minutes. Then the DNA-cellulose or plain cellulose was washed four times with the same buffer.
15. M. Katsumata, M. K. Baker, A. S. Goldman, D. L. Gasser, *Immunogenetics* **13**, 219 (1981); M. Katsumata, M. K. Baker, A. S. Goldman, *Biochim. Biophys. Acta* **676**, 245 (1981).
16. W. M. Burnham, L. Spero, M. M. Okasaki, B. K. Madras, *Can. J. Pharmacol.* **59**, 402 (1981).
17. B. Hammond, B. S. Katzenellenbogen, N. Krauthammer, J. McConnel, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 6641 (1979).
18. C. Gupta and A. S. Goldman, *Science* **216**, 994 (1982).
19. M. Katsumata, C. Gupta, A. S. Goldman, in preparation.
20. J. L. Humes, R. J. Bonney, L. Pelus, M. E. Dahlgren, S. J. Sadowski, F. A. Kuehl Jr., P. Davies, *Nature (London)* **269**, 149 (1977).
21. Supported in part by grants DE-4622, DE-5041, and DE-5592 from the National Institute of Dental Research.

21 September 1982

Eukaryotic Transcriptional Regulation and Chromatin-Associated Protein Phosphorylation by Cyclic AMP

Abstract. *Cyclic adenosine monophosphate (AMP) analogs or agents that increase intracellular cyclic AMP rapidly stimulate transcription of the prolactin gene in a line of cultured rat pituitary cells. This effect is correlated with the phosphorylation of a chromatin-associated basic protein designated BRP. These data are consistent with the postulate that increased intracellular cyclic AMP concentrations induce rapid transcriptional effects on specific genes in eukaryotes, mediated by direct or indirect phosphorylation of a specific chromatin-associated protein or proteins.*

Adenosine 3′,5′-monophosphate (cyclic AMP) is an important regulatory molecule serving to control diverse biochemical events in both prokaryotic and eukaryotic organisms. In prokaryotes, it acts as an allosteric effector allowing a specific gene activator protein (termed either CAP or CRP) to bind to specific DNA sequences and thus regulate transcription of several catabolite-sensitive operons (*1*). In eukaryotes, it is believed to act as a "second messenger" through which intracellular events are controlled by external stimuli, including various polypeptide hormones. Both the discovery that cyclic AMP receptor protein is associated with a catalytic subunit in higher eukaryotes (*2*) and studies of cyclic AMP–resistant cell lines (*3*) suggest that the cyclic AMP–dependent protein kinase is responsible for many, and possibly all, of the cyclic AMP–mediated effects. One critical consequence of an increase in cyclic AMP in eukaryotes is the increased biosynthesis of specific proteins, which is invariably associated with an increase in their encoding messenger RNA's (mRNA's) (*4, 5*). Because of the nuclear location of eukaryotic genes, it is necessary to ascertain whether cyclic AMP exerts direct, specific transcriptional effects and how such regulation might occur. Recent advances in recombinant DNA technology allow potential regulation of specific genes by cyclic AMP to be critically assessed.

Cyclic AMP has been shown to overcome the inhibitory effects of a dopamine agonist on prolactin gene transcription in pituitary cells, implying its potential as a gene regulator (*6*). We now report the use of cloned prolactin DNA sequences to demonstrate directly that an elevation of intracellular cyclic AMP is associated with a rapid increase in prolactin gene transcription in a clonal line of rat pituitary cells. We further identify the concomitant phosphorylation of a 23-kilodalton chromatin-bound basic protein. The association of these events suggests that the pathway mediating cyclic AMP regulation may involve phosphorylation of a nuclear protein, which in turn regulates transcription of specific genes.

The cultured clonal cell line (GH) provided a system in which to study the transcriptional regulation of the prolactin and growth hormone genes by both polypeptide and steroid hormones (*7, 8*). Because many of these hormones are thought to act via second messengers, prolactin gene expression was investigated to evaluate potential regulatory effects produced by elevation of intracellular cyclic AMP. Transcription rates were determined in isolated nuclei by elongating nascent RNA transcripts in the presence of [^{32}P]uridine triphosphate. Labeled prolactin RNA transcripts were quantified by hybridization to an immobilized intervening sequence subclone under DNA excess hybridization conditions (*9*), as described (*10*). Based on α-amanitin sensitivity, all the hybridized labeled prolactin RNA products appear to represent polymerase II transcripts.

