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Increased RNA Synthesis in Nuclei Isolated from Rat Liver Tissue Slices Incubated with Cyclic Adenosine 3′,5′-Monophosphate or Glucagon[1]

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Incubation of rat liver tissue slices with cyclic adenosine 3′,5′-monophosphate (cyclic AMP) for 30 min results in a dose-dependent increase in RNA synthesizing capacity of nuclei prepared from these slices, with a doubling of synthetic rate observed at 10^{-7} M cyclic AMP. The cyclic AMP effect is observed when RNA polymerase activity is measured either in the presence of Mg^{2+} and low ionic strength, or Mn^{2+} and high ionic strength. Experiments employing saturating amounts of exogenous bacterial RNA polymerase suggest that the cyclic AMP-induced stimulation occurs primarily at the level of template activity. Other cyclic nucleotides tested in the same manner are ineffective in stimulating RNA synthesis by tissue slice nuclei. In addition to cyclic AMP, adenosine 5′-monophosphate (5′-AMP) consistently produced small increases in nuclear RNA synthesis although never of the magnitude seen with the cyclic nucleotide.

An increased capacity for RNA synthesis is also seen in nuclei isolated from liver slices incubated with glucagon at concentrations from 0.5 μg/ml to 50 μg/ml. A maximal stimulation of approximately twofold occurs at a glucagon concentration of 1.0 μg/ml. Liver slices incubated with optimal concentrations of cyclic AMP and glucagon simultaneously show that the effects of the two agents on RNA synthesis are not additive.

The results indicate that cyclic AMP at physiological concentrations can stimulate RNA synthetic capacity *in vitro*, and that the effect mimics a similar response to glucagon. Since it is known that glucagon causes an increase in liver concentrations of cyclic AMP and a subsequent induction of some liver enzymes, it is suggested that cyclic AMP-mediated control of RNA synthesis may be involved in such regulation.

Cyclic adenosine 3′,5′-monophosphate (cyclic AMP)[3] is now known to be an important factor involved in the control of RNA synthesis in bacterial systems (1). Recent evidence has led to the proposal that a cyclic AMP-protein complex binds to *E. coli* DNA and serves as a positive signal for the initiation of transcription of the genes for a number of inducible enzymes (2). Since cyclic AMP is known to mediate the effects of a large number of hormones in higher organisms (3), it seems of interest to determine whether any of

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[3] Abbreviations used: cyclic AMP, or cAMP, adenosine 3′,5′-monophosphate; cyclic GMP, guanosine 3′,5′-monophosphate; cyclic UMP, uridine 3′,5′-monophosphate; cyclic CMP, cytidine 3′,5′-monophosphate; KRP, 0.14 M NaCl, 5 mM KCl, 2.3 mM $CaCl_2$, 1.3 mM $MgSO_4$, 0.01 M Na_2HPO_4, pH 7.4; TMS, 0.01 M Tris–HCl, 4 mM $MgCl_2$, 0.25 M sucrose, pH 8.3; TCS, 0.01 M Tris–HCl, 3 mM $CaCl_2$, 0.25 M sucrose, pH 8.3.

these responses also involve the control of RNA synthesis by this cyclic nucleotide.

Recently some evidence has begun to accumulate which supports the possibility that cyclic AMP does play a role in controlling gene transcription in eukaryotic cells. For example, it has been shown in a number of different systems that cyclic AMP causes an increase in the rate of incorporation of radioactive precursors into RNA (4–15). One system which has been extensively studied and serves as a good model in this regard is the stimulation of mammalian liver by glucagon, a hormone whose actions are known to be mediated at least in part by cyclic AMP (3). Administration of glucagon or cyclic AMP has been shown to cause the induction of a specific set of enzymes in rat liver (16), and inhibition of RNA synthesis by actinomycin D blocks such effects on at least one enzyme (17). Furthermore, Langan (18, 19) has observed that phosphorylation of liver f_1 histone is stimulated by both glucagon and cyclic AMP, a finding which has led him to suggest that this histone modification may lead to a specific stimulation of transcription of the genes coding for some glucagon-inducible enzymes.

