Regulation of the Cytosolic Aspartate Aminotransferase Housekeeping Gene Promoter by Glucocorticoids, cAMP, and Insulin†

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ABSTRACT: The cytosolic aspartate aminotransferase (cAspAT) is a ubiquitous enzyme that displays liver-specific hormonal regulation. In the hepatoma cell line Fao, both the activity and the mRNA level of cAspAT are increased by glucocorticoids. This effect is potentiated by cAMP and inhibited by insulin. Using *in vivo* run-on experiments, we showed that these effectors act at the transcriptional level. A cAspAT gene fragment containing 2405 bp of the promoter was sequenced. Deletion fragments of this promoter were inserted upstream of the CAT gene, and the regulation of their activity was assayed following transfection in Fao cells. Stable transfection experiments established that the construct including the entire 2.405-kb fragment undergoes positive regulation by glucocorticoids and cAMP and negative regulation by insulin similar to the regulation of the endogenous gene. A physical separation of the positive and negative control elements is suggested by the fact that cAMP acted on the −682/−26-bp fragment (a 2-fold increase of the stimulation by dexamethasone), whereas the negative regulation by insulin (50% of the stimulation by dexamethasone) required the −1983/−1718-bp fragment. Both regions were required for maximal glucocorticoid activity (6–9-fold increase of CAT activity). We conclude that at least two regulatory regions, a proximal and a distal one, are required for full hormonal regulation of the cAspAT gene.

Aspartate aminotransferase (E.C. 2.6.1.1) is present in most animal cells as two isoenzymes, one cytosolic and the other mitochondrial (Christen et al., 1985). Among its various functions (Christen et al., 1985; Cooper et al., 1985), aspartate aminotransferase is part of the malate–aspartate "shuttle" present in all cells (Cooper et al., 1985). It also plays a tissue-specific role in gluconeogenesis in the liver, as the oxaloacetate that it produces can enter the gluconeogenic pathway (Horio et al., 1988). This pathway is under the control of several hormones. In a previous paper (Pavé-Preux et al., 1988), we demonstrated that hydrocortisone specifically stimulates the cytosolic isoenzyme in the rat liver and kidney but is ineffective in the heart and the brain. We have also shown that the activity and the mRNA level of the cytoplasmic AspAT (cAspAT)[1] in the Fao rat hepatoma cell line are increased by glucocorticoids; this effect is potentiated by cAMP and inhibited by insulin. However, cAMP and insulin alone have no effect on the basal activity or on the mRNA level of the enzyme (Barouki et al., 1989). The effects of these hormones are probably responsible for the stimulation of the enzyme activity in the liver after a protein-rich diet or during starvation (Horio et al., 1988; Katunuma et al., 1966). Recently, the cloning of the proximal 5′ end of the gene coding for the rat cAspAT allowed us to analyze its promoter. Although it has the classical characteristics expected for a housekeeping gene promoter (the absence of a TATA box, a high G + C content, the presence of Sp1 sites and multiple initiation sites), the cAspAT promoter also contains CCAAT boxes and glucocorticoid responsive elements (GREs) (Pavé-Preux et al., 1990), which is unusual for this type of promoter. Two putative GREs are found at positions −585/−571 and −462/−442, and a hemipalindrome can also be detected at position −382. These dual properties of the cAspAT promoter correlate well with the metabolic pathways in which the enzyme participates. Some pathways are constitutive with ubiquitous expression of the enzyme (Cooper et al., 1985), whereas other pathways are metabolically and hormonally regulated in a tissue-specific manner (Pavé-Preux et al., 1988).

In this study, we have investigated the hormonal control elements of the cAspAT gene promoter. Using *in vivo* run-on experiments, we have demonstrated that the regulation of the enzyme activity by glucocorticoids, insulin, and cAMP occurs mainly at the transcriptional level. Positive and negative hormonal regulation similar to that of the endogenous cAspAT gene was also demonstrated with chimeric plasmids constructed from the bacterial CAT gene under the control of various parts of the cAspAT promoter. Two different promoter regions were found to contain the hormonal regulatory sites: a proximal region mediates the action of cAMP, whereas a distal one mediates the insulin effect. Both regions are required for maximal glucocorticoid activity.

EXPERIMENTAL PROCEDURES

Cell Culture. The rat hepatoma clone Fao is derived from the H4IIEC3 line of the Reuber H35 hepatoma (Deschatrette & Weiss, 1974). Cells were maintained as described (Pavé-Preux et al., 1990) and were treated, as indicated, with the various hormones and drugs. For transcription experiments, 60×10^6 cells were treated for 2 h with the various effectors. The human hepatoma cell line HepG2 (Knowles et al., 1980)

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[1] Abbreviations: cAspAT, cytosolic aspartate aminotransferase; CAT, chloramphenicol acetyltransferase; bp, base pair; kb, kilobase; GRE, glucocorticoid responsive element; IRS, insulin responsive sequence; IRE, insulin responsive element; SRE, serum responsive element; TK, thymidine kinase; RSV, Rous sarcoma virus; β-gal, β-galactosidase; PEPCK, phosphoenolpyruvate carboxykinase; GADPH, glyceraldehyde-3-phosphate dehydrogenase; GH, growth hormone; PCR, polymerase chain reaction; SE, standard error; 8Br-cAMP, 8-bromoadenosine 3′,5′-cyclic monophosphate.

