REVIEW ARTICLE

Applications of recombinant DNA technology to studies of metabolic regulation

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Introduction

Studies of the regulation of metabolic pathways encompass a wide variety of experiments aimed at answering a range of questions. The initial studies of a pathway, at the level of the organelle, cell, tissues or organism, should identify the factors (such as availability and type of food or substrate, availability of oxygen, hormonal stimuli) that affect the rate of the pathway. Subsequent studies are aimed at revealing how these factors affect the pathway in molecular terms, and include:

(a) Identification of the enzymes that contribute most significantly to the regulation of the pathway.

(b) Analysis of the mechanisms by which the activities of these enzymes are controlled. These mechanisms could affect either the activities of pre-existing enzymes (by allosteric effects or covalent modification) or the amount of enzyme protein (by altering rates of enzyme synthesis or degradation).

(c) Analysis of the roles played by the control mechanisms *in vivo*.

Great progress in studies of metabolic regulation has been made by workers using conventional techniques (e.g. Cohen, 1983; Denton & Pogson, 1976; Newsholme & Start, 1973). However, recombinant DNA techniques are now playing an increasing role and are indeed allowing us to probe regulatory mechanisms in remarkable detail. They are proving important in each of the three general areas outlined above. Moreover, techniques are being developed that will allow analysis or manipulation of regulation in other ways. Our intention in this review is not to give a comprehensive list of all of the contributions of recombinant DNA technology to the field of metabolic regulation, but rather to indicate the great and diverse potential of these techniques by considering selected examples.

Measurement of flux control coefficients and uses of adjustable expression vectors

Many studies of metabolic regulation have involved the qualitative concept of a 'rate-limiting step' and, intelligently applied, this concept proved a very useful simplification. However several groups (e.g. Kacser & Burns, 1973; Heinrich & Rapoport, 1974; Groen *et al.*, 1982) have stressed that rarely (or never) is a single enzyme truly rate-limiting in a pathway, and that it is important to study regulation quantitatively by measuring the flux control coefficients of the enzymes in the pathway. The flux control coefficient of a step in a

pathway is defined as the fractional change in flux through the pathway induced by a fractional change in the capacity of the enzyme under consideration. In general, measurement of flux control coefficients is not easy, but it has been achieved in some favourable cases, for example by the use of an irreversible inhibitor of the adenine nucleotide translocator in experiments on isolated mitochondria (Groen *et al.*, 1982) or of several fungal heterokaryons with different levels of one enzyme activity (Kacser & Burns, 1973).

The work of Walsh & Koshland (1985) on *E. coli* citrate synthase (EC 4.1.3.7) afforded the first example of how recombinant DNA techniques can be used in the measurement of flux control coefficients. The control of *E. coli* citrate synthase is very interesting because this enzyme lies at the branchpoint of oxidative, lipogenic and anaplerotic pathways. The regulatory properties of the enzyme *in vitro* have been studied intensively (e.g. Weitzman, 1981). The enzyme responds sigmoidally to acetyl-CoA. Both NADH and 2-oxoglutarate are allosteric inhibitors and these effectors are thought to be physiologically significant (Weitzman, 1981). Owing to the difficulties of measuring metabolite levels and the flux rate through citrate synthase in bacterial cells, our views of the regulatory role of citrate synthase have largely been inferred from the properties of the enzyme *in vitro*. Walsh & Koshland (1985), however, sought to measure the flux control coefficient of citrate synthase in the tricarboxylic acid cycle directly.

The *E. coli* citrate synthase gene (*gltA*) had been cloned by Guest (1981) by screening the Clarke–Carbon gene bank for complementation of a *gltA*[−] mutation. Walsh & Koshland (1985) constructed a recombinant plasmid with the *gltA* gene behind the *tac* promoter; this is an efficient promoter, derived from *trp* and *lac* promoter sequences, that is repressed by the *lac* repressor (see DeBoer *et al.*, 1983). The plasmid also contained the *lacI*^q gene which expresses high levels of the *lac* repressor (Calos, 1978). This ensured that the *gltA* gene was repressed in the absence of inducer. The plasmid was inserted into a *gltA*[−] mutant. The intracellular level of citrate synthase could then be manipulated by altering the concentration of the non-metabolizable inducer isopropyl β-D-thiogalactopyranoside in the medium. Walsh & Koshland (1984) had already developed the methodology required to calculate the flux rates through individual steps of the tricarboxylic acid cycle and the glyoxylate bypass. They applied these techniques to cells containing various levels of citrate synthase and found that, on a glucose plus acetate medium, the flux through citrate synthase did not change while its activity was

varied between 8% and 105% of the wild-type level. Thus in these conditions the flux control coefficient of citrate synthase is extremely low. In contrast, on an acetate medium, flux through citrate synthase increased approximately linearly with citrate synthase activity over the range 10–160% of the wild-type activity. However, extrapolation of the flux–activity relationship implied that a finite flux would be observed at zero activity. This is clearly not possible, so a different relationship between flux and citrate synthase activity must obtain in the range 0–10% of the wild-type activity. Thus, while citrate synthase clearly must play a significant role in regulating flux through the tricarboxylic acid cycle during growth on acetate, control must also be exerted at other steps.

The use of adjustable expression vectors clearly has great potential for organisms such as *E. coli*, where relevant mutants are available, many genes of interest have been cloned and the methodology required to control the expression of inserted genes is well developed. The technique should also become applicable to mammalian cells in culture. Methods for the introduction of suitable genes by transfection or using viral vectors are already available and adjustable expression is possible using, for example, the mouse mammary tumour virus or metallothionein promoter regulatory regions which are sensitive to dexamethasone. Indeed, a vector based on the former system is now available commercially.

A recent example relevant to metabolic regulation is the work of Wakelam *et al.* (1986) on the function of the human *N-ras* gene. This gene is one of a family that encode the plasma membrane protein p21, whose only known biochemical property is a high-affinity GTPase activity. A recombinant vector was constructed in which a normal human *N-ras* gene was placed downstream of the promoter regulatory region of the mouse mammary tumour virus long terminal repeat and NIH 3T3 cells were transfected with this vector. In one resulting cell line the level of p21^{*N-ras*} could be controlled by altering the level of dexamethasone; at high levels of the inducer, the cells were transformed. Using this adjustable expression system, Wakelam *et al.* (1986) showed that cells expressing the *N-ras* gene at high levels responded to some growth factors (e.g. bombesin, bradykinin) with a marked increase in inositol phosphate accumulation. Increasing concentrations of dexamethasone not only increased the level of p21^{*N-ras*} but also increased the amount by which bombesin stimulated inositol phosphate production. This led Wakelam *et al.* (1986) to suggest that p21^{*N-ras*} is a coupling protein akin to or identical with the putative guanine nucleotide regulatory protein G_p that is thought to be responsible for receptor-mediated stimulation of inositol phospholipid metabolism. This assignment is important not only in identifying one of the components of the inositol phospholipid system, but also in throwing some light on the coupling between receptors and the phospholipase C responsible for inositol phospholipid breakdown. Since the expression of p21^{*N-ras*} gave a marked increase in receptor-stimulated inositol phosphate generation in NIH 3T3 cells, it seems that in these cells the coupling protein (G_p) concentration must be limiting.

