Role of Cyclic AMP in the Control of Cell-Specific Gene Expression

Wolfgang Schmid, Doris Nitsch, Michael Boshart, and Günther Schütz

Genes have to be expressed in specific cell types at appropriate times of development dependent on external signals. cAMP signaling occurs in all cells, thus raising the question of how this signal transduction pattern is integrated into mechanisms determining cell-specific gene expression. We have analyzed expression of the tyrosine aminotransferase gene as a model to study the basis of this cell type specificity of hormone induction. We found that cell-type-specific expression is generated by combined action of cAMP signal-dependent and liver cell-specific transcription factors. The interdependence of the cAMP response element and an element determining liver cell specificity enables a gene to respond to an ubiquitous signal in a cell-specific manner. (Trends Endocrinol Metab 1993;4:204–209)

Extracellular signals influence gene expression by utilizing a limited number of intracellular signaling pathways. Certain hormones (for example, steroids and thyroid hormone) associate with cytoplasmic or nuclear receptor proteins, which are activated upon ligand binding to sequence-specific transcription factors [reviewed by Beato (1989)]. On the other hand, neurotransmitters, growth factors, and peptide hormones bind specifically to cell-surface receptors. This binding leads to increases in the levels of intracellular Ca^{2+} and/or synthesis of second messengers, which in turn may activate protein kinases and phosphatases within the cell (Berridge 1993). Alternatively, binding may initiate signal transduction by activation of membrane receptors, which either have intrinsic tyrosine kinase activity or the ability to recruit cytosolic tyrosine kinases (Schlessinger and Ullrich 1992). Either cascade can then lead to functional modifications of intracellular target proteins.

One of the best-studied examples of membrane-generated signal transduction is the activation of protein kinase A (PKA). Ligand binding to a membrane receptor activates, via G proteins, adenylate cyclase, which catalyzes an increase in the levels of cAMP. PKA is composed of two regulatory (R) and two catalytic (C) subunits [reviewed by Taylor et al. (1990)]. Binding of two cAMP molecules to each regulatory subunit of the holoenzyme liberates the C subunit, the active form of the enzyme, by reversible dissociation of the inactive R_2C_2 complex. At present, four R subunits (RIα, RIβ, RIIα, and RIIβ) and three C subunits (Cα, Cβ, and Cγ) have been identified (Taylor et al. 1990). The regulatory subunits α are constitutively expressed in most tissues, whereas the regulatory subunits β are predominant in brain, testis, and ovary. In contrast to the holoenzyme, which is cytoplasmic and/or membrane associated, the free C subunit possesses a potential nuclear localization signal and can be rapidly and reversibly translocated to the nucleus (Meinkoth et al. 1990).

Stimulation of PKA clearly has pleiotropic effects, as the C subunit can phosphorylate a plethora of different proteins. Phosphorylation of target proteins that are present in many cell compartments can positively or negatively affect their activity. We restrict this review to the role of cAMP-induced modification of transcription factors. Since the role of cAMP in gene regulation has been extensively studied in liver, we primarily discuss examples of transcriptional regulation of genes involved in gluconeogenesis and glycolysis.

• cAMP Activates a Family of Sequence-Specific Transcription Factors

Recent work has shown that many cAMP-regulated genes have in their control region a short perfect or imperfect palindromic sequence termed CRE (*c*AMP-*r*esponsive *e*lement). Such a CRE was first identified in the somatostatin promoter by Montminy et al. (1986), and a protein binding to that sequence, a CRE-binding protein, CREB, was described (Montminy and Bilezikjian 1987). Cloning of the cDNA for CREB identified it as a member of the bZip family (Hoeffler et al. 1988, Gonzalez et al. 1989) of transcription factors that have a characteristic leucine zipper adjacent to a basic domain. These transcription factors have been shown to interact as dimers with palindromic binding sequences. The sequence of the leucine zipper domain (Zip) specifies formation of homo- or heterodimers, whereas the basic DNA-binding domain (b) contacts the DNA [reviewed by Kerppola and Curran (1991)].

