

# Short Exposure to Actinomycin D Induces "Reversible" Translocation of Protein B23 as Well as "Reversible" Inhibition of Cell Growth and RNA Synthesis in HeLa Cells<sup>1</sup>

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## ABSTRACT

HeLa cells were grown in medium containing various amounts of actinomycin D for various times. Cellular localization of protein B23 was detected using an immunofluorescence technique. Bright nucleolar fluorescence was observed in untreated cells. A shifting of nucleolar to nuclear fluorescence was observed with increasing doses of actinomycin D and longer incubation times. The degree of translocation of protein B23 from nucleoli to nucleoplasm is dependent on the amount of the drug used and the duration of incubation.

Short exposure (0.5 h) of HeLa cells to actinomycin D (0.01–0.25  $\mu\text{g}/\text{ml}$ ) induced "reversible" translocation of protein B23, inhibition of cell growth, and RNA synthesis. A majority of cells (>75%) treated with actinomycin D (0.01–0.25  $\mu\text{g}/\text{ml}$ ) for 0.5 h still retained bright nucleolar fluorescence. A shifting of nucleolar to nuclear fluorescence as well as inhibition of cell growth and RNA synthesis were observed within 6 h after the removal of the drug. However, at the extended periods (>24 h) after drug removal, RNA synthesis and cell growth resumed at the normal rate, and protein B23 relocated from nucleoplasm to nucleoli. This is in contrast to the results obtained from the experiments using higher doses (1  $\mu\text{g}/\text{ml}$ ; 0.5 h) or longer (0.25  $\mu\text{g}/\text{ml}$ ; 2 h) exposure of HeLa cells to actinomycin D, which induced irreversible B23 translocation as well as irreversible inhibition of cell growth and RNA synthesis. These results indicated that actinomycin D can be a reversible inhibitor depending on the drug extracellular concentrations and exposure times. Our results also indicated that "B23 translocation" is closely associated with states of cell growth and inhibition of RNA synthesis. "B23 translocation" may therefore be a simple and rapid method for assessing the inhibition of cell growth in response to antitumor therapy.

## INTRODUCTION

Actinomycin D is an antitumor antibiotic with known activity against a variety of pediatric malignant tumors (1, 2). Actinomycin D is known to inhibit RNA synthesis (3). High doses (2.0  $\mu\text{g}/\text{ml}$ ) of actinomycin D block the transcription of all RNA species, while low doses (40.0 ng/ml) cause a preferential inhibition of rRNA synthesis (4, 5). It was reported that actinomycin D inhibits transcription by binding to the DNA template (6). Trask and Muller (7) recently have proposed that topoisomerase I contributes to actinomycin D-induced inhibition of transcription in nucleoli. Actinomycin D stabilizes and extends the half-life of the covalent topoisomerase I-DNA complex. Our previous studies have demonstrated that when cells are treated with actinomycin D (8) or certain other antitumor drugs, such as doxorubicin (9), toyocamycin (10), and mitomycin (11), phosphoprotein B23 translocates from the nucleolus to the nucleoplasm. Protein B23 is particularly sensitive to these agents. Other nucleolar proteins, such as protein

C23 and fibrillarlin, do not translocate under the same conditions (12). Experiments using luzopeptin analogues (BBM 928A, -B, -C, and -D) (13, 14) with different antitumor activities (15) have shown that "B23 translocation" and inhibition of RNA synthesis correlate well with the antitumor activities of luzopeptins (16). These studies indicate that the "B23 translocation assay" is a simple and rapid method to determine the efficacy of antitumor agents.

Clinically, actinomycin D generally has been administered using a 5-day divided dose schedule (1). Many experimental data suggest that a single-dose actinomycin D schedule is more effective than a divided dose schedule (17). Clinical studies in adults with malignant melanoma (18) and children with relapsed acute lymphoblastic leukemia (19) demonstrated that single dose drug accumulation is well tolerated. The present study was therefore undertaken to provide information on the dose/time response of protein B23 translocation, cell growth, and RNA synthesis to actinomycin D. The reversibility of these effects upon removal of actinomycin D was also analyzed.

## MATERIALS AND METHODS

**Cells.** HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, and antibiotics in a 5% CO<sub>2</sub> humidified incubator at 37°C.

