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Altered subcellular distribution of nucleolar protein fibrillarin by actinomycin D in HEp-2 cells[1]

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KEY WORDS dactinomycin; nuclear proteins; fluorescence antibody technique; immunoblotting

ABSTRACT

AIM: To study the effects of actinomycin D on subcellular distribution of nucleolar protein fibrillarin in HEp-2 (human esophageal epithelial type 2) cells, and molecular mechanisms for maintenance of fibrillarin in nucleolus. **METHODS:** Indirect immunofluorescence assay was employed to investigate subcellular distribution of nucleolar protein fibrillarin and immunoblotting analysis was used to detect the total cellular amount of fibrillarin. **RESULTS:** Control cells with no drug treatment showed bright clumpy nucleolar staining, which indicated that fibrillarin decorated the nucleolus only. Treatment with actinomycin D caused dislocation of fibrillarin from nucleoli to nucleoplasm with numerous stained small nucleoplasmic entities. Immunoblotting showed that neither total cellular amount of fibrillarin nor the integrity of fibrillarin was changed upon the treatment. The dislocation of fibrillarin in cells treated at a lower concentration (0.05 mg/L) of actinomycin D, was totally reversible after removal of the drug from the medium. However, this reversion was not observed at a high drug concentration (1 mg/L). **CONCLUSION:** Actinomycin D induced dislocation of fibrillarin from nucleoli to nucleoplasm in HEp-2 cells. The retention of fibrillarin within the nucleolus was related to active RNA synthesis.

INTRODUCTION

The nucleolus of eukaryotic cells is the site of ribosome assembly where synthesis and processing of the rRNA precursor molecules (pre-rRNAs) as well as their coordinate assembly with specific ribosomal and nonribosomal proteins to form preribosomal particles took place[1]. Besides the small nucleolar RNAs (snRNAs), several proteins, including fibrillarin and B23, are found in the nucleolus[2].

Fibrillarin (B36, NOP1) is an abundant nucleolar protein which plays a role in pre-rRNA processing. It has a molecular weight of 34 kDa and is localized both in the fibrillar center and dense fibrillar center of the nucleolus[3]. The similarity in primary structure from a number of species indicates that fibrillarin has been structurally and functionally conserved during evolution[4]. It serves as an important component in nucleolar function and is required for multiple events leading to rRNA maturation and ribosome-subunit assembly.

The antitumor antibiotic actinomycin D is the most widely used inhibitor to RNA synthesis[5]. It is able to bind to duplex DNA and inhibit the progression of DNA-dependent RNA polymerase in all organisms[6]. Nucleolar protein B23, a marker protein for the granular center of the nucleolus, is known to translocate from nucleoli to other regions of the nucleus after treatment with ac-

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tinomycin D[7-10]. In contrast, the distribution of some other nucleolar proteins such as fibrillarin in human HeLa cells is unchanged. Similarly, no alteration of subcellular localization of fibrillarin was observed in actinomycin D-treated PtK2 (rat kangaroo kidney epithelial) cells[11] and 3T3 (mouse macrophage) cells[12] as well. Although one paper showed the dislocation of fibrillarin from nucleoli to nucleoplasm by immunoblotting the fractions of frog *Xenopus* laevis treated with actinomycin D[13], there is no report showing *in situ* alteration of subnuclear distribution of fibrillarin after this drug treatment.

In this study, the effects of actinomycin D on subcellular distribution of nucleolar protein fibrillarin in human HEp-2 cells, and molecular mechanisms for nucleolar maintenance of fibrillarin were investigated.

MATERIALS AND METHODS

Cells HEp-2 cells were grown in complete RPMI-1640 containing 10 % fetal calf serum and supplemented with *L*-glutamine (2 mmol/L), salt pyruvat (1 mmol/L), 1 % non essential amino acids, and streptomycin (10 mg/L) in a 10 % CO_2 moist incubator at 37 ºC.

