THE BINDING OF ACTINOMYCIN D-^{3}H TO HETEROCHROMATIN AS STUDIED BY QUANTITATIVE HIGH RESOLUTION RADIOAUTOGRAPHY

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INTRODUCTION

A few studies have been carried out on the intracellular distribution of actinomycin D-^{3}H all of them at the level of light optical radioautography since the specific activity of the labeled antibiotic did not permit its ultrastructural localization. These studies have shown that actinomycin D-^{3}H is located in the nucleus (1, 4, 8) and is associated with the deoxyribonucleoprotein fraction (10) and that treatment with DNAse results in the disappearance of silver grains while RNase has no effect (12). Since the selective cytological target effect of actinomycin D is a segregation of nucleolar components (24), an attempt was made to localize the silver grains in the nucleolar region (19). The availability of an actinomycin D with a high specific activity makes possible the study of its distribution in cultured cells by means of quantitative high-resolution radioautography.

MATERIAL AND METHODS

The cell line (BHK) used for these experiments is derived from the kidneys of Syrian hamsters. The cells are grown in prescription bottles with a modified Eagle's solution. 48 hr after subculture, the cells had formed a monolayer and were treated with actinomycin D-^{3}H, a product of Schwarz BioResearch Inc. (Orangeburg, New York), prepared by incorporation of L-methionine-methyl-^{3}H into *Streptomyces antibioticus*. The radiochemical purity checked by paper chromatography is 95% at area D; the specific activity is 3.8 c/mmole.

The tritiated antibiotic was added to the culture medium to a concentration of 75 μc/ml for 10, 20, 30, and 60 min. After removal of the radioactive medium, the cells were fixed in situ by addition of 2.5% glutaraldehyde for 1 hr followed by 2% osmium tetroxide for 1 hr, each fixative being in Veronal-acetate buffer at pH 6.1. Careful washing was carried out with the same buffer. The cells were then scraped off the glass wall and centrifuged at 10,000 *g* for 15 min. The pellet was cut into small cubes and post-fixed overnight at room temperature in Ryter-Kellenberger's fixative, buffered with veronal at pH 6.1, and supplemented with 0.5% uranyl acetate. This treatment allowed better preservation of the cells and enhanced their contrast. The specimens were dehydrated in graded alcohols and embedded in Epon.

Quantitative Radioautography

Uniform sections (silver) were obtained with the LKB ultramicrotome and transferred to a glass slide

previously covered with collodion. The sections were then stained with a saturated aqueous solution of uranyl acetate for 1 hr, followed by lead citrate for 15 min, and vacuum-coated with carbon (22). The slides were then coated by dipping (9) with either Ilford L4 or the Gevaert NUC 307 (Anvers, Belgium) emulsion diluted 1:4 in distilled water and were exposed for 6 wk. Development was done in a freshly prepared bath of D19 (Kodak, Eastman Kodak Co., Rochester, N. Y.) for 5 min at 18°C. After fixation with buffered hyposulfite, the slides were dried, rinsed, and a 200-mesh grid was slid under the sections by the method of floating off the collodion membrane. The radioautograms were photographed at 80 kv with a Siemens Elmiskop I at a uniform magnification of 6,000 with an objective aperture of 50 μ.

Two methods were used for quantitation. The concentration of the radioactivity over the nucleolus, nucleus, and cytoplasm was estimated by counting the grains on the photographic plate, and the surface approximated by superposition of a transparent sheet calibrated in square microns (μ^2).

The second method was used to determine the distribution of the radioactivity inside the nucleus, i.e. between the nucleolus-associated chromatin, the heterochromatin, the euchromatin, and the nucleolar body. The plates were developed on 18 × 24 Kodak paper G2 and the outlines of the structures were traced on paper and cut out. Such a development allows better distinction between grains should they occur in cluster. The surface was estimated by weight and converted into μ^2. With the resolution of the emulsion Ilford L4 (3, 21), most of the cellular structures are large enough to permit identification of a radioactive emission.