**Antibodies.** The monoclonal antibody to protein B23 (37/5.1) was produced by *in vitro* fusion techniques (20). Antibodies were collected from the hybridoma cell culture medium and the immunoglobulin fraction was concentrated by ammonium sulfate precipitation.

**Immunofluorescence.** HeLa cells were fixed in 2% electron micrograph-grade formaldehyde in PBS<sup>3</sup> for 20 min at room temperature. The cells were permeabilized with acetone at -20°C for 3 min. After a wash with PBS, the fixed cells were incubated with the monoclonal antibody (diluted 1:30) at 37°C for 1 h. Then cells were washed four times for 10 min each in PBS and incubated with fluorescein-conjugated affinity-purified goat anti-mouse IgG (diluted 1:20 with PBS) at 37°C for 35 min. The cells were then washed four times for 10 min each with PBS and mounted in 50% glycerol in PBS (pH 9).

**Cell Growth Determination.** HeLa cells (approximately  $1 \times 10^5$ ) cultured on slides were incubated with actinomycin D (Sigma Chemical Co.) for 30 min. Cells were then washed 3 times with PBS before fresh medium was added. Cultures were incubated at 37°C. At various times after the washing procedure, cultures were harvested. Cell numbers were obtained by counting cell suspensions in a Coulter electronic particle counter.

**[<sup>3</sup>H]Uridine Incorporation Determination.** HeLa cells (approximately  $1 \times 10^5$ ) were preincubated with actinomycin D for 30 min. Cells were then washed 3 times with PBS before [<sup>3</sup>H]uridine was added. They were further incubated at 37°C for various intervals. The cells were scraped from slides and collected in centrifuge tubes, washed with PBS, and precipitated with 1.0 ml of trichloroacetic acid (0°C). The pellets were then washed 3 times with cold 10% trichloroacetic acid. The residues were solubilized in 1 N NaOH and the radioactivity of each sample was determined in a Packard liquid scintillation counter after 5.0 ml of Aquasol were added.

<sup>3</sup> The abbreviation used is: PBS, 8.5 mM Na<sub>2</sub>HPO<sub>4</sub>-1.6 mM NaH<sub>2</sub>PO<sub>4</sub>-0.145 M NaCl, pH 7.2.

Received 3/5/90; accepted 5/29/90.

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<sup>1</sup> This work is supported by Chang Gung Research Grant CMRP 275 and National Science Council Grant NSC79-0412-B182-44, Republic of China.

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Table 1 "B23 translocation" in HeLa cells treated with actinomycin D

HeLa cells were cultured on slides. Doses of actinomycin D were added to the culture medium. The localization of protein B23 was determined after various times of incubation.

Dose ( $\mu\text{g/ml}$ )	Time (h)	% of cells in each translocation pattern <sup>a</sup>		
		A	B	C
0.01	0.5	100	0	0
	1.0	56	44	0
	1.5	24	76	0
	2.0	0	95	5
	4.0	0	5	95
0.05	0.5	90	10	0
	1.0	22	78	0
	1.5	0	51	49
	2.0	0	32	68
	4.0	0	0	100
0.25	0.5	78	22	0
	1.0	0	88	12
	1.5	0	17	83
	2.0	0	0	100
	4.0	0	0	100
1.0	0.5	8	92	0
	1.0	0	78	22
	1.5	0	5	95
	2.0	0	0	100
	4.0	0	0	100