Elevation of cyclic AMP by addition of the cyclic AMP analogs, 8-bromo–cyclic AMP or dibutyryl cyclic AMP, to cell cultures increased transcription of the prolactin gene four- to fivefold above that in corresponding unstimulated cells (Fig. 1A). A butyrate control was included since butyrate can be generated by metabolism of dibutyryl cyclic AMP (*11*). Addition of forskolin, a diterpene

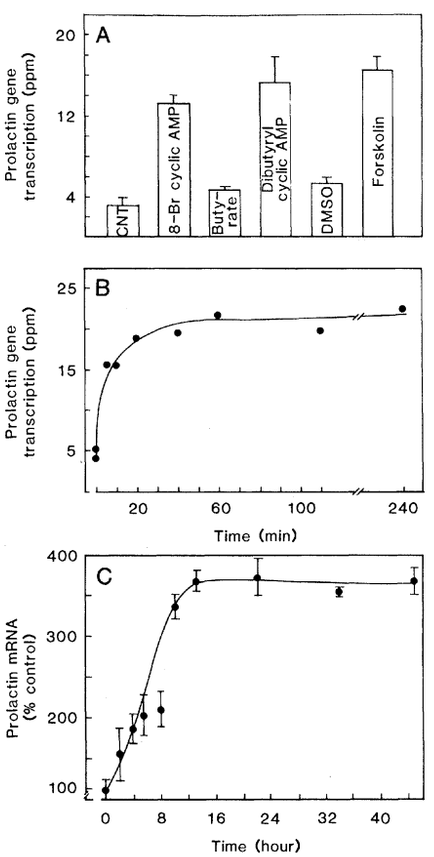

Fig. 1. (A) Effect of elevated cyclic AMP on prolactin gene transcription. Prolactin gene transcription rates were measured by quantifying specific prolactin transcripts as follows. Elongating nascent RNA chains were isolated from nuclei prepared from GH_4 cells that had been incubated with 8-bromo (*8-Br*)–cyclic AMP (2.5 m*M*), butyrate (1 m*M*), dibutyryl cyclic AMP (1 m*M*), forskolin (10^{-6}*M*), or dimethyl sulfoxide (*DMSO*) (0.1 percent as solvent control for forskolin) or in the absence of added agents (*CNT*) for 60 minutes prior to isolation of nuclei. (B) Time course of forskolin (10^{-6}*M*) stimulation of prolactin gene transcription. Forskolin was added in ethanol at a final concentration of 0.5 percent, which was determined to exert no effect on prolactin gene transcription. (C) Time course of prolactin mRNA accumulation following transcriptional stimulation by forskolin (10^{-6}*M*). RNA was prepared by phenol-chloroform extraction and prolactin mRNA was quantified by immobilization of "diazotyzed paper" (DBM) and DNA-excess hybridization with the use of a cloned, nick-translated prolactin complementary DNA probe, as described (*13*). Each point in panels A, B, and C is the average (± standard error of the mean) of triplicate hybridizations per group. Each group consists of four to six plates (3×10^7 cells). The triplicate determination in (B) differed by less than 5 percent. This experiment was representative of six experiments of similar design giving comparable data.

0036-8075/82/1224-1315$01.00/0

that specifically and rapidly activates the catalytic subunit of adenylate cyclase with resultant large increases in cellular cyclic AMP concentrations (*12*), also produced a four- to fivefold stimulation. Altering the concentration of these agents over a 100-fold range produced no additional induction, suggesting that the observed four- to fivefold stimulation represents the maximal increase in prolactin gene transcription resultant from the increase in cyclic AMP.