Antibodies Goat antibody against fibrillarin (Santa Cruz) and mouse monoclonal anti-B23 antibody (Sigma) were used as primary antibodies for indirect immunofluorescence. Goat antibody was also used as primary antibody against fibrillarin for immunoblotting. As secondary antibodies goat anti-mouse fluorescein-conjugated IgG, rabbit anti-goat fluorescein-labeled IgG, and peroxidase-conjugated rabbit anti-goat IgG (Jackson Immuno-Research Laboratories) were used.

Actinomycin D treatment Actinomycin D was obtained from Sigma company. Stock solution of actinomycin D was prepared in ethanol. It was diluted at least 2500-fold and prepared freshly before addition to cells.

Indirect immunofluorescence assay HEp-2 cells were grown on coverslips in petri dishes. After fixation and permeabilization, primary antibodies were diluted at 1:200 and 1:20 in PBS for anti-fibrillarin and anti-B23 antibodies, respectively, and incubated with the cells in a moist chamber for 1 h at room temperature (RT). After three washes in PBS, secondary antibodies were incubated with the cells for 30 min at RT. The slides were viewed under a fluorescence microscope (Leitz).

Western blot Total HEp-2 cell extract was separated by 10 % non-gradient SDS-PAGE and the proteins were electronically transferred onto a nitrocellulose membrane (NC). The NC membrane was soaked in blocking solution (4 % nonfat dried milk and 0.1 % Tween-20 in PBS) at RT for 1h. It was then incubated with goat antibody against fibrillarin (1:200 diluted in blocking solution) for 1 h at RT. After washing in PBS-T buffer (0.5 % Tween-20 in PBS), the NC membrane was incubated for 45 min with peroxidase-conjugated rabbit anti-goat IgG, diluted at 1:5,000 in blocking solution. The NC membrane was then washed with PBS-T and stained with the ECL Western blotting detection system according to the manufacturer's instructions (Amersham Life Science).

RESULTS

Dislocation of fibrillarin after actinomycin D treatment in HEp-2 cells Treatment of HEp-2 cells with actinomycin D altered the immunofluorescence pattern observed for nucleolar protein fibrillarin. Control cells with no drug treatment gave bright clumpy nucleolar staining (Fig 1A). Treatment with actinomycin D (0.05, 0.1, and 1 mg/L, respectively) at 37 ºC for 4 h resulted in dislocation of fibrillarin, eg, a marked decrease in nucleolar staining. However, a bright nucleo-

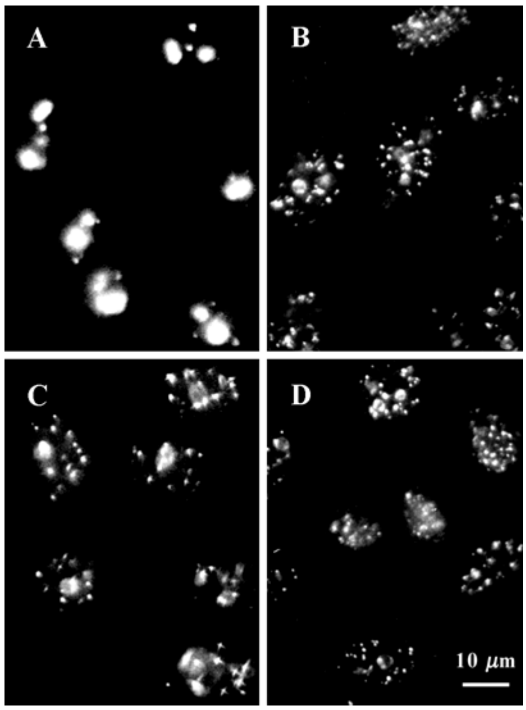

Fig 1. Immunofluorescence of fibrillarin in HEp-2 cells untreated (A) or treated with actinomycin D 0.05 mg/L (B), 0.1 mg/L (C), or 1 mg/L (D) for 4 h.

plasmic staining was visualized in these cells (Fig 1B, 1C, and 1D).