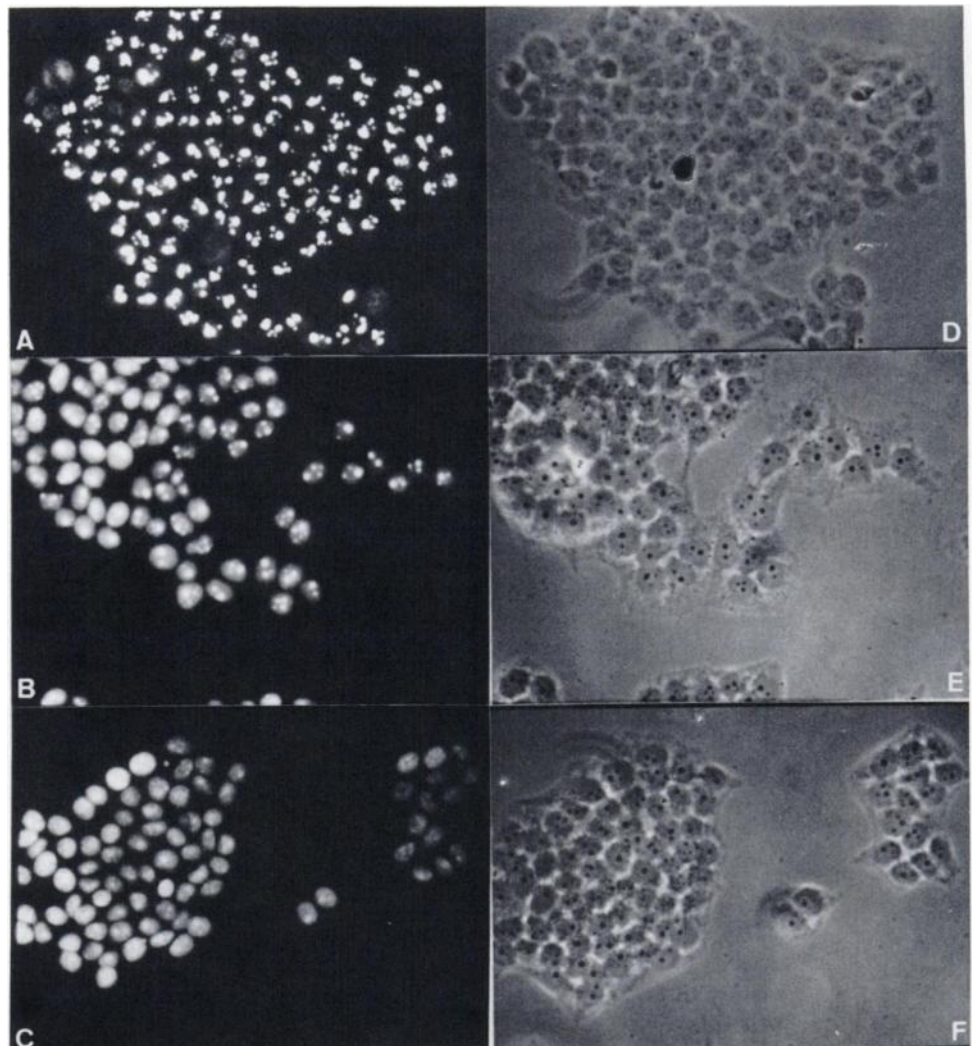
<sup>a</sup> A, cells showed bright nucleolar fluorescence with little or no nuclear fluorescence; B, both nuclear and nucleolar fluorescence were observed in cells; C, cells showed homogeneous nuclear fluorescence with no distinct nucleolar fluorescence. Triplicate 100-cell counts were performed. Values are representative of the results obtained in several (at least two) replications of the experiment.

## RESULTS

**Time and Dose-Response Studies.** Table 1 shows the resulting immunofluorescence patterns of HeLa cells after incubation with various amount of actinomycin D for various times. Control cells with no drug treatment gave bright nucleolar fluorescence (A pattern, Fig. 1, A), which indicated that there was no B23 translocation. After treatment with actinomycin D (0.01  $\mu\text{g/ml}$ ; 1 h), 44% of the cells began to show B23 translocation with both nuclear and nucleolar fluorescence (B pattern, Fig. 1B), while 56% of the cells retained bright nucleolar fluorescence (A pattern). At 0.05  $\mu\text{g/ml}$  of actinomycin D (1.5 h treatment), 49% of the cells showed complete nuclear fluorescence (C pattern, Fig. 1C). The percentage of cells with complete B23 translocation was shown to increase with higher doses (0.25–1  $\mu\text{g/ml}$ ) and longer incubation times (2–4 h). These results indicated that the degree of translocation of phosphoprotein B23 from nucleoli to nucleoplasm in HeLa cells is dependent on the amount of drug used and the duration of incubation.

