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Cortisone Stimulation of Nucleolar RNA Polymerase Activity

(rat/actinomycin D)

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ABSTRACT **Four hours after intraperitoneal injection of cortisone acetate into rats, hepatic nuclei had an enhanced ability to incorporate labeled nucleoside triphosphates into RNA *in vitro*. When the nuclei were further fractionated into nucleolar and extranucleolar (nucleoplasmic) fractions, it was found that this hormonal effect was localized exclusively in the nucleolar fraction. This increased transcriptive activity potentially could be due to either (*a*) an increased availability of the DNA template or (*b*) an increase in the amount or catalytic efficiency of the RNA polymerase, or both. In order to distinguish between these possibilities, actinomycin D was used to inhibit the template function of endogenous nucleolar DNA and a synthetic template, polydeoxycytidylate, which is insensitive to actinomycin D and codes for polyriboguanylate synthesis, was used to evaluate RNA polymerase activity. These studies indicate that the increased RNA synthesis evident in nucleoli isolated from livers of rats four hours after cortisone treatment is largely a consequence of elevated levels of RNA polymerase.**

Almost a decade ago, this laboratory reported a marked enhancement of rat liver RNA synthesis *in vivo* after glucocorticoid administration (1). Various investigators have since confirmed and extended this finding, demonstrating that ribosomal, transfer, and messenger RNA synthesis are all stimulated by glucocorticoid (2–10). The biochemical mechanisms underlying this hormonal regulation of transcription, however, remain uncertain. It is well established that, in eukaryotic cells, the nucleolus is the site of ribosomal RNA synthesis (11–13). As stimulation of ribosomal RNA synthesis is one of the most pronounced, although not the earliest (9), responses to glucocorticoids, we used isolated nucleoli to study the mechanism by which this hormone regulates gene transcription.

Increased transcriptive activity in the nucleolus may reflect either increased template availability of the nucleolar genome, increased amount and/or efficiency of the RNA polymerase, or both. To elucidate the nature of glucocorticoidal action, we have tried to differentiate between these possibilities. Actinomycin D is a potent inhibitor of DNA-dependent RNA transcription. Its inhibitory action results from specific binding of the antibiotic to deoxyguanosine moieties, and its intercalation into endogenous double-stranded DNA (14). Thus, by the use of actinomycin D, we inactivated the endogenous nucleolar template and replaced it with a synthetic template, polydeoxycytidylate, which is resistant to inhibition by actinomycin D. Using actinomycin D and a fixed amount of synthetic deoxynucleotide template, we measured the RNA polymerase activity in hepatic nucleoli derived from control and cortisone-treated animals. We found that cortisone stimulation of nucleolar RNA synthesis is, to a considerable degree, independent of template availability and reflects hormonally elevated activities of RNA polymerase.

MATERIALS AND METHODS

Experimental animals and cortisone administration

Male Sprague–Dawley rats of about 200 g body weight were starved overnight and were divided into two groups of 3 or 4 animals each. The hormone-treated group received a single intraperitoneal injection of cortisone acetate suspension (Upjohn Co.), 5 mg/100 g body weight, 4 hr before they were killed; the control group received an equivalent volume of 0.9% saline.

Isolation of rat-liver nuclei, nucleoli, and extranucleolar fractions

The isolation procedure is essentially that of Muramatsu, *et al.* (15). Rats were stunned by a blow on the head and exsanguinated. The livers were excised after perfusion with cold 0.25 M sucrose–3.3 mM $CaCl_2$ solution. All subsequent operations were conducted at 0–4°C.

18 g of liver from each group were used and homogenized in 10 volumes of 2.3 M sucrose–3.3 mM $CaCl_2$ in a glass Potter–Elvehjem homogenizer, loosely fitted with a Teflon pestle. The homogenate was filtered through cheese cloth and centrifuged at 40,000 × *g* for 1 hr. The nuclear pellet was then suspended in 18 ml of 0.34 M sucrose solution; a 3-ml aliquot from each group was saved as the intact nuclear fraction. The remainder of the nuclear suspension was sonicated with a Branson sonifier (Model no. S-110), equipped with a fine probe, until virtually all nuclei were broken (about 2 min, at a setting of 5). The sonicate was layered over 15 ml of 0.88 M sucrose and centrifuged at 2000 × *g* for 20 min at 0°C. The pellet contained the highly purified nucleoli and the supernatent contained the extranucleolar fraction. The purity of the isolated nucleoli was verified by microscopic examination.