The essential requirements for quantiative radioautography (9) with respect to the uniformity of the sections, the layering, exposition, and development of the emulsion were carefully respected. All quantitations were done with the Ilford L4-coated radioautographs; the background averaged 2 grains per 100 μ^2.

RESULTS

General Observations

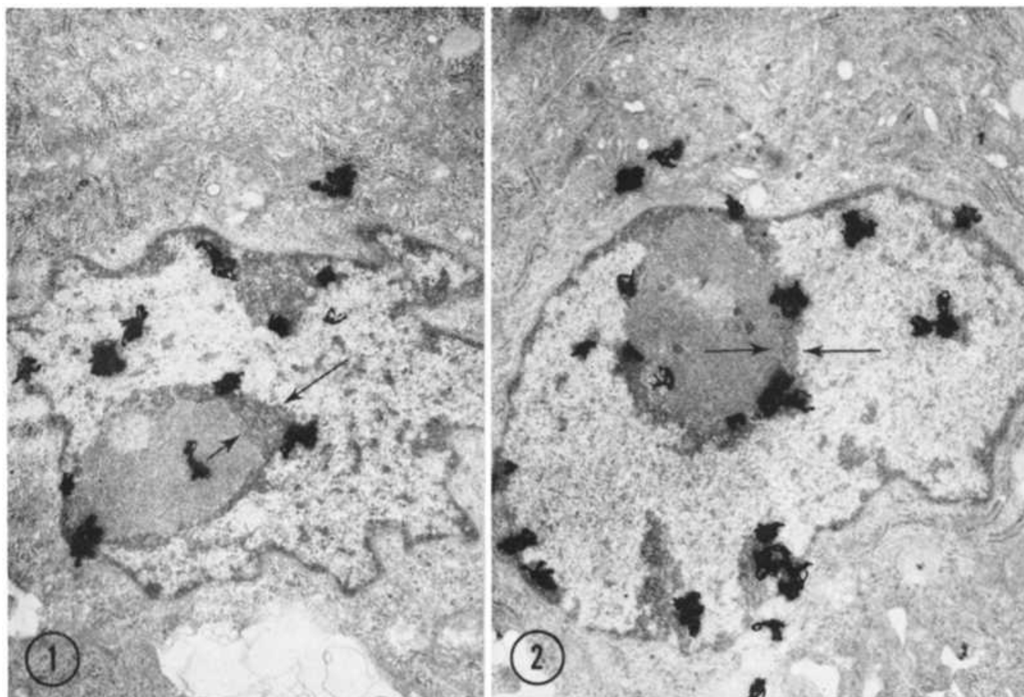
The triple fixation procedure described here allows an excellent contrast and preservation of the cellular ultrastructure of cultured cells and is highly adapted for the purpose of high resolution radioautography. The staining of such fixed cultured cells can be done before layering the emulsion, and there is no need for a removal of the gelatin, either with alkaline or proteolytic digestion (17), in order to thin down the membranes for postexposure staining. As pointed out by Granboulan (9), removal of the gelatin is a delicate procedure and must be adapted by trial and error. Though the procedure may possibly cause displacement or loss of silver grains (3), it was, until now, necessary in cultured cells in which contrast is never so strong as in tissues. The triple fixation makes gelatin removal unnecessary and preserves remarkably well the nuclear structures.

The heterochromatin appears as contrasted masses located along the nuclear membrane or as scattered patches in the nucleoplasm: it will be referred to here in the same sense defined by Heitz (11) as part of the chromosome which retains its dense staining after cell division. Part of the heterochromatin surrounds the nucleolar body with intranucleolar branches: it will be referred to as the nucleolus-associated chromatin; the behavior of this nucleolar chromatin has been shown to be different from that of the rest of the heterochromatin (5, 14). The other portion of the chromosomes, the euchromatin, appears in the form of swollen masses of low electron opacity filling part of the interchromatinic zones of the nucleus. The outlines of heterochromatin, nucleolus-associated chromatin, and nucleolus are well demarcated and allow quantitation for the localization of actinomycin D–^{3}H (Figs. 1–4).