It later became clear that several CRE-binding proteins exist, called ATFs for *a*ctivating *t*ranscription *f*actors (Hai et al. 1989), which form a subfamily of bZip proteins. This subfamily is related to the AP1 subfamily (Ziff 1990) that binds to the TPA response elements (TRE), a palindromic sequence highly related to the CRE, which transduces signals via the protein-kinase-C pathway (among others). Only a few members of the ATF family appear to be involved in cAMP signaling. Besides CREB, these are ATF1 (Hai et al. 1989, Hurst et al. 1991) and CRE-modulating protein or CREM (Foulkes et al. 1992, Del-

Wolfgang Schmid and Günther Schütz are at the German Cancer Research Center, Division Molecular Biology of the Cell I, 69120 Heidelberg; and Michael Boshart is at the Max Planck Institute for Biochemistry, Gene Center, 82152 Martinsried, Germany; and Doris Nitsch is at the Masaryk Memorial Cancer Institute, Department of Cellular and Molecular Oncology, 65653 Brno, Czech Republic.

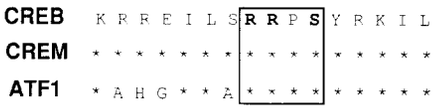

Figure 1. A protein-kinase-A (PKA) recognition site is conserved in CREB, CREM, and ATF1. Conserved amino acids are indicated by *asterisks*. The PKA recognition sequence is *boxed*, with the characteristic amino acids *highlighted*. The phosphorylated serine residue is in CREB ser$^{119/133}$, in CREMτ ser^{117}, and in ATF1 ser^{63}.

mas et al. 1992). All three proteins, which are strongly related in sequence and which can form heterodimers, have in their conserved region a sequence motif (see Figure 1) recognized by PKA (Gonzalez and Montminy 1989) and by the calmodulin-dependent protein kinases II (Sheng et al. 1991). Cells may have more than one CRE-binding protein. For example, incubation of hepatoma cell nuclear extract with antibodies directed against a CREB peptide containing the phosphorylated serine$^{119/133}$ residue immunoprecipitated three proteins, which were identified as phosphorylated CREB, ATF1, and, putatively, CREM (Ganss et al. unpublished).

Phosphorylation by PKA is necessary for transcriptional activation of these factors (Gonzalez and Montminy 1989, Lee et al. 1990, Hurst et al. 1991, Foulkes et al. 1992) and, in the case of CREB, also increases CRE binding (Nichols et al. 1992). The crucial role of CREB phosphorylation is underscored by the recently elucidated attenuation of CREB activity by dephosphorylation by protein phosphatase 1 (Hagiwara et al. 1992). Similarly, in the extinguished state of hepatoma × fibroblast hybrids (see below), inactivity of cAMP-controlled genes was strongly correlated with lack of CREB phosphorylation by PKA (Boshart et al. 1991). Furthermore, in experiments in which a nonphosphorylatable transdominant-negative CREB mutant was expressed in transgenic mice under the control of the pituitary-specific GH gene promoter, a marked mutant phenotype was observed (Struthers et al. 1991). The transgenic mice showed a strong depletion of somatotrophic cells in the pituitary anterior lobe and developed as dwarfs. The mutant CREB acts as a transdominant-negative inhibitor by competitive inhibition of DNA binding of phospho-CREB and other CRE-binding proteins, or by forming inactive heterodimers.

• Interdependency of cAMP-Responsive and Cell-Specific Elements Generates a Signal-Dependent Switch

While the cAMP–PKA signaling pathway is operative ubiquitously and members of the CREB family activated by this signaling pathway are present in most cells, many of the cAMP-controlled genes are expressed in only one or a few cell types. For example, the enzyme tyrosine aminotransferase (TAT), which can be induced by glucocorticoids and cAMP, is expressed exclusively in liver parenchymal cells (Hargrove and Granner 1985). How is this cell-specific restriction achieved in view of the ubiquitous activity of the cAMP signal-transducing system? Detailed analysis of the control elements of the TAT gene by gene transfer experiments and by in vitro and in vivo protein–DNA-binding studies have helped to clarify this question.