Enzyme structure, function and regulation

For a full understanding of the regulation of any metabolic pathway, it is essential that the relevant properties of the regulatory enzymes be understood at

the molecular level. Studies using recombinant DNA techniques can have a very significant impact in several different ways. These include: (i) facilitating the isolation of proteins in large amounts by over-expression of cloned genes, (ii) allowing sequence information to be obtained readily for proteins available in only small amounts, (iii) throwing new light on structure–function relationships, particularly by the use of protein engineering, and (iv) analysis of the expression and roles of multi-gene families. There are many examples of such contributions, particularly of over-expression and sequencing, in the literature.

Isocitrate dehydrogenase kinase/phosphatase. One recent example concerns the structure, organization, properties and role of the bifunctional enzyme isocitrate dehydrogenase kinase/phosphatase of *E. coli*. This enzyme is one member of a recently recognised group of bifunctional regulatory enzymes; other examples are 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase (e.g. El-Maghrabi *et al.*, 1982; Van Schaftingen *et al.*, 1982) and the regulatory protein of maize pyruvate, phosphate dikinase (Burnell & Hatch, 1985). The structural organization, metabolic roles and evolution of these bifunctional proteins pose fascinating problems. Attempts to answer these questions for isocitrate dehydrogenase kinase/phosphatase were hampered by the small amounts of protein available, so the kinase/phosphatase gene was an obvious target for cloning.

The metabolic role of isocitrate dehydrogenase kinase/phosphatase is illustrated in Fig. 1. When micro-organisms grow on acetate on sole carbon source, they use the glyoxylate bypass to generate the precursors required for biosynthesis (Kornberg, 1966), and cell growth is therefore absolutely dependent on flux through this pathway. The competition between isocitrate dehydrogenase and isocitrate lyase for the available isocitrate is resolved, in *E. coli*, by partial inactivation and phosphorylation of isocitrate dehydrogenase (Bennett & Holms, 1975; Borthwick *et al.*, 1984; Garnak & Reeves, 1979; LaPorte *et al.*, 1984). Isocitrate dehydrogenase kinase/phosphatase, a dimer of subunit M_r 66000, can catalyse both the phosphorylation of isocitrate dehydrogenase, using ATP as the phosphoryl group donor, and its dephosphorylation, releasing P_i (LaPorte & Koshland, 1982; Nimmo *et al.*, 1984). Its activities are controlled by a number of metabolites, adenine nucleotides and biosynthetic precursors, most of which inhibit the kinase activity and activate the phosphatase activity (LaPorte & Koshland, 1983; Nimmo & Nimmo, 1984); these metabolites are believed to control the phosphorylation state of isocitrate dehydrogenase *in vivo* and thus the division of carbon flux between the tricarboxylic acid cycle and the glyoxylate bypass (e.g. Nimmo, 1984).

Preliminary work (LaPorte *et al.*, 1985) showed that the kinase/phosphatase gene, termed *aceK*, was part of the glyoxylate bypass operon, downstream of the genes encoding malate synthase A (*aceB*) and isocitrate lyase (*aceA*) and the entire operon has now been cloned (LaPorte & Chung, 1985; El-Mansi *et al.*, 1987). LaPorte & Chung (1985) subcloned the *aceK* gene in an expression vector. The resulting plasmid, in an *aceK*[−] host, directed the synthesis of large amounts of active kinase/phosphatase. LaPorte & Chung (1985) then located the kinase/phosphatase coding region by deletion mapping

Fig. 1. Roles of the glyoxylate bypass and isocitrate dehydrogenase kinase/phosphatase during growth on acetate

The enzymes of the glyoxylate bypass are: 1, isocitrate lyase, 2, malate synthase A. Heavy arrows represent fluxes to biosynthesis; $\ominus$ represents inhibition and $\oplus$ represents activation. Abbreviation: ICDH, isocitrate dehydrogenase.

and found that the coding sequence was some 1800 bp in length. Since a gene of this length can encode only one protein of subunit M_r 66000, this work provided the first good evidence that the kinase/phosphatase contains only a single type of subunit and that both activities must therefore be carried on the same polypeptide chain. The deletion mapping produced another interesting result: even quite short deletions at either end of the putative coding region gave a sharp and parallel decline in both activities. This might argue against the existence of two independently folding domains, one carrying each activity. However further work on the protein is required, and the availability of much larger amounts of pure enzyme will make such work much easier. Sequencing of the *aceK* gene will throw further light on the organization of the kinase/phosphatase and will also allow the first comparison of a bacterial protein kinase with similar enzymes from higher organisms.

The isocitrate dehydrogenase system also affords examples of the uses of recombinant DNA techniques in testing hypotheses concerning metabolic regulation. LaPorte *et al.* (1985) constructed an *E. coli* strain which contained a multicopy plasmid bearing the gene (*icd*) that codes for isocitrate dehydrogenase. When this strain was grown on acetate, it expressed 15 times as much total isocitrate dehydrogenase (phosphorylated plus dephosphorylated) as did control strains. However the phosphorylation state of isocitrate dehydrogenase was considerably increased so that the amount of active (unphosphorylated) enzyme remained approximately constant. This is precisely the behaviour predicted by the current model for the role and regulation of the phosphorylation system (El-Mansi *et al.*, 1985, 1986; Nimmo, 1984).

Goldbeter & Koshland (1981) noted that if either or both of the activities responsible for the reversible covalent modification of a protein are close to saturation with their protein substrate (i.e. in the zero-order range) the system can respond (in terms of the degree of modification of the protein substrate) much more sensitively than if both the converter enzymes are far from saturation (i.e. in the first-order range); they used the term 'zero-order ultrasensitivity' to describe this effect.

LaPorte & Koshland (1983) showed that the

phosphorylation of isocitrate dehydrogenase *in vitro* exhibited 'zero-order ultrasensitivity'; the phosphorylation state of the enzyme became ultrasensitive to changes in the concentration of 3-phosphoglycerate, an effector of the kinase/phosphatase, when the enzyme concentration was sufficiently high to saturate the phosphatase activity. It was suggested that 'zero-order ultrasensitivity' may be of general importance in enhancing the sensitivity of covalent modification systems. It should be possible to test whether this effect is significant *in vivo* in the case of isocitrate dehydrogenase by using the adjustable expression system described for citrate synthase above.

3-Hydroxy-3-methylglutaryl-coenzyme A reductase.

The recent advent of protein engineering techniques affords new scope for studies of metabolic regulation in that specific mutations can be constructed and their effects analysed *in vitro* or in intact cells. Several stringent criteria should be satisfied before such work can be attempted. The gene in question must be cloned and sequenced, and expression systems must be available (preferably including a system in a homologous cell type). Ideally, mutants in the target protein should be available, and there must be sufficient information already available about the structure and function of the protein that engineering experiments can be designed rationally. The mutant and wild-type proteins must be compared to ensure that the mutation does not cause gross distortions of the folding or conformation of the protein. These criteria have been met in a number of cases and fascinating work using protein engineering is beginning to appear.