Assay for RNA polymerase activity

The reaction mixture for measurement of the Mg^{+2}-activated polymerase was adjusted to a final volume of 0.65 ml and contained: 0.15 ml of nuclei, nucleolar, or extranucleolar suspension (nucleoplasm) representing the equivalent of 0.15, 1.1, and 0.15 g of liver, respectively; 50 μmol Tris.HCl (pH 8.2); 5 μmol $MgCl_2$; 1.0 μmol of ATP; and 0.05 μmol (each) of CTP, UTP, and GTP. 0.1 μCi of [8-^{14}C]GTP (Schwarz Bioresearch, Inc. 40 Ci/mol) and 2 μCi of [5-^{3}H]-

TABLE 1 *The effect of cortisone administered in vivo on the RNA polymerase activities of isolated hepatic nuclear, nucleolar, and extranucleolar fractions*

	(Specific activity of RNA polymerase*) [^{3}H]UTP			[^{14}C]GTP			U/G Ratio	
	Control	Cortisone†	%	Control	Cortisone	%	Control	Cortisone
				Mg^{+2}-Activated				
Nuclear	1303 ± 57	1481 ± 195	+14	2184 ± 219	3370 ± 291	+54	0.60	0.44
Nucleolar	1369 ± 362	2127 ± 536	+55	3399 ± 719	5771 ± 887	+70	0.40	0.37
Extranucleolar	1665 ± 90	1694 ± 99	+2	874 ± 148	1016 ± 176	+16	1.9	1.7
				$Mn^{+2}/(NH_4)_2SO_4$-Activated				
Nuclear	1783 ± 119	1819 ± 180	+2	4444 ± 292	3658 ± 314	−18	0.40	0.50
Nucleolar	1527 ± 111	1265 ± 270	−17	2724 ± 174	3702 ± 314	+36	0.56	0.34
Extranucleolar	1079 ± 10	926 ± 24	−14	1660 ± 275	1700 ± 348	+2	0.65	0.54

* Values are given as mean pmol of nucleoside monophosphate incorporated/mg RNA ± SE of duplicate determinations in each of three replicate experiments.

† Cortisone acetate suspension, 5 mg/100 g body weight, was intraperitoneally administered 4 hr before the animals were killed.

UTP (New England Nuclear, 15.9 Ci/mmol) were added singly, or combined (as indicated). The reaction mixture for $Mn^{+2}/(NH_4)_2SO_4$-activated polymerase was identical with the Mg^{+2}-containing assay medium, except that 2.2 μmol of $MnCl_2$ and 0.2 mmol of $(NH_4)_2SO_4$ were substituted for the $MgCl_2$, and the pH of the medium was 7.5 (16). After equilibration at 37°C, the reaction was initiated by addition of nuclei, nucleoli, or extranuclear fraction and was further incubated for 15 min. The reaction was halted by transfer to chipped ice, followed by immediate addition of 5 ml of cold 10% trichloroacetic acid. The acid-insoluble material was collected on Millipore filters (24 mm, type HA), which were then exhaustively washed three times with 10 ml of 5% cold trichloroacetic acid and once with 5 ml of 60% ethanol. The filters were dried in air and counted (17) in 10 ml of Bray's solution (18).

RNA and DNA determinations

RNA determinations were as described (19). DNA was hydrolyzed, after the removal of RNA, with 5% trichloroacetic acid in a boiling-water bath for 20 min. DNA concentration was determined by absorbance at 260 nm, with 5% trichloroacetic acid as blank, on the assumption that an absorbance of 1.0 at 260 nm (1-cm light path) corresponds to 32.0 μg of DNA/ml (20).

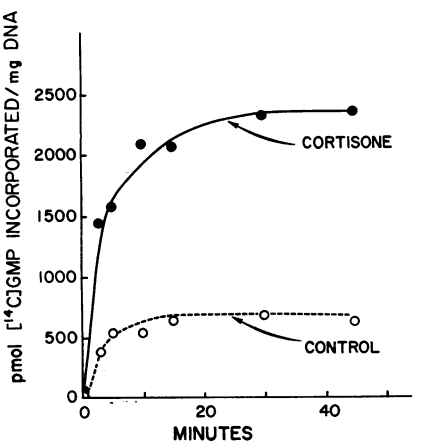

FIG. 1. Time course of activity of the Mg^{+2}-activated rat-liver nucleolar RNA polymerase.