If Langan's hypothesis is correct, then cyclic AMP should cause an increase in the rate of RNA synthesis in rat liver, although the magnitude of this effect might not be very great due to the small number of enzymes whose synthesis is induced by this nucleotide. Consistent with this prediction, positive effects of cyclic AMP on RNA synthesis in rat liver have been observed both by injecting cyclic AMP *in vivo* (10) and by incubating cell-free systems with cyclic AMP *in vitro* (9, 12). Unfortunately, the *in vivo* experiments are complicated by the possibilities of secondary effects when injecting whole animals with cyclic AMP, while most of the *in vitro* experiments with isolated nuclei required relatively high concentrations of cyclic AMP to produce effects.

In order to try to overcome these difficulties, we have chosen to study RNA synthesis in nuclei obtained from liver slices which have been incubated with cyclic AMP or glucagon. The present studies show that in such a system low concentrations of both cyclic AMP and glucagon produce significant increases in the ability of nuclei to synthesize RNA. Since these two substances do not give additive effects when administered together, it is suggested that the stimulation of RNA synthesis by glucagon is mediated by its elevation of cyclic AMP levels and that the resulting effects on RNA synthesis may be involved in the mechanism of action of this peptide hormone.

MATERIALS AND METHODS

Preparation and incubation of liver slices. Adult male Sprague-Dawley rats were fasted 18–20 hr prior to the start of each experiment. The rats were sacrificed by decapitation and the livers perfused with 15 ml of ice-cold 0.14 M NaCl. Liver slices were prepared at 4°C with a Stadie-Riggs microtome (Arthur Thomas) and placed in 50 ml of Krebs Ringer phosphate (KRP) buffer (0.14 M NaCl, 5 mM KCl, 2.3 mM $CaCl_2$, 1.3 mM $MgSO_4$, 0.01 M Na_2HPO_4, pH 7.4). Just before incubation, control slices were transferred into 50 ml of fresh KRP. Experimental slices were placed into 50 ml KRP containing various concentrations of cyclic nucleotides and/or glucagon. Slices were incubated at 37°C for 30 min with constant shaking. At the end of this period, the supernatant medium was decanted and the pH read.

Isolation of nuclei. All operations were carried out at 4°C. Each 5 g of liver (slices or fresh) was homogenized with a Dounce homogenizer (4 strokes with loose pestle and 3 strokes with tight pestle) in 3 vol of 0.32 M sucrose/3 mM $MgCl_2$. The homogenates were spun at 1,000*g* for 15 min, and the resulting pellets suspended in 40 ml of 2.4 M sucrose/1 mM $MgCl_2$ with a Sorvall Omnimixer. The preparations were centrifuged at 105,000*g* for 1 hr, the nuclear pellets resuspended and washed two times in TMS (0.01 M Tris–HCl, 0.25 M sucrose, 4 mM $MgCl_2$, pH 8.3), and finally suspended in 2 vol of TMS.

Measurements of RNA synthesis by isolated nuclei. Assays for magnesium-activated RNA synthesis were performed by incubating 0.1 ml of nuclei (400–600 μg DNA) in a final volume of 0.5 ml of reaction medium containing 8 mM Tris-HCl, pH 8.3, 0.2 M sucrose, 4 mM $MgCl_2$, 0.2 μCi [8-^{14}C]-ATP (30 mCi/mmole), and 1.8 mM each CTP, GTP, and UTP. For measurements of manganese-activated RNA synthesis, nuclei were washed in TCS (0.01 M Tris–HCl, 3 mM $CaCl_2$, 0.25 M sucrose, pH 8.3) instead of TMS, and 0.1-ml aliquots of nuclear suspension were then incubated in a final volume of 0.5 ml of reaction medium containing 8

mM Tris–HCl, pH 8.3, 0.2 M sucrose, 1.6 mM $CaCl_2$, 0.6 mM $MnCl_2$, 40 mM $(NH_4)_2SO_4$, 0.2 μCi [8-^{14}C]-ATP (30 mCi/mmole), and 1.8 mM each CTP, GTP, and UTP. In both cases, 0.1-ml samples were taken at 1, 3, and 5 min, and incorporation of radioactivity into acid-insoluble material determined as described elsewhere (10).

