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The Effect of Inhibitors of Transcription and Translation on Chromosomal Proteins

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SUMMARY

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After the administration of actinomycin D, α-amanitin and D-galactosamine, the [^{3}H]-leucine incorporation into total rat liver proteins and into nonhistone chromosomal proteins decreased to about 50% and 20% of controls, respectively. At a low dose of cycloheximide the [^{3}H]leucine incorporation into nonhistone chromosomal proteins was unaffected, while the incorporation into total liver proteins was decreased to 35% of controls. The nonhistone chromosomal protein to DNA ratios changed to 94%, 78%, 149% and 66% of the controls after actinomycin D, α-amanitin, cycloheximide and D-galactosamine, respectively. SDS-polyacrylamide gel electrophoresis showed different changes in the nonhistone chromosomal protein patterns after the administration of actinomycin D, α-amanitin, cycloheximide and D-galactosamine. The findings are discussed with respect to possible mechanisms involved in liver cell death.

INTRODUCTION

The regulation of gene activity in eukaryotes is still largely unknown. It is important to the understanding of differentiation processes, eukaryotic development, hormone action, and abnormal processes such as cancer. To elucidate the mechanism(s) of gene regulation in eukaryotes, studies have been carried out with systems in which the gene activity is increased, e.g., in cancer cells or hormone treated cells. There is evidence from many studies that the nonhistone chromosomal proteins play a key role in the regulation of gene activity (1, 2).

Although a large number of publications on nonhistone chromosomal proteins exist (for review see reference 1) only a few studies of the effect(s) of various inhibitors of transcription and/or translation are known (3-5). Therefore, nonhistone chromosomal proteins from rat liver chromatin were analyzed after the administration of several well known inhibitors. Actinomycin D (6, 7), α-amanitin (8), and D-galactosamine (9, 10) were used as inhibitors of transcription. Cycloheximide (11) served as an inhibitor of translation.

MATERIALS AND METHODS

D-Galactosamine hydrochloride, cycloheximide, actinomycin D, Triton X-100, SDS[1] were purchased from C. Roth OHG (Karlsruhe, Germany), calf thymus DNA was obtained from Sigma Chemical Company (St. Louis, Mo.), sucrose (ribonuclease free) was from Schwarz/Mann (Orange-

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[1] The abbreviations used are: $PheMeSO_3F$, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate.

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burg, USA). [5-^{3}H]orotic acid (24 Ci/mmol) and [4,5-^{3}H]leucine (53 Ci/mmole) were obtained from the Radiochemical Centre (Amersham, England), α-amanitin was generously supplied by Prof. Th. Wieland (Max Planck Institut, Heidelberg). All chemicals were of highest purity grade available.

Animals. Male Wistar rats (180–200 g) (Ivanovas, Kisslegg, Germany) had free access to water and a carbohydrate-rich, 20% protein diet (Altromin R, Altromin GmbH, Lage/Lippe, Germany). All initial injections were given between 8 and 9 a.m. The animals were fasted overnight prior to an experiment.

Isolation of nuclei. Nuclei were prepared by the method of Blobel and Potter (12) and washed with 1% (v/v) Triton X-100 in 0.25 M sucrose, 25 mM KCl, 5 mM $MgCl_2$, 50 mM Tris-HCl (pH 7.5).

Chromatin preparation. Chromatin was prepared according to Spelsberg and Hnilica (13). The Triton X-100 treated nuclei were washed successively with 0.08 M NaCl, 0.02 M EDTA, pH 6.3 (three times); 0.15 M NaCl, 10 mM EDTA, pH 6.0 (twice); and 1.5 mM NaCl, 0.15 mM citrate, pH 7.0 (twice). The final chromatin preparation was in 1.5 mM NaCl, 0.15 mM sodium citrate, pH 7.0. Shearing of chromatin was avoided during its preparation.

Determination of histones, nonhistone proteins, RNA, and DNA. The nonhistone protein fraction examined in the experiments to be described is defined as follows. Chromatin was extracted with 0.25 N H_2SO_4. This extract contained all histones and in addition some acid soluble chromosomal proteins. The latter represented 18% of protein in the sulfuric acid extract. The acid extracted "chromatin" was dissolved in 0.1 N NaOH, resulting in a solution that contained DNA, RNA, and acid-insoluble chromosomal proteins (i.e., nonhistone chromosomal protein fraction).