Most of silver grains are found on the condensed portion of the chromatin and on the nucleolus-associated chromatin (Fig. 1). Occasionally a ring of silver grains occurs around the nucleolar body (Fig. 2). As treatment progresses, the number of grains increases with little change in their localization (Figs. 3–4).

Distribution of Actinomycin D–^{3}H in BHK Cells

Table I shows the distribution of the radioactivity in the nucleus, nucleolus (with its associated chromatin), and cytoplasm of BHK cells treated with actinomycin D–^{3}H for increasing periods of time. The number of silver grains reported on the surface in μ^2 indicates that the concentration of radioactivity is always higher in the nucleolus than in the nucleus while the radioactivity in the cytoplasm is negligible. However, with longer treatment the ratio of the concentration of radioactivity in the nucleolus to that in the nucleus tends to decrease towards a value of 1, while the total radioactivity increases remarkably. There is no appreciable change in the radioactivity of the cytoplasm even after a treatment of 1 hr (Fig. 5).



FIGURES 1 and 2 BHK cells treated with actinomycin D-^{3}H for 10 min (Fig. 1) and 20 min (Fig. 2). The silver grains are located mostly on the condensed portion of the nuclear chromatin, and around the nucleolar body, on the nucleolus-associated chromatin (arrows). A few silver grains are seen in the cytoplasm close to the nuclear membrane. Triple fixation. Uranyl acetate and lead citrate. Ilford L4 emulsion. × 15,000.

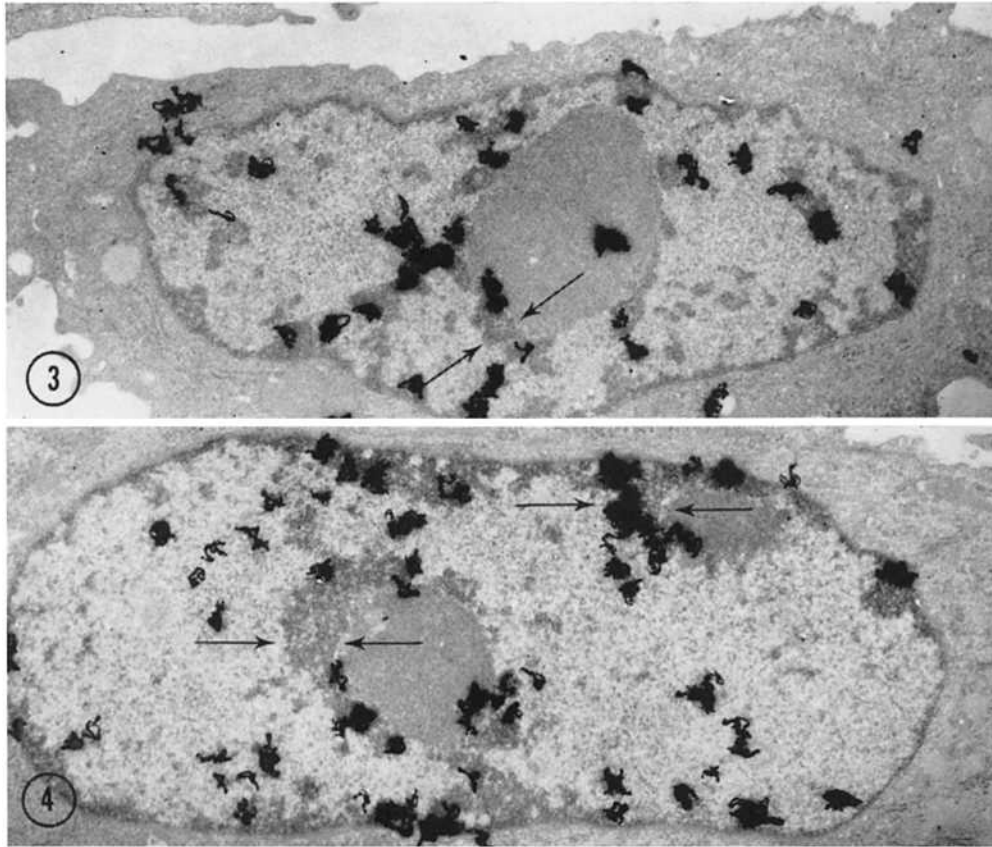
Distribution of Actinomycin D-^{3}H in the Nucleus