Because of its stability, high solubility, and rapidity of action, forskolin was chosen as a convenient agent for further analysis of the cyclic AMP regulation of prolactin gene expression (Fig. 1A). Forskolin increased transcription of the prolactin gene with a half-time to maximal stimulation of < 5 minutes (Fig. 1B). Because the probe used to quantify transcription is in the third intervening sequence, > 5 kilobase pairs 3′ with respect to the 5′ transcription initiation site, the elongation assay will reflect events occurring 1 to 2 minutes after the initiation of cyclic AMP–induced transcriptional effects. Therefore, the transcriptional effects by forskolin must begin within seconds to minutes. These data are suggestive of a direct cyclic AMP effect rather than a process secondary to cyclic AMP–induced alteration of cell metabolism. There is no attenuation of the induction over the initial 10 hours, during which this increase in cyclic AMP is maintained. This is in contrast to the effects of the thyrotropin-releasing hormone (TRH) on prolactin gene transcription, which is characterized by an initial burst of transcriptional stimulation followed by a rapid, marked attenuation (*13*).

The consequence of the increased prolactin gene transcription following the addition of forskolin is an increase in prolactin mRNA with the rate of accumulation similar to that observed after the addition of TRH or EGF (epidermal growth factor) (Fig. 1C). Although the half-life of prolactin mRNA has not been reported, the steady-state stimulation of transcription in response to cyclic AMP implies that the half-time to maximal RNA accumulation (approximately 6 hours) corresponds to the half-life of prolactin mRNA in the cyclic AMP–induced cells.

If cyclic AMP exerts its effects in eukaryotes via activation of cyclic AMP–dependent protein kinase, then it is possible that the rapid effects of prolactin gene transcription, which occur within seconds to minutes, are mediated by phosphorylation of a nuclear protein (or proteins). Multiple types of two-dimensional gel analyses, designed to display different subsets of nuclear proteins, were performed to evaluate this hypothesis. Surprisingly, a single nuclear protein was identified which is phosphorylated in parallel with the cyclic AMP–induced effects on prolactin gene transcription. To examine this protein further, we incubated GH_4 cells for 5 hours in a phosphate-free medium (*13*) containing [^{32}P]orthophosphate (0.5 mCi/ml) to fix the specific activity of the adenosine triphosphate (ATP) pool; 20 minutes before the cells were harvested, forskolin (10^{-6} *M*) was added to appropriate cultures. Chromatin was prepared from isolated nuclei, and acid-solubilized proteins were precipitated with acetone and subjected to two-dimensional gel electrophoresis with the use of an acid urea–Triton polyacrylamide gel for the first dimension and sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis for the second dimension (*14*).

As shown in Fig. 2, A and B, the phosphorylation of a discrete basic nuclear protein is stimulated more than tenfold after elevation of cellular cyclic AMP by forskolin or the addition of cyclic AMP analogs. No other basic proteins, including the major H1 histone, the minor H1 isotypes, or the H3 histones were phosphorylated in response to cyclic AMP, nor has a rapid cyclic AMP–induced phosphorylation of specific acid nuclear proteins yet been identified. This relatively minor nuclear protein, with a molecular weight of approximately 23,000, therefore represents a potential substrate of the catalytic subunit of the cyclic AMP–dependent protein kinase. This phosphoprotein co-migrates on denaturing SDS gel electrophoresis with the major H1 histone, but migrates more slowly in acid urea-Triton gels. It is initially best classified as a basic chromatin-associated regulated phosphoprotein, which we now refer to as BRP. It is likely that this or a related protein is present and exhibits regulated phosphorylation in other tissues and cell types. Nuclear proteins with similar properties may be present in several types of neural tissue (*14, 15*), and perhaps other tissues as well. Because of the ubiquity of the elements of the cyclic AMP regulatory system, we suggest that BRP or BRP-related proteins may be present in many hormonally responsive cells (*16*). It becomes important to ascertain whether BRP can act as a direct substrate of the cyclic AMP–dependent protein kinase. In order to address this question, chromatin was prepared and incubated with a homogeneous preparation of the catalytic subunit of cyclic AMP–dependent protein kinase in the presence of ^{32}P-labeled ATP (*14*). Addition of the catalytic subunit increased the labeling of BRP (Fig. 2, C and D). However, since chromatin may contain associated protein kinase activity (Fig. 2C), it remained possible

