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Gene expression and cAMP

(cyclic nucleotides/hormonal regulation/protein kinase/cAMP-binding proteins)

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ABSTRACT **By comparing the 5′-flanking region of the porcine gene for the urokinase form of plasminogen activator with those of other cAMP-regulated genes, we identify a 29-nucleotide sequence that is tentatively proposed as the cAMP-regulatory unit. Homologous sequences are present (*i*) in the cAMP-regulated rat tyrosine aminotransferase, prolactin, and phospho*enol*pyruvate carboxykinase genes and (*ii*) 5′ to the transcription initiation sites of cAMP-regulated *Escherichia coli* genes. From this we conclude that the expression of cAMP-responsive genes in higher eukaryotes may be controlled, as in *E. coli*, by proteins that form complexes with cAMP and then show sequence-specific DNA-binding properties. The complex formed by cAMP and the regulatory subunit of the type II mammalian protein kinase might be one candidate for this function. Based on several homologies we suggest that this subunit may have retained both the DNA-binding specificity and transcription-regulating properties in addition to the nucleotide-binding domains of the bacterial cAMP-binding protein. If this were so, dissociation of protein kinase by cAMP would activate two processes: (*i*) protein phosphorylation by the catalytic subunit and (*ii*) transcription regulation by the regulatory subunit.**

Changes in protein phosphorylation are an important mechanism for modulating cellular activities in response to stimuli that alter the concentration of cAMP. Two groups of enzymes are responsible for cAMP-dependent protein phosphate metabolism—namely, the cAMP-dependent protein kinases (ref. 1, and see references in ref. 2) and the less well-studied protein phosphatases (reviewed in ref. 2). The link between cAMP and protein phosphorylation must have been established early in eukaryote evolution since, in addition to their distribution throughout the animal kingdom (3, 4), cAMP-dependent protein kinases have also been found in eukaryotic microorganisms, such as cellular slime molds (5) and fungi (see ref. 6). These enzymes invariably consist of two types of subunits dissociable by cAMP: catalytic (C) subunits that phosphorylate selected hydroxyamino acid residues in protein substrates, and regulatory (R) subunits that contain the cAMP-binding sites and repress catalytic activity in the undissociated complex (see references in refs. 2 and 6).

Many proteins are phosphorylated by cAMP-dependent kinases, and it is clear from a large body of data that this reaction is a versatile mechanism for integrating the activities of different metabolic pathways. This is illustrated by the classical changes in muscle and liver glycogen metabolism following exposure to epinephrine and glucagon, respectively. Here the hormone-induced rise in cAMP yields the desired physiological result—an increase in blood sugar—by means of two simultaneous and distinct series of protein phosphorylations, one of which reduces glycogen synthesis, while the other enhances breakdown (7). Additional examples of integrated responses that require coordinated cAMP-dependent protein phosphorylations include triglyceride metabolism in adipose tissue (8, 9), steroid metabolism in adrenal cortex (10), and hormone production in other endocrine glands (11). The weight of this and other evidence underlies the generally accepted view that the effects of cAMP in eukaryotes are mediated exclusively through protein phosphorylation.

In bacteria the sole known action of cAMP is to induce and repress specific genes; here the cyclic nucleotide binds to a receptor protein (catabolite repressor protein), crp, or cAMP-binding protein (CAP), and the resulting complex can then interact with certain DNA sequences that influence genetic transcription at nearby promoters (reviewed in ref. 12). Gene expression in higher eukaryotes is also stimulated by raising intracellular cAMP: increased levels of mRNA have been reported for tyrosine aminotransferase (TAT) (13), prolactin (14), and phospho*enol*pyruvate (*P-enol*pyruvate) carboxykinase (15), and plasminogen activator (16), although the mechanism by which cAMP enhances transcription in these cells is not yet known. What bacteria have in common with mammals in terms of cAMP-related function is modulation of gene expression; bacteria lack cAMP-dependent protein phosphorylation, which, in a regulatory context, seems to have been a later evolutionary development.