Expression of the TAT gene is controlled by three liver-specific enhancers. One enhancer region (at −3.6 kb) mediates the cAMP response (Boshart et al. 1990), while the enhancer at −2.5 kb is responsive to glucocorticoids (Jantzen et al. 1987). A detailed analysis of the cAMP-responsive enhancer by clustered point mutations demonstrated that it consists of two essential elements [see Figure 2 (Boshart et al. 1990)], one of which is an imperfect CRE palindrome. cAMP administration leads to transcriptional activation of TAT that closely correlates with increased protein–DNA interaction at the CRE in vivo, as revealed by genomic footprinting (Weih et al. 1990). CREB binds to the TAT–CRE, as shown with CREB purified from rat liver and brain or generated by expression in *Escherichia coli* (Nichols et al. 1992). The other important element of the −3.6-kb enhancer interacts with the liver-enriched transcription factor HNF4 (Nitsch et al. 1993b), a member of the steroid receptor superfamily that controls the expression of several liver-specific proteins (Sladek et al. 1990). Either element, tested in front of a heterologous promoter, is inactive, but their combination restores cell-specific and cAMP-dependent expression as observed in the native TAT gene enhancer [Figure 3, Boshart et al. 1990)]. This combination of two interdependent modules generates a sensitive switch, the activity of which depends on both the presence of the cell-specific factor and the amount of active CREB. This organization of cell-specific and signal-dependent elements should be contrasted with that of constitutively active enhancers. For example, mutational analysis of the SV40 enhancer showed that its activity is generated by multiple elements, none of which is essential for overall activity (Zenke et al. 1986, Fromental et al. 1988).

Analysis of control elements in other cAMP-inducible genes has also revealed this principle of modular organization of interdependent elements for generation of enhancers that operate as a cell type-specific switch. A few well-studied examples are briefly discussed.

The regulation of phosphoenolpyruvate carboxykinase (PEPCK) gene expression, which in many respects resembles TAT gene regulation, has been recently reviewed in great detail (Lucas and Granner 1992, McGrane et al. 1992). PEPCK is expressed in liver, kidney, and fat cells in dependence of cAMP and glucocorticoid, which upregulate, and of insulin, which downregulates its transcription. The regulatory region extending from −460 to +73 contains CREs and GREs (glucocorticoid response element) intermingled with binding sites for the liver-enriched factor HNF1 and members of the C/EBP family. On the other hand, L-type pyruvate kinase is negatively controlled by glucagon and cAMP. Experiments in transgenic mice have demonstrated that the sequences required for liver-specific and hormone-controlled expression reside between positions −183 and +11. This region contains binding sites for the liver-enriched factors HNF1 and HNF4, and an element that, in cooperation with the HNF4-binding site, confers repression by cAMP and induction by insulin and glucose (Bergot et al. 1992, Cuif et al. 1992). Interestingly, the latter element shows no homology with a CRE. The somatostatin gene is expressed in brain, in endocrine cells of the pancreas, and in the gastrointestinal tract. Pancreas-specific expression depends on an upstream sequence with a composite structure, which binds to the pancreas-specific transcription factor Isl-1 (Vallejo et al. 1992a, Leonard et al. 1992) and to a CRE-binding protein (Vallejo et al. 1992b). This upstream enhancer element interacts synergistically with a downstream CRE first identified by Montminy et al. (1986). The α-subunit glycoprotein is expressed in placental trophoblast cells and in the anterior pituitary, coordinately