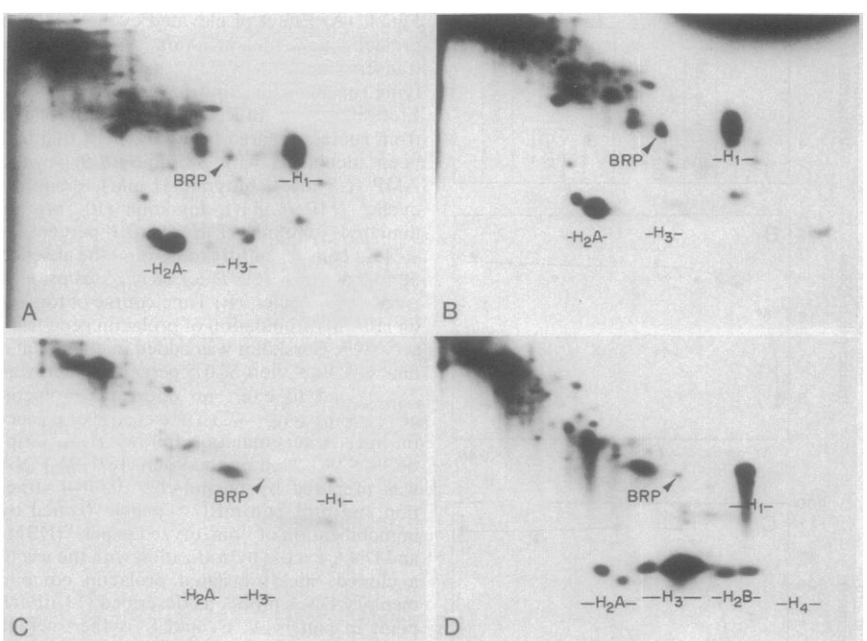

Fig. 2. Phosphorylation of a basic nuclear protein in response to alterations of intracellular cyclic AMP. Autoradiograms are shown of the two-dimensional gels of basic proteins prepared from chromatin from unstimulated GH_4 cells (A) or from cells treated with forskolin (10^{-6} *M*) for 20 minutes prior to harvest (B); or from purified chromatin from unstimulated cells incubated in the presence (D) or absence (C) of a homologous preparation of the subunit of cyclic AMP–dependent protein kinase (20 μg/ml).

that a kinase cascade effected BRP phosphorylation. At least potentially, BRP can represent a direct substrate for the cyclic AMP–dependent protein kinase, since BRP purified by acid solubilization, which destroys endogenous kinase activity, serves as direct substrate for the catalytic subunit of the cyclic AMP–dependent protein kinase. Phosphorylation by an isolated protein kinase in vitro of course does not prove that it serves as a substrate in vivo. For example, the major H1 histone isotype serves as an in vitro substrate but exhibits no cyclic AMP–dependent phosphorylation in the intact cell. Furthermore, it is not unequivocally established that the catalytic subunit of the cyclic AMP–dependent kinase can translocate to the nucleus (*11*, *17*).

The determination of the primary sequence of BRP and characterization of the phosphorylated site (or sites) requires a purification of this low-abundance protein, which is present at approximately 1 percent of the mass of the major H1 histone isotype. This protein is clearly distinct from the developmentally regulated histone isotype referred to as $H1^0$ (*18*).

These data are most compatible with a model in which the holoenzyme resides in, or the activated catalytic subunit of the cyclic AMP–dependent protein kinase translocates to, the nucleus and phosphorylates at least one minor basic chromosomal protein, which regulates the transcription of specific genes. In this model, cyclic AMP would exert a transcriptional effect, as it does in prokaryotes (*1*), but effects of the cyclic AMP–receptor complex on gene transcription would thus be indirect, mediated by phosphorylation of chromatin-associated proteins. Alternatively, a "kinase cascade" could be involved. It is possible that the content, genomic distribution, or covalent modification of BRP (or all) directly or indirectly determines the altered binding of RNA polymerase leading to increased transcription of a restricted set of loci, including the prolactin gene, in a fashion analogous to the effects of cyclic AMP–CRP binding to specific sites in the bacterial genome. Such a model can potentially be tested with current biochemical and molecular biological techniques.

