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APPARENT PROTEIN KINASE ACTIVITY IN OLIGODENDROGLIAL CHROMATIN AFTER CHRONIC MORPHINE TREATMENT

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Abstract—The oligodendroglial nuclei were purified from mouse brain with more than 97 per cent homogeneity. Cyclic AMP-independent phosphorylation of non-histone protein in oligodendroglial chromatin was studied. Morphine sulfate, *in vitro*, had no effect on phosphorylation. However, chronic morphine treatment resulted in an increase of phosphorylation in high molecular weight regions of sodium dodecylsulfate (SDS) electrophoresis gel. The increase was not the result of a decrease in phosphoprotein phosphatase activity.

Chronic morphine treatment has been shown to alter chromatin template activity in oligodendroglial nuclei [1]. It is believed that the non-histone proteins are responsible for the change, since the removal of histones does not eliminate the chronic morphine effect on chromatin template activity. The non-histone proteins in cell nuclei have been implicated in regulation of DNA template activity in chromatin [2, 3]. The heterogeneity of these proteins makes it difficult to study each protein with respect to its function and regulation. However, it would be helpful to study the phosphorylation of these proteins, since the phosphorylation of the nuclear proteins, especially non-histone proteins, has been suggested as a means of positive gene regulation. The present study describes the property of apparent protein kinase activity in oligodendroglial-rich chromatin isolated from mice after prolonged treatment of morphine.

MATERIALS AND METHODS

Animals

ICR mice (male, 20–25 g) from Simonsen Laboratories, Gilroy, Calif., were used in all experiments.

Materials

γ-[^{32}P]ATP (tetratriethylammonium salt, 20–40 Ci/m-mole) was purchased from New England Nuclear Co.

Preparation of morphine-induced tolerant mice

The animals were rendered tolerant to morphine by the implantation of a 75-mg morphine pellet for a period of 72 hr [4]. The control group received placebo pellets.

Preparation of nuclei

The procedure was the same as described by Blobel and Van Potter [5] with some modification. All animals were sacrificed by decapitation. Brains were removed immediately and kept in cold 0.25 M sucrose–TKM buffer (Tris–HCl, 50 mM, pH 7.5, 23°; $MgCl_2$, 10 mM; and KCl, 25 mM). All procedures were carried out at 4° except where specified. The brains were homogenized with 2 vol. of 0.25 M sucrose–TKM buffer in a Potter–Elvehjem homogenizer, using ten strokes at 2000 rev/min with a tightly fitted Teflon pestle (Thomas Co., clearance 150 μm). The homogenate was filtered through four layers of cheesecloth and thoroughly mixed with an equal volume of 2.3 M sucrose in TKM buffer. A 24-ml portion of homogenate was underlayed with 12 ml of 2.3 M sucrose–TKM buffer and centrifuged at 96,300 *g* for 90 min. After the nuclei were isolated, the chromatins were purified according to the method of Spelsberg and Hnilica [6] and modified by Lee *et al.* [1].

DNA and protein determination

DNA and protein contents were measured according to the methods of Lowry *et al.* [7] and Ceriotti [8] respectively. The protein:DNA ratio is 1:1 for both placebo and tolerant groups.

Microscopy of nuclear preparations

Each nuclear preparation was examined under a Leitz Wetzlar microscope. The nuclear pellet was fixed with 10% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, rapidly dehydrated with increasing concentrations of ethanol (50, 70 and 95%), and embedded in paraffin wax containing picolyte resin. The sample was sectioned on a microtome to a thickness of 5 μm or less, stained with 0.1% cresyl violet, and examined.

Assay of phosphorylation in vitro

The incubation mixture contained potassium phosphate buffer, 50 mM, pH 6.5 or 7.3; EGTA, 0.3 mM; $MgCl_2$ 10 mM; NaF, 10 mM; γ-[^{32}P]ATP, 5 μM

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(500,000-800,000 cpm/0.2 ml of reaction mixture); and chromatin and water to a total volume of 0.2 ml. The reaction was initiated by the addition of ATP and incubated at 30° for 5 min. Two ml of 5% TCA was added to terminate the reaction. To the reaction mixture was then added 100 μg bovine serum albumin and it was allowed to stand in ice for 10 min. The precipitate was filtered through a GFC filter (Whatman) and washed four times with 9 ml trichloroacetic acid (TCA) (5%). The dried filters were then counted with 10 ml Scintiverse (Fisher Chemical Co.) in a Beckman LS-100 liquid scintillation counter.