One example concerns the turnover of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (EC 1.1.1.34). This is an integral protein of the endoplasmic reticulum that catalyses a regulatory step in the biosynthesis of cholesterol (Fig. 2). The amount of HMG-CoA reductase protein in cultured cells is reduced by a factor of 10 in the presence of cholesterol, delivered on low density lipoprotein via receptor-mediated endocytosis, or cholesterol derivatives (e.g. Faust *et al.*, 1982; Edwards *et al.*, 1983). The decrease is attributable to both suppressed transcription of the HMG-CoA reductase gene and accelerated degradation of the reductase

Fig. 2. Some aspects of the regulation of HMG-CoA reductase

⊕ and ⊖ represent activation and inhibition respectively. Abbreviation: LDL, low-density lipoprotein.

protein (Faust *et al.*, 1982; Edwards *et al.*, 1983; Chin *et al.*, 1985). The rate of turnover of the enzyme is much higher than those of other endoplasmic reticulum proteins, suggesting that there may be a specific mechanism for degradation of the reductase (Schimke, 1975).

cDNA for HMG-CoA reductase has been cloned and sequenced (Chin *et al.*, 1984). This allowed development of an expression system for the cloned gene in cultured cells and also gave information about the probable domain structure of the protein. The reductase cDNA was cloned in a plasmid vector, downstream of the SV40 promoter, which was used to transfect a Chinese hamster ovary cell line lacking reductase activity. The plasmid integrated into the genome in multiple copies and directed the synthesis of active reductase (Chin *et al.*, 1985). Sequencing at the cDNA level showed that the reductase (subunit M_r 97000) comprised 887 amino acid residues (Chin *et al.*, 1984) and suggested the existence of two distinct regions in the protein. Residues 10–339 comprised very hydrophobic sequences alternating with short hydrophilic ones. Seven potential membrane-spanning regions were identified and this region of the protein is clearly attached to the endoplasmic reticulum. In contrast, the C-terminal two-thirds of the protein is relatively hydrophilic. Protease treatment of endoplasmic reticulum membranes cleaves the polypeptide in the region of residues 450–470 and gives an enzymically active soluble fragment of apparent M_r 53000. On hypo-osmotic incubation of cells, an endogenous protease cleaves at around residue 370 to give a slightly larger active fragment that can be released from the endoplasmic reticulum by washing in 0.1 M- Na_2CO_3 (Faust *et al.*, 1982; Liscum *et al.*, 1983, 1985). Thus the protein comprises an N-terminal membrane-bound domain and a C-terminal water-soluble domain containing the active site.

Gil *et al.* (1985) set out to test the hypothesis that the membrane-bound domain might be involved in controlling degradation of the enzyme. Using oligonucleotide-primed mutagenesis, they constructed a plasmid in which the nucleotides coding for amino acids 10–341 had been deleted. The deletion was in frame so that in the product of the mutated gene the first, hydrophilic, nine residues should be fused to the water-soluble domain (residues 342–887). The cell line lacking reductase activity was transfected with this plasmid. A resulting clone of cells was able to grow in the absence of plasma lipoproteins or mevalonate, indicating that the cells contained functional HMG-CoA reductase. The

cells had integrated multiple copies of the plasmid and contained a truncated form of the reductase of subunit M_r 60000, the expected value. Some 90% of the total reductase was in the soluble fraction of the cells, and the truncated reductase was very similar to the full-length enzyme in terms of its K_m for HMG-CoA and its sensitivity to a competitive inhibitor. Thus the deletion generated a soluble, truncated form of reductase with apparently unaltered kinetic properties.

The degradation of the truncated reductase was then compared with that of full-length enzyme by immunochemical and labelling techniques, using cells transfected with plasmids containing either the truncated or the full-length cDNA. These studies showed that in the absence of sterols the half-life of the truncated enzyme was over 5-fold higher than that of the full-length enzyme, although the rates of turnover of total protein in the two cell lines were the same. Moreover, while sterols considerably increased the rate of degradation of the full-length enzyme, they had no effect on the degradation of the truncated form. These studies show that the membrane-bound domain of HMG-CoA reductase influenced the degradation of the enzyme and the stimulation of this process by sterols. It is not yet clear whether this domain plays a direct role in degradation (for example, it may bind cholesterol or a related compound and this may trigger degradation) or whether it merely allows the reductase to interact with a membrane-associated, sterol-binding component that initiates or controls degradation. However, the use of recombinant DNA techniques by Gil *et al.* (1985) has given a first insight into how the structure of HMG-CoA reductase is related to control of its degradation, which is an important factor in the control of cholesterol biosynthesis.

The insulin receptor. A further illustration of the potential value of protein engineering is afforded by recent work on the insulin receptor. Insulin can elicit a wide variety of effects in target cells and the nature of signal transduction by the insulin receptor is a topic of intense interest. The insulin receptor is synthesized as a single polypeptide of subunit M_r about 180000. Disulphide-linked dimers are glycosylated, cleaved to yield α and β subunits and inserted into the plasma membrane. A schematic view of the insulin receptor, based on its cDNA sequence and many conventional studies and indicating various functional domains, is shown in Fig. 3. The discovery that the receptor can become phosphorylated on serine and tyrosine residues in the β subunit and that the β subunit possesses a kinase activity that can

Fig. 3. Schematic diagram of the insulin receptor

The hatched area represents the cytoplasmic tyrosine kinase domain and the stippled area the transmembrane region. The numbers on the right indicate aminoacid residues, using the numbering of Ebina *et al.* (1985).

phosphorylate exogenous proteins on tyrosine residues has focused attention on the possible roles of these phenomena in the functions of the insulin receptor (e.g. Gammeltoft & van Obberghen, 1986). The cloning and sequencing of human insulin receptor cDNA allowed identification of a domain in the β subunit that is thought to be located on the cytoplasmic side of the plasma membrane and to have tyrosine-specific protein kinase activity (Ebina *et al.*, 1985; Ullrich *et al.*, 1985). Ellis *et al.* (1986) have recently reported the first results of protein engineering experiments aimed at elucidating structure/function relationships in the insulin receptor.

Several sites in the insulin receptor can be phosphorylated, and at least some of the sites labelled *in vivo* are apparently not the same as those labelled *in vitro* (e.g. White *et al.*, 1985). None of the phosphorylated sites have been positively identified, but Ellis *et al.* (1986) pinpointed two tyrosine residues [1162 and 1163 in the sequence numbering of Ebina *et al.* (1985)] which are in the tyrosine kinase domain and in positions analogous to the major sites of autophosphorylation in pp60^{v-src} and they therefore studied the effects of converting one (Tyr-1162) or both of these to phenylalanine residues, which cannot be phosphorylated.

Wild-type or mutated human insulin receptor cDNAs were cloned behind the SV40 early promoter in plasmids which were used to transfect Chinese hamster ovary cells. The host cells, of course, express their own insulin receptors, but the transfected cell lines expressed the human receptor at 250–1000 times the level of the endogenous receptor, so the lack of a receptor-minus mutant to act as host was overcome. To check that the mutations did not grossly distort the overall conformation of the human receptor, Ellis *et al.* (1986) showed that the affinities of the wild-type and mutant receptors for insulin were very similar and that the wild-type and mutant receptors were recognized by several classes of monoclonal antibodies specific for the cytoplasmic domain of the receptor β -subunit.

