Enhanced Template Activity in Chromatin from Adrenal Medulla After Phosphorylation of Chromosomal Proteins

Abstract. *Translocation of protein kinase to the nucleus had been implicated earlier in the transsynaptic control of gene expression mediated by cholinergic nerves in adrenal medulla. Phosphorylation of chromosomal proteins by adenosine 3',5'-monophosphate–dependent protein kinase and adenosine 3',5'-monophosphate enhances the template activity of chromatin from adrenal medulla. When homologous RNA polymerase II is used the relative activation is greater than that obtained with* Escherichia coli *RNA polymerase. The substrate for such phosphorylation does not seem to be RNA polymerase II. Phosphorylation of specific acidic protein probably mediates this enhancement of template activity.*

Regulation of gene expression in eukaryotes involves a variety of mechanisms (*1*). The study of gene regulation in eukaryotic tissue is often complicated by the presence of heterogeneous cell populations and mitosis. In adrenal medulla, the majority of cells are chromaffin cells, which show little or no mitotic activity. These cells are innervated exclusively by cholinergic neurons, which, by releasing acetylcholine, can regulate gene expression in postsynaptic cells (*2*). The transsynaptic induction of tyrosine 3-monooxygenase has provided a model for studying transsynaptic regulation of gene expression in adrenal medulla (*2*). This induction is mediated by the following sequence of events. Concurrent with stimulation of postsynaptic acetylcholine nicotinic receptors there is a tenfold increase in the amount of adenosine 3',5'-monophosphate (cyclic AMP) in adrenal medulla (*3*). When the cyclic AMP increase persists longer than 1 hour the cyclic AMP–dependent protein kinase in adrenal medulla cytosol is activated for several hours (*3*). Three hours after stimulus application the free catalytic subunits of protein kinase are translocated from the cytosol to the particulate fraction, and the activity extracted from nuclei is doubled by 7 hours after stimulus application (*4*). After protein kinase translocation, in vivo synthesis of RNA containing polyadenylic acid [poly(A)] and RNA synthesis dependent on RNA polymerase II in isolated nuclei are increased (*5*, *6*). By 10 hours the rate of tyrosine 3-monooxygenase biosynthesis begins to increase, and enzyme activity in the medulla is about doubled by 24 hours (*3*, *7*).

