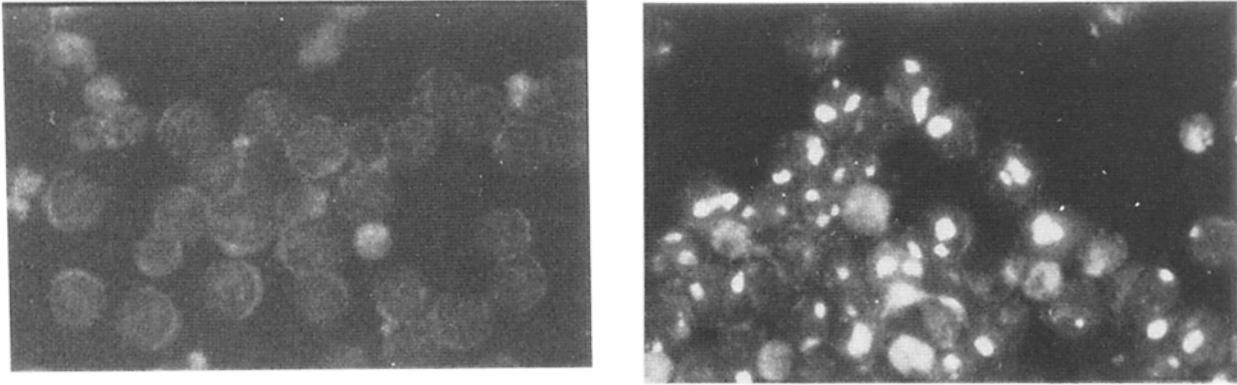
**Figure 8.** Kinetics of the synthesis of numatrin and of DNA in quiescent Swiss 3T3 fibroblasts stimulated by serum and the B subunit of cholera toxin. Quiescent Swiss 3T3 fibroblasts were incubated with FCS (10%) and the B subunit of cholera toxin (1  $\mu$ g/ml) for various periods of time. 2 h before the end of the incubation periods the cells were labeled with either [<sup>35</sup>S]methionine (150  $\mu$ Ci/ml for 2 h) or with [<sup>3</sup>H]thymidine (1  $\mu$ Ci/ml for 2 h). [<sup>3</sup>H]thymidine incorporation was analyzed as described in Materials and Methods. <sup>35</sup>S-labeled nuclear proteins (equal amounts) were analyzed by two-dimensional gel electrophoresis. Numatrin was identified by autoradiography and its radioactivity was measured directly in the gels. ●, cpm in numatrin. ○, [<sup>3</sup>H]thymidine incorporation in DNA.

of discrete genes. In view of these findings, the failure of the calcium ionophore to induce the synthesis of numatrin indicates that an increase in the intracellular  $Ca^{+2}$  level is not sufficient for the induction of numatrin synthesis while it is sufficient for the induction of other nuclear proteins. In this regard it is pertinent that A23187 when administered alone does not induce DNA synthesis in BALB/c Swiss 3T3 fibroblasts (20). It is intriguing to speculate that the failure of A23187 to induce DNA synthesis (in spite of its reported effect on c-myc and c-fos mRNA levels) is due to its failure to induce the expression of other important growth regulated proteins such as numatrin which might be essential for induction of mitogenesis. These results further indicate that the increase in the synthesis of numatrin may be more closely correlated with cellular commitment for mitogenesis as compared with other biochemical parameters of cellular activation.

Kinetic studies of numatrin synthesis in lymphocytes (12, 13) and in fibroblasts (Fig. 8) have shown that the increase in numatrin synthesis occurred rapidly after mitogen stimulation at early G1 phase and was gradually reduced during S phase. This observation raised questions regarding the kinetics of abundance of numatrin: is numatrin abundance (relative to other proteins) increased in parallel with its increased synthesis during G1? Is the cellular level of numatrin