RNA was determined according to Fleck and Munro (14) using yeast RNA as standard (A_{260} = 1.000 at 42 μg RNA/ml), DNA was estimated according to Ceriotti (15) with calf thymus DNA as standard. Protein determinations were performed according to Lowry et al. (16) with bovine serum albumin as standard. Total liver RNA was determined according to Bresnick (17).

Determination of [^{3}H]leucine incorporation into proteins. Determination of incorporation into total liver proteins followed the method of Mans and Novelli (18) precipitating 100 μl of liver homogenate on Whatman 3 MM filter paper disks. After successive washes in trichloroacetic acid, ether/ethanol, and a final wash in ether, the filters were dried and counted in 10 ml scintillation fluid (Rotiszint 11 from C. Roth, Karlsruhe, Germany) composed of 5 g 2,5-diphenyloxazole, 0.2 g 1,4-bis(2-[5-phenyloxazolyl])benzene, and 867 g toluene. Incorporation into chromosomal proteins was determined by suspending samples of 0.1 and 1.0 ml in 10 ml of scintillation fluid (Rotiszint 11: Triton X-100 [2:1]) and counting in a liquid scintillation spectrophotometer after 12 hours at 4° in the dark. The counting efficiencies for [^{3}H]leucine were 10% in the case of total liver proteins and 25% for chromosomal proteins, respectively.

Polyacrylamide gel electrophoresis. SDS slab gel electrophoresis was carried out as detailed by King and Laemmli (19) using 160 × 140 × 1 mm separation gels (10% acrylamide) and a spacer gel (3.0% acrylamide) of 10 mm length. The electrode buffer was composed of 0.1% SDS, 0.025 M Tris/HCl, and 0.192 M glycine, pH 8.3. Electrophoresis was carried out at 40 mA/slab gel until the tracking dye had reached the last 4 mm of the lower gel (2 hr at 20°). Gels were stained with 0.25% Coomassie Brilliant Blue R 250 in methanol:acetic acid:water (10:2:10) and destained with methanol:acetic acid:water (1:2:17). Densitometry of stained gels was performed at 550 nm with a Gilford Model 2400 densitometer.

Preparation of samples. Three milliliters of chromatin was centrifuged for 10 min at 40,000 × *g* and the pellet dissolved in a 5 M urea, 2 M NaCl, 10 mM Tris-HCl solution, pH 8.3. $PheMeSO_3F$ (1 mM) was added to inhibit proteinase activities. Chromatin dissociation was carried out at 4° for 10 min by frequent stirring. The dissociated chromatin was centrifuged for 15 hr at 300,000 × *g* to remove the DNA. The supernatant was dialyzed against 0.1% SDS, containing

0.1% 2-mercaptoethanol at room temperature. The dialyzed chromosomal proteins were lyophilyzed and stored at −25°. Prior to electrophoresis, the lyophilyzed chromosomal proteins were dissolved in 200–400 μl of distilled water and the protein concentration was determined. For acrylamide gel electrophoresis 2-mercaptoethanol, glycerol, and Bromophenol Blue were added to final concentrations of 1%, 10%, and 0.001%, respectively. Samples of 60 μg of chromosomal proteins in a volume of 10 μl were applied to the gels.

Amino acid analysis. Animals were stunned, decapitated, and exsanguinated. The livers were freeze-clamped and pulverized with a mortar in the presence of liquid N_2. Four ml of 0.7 N $HClO_4$ were added to 1 g of liver powder and homogenized in a Potter Elvehjem homogenizer at 0°. After centrifugation at 12,000 × *g* for 10 min, the pellet was reextracted with 2 ml of 0.7 N $HClO_4$. The combined supernatants were neutralized with 10 N KOH and $KHCO_3$, aliquots were lyophilyzed and used for amino acid analysis, performed with a Beckman model Biotronik LC 600 automatic amino acid analyzer.