In the eukaryotic system that we have studied—plasminogen activator (urokinase, uPA) synthesis in cultures of a porcine kidney cell line (LLC-PK_1)—increases in cAMP strongly induce uPA production (17): (*i*) higher rates of uPA transcription can be detected in <20 min (ref. 16; unpublished data); (*ii*) the level of specific uPA mRNA rises from as few as 5–10 molecules per cell in the basal state to 1800 or more in fully induced cells (unpublished data); (*iii*) uPA induction is a "primary" effect of cAMP in the sense that increased transcription is unaffected in cells pretreated with high levels of cycloheximide (ref. 16; unpublished data). Induction is thus independent of new protein synthesis and cAMP-activated uPA gene expression must be based on preexisting molecules. How might induction occur?

The most economical explanation, at first sight, would merely invoke the well-defined cAMP-dependent protein kinases: particular chromatin or DNA-binding proteins, whose activity was controlled by phosphorylation, would determine the transcription state of cAMP-responsive genes. Nonetheless, some recent observations on several hormone-regulated genes provide reasons to consider a possible alternative, analogous to the way in which cAMP modulates gene expression in bacteria without protein phosphorylation.

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Abbreviations: bp, base pairs; uPA, urokinase form of plasminogen activator; TAT, tyrosine aminotransferase; CAP, cAMP-binding protein; *P-enol*pyruvate, phospho*enol*pyruvate; R and C subunits, regulatory and catalytic subunits, respectively, of cAMP-dependent protein kinase.

HYPOTHESIS

We propose the following working hypothesis to account for cAMP regulation of transcription in higher eukaryotes. We suggest that dissociation of protein kinase by cAMP activates both subunits, each for a distinct function: the C subunit, freed of the R subunit, can function enzymatically to phosphorylate a wide range of proteins. Concurrently, the R subunit, once activated by bound cAMP, would be capable of modulating transcription, like bacterial CAP, by interacting with specific regulatory sequences in DNA. This dual activation process would permit cAMP to function as a "second messenger" and to coordinate cellular responses at two levels, gene expression (by the R subunit) and enzyme activity (by the C subunit). The supporting data and arguments, and some implications of this view, are outlined below.

uPA expression in LLC-PK_1 cells is induced by the cAMP-elevating hormones calcitonin and vasopressin (16, 17) and by phorbol esters acting through a cAMP-independent pathway (unpublished data); it is repressible by glucocorticoids (ref. 17 and unpublished observations). This variety of responses prompted us to clone and sequence both the porcine uPA cDNA (18), which closely resembles its human homologue (19, 20), and the gene (18) with a view to understanding the basis of hormone regulation. The 5'-flanking region of the gene contains three potential "TATA boxes," two presumptive glucocorticoid-binding sites (21), and other repeated sequences of undefined function. It also contains a 29-nucleotide sequence that we propose as a mediator of cAMP-regulated transcription (Table 1). This begins 395 base pairs (bp) upstream of the 5'-cap site on uPA mRNA and it is noteworthy because (*i*) two comparable and highly homologous sequences are present in the 5'-flanking regions of the rat TAT (22) and rat prolactin (23) genes, respectively (Table 1). Further, the recently (24) described 547-nucleotide-long 5'-flanking segment that contains the determinants for cAMP regulation of *P-enol*pyruvate carboxykinase expression also includes two regions that show homology with the uPA reference sequence. The transcription of all three of these genes, like that of uPA, is stimulated by hormones that raise intracellular cAMP*. (*ii*) A particularly revealing homology in *Escherichia coli* DNA is located in the *gal* operon, 29 bp upstream of the *gal* transcription initiation site (Table 1). This sequence mediates the induction of *gal* transcription by binding the complex already mentioned, CAP–cAMP.

*The human uPA gene also contains a similar region, 69% homologous to the porcine uPA sequence at positions (−311 to −280) upstream of the transcription initiation site (unpublished observations).

DNA interaction sites where the CAP–cAMP complex may regulate gene expression have been identified and characterized in detail by means of several independent and converging experimental approaches (reviewed in ref. 12). Nucleotide sequences have been determined by DNase footprinting of numerous sites (see ref. in 12) because they are protected by bound complex against enzymic attack; the length of protected segments, which contain 5' TGTGA 3' as an almost constant feature, varies in the range of 16–33 nucleotides (see ref. 12 and references therein). In Table 2 are assembled sequences surrounding the common pentanucleotide unit in 13 bacterial binding sites, at 6 of which a positive transcriptional response to CAP–cAMP either has been established or is likely; also presented for comparison are the corresponding segments of the four eukaryotic genes. Sequences have been aligned with their common units in register, and homology has been assessed with reference to *gal*, uPA, and an *E. coli* consensus sequence. It can be seen that (*i*) with one exception (*ara2*), there is a variable but always significant homology among all of the sequences and (*ii*) there is considerable homology between bacterial and mammalian sequences: both the range and average degree of homology are similar with either *gal* or uPA as reference. The high level of homology between two mammalian sequences—uPA and *P-enol*pyruvate carboxykinase I*—is evident.