Disc gel electrophoresis

Preparation of chromatin protein for electrophoresis. The reaction mixture for gel electrophoresis was the same as described above that [^{32}P]ATP, 2 Ci/mmole, was used and the total volume was 0.4 ml. After the chromatin protein (300 μg) had been phosphorylated *in vitro*, the reaction was terminated and proteins were dissociated with the addition of solid urea and NaCl to a final concentration of 5 and 2 M respectively. The phosphorylated chromatin was dialyzed against 8 M urea (deionized), 0.1% β-mercaptoethanol, and 0.01 M Na phosphate buffer, pH 7.0, at 4° overnight. The buffer was changed three times during the course of overnight dialysis. The dissociated chromatin proteins were then dialyzed against the same buffer plus 1% sodium dodecylsulfate (SDS). The dialyzed sample (150 μg protein) was incubated at 37° for 3 hr to insure complete dissociation, mixed with 10 μl Coomassie Brilliant Blue (100 μg/ml) and 10 μl of concentrated β-mercaptoethanol, and allowed to stand for 1 hr at room temperature before being applied to the acrylamide gel for electrophoresis.

Acrylamide gel. The 7.5% acrylamide gels were prepared according to the method of Bhorgee and Pederson [9]. The final concentrations in the separation gels were as follows: acrylamide, 7.5%; *N,N'*-bismethylene acrylamide, 0.28%; sodium dodecylsulfate, 0.1%; sodium phosphate buffer, 0.1 M, pH 7.0; urea, 0.5 M; and EDTA, 5 mM. The gels were polymerized by the addition of 0.05% tetramethylenediamine and 0.1% ammonium persulfate. The gels were 14 cm in length and 7.5 mm in diameter. The spacer gels contained 2.5% acrylamide and 0.01 M sodium phosphate buffer, pH 6.0. The length of the spacer gels was 2 cm. The electrode buffer contained 0.1 M sodium phosphate buffer, pH 7.0; 0.1% SDS; and 5 mM EDTA. Electrophoresis was carried out with a current of 10 mA/gel and terminated when the tracking dye migrated 7 cm from the top of the separating gel (about 7 hr). Proteins were fixed and stained according to the method of Fairbanks *et al.* [10].

RESULTS

Morphology of nuclear preparations

The procedure used in this study to purify nuclei resulted in a morphologically pure population (Fig. 1). Using the criteria described by Austoker *et al.* [11], or nuclei resemble oligodendroglial nuclei, being small and having round dense nuclei with tightly packed chromatin and peripheral nucleoli. In one typical preparation, out of 1257 total counts of nuclei, there were 37 nuclei of neuronal origin. The yield of the nuclei from this procedure was low (DNA recovery was 10 per cent of brain homogenate), but very little contamination by other nuclei was observed. It was found that the protein kinase activity from neuronal-rich nuclei was quite different from that of oligodendroglial nuclei (to be published elsewhere). Since our interest was in the protein kinase activity of pure oligodendroglial nuclei, we thus accepted the low yield of nuclei for high purity. In order to ascertain the purity of all the preparations,