This second step in the quantitation was designed for the comparison of the radioactivity in the different nuclear compartments; the hetero- and euchromatin, the nucleolus-associated chromatin, and the nucleolar body (Table II). The surface was approximated by the cut-and-weight technique as described above. The comparable results for grain counts and surface approximation in both experiments are noteworthy. The bulk of the radioactivity is concentrated equally in the nuclear heterochromatin and nucleolus-associated chromatin, and little activity is evident in the euchromatin or the nucleolar body. The kinetic study (Fig. 6) shows that the ratio of the concentration of radioactivity in the nucleolus-associated chromatin to that in the heterochromatin is always close to 1, whatever the duration of treatment, while there is a slow increase in the activity of the euchromatin as treatment progresses. The uptake of the radioactivity by the nucleolar body remains always low and negligible.

DISCUSSION

It is not within the scope of this study to discuss the value of high-resolution radioautography as a quantitative tool for the localization of drugs at the ultrastructural level. Several authors have already analyzed the problem and excellent reviews of the question are available (3, 23). Droz and Bergeron estimate that when all the conditions are respected reproducible results can be obtained with a standard deviation less than 10% (2).

The localization of actinomycin D-^{3}H in the nuclear region of BHK cells is confirmed in the present work and does not come as a surprise. The amount of radioactivity in the cytoplasm remains always negligible, whatever the duration of treatment. Moreover, the majority of the cytoplasmic silver grains were within a range of 0.1–0.3 μ of



FIGURES 3 and 4 BHK cells treated with actinomycin D-^{3}H for 30 min (Fig. 3) and 60 min (Fig. 4). The number of silver grains has increased both in the condensed chromatin (Fig. 3) and in the nucleolus-associated chromatin (arrows) (Fig. 4). The dispersed chromatin shows some activity in Fig. 4. Same technique as for Figs. 1 and 2. × 12,000.

TABLE I

Distribution of the Radioactivity in BHK Cells Treated with Actinomycin D-^{3}H for Various Periods of Time

	Nucleolus			Nucleus			Cytoplasm		
Tm*	GC	S	CR	GC	S	CR	GC	S	CR
10	110	62.5	1.74	322	459	0.7	196	960	0.2
20	122	70.0	1.74	442	485	0.91	150	800	0.18
30	183	65.5	2.8	672	437	1.5	217	1038	0.23
60	246	39.0	6.3	1820	427.5	4.25	230	728	0.3

* Tm = time in minutes. The concentration of the radioactivity (CR) represents in each experiment the ratio of the total grain count (GC) in 20 cells to the surface (S) in square microns measured by the calibrated transparent sheet method.

the nuclear membrane so that a high probability exists that they result from β-particles emitted by a tritium source located in the nucleus. No special radioactivity was found in mitochondria: BHK cells have small oval-shaped mitochondria with rudimentary cristae and rare DNA fibers. The number of silver grains increases considerably as a function of duration of treatment with isotope,

thus indicating that more binding sites become available as treatment progresses. The same results have been obtained by Harbers et al. (10) with actinomycin C-^{14}C localization in ascites tumor cells.

In the nucleus, actinomycin D-^{3}H is located preferentially in the nucleolus if the concentration of the radioactivity in the nucleolus with its associated chromatin is simply compared to that in the whole nucleus. This ratio decreases from 2.54 for a 10-min treatment to 1.48 for a 60-min treatment as the concentration of the radioactivity increases proportionally in the nucleus. However, a simple look at Figs. 1–4 shows that (*a*) the radioactivity is located on the nucleolus-associated chromatin, (*b*) the number of silver grains appears as significant in the condensed portion of the nuclear chromatin or heterochromatin, and (*c*) little radioactivity is present in the swollen and diffuse euchromatin, at least for short pulses. Since the nucleolus-associated chromatin is part of the heterochromatin, it seemed of immediate interest to compare the concentrations of radioactivity in both of them as opposed to the euchromatin and to the nucleolar body.