Altered subcellular localization of nucleolar protein B23 in HEp-2 cells Nucleolar protein B23, which is known to relocalize after actinomycin D treatment in many cell types, was also examined by indirect immunofluorescence in HEp-2 cells. The staining for B23 was concentrated in nucleoli of untreated cells (Fig 2A), but translocated diffusely throughout the nucleus after treatment with actinomycin D (Fig 2B).

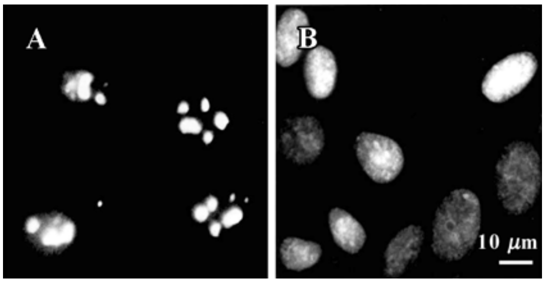

Fig 2. Actinomycin D alters the immunofluorescence pattern of B23 in HEp-2 cells. A) cells without treatment; B) cells treated with actinomycin D 0.05 mg/L for 4 h.

Fibrillarin content does not change upon treatment with actinomycin D After treatment with actinomycin D, HEp-2 cells were washed and lysed under denaturing conditions. Protein amount was compared by immunoblotting analysis after seperation on a 12 % SDS-PAGE. Immunoblotting demonstrated no change in total cellular fibrillarin content (Fig 3). Thus, the actinomycin D treatment did not affect the net amount of fibrillarin, whereas it did change the cellular distribution of fibrillarin.

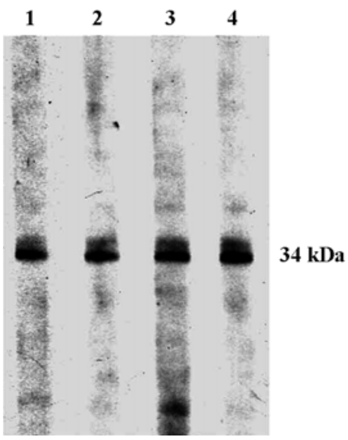

Fig 3. Immunoblotting of fibrillarin in untreated (1) and actinomycin D 0.05 mg/L (2), 0.1 mg/L (3), or 1 mg/L (4) treated HEp-2 cells.

Dislocation of fibrillarin is reversible Additional experiments were undertaken to assess the persistence and the reversibility of the actinomycin D-induced dislocation of fibrillarin. When HEp-2 cells were incubated with actinomycin D 0.05 mg/L for 4 h, dislocation of fibrillarin was observed in every cell (Fig 4A). Removal of the drug from the culture medium allowed the reaccumulation of fibrillarin in nucleoli in every cell (Fig 4B). However, reaccumulation was not observed (Fig 4D) after treatment with a high concentration of actinomycin D (1 mg/L for 1 h).

DISCUSSION

Fibrillarin is a component of terminal balls at the 5' end of the nascent rRNA transcripts[14], and its presence in the nucleoli might depend in part on the presence of the initial rRNA precursors[12]. Without destroying the cells, we demonstrated redistribution of nucleolar protein fibrillarin from nucleoli to nucleoplasm (Fig 1) after treatment of HEp-2 cells with actinomycin D. Actinomycin D may displace RNA polymerases by binding to DNA thereby inhibitting RNA synthesis[15]. Furthermore, actinomycin D-induced decrease in nucleolar fibrillarin occurred without any alteration in fibrillarin protein content and without cleavage of fibrillarin (Fig 3). These results may reflect redistribution of fibrillarin from nucleoli to nucleoplasm when transcription is inhibited.

Another nucleolar protein B23, which translocates from nucleoli to nucleoplasm upon treatment with RNA synthesis inhibitors in some cell types such as HeLa cells[7-10] and P388D1 cells[16], was also translocated in HEp-2 cells in the present study (Fig 2).