Nuclear DNA concentrations were determined by a modified indole procedure (20). Insulin-free glucagon (0.0000025%; 0.6 μU/mg) was kindly supplied by David Brennan of Eli Lilly Co., Indianapolis, IN.

RESULTS

RNA synthesis in nuclei isolated from liver slices. It is known that rat liver nuclei carry on RNA synthesis *in vitro* and that transcription of chromatin prepared from rat liver nuclei results in the production of an RNA complement similar to that transcribed *in vivo* (21–23). However, because of the time needed to prepare and incubate liver slices, and to isolate nuclei from these slices, it seemed necessary to determine at the outset whether RNA synthesis in nuclei isolated from such slices occurred at a rate comparable to that of nuclei prepared directly from rat liver.

As is shown in Fig. 1, nuclei isolated from rat liver slices which had been incubated for 1 hr prior to nuclear isolation exhibit a rate of RNA synthesis comparable to that of nuclei isolated directly from liver. Therefore, it is assumed that the nuclei used in this set of experiments are not only viable, but are also comparable to standard rat liver nuclei in regard to both rate of nucleotide incorporation and total amount of RNA synthesized.

Effects of nucleotides on RNA synthesis. When rat liver slices are incubated for 30 min in the presence of varying concentrations of cyclic AMP, nuclei isolated from these slices are found to exhibit an increased rate of RNA synthesis (Fig. 2). The optimal effect is observed at a cyclic AMP concentration of 10^{-7} M, where a doubling of the rate of RNA synthesis occurs. Some stimulatory effect can be observed at cyclic AMP concentrations as low as 10^{-9} M, and in the presence of 1 mM theophylline optimal stimulation was found to occur with cyclic AMP concentrations as low as 10^{-8} M.

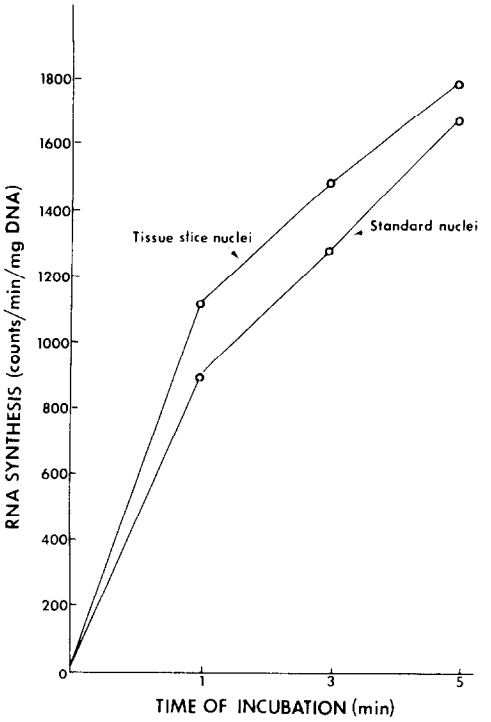

FIG. 1. Comparison of rates of RNA synthesis in nuclei obtained from rat liver slices versus nuclei obtained directly from fresh liver ("standard nuclei"). Liver slices were prepared as described in the text and incubated for 1 hr at 37°C prior to isolation of nuclei. RNA synthesis was measured in the magnesium-containing medium. Each point is the average of 5 samples of 0.1 ml each; standard deviations for each set of points show no significant differences between standard and slice nuclei.

In order to determine whether the observed stimulation of RNA synthesis is specific for cyclic AMP, we have tested the effects of a variety of other nucleotides in this system. Incubation of liver slices with 5′-AMP causes a small, but reproducible, stimulation of RNA synthesis (Fig. 3). This effect is never near the magnitude of that seen with cyclic AMP, although concentrations as high as 10^{-3} M have been tested. No other 5′- or cyclic nucleotides tested in this system have been found to produce any significant alterations of RNA synthesis (Fig. 4).

