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EFFECTS OF ACTINOMYCIN D ANALOGS ON NUCLEOLAR PHOSPHOPROTEIN B23 (37,000 DALTONS/pI 5.1)

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Abstract—Localization of protein B23 in HeLa cells after treatment with actinomycin D and its analogs was studied using indirect immunofluorescence. Bright nucleolar fluorescence was observed in control HeLa cells. After treatment with actinomycin D (250 ng/ml) for 2 hr, a uniform nucleoplasmic fluorescence was observed. Similar results were obtained with the actinomycin analogs, actinomycin Z5 and actinomycin K2T. Only after a much longer incubation (24 hr) with actinomycin 4-4′-gly was nucleoplasmic fluorescence observed. Actinomycin D, actinomycin Z5, and actinomycin K2T inhibited [^{3}H]uridine incorporation into the trichloroacetic acid insoluble fraction of HeLa cells with IC_{50} values of 9.5 ± 3.2, 59.1 ± 19.6 and 1423.3 ± 212.2 ng/ml respectively. No inhibition of [^{3}H]uridine incorporation was observed using actinomycin 4-4′-gly (2000 ng/ml, 2-hr incubation). The order of potency for the loss of nucleolar fluorescence and the concurrent increase in nucleoplasmic fluorescence was actinomycin D > actinomycin Z5 > actinomycin K2T > actinomycin 4-4′-gly, which correlated with the order of their IC_{50} values for inhibition of [^{3}H]uridine incorporation. Studies of the effects of actinomycin D and its analogs on RNA synthesis and localization of protein B23 indicated that there is a direct relationship between the B23 "translocation" from nucleolus to nucleoplasm and the inhibition of RNA synthesis. At 45–55% inhibition of RNA synthesis, both nuclear and nucleolar B23 immunofluorescence were observed. At 75–85% inhibition, only a uniform nucleoplasmic fluorescence was observed.

The nucleolus, a distinct biochemical and morphological entity, is the site of synthesis and assembly of ribosomal subunits [1–4]. Many studies have been done on the biosynthesis and metabolism of rRNA and r-proteins [5–16]. Although the exact mechanism of the biogenesis of ribosomes is not yet known, it has been suggested that nucleolar RNA synthesis occurs in fibrillar centers (the location of nucleolar DNA) and that the newly formed rRNP migrates from nucleolar fibrillar regions to the nucleolar granular regions, where preribosomal particles are found [16]. Much interest has developed as the the functions and the roles of nucleolar proteins in ribosome biosynthesis. Two major nucleolar phosphoproteins, designated as B23 (mol. wt/pI = 37,000 daltons/5.1) and C23 (mol. wt/pI = 110,000 daltons/5.1), have been purified and characterized [17–19]. The finding that protein C23 is the silver-binding protein associated with the NOR region of chromosomes has suggested that protein C23 may function in rRNA gene regulation [13–15, 20]. Immunoelectron microscopic studies have shown that protein B23 exhibits a generalized immunostaining pattern, primarily in the granular regions of the nucleolus [20]. This finding indicates that proteins B23 may be associated with preribosomal particles, and involved in the process of maturation of ribosomal structures. However, the exact function of protein B23 is not yet known.

Recent studies on nucleolar phosphoprotein B23 (37/5.1) demonstrated that actinomycin D blocked the binding of a monoclonal antibody to protein [21], suggesting that the action of actinomycin D may alter the function of protein B23. The present study was undertaken to provide information on the cellular distribution of protein B23 in HeLa cells treated with actinomycin D and actinomycin D analogs. The correlation of localization of protein B23 with RNA synthesis was also analyzed.

MATERIALS AND METHODS

Drugs. Actinomycin D was purchased from the Sigma Chemical Co. Actinomycin K2T (the proline residues of actinomycin D are replaced by *trans*-4-methyl-prolines) and actinomycin 4-4′-gly (the sarcosine residues of actinomycin D are replaced by glycines) were provided by Dr. Anthony Mauger, The Washington Hospital Center, Washington, DC, U.S.A. Actinomycin Z5 ([α,βPro(5Me)^3, Opr(5Me)^3, Me-Ala^5]-Val$_2$-actinomycin) was a gift from Dr. Matthew Suffness, National Institutes of Health, Bethesda, MD.

Radioactive material. [^{3}H]Uridine (sp. act. = 16 Ci/mmole) was purchased from ICN (Chemical and Radioisotope Division).

Culture materials. Minimum essential medium (Eagle's), fetal calf serum, glutamine and penicillin-streptomycin solutions were purchased from the Grand Island Biological Co., Grand Island, NY. Petri plates and flasks were purchased from the Corning Glass Co., Corning, NY. Fluorescent antibody microslides were obtained from Dickinson & Co., Oxnard, CA.