RESULTS

Protein synthesis was measured by the incorporation of [^{3}H]leucine into total liver proteins and into nonhistone chromosomal proteins after the administration of actinomycin D, α-amanitin, cycloheximide and D-galactosamine (Table 1).

The inhibition of [^{3}H]leucine incorporation was similar for all the drugs, except for cycloheximide, which showed the greatest decrease in [^{3}H]leucine incorporation into the total liver proteins and the lowest inhibition of incorporation into nonhistone chromosomal proteins. This effect was even more evident when cycloheximide was administered only once at a dose of 2 mg/kg body weight. Under these conditions nonhistone chromosomal protein synthesis was unchanged, while total protein synthesis was diminished to 35% of controls. Conclusions on alterations in the rate of protein synthesis, based on changes in the rate of incorporation of an amino acid, should always consider changes in the pool size and thus specific radioactivities of the pool. Therefore, leucine pools and specific radioactivities were measured (Table 2) and used to correct the values for protein synthesis after the administration of the various drugs (Table 1).

Table 3 summarizes the results obtained after measuring the effect of actinomycin D, α-amanitin, cycloheximide, and D-galac-

TABLE 1

Effect of actinomycin D, α-amanitin, cycloheximide and D-galactosamine on the incorporation of [4,5-^{3}H]leucine into total liver proteins and the nonhistone chromosomal proteins

Actinomycin D (2 mg/kg), α-amanitin (0.5 mg/kg), cycloheximide (a) (2 mg/kg) and D-galactosamine (800 mg/kg) were injected intraperitoneally into animals fasted overnight. In the case of α-amanitin and cycloheximide (b), a second injection was given after 3 hr. The animals were killed 6 hr after the first injection. The experimental details for the isolation of the nonhistone chromosomal protein fraction are given in MATERIALS AND METHODS. One hour before the animals were sacrificed 1.0 mCi [^{3}H]leucine/kg body weight was injected intraperitoneally. The specific radioactivities of total liver proteins (cpm/mg protein), and nonhistone proteins (cpm/mg DNA) were determined. The data are means of 2 determinations from 2 different animals, with percentages of corresponding control values in parenthesis. The values in parentheses, marked with *, were obtained after taking the various leucine pools (Table 2) into consideration.

	Specific radioactivity					
	Control	Actinomycin D	α-Amanitin	Cycloheximide (a)	Cycloheximide (b)	D-galactosamine
Total liver proteins	2,008	1,285	1,064	863	522	1,084
	(100)	(64)	(53)	(43)	(26)	(54)
		(64)*	(59)*	(35)*	(18)*	(47)*
Nonhistone proteins	6,482	1,231	1,490	6,936	2,528	1,167
	(100)	(19)	(23)	(107)	(39)	(18)
		(19)*	(25)*	(90)*	(27)*	(16)*

TABLE 2

Specific radioactivity of leucine in rat liver after administration of actinomycin D, α-amanitin, cycloheximide, and D-galactosamine

The data in the first two columns are given as μmol or cpm per g of freeze clamped liver, respectively. For experimental details see legend to Table 1 and MATERIALS AND METHODS. Cycloheximide was given at a dose of 2 × 2 mg per kg. The data are means of 2 determinations.

	μmol/g	10^{-3} × cpm/g	Specific radioactivity
			10^{-6} cpm/μmol
Control	0.17	89	0.52
Actinomycin D	0.17	88	0.52
α-Amanitin	0.17	80	0.47
Cycloheximide	0.20	154	0.77
D-Galactosamine	0.11	66	0.60

tosamine on the incorporation of [^{3}H]orotate into total liver RNA. Under the conditions in which the synthesis of nonhistone chromosomal proteins is depressed to the same extent after administration of the drugs (Table 1), a remarkable difference is found for total RNA synthesis for actinomycin D, α-amanitin, and D-galactosamine on the one hand and cycloheximide on the other. This indicates that cycloheximide at the dosage used (2 × 2 mg/kg) does not affect total RNA synthesis. When total RNA per mg DNA was determined after administration of the drugs, only small changes could be detected (Table 4). Whereas the amount of chromosomal RNA per mg of DNA decreased in the experiments with actinomycin D, α-amanitin, and D-galactosamine, a drastic increase was found after cycloheximide.