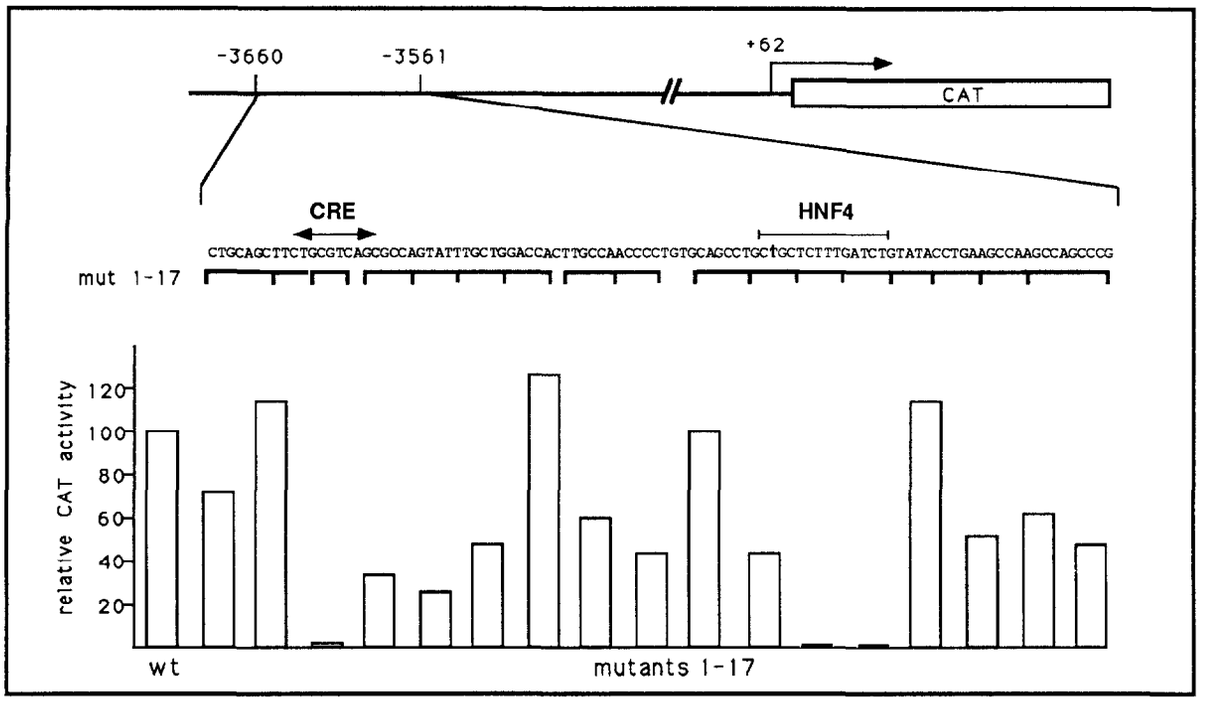

Figure 2. Mutational analysis of the enhancer of the TAT gene by clustered point mutations shows that synergism of two essential elements is required for cell-specific and cAMP-dependent expression. The TAT upstream sequence from -3651 to -3660 is shown. The position of the CRE is indicated by *arrows*, and the position of the HNF4 binding site is *overlined*. The positions of the clustered point mutations are shown by *brackets*. Hepatoma cells were transfected with either the wild-type (wt) construct TATCAT or with the 17 mutants (Boshart et al. 1990). CAT activity is given as arbitrary units, setting wt expression to 100.

with the β-subunit of the hormones hCG, LH, FSH, and TSH. Expression in the placenta requires a CRE and an upstream element, which is functionally dependent on the CRE (Delegeane et al. 1987, Jameson et al. 1989). Pituitary-specific expression is CRE independent (Bokar et al. 1989).