Using partially purified receptor preparations, Ellis *et al.* (1986) found that both mutations increased several-fold the autophosphorylation of the receptor in the absence of insulin, but decreased it in the presence of insulin. Thus the stimulation of autophosphorylation by insulin was severely reduced (for the single mutant) or eliminated (for the double mutant). The two mutations also reduced or abolished the ability of insulin to stimulate the phosphorylation of an exogenous substrate

by partially purified receptor. In intact cells, the mutations considerably decreased the extent of insulin-stimulated tyrosine phosphorylation of the receptor and the sensitivity of 2-deoxyglucose uptake to insulin, and partially reduced the maximum effect of insulin on the rate of uptake.

Identification of the sites phosphorylated in the wild-type and mutant receptors in different conditions is clearly essential. The data of Ellis *et al.* (1986) are compatible with the view that tyrosine residue(s) 1162 and/or 1163 are sites of autophosphorylation. Using protein chemical techniques, Stadtmayer & Rosen (1986) arrived at a similar conclusion by showing that a 12-residue peptide corresponding to residues 1154–1165 was a good substrate for the kinase activity of the insulin receptor and that the residue phosphorylated was Tyr-1162. Moreover, experiments with anti-peptide antibodies revealed that one of the sites phosphorylated in intact cells in response to insulin was in a tryptic peptide containing tyrosine residues 1158, 1162 and 1163. Phosphorylation of this region *in vitro* shows the same time-course as the activation of the receptor kinase (Herrera & Rosen, 1986). However several other sites can also be phosphorylated and the mutations introduced by Ellis *et al.* (1986) seem to render some of these more accessible to phosphorylation *in vitro*. The results allow two important conclusions to be drawn; first, autophosphorylation of Tyr-1162 and/or 1163 is important in regulation of the tyrosine kinase activity of the insulin receptor, and second that this activity plays a role in mediating insulin's effect on glucose uptake. The latter point receives support from the recent demonstration that autophosphorylation of the receptor precedes activation of glucose uptake (Kohanski *et al.*, 1986). The ability to generate other site-specific mutants of the insulin receptor and to study their behaviour in intact cells should greatly facilitate studies of the molecular basis of signal transduction by the insulin receptor.

Analysis of multigene families. Some enzymes involved in metabolic regulation consist of a set of related proteins encoded by a multigene family. The degree of resolution afforded by currently available techniques of protein purification is not always sufficient to resolve the members of such a set. Recombinant DNA techniques can be of great value in the analysis of such systems, for example in determining the number of genes involved and investigating the tissue specificity of the expression of the genes.

Protein kinase C is thought to play a key role in many signal transduction processes by virtue of its activation by diacylglycerol, which can be generated by receptor-mediated activation of phospholipase C (e.g. Nishizuka, 1984). Purified protein kinase C was originally regarded as a single entity (e.g. Kikkawa *et al.*, 1982; Parker *et al.*, 1984; Wise *et al.*, 1982), although recently three distinct forms of the rat brain enzyme were separated chromatographically (Huang *et al.*, 1986).

Recently several groups have isolated and sequenced cDNA clones of protein kinase C and this work has revealed the existence of a multigene family. Screening of a bovine brain cDNA library showed the existence of at least three related cDNA species encoding highly homologous protein kinases (Coussens *et al.*, 1986; Parker *et al.*, 1986). Similarly, three distinct species were found by screening of a human foetal brain cDNA

library, and the corresponding genes were assigned to different human chromosomes (Coussens *et al.*, 1986). Based on the results obtained by probing Southern blots of bovine or human chromosomal DNA, Coussens *et al.* (1986) suggested the possible existence of several additional protein kinase C-related genes.

Multiple protein kinase C cDNAs have also been identified in rat (Knopf *et al.*, 1986; Ono *et al.*, 1986) and rabbit (Ohno *et al.*, 1987) brain cDNA libraries. Two of the cDNAs differ only in the region encoding the C-terminal 50 residues and the 3' untranslated region; the two corresponding mRNAs probably arise from alternative splicing of a single gene transcript (Ono *et al.*, 1986; Ohno *et al.*, 1987). Analysis of 'Northern' blots showed that three rabbit protein kinase C mRNAs differed in their tissue distribution (Ohno *et al.*, 1987).

The current picture in this fast-moving field is thus of the existence of multiple protein kinase C genes. These encode related proteins, which probably have subtly different properties and functions, and are expressed in a tissue-specific manner. This clearly has important implications for our understanding of the specificity and mechanisms of signal transduction.

The existence of two major classes (Type I and Type II) of cyclic AMP-dependent protein kinase is well known; these contain distinct regulatory subunits (RI and RII respectively) which were thought to interact with a single type of catalytic subunit (e.g. Krebs & Beavo, 1979). However, screening of cDNA libraries has now shown the existence in mouse and bovine tissue of two isoforms of the catalytic subunit, termed $C\alpha$ and $C\beta$ (Showers & Maurer, 1986; Uhler *et al.*, 1986). By using a quantitative solution hybridization assay, Uhler *et al.* (1986) found that the ratio of $C\alpha$ mRNA to $C\beta$ mRNA was tissue-specific, varying from about 1 (spleen) to about 20 (testis). The functional significance of the existence and differential expression of two distinct catalytic subunits is not known, although Uhler *et al.* (1986) speculated that they may have different substrate specificities. They also postulated that $C\alpha$ and $C\beta$ do not represent Type I- and Type II-specific catalytic subunits but that $C\alpha$ interacts with both types of regulatory subunit while $C\beta$ may interact preferentially with a distinct regulatory subunit that has yet to be characterized.

These studies illustrate the power of recombinant DNA techniques in the analysis of multienzyme families. However the most important questions as regards metabolic regulation must involve the functional differences between the various members of a set of related enzymes. Such questions can only be addressed at the protein level. In this connection, expression of the different isoenzymes in appropriate host/vector systems could lead to the isolation of pure proteins in quantities that would permit the necessary enzymological characterization.

Identification of the levels at which enzyme synthesis is regulated

Methods. Measurements of enzyme synthesis and degradation using conventional techniques (e.g. labelling and chase experiments, immunoprecipitation, use of inhibitors of protein or RNA synthesis etc.) have played a very significant part in studies of metabolic regulation in higher organisms, and many important examples of enzyme induction are known. In such cases, recombinant

DNA techniques can be used to pinpoint the level at which enzyme synthesis is controlled and the mechanisms that are involved. Two techniques in particular should be mentioned here. First, specific mRNAs can be quantitated easily by dot-blot hybridization with a specific labelled DNA probe, for example using total cellular RNA, nuclear RNA or poly(A)-containing RNA. Second, rates of synthesis of specific mRNAs can be measured by a transcription elongation or 'run-off' assay (McKnight & Palmiter, 1979). In this technique, nuclei are isolated and then incubated with the requirements for RNA synthesis, including an appropriately labelled nucleotide. Labelled RNA is isolated and the incorporation into a specific mRNA is quantified by measuring the amount of label that hybridizes to a specific, immobilized DNA probe. Incorporation into total RNA is measured in the same experiment and the rate of specific mRNA synthesis is usually expressed as a proportion of the total. Most of the RNA labelled during incubation of isolated nuclei is formed by elongation of nascent molecules, and this assay thus reflects the rate of synthesis of the specific mRNA at the time of isolation of the nuclei. These methods can be applied to isolated cells or intact animals subjected to appropriate stimuli. Comparison of the effects of a stimulus on the rate of specific mRNA synthesis and the cellular level of the mRNA can then, in principle, reveal whether effects are mediated at the level of gene transcription, mRNA stability, or transcript processing.