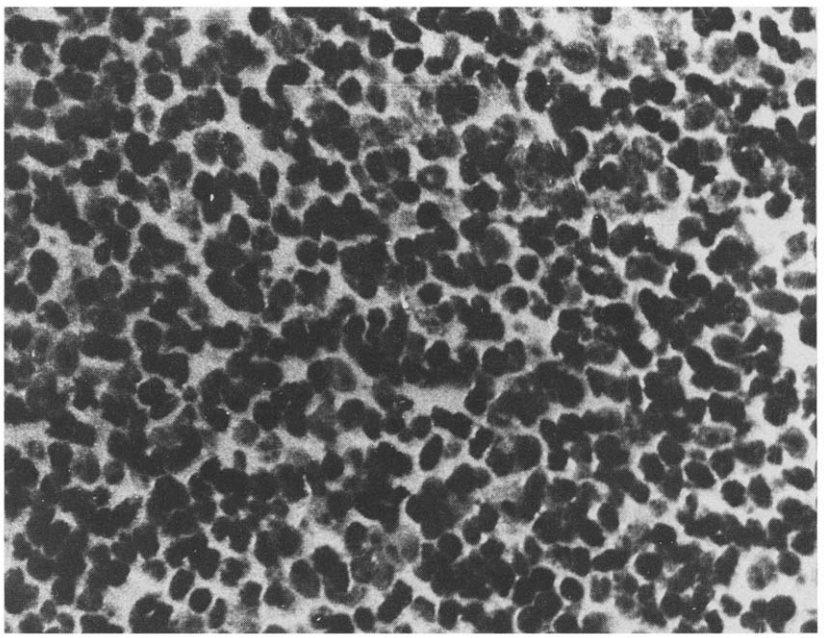

Fig. 1. Stained oligodendroglial nuclei from mice brain. The nuclear population was fixed and stained as described in Methods. The photograph was taken under a Leitz Wetzlar microscope at a magnification of 750.

light microscopic examination was performed for each nuclear preparation. Only the preparations with less than 5 per cent visible contamination were used to study protein kinase activity.

Protein kinase activity in vitro

Figure 2 shows that the activity of protein kinase isolated from mouse brain chromatin is linear up to 10 min. The zero time count was always less than 10 per cent of the total counts and has been subtracted. The pH optimum of the enzyme was quite broad; in the range tested (pH 6.0 to 8.0), there was no obvious peak. Therefore, in routine assay, pH 6.5 was used. The γ-[^{32}P]ATP incorporation was linear with increasing concentration of ATP up to 5 μM.

The enzyme may use its endogenous chromatin proteins as substrate. Additional histone (type II from calf thymus, Sigma Chemical Co.) 40 μg/0.2 ml did not increase the amount of γ-[^{32}P]ATP incorporation. However, additional casein (40 μg/0.2 ml) resulted in 50–60 per cent more phosphorylation. The enzyme was active when chromatin was freshly prepared; however, within a couple of days of storage at $-10°$, more than 80 per cent of the activity was lost. Therefore, assay of the protein kinase activity immediately after chromatin preparation is important.

Table 1 shows that the protein kinase activity from chromatin isolated from morphine-induced tolerant mice was 65 per cent higher than that of the control group. Addition of morphine sulfate (10^{-4} to 10^{-7} M or cAMP (10^{-5} to 10^{-7} M) *in vitro* had no significant effect on protein phosphorylation (Table 2).

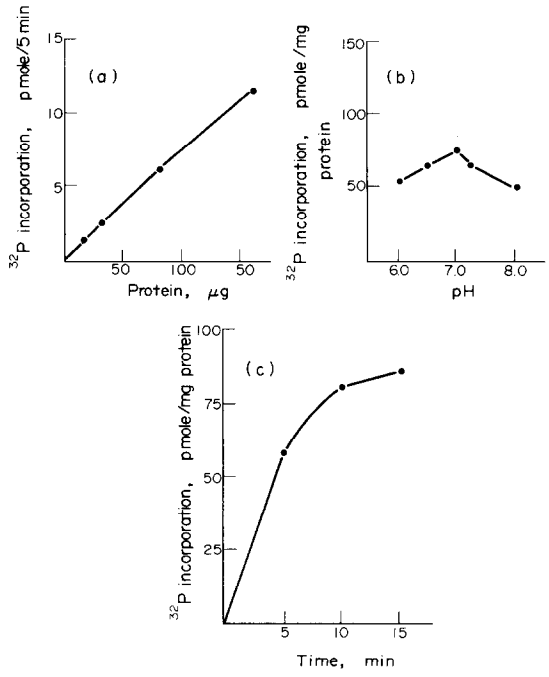

Fig. 2. Some properties of the apparent protein kinase activity in oligodendroglial-rich chromatin. The reaction mixture is as described in Methods except that in panel a various concentrations of chromatin proteins were added, in panel b, various pH values of potassium phosphate buffer were used and in panel c various time intervals if incubation were used.

Table 1. Effect of chronic morphine treatment on chromatin protein kinase activities

Treatment	^{32}P incorporated (pmoles/mg protein)
Non-tolerant	54.9 ± 3.47 (4)*
Non-tolerant + 40 μg casein/reaction	81.2
Tolerant	90.9 ± 10.23 (4)*
Tolerant + 40 μg casein/T	112.3

* $P < 0.01$. Number in parentheses is the number of experiments performed.

