COMPARISON ON EFFECTS OF PHENOBARBITAL AND NICOTINE ON NUCLEAR PROTEIN SYNTHESIS IN RAT LIVER

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1. Introduction

Recently, effects on the synthesis of nuclear proteins have been reported to occur after the administration of agents which are capable of producing enzyme induction in animal cells. Bresnick [1] has reported that administration of 3-methylcholanthrene to rats produced an increase in the synthesis of a species of hepatic nuclear protein. Stein and Baserga [2] have shown that acidic nuclear protein formation was elevated in mouse salivary glands 2 hr after isoproterenol administration. In addition, an increased synthesis of specific non-histone nuclear proteins has been reported to occur in rat liver after phenobarbital injection [3] or after hydrocortisone administration [4] and in rat uterus after estradiol treatment [5]. In general, these elevations of nuclear protein formation occurred early in the induction process, e.g. prior to increases in enzyme synthesis. This evidence suggests that alteration of synthesis and turnover of specific non-histone nuclear proteins is a crucial event in activation of the flow of genetic information in mammalian cells challenged with agents that produce proliferative or enzyme inductive responses in these cells.

Chronic administration of nicotine to rats in doses of 4–5 mg/kg/day has been shown to increase the activity of a number of hepatic microsomal drug metabolizing enzymes [6]. The increased activity after nicotine treatment appeared to be due to an increase in enzyme synthesis [6, 7]. The present investigation was undertaken to determine if chronic administration of nicotine to rats, with doses that produce induction of microsomal enzymes, would lead to an increased synthesis of non-histone nuclear proteins. The effects of nicotine treatment were compared to those of inducing doses of phenobarbital.

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2. Methods

Male rats (Spartan Farms, Lansing, Michigan) weighing 70–80 g were employed in the study. For the phenobarbital experiments, 7 animals were injected intraperitoneally with phenobarbital (75 mg/kg) and 7 animals with 0.9% NaCl 2 hr prior to pulse labeling for 40 min with an i.p. dose (200 μCi) of leucine-4,5-^{3}H (58.2 Ci/mmole; New England Nuclear Corp.) in the case of the phenobarbital-treated rats or 20 μCi leucine-^{14}C (225 mCi/mmole; New England Nuclear Corp.) in the case of the controls. After sacrifice, the livers from the control and treated groups of animals were pooled, homogenized, and the nuclear proteins extracted as described below.

Another group of rats was treated chronically with nicotine in the drinking water as previously described [6]. The concentration was adjusted to deliver a dose of approximately 5 mg/kg/day for a 7 day period. Control rats were placed in the same environment with access to untreated drinking water. At the end of one week, 7 control and 7 nicotine-treated animals were each injected with a pulse dose of leucine-^{3}H (340 μCi) and sacrificed 40 min later. An increased dose of labeled leucine was employed in this latter

experiment in an attempt to increase the specific activity of the nuclear protein fraction. The liver preparations from the control group were pooled, as were the livers from the treated group, after homogenization.

All animals were sacrificed between 8–9 a.m. After sacrifice the livers were removed and homogenized in 4 volumes of ice-cold 0.44 M sucrose in a Dounce homogenizer (4 strokes with a "loose" and 3 strokes with a 'tight' pestle). Nuclei were isolated by the procedure of Chaveau et al. [8]. A 'non-histone' fraction of nuclear proteins was extracted with 1 M NaCl as previously described [3]. The 1 M NaCl extracts were then dialyzed to 0.14 M NaCl, and the flocculent precipitate of nucleohistone was removed by centrifugation at 25,000 *g* for 30 min in a Sorvall refrigerated centrifuge.