**Figure 9.** The effect of calcium ionophore A23187 on the synthesis of nuclear proteins in Swiss 3T3 murine fibroblasts. Quiescent Swiss 3T3 fibroblasts were stimulated with calcium ionophore A23187 (0.5 and 1  $\mu$ M) for 4 and 8 h and then labeled with [<sup>35</sup>S]methionine (200  $\mu$ Ci/ml for 90 min). Nuclear proteins were analyzed by two-dimensional gel electrophoresis. *A*, nontreated cells; *B*, cells stimulated with A23187 (1  $\mu$ M) for 8 h; *C*, A23187 (0.5  $\mu$ M), 4 h; *D*, A23187 (1  $\mu$ M), 4 h; and *E*, ionomycin (1  $\mu$ M), for 4 h. Arrows indicate numatrin. Circles indicate a protein at 80 kD/pI-5 whose synthesis was stimulated by A23187 and ionomycin.

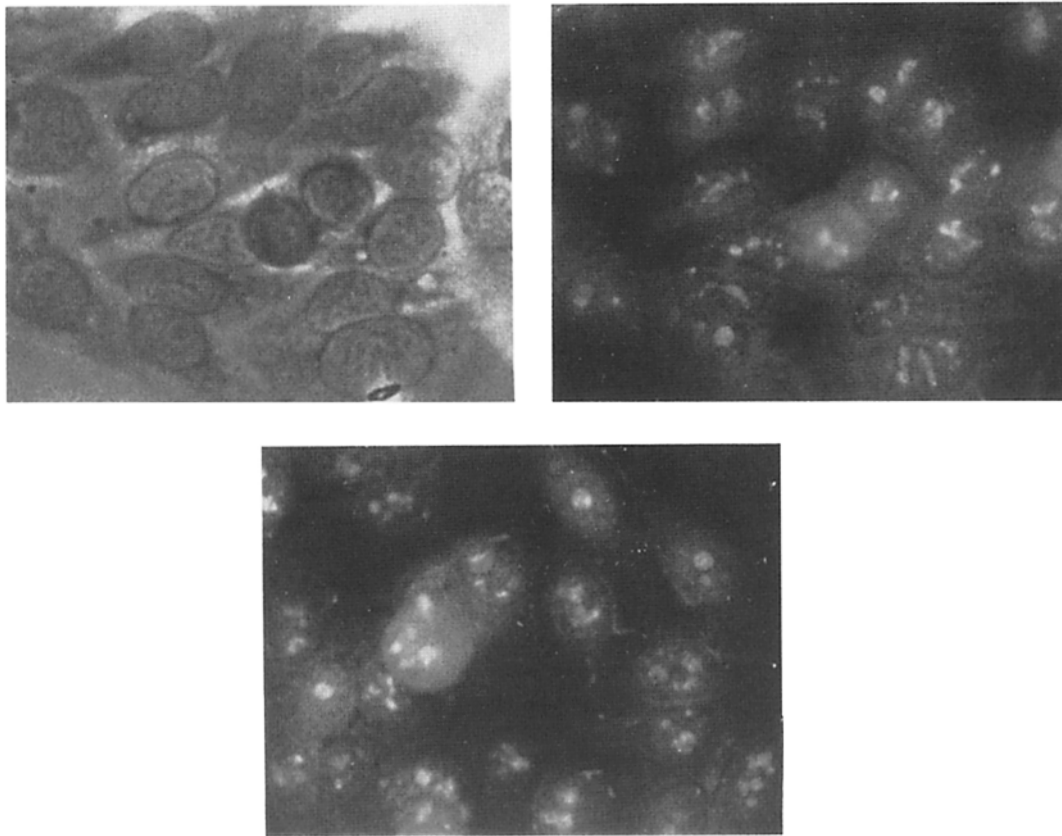


**Figure 10.** Indirect immunofluorescence detection of numatrin in anti-Ig-stimulated B lymphocytes. B lymphocytes were stimulated with anti-Ig (10  $\mu\text{g/ml}$ ) and 8-mercaptoguanosine for 16 h and then cytocentrifuged onto slides, fixed, and exposed to indirect immunofluorescence assay as described. *Left*, resting cells; *right*, mitogen-stimulated cells.

maintained elevated during S phase or is it rapidly degraded during this stage? Using a specific anti-numatrin antibody in immunoblot analysis we were now able to examine also the kinetics of numatrin abundance during various periods of the cell cycle. We showed that mitogen stimulation induced not only an increase in the synthesis but also an increase in the abundance of numatrin that was detected at early G1, reached

a plateau at the onset of S phase, maintained elevated during S, and declined at the end of S phase.