Malic enzyme and fatty acid synthase. Application of these techniques has shown that control of mRNA abundance is often exerted at more than one level and that in several cases a large part of the control occurs at a post-transcriptional level. For example, the malic enzyme (EC 1.1.1.40) activity and mRNA content of rat liver is increased 7–8-fold when the animals are fed on a high carbohydrate diet; this effect is tissue-specific (Dozin *et al.*, 1986). The high carbohydrate diet had no effect on the rate of transcription of the malic enzyme gene. The simplest hypothesis compatible with the data was that carbohydrates affected the rate of degradation of cytoplasmic malic enzyme mRNA, although a more complex mechanism involving changes in both degradation of nuclear malic enzyme mRNA and its transport to the cytoplasm could not be ruled out (Dozin *et al.*, 1986).

Dietary control of malic enzyme synthesis in ducklings affords a more complex example in which both transcriptional and post-transcriptional effects are involved (Goldman *et al.*, 1985). The abundance of malic enzyme mRNA is some 20-fold higher in the livers of carbohydrate-fed ducklings than in those of starved ducklings. Feeding only increased the rate of transcription of the malic enzyme gene by 2–3-fold. The half-life of the malic enzyme mRNA was estimated by monitoring either the kinetics of its decay after the onset of starvation or the kinetics of its appearance on re-feeding. The results showed that starvation reduced the half-life from 3–5 h to about 1 h. In combination, the effects on transcription and mRNA stability could account for the change in mRNA abundance and there was no evidence for regulation of nuclear processing steps (Goldman *et al.*, 1985). Feeding of starved ducklings also increased the abundance of fatty acid synthase mRNA in liver, by some 10-fold (Back *et al.*, 1986a). In this case, however,

Fig. 4. Hormonal control of liver pyruvate kinase (L-PK) and phosphoenolpyruvate carboxykinase (PEPCK)

Changes in the amounts of mRNAs are indicated by the vertical arrows.

most of the effects can be accounted for by changes in the rate of transcription of the fatty acid synthase gene. It is thus very interesting to note that, although both malic enzyme and fatty acid synthase are involved in fatty acid synthesis *de novo* and their biosyntheses are co-ordinated under many physiological conditions, the mechanisms involved in the response to nutritional changes are rather different for the two enzymes.

Co-ordinate regulation of the level of mRNAs for malic enzyme and fatty acid synthase is seen with the hormones tri-iodothyronine and glucagon (Goodridge *et al.*, 1986). In isolated chick embryo hepatocytes incubated in a serum-free medium containing insulin, tri-iodothyronine caused a greater than 10-fold increase of both mRNAs, while further addition of glucagon decreased the amount of malic enzyme mRNA by 90–95% and that of fatty acid synthase mRNA by 50%. The regulation was judged to be predominantly post-transcriptional for both hormones, since transcription rates measured by 'run-off' assays in the presence and absence of hormones did not vary greatly. For malic enzyme increased degradation of mRNA in response to glucagon was implicated. The half-life of malic enzyme mRNA, measured in cells incubated with tri-iodothyronine and either actinomycin D or α -amanitin to inhibit transcription, was 8–11 h. The half-life in cells incubated with tri-iodothyronine and glucagon was less than 2 h. However, this 5-fold change in half-life was not sufficient to account for the large change in the amount of malic enzyme mRNA observed in glucagon-treated cells and therefore other levels of post-transcriptional regulation must also be involved (Back *et al.*, 1986b; Goodridge *et al.*, 1986).

Pyruvate kinase. Besides identifying the levels at which control is exerted, recombinant DNA techniques can also uncover new sites of regulatory control. For example it has recently been shown that glucagon can alter the quantity of the mRNA for the glycolytic enzyme L-type pyruvate kinase (ATP: pyruvate phosphotransferase, EC 2.7.1.40). The action of glucagon and its intracellular mediator cyclic AMP at the post-translational level on L-type pyruvate kinase is well known (Engstrom, 1980). Phosphorylation catalysed by cyclic AMP-dependent protein kinase inhibits the enzyme activity and thus stimulates gluconeogenesis (Fig. 4). In this respect it is

interesting that the isolation and sequencing of cDNA clones for L-type pyruvate kinase identified the position of the phosphorylated serine near the *N*-terminus of the molecule and showed that muscle pyruvate kinase, which is not regulated by phosphorylation, does not possess this *N*-terminal regulatory region (Lonberg & Gilbert, 1983; Ionue *et al.*, 1986; Lone *et al.*, 1986).

The discovery that glucagon can also act at a pretranslational level to control L-type pyruvate kinase activity came from quantification of L-type pyruvate kinase mRNA with the use of cDNA probes under different dietary and hormonal conditions. Hybridizable L-type pyruvate kinase mRNA is barely detectable in starved animals, but can be readily detected in animals fed on laboratory chow and feeding of a high carbohydrate diet increases this level still further (Noguchi *et al.*, 1983; Simon *et al.*, 1983). Administration of glucagon or analogues of its second messenger, cyclic AMP, to animals fed on laboratory chow or on a high-carbohydrate diet abolishes the induction of L-type pyruvate kinase mRNA, and exogenous insulin cannot reverse the inhibitory action of glucagon (Munnich *et al.*, 1984; Noguchi *et al.*, 1985).

Measurements of transcription rates using 'run-off' assays show that a high-carbohydrate diet stimulates an increase in the rate of L-type pyruvate kinase gene transcription, while administration of glucagon or analogues of cyclic AMP results in inhibition of the transcription rate. The latter effect is rapid (complete within 15 min), suggesting that inhibition is the result of a 'direct effect' of cyclic AMP or cyclic AMP-dependent protein kinase on the initiation of transcription. However, no significant decrease in the level of L-type pyruvate kinase mRNA is observed for 1 h after glucagon administration and the decay thereafter is more complex than the pseudo-first-order kinetics expected if glucagon were only inhibiting the transcription rate. Therefore, in addition to its effect on transcription, glucagon may also act at the post-transcriptional level, decreasing the stability of L-type pyruvate kinase mRNA (Vaulont *et al.*, 1986). In summary, recent studies using recombinant DNA probes reveal at least one and probably two pretranslational sites of glucagon/cyclic AMP regulation of L-type pyruvate kinase. These complement the regulation by post-translational phosphorylation, which was previously thought to be the only mode of action of these effectors on this enzyme.

Detailed analyses of hormonal effects on transcription

Application of recombinant DNA techniques to the study of certain regulatory enzymes is proving very valuable in probing mechanisms of hormonal control and in identifying those regions of genes that are involved in hormonal responses. A technique that has found wide application here involves ligation of the putative control region of a gene (usually the 5' end) to an indicator gene (e.g. the herpes virus thymidine kinase gene and bacterial neomycin-resistance or chloramphenicol acetyltransferase genes). The fused gene is transfected into hormonally responsive cells and either transient or stable expression of the linked indicator gene is examined under different hormonal conditions (for a detailed review see Wynshaw-Boris *et al.*, 1986a). If hormonal control is observed, mutants of the fusion gene can be constructed with deletions in the putative control region. Examination of the expression of these mutants in different hormonal conditions can define more precisely the DNA sequences that are essential to the hormonal response. The approach has been used very effectively in studies of the sequences involved in the responses of certain enzymes to cyclic AMP and glucocorticoids.