The temporal correlation between the increase in transcription of RNA con-

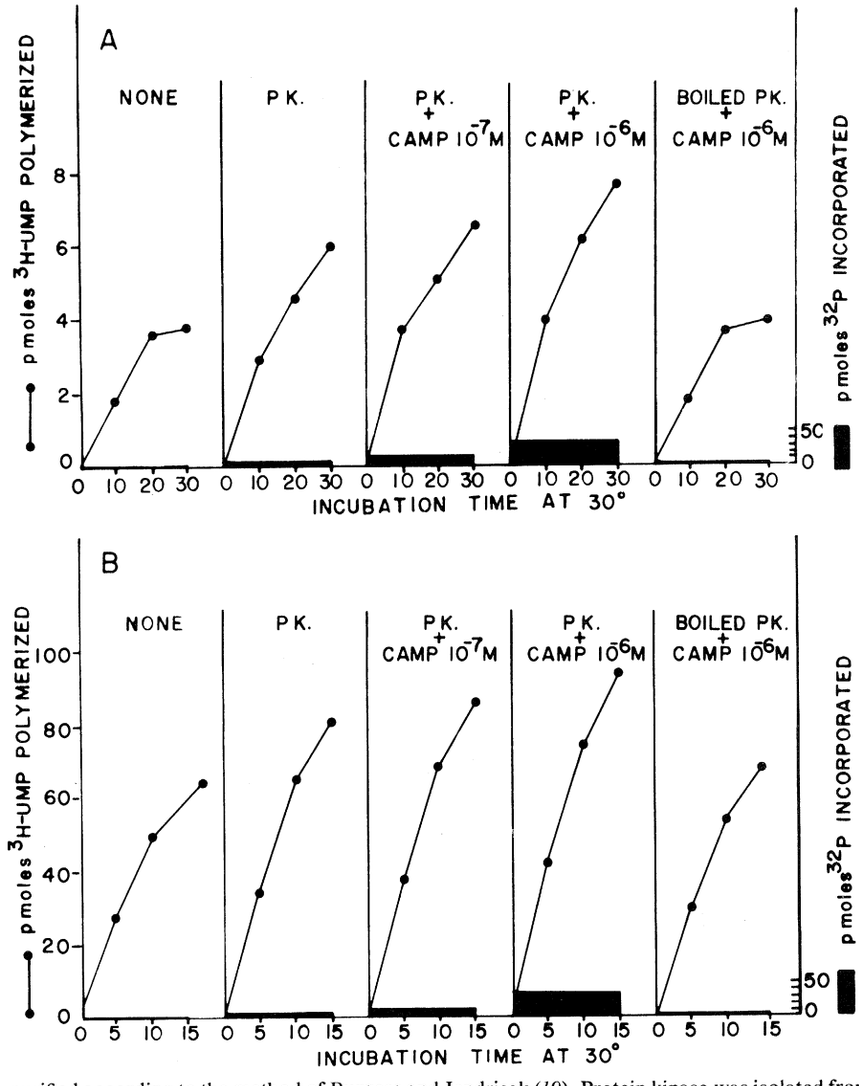

Fig. 1. Effects of protein kinase and cyclic AMP on the template activity of chromatin isolated from beef adrenal medulla. Chromatin was first incubated in various phosphorylating conditions and then transcribed by medullary RNA polymerase II (A) or by *E. coli* RNA polymerase (B). Chromatin containing 28 μg of DNA and 60 μg of protein was phosphorylated by incubation at 30°C for 10 minutes in 180 μl of medium including 50 m*M* tris(hydroxymethyl)aminomethane hydrochloride (*p*H 6.5), 5 m*M* $MgCl_2$, 10 m*M* NaF, 2.5 m*M* theophylline, 125 μ*M* ATP containing [γ-^{32}P]ATP (18.0 c/mmole), 80 μg of protein kinase (*PK*) where indicated, and the indicated concentration of cyclic AMP (*CAMP*). After incubation, 37-μl portions were transferred as the source of chromatin template to a second medium for assay of RNA synthesis. The reaction mixture, described by Jungmann *et al.* (*13*), contained 20.5 μg of RNA polymerase II protein or 37.2 μg of *E. coli* RNA polymerase protein. The final concentration of $(NH_4)_2SO_4$ in the incubation mixture was 100 m*M*. When *E. coli* RNA polymerase was used, Mn^{2+} was replaced by 10 m*M* $MgCl_2$. After the second incubation, the cold TCA-insoluble material was collected and washed on a Millipore filter (HA 0.45). The precipitates were dissolved, and ^{3}H and ^{32}P disintegrations were counted in Aquasol. Specific activities for [^{3}H]UTP and [^{32}P]ATP were 93 and 32 count/min per picomole, respectively. Counts obtained at zero time (background) were subtracted. Nuclei were isolated from fresh bovine adrenal medulla as reported (*18*). Chromatin was purified by the method of Spelsberg and Hnilica (*8*). RNA polymerase II was purified from medullary nuclei according to the procedures of Jungmann *et al.* (*13*) except that diethylaminoethyl (DEAE) cellulose instead of DEAE Sephadex was employed. The third enzyme peak, which was abolished completely by α-amanitin (1 μg/ml), was the source of RNA polymerase II. *E. coli* RNA polymerase containing sigma factor (Miles Laboratories) was purified according to the method of Burgess and Jendrisak (*19*). Protein kinase was isolated from bovine heart as described by Kuo and Greengard (*20*). DNA was determined by ethidium bromide binding (*21*), and protein was measured by the method of Lowry *et al.* (*22*).

taining poly(A) (*5*, *6*) and the activation and translocation of protein kinase to nuclei (*4*) suggests that the enhanced RNA synthesis is mediated by phosphorylation of nuclear proteins involved in transcriptional regulation. We present evidence here that template activity of chromatin isolated from bovine adrenal medulla can be enhanced by prior incubation of the chromatin with protein kinase, adenosine triphosphate (ATP), and cyclic AMP to allow the phosphorylation of chromosomal proteins. However, when RNA polymerase II from adrenal medulla is incubated under analogous phosphorylating conditions, the enzymatic activity remains unchanged.