We believe that the switchlike properties of the combination of cAMP-responsive and cell-specific elements represent a widely used principle in the control of gene activity. The glucocorticoid-dependent enhancer of the TAT gene also illustrates such an organization. The glucocorticoid receptor synergizes with members of the HNF3 and C/EBP families (Nitsch et al. 1993b). The nature of these enhancers enables a better understanding of the perinatal activation of gluconeogenic genes like TAT and PEPCK (Greengard 1970). Before birth, owing to low fetal levels of glucocorticoids and glucagon/cAMP, the enhancers are inactive, even though all components required for activation are already present. The inactivity of the CRE may be further enforced by high levels of free PKA-RIα subunit (see below) and of insulin in the prenatal period, which antagonize the activity of the CRE (Ganss and Weih unpublished). The postnatal hypoglycemia leads to elevation of glucagon and glucocorticoid levels, lowers insulin concentration, and thereby strongly induces synthesis of TAT and other gluconeogenic enzymes.

- **The CRE Is a Target for Extinction of Gene Expression in Somatic Cell Hybrids**

The crucial role of CREs for the cell-specific expression of cAMP-regulated genes is underlined by analysis of the ex-

construct	forskolin	relative CAT activity			
		Hepatoma		Fibroblast	
		–	+	–	+
TK CAT		1.0	1.0	1.0	1.0
CRE HNF4 TK CAT		15.0	80.0	0.9	0.8
~~CRE~~ HNF4 TK CAT		1.5	2.7	ND	ND
CRE ~~HNF4~~ TK CAT		1.5	1.6	ND	ND

Figure 3. Interdependence of a CRE and a liver cell-specific element—the basis of the cAMP-dependent enhancer. A combination of oligonucleotides containing either wild-type or mutated CRE and HNF4 motifs was cloned in front of the HSV-thymidine kinase promoter and assayed for CAT activity after transfection in hepatoma or fibroblast cells in the absence or presence of cAMP. ND, not determined.