**Translocation and Relocation of Protein B23.** Localization of protein B23 in actinomycin D-treated HeLa cells (0.5-h treatment) upon removal of drug was studied. Most of the cells (>75%) treated with various doses of actinomycin D (0.01–0.25  $\mu\text{g/ml}$ ) for 0.5 h still retained bright nucleolar fluorescence (A pattern). Surprisingly, gradual shifting of nucleolar to nuclear fluorescence (B and C patterns) was observed within 6 h after removal of the drug. Table 2 summarizes the quantitative

Fig. 1. Immunofluorescence and phase contrast pictures of HeLa cells after treatment with actinomycin D. HeLa cells were grown on slides. Actinomycin D was added, and cultures were incubated at 37°C for various times before the cells were fixed and immunostained by protein B23 antibody. A, control cells without drug treatment; only nucleolar fluorescence was observed. B, cells treated with actinomycin D (0.01  $\mu\text{g/ml}$ ) for 2 h, both nuclear and nucleolar fluorescence were observed. C, maximum effect after treatment with actinomycin D (0.25  $\mu\text{g/ml}$ ; 2 h) in which only nuclear fluorescence was observed. D, E, and F, phase contrast pictures.



**Table 2** Localization of protein B23 upon removal of actinomycin D after 0.5 h treatment

HeLa cells were cultured on slides. Doses of actinomycin D were added to the culture medium. After 30 min of incubation, the drug was removed (washed 3 times with PBS). Localization of protein B23 was then determined at various times after removal of drug.

Dose (μg/ml)	Time (h)	% of cells in each translocation pattern <sup>a</sup>		
		A	B	C
0.01	0	100	0	0
	0.5	100	0	0
	6.0	37	63	0
	24.0	75	25	0
	30.0	88	12	0
	40.0	91	9	0
0.05	0	90	10	0
	0.5	85	15	0
	6.0	0	81	19
	24.0	21	73	19
	30.0	61	39	0
	40.0	73	27	0
0.25	0	79	21	0
	0.5	15	85	0
	6.0	0	18	82
	24.0	0	57	43
	30.0	16	72	12
	40.0	32	68	0
1.0	0	0	95	5
	0.5	0	15	85
	6.0	0	0	100
	24.0	0	0	100
	30.0	0	0	100
	40.0	0	0	100

<sup>a</sup> Classification of immunofluorescence pattern is defined in Table 1, Footnote a. Triplicate 100-cell counts were performed. Values are representative of the results obtained in several (at least two) replications of the experiment.

**Table 3** Localization of protein B23 upon removal of actinomycin D after 2.0 h treatment

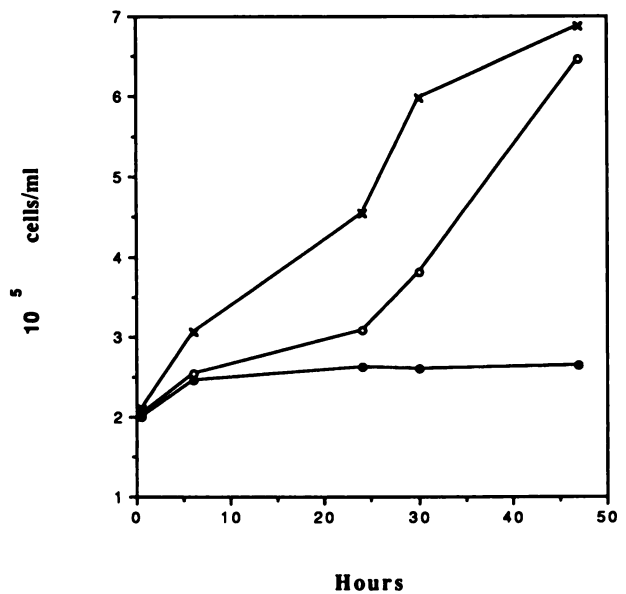
HeLa cells were cultured on slides. Doses of actinomycin D were added to the culture medium. After 2 h of incubation, the drug was removed (washed 3 times with PBS). Localization of protein B23 was then determined at various times after removal of drug.