GEOFFREY H. MURDOCH
MICHAEL G. ROSENFELD
Eukaryotic Regulatory Biology Program (M-013), University of California, San Diego, La Jolla 92093
RONALD M. EVANS
Tumor Virology Laboratory, Salk Institute, San Diego, California 92138

References and Notes

1. B. de Crombrugghe, B. Chen, W. C. Anderson, P. Nissley, M. Gottesman, I. Pastan, R. L. Perlman, *Nature (London)* **231**, 139 (1971); G. Zubay, D. Schwartz, J. Beckwith, *Proc. Natl. Acad. Sci. U.S.A.* **66**, 104 (1970); E. Englesberg and G. Wilcox, *Annu. Rev. Genet.* **8**, 219 (1974); A. Peterkofsky, *Adv. Cyclic Nucleotide Res.* 7, 1 (1976).
2. D. A. Walsh, J. P. Perkins, E. G. Krebs, *J. Biol. Chem.* **243**, 3763 (1968); J. F. Kuo and P. Greengard, *Proc. Natl. Acad. Sci. U.S.A.* **64**, 1344 (1969); G. N. Gill, *J. Cyclic Nucleotide Res.* **3**, 153 (1977).
3. P. A. Insel, H. R. Bourne, P. Coffino, G. M. Tomkins, *Science* **190**, 896 (1975); R. Simantov and L. Sachs, *Eur. J. Biochem.* **59**, 89 (1975).
4. T. Noguchi, M. Diesterhaft, D. Granner, *J. Biol. Chem.* **253**, 1332 (1978); M. J. Ernest and P. Feigelson, *ibid.*, p. 319; M. Cimbala, W. Lamers, K. Nelson, J. Monahan, H. Warren, R. Hanson, *ibid.* **257**, 7629 (1982).
5. J. G. Williams, A. S. Tsang, H. Mahbubani, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 7171 (1980).
6. R. A. Maurer, *Nature (London)* **294**, 94 (1981).
7. G. A. Evans, D. N. David, M. G. Rosenfeld, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 1294 (1978); M. A. Shupnik, L. A. Baxter, L. R. French, J. Gorski, *Endocrinology* **101**, 729 (1979); R. A. Maurer, *J. Biol. Chem.* **255**, 8092 (1980); P. S. Dannies and A. H. Tashjian, Jr., *Biochem. Biophys. Res. Commun.* **70**, 1180 (1976).
8. J. A. Martial, J. D. Baxter, H. M. Goodman, P. H. Seeburg, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 1816 (1977); R. J. Tushinski, P. Sussman, Y.-Y. Yu, F. C. Bancroft, *ibid.*, p. 2357; M. M. Harpold, P. R. Dobner, R. Evans, F. C. Bancroft, J. D. Darnell, *Nucleic Acids Res.* **6**, 3133 (1979); L. E. Shapiro, H. H. Samuels, B. M. Yaffee, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 45 (1978).
9. G. S. McKnight and R. D. Palmiter, *J. Biol. Chem.* **254**, 9050 (1979).
10. G. H. Murdoch, E. Potter, A. K. Nicolaisen, R. M. Evans, M. G. Rosenfeld, *Nature (London)*, in press.
11. M. Castagna, W. K. Palmer, D. A. Walsh, *Eur. J. Biochem.* **55**, 193 (1975).
12. K. B. Seamon and J. W. Daly, *J. Cyclic Nucleotide Res.* **7**, 201 (1981).
13. E. Potter, A. K. Nicolaisen, E. S. Ong, R. M. Evans, M. G. Rosenfeld, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 6662 (1981).
14. U. K. Laemmli, *Nature (London)* **227**, 680 (1970); M. M. Sanders, V. E. Groppi, Jr., E. T. Browning, *Anal. Biochem.* **103**, 157 (1980); A. Zweidler, *Methods Cell Biol.* **17**, 223 (1978).
15. M. M. Yu, N. W. Tolson, G. Guroff, *J. Biol. Chem.* **255**, 10481 (1980); S. Halegoua and J. Patrick, *Cell* **22**, 571 (1980).
16. T. A. Langan, *Adv. Cyclic Nucleotide Res.* **3**, 99 (1973).
17. A. Guidotti, D. M. Chuang, R. Hollenbeck, E. Costa, *ibid.* **9**, 185 (1978); S. L. Keely, Jr., J. D. Corbin, C. R. Park, *Proc. Natl. Acad. Sci. U.S.A.* **72**, 1501 (1975).
18. S. Panyim and R. Chalkley, *Biochem. Biophys. Res. Commun.* **37**, 1042 (1969); F. Varricchio, *Arch. Biochem. Biophys.* **254**, 11264 (1979); R. Gjerset, C. Gorka, S. Hasthorpe, J. J. Lawrence, H. Eisen, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 2333 (1982).
19. We thank Rodrigo Franco and Marian Waterman for discussion and suggestions regarding the manuscript and Susan Taylor for the cyclic AMP–dependent protein kinase. Supported by grants from the National Institutes of Health.