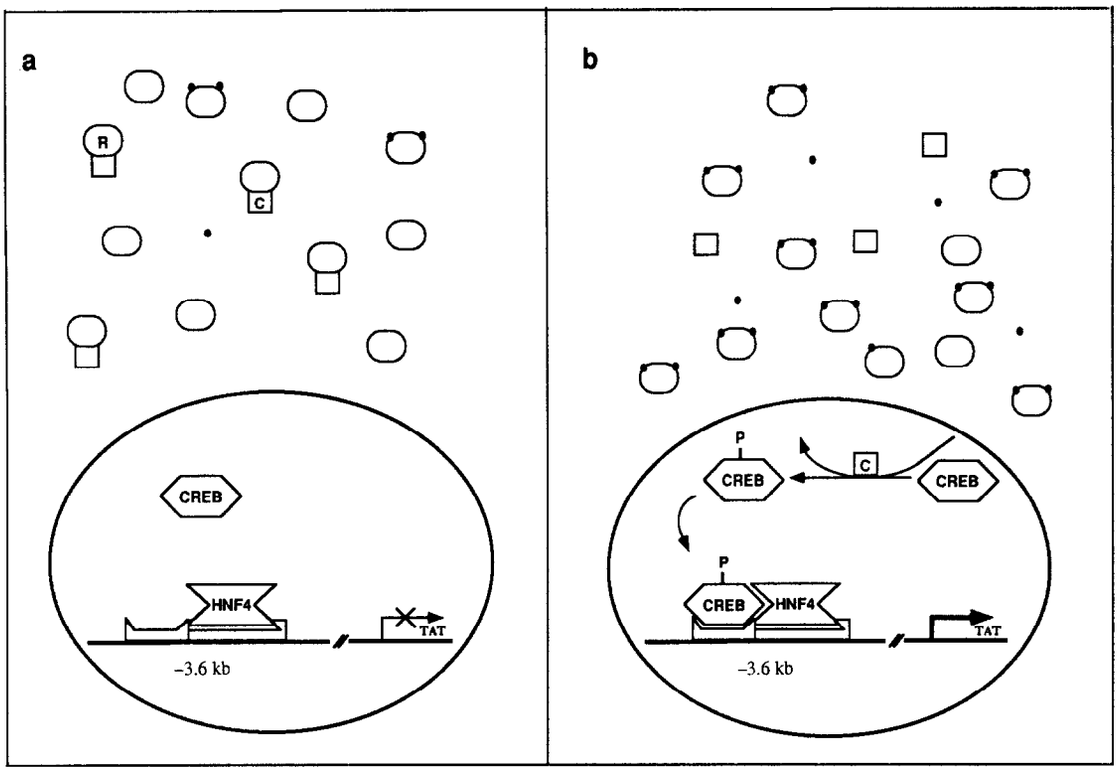

Figure 4. cAMP overcomes extinction by TSEI-RIα. **(a)** In the extinguished state, protein kinase A (PKA) is completely in the inactive R_2C_2 configuration. HNF4 alone is not sufficient for transcription. **(b)** Binding of cAMP to the regulatory subunit of the inactive R_2C_2 holoenzyme liberates the catalytic subunit that is translocated into the nucleus. Phosphorylation of CREB by the catalytic subunit leads to its binding to the CRE and activates transcription in collaboration with HNF4. For simplicity, the subunit structure of PKA is ignored.

tinction phenomenon (Boshart et al. 1991, Nitsch et al. 1993a). Extinction of gene activity in somatic cell hybrids is a multifactorial process that leads to the loss of cell-specific gene products of either parent after fusion of distinctly differentiated somatic cells (Davidson 1974, Weiss et al. 1988, Gourdeau and Fournier 1990). In rat hepatoma × mouse fibroblasts hybrids, a gene causing TAT extinction was localized on a distinct part of the human chromosome 17 (Killary and Fournier 1984, Leach et al. 1989). This locus, called *TSE1* (*t*issue-specific *e*xtinguisher), drastically reduces the expression of several liver-specific genes that are all regulated by cAMP (Thayer and Fournier 1989, Ruppert et al. 1990). Remarkably, repression by *TSE1* is reversible by cAMP, which suggests that *TSE1* may directly interfere with cAMP-dependent signaling. The target of *TSE1* repression has been identified as the CRE (Boshart et al. 1990). Functional antagonism of *TSE1* and cAMP was observed at the levels both of in vivo protein binding to the CRE and of PKA-specific phosphorylation of CREB (Boshart et al. 1990).

Further experiments have shown that the regulatory subunit RIα of PKA is identical with *TSE1* (Boshart et al. 1991, Jones et al. 1991). Both the level of cAMP and the levels of *TSE1*–RIα and the other regulatory subunits determine the equilibrium between inactive holoenzyme and free R and C subunits. In nonstimulated hepatoma cells, where *TSE1*–RIα is expressed at a very low level, the basal level of cAMP leads to only low activation of PKA. In hybrid cells, high levels of *TSE1*–RIα sequester the low level of cAMP present in nonstimulated cells, thus shifting the equilibrium toward the inactive R_2C_2 configuration. An increase in cAMP concentration then activates PKA by liberation of C subunits (Figure 4).

As already mentioned, the TAT gene belongs to a group of genes that are upregulated around birth. It is appealing to speculate that the *TSE1*–RIα gene is prenatally active in hepatocytes and that its expression is downregulated around birth, thus allowing expression of cAMP-dependent gluconeogenic enzymes. Consistent with this speculation, *TSE1* activity in a hepatoma line showing fetal characteristics is high compared with differentiated hepatoma cells (Gourdeau et al. 1989). This finding has been strengthened by direct determination of RIα mRNA levels in fetal and differentiated hepatoma cells (Faust, Boshart, Schütz, and Weiss unpublished).

• Conclusion

Modular organization of control regions offers a versatile and economical mechanism to create a large number of differentially regulated enhancers by combinatorial use of a much smaller number of regulatory units. The combination of ubiquitously active, hormone-dependent elements with cell-specific elements generates enhancers whose expression is restricted by the presence of cell-specific proteins and that are tuned by the activity of the hormone-dependent transcription factor. Transgenic experiments to show that this principle is important during developmental activation of liver genes are under way.

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