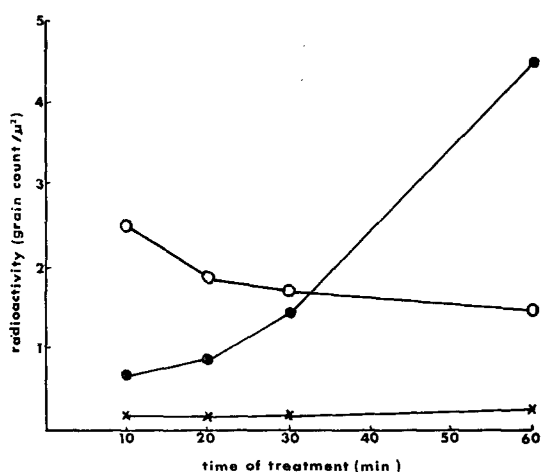


FIGURE 5: Kinetic analysis of the radioactivity as seen in Table I. The concentration of the total radioactivity in the nucleus (●——●) increases as treatment progresses in time, while that of the cytoplasm (×——×) remains constantly low. The ratio $\frac{\text{nucleolar}}{\text{nuclear}}$ concentration of the radioactivity (○——○) tends to decrease towards a value of 1.

With this second method of quantitation, the results are clear. The radioactivity is concentrated equally in the nucleolus-associated chromatin and in the heterochromatin, whatever the duration of treatment. The radioactivity in the euchromatin and the nucleolar body remains extremely low although the activity in the euchromatin increases slowly for long pulses.

The fact that actinomycin D-^{3}H is located mostly in condensed masses of heterochromatin whether it is associated with the nucleolus or not is significant, and two explanations for this can be proposed: (*a*) The first deals with the amount of DNA found in both forms of chromatin. The presence of more silver grains in heterochromatin could be interpreted in terms of more DNA available for the binding with the tritiated antibiotic. There are little data and much controversy concerning the amount of DNA in both the hetero- and euchromatin because of the difficulty of isolating them. But it has been claimed that there is more DNA in hetero- than in euchromatin, as demonstrated in *melanopus* by cytophotometric methods (13) and in calf thymus lymphocytes by biochemical methods (7). Whether these measurements remain valid for cultured cells in exponential growth has not been demonstrated. (*b*) A second

TABLE II

Distribution of the Radioactivity within the Nuclear Structures of BHK Cells Treated with Actinomycin D-^{3}H

	Heterochromatin			Nucleolus-Ass. Chromatin			Euchromatin			Nucleolar Body		
Tm	GC	S	CR	GC	S	CR	GC	S	CR	GC	S	CR
10	175	18	1.95	71	6	2.01	54	67	0.14	7	9	0.14
20	169	15	2.07	62	6	1.90	60	69	0.16	5	10	0.09
30	419	22	3.2	140	8	3.47	133	61	0.38	14	9	0.28
60	620	20	6.2	222	6	6.3	433	67	1.2	20	7	0.57

The structures were traced on paper, cut out, and their surface (S) is expressed here in %, estimated by weight. The concentration of the radioactivity (CR) represents the ratio of the total grain count (GC) in 15 cells to the surface (S) in square microns.