Since nuclear RNA synthesis is now known to be catalyzed by at least two distinct RNA polymerase activities, one activated

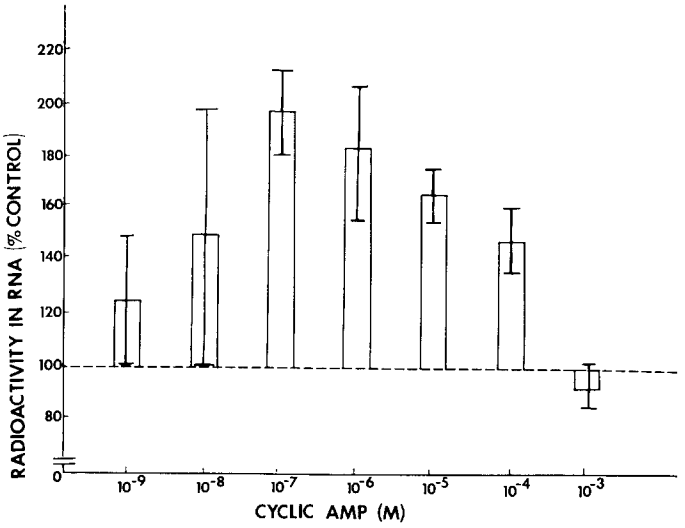

FIG. 2. Effects of incubating rat liver slices with varying concentrations of cyclic AMP on rates of RNA synthesis in nuclei isolated from these slices. Cyclic AMP was included during a 30-min incubation, after which nuclei were isolated and RNA synthesis measured in the magnesium-containing medium during a 3-min incubation. The data represent averages obtained from at least two, and in some cases, up to five experiments. The vertical lines are standard deviations. The control value in this experiment represents 727 cpm/mg DNA.

by magnesium and one by manganese (24–26), we compared the effects of cyclic AMP on RNA synthesis under each of these conditions. As is shown in Fig. 5, both magnesium- and manganese-activated RNA synthesis are stimulated by cyclic AMP.

One common way to determine whether an observed stimulation of RNA synthesis is caused via an effect on RNA polymerase or chromatin template activity is to incubate with saturating amounts of exogenous bacterial RNA polymerase. As is seen from the data in Table I, cyclic AMP induces a comparable percentage increase in RNA synthesis in nuclei incubated with exogenous RNA polymerase. These results suggest that the effect of the cyclic nucleotide may be at the level of chromatin template activity rather than a stimulation of endogenous RNA polymerase, although alternative explanations are also possible.

Effects of glucagon and epinephrine on RNA synthesis. When rat liver tissue slices are incubated in the presence of varying concentrations of glucagon, nuclei isolated from these slices are also found to exhibit an increased rate of RNA synthesis (Fig. 6). Again the response is found to be concentration dependent, with near optimal effects being observed with hormone concentrations as low as 1.0 μg/ml. It is of interest to note that in the case of both cyclic AMP and glucagon, the maximum increases in RNA synthesis seen are of the same magnitude, approximately a doubling of control values. In order to rule out the possibility that the stimulation of RNA synthesis seen with glucagon was caused by insulin contamination, "insulin-free" glucagon (<0.0000025% insulin) was obtained from Eli Lilly Co. and tested in this system. Under such conditions, a similar stimulation of RNA synthesis was observed.