Doses (μg/ml)	Time (h)	% of cells in each translocation pattern <sup>a</sup>		
		A	B	C
0.01	0	24	76	0
	0.5	10	90	0
	6.0	0	70	30
	24.0	0	82	18
	30.0	27	73	0
	40.0	54	46	0
0.05	0	0	21	79
	0.5	0	12	88
	6.0	0	5	95
	24.0	0	22	78
	30.0	0	19	81
	40.0	0	17	83
0.25	0	0	0	100
	0.5	0	0	100
	6.0	0	0	100
	24.0	0	0	100
	30.0	0	0	100
	40.0	0	0	100
1.0	0	0	0	100
	0.5	0	0	100
	6.0	0	0	100
	24.0	0	0	100
	30.0	0	0	100
	40.0	0	0	100

<sup>a</sup> Classification of immunofluorescence pattern is defined in Table 1, Footnote a. Triplicate 100-cell counts were performed. Values are representative of the results obtained in several (at least two) replications of the experiment.

analysis of these studies. At 0.25 μg/ml of actinomycin D (0.5-h treatment), 79% of the cells retained bright nucleolar fluorescence (A pattern). At 6 h after drug removal, a majority of the cells (82%) showed complete nuclear fluorescence. However, at the extended periods (>24 h) after drug removal, protein B23 began to relocate from nucleoplasm to nucleoli. A shifting of nuclear to nucleolar fluorescence was observed. At 40 h after removal of actinomycin D (0.25 μg/ml), 68% of the cells showed both nuclear and nucleolar fluorescence while 32% of the cells showed bright nucleolar fluorescence. These results strongly suggested that short exposure of cells to actinomycin D could induce only "reversible translocation." This is in contrast to the results obtained from the experiments of higher doses (1 μg/ml; 0.5 h) or longer (0.05–1.0 μg/ml; 2 h) (Table 3) exposure of HeLa cells to actinomycin D, of which the induced B23 translocation is irreversible.

**Cell Growth and RNA Synthesis.** Other measures we took in our studies are determination of cell growth and incorporation of [<sup>3</sup>H]uridine into acid-insoluble material, which is generally considered to quantify RNA synthesis and inhibition of RNA synthesis. Figs. 2 and 3 show the results of cell growth and RNA synthesis in actinomycin D-treated HeLa cells (0.25 μg/ml; 0.5 h) upon removal of drug, respectively. Similar to the results of translocation studies, short exposure of cells to actinomycin D (0.25 μg/ml; 0.5 h) did cause slowdown of cell growth and inhibition of RNA synthesis within the period of 6 h after drug removal. Recovery of cell growth and RNA synthesis were also observed at the extended period (>24 h) after drug removal. Treatment with high doses of actinomycin D (1 μg/ml; 0.5 h), on the other hand, caused severe inhibition of cell growth and RNA synthesis. Recovery of such inhibitions (Figs. 2 and 3) and relocation of protein B23 from nucleoplasm to



**Fig. 2.** Cell growth in HeLa cells after short exposure to actinomycin D. HeLa cells cultured on slides were incubated with actinomycin D for 30 min. Cells were then washed 3 times with PBS before fresh medium was added. Cultures were incubated at 37°C. At various times after the washing procedure, cultures were harvested. Cell numbers were obtained by counting cell suspensions in a Coulter electronic particle counter. Points, mean of duplicate slide samples. Values are representative of the results obtained in several (at least two) replications of the experiments. x, control cells without drug treatment; o, 0.25 μg/ml actinomycin D treatment; ●, 1 μg/ml actinomycin D treatment.

nucleoli (Table 2) were not observed upon removal of drug. These results indicated that exposure of cells to high doses of actinomycin D would cause irreversible effects on cell growth, RNA synthesis and translocation of protein B23. Our results