In order to determine whether or not the increase in phosphorylation of chromatin protein isolated from tolerant animals was due to a decrease in phosphoprotein phosphatase activity, the cold ATP dilution procedure was used. γ-[^{32}P]ATP-dependent phosphorylation was preincubated for 10 min. Non-radioactive ATP (5 mM) was added to one-half of the reaction mixture and the same volume of water was added to the other half. Aliquots were withdrawn at various times and chromatin proteins were analyzed for their ^{32}P content. Figure 3 shows that dephosphorylation was evident in both the placebo and the tolerant groups. At 20 min, dephosphorylation was identical for both preparations. At 60 min, in chromatin isolated from the placebo and the tolerant groups, 31 and 18 per cent of the ^{32}P were lost respectively. However, in the absence of cold ATP, chromatin isolated from tolerant animals increased phosphorylation by 56 per cent at 60 min, whereas in chromatin isolated from placebo, phosphorylation increased less than 20 per cent. This result indicated that protein kinase was responsible for the increase of phosphorylation in chromatin protein isolated from tolerant animals.

Electrophoresis of the phosphorylated chromatin proteins

Nuclear chromatin comprises a heterogeneous mixture of proteins differing in molecular weight, amino acid composition and degree of phosphorylation. The heterogeneity of molecular sizes is indicated by the differences in electrophoretic mobility in SDS-polyacrylamide gel. The degree of phosphorylation can also be measured in the gel with ^{32}P-labeled chromatins.

Figure 4 reveals the complicated banding pattern of chromatin protein subunits. There are at least thirty different multiple polypeptide bands ranging in molecular weight from 15,000 to 200,000 daltons calculated from proteins with known molecular weights. The most prominent bands after staining with Coomassie Brilliant Blue were the histone proteins. It has been reported that two of the histone proteins were located in the middle of the gel (slices 19–22) and two more were observed in the lower part of the gel (slices 30–36) [9]. In this study, the specific activity of ^{32}P-labeling was low in the histone protein-rich area. In agreement with the findings of Rickwood *et al.* [12], this indicated that histone proteins were a rather weak substrate for this protein kinase reaction. The other bands of the gels were non-histone protein bands. The high molecular weight region (slices 1–5)

Table 2. Effect of morphine and cAMP on phosphorylation of chromatin protein *in vitro*

Treatment	^{32}P incorporated (pmoles/mg protein)
Control	65.8 ± 4.2
Control + morphine sulfate, 10^{-7} M	64.5 ± 1.9
Control + morphine sulfate, 10^{-6} M	56.8 ± 2.3
Control + morphine sulfate, 10^{-5} M	64.8 ± 4.5
Control + morphine sulfate, 10^{-4} M	58.4 ± 4.7
Control + cAMP, 10^{-5} M	72.2 ± 5.0
Control + cAMP, 10^{-6} M	68.8 ± 2.0
Control + cAMP, 10^{-7} M	69.2 ± 5.5

represents a series of very finely separated high molecular weight protein subunits. Although the bands were rather light after staining with Coomassie Blue, it was quite clear that they are highly phosphorylated (Fig. 4). The degree of phosphorylation in this region was about 74 per cent higher in the tolerant group than in the control group (Table 3), although the electrophoretic patterns between those two groups were similar.

Since there was no visible protein staining, the ^{32}P-labeling was quite significant in slices 38–46 (molecular weight about 7000 daltons). The intensity of phosphorylation was about the same between chromatins isolated from tolerant animals and controls. However, the band moved slightly faster in the tolerant group (Fig. 5). The shifting in mobility is a true phenomenon, since we have repeated it more than five times in five different nuclei preparations and found it to be reproducible each time. When morphine sulfate (1 mM) was added during phosphorylation *in vitro* of chromatin proteins isolated from control or tolerant animals, the electrophoretic pattern of this shift was unaffected.

Cyclic AMP did not stimulate the phosphorylation of chromatin protein *in vitro*. Electrophoretic patterns of the chromatin proteins phosphorylated with or without the presence of cAMP (5 μM) were similar, except in slices 38–46. Figure 6 shows that this peak shifted when phosphorylated in the presence of cAMP. This shift was observed in both types of chromatins.