The administration of actinomycin D, α-amanitin, cycloheximide and D-galactosamine in dosages as described above led to an inhibition of nonhistone chromosomal protein synthesis to about 16–27% of control (Table 1) and of RNA synthesis to 7, 28, and 25% of controls with actinomycin D, α-amanitin, and D-galactosamine, respectively, whereas a slight stimulation of RNA synthesis was found after cycloheximide administration (Table 3). Under these particular experimental conditions, the effect(s) of the various drugs on individual nonhistone chromosomal proteins were examined. Figure 1 shows the patterns of nonhistone chromosomal proteins after administration of actinomycin D, α-amanitin, cycloheximide, or D-galactosamine and electrophoretic separation on SDS-polyacrylamide gels. After the administration of D-galactosamine and α-amanitin, remarkable decreases in the intensities of the non-

TABLE 3

Effect of actinomycin D, α-amanitin, cycloheximide, and D-galactosamine on the incorporation of 5-[^{3}H]orotate into total liver RNA

Actinomycin D, α-amanitin, cycloheximide and D-galactosamine were injected as described in the legend to Table 1. Thirty minutes before the animals were killed 0.5 mCi 5-[^{3}H]orotate/kg were injected intraperitoneally. Total liver RNA was isolated according to Bresnick (17).

Control	Actinomycin D	α-Amanitin	Cycloheximide	D-galactosamine
Specific radioactivity (cpm/mg wet weight)				
1364	97	388	1552	
(100)	(7)	(28)	(114)	(<25)[a]

[a] The value given is taken from Keppler et al. (10).

TABLE 4

Effect of actinomycin D, α-amanitin, cycloheximide, and D-galactosamine on total liver RNA and chromosomal RNA

Actinomycin D, α-amanitin, cycloheximide and D-galactosamine were administered as described in the legend to Table 1. Total liver RNA was determined according to Bresnick (17). For the determination of chromosomal RNA, chromatin was used. The data are means of 2 determinations from 2 different animals with percentages of corresponding control values in parenthesis.

	Control	Actinomycin D	α-Amanitin	Cycloheximide	D-Galactosamine
Total liver RNA (mg/mg DNA)	2.59	2.50	2.68	3.33	2.33
	(100)	(97)	(103)	(129)	(90)
cRNA (mg/mg DNA)	0.143	0.092	0.110	0.216	0.072
	(100)	(64)	(77)	(151)	(50)

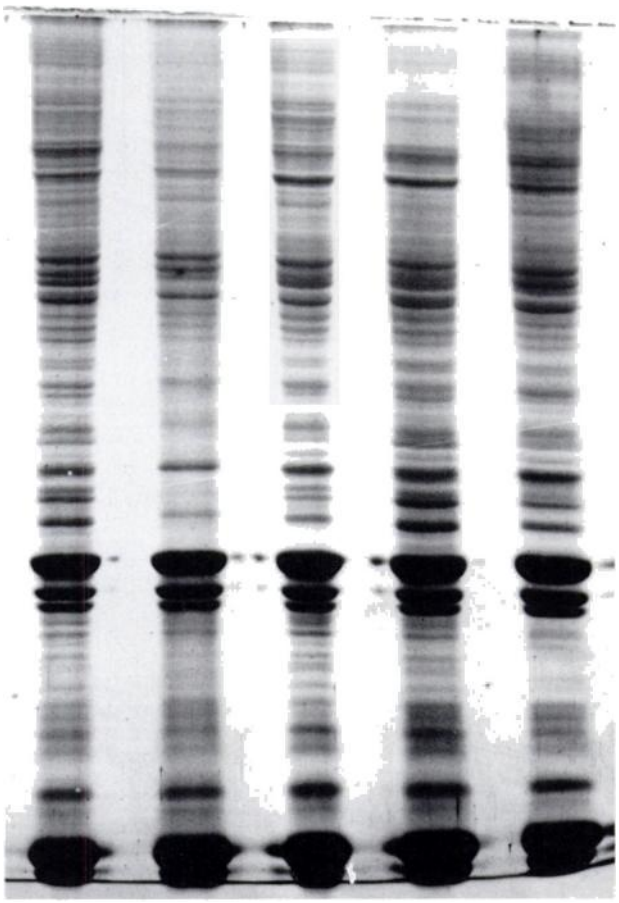

FIG. 1. *SDS-polyacrylamide gel electrophoretic patterns of chromosomal proteins after administration of D-galactosamine, α-amanitin, cycloheximide and actinomycin D.*