Polyacrylamide gel electrophoresis was carried out on 10% acrylamide gels with a running buffer of 0.1 M sodium phosphate (pH 7.2), containing 0.1% sodium dodecyl sulfate, by the method of Platz et al. [9]. Protein samples (40–50 μg) previously diluted with a buffer containing 0.01 M sodium phosphate, pH 7.2, 0.1% SDS, and 0.1% β-mercaptoethanol were layered on the gels. Electrophoresis was carried out with a current of 6 milliamps per tube. The protein bands were stained with 0.25% Coomassie brilliant blue in methanol–acetic acid–water (5:1:5). The gels were then destained and scanned at 550 nm in a Gilford Model 2400 recording spectrophotometer with a linear transport attachment. Three gels of each group were aligned according to the pattern of protein bands; consecutive transverse slices (1.2 mm each) were then made and 2 corresponding slices from each gel (total of 6 slices) were placed in scintillation counting vials. The gel slices were then dissolved in 1 ml of 30% hydrogen peroxide at 50° for 12 hr. Ten ml of a mixture containing 6 parts Triton X-100 and 7 parts toluene scintillator was added to each vial, and the samples were analyzed for radioactivity in a Packard Tri Carb liquid scintillation spectrometer.

Radioactivity of the total protein extracts was determined by adding 1 ml aliquots of extract to 10 ml of Scintisol–toluene phosphor mixture (1:5) and counting as above. Protein was determined by the method of Lowry et al. [10].

3. Results and discussion

In order to observe an increase in the specific activities of hepatic microsomal drug-metabolizing enzymes after nicotine administration to rats, it is necessary to administer the drug over a period of several days [6]. Therefore, in order to determine whether there might be a relationship between nuclear protein synthesis and elevated enzyme activity, similar to that observed after doses of phenobarbital which induce microsomal enzyme formation [3], nicotine was administered in the drinking water for 1 week at a dose (5 mg/kg/day) previously observed to increase microsomal enzyme activity.

There was an increase in the incorporation of labeled leucine into the total non-histone nuclear protein fraction after a 40 min pulse in the nicotine-treated rats (control = 13,627 cpm/mg and treated = 16,810 cpm/mg protein). Doses of nicotine of this magnitude do not produce an increased uptake of labeled amino acid into the acid soluble pool. Fig. 1 illustrates the absorbancy tracing of an electrophoretic run of hepatic nuclear proteins. There was no difference between the absorbancy profile of the proteins extracted from nicotine-treated and control

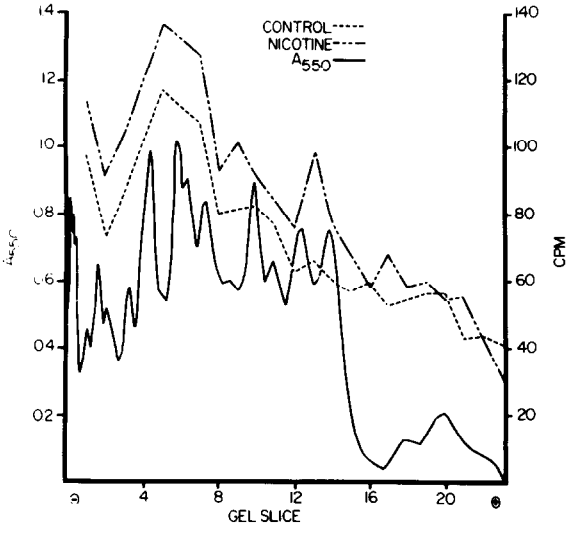

Fig. 1. Polyacrylamide gel electrophoresis of hepatic nuclear protein samples from control and nicotine-treated rats. The same amount of protein (47 μg) was layered on each gel. Gels were prepared and analyzed as described in the Methods.

animals. The radioactivity contained in the major peaks was generally higher in the nicotine-treated samples, in agreement with the higher specific activity observed in the total nuclear protein extract from nicotine-treated rats. However, when the ratio of cpm-treated/cpm-control was plotted from the radioactivity contained in the gels, there was an apparent selectivity to the labeling pattern (fig. 2). The most pronounced peak was 34 mm from the origin. For comparison, the ratios for a similar experiment, in which 40 min pulses of labeled leucine were given 2 hr after an 'enzyme-inducing' dose of phenobarbital (75 mg/kg) or 0.9% NaCl, are plotted on the same scale. In these latter experiments, the double-label technique was employed and an increased labeling of a peak approximately 10–12 mm down the gels was observed. This distance of migration of the labeled peaks was repeatedly observed when the extracts from the nicotine and phenobarbital experiments were rerun on polyacrylamide.