17 September 1982

Chromosomal Assignment of the Endogenous Proto-Oncogene C-*abl*

Abstract. *Abelson murine leukemia virus (A-MuLV) is a replication-defective retrovirus that transforms lymphocytes of the B-cell lineage. This virus is a recombinant between the parental Moloney murine leukemia virus and a cellular gene termed C-*abl. *By analysis of a series of mouse × Chinese hamster hybrid cell lines containing various mouse chromosomes, we have mapped the C-*abl *gene to mouse chromosome 2.*

Abelson murine leukemia virus (A-MuLV) is one of many replication-defective transforming retroviruses. Like most such viruses, A-MuLV is a hybrid of elements of a replication-competent leukemia virus and sequences derived from the normal mouse genome. These sequences, contiguous in the virus, are spaced apart by intervening sequences in the mouse genome, spanning more than 20 kilobases (kb) of DNA. The mouse gene has been termed C-*abl*, and its viral homolog V-*abl* (*1*). A-MuLV is unusual in its tissue specificity; it transforms fibroblasts and immature lymphocytes of the B-cell lineage, but not cells of the T-

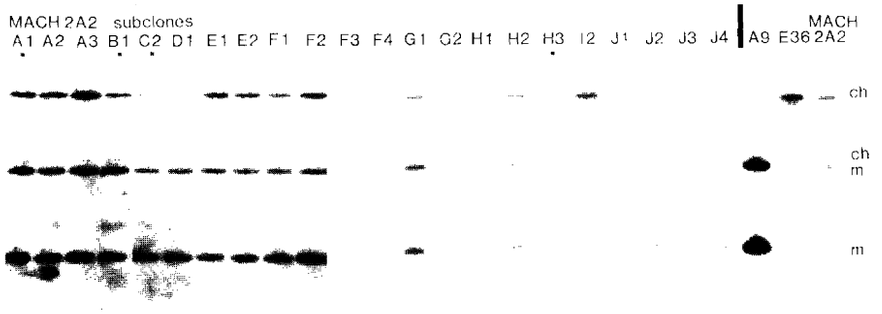

Fig. 1. Detection of C-*abl* DNA fragments in mouse (A9), Chinese hamster (E36), and hybrid cell genomic DNA. DNA digested with Hind III enzyme was fractionated by electrophoresis in agarose, transferred to nitrocellulose, and hybridized with pAB3sub3 DNA isotopically labeled by nick-translation in vitro. The four subclones of hybrid MACH 2A2 subjected to detailed karyotic and isoenzyme analysis (Table 2) are indicated (■); *ch*, Chinese hamster; *m*, mouse.

0036-8075/82/1224-1317$01.00/0