Since glucagon is known to elevate cyclic AMP levels in rat liver (3), it is possible that the stimulation of RNA synthesis seen with glucagon is in fact mediated by this increase in cyclic AMP, which we have already seen can stimulate RNA synthesis. On the other hand, it is conceivable that glucagon is stimulating RNA synthesis via a mechanism independent from cyclic AMP. It is easy to distinguish between these

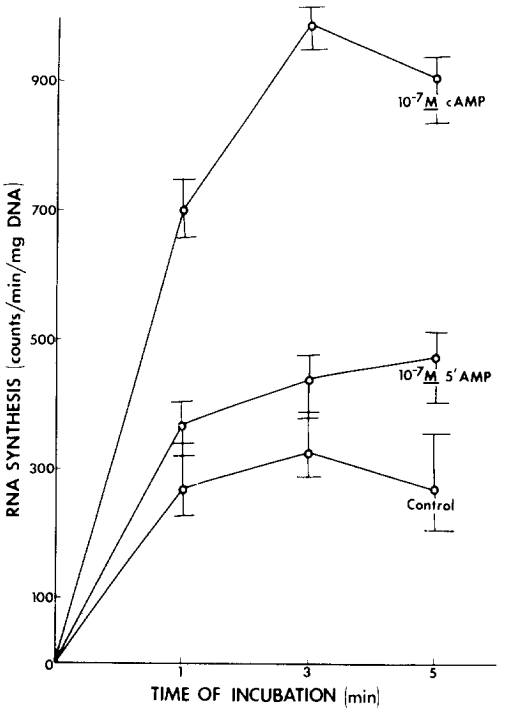

FIG. 3. Comparison of rates of RNA synthesis in nuclei obtained from rat liver slices incubated with cyclic AMP or 5′-AMP. Slices were incubated for 30 min with 10^{-7} M nucleotide, after which nuclei were isolated and RNA synthesis measured in the magnesium-containing medium. A small, but reproducible stimulation occurs with 5′-AMP, although the magnitude of the effect never approaches that seen with cyclic AMP. Vertical lines are standard deviations.

possibilities experimentally, since, if glucagon is acting on RNA synthesis independently of cyclic AMP, the effects of these two agents should be additive. As is shown in Fig. 7, simultaneous addition of glucagon and cyclic AMP to tissue slice incubations stimulates RNA synthesis in isolated nuclei to no greater extent than the presence of either agent alone.

If glucagon is stimulating RNA synthesis via a cyclic AMP-mediated mechanism, then one might expect other agents which elevate cyclic AMP levels to also enhance RNA synthesis in this system. Consistent with this prediction, we found that epinephrine at a concentration of 1.0 μg/ml caused a 60% stimulation of the rate of RNA synthesis.

TABLE I

EFFECTS OF EXOGENOUS RNA POLYMERASE ON CYCLIC AMP-INDUCED STIMULATION OF RNA SYNTHESIS[a]

Condition	Radioactivity in RNA (cpm/mg DNA)		Stimulation (% control)
	Control	+ cyclic AMP	
A. Mg^{2+}-containing assay			
Complete system	1,296	2,460	190
+ *E. coli* RNA polymerase	2,592	4,780	183
B. Mn^{2+}-containing assay			
Complete system	6,750	8,775	130
+ *E. coli* RNA polymerase	11,400	22,100	194

[a] Nuclei were prepared from rat liver slices incubated for 30 min with or without 10^{-6} M cyclic AMP as described in Methods. Assay for RNA synthesis was done by labeling for 3 min in either magnesium- or manganese-containing media. In cases where the effects of exogenous RNA polymerase were tested, 12 units (a saturating amount) of *E. coli* RNA polymerase were added.

DISCUSSION

Although firm evidence exists that cyclic AMP mediates the action of glucagon in rat liver, little is known about the mechanism by which cyclic AMP activates the synthesis of glucagon-inducible enzymes. The present experiments lend support to the possibility that at least part of the effects of cyclic AMP are mediated via effects on gene transcription. We have found that incubation of rat liver slices with concentrations of cyclic AMP in the physiological range can cause dramatic increases in the capacity for RNA synthesis in nuclei isolated from these slices. Although others have been able to observe such effects in liver slices only with the dibutyryl derivative of cyclic AMP (12), they did not measure RNA synthesis directly in isolated nuclei as was done in the present studies. The virtual absence of any comparable effects for a wide range of other nucleotides tested in our system suggests that the effect is highly specific for cyclic AMP. The only other nucleotide which causes any significant enhancement of

RNA synthesis is 5′-AMP, and its effect is much less dramatic than that observed with cyclic AMP. Since it is known that 5′-AMP can activate adenyl cyclase in brain tissue (27), it is possible that even the effects of 5′-AMP in our system are mediated via cyclic AMP.