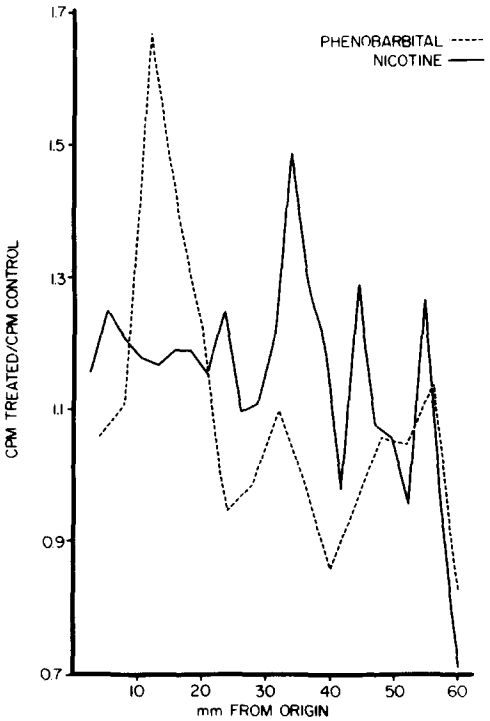

Fig. 2. Comparison of relative migrations of labeled nuclear protein fractions whose synthesis is stimulated by either phenobarbital or nicotine administration. Gels were prepared and analyzed as described in the Methods.

Shelton and Allfrey [4] have observed a similar finding in rat liver after the administration of hydrocortisone, i.e. a selective increase in synthesis of a specific acidic nuclear protein observed with a 60 min pulse of leucine-^{3}H. This increased synthesis was evident 2–3 hr after hydrocortisone administration and was increased to 200% above the control by 7–8 hr after drug treatment. It therefore appears that various agents which lead to increased enzyme synthesis in liver produce a prior of concomitant elevation in the synthesis of specific non-histone nuclear proteins. Since these proteins may be involved in control of gene readout [11], it is important to understand what factors control the synthesis, turnover, and function of this group of nuclear proteins.

On the basis of the available evidence, some of which is detailed above, we have proposed a schematic model for the control of gene readout in mammalian cells (fig. 3). The model proposes that various hormones, inducing agents, and chemical carcinogens enter the cell nucleus (perhaps via a protein carrier as has been implicated for estradiol) [12], and then interact with specific 'acidic' nuclear protein receptors (ANP used here simply to mean non-histone protein). The altered nuclear protein then becomes a substrate for a specific phosphorylation reaction. This reaction is probably mediated by 3′,5′-cyclic AMP in the case of certain hormones [13]. Thus, cyclic AMP may act in a 'permissive' way in the gene activation events which follow. Phosphorylation of the ANP-inducer complex 'activates' it in such a way that it now becomes an active initiator for RNA polymerase at a specific gene site. Once the gene activation event has been initiated, the 'used' phosphoprotein initiator is released. This may occur subsequent to the formation of the first phosphodiester bond in the RNA chain (the phosphoprotein binding site on polymerase may be at or near the triphosphate binding site of the enzyme).

The phosphoprotein initiator could then be dephosphorylated via a phosphatase. Subsequently, it could be reactivated by the phosphorylation step and utilized again as an initiator for RNA polymerase. This would seem to be the most economical way for the cell to maintain a particular level of a given initiator. However, it is also visualized that a significant portion of the 'used' phosphoprotein is metabolized after its release in order to provide for a