The marked decline in numatrin amount at the end of S phase suggests that numatrin may be rapidly degraded or translocated from the nuclei at the end of S phase. The maximal abundance of numatrin at the onset of S phase as well as the marked diminution in the amount of numatrin at the



**Figure 11.** Indirect immunofluorescence detection of numatrin in KB cells and in Hep2 cells. Indirect immunofluorescence assays of fixed cells were done as described in Materials and Methods. *Left top*, KB cells (phase); *right top*, KB cells (fluorescence picture of the same field as in left); *bottom center*, Hep2 cells.

end of S phase indicate that the cellular abundance of numatrin is closely correlated with S phase of the cell cycle, suggesting that possible involvement of numatrin in processes associated with DNA replication should be considered. Taken collectively with previous results it may be suggested that the rapid increase in the synthesis of numatrin at early G1 phase prepares the cells to enter and transverse S phase. This possibility is in accord with the observation that the increase in the synthesis of numatrin is exclusively associated with the effect of stimuli that induce DNA synthesis but is not associated with the effect of certain well-known B cell activators that do not induce DNA synthesis (12).

Numatrin was also found to be markedly abundant in various lymphoma cells regardless of their cellular origin, suggesting that numatrin similar to nuclear oncogene products such as *myc*, *fos*, and p-53 (review reference 2) is overexpressed in malignant cells. This conclusion was further supported by the abundance of numatrin in other malignant cells such as human epidermoid carcinoma and human hepatoma cells (Fig. 11). Taken collectively, these results suggest that numatrin may be involved in regulation of cellular growth in various normal and neoplastic cell types.

The ubiquitous nature of numatrin and its distinct nucleolar localization in mitogen-stimulated B cells and in other malignant cells are in agreement with the recent finding that numatrin is identical to the nucleolar phosphoprotein B23 (14). Protein B23 has been described as a nucleolar phosphoprotein in 1974 (15, 16). Later biochemical studies in HeLa cells showed that part of protein B23 was associated with preribosomal ribonucleoprotein particles (28) and that translocation of the protein B23 from the nucleolus to the nucleoplasm was observed upon serum deprivation of (8) and in response to actinomycin D or toyocamycin treatment of HeLa cells (28). Recently, protein B23 has been shown to be a hexamer composed of four  $\alpha$  and two  $\beta$  monomers (29). While the function of B23 is not yet known, its localization in the nucleolus and its possible association with preribosomal ribonucleoprotein particles led to the suggestion that B23 might be involved in ribosome assembly and/or processing of ribosomal RNA.

Earlier observations have shown that the amount of B23 is elevated in rat hypertrophic and hematoma cells as compared with normal rat liver cells (1, 5) suggesting that the amount of B23 correlates with cell growth. This observation is in accord with the studies of numatrin. Furthermore, the studies of numatrin provide the first evidence to show that a marked and rapid induction in the synthesis of B23 (numatrin) may be an early signal that occurs at G1 phase of the cell cycle, precedes the induction of DNA synthesis, and is closely correlated with receptor-mediated induction of mitogenesis in various cell systems. As previously suggested (14) the identification of numatrin as protein B23 further calls attention to the nucleolus as a specific nuclear site that might bear a crucial role in early propagation of the mitogenic signal.

Finally, studies of this work demonstrate that while the kinetics of numatrin/B23 synthesis is correlated with G1 phase, the kinetics of its accumulation in the cells closely correlates with S phase. This observation suggests that a possible role of numatrin/B23 in S phase-associated processes such as DNA replication warrants further investigation.

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