Chemicals. EM-grade formaldehyde solutions were purchased from Polysciences, Inc., Warrington, PA. Sodium phosphate monobasic, sodium

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phosphate dibasic, acetone and trichloroacetic acid were obtained from the Fisher Scientific Co., Pittsburgh, PA; and Bio-Rad dye reagent from Bio-Rad Laboratories, Richmond, CA.

Cells. HeLa S-3 cells were grown in Eagle's minimum essential medium supplemented with 10% fetal calf serum, glutamine and antibiotics in a 5% CO_2 humidified incubator at 37°. For immunofluorescence studies, cells were grown on slides in a Petri dish.

Antibodies. The monoclonal antibody to protein B23 (37/5.1) was produced by *in vitro* fusion techniques [22]. Antibodies were collected from the hybridoma cell culture medium and purified by ammonium sulfate precipitation.

Immunofluorescence. HeLa cells were fixed in 2% EM-grade formaldehyde in PBS (phosphate-buffered saline) for 20 min at room temperature. The cells were permeabilized with acetone at −20° for 3 min. After a wash with PBS, the fixed cells were incubated with the monoclonal antibody (diluted 1:16) at 37° for 1 hr. 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° 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).

[^{3}H]*Uridine incorporation determination.* HeLa cells (10^6) were preincubated with actinomycin D or its analogs for 30 min; after [^{3}H]uridine (2.5 μCi/ml) was added, they were incubated at 37° for various intervals. The cells were collected in centrifuge tubes, washed with PBS, and precipitated with 1.0 ml of 10% trichloroacetic acid (O^0). The pellets were then washed three times with 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 was added.

Protein determination. Protein was determined by the Bio-Rad protein assay [23].

RESULTS

Localization of protein B23. The effects of actinomycin D and its analogs on the localization of nucleolar phosphoprotein B23 were examined in HeLa cells using indirect immunofluorescence. In control HeLa cells, bright nucleolar fluorescence, but little or no nuclear fluorescence, was observed after immunostaining with the anti-protein B23 antibody (Fig. 1a). After treatment with actinomycin D (250 ng/ml) for 2 hr, a uniform nucleoplasmic fluorescence was observed (Fig. 1c). Similar results were obtained with the actinomycin analog actinomycin Z5. When HeLa cells were treated with reduced amounts of actinomycin D (10 ng/ml), actinomycin Z5 or K2T (50 ng/ml) for 2 hr, both nucleoplasmic and nucleolar fluorescence were observed (Fig. 1b). A longer incubation time (24 hr) at a higher dose (250 ng/ml) was required for actinomycin 4-4′-gly to produce nucleoplasmic fluorescence. Immunostaining of the actinomycin D treated cells (250 ng/ml; 4 hr) with anti-protein C23 antibody showed no decrease in the bright nucleolar fluorescence by comparison with the controls.

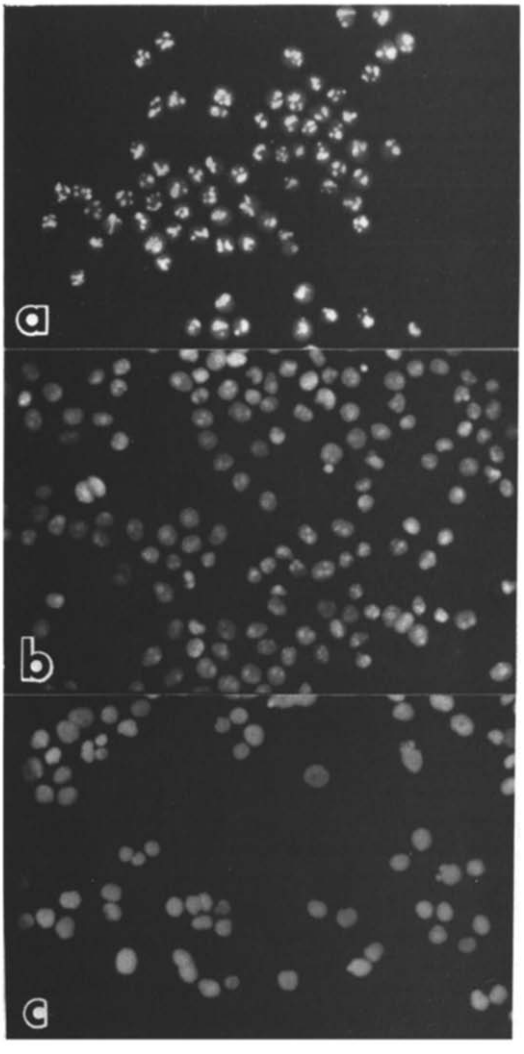

Fig. 1. Studies of the effect of actinomycin D and its analogs on the cellular localization of protein B23. HeLa cells were grown on slides. Actinomycin D or an analog was added, and cultures were incubated at 37° for various times before the cells were fixed and immunostained by protein B23 antibody. Key: (a) control HeLa cells; only nucleolar fluorescence was observed; (b) after treatment with actinomycin D (10 ng/ml, 2 hr), actinomycin Z5 (50 ng/ml; 2 hr) or actinomycin K2T (50 ng/ml; 2 hr), both nuclear and nucleolar fluorescence were observed; and (c) maximum effect after treatment with actinomycin D (250 ng/ml, 2 hr) in which only nuclear fluorescence was observed.