Chromatin was isolated by the method of Spelsberg and Hnilica (*8*) with slight modification. The chromatin was washed with 0.35*M* NaCl to minimize the activity of protein kinases and RNA polymerases. It was incubated at 30°C for 10 minutes in a phosphorylation medium and then transferred to a second mixture containing an excess amount of exogenous RNA polymerase, which would allow the expression of RNA transcription (Fig. 1). Thus, the zero times in Fig. 1 represent the beginning of uridine monophosphate (UMP) incorporation into RNA and completion of the phosphorylation reaction.

When chromatin alone was present during the first incubation, little ^{32}P was incorporated into material insoluble in trichloroacetic acid (TCA). Addition of protein kinase from bovine heart and $10^{-6}M$ cyclic AMP resulted in a 20-fold increase in the incorporation of ^{32}P into chromosomal proteins. Concomitantly, template activity of chromatin transcribed by RNA polymerase II from adrenal medulla increased about 100 percent (Fig. 1A). A lower cyclic AMP concentration ($10^{-7}M$) in combination with protein kinase in the first medium produced a smaller stimulatory effect on RNA transcription and less than a tenfold increase in phosphorylation. Prior incubation with protein kinase alone increased the template activity by 50 percent and stimulated the phosphorylation of chromatin by only 100 percent. Boiled protein kinase together with $10^{-6}M$ cyclic AMP produced little or no effect on transcription and phosphorylation in this system. In another experiment (Fig. 1B), which was similar except that the medullary RNA polymerase was replaced by *Escherichia coli* RNA polymerase, the chromatin template was more efficiently transcribed than it was by the homologous polymerase. This confirmed reports that the bacterial RNA polymerase has a higher affinity for the chromatin template (*9*, *11*). However, the relative increase in chromatin template activity resulting from prior treatment with protein kinase alone or with cyclic AMP was considerably smaller than that found in the homologous system. There is evidence that mammalian RNA polymerase, unlike prokaryotic RNA polymerase, binds to and initiates RNA synthesis at different sites on chromatin (*10–12*). Thus, our results suggest that enhanced transcriptional activity may involve the phosphorylation of specific chromatin proteins that facilitate the binding of eukaryotic RNA polymerase to specific loci on the template.