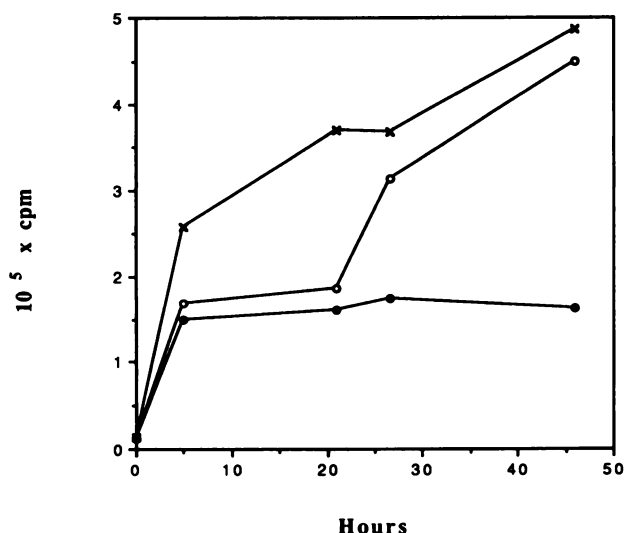


Fig. 3. RNA synthesis in HeLa cells after short exposure to actinomycin D. HeLa cells cultured on slides were incubated with actinomycin D for 30 min. Cells were then washed 3 times with PBS before fresh medium and [<sup>3</sup>H]uridine were added. Cultures were incubated at 37°C. At various times after the washing procedure, cultures were harvested and [<sup>3</sup>H]uridine incorporation was determined. Points, mean of duplicate slide samples. Values are representative of the results obtained in several (at least two) replications of the experiments. x, control cells without drug treatment; O, 0.25 µg/ml actinomycin D treatment; ●, 1 µg/ml actinomycin D treatment.

strongly indicate that "B23 translocation" is closely associated with states of cell growth and inhibition of RNA synthesis. "B23 translocation" may therefore be a simple and rapid method for assessing the inhibition in cell growth in response to antitumor therapy.

## DISCUSSION

Our results demonstrate that the degree of actinomycin D-induced translocation of phosphoprotein B23 from nucleoli to nucleoplasm is dose and time dependent. Upon actinomycin D treatment, B23 gradually translocates from nucleoli to nucleoplasm. It is possible that actinomycin D diffuses passively into the cells exerting an inhibitory effect on nucleoli. Diffusion may proceed as a first-order process as long as the intracellular concentration of free drug is maintained at a much lower level than the extracellular concentration. In our actinomycin D short exposure experiments (<0.25 µg/ml; 0.5 h), we observed slowdown of cell growth, inhibition of RNA synthesis, and translocation of protein B23 from nucleoli to nucleoplasm at 6 h after removal of the drug. It is interesting to note that such treated cells could recover well from those inhibitions. At extended periods (>24 h) after removal of the drug, cells resume their abilities to grow and to synthesize RNA. Protein B23, accordingly, relocates from nucleoplasm to nucleoli. These findings suggest that small doses of actinomycin D diffusing into the cells after short exposure could only induce "reversible" inhibitory effects on cell growth, RNA synthesis, and translocation of protein B23. Trask and Muller (7) recently proposed that topoisomerase I contributes to actinomycin D inhibition of transcription. Actinomycin D treatment of nuclei for a short time extended the half-life of the covalent topoisomerase I-DNA complex. Topoisomerase I, however, can still carry the resealing step in the presence of the drug; thus, actinomycin D does not freeze the topoisomerase-DNA intermediate into a permanent and irreversible complex. Our present studies indicate that actinomycin D can be a reversible inhibitor of RNA synthesis depending on the extracellular drug concentrations

and exposure times. These studies have important therapeutic implications. Exposure of the tumor to the drug for a short period of time may not be effective enough for control of the tumor growth. The inhibition of the drug efflux rate offers a simple explanation of the known synergism between anthracyclines and dipyridamole in promoting therapeutic efficacy (21, 22). The direct dependence of the inhibitory effect of actinomycin D on extracellular drug concentration and exposure time suggests that the increased cytotoxicity could be obtained by maintaining constant blood level of the drug for extended periods. At divided low doses, B23 translocation, inhibition of cell growth, and RNA synthesis may not be complete and could be reversible. It is therefore a single dose actinomycin D schedule may be more effective than a divided dose schedule, which is in agreement with many other experimental data (17).

The cause-effect relationship of B23 translocation and anti-tumor activity is not known. Our previous studies (9, 23) indicated that protein B23 binds to certain elements in the nucleolus (pre-rRNA, proteins, or matrix structure) and plays an essential role in ribosome synthesis. Our present studies have significantly demonstrated that "B23 translocation" is closely associated with states of cell growth and inhibition of RNA synthesis. It is possible that, when cells are treated with antibiotics (actinomycin D, toyocamycin, and luzopeptin), RNA synthesis, RNA processing, and cell growth are inhibited and protein B23 loses its binding target in the nucleolus and diffuses into the nucleoplasm.

In conclusion, "B23 translocation" as observed by immunofluorescence may well be a simple, rapid, and important method for: (a) detection of growth inhibition-stimulation of cells; (b) selection of effective drugs for patients; (c) monitoring anticancer drug efficacy in patients and providing more efficient chemotherapy schedules.

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