Table 1 summarizes the effects of actinomycin D and its analogs on the localization of protein B23. The order of potency for the reduction of nucleolar and the concurrent increase in nucleoplasmic fluorescence was actinomycin D > actinomycin Z5 > actinomycin K2T > actinomycin 4-4′-gly.

Effects on RNA synthesis. Inhibition of RNA synthesis was studied with HeLa cells treated with actinomycin D and its analogs. RNA synthesis was measured as the incorporation of [^{3}H]uridine into trichloroacetic acid insoluble material. To estimate the relative potency of actinomycin D and its analogs

Table 1. Effect of actinomycin D and its analogs on the cellular localization of protein B23

		Immunofluorescence			
		Dose (ng/ml) 50		250	
Drug	O	2 hr	24 hr	2 hr	24 hr
Actinomycin D	A	C	C	C	C*
Actinomycin Z5	A	B	C	C	C*
Actinomycin K2T	A	B	B	B	C
Actinomycin 4-4′-gly	A	A	B	A	C

HeLa cells were cultured on slides in minimum essential medium (Eagle's). Actinomycin D or its analogs was added for 2 or 24 hr before the cells were fixed and immunostained by protein B23 antibody. Viability of the cells was over 95% under these conditions except for those marked with an asterisk. A = Over 90% of the cells showed bright nucleolar fluorescence with little or no nuclear fluorescence (Fig. 1a). About 1200 cells were counted. B = Over 90% of the cells showed both nuclear and nucleolar fluorescence (Fig. 1b). About 1200 cells were counted. This is an intermediate phenomenon between A and C. C = Over 95% of the cells showed homogeneous nuclear fluorescence. No distinct nucleolar fluorescence was observed (Fig. 1c). About 1200 cells were counted.

* The viability of the cells was 40%.

in terms of RNA synthesis inhibition, the IC_{50} values were determined using a 2-hr incubation interval. The IC_{50} values of actinomycin D, Z5 and K2T were 9.5 ± 3.2, 59.1 ± 19.6 and 1423.3 ± 212.2 ng/ml respectively (Table 2). Actinomycin 4-4′-gly did not inhibit the [^{3}H]uridine incorporation at a concentration of 2000 ng/ml (Table 2). The order of their IC_{50} values for inhibition of RNA synthesis correlated with the order of potency for the loss of nucleolar and the concurrent increase of nucleoplasmic fluorescence.

Time course study of effects on RNA synthesis and localization of protein B23. Table 3 shows the effects of actinomycin D and its analogs on the RNA synthesis and the cellular localization of protein B23 at various time intervals. Using doses equivalent to their IC_{50} values, actinomycin D, Z5 and K2T caused 45–55% inhibition of RNA synthesis after 1–2 hr of incubation. At those times, both nucleolar and nuclear fluorescence were observed (result is similar to those shown in Fig. 1b). At 4 hr of incubation with actinomycin D and its analogs, about 75% inhibition of RNA synthesis was observed. During this time, the cells showed only nucleoplasmic fluorescence (result is similar to those shown in Fig. 1c). These results indicate that a direct relationship exists between actinomycin-induced protein B23 "translocation" from the nucleolus to nucleoplasm and inhibition of RNA synthesis.

DISCUSSION

Actinomycin D is known to inhibit RNA synthesis [5]. High doses (2.0 μg/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 [24, 25]. It was reported that actinomycin D inhibits transcription by binding to the DNA template [26]. However, the mechanism of production of nucleolar segregation by the actinomycin D is not yet known. The present studies show that the inhibition of RNA synthesis by actinomycin D and its analogs was associated with translocation of phosphoprotein B23 from nucleoli to the nucleoplasm. These results (Table 2) also correlate with the antimicrobial effects of actinomycin D analogs [27]. Earlier studies by Busch *et al.* [27] indicated that binding of actinomycin D to protein B23 might occur.

The present studies demonstrate that a modification of amino acid residues of actinomycin D (e.g. both sarcosines replaced by glycine or both L-prolines replaced by *trans*-4-methyl-prolines) affected the degree of RNA synthesis inhibition and the translocation of protein B23. This effect may be due to the changes in drug conformation or to decreases in binding forces that are responsible for the recognition and preferential binding of the pentapeptide side chains of actinomycin D to rDNA.