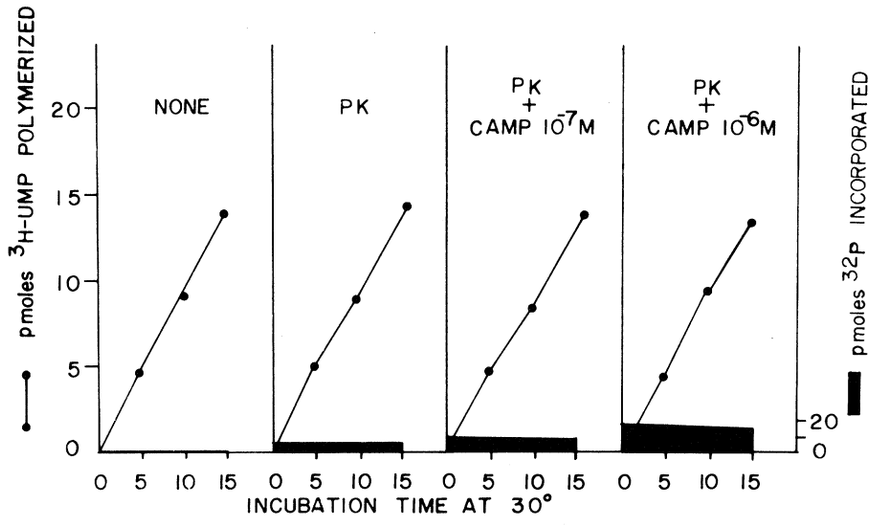

Fig. 2. Effect of protein kinase and cyclic AMP on the activity of RNA polymerase II from adrenal medulla. RNA polymerase II containing 50 μg of protein was incubated in a volume of 360 μl in the various phosphorylating media described for Fig. 1, except that 160 μg of heart protein kinase was used where indicated. A 75-μl portion was pipetted and used as the source of polymerase in the second incubation mixture for RNA synthesis as in Fig. 1; 40 μg of calf thymus DNA was the polymerase template. The assay for incorporation of ^{32}P into protein and [^{3}H]UMP into RNA was described in Fig. 1. The specific activities of [^{3}H]UTP and [^{32}P]ATP were 91.5 and 42.7 count/min per picomole, respectively.

The RNA polymerases I and II isolated from bovine ovary (*13*) and thymus (*14*) and from rat liver (*15*) can be activated by protein kinase through the phosphorylation of proteins present in this enzyme preparation. Furthermore, the sigma factor of *E. coli* RNA polymerase has been reported to be a substrate for mammalian protein kinase (*16*). Whether the adrenal medulla RNA polymerase II can be activated by phosphorylation was examined in the experiment described in Fig. 2. The polymerase was first incubated under conditions identical to those shown in Fig. 1, and enzymatic activity was assayed with excess calf thymus DNA. No significant change in the polymerase activity was detected when the polymerase proteins were phosphorylated by prior treatment with protein kinase alone or with protein kinase and cyclic AMP. Similar results were obtained when the homologous protein kinase isolated from cytosol or nucleus of bovine adrenal medulla was used (not shown). Since the polymerase II preparation was only partially purified, it is uncertain whether the phosphorylation shown in Fig. 2 occurred on the polymerase itself or on some contaminating nuclear protein. Nevertheless, our results indicate that the polymerase activity was not stimulated by cyclic AMP and protein kinase. Under similar conditions, Jungmann *et al.* (*13*) reported a tenfold increase in polymerase II activity in bovine ovary. The reason for this discrepancy is unknown, but may be related to a difference in mitotic activity, which is minimal in medullary chromaffin cells but is elevated in ovary, thymus, and liver cells. It is conceivable that the activity of RNA polymerase II can be regulated by phosphorylation and dephosphorylation in certain tissues but not in adrenal medulla. Alternatively, our preparation of RNA polymerase II from beef adrenal medulla might have been maximally phosphorylated in vivo and therefore could not be activated in vitro. However, our present results, showing that the medullary RNA polymerase II cannot be enhanced by cyclic AMP–dependent protein kinase, are consistent with our previous observation that the activity of RNA polymerase II from rat adrenal medulla is unchanged during the transsynaptically induced synthesis of RNA containing poly(A) (*5*). The in vitro enhancement of template activity by protein kinase and cyclic AMP reported here strongly suggests that transsynaptic

regulation of gene transcription involves a release of chromatin template restriction by the phosphorylation of some chromosomal protein; the phosphorylation appears to be mediated by a protein kinase which has been translocated to the medullary nucleus.

Only 20 percent of the ^{32}P incorporated into chromatin in our in vitro system could be extracted with $0.4N$ H_2SO_4, which indicates that most of the phosphorylation probably was in the nonhistone protein fraction. Purified acidic nuclear phosphoproteins isolated from a number of tissues bind to homologous DNA and enhance RNA synthesis in vitro (*17*). Thus, the augmented template activity observed in our system may have resulted from the phosphorylation of acidic chromatin proteins. The lack of quantitative correlation between the extent of RNA transcription and the degree of protein phosphorylation (Fig. 1) may suggest that phosphorylation of a specific substrate is involved in gene activation. Indeed, our recent data demonstrate a differential phosphorylation of acidic chromosomal proteins under the various conditions described in Fig. 1. It will be of interest to identify chromosomal proteins functioning as a physiological substrate for the catalytic subunits of protein kinase which are translocated during the transsynaptic induction of tyrosine 3-monooxygenase in rat adrenal medulla.