With the evolutionary interval separating bacteria and mammals in mind, these homologies seem impressive: first, there is strong genetic and biochemical evidence that several of the bacterial sequences given in Table 2 are implicated in regulatory phenomena controlled by CAP–cAMP (12); and second, the mammalian sequences, like their bacterial counterparts, are located in regions likely to be of regulatory significance—5' to the promoter segments of cAMP-responsive genes. We therefore tentatively suggest that, at least for the four mammalian genes considered here, genetic regulation by cAMP in higher eukaryotes may also be mediated by a CAP-like, cAMP-dependent, DNA-binding protein that interacts at these sites.

Two general mechanisms suggest themselves for modulating transcription by means of cAMP-requiring and DNA-binding molecules. The first would involve one or more proteins analogous to bacterial CAP: protein(s) complexing cAMP reversibly (as a function of its intracellular concentration) would undergo ligand-dependent conformational changes that permitted sequence-specific interactions with DNA. Elaborations of this theme that allowed for concurrent and specific regulation of different classes of genes within a single cell type or for tissue specificity in the pattern of cAMP-regulated transcription (or both) can easily be imagined.

From a second and more interesting viewpoint the R subunits of the conventional cAMP-dependent protein

Table 1. Sequence homologies in cAMP-regulated genes

	Ref.	5'		3'	% homology: With porcine uPA	% homology: With rat TAT
Porcine uPA	18	−395	GAAAGGGTGAGAAAGAGCTGATTGAGGGG	−367	—	76
Rat TAT	23	−152	.TTG.....C...A..G..G....	−124	76	—
Rat prolactin	24	−107	CTT.AT.ACG....T..A.....G.A..	−79	59	59
Rat *P-enol*pyruvate carboxykinase						
I	25	−488	C....TC..CA.C..CCGA.T.G.CG...	−516	52	35
II	25	−412	.GTGTTT...C..CC...A.CCACT..CA	−384	41	55
E. coli gal	12	−29	A....T....C.TG..ATAA...AGT..A	−57	55	45

Homology in 5'-flanking regions of eukaryotic and prokaryotic sequences upstream of the respective transcriptional promoters in genes regulated by cAMP. The degree of homology with porcine uPA and with rat TAT, expressed as % of nucleotide identity, is shown for each case. The numbers beside each sequence represent the distance from the mRNA cap site for eukaryotic genes and the distance from the transcription initiation site for *E. coli gal*. Nucleotide identity with porcine uPA is represented by dots.

Table 2. Sequence homologies among genes regulated by cAMP

		% homology		
		With *gal*	With uPA	With *E. coli* consensus
gal	AAAGTGTGACATGGA	—	73	93
lacl	TT.A.....GT.A.C	53	67	67
araBAD	.....GCC.T	73	60	80
araC	.....CTATAATC	47	53	60
malT	G..T.....CA.T	67	60	73
catl	...A..A...G....	80	53	87
deol	T..T.....TG..T.	67	47	73
cat2	T.CC.....GGAAG	47	40	60
lac2	G..T.....GCG.AT	53	47	47
deo2	.TTA.T...ACCA..	47	47	53
ara2	CTGCC....TTATAG	27	33	27
ompA	C.....A..ACC	60	47	80
pBR-P_4	GCG.....A..ACC	53	47	67
Porcine uPA	....G....G.AA..	73	—	80
Rat TAT	TTG.G....G.AAC.	47	73	53
Rat prolactin	TT.A..ACGG.AAT.	33	47	40
Rat *P-enol*pyruvate carboxykinase				
I	.....C..CA.CA.C	60	53	73
II	GTGT.T.....ACC.	47	40	47
E. coli CAP-binding consensus	AAAGTGTGACATAGA G C	—	—	—