It is of interest to note that concentra-

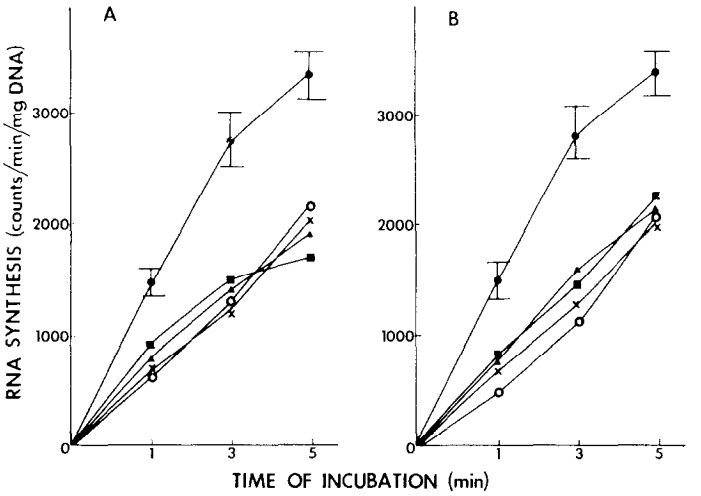

FIG. 4. Comparison of rates of RNA synthesis in nuclei obtained from rat liver slices incubated with various nucleotides at 10^{-6} M. Experiments were performed as described in Fig. 3; A. cyclic AMP (●– –●), cyclic GMP (○ —○), cyclic UMP (▲ —▲), cyclic CMP (■– –■), control (×——×). B. cyclic AMP (●– –●), 5′-GMP (○– –○), 5′-UMP (▲——▲), 5′-CMP (■——■), control (×— –×). Note that of all these nucleotides tested, only cyclic AMP causes a significant stimulation of RNA synthesis. Vertical lines are standard deviations.

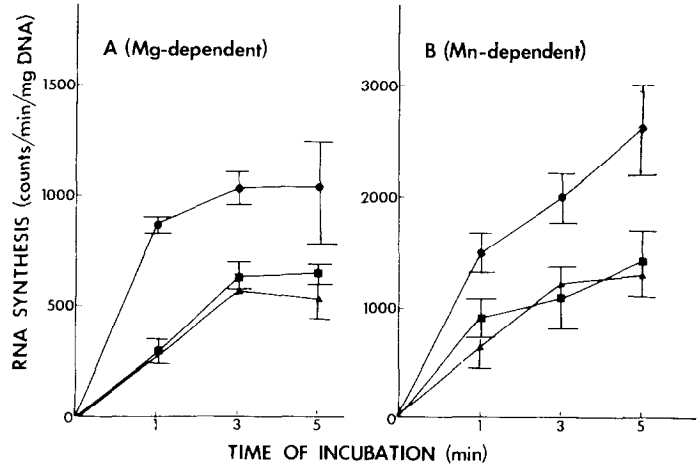

FIG. 5. Comparison of the effects of cyclic AMP on magnesium- and manganese-dependent RNA synthesis. Rat liver slices were incubated with cyclic AMP for 30 min, nuclei isolated, and their capacity for RNA synthesis measured in either magnesium- or manganese-containing media as described in the text. Effects of 10^{-7} M (●——●) and 10^{-3} M (▲——▲) cyclic AMP are compared to control values (■——■). Note that a concentration-dependent stimulation of both types of RNA synthesis occurs. Vertical lines are standard deviations.