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Cross-Linking of DNA in situ as a Probe for Chromatin Structure

Abstract. *The photochemical cross-linking of DNA in situ in chromatin is blocked over short intervals. Electron microscopy of DNA cross-linked in chromatin reveals the lengths of protected regions and provides a map of their sites along the DNA. Protected regions occur most frequently in tandem and have a basic length of 160 to 200 base pairs.*

A chemical probe for chromatin structure ideally should possess two properties. (i) It should be capable of permeating cells and acting in vivo to avoid isolation artifacts; and (ii) it should involve a covalent interaction with the chromatin components so that the product provides a stable record of the specificities of this interaction. Photochemical cross-linking of DNA in chromatin by derivatives of psoralen meets these criteria.

Psoralens are a class of medically important furocoumarins known for their ability to photosensitize mammalian skin (*1*) (Fig. 1). Psoralen and its derivatives in conjunction with ultraviolet light of long wavelength (320 to 380 nm) can covalently cross-link pyrimidines in opposite strands of the DNA double helix (*1, 2*). Because cellular and nuclear membranes are permeable to psoralens, this reaction can take place in DNA in situ in chromatin, isolated nuclei, or intact living cells (*1, 3*).

We have developed a method for determining the sites of cross-links in DNA by electron microscopy. The DNA is prepared for microscopy as follows: it is exhaustively denatured by heat for 2 hours at 70°C in a mixture of 10 percent formaldehyde, $0.02M$ sodium carbonate, pH 7.0, $0.005M$ EDTA. Denatured regions are prevented from renaturing by their reaction with formaldehyde (*4*). A portion of the sample is then spread on a cytochrome protein film by the Kleinschmidt technique (*5*) with the formamide conditions of Davis *et al.* (*6*) for visualizing both (denatured) single strands and double strands of nucleic acid. Cross-linked regions thus have the appearance of double-stranded DNA while regions protected from cross-linking appear as open loops with single-stranded arms (Fig. 2b).

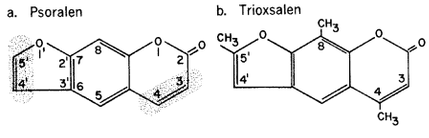

Fig. 1. (a) The general structure of psoralens. After intercalation into the DNA double helix, psoralens photoreact covalently with pyrimidines via the bonds in the shaded areas (*2*). (b) The structure of 4,5′,8-trimethylpsoralen (trioxsalen), which is the psoralen derivative used in this study.

By this technique we have observed the sites of cross-linking in DNA that undergoes photoreaction in situ and is subsequently purified. The overall design of the experiment is shown in Fig. 3. Spaced regions of each DNA molecule are often found to be so heavily cross-linked that the distance between cross-links is not detected by our technique (*7*). These regions alternate with stretches of 50 or more base pairs, which are protected from cross-linking. The protection of regions of DNA from cross-linking in situ appears not to be the result of base sequence heterogeneity since equivalent cross-linking of deproteinized DNA is uniform and nearly complete (Fig. 2a). The cross-linking pattern therefore must result from DNA conformation or interaction with proteins in chromatin.

The percentage of DNA in protected regions, the lengths of such regions, and their loci along the molecule may be determined. The fraction of DNA in protected regions is dependent on the extent of photochemical cross-linking; but even when the cross-linking is taken to a saturating level, a minimum of 60 percent of the DNA remains protected. This is comparable to the 50 to 87 percent of DNA that was protected from degradation in several studies of (limit) digestion of DNA in chromatin by exogenous nuclease (*8–10*).

The lengths of regions protected from cross-linking form a distribution as