Homologies in 5'-flanking regions of prokaryotic and eukaryotic genes. The prokaryotic data were derived from DNase footprinting studies in which the indicated sequences were protected by binding the CAP–cAMP complex; the complex is known to influence transcription at sequences 1–4 and is likely to do so at sequences 5–7; CAP–cAMP binds to sequences 8–13 but with no known regulatory consequences. The prokaryotic sequences are taken from refs. 12, 25, and 26, and the eukaryotic sequences are from Table 1. The *E. coli* consensus assignment is defined by the base that appears at the indicated position at the highest frequency. Dots indicate nucleotide identity with the *gal* sequence.

kinases could themselves be envisaged as cAMP- and DNA-binding molecule(s), the vertebrate counterparts of bacterial CAP. It has been pointed out (27) that the cAMP-binding domain of CAP shows extensive homology with two regions of the RII subunit of bovine cardiac protein kinase that are therefore presumed to function in cAMP binding. Inspection of the RII amino acid sequence (28) indicates retention of two additional features that are consistent with the notion of DNA binding: one of these is an undecapeptide (^{347}*A* Y A V *G* D V K C L *V*^{357}), in which the italicized residues have preserved the alignment found in a sequence that is highly conserved among prokaryote DNA-binding proteins (29), including CAP (residues 174–189 in the DNA-binding domain (30, 31); here the glycine residue is thought to form a bend in the polypeptide chain separating two helical domains that can interact with DNA. A second point emerges from a comparison of the carboxyl portion of CAP (residues 100–210) (30, 31) (which is thought to mediate DNA binding) with the amino-terminal segment of RII (residues 1–120) (28). These domains, though devoid of discernible homology in amino acid sequence, contain 18 and 33 positively charged amino acids, respectively, of which 4 can be aligned in register [CAP: K R R K (positions 101, 124, 143, and 189, respectively) with RII: H R R R (positions 2, 24, 43, and 89, respectively)]. These characteristics, though not in themselves convincing, imply that elements of secondary structure able to promote DNA binding may also have been conserved in R subunits. A final significant point about the RII subunit structure, brought to our attention by Sidney Strickland, concerns the sequence of four positively charged amino acids, ^{243}K K R K^{246}. From the work of Kalderon *et al.* (36) on simian virus 40 T antigen, the controlled exposure of this sequence might provide a signal for regulating the transport of the protein into the nucleus.

Along with the previously cited features of secondary structure and amino acid sequence, considerations related to cellular evolution reinforce the idea that R subunits may have retained the bifunctionality of CAP (i.e., both cAMP and DNA binding). In the transition from prokaryotic to eukaryotic cellular organization, the differentiation of nuclear and cytoplasmic environments, and hence the physical segregation of genetic and cytoplasmic functions, created two milieus, each capable of responding to external stimuli. Since transcription and RNA processing are relatively slow, the time constant of most nuclear responses (minutes) is much longer than that of the more rapid cytoplasmic reactions (milliseconds to seconds), and mechanisms that coordinated the two sets of responses would obviously be advantageous. On the assumption that bacteria represent a primitive stage of cellular evolution and organization, the primordial function of cAMP as an intracellular messenger was the modulation of gene expression in response to changing extracellular conditions. It is clear from studies of several genes that this function has persisted in higher eukaryotes. The regulation of gene expression by cAMP in bacteria is achieved by modulating the DNA-binding potential of a single protein—CAP—and the homologies between CAP and the R subunit indicate that a large part of this structure has, with some modifications, been conserved during evolution.

To accommodate the currently prevailing view that protein phosphorylation is the sole transducer of cAMP function in higher eukaryotes would require (*i*) the concurrent evolutionary retention of a large part of CAP structure and activity as a cAMP-binding protein and loss of the DNA-binding function of the CAP–cAMP complex and (*ii*) the replacement of the genetic regulatory functions of CAP–cAMP by a new, but still cAMP-related, pathway based on protein phosphorylation. In the absence of some direct supporting evidence we consider this sequence of events unlikely and wish to