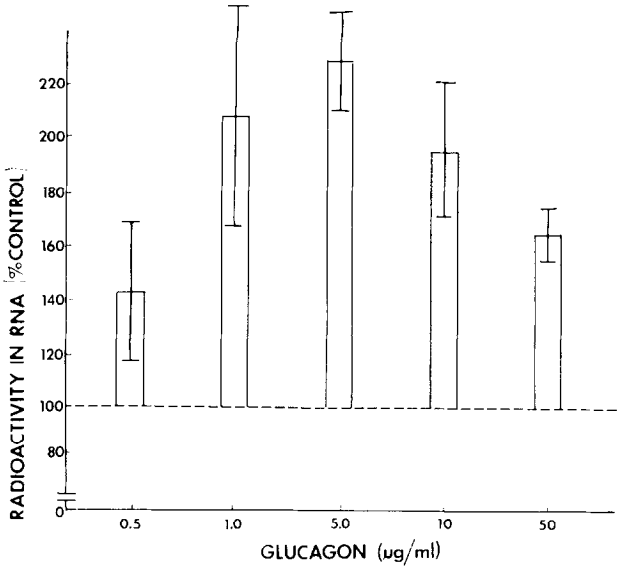

FIG. 6. Effects of incubating rat liver slices with varying concentrations of glucagon on rates of RNA synthesis in nuclei isolated from these slices. Glucagon was included during a 30-min incubation, after which nuclei were isolated and RNA synthesis measured in the magnesium-containing medium for 3 min. The control value in this case represents 400 cpm/mg DNA. Vertical lines are standard deviations.

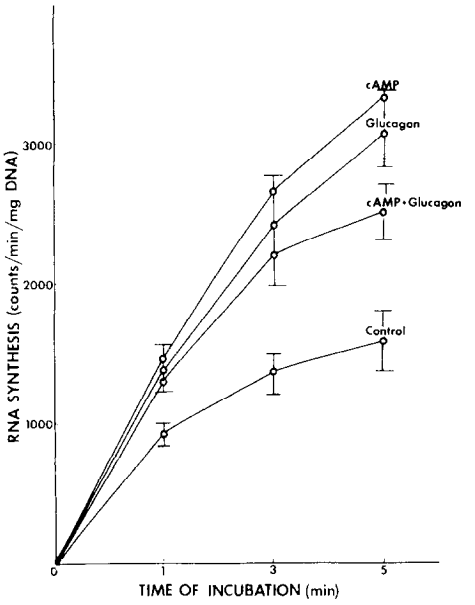

FIG. 7. Effects of cyclic AMP and glucagon on RNA synthesis. Rat liver slices were incubated for 30 min with 10^{-5} M cyclic AMP, 5.0 μg/ml glucagon, or both. Nuclei were isolated, and RNA synthesis was measured in the magnesium-containing medium. Note that the effects of cyclic AMP and glucagon are not additive. Vertical lines are standard deviations.

tions of cyclic AMP higher than 10^{-6} M in the slice incubation medium produce submaximal increases in nuclear RNA synthesis. It is a possibility that the intracellular concentrations of cyclic AMP generated by these high incubation dosages may activate some sort of a negative feedback system, i.e., high levels of cyclic AMP may activate phosphodiesterase activity (28) or some as yet unknown inhibitor of cyclic AMP action. Little concrete evidence is available to explain how responses to cyclic AMP are turned off, but data such as described here imply that regulation at this point may be as complex as activation of the entire system.

Several aspects of these experiments suggest that a physiologically relevant response is being observed. In addition to the above-mentioned specificity of the effect for cyclic AMP and its effectiveness at low concentrations, another significant observation in this regard is the fact that low levels of glucagon produce a stimulation of RNA synthesis similar to that seen with cyclic AMP and that optimal doses of glucagon and cyclic AMP in combination produce an effect no greater than either

alone. Such data are consistent with the fact that cyclic AMP is known to be a mediator of glucagon action *in vivo* and suggest the possibility that the mechanism of action of glucagon involves at least in part a cyclic AMP-mediated stimulation of RNA synthesis. Glucagon has several actions in mammalian liver (3), but the one which seems most likely to involve RNA synthesis is the induction of a series of enzymes. The fact that cyclic AMP itself, when directly applied, can also induce the formation of these same enzymes (16, 17) further supports the possibility that a cyclic AMP-induced activation of gene transcription is involved in glucagon action. In support of this general idea, in at least one case (15) cyclic AMP has been shown to stimulate synthesis of RNA rich in poly(A), which is thought to be a characteristic of messenger RNA.