At low concentrations (0.04-0.05 mg/L), actinomycin D inhibits transcription mediated by RNA polymerase I in the nucleolus but not transcription by RNA polymerase II and III[5,17]. Reaccumulation of fibrillarin from nucleoplasm to nucleoli was observed upon removal of the lower concentration (0.05 mg/L) of actinomycin D (Fig 4). It extended the results described above by the finding that the effect on subcellular redistribution of fibrillarin induced by low concentrations of actinomycin D was reversible. However, even after extended period of drug removal, dislocation of fibrillarin was irreversible upon challenge of cells with high concentrations of actinomycin D which caused severe in-

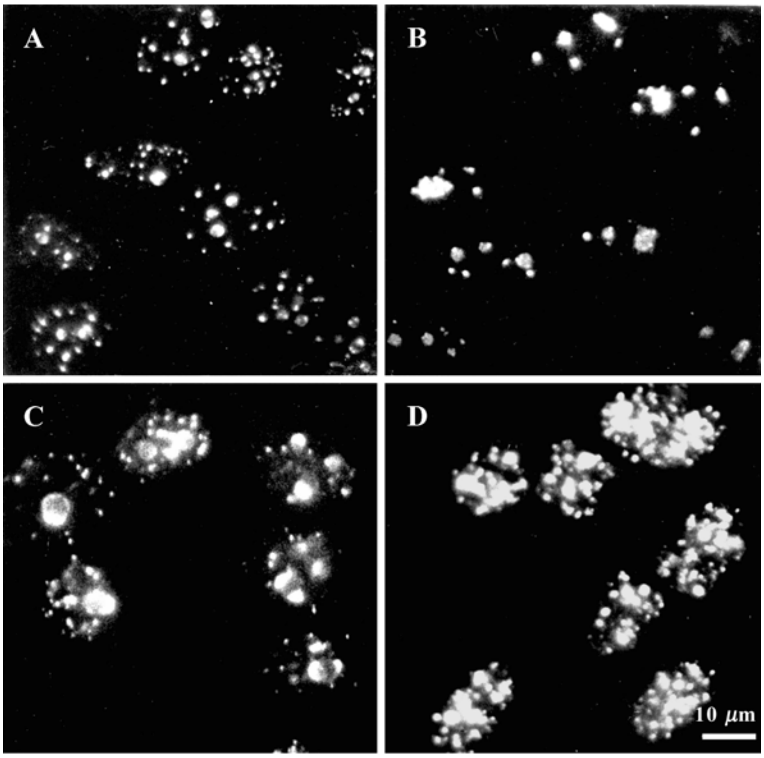

Fig 4. Localization of fibrillarin in HEp-2 cells after removal of actinomycin D. A) cells treated with actinomycin D 0.05 mg/L for 4 h; B) cells treated with actinomycin D 0.05 mg/L for 4 h, washed, and incubated for 24 h in drug-free culture medium; C) cells treated with actinomycin D 1 mg/L for 1 h; D) cells treated with actinomycin D 1 mg/L for 1 h, washed, and incubated for 27 h in drug-free culture medium.

hibition of RNA synthesis.

The dense fibrillar center and the edge of the fibrillar center are the nucleolar regions where transcription takes place. Concerning the similar nucleolar localization of fibrillarin and transcription sites, the observation that even at high concentrations of actinomycin D, only some but not all of fibrillarin dislocated to the nucleoplasm revealed direct evidence that presence of fibrillarin in the nucleolus was partially dependent on active transcription. Since transcription status varied in different cell types, active transcription might be crucial to the dislocation of fibrillarin.

In conclusion, our results showed the *in situ* evidence that actinomycin D did induce dislocation of fibrillarin in HEp-2 cells. Different results from other cells suggested that dislocation of fibrillarin by actinomycin D appeared to be cell type-specific but not species-specific. Moreover, the results also indicated that the retention of fibrillarin within nucleolus was related to rRNA synthesis.

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