Slab gel electrophoresis was performed as described in MATERIALS AND METHODS. In each case 60 μg of chromsomal protein were applied to the gels. (A) Control; (B) D-galactosamine; (C) α-amanitin; (D) cycloheximide; and (E) actinomycin D were injected, as described in the legend to Table 1.

histone chromosomal proteins were observed (Fig. 1, B and C). In the case of cycloheximide, however, a general increase in intensity of the protein bands was found (Fig. 1, D); after the application of actinomycin D, the nonhistone chromosomal protein pattern was essentially unchanged (Fig. 1, E).

A more detailed analysis of the nonhistone chromosomal protein patterns of Fig. 1 is obtained after densitometry (Fig. 2). The three bands in group E represent histone H1, and group G the remaining unresolved 4 histones (H2A, H2B, H3 and H4). The most prominent decreases in intensity of protein bands after D-galactosamine or α-amanitin administration were found in the groups A, C and D in the case of D-galactosamine and in groups C and D in the case of α-amanitin. Slight changes of the protein bands in groups B and C were obtained after actinomycin D administration. Cycloheximide, on the other hand, gave rise to a marked increase in the intensities of

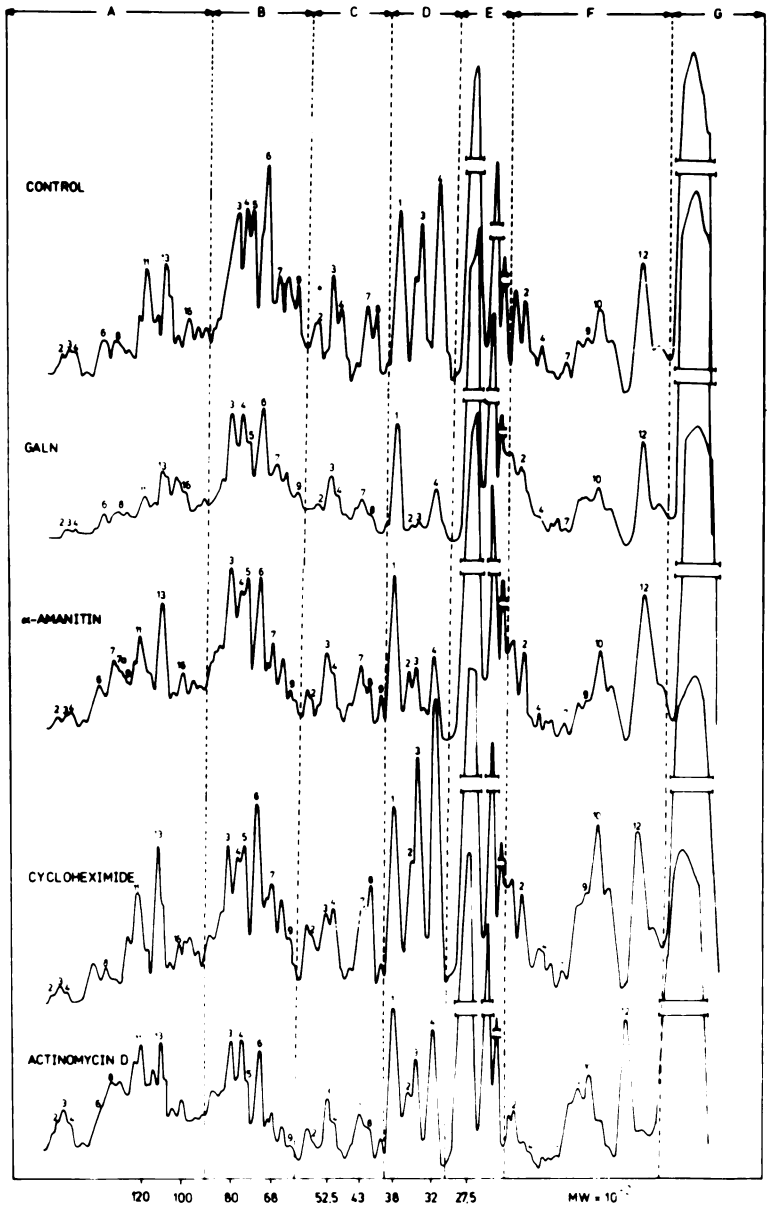

FIG. 2. Densitometer tracings of the stained SDS-polyacrylamide gels of Fig. 1. Abbreviation: GalN, D-galactosamine.