RESULTS

Previous studies have established that the stimulation of rat liver RNA synthesis *in vivo* that occurs a few hours after a single injection of cortisone is predominately G-rich ribosomal RNA (1,2, 9,10,21). The present study confirms this finding in an *in vitro* system (Table 1). Furthermore, 4 hr after injection of cortisone, the hormonal stimulation of nucleotide incorporation into nuclear RNA is localized solely in the nucleolus, which is known to be the site for ribosomal RNA synthesis in eukaryotic cells (11–13). This result is illustrated in these double-label experiments by the low ratio of incorporation of U to G manifested by the hormonally-stimulated nuclei and nucleoli. The extranucleolar fraction, on the other hand, is currently considered to be the site for messenger RNA synthesis and, indeed, has appropriately incorporated labeled nucleotides with a high U to G ratio into RNA, in agreement with radioautographic observations (22). The presence of $Mn^{+2}/(NH_4)_2SO_4$ lowered, or entirely abolished, the hormonal effect in whole nuclear and nucleolar preparations, as observed with this or other hormones (23–27).

Fig. 1 shows the rate of precurser incorporation by the Mg^{+2}-activated RNA polymerase of both control and hormone-treated nucleolar fractions with endogenous template. It is clear that both preparations had similar incorporation rates, reaching maximum incorporations at 10–15 min. These results are similar to the report by Ro *et al.* (28), who used isolated normal rat-liver nucleoli. Cortisone administration *in vivo* resulted in a 2-fold or greater stimulation of RNA synthesis in the nucleoli throughout the incubation period.

To differentiate between the possibilities that the increased nucleolar RNA synthesis observed *in vitro* after cortisone administration *in vivo* could be the result of (*a*) hormonal activation or derepression of the nucleolar genome, or (*b*) an increased amount or efficiency of the RNA polymerase by the hormone, or (*c*) both of these mechanisms, the following experiments were done. The RNA polymerase activities of the control and the hormone-treated nucleolar fractions

TABLE 2 *Effect of cortisone on rat-liver nucleolar RNA polymerase in the absence and presence of exogenous template*

Expt.	Additions			Specific Activity of RNA Polymerase			
	Cortisone*	Actin.-D†	Poly dc‡	pmol of [^{14}C]GMP incorporated/mg RNA	%	pmol of [^{14}C]GMP incorporated/mg DNA	%
1	−	−	−	2172		750	
					+57		+83
	+	−	−	3400		1370	
	−	+	−	533		184	
					+20		+40
	+	+	−	640		258	
	−	+	+	2171		750	
					+38		+61
	+	+	+	3000		1210	
2	−	−	−	2154		708	
					+130		+422
	+	−	−	4949		3696	
	−	+	−	448		147	
					+61		+267
	+	+	−	722		540	
	−	+	+	2014		662	
					+83		+316
	+	+	+	3684		2751	

RNA polymerase activity was assayed in nucleoli containing 25–30 μg of DNA in the Mg^{+2}-activated reaction medium incubated for 15 min at 37°C.

* Cortisone acetate suspension (Upjohn Co.), 5 mg/100 g body weight, was injected intraperitoneally 4 hours before the animals were killed.

† Actinomycin D (Nutritional Biochemicals Co.), 5 μg in 25 μl of H_2O, was added at zero time to the incubation mixture.

‡ Polydeoxycytidylate, 51.9 μg in 100 μl of H_2O, was added at zero time to the incubation medium. This was a gift of Dr. N. Goodman of Dr. Spiegelman's laboratory.

were measured with exogenous synthetic polydeoxycytidylate as the template in the presence of actinomycin D, an inhibitor of the function of endogenous nucleolar template. The results of two typical experiments are presented in Table 2. Actinomycin D treatment inhibited RNA synthesis with endogenous template by 75–85%. Noteworthy is the finding that cortisone stimulates nucleolar RNA synthesis most strongly when a constant amount of exogenous polydeoxycytidylate is present as template. In each experiment, however, there was a slightly greater hormonal stimulation of GMP incorporation into RNA in the presence of the native nucleolar template then with polydeocycytidylate as template. Thus, in addition to its enhancement of nucleolar RNA polymerase, cortisone may further stimulate transcription due to increased availability (derepression) of the endogenous DNA template of the nucleolar genome.