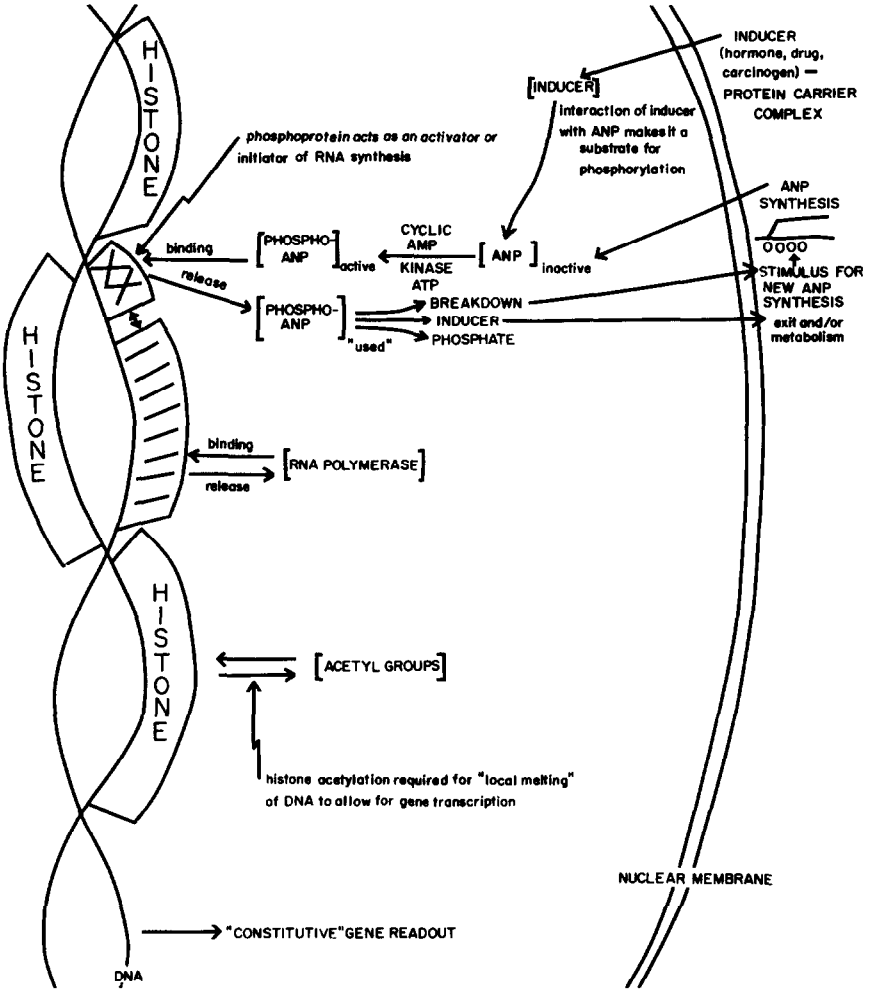

Fig. 3. Schematic model for the control of gene readout in mammalian cells. ANP = acidic nuclear protein; [XX] = activated phosphoprotein initiator; [/////////] = RNA polymerase. The direction of gene readout is visualized as proceeding from the initiator site and is determined by the orientation of the initiator and RNA polymerase.

distinct shut-off point and to prevent a continued re-initiation of the activation event. This would also explain stimulation of the synthesis of specific nuclear proteins that has been observed after the administration of inducing agents [3–5]. Thus the metabolism and breakdown of the nuclear protein involved in the induction process is a signal for the re-synthesis of that protein by the synthetic machinery in the cytoplasm. In this way the cell very carefully regulates the level of specific non-histone nuclear proteins. The binding of RNA polymerase is visualized as being mediated via the activated initiator protein. Once transcription is completed the polymerase is released.

In this model, chemical alteration of histone by acetylation (and perhaps by phosphorylation) is seen as a concomitant, permissive event allowing for the local 'melting out' of the DNA chain which may be involved in the subsequent base-pairing events between DNA and nascent RNA chains. This might be brought about by alteration of the steric and/or ionic properties of the histone molecule. In any event, histone is not visualized as actually being released from chromatin since this would imply that the synthesis

of new proteins, not usually synthesized in that cell type, would be initiated – an observation inconsistent with most of the data on enzyme induction in differentiated cells. The genes which require specific activation would be readout only at a reduced or very slow rate in the non-induced state. A portion of the genome does not require specific activation and would be involved in 'constitutive' gene readout.

The differences between agents which produce a more 'physiological' response (e.g. hormones) and those such as carcinogens, which may produce more 'irreversible' biochemical changes in gene readout, may reside in the tightness of binding of the agent with ANP and the subsequent affinity of the inducer-ANP complex for DNA. It is known, for example, that certain polycyclic hydrocarbons and other carcinogens (e.g. nitrosamines) may form covalent bonds with proteins and nucleic acids. This might then result in a more permanent type of altered gene readout of normally only minimally active genes.

This model, based on recent experimental evidence, offers a rationale by which the role of nuclear proteins in control of gene activity can be systematically examined.

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