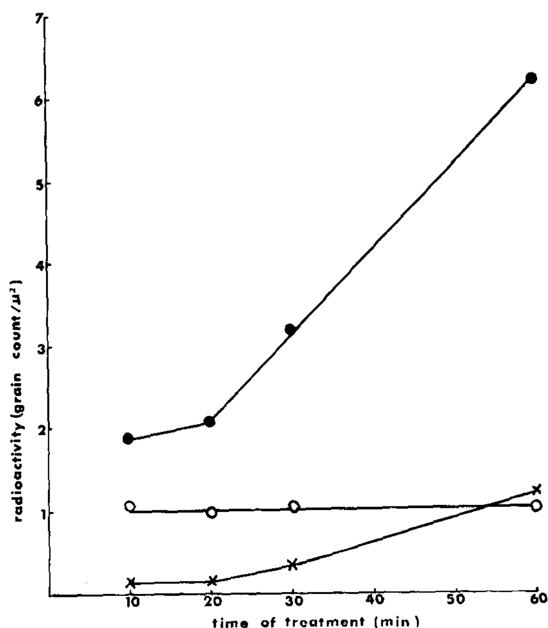


FIG. 6 Kinetic analysis of the radioactivity within the nuclear structures as seen in Table II. The concentration of the radioactivity in the heterochromatin (●——●) is much more significant than in the euchromatin (X——X). But the ratio of the concentration of the radioactivity in the nucleolus-associated chromatin to that in the nuclear hetherocromatin (O——O) remains close to 1, whatever the treatment time may be.

explanation would be to interpret our results in terms of preferential binding. Heterochromatism, as pointed out by Ris (18), is a matter of differential coiling of chromosomal parts during the interphase and is characterized by an increased stability of the DNA helix with no evidence of strand separation or RNA transcription (7). On the contrary the euchromatin consists of swollen masses filling the interphase nucleus (16) in which the DNA molecules are in the form of extended microfibrils with local strand separation and active RNA transcription. According to Frenster (6), molecular species that stimulate RNA synthesis would bind preferentially to the single-stranded loops of DNA in the euchromatin. This could stabilize the strand separation and favor active transcription. Conversely inhibitors of RNA synthesis like actinomycin D, could bind to the tight double-stranded form of DNA of the heterochromatin, tending to stabilize the DNA against strand separation and to depress RNA synthesis. Our findings can also be interpreted according to this view; actinomycin D-^{3}H bound to the repressed heterochromatin would tend to prevent the strand separation of the DNA, thus inhibiting RNA transcription.

However, this interpretation does not take into account the fact that the most active site of RNA synthesis is in the nucleolus (15) and that the nucleolus-associated chromatin is part of the condensed heterochromatin (20). Moreover, actinomycin D, according to morphological criteria, affects selectively the nucleolus, with segregation and exhaustion of the nucleolar components (24), and its biochemical effect is a blocking of large molecular species of RNA precursors of ribosomal RNA (15). Therefore, the hypothesis concerning the state of repression for heterochromatism would not be true for the nucleolus-associated chromatin. More information is needed on the nucleolus-associated chromatin to clarify this problem. According to Sirlin (25), one has to make a distinction between the nucleolar organizer, which contains the cistrons for ribosomal RNA, and the DNA-histone regions penetrating in various degrees into the nucleolar body and forming the nucleolus-associated chromatin. Only a short polycistronic portion of the DNA within the nucleolar chromatin would be active for RNA synthesis, while most of it is in the form of repressed heterochromatin. The contradiction would thus be more apparent than real. The fact that we do not find any differences between heterochromatin and nucleolar chromatin for the binding of actinomycin D-^{3}H then simply reflects the fact that *most* of the nucleolus-associated chromatin is in the same physical and functional stage as heterochromatin. And it would be likely that the first active portion of the genome to be affected by actinomycin D is the derepressed nucleolar organizer.

SUMMARY

Hamster fibroblasts (strain BHK) were treated with tritiated actinomycin D for 10, 20, 30, and 60 min for study of the sites of binding of actinomycin D-^{3}H by quantitative high resolution radioautography. The results indicate a strong nucleolar and nuclear labeling which is proportional to the duration of treatment. The distribution of the radioactivity inside the nuclear compartments (heterochromatin, nucleolus-associated chromatin, euchromatin, and nucleolar body) indicates that actinomycin D-^{3}H is bound equally

to nuclear heterochromatin and nucleolus-associated chromatin, while little activity is seen in the euchromatin and the nucleolar body. Whether this reflects a difference in the amount of DNA in each compartment or a preferential binding is discussed.

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