Cytosolic phosphoenolpyruvate carboxykinase. As a key regulatory enzyme in gluconeogenesis, cytosolic hepatic phosphoenolpyruvate carboxykinase (PEPCK) [GTP:oxaloacetate carboxy-lyase (transphosphorylating), EC 4.1.1.32] is subject to hormonal control (Fig. 4). The amount of PEPCK mRNA and protein is increased by glucagon (acting via cyclic AMP) and by glucocorticoids and decreased by insulin. Isolation of cDNA probes (Yoo-Warren *et al.*, 1981; Beale *et al.*, 1982) made possible direct quantification of PEPCK mRNA and established that the hormonal effects are brought about by changes in mRNA concentration (Cimbala *et al.*, 1982; Beale *et al.*, 1982; Granner *et al.*, 1983; Granner & Beale, 1985). 'Run-off' transcription assays clearly demonstrate that cyclic AMP and glucocorticoids increase the rate of transcription of the PEPCK gene (Lamers *et al.*, 1982; Sasaki *et al.*, 1984). However, cyclic AMP may also alter PEPCK gene expression at a post-transcriptional step and, in the case of glucocorticoids, measurements of the half-life of the mRNA have shown that some control is also exerted at the level of mRNA stability (Granner & Beale, 1985).

The gene for PEPCK has been cloned and sequenced (Beale *et al.*, 1985; Yoo-Warren *et al.*, 1983). Investigations of hormone action on transcription have concentrated on the 5' end of the gene as being the most likely region to contain DNA sequences involved in hormonal responses. Wynshaw-Boris *et al.* (1986b) constructed a fusion gene containing the promoter regulatory region (−548 to +73, relative to the transcription start site) of the PEPCK gene immediately 5' to the thymidine kinase gene with its own promoter. After transfection into rat hepatoma FTO-2B cells, the transcription of the PEPCK-thymidine kinase fusion gene was regulated by dibutyryl cyclic AMP and the synthetic glucocorticoid dexamethasone but not by insulin. A smaller fragment of the promoter regulatory region (−416 to −61) which did not contain the PEPCK promoter or the start site of transcription also responded to dibutyryl cAMP and glucocorticoids when ligated to the thymidine kinase gene. The regulation was in-

dependent of the orientation of the promoter regulatory region relative to the transcription of the thymidine kinase gene. The fragment was effective at a variety of locations relative to the thymidine kinase promoter. Placing it 3' to the thymidine kinase gene in either orientation still resulted in a hormonal response, although the magnitude of the response was lower.

In order to locate the cyclic AMP and glucocorticoid regulatory elements more precisely, deletion mutants of the PEPCK promoter regulatory region (−355 to +73) linked to the neomycin-resistance gene or the thymidine kinase gene were stably transfected into FTO-2B hepatoma cells and tested for hormonal responses (Short *et al.*, 1986). Mutants with the region −355 to −109 deleted were still sensitive to dibutyryl cyclic AMP and dexamethasone. Loss of the dibutyryl cyclic AMP sensitivity but not the glucocorticoid response occurred when the deletion in the PEPCK promoter regulatory region extended to position −68. The smallest fragment found to elicit a response to both dibutyryl cyclic AMP and dexamethasone, albeit less than maximum, was a synthetic 47 bp fragment corresponding to base pairs −62 to −108 in the rat PEPCK gene. These results are taken to indicate the presence of a cyclic AMP regulatory element located between positions −108 to −62 and two glucocorticoid regulatory regions, the first between positions −108 and −62 and the second between positions −68 and +73, either one of which can confer sensitivity to dexamethasone.

Inspection of the DNA sequence in the glucocorticoid-sensitive regions revealed the element TGTCCTCCC at positions −48 to −40, which is identical to the core sequence within the glucocorticoid regulatory element of the human metallothionein II^A gene (Karin *et al.*, 1984), and a further sequence AGCCTCC at −76 to −70 which shows only partial homology to the core sequence. Inspection of the nucleotide sequence of the PEPCK gene in the cyclic AMP-responsive region reveals a 12 bp sequence CTTACGTCAGAG at positions −91 to −80 which shows homologies with sequences in similar positions in other genes regulated by cyclic AMP. This sequence is virtually identical to 12 bp of DNA in the rat preprosomatostatin gene element (−60 to −29) that has also been implicated by expression studies in the response to cyclic AMP (Montminy *et al.*, 1986). Analysis of the expression of the human proenkephalin gene (Comb *et al.*, 1986) has shown that a 37 bp DNA fragment is essential for the regulation of this gene by cyclic AMP

Table 1. Sequence homologies in genes whose expression is regulated by cyclic AMP

Sequences occur in regions that have been shown by expression studies to respond to cyclic AMP. They have been aligned to maximize homology. The data are taken (from top to bottom) from Short *et al.* (1986), Montminy *et al.* (1986) and Comb *et al.* (1986).

Gene	Sequence
Rat PEPCK	−91 C T T A C G T C A G A G −80
Rat prepro-somatostatin	−49 C T G A C G T C A G A G −38
Human pro-enkephalin	−93 C T G − C G T C A G C T G C A G −79

and that a section of it bears significant homology with the sequences in the cyclic AMP regulatory elements of the PEPCK and somatostatin genes. The homologies are illustrated in Table 1.

It has been suggested that the regulation of gene expression by cyclic AMP in higher eukaryotes may be controlled by a mechanism homologous to that in prokaryotes where cyclic AMP binds to a cyclic AMP receptor protein and the resulting complex can interact with certain DNA sequences that influence transcription at nearby promoters (de Crombrughe *et al.*, 1984). For this reason sequences in eukaryote genes regulated by cyclic AMP were compared with the receptor-protein-binding sequences of prokaryotes and two regions of homology, at -516 to -488 and -412 to -384 in the PEPCK gene, were identified (Nagamine & Reich, 1985). However, no functional tests have yet been carried out with these regions and therefore the significance of these homologies remains to be assessed.

The cyclic AMP receptor protein belongs to a family of prokaryotic proteins all of which bind as dimers to DNA sequences that have two-fold symmetry (Anderson *et al.*, 1985). It may be significant that the cyclic AMP regulatory element in the somatostatin gene contains a 10 bp palindrome at positions -49 to -40. However, functional studies indicate that, although the palindrome is a component of the regulatory region, it does not by itself confer cyclic AMP inducibility. GC-rich regions found at the 5' end, and the AG dinucleotides found at the 3' end, of the palindrome may also be essential, since they are present in the cyclic AMP-responsive elements of other genes.