The function of protein B23 and the mechanism of the translocation from nucleoli to nucleoplasm is not yet known. It is possible that the movement of protein B23 may be due to loss of binding sites in

Table 2. Summary of the activities of actinomycin D and its analogs

Drug	Antimicrobial activities (%)	IC_{50}* (ng/ml) (RNA synthesis inhibition)	Translocation of protein B23 from nucleoli to nucleoplasm
Actinomycin D	100	9.5 ± 3.2	++++†
Actinomycin Z5	ND‡	59.1 ± 19.6	+++
Actinomycin K2T	50	1423.3 ± 212.2	++
Actinomycin 4-4′-gly	0	No inhibition at 2000	+

* HeLa cells cultured in minimum essential medium (Eagle's) were preincubated with various doses (5–2000 ng/ml) of actinomycin D, Z5, K2T or 4-4′-gly for 30 min before [^{3}H]uridine was added. Cultures were incubated at 37° for 2 hr, and [^{3}H]uridine incorporation was determined. The dose of drug that produced 50% inhibition is assigned the IC_{50} values. The IC_{50} values in this table represent the mean ± S.D. of four experiments.

† ++++ = most potent, + = least potent.

‡ Not determined.

Table 3. Time course of the effect of actinomycin D and its analogs on the RNA synthesis and location of protein B23

Drug	Time (hr)	Inhibition of RNA synthesis* (%)	Immuno-fluorescence†
Actinomycin D	0	0	A
(10 ng/ml)	1	47.5 ± 5.2‡	B
	2	55.0 ± 4.3§	B
	4	88.8 ± 5.6‖	C
Actinomycin Z5	0	0	A
(40 ng/ml)	1	46.7 ± 3.9‡	B
	2	54.5 ± 3.8§	B
	4	77.1 ± 4.9‖	C
Actinomycin K2T	0	0	A
(1400 ng/ml)	1	46.8 ± 4.7‡	B
	2	50.7 ± 6.1§	B
	4	75.5 ± 3.0‖	C

HeLa cells were cultured on slides. An equivalent dose (IC_{50}) of actinomycin D or its analogs was added to the culture medium for 1, 2 and 4 hr. The cellular incorporation of [^{3}H]uridine and the localization of protein B23 were then determined. Viability of the cells was over 95% under these conditions.

* Percent of RNA synthesis inhibition was calculated as

$$100 \times \left[1 - \frac{[^3\text{H}]\text{uridine uptake in treated HeLa cells}}{[^3\text{H}]\text{uridine uptake in control HeLa cells}}\right]$$

The values represent the mean ± S.D. of three experiments.

† A = Over 90% of the cells had bright nucleolar fluorescence with little or no nuclear fluorescence (Fig. 1a). About 1200 cells were counted. B = Over 90% of the cells had both nuclear and nucleolar fluorescence (Fig. 1b). About 1200 cells were counted. This is an intermediate phenomenon between A and C. C = over 95% of the cells had homogeneous nuclear fluorescence but no distinct nucleolar fluorescence was observed (Fig. 1c). About 1200 cells were counted.

‡ There were about 1.0×10^6 cpm [^{3}H]uridine incorporated/1.0 mg protein in control HeLa cells.

§ There were about 2.0×10^6 cpm [^{3}H]uridine incorporated/1.0 mg protein in control HeLa cells.

‖ There were about 4.0×10^6 cpm [^{3}H]uridine incorporated/1.0 mg protein in control HeLa cells.

nucleoli in the pre-segregation stage. It was reported by Jordan and McGovern [28] that, after a treatment of human diploid fibroblasts with 80 ng/ml of actinomycin D for 1 hr, the nucleoli developed the characteristic segregation, but the degree of segregation was less pronounced than that observed with higher doses. However, the present study indicates that, in drug-induced decreases in rRNA synthesis, protein B23 was translocated from nucleoli to nucleoplasm. Preliminary studies (Chan *et al.* manuscript in preparation) suggest that there may be two forms of protein B23 in nucleoli, of which only one may be found in the nucleoplasm during serum deprivation. The nucleoplasmic form of protein B23 may be derived from the nucleolar form, which, after synthesis in the cytoplasm, may undergo processing such as phosphorylation, amidation or a conformational change before entering the nucleoli. It is also possible that there is an equilibrium governing the translocation of protein B23. Under normal conditions, protein B23 is localized in nucleoli, whereas under conditions that decrease growth the equilibrium may shift and protein B23 may not enter nucleoli but may remain in the nucleoplasm. The exact mechanisms of translocation of protein B23 to the nucleoplasm and its role in biosynthesis of rRNA require further investigation.

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