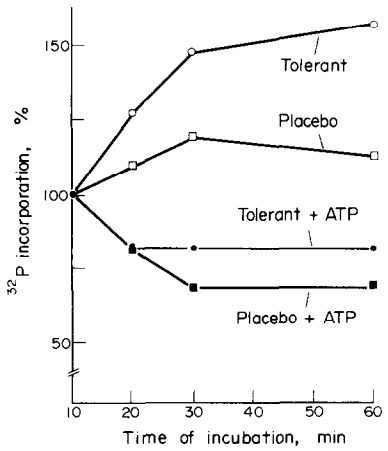

Fig. 3. "Turnover" of previously incorporated [^{32}P]phosphate in chromatin proteins. Chromatin proteins were incubated for 10 min in the presence of [^{32}P]ATP as described in Methods. Retention of ^{32}P was measured after the addition of 5 mM of non-radioactive ATP.

DISCUSSION

The data presented in this study demonstrate the apparent protein kinase activity in oligodendroglial-rich chromatin. Since it is unclear whether or not the protein kinase(s) is a separate protein(s) or the kinase activity is inherent in the phosphoproteins themselves [9], we decided to refer to the activity as "apparent protein kinase activity". The addition of casein has been shown to increase ^{32}P-labeling in TCA-precipitable materials. Since casein is not a natural component of brain tissue, the significance of

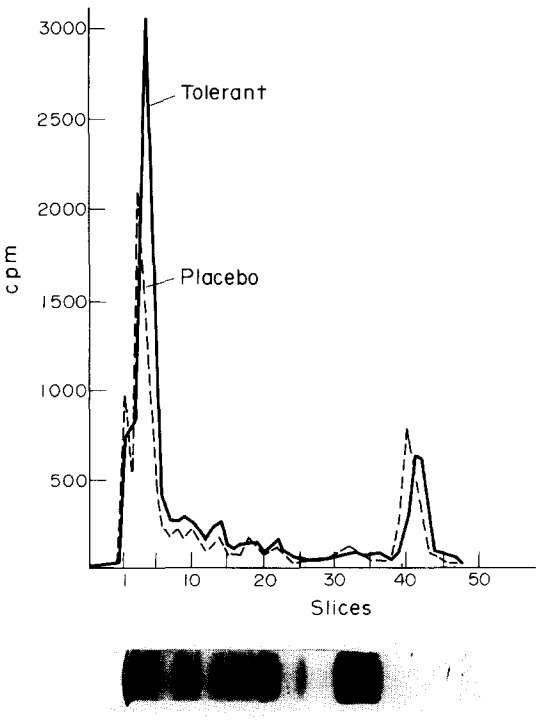

Fig. 4. SDS acrylamide gel electrophoresis of mice brain chromatins. The chromatins from placebo and morphine-tolerant mice were phosphorylated and electrophoresed as described in Methods. Gels were stained by Coomassie Brilliant Blue. Gel origin is at left.

Table 3. Degree of phosphorylation in high molecular weight region

Treatment	Activity (cpm)
Placebo	1675 ± 154 (4)*
Tolerant	2925 ± 142 (4)*

* $P < 0.001$. Number in parentheses is the number of experiments performed.

the stimulation of phosphorylation is unclear. There is no obvious optimum pH for this enzyme activity indicating that several enzymes may be responsible for the phosphorylation. Kish and Kleinsmith [13] have reported the separation of protein kinase activity into twelve distinct enzyme fractions in beef liver chromatin. This possibility of multiple enzymes would also have to be carefully examined in oligodendroglial-rich chromatin preparation. Unlike the histone kinase, this protein kinase activity was very labile; 80 per cent of the activity was lost within only a few days of storage. This presents a serious problem in further purification of the enzymes. A search for methods to stabilize the activity is now in progress.

Chronic morphine treatment resulted in an increase in total apparent protein kinase activity. It was evident in cold ATP dilution experiments that the increase in phosphorylation of chromatin in tolerant animals was, indeed, due to "apparent protein kinase activity". Compared to the placebo group, although the turnover rate of ^{32}P was slower in chromatin protein isolated from tolerant animals, the phosphorylation rate in the absence of cold ATP was considerably higher at all time periods. These results suggest that this may be at least partially due to the increase in protein kinase activity.