The regulatory subunit (RII) of cyclic AMP-dependent protein kinase bears significant sequence homology to the cyclic AMP receptor protein of *E. coli* and it has been proposed that RII may bind to eukaryote genes sensitive to cyclic AMP and regulate transcription (Weber *et al.*, 1982; Nagamine & Reich, 1985; Constantinou *et al.*, 1985). An equally plausible mechanism for the regulation is the phosphorylation of chromosomal proteins by the catalytic subunit of cyclic AMP-dependent protein kinase (Waterman *et al.*, 1985). However, a recent study demonstrates a specific requirement for type II cyclic AMP dependent protein kinase in one case of transcriptional regulation of gene expression by cyclic AMP. The response of the preprosomatostatin gene promoter regulatory region to cyclic AMP was greatly reduced in a mutant rat pheochromocytoma cell line deficient in type II cyclic AMP-dependent protein kinase but with normal levels of the type I enzyme (Montminy *et al.*, 1986). Since the catalytic subunit is common to both type I and type II protein kinases, these results may implicate the RII subunit in the mechanism of transcriptional regulation of gene expression by cyclic AMP in eukaryotes.

The effect of insulin on the transcription of the PEPCK gene has been well documented using 'run-off' transcription assays (Sasaki *et al.*, 1984; Granner & Andreone, 1985). Insulin inhibits the basal transcription of the PEPCK gene in rat hepatoma H4IIE cells and over-rides the stimulatory actions of cyclic AMP and glucocorticoids. The effects are exerted at physiological concentrations of insulin (1 pM-1 nM), occur rapidly (the initial effects are seen within 5 min), are reversible, do not require ongoing protein synthesis and do not involve a decrease in cyclic AMP concentration. These

facts have led to the suggestion that insulin acts on a regulatory transcription factor, although not on RNA polymerase II nor on any general transcription factor, since the effect on PEPCK transcription is very specific and total gene transcription is not affected. However, sequences responsive to insulin do not appear to be located within 2.1 kb of the PEPCK gene 5' to the transcription start site, and it seems unlikely that they are located further upstream, since a transcriptionally active region of unknown function is present that is not regulated by insulin (Short *et al.*, 1986). Transcribed sequences within the PEPCK gene have not yet been tested for response to insulin.

A recent interesting finding is that the action of insulin on the PEPCK gene is mimicked by the phorbol ester, phorbol myristate acetate, and by diacylglycerol. Since these compounds are both activators of protein kinase C, it has been suggested that a phosphorylation-dephosphorylation modification of regulatory transcription factor(s) may be involved (Granner *et al.*, 1986; Chu & Granner, 1986). Thus covalent modification may play a role in this action of insulin as it does in many others.

Tyrosine aminotransferase and tryptophan oxygenase. Tyrosine aminotransferase (L-tyrosine:2-oxoglutarate aminotransferase, EC 2.6.1.5) and tryptophan oxygenase (EC 1.13.11.11), which catalyse the rate-limiting steps in tyrosine and tryptophan catabolism respectively and possibly supply substrates for gluconeogenesis, are classical examples of enzymes that undergo hormonal induction. Glucocorticoids induce both tyrosine aminotransferase and tryptophan oxygenase, while tyrosine aminotransferase is also subject to regulation by insulin, glucagon and its intracellular mediator cyclic AMP. Isolation of cDNA probes for both enzymes (Scherer *et al.*, 1982; Schmid *et al.*, 1982) allowed direct quantification of their mRNAs and made the measurement of transcription rates using 'run-off' assays feasible. These studies convincingly confirm that glucocorticoids increase the rate of transcription of both these enzymes, that cyclic AMP increases the rate of transcription of tyrosine aminotransferase and that the combined effect of glucocorticoids and cyclic AMP results in higher mRNA concentrations than either inducer alone, implying that these two effectors act by distinct mechanisms (Danesch *et al.*, 1983; Hashimoto *et al.*, 1984).

Isolation and sequencing of the genes for tyrosine aminotransferase and tryptophan oxygenase (Shinomiya *et al.*, 1984) has led to further characterization of the 5' ends of the genes in order to obtain information about possible regulatory sequences. One method of analysis is to examine the sensitivity of the genes to digestion by DNAase I in isolated nuclei. DNAase I degrades actively transcribed chromatin much faster than it does non-transcribed sequences. However, the degradation is not uniform and some sites are extremely sensitive to DNAase I cleavage. These DNAase I hypersensitive sites can be identified using an appropriately labelled probe (for a review, see Elgin, 1981). Becker *et al.* (1984) found DNAase I hypersensitive sites near the promoters of the tyrosine aminotransferase and tryptophan oxygenase genes in rat liver cells and additional cleavage sites further upstream. All these hypersensitive sites are absent from kidney cells, which do not express tyrosine aminotransferase and tryptophan oxygenase. More inter-

esting is the detection of an extra DNAase I hypersensitive site upon induction with dexamethasone. This site is about 2.4 kb upstream of the start of transcription of the tyrosine aminotransferase gene and suggests that glucocorticoid response elements might be located far upstream of the start site of transcription (Schutz *et al.*, 1986).

In order to determine whether these 5' regions of the tyrosine aminotransferase and tryptophan oxygenase genes are important for the hormonal control by glucocorticoids, fusion genes were constructed containing the 5' flanking region of the aminotransferase or oxygenase genes ligated to the coding region of the bacterial chloramphenicol acetyltransferase gene (Schutz *et al.*, 1986). Both fusion genes show glucocorticoid-regulated expression of chloramphenicol acetyltransferase in hepatoma cells. Deletion mutants of these constructs were used to ascertain the location of the regulatory region(s). Strong induction of expression by glucocorticoids is observed as long as 1930 nucleotides remain of the 5' flanking region of the tryptophan oxygenase hybrid gene. Deletion of the 5' flanking region up to position -600 results in considerable but not complete loss of inducibility. Further deletion to position -314 abolishes all inducibility. These studies thus locate two control regions in the 5' flanking region of the tryptophan oxygenase gene.

Analysis of deletion mutants of the tyrosine aminotransferase-chloramphenicol acetyltransferase hybrid gene showed that approximately 20-fold induction of expression by dexamethasone occurred as long as 2950 nucleotides of the 5' flanking region of the hybrid gene remained. Deletion of the 5' flanking region up to position -2474 curtailed this inducibility by dexamethasone to approx. 2-fold and thus identified an important control element between -2.9 kb and -2.4 kb (Schutz *et al.*, 1986). This region is the same as that which showed increased DNAase I hypersensitivity in response to glucocorticoids.

Inspection of the sequence in this region revealed the presence of the hexanucleotide 5'TGTTCT3' at positions -2611 and -2500, and, in the opposite orientation, at position -2440. This sequence is found four times within the region of the mouse mammary virus long terminal repeat that is known to interact with the glucocorticoid receptor protein (Schneidereit *et al.*, 1983; Payvar *et al.*, 1983) and once within the element of the vitellogenin II gene that is known to interact with the oestrogen receptor (Jost *et al.*, 1984). In order to examine whether the far upstream region of the tyrosine aminotransferase gene at approx. -2.5 kb was functional as a glucocorticoid receptor binding site, binding studies with the glucocorticoid receptor and the 5' deletion mutants were performed. Labelled fragments which spanned the 5' flanking region of the deletion mutants were examined for the ability to bind the purified glucocorticoid receptor on a filter in the presence of increasing amounts of calf thymus DNA to prevent non-specific binding. These studies showed that the glucocorticoid receptor interacts preferentially with a deletion mutant ending at position -2561, which contains two 5'TGTTCT3' sequences, but not with deletion mutants ending at positions -1890 or -1300 (Schutz *et al.*, 1986).