the bands of groups C, D, and F. Since the same amounts of chromosomal proteins were applied to the gels, the changes related to milligrams of DNA cannot be visualized from Figs. 1 and 2. Nonhistone chromosomal protein to DNA ratios of 1.0, 0.94, 0.78, 1.49 and 0.66 were obtained for the control, actinomycin D, α-amanitin, cycloheximide and D-galactosamine, respectively, after planimetry of the areas under the peaks in the densitometer tracings.

DISCUSSION

The fact that the [^{3}H]leucine incorporation into nonhistone chromosomal proteins after actinomycin D, α-amanitin, and D-

galactosamine was much more decreased than incorporation into total liver proteins (Table 1) is very likely due to the high degradation rates of nonhistone chromosomal proteins. Dice and Schimke have shown that nonhistone chromosomal proteins have rather high turnover rates (20). In the case of cycloheximide, however, particularly when only a single dose of 2 mg/kg had been administered (Table 1), it was surprising that the incorporation of [^{3}H]leucine into nonhistone chromosomal proteins was essentially unchanged, whereas a decrease to about 35% of control was observed in the case of total liver proteins. These findings cannot be explained at the present time. One could speculate that the nonhistone chromosomal protein synthesis occurs on ribosomes with a different sensitivity to the action of cycloheximide. Furthermore, the results could also be explained by a site of protein synthesis, not affected by cycloheximide. In this respect protein synthesis within nuclei that has been described by several authors (21–26) should be mentioned. The anomalous effect of cycloheximide on nonhistone chromosomal protein synthesis agrees with the findings of Rothblum *et al.* (3). These authors found a slight stimulation of total liver protein synthesis and a 4-fold increase of [^{3}H]leucine incorporation into nonhistone chromosomal proteins 24 hr after the administration of a single dose of 2 mg cycloheximide per kg of body weight.

When the effects of actinomycin D, α-amanitin, cycloheximide, and D-galactosamine on the nonhistone chromosomal proteins were studied by one dimensional SDS-polyacrylamide gel electrophoresis, changes were found for all inhibitors used (Figs. 1 and 2). In spite of the fact that nonhistone chromosomal protein synthesis was decreased to the same extent for all inhibitors (Table 1), an increase in the nonhistone chromosomal protein content was found after cycloheximide (Figs. 1 and 2). This effect can be explained only by an inhibition of protein degradation and residual protein synthesis. Inhibition of protein degradation after cycloheximide has been described by several authors (27–29).

The marked decrease in the nonhistone chromosomal protein content after administration of α-amanitin and D-galactosamine on the other hand, may be explained by increased degradation and/or loss of nonhistone chromosomal proteins, since after actinomycin D administration only a slight decrease in nonhistone chromosomal proteins was found. It should be mentioned that α-amanitin and D-galactosamine, unlike actinomycin D and cycloheximide, administered in dosages described lead to liver cell death (30–34). It is possible that a marked decrease of nonhistone chromosomal proteins after α-amanitin and D-galactosamine administration is a prerequisite for the development of liver cell injury.

It is well known that the primary transcription products of chromatin, complexes of heterogeneous nuclear RNA, and corresponding proteins are associated with chromatin (35). Although our method of isolation of chromatin involves Triton X-100 treatment, which should remove ribonucleoprotein complexes (36), and washing with 0.14 M NaCl, a salt concentration that is used for the extraction of ribonucleoprotein complexes (35), presently one cannot determine precisely whether the effects of the inhibitors studied are effects exclusively on nonhistone chromosomal proteins, ribonucleoprotein particle proteins, or both.

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