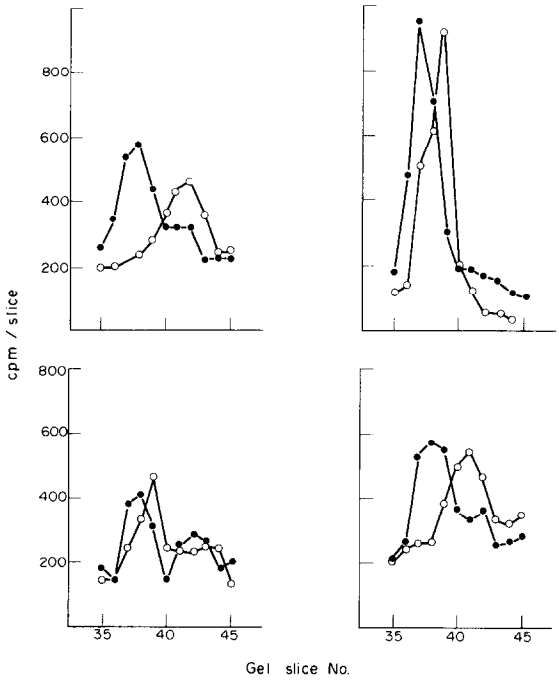

Fig. 5. Phosphorylation in electrophoresis gel slices 35–45 from four different chromatin preparations. The chromatins from placebo and morphine-tolerant mice brain were phosphorylated and electrophoresed as described in Methods. Results from four different chromatin preparations are presented. Gel origin is at left. Key: ●, placebo; ○, tolerant.

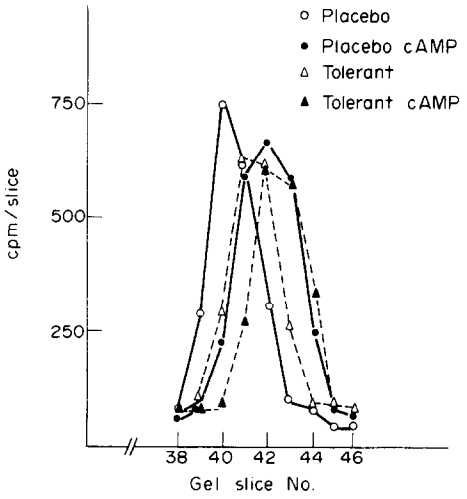

Fig. 6. Effect of cAMP *in vitro* in phosphorylation in electrophoresis gel slices 38–45. The chromatins from placebo and morphine-tolerant mice brain were phosphorylated in the absence or presence of cyclic AMP (5×10^{-6} M), respectively, and electrophoresed as described in Methods. Gel origin is at left.

The phosphorylation of proteins occurred mainly in the acidic protein region of chromatin proteins. Electrophoresis of SDS-acrylamide gel revealed that chronic morphine treatment increased the ^{32}P incorporation in this area. It is not known if the gel slices still contain protein kinase activity. Furthermore, the ability of a one-dimensional dodecylsulfate-electrophoresis system to demonstrate the true complexity of chromatin non-histone proteins is limited [14], therefore requiring more detailed work to elucidate this high molecular weight region. In slices 39–45, where there was no visible protein stain, significant phosphate labeling was observed. It is interesting to note that this band was similar in position to the one reported by MacGillivray and Rickwood [14]. Chronic morphine treatment shifted this band slightly; it is not known if this was the result of a change in peptide molecular weight.

It has been suggested that phosphorylation of chromatin proteins would normally increase chromatin template activity [15]. Therefore, our observation may represent one of the positive controls of gene expression. The addition of morphine sulfate *in vitro* had no effect on ^{32}P-labeling of protein, indicating that morphine did not directly interfere with protein phosphorylation. Therefore, the effect of chronic morphine treatment *in vivo* may be via some other mechanism, indirectly affecting protein phosphorylation of chromatin proteins.

In summary, we have demonstrated apparent protein kinase activity in oligodendroglial-rich chromatin. The activity was unaffected by cAMP or morphine sulfate *in vitro*. Chronic morphine treatment resulted in increased phosphorylation which may be

duc to protein kinase activity rather than to a decrease of phosphoprotein phosphatase activity.

The increase was located primarily in high molecular weight regions of the SDS gel. We reported before [1] that the oligodendroglial-rich template activity increased in chronic morphine-treated animals; the increase in phosphorylation of non-histone proteins observed in this study may be related. Further studies are in progress in this laboratory to educidate the phosphorylation reaction and its relationship to chromatin template activities in different types of nuclei.

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