propose, instead, the following alternative. With the preceding homologies in mind, we suggest that the primordial biological role of cAMP in regulating genetic transcription has persisted in higher eukaryotes in the form of the R subunit of protein kinase, this protein having retained its original DNA-binding properties. Protein phosphorylation, and the relevant enzymes [the presumed precursor(s) of the present C subunit], would first have emerged independently as a regulatory mechanism predominantly concerned with processes other than gene expression during the evolution of the cytoplasm as a separate compartment in early eukaryotes. The kinase might initially have been cAMP independent and under some other form of control. Subsequent evolutionary modifications that promoted the reversible physical association of the two proteins would ultimately have led to the current structure of the cAMP-dependent protein kinases. This structure permits simultaneous activation of both types of subunits upon dissociation by cAMP and creates a mechanism for coordinating their function in two distinct domains—the one primarily nuclear and gene regulatory for the R subunit and the other chiefly cytoplasmic for the C subunit. However, we do not suggest that, at this stage of evolution, the functions of the C and R subunits are exclusively cytoplasmic and nuclear, respectively; cAMP-dependent phosphorylation of nuclear proteins is well established, and additional undiscovered functions for each subunit are not meant to be excluded (32).

During their recent revealing studies of rat *P-enol*pyruvate carboxykinase, Wynshaw-Boris *et al.* (24) considered the possibility that regulatory sequences in cAMP-modulated mammalian and bacterial genes might be homologous and proposed a sequence—AAAGTTTAGTCAA (positions −262 to −248)—that required extensive optimization to obtain significant homology with either bacterial CAP-binding or 5′-flanking sequences of mammalian cAMP-regulated genes. This sequence does not seem to be the best candidate for mediating cAMP effects on transcription because the porcine uPA gene shows no homology in its 5′-flanking region [although a homologous sequence—AAAGTTTAAA (positions 4210–4219)—is located in intron J, far downstream of the transcription initiation site, where it is unlikely to be of regulatory significance]. In contrast, the 5′-flanking region of *P-enol*pyruvate carboxykinase contains two stretches that resemble our proposed regulatory sequence, one of which (*P-enol*pyruvate carboxykinase I, Tables 1 and 2) is highly homologous with uPA, *gal*, and the bacterial consensus.

From the data summarized in Table 2 and elsewhere (12) it is clear that there is considerable variation and hence some lowering of homology among sequences that bind CAP–cAMP and mediate the gene-regulating functions of the complex. For units of length of 15 (Table 2) or 29 nucleotides (Table 1) we can expect a high frequency of sequences showing homologies in the range of 50–60%; indeed, in a survey of 7.5 kilobases of *E. coli* DNA we found twenty-one 29-mers that were 52% homologous with the *gal* CAP–cAMP-binding sequence, of which 12 were present in 1.9 kilobases of the tryptophanase gene alone. Likewise, in 40 kilobases of mammalian DNA, there were, on average, 70, 27, and six 29-mers per 10 kilobases at 52%, 55%, and 59% homology, respectively. These findings are not inconsistent with our hypothesis because the ability of a particular sequence to bind effector proteins and mediate regulatory signals in bacteria and eukaryotes probably depends on numerous factors. These might include the accessibility of the sequence, the proximity of suitable structures or sequences permitting interactions with other laterally located DNA-binding proteins, and the positioning of the potential binding sequence in regions that are relevant to the regulation of gene expression. These considerations make it difficult to evaluate our proposal by assessing the location and frequency of homologous sequences alone. In this context it would also be of interest to determine whether the putative binding sequence is absent from the 5′-flanking sequences of mammalian genes that are unaffected by cAMP but, with the complexities of life cycle and tissue specificity in mind, there is as yet no way that any particular gene can be certified as cAMP unresponsive in all cells of a higher vertebrate.

Even if our hypothesis should prove to be correct in its essentials, it seems likely that the regulation of gene expression by cAMP in higher eukaryotes will not be as simple as it appears to be in bacteria. From the examination of several tissues, including sperm, it appears that porcine uPA is not involved in genomic rearrangements (unpublished observations), suggesting that this gene is not controlled by the tissue-specific introduction of regulatory sequences. The pattern of uPA gene expression is complex since, depending on cell type, it can increase, decrease, or remain silent in response to rises in cAMP†; hence, the mere presence of a regulatory sequence might not be sufficient to determine a particular response.

The mechanism that we envisage for cAMP-mediated coordination of nuclear and cytoplasmic responses can be tested experimentally—in particular, by transfecting cells with suitable genetic constructs; it might have parallels in processes regulated by some other hormones.

†Increases in cAMP stimulate uPA expression in adrenal cells (33), repress uPA in granulocytes, monocytes, and macrophages (34, 35), and produce no detectable effect in ovarian granulosa (S. Strickland, personal communication) and Sertoli cells (I. Fritz, personal communication).

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