DNAase I footprinting studies (Galas & Schmitz, 1978), which involved DNA sequence analysis before and after binding of the partially purified glucocorticoid

complex to the relevant DNA fragment *in vitro*, localized the receptor binding sites more precisely (Schutz *et al.*, 1986). *In vitro* dimethyl sulphate protection experiments were used to complement these analyses (Becker *et al.*, 1986). Guanine bases in DNA are selectively methylated by dimethyl sulphate. If this reaction is allowed to proceed after binding of the glucocorticoid receptor to a DNA fragment, any G residues that interact with the receptor will not be accessible to the reagent. Sequence analysis using piperidine cleavage identifies the protected guanine bases. In addition it identifies guanine bases subject to enhanced attack by dimethyl sulphate, a phenomenon which is postulated to arise from a local increase in reagent concentration in hydrophobic pockets arising from the interaction of the receptor with the DNA. A further valuable technique, genomic footprinting, developed by Church & Gilbert (1984), allows the identification of guanine residues protected from and subject to enhanced attack by dimethyl sulphate as the result of binding of the hormone-receptor complex to the DNA *in vivo*.

The outcome of these footprinting analyses *in vitro* and *in vivo* on the tyrosine aminotransferase gene (Becker *et al.*, 1986) is that two sequences protected by the hormone-receptor complex *in vitro* have been identified at around -2.5 kb, together with G residues around this region that show changes in reactivity to dimethyl sulphate on binding of the hormone-receptor complex *in vitro* and *in vivo*. Fig. 5 summarizes the main results. By comparison with other glucocorticoid-regulated genes on which similar studies have been carried out, this receptor-binding region is located unusually far upstream. However, the presence of the 5'TGTTCT3' sequences lends support to the functional studies which identify the -2.5 kb region as a glucocorticoid regulatory element. The lack of more extensive sequence homologies with the 5' regions of other glucocorticoid-regulated genes makes it unlikely that the glucocorticoid-receptor complex recognition site is a unique DNA sequence (for review, see Groner *et al.*, 1984). Nevertheless, recombinant DNA techniques are playing a crucial role in delineation of features necessary for the response of genes to glucocorticoids.

Summary and perspectives

It is clear that recombinant DNA techniques are already being applied in studies of metabolic regulation in a wide variety of ways. They can be used in the measurement of flux control coefficients which lead to the identification of the regulatory step(s) in metabolic pathways *in vivo*. The sequences of many regulatory enzymes have been determined via their cDNAs, and such information is essential for a complete understanding of their regulation. In this connection, deletion and site-directed mutagenesis can help to define the regions of a protein connected with a particular function or regulatory property. The existence of previously undetected isoenzymes has been revealed by analysis of cDNA libraries. Use of recombinant DNA techniques to probe the mechanisms of enzyme induction is allowing identification of the level(s) at which control is exerted, and dissection of the mechanisms by which hormones act to alter mRNA levels of regulatory enzymes.

Recombinant DNA techniques also afford several other exciting prospects for the future. These could

Fig. 5. Some features of the 5' flanking region of the tyrosine aminotransferase gene

The TGGTTCT sequences are boxed. Hatched regions indicate glucocorticoid receptor binding sites as shown by DNAase I footprinting experiments *in vitro*. Dexamethasone-dependent changes determined by genomic footprinting studies are indicated by arrows. These indicate guanine residues whose reactivity to dimethylsulphate is reduced (↓) or increased (↑) as a result of binding of the dexamethasone-glucocorticoid receptor complex *in vivo*. The sequence is numbered relative to the transcription start site. Data are from Becker *et al.* (1986).

include the use of antisense RNA as a tool to control the levels of regulatory enzymes and the use of retroviral vectors for efficient gene transfer.

Antisense RNA is a transcript which is complementary to all or part of a target RNA sequence, which usually codes for protein. Its natural occurrence in prokaryotes is now well documented (Green *et al.*, 1986) and it seems to have a regulatory function, brought about by the hybridization of the antisense RNA to the sense transcript. Artificial antisense RNA has been used to regulate the expression of certain genes in eukaryotes. Two general techniques have been used here. The first, microinjection of the antisense RNA, inhibits translation of the target RNA. The second, transformation with a plasmid containing part or all of the target gene inserted in the reverse orientation between a promoter and a polyadenylation signal, results in a deficit of the target mRNA in the cytoplasm. Such methods could be used to control the expression of regulatory enzymes. However some work suggests that complete inhibition may be difficult to achieve. It has been reported for the *H-ras* oncogene that a 10–20-fold excess of antisense RNA over sense *H-ras* does not completely suppress *H-ras* expression or the transformed phenotype (Salmons *et al.*, 1986), nor does a 300-fold excess of antisense thymidine kinase RNA over the sense RNA abolish thymidine kinase activity (Kim & Wold, 1985). Nevertheless, the uses of a vector with an inducible promoter for the production of antisense RNA would have the potential for investigation of the function, the differential and/or the temporal expression of genes whose complete loss by mutation would be lethal.

An alternative approach is the use of synthetic oligonucleotides complementary to short stretches of the target RNA. HTLV-III virus (HIV) gene expression has been inhibited by up to 95% using one 20-base oligonucleotide and the possible occurrence of synergistic effects of two antisense oligonucleotides holds an exciting prospect (Zamecnik *et al.*, 1986).

Retroviral vectors provide a potentially very powerful tool for the introduction of genes into eukaryotic cells. The genetic information required for production of packagable RNA is located at the 5' and 3' ends of the viral genome. This allows the *gag-pol-env* genes to be replaced by other genes, including selectable markers. Such recombinant retroviruses are defective for replication. However pure stocks of recombinant virions can be produced by using 'helper' cells containing an integrated provirus that provides all the *trans* functions necessary for the encapsidation of a recombinant genome yet is unable to encapsidate its own RNA (e.g. Cone & Mulligan, 1984; Mann *et al.*, 1983). Transfection of such cells with a suitable retrovirus-based vector (e.g. Cepko *et al.*, 1984) leads to the production of the recombinant virions which can be used to infect a wide variety of recipient cells in an efficient process. Usually, a single intact copy of the recombinant provirus becomes integrated at random into the genome of the recipient cell and expresses the 'foreign' protein(s) stably. The expression of genes introduced into cells by retroviral infection is more efficient than that of genes introduced by DNA transfection (Hwang & Gilboa, 1984). Retroviral vectors therefore offer a number of advantages over other methods for the introduction of genes into eukaryotic cells. It seems likely that these vectors will increasingly be used to facilitate controllable expression of regulatory enzymes or to introduce mutated versions of regulatory proteins into eukaryotic cells. The protein product of an introduced gene can, of course, be 'targetted' to a specific subcellular compartment by use of an appropriate signal sequence.

Thus recombinant DNA techniques are already playing a significant role in studies of metabolic regulation and further exciting applications are in prospect. These developments seem certain to have a very great impact on our understanding of metabolic regulation at the molecular level.

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