DISCUSSION

One of the challenging problems in modern molecular biology concerns the mechanism of the hormonal regulation of gene expression. Studies of hormonal action *in vivo* are useful in establishing the basic physiological phenomena; in general, however, *in vivo* conditions are too complex to permit definitive insights into the underlying molecular processes. *In vitro* studies unsupported by biological observations, on the other hand, confront the uncertainty of their physiological relevance. Our early observations of hormonally enhanced precursor incorporation into hepatic RNA *in vivo* have been adequately confirmed and now are generally accepted as indicating that glucocorticoids regulate rat-liver RNA synthesis (1–10). It has been observed, furthermore, that 4 hr after cortisone treatment, the synthesis of G-rich ribosomal RNA is preferentially stimulated (9, 10, 21). Since the nucleolus is the site of ribosomal RNA synthesis in eukaryotic cells (11–13), *in vitro* studies with isolated nucleoli offer a convenient system to examine hypotheses concerning biochemical events underlying hormonal regulation of gene expression.

That the rat-liver nucleoli used in the present study constitute a physiologically relevant system is attested to by the following facts: (*a*) this preparation is capable of polyribonucleotide synthesis, (*b*) transcription of the endogenous nucleolar template is sensitive to inhibition by actinomycin D, (*c*) the RNA synthesized is G-rich; its base composition resembles that of ribosomal RNA, and (*d*) after cortisone treatment *in vivo*, these isolated hepatic nucleoli have an elevated rate of RNA synthesis similar in nature and magnitude to that observed *in vivo* (9).

Using this system, we have explored the question as to whether the enhanced gene transcription observed after glucocorticoid administration is due to hormonal derepression of the genome, with consequent increase in DNA template availability, or to hormonal enhancement in the RNA polymerase complex that transcribes this portion of the genome. Actinomycin D was added to inhibit endogenous DNA template activity. Addition of the exogenous template, polydeoxycytidylate, which lacks guanine and is insensitive to actinomycin D, restored incorporation of its complementary base, GTP, into a polymer. Hepatic nucleoli derived from glucocorticoid-treated animals have a markedly

enhanced transcriptive activity in the presence of the exogenous synthetic template, as well as with endogenous nucleolar DNA. Polydeoxycytidylate-stimulated RNA synthesis does not seem to be an indirect consequence of the release of the native template, since under the influence of actinomycin D, in the absence or presence of polydeoxycytidylate, [^{3}H]UTP was not incorporated at all (unpublished data). Since the effectiveness of cortisone persisted when endogenous template activity was bypassed in this manner, we conclude that the hormonal stimulation of nucleolar RNA synthesis is largely due to an augmentation of the activity of the nucleolar RNA polymerase. It seems possible that some gene derepression of endogenous DNA may also be elicited by glucocorticoid, which would account for the slightly reduced responses to the hormone by exogenous, as compared with endogenous, templates (Table 2). These conclusions are compatible with earlier studies by Barnabei *et al.* indicating that puromycin treatment *in vivo* abolished the hormonally increased RNA synthesis (23), and with recent reports (29, 30) that the catalytic activity of a nuclear RNA polymerase fraction increased after glucocorticoid treatment *in vivo*. However, we must also take cognizance of the report of Dahmus and Bonner (31) that 4 hr after administration of hydrocortisone *in vivo*, isolated rat-liver chromatin showed a 10–35% increase in template activity when tested with *Escherichia coli* RNA polymerase. This may be analogous to the minor enhancement in rat-liver nucleolar template efficiency that seems to be evoked by cortisone in the present investigation. Other studies, using either isolated whole nuclei or aggregate enzyme preparations, are intrinsically incapable of distinguishing between hormone effects at the level of template activation or on the polymerase enzyme itself (32–34). The present investigation indicates that hormonal induction of, or enhanced catalytic efficiency of, the nucleolar RNA polymerase enzyme *per se* is largely responsible for the augmentation by glucocorticoids of ribosomal RNA synthesis. Gene derepression by the hormone exerts, at most, a quantitatively subordinate role.

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