This brings us to the question of possible mechanisms which might explain how cyclic AMP can activate gene transcription in this system. Langan (18, 19) has suggested that cyclic AMP-activated phosphorylation of f_1 histones causes a change in chromatin template activity, leading in turn to an activation of transcription of the genes coding for the glucagon-inducible enzymes. Such a model would predict only small increases in overall RNA synthesis following cyclic AMP administration, and yet the present data show that under optimal conditions a doubling of the rate of nuclear RNA synthesis can be produced by cyclic AMP. Such increases are obviously beyond what would be needed to account for the synthesis of those messenger RNAs coding for a small number of inducible enzymes.

Several possibilities can be considered which might account for such unexpectedly large increases in nuclear RNA synthesis. One obvious possibility is that the synthesis of species of RNA other than the specific messages for the glucagon-inducible enzymes are involved. Jost and Sahib (19) have shown that the synthesis of both preribosomal and heterodisperse nuclear RNAs are stimulated when isolated nuclei are incubated with dibutyryl cyclic AMP. The present experiments show that both magnesium- and manganese-activated RNA polymerase activities are increased by incubation of liver slices with cyclic AMP, a finding which is also consistent with the stimulation of synthesis of ribosomal as well as nonribosomal RNA species. Another complication which is relevant when considering the magnitude of the observed stimulation of RNA synthesis is the well-established finding that only a small portion of the total amount of RNA synthesized on chromatin ever leaves the nucleus to become involved in the process of cytoplasmic protein synthesis (29). Thus it is difficult to make any direct comparisons between the overall rate of nuclear RNA synthesis and the amount of RNA which eventually functions as cytoplasmic messages for the synthesis of inducible enzymes.

Recent evidence suggests several other possible mechanisms for the cyclic AMP-induced stimulation of RNA synthesis, in addition to the previously mentioned model based on cyclic AMP-mediated stimulation of histone phosphorylation. For example, cyclic AMP has recently been shown to stimulate the phosphorylation of nonhistone chromatin proteins in rat liver (30), and the existence of multiple nuclear protein kinases with differing substrate specificities and dependencies on cyclic AMP has been demonstrated (31, 32). Thus, the possibility arises that cyclic AMP may have multiple effects in both stimulating and inhibiting (31, 32) the phosphorylation of specific nonhistone proteins, and in view of the recent evidence suggesting a role of nonhistone chromatin proteins in the regulation of gene transcription, such phosphorylation reactions may be involved in regulating RNA synthesis (33–37). Another possible effect of cyclic AMP is directly on RNA polymerase itself, since there has been some suggestion in the literature that a cyclic AMP-stimulated phosphorylation of a bacterial RNA polymerase factor may activate the enzyme for transcription (38). In this regard, however, our data with exogenous RNA polymerase suggest that the primary effect is not at the level of RNA polymerase activity. Finally, there is some evidence from eukaryotic cells for an effect of a cyclic AMP-protein complex

directly at the level of the DNA template (12), analogous to the situation found in bacteria (2). Thus, a variety of possible mechanisms exist which might explain the effects of cyclic AMP on RNA synthesis. Indeed, it is quite possible that more than one type of mechanism might be at work.

The present experiments thus demonstrate that cyclic AMP can dramatically stimulate the ability of rat liver nuclei to synthesize RNA and suggest that such a mechanism is involved in the action of glucagon on this tissue. These results do not imply that all the actions of these agents are mediated at a transcriptional level, and indeed there is good evidence that cyclic AMP also acts at the post-transcriptional level (16, 39, 40). The present experiments make it clear, however, that any complete model of how this cyclic nucleotide acts in higher organisms must take into account the observed effects at the level of gene transcription.

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