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was cultured under the same conditions as described in Pavé-Preux et al. (1990).

Run-On Experiments. Nuclei were prepared as described (Ausubel et al., 1987). Nascent RNA transcripts were labeled with [α-^{32}P]UTP, isolated, and quantitated according to the technique of Vaulont et al. (1984) with some modifications (Antras et al., 1991). The labeled RNA (6×10^6 cpm) was hybridized to Hybond nylon filters (Amersham) containing 10 μg of either the linearized plasmid 4B21 containing the cAspAT cDNA (Pavé-Preux et al., 1988), the linearized actin cDNA plasmid (Alonso et al., 1986), or the linearized vector alone according to Antras et al. (1991). The nylon filters were washed at 65 °C, for 1 h, once in 2× SSC and once in 1× SSC (0.15 M NaCl, 0.015 M sodium citrate) and were then treated for 30 min at 37 °C in 1× SSC containing 10 μg/mL of RNase A. The final wash was carried out in 1× SSC for 1 h at 37 °C. Autoradiography of the filters was then performed using Amersham Hyperfilm MP, and quantification of the signal was achieved by densitometric analysis.

Sequencing of the 5′ Region of the Gene. A 5.8-kb *Eco*RI fragment of the gene was subcloned into pGEM4Z (Promega). A 2405-bp fragment of promoter was sequenced according to Sanger et al. (1977), using the DNA polymerase Sequenase (U.S. Biochemical Corp.). Oligonucleotides, used as sequencing primers, were synthesized on an Applied Biosystems synthesizer.

Plasmids. The various plasmids were constructed by inserting the desired fragment of the cAspAT gene promoter into the polylinker of pMW-1, a pUC18 derivative carrying the CAT reporter gene, as reported previously (Pavé-Preux et al., 1990). The p–2405/–26CAT and the p–682/–26CAT constructs were obtained by subcloning the *Kpn*I/*Xho*I and the *Pst*I/*Xho*I fragments, respectively, in front of the CAT gene. Other deletion plasmids were obtained by subcloning *Kpn*I/*Pst*I-cut PCR products into the corresponding sites of the p–682/–26CAT construct. For the PCR experiments, the matrix was the p–2405/–26CAT construct. In the amplification reaction, the downstream oligonucleotide was complementary to the cAspAT promoter sequence (–493/–513) and was therefore downstream of the *Pst*I site. The upstream oligonucleotide was complementary to the 9 or 13 base pairs of the 5′ end of the desired deletion flanked by a *Kpn*I site and 8 nucleotides in the 5′ direction.

The mutation of the 10-bp fragment identical to the IRS of the PEPCK gene was created by a double PCR experiment. Four oligonucleotides were designed to introduce the mutation: oligo1, 5′-AGTTCCTTGGTGGGGGTTGAGAAGAC-3′; oligo2, 5′-GTCTTCTCAACCCCCACCAAGGAACT-3′; oligo3, 5′-AAGTTGGGTACCAAAGGGTTTATTTGG-3′, complementary to sequence –2629/–2603; and oligo4, 5-CCTGCGCACGTTCCTGCGTTA-3′, complementary to sequence –493/–513. Briefly, PCR products were obtained from amplifications between oligonucleotides 1 and 3 on the one hand and oligonucleotides 2 and 4 on the other hand, using a p–2913/–26CAT construct as a matrix. After one phenol and two chloroform extractions, the PCR products were precipitated with 1 μL of 1 mg/mL dextran, 2 M ammonium acetate, and 0.6 vol of 2-propanol. After being washed in 70% ethanol, each DNA sample was resuspended in 20 μL of water. Five microliters from each PCR product were mixed, and a new amplification was performed using oligonucleotides 3 and 4. The new PCR product was treated as described above and digested with the restriction enzymes *Kpn*I and *Pst*I. The digested product was subcloned into the p–682/–26CAT construct digested with the same enzymes. The mutated constructs were sequenced according to Sanger et al. (1977) as described above. The pTK plasmid (Edlund et al., 1985), the pRSV-β-gal plasmid (Edlund et al., 1985), and the pSV2neo plasmid (Southern & Berg, 1982) were described elsewhere.

Transfection Experiments. One day prior to the transfection, Fao cells (1.5×10^6 cells/10-cm dish) or HepG2 cells (10^6 cells/10-cm dish) were seeded into the usual culture medium containing fetal calf serum (Barouki et al., 1989). Ten milliliters of fresh medium with serum was added to the cells 2–3 h before the transfection. The expression plasmid (10 μg) and either the reference plasmid pRSV-β-Gal (10 μg), for transient transfections, or the pSV2neo plasmid (2 μg), for permanent transfections, were introduced into the cells by the calcium phosphate coprecipitation technique followed by a glycerol shock (Wigler et al., 1979). For transient transfections, following the glycerol shock, 10 mL of fresh medium containing serum (and containing, when appropriate, the hormones) was added to the cells. The cells were then cultured for either 18 or 40 h. For permanent transfections, 10 mL of fresh medium with serum was added after the glycerol shock. Two days later, the cells were split 1:5 and allowed to grow for 24 h prior to the addition of the neomycin analog G418 (GIBCO; 250–500 μg/mL, depending on the batch). The medium was changed every three days. Two to three weeks later, the surviving cells were harvested and pooled for CAT assay. When needed, the cells were treated for 24–40 h with the relevant hormones, as described in the legends to the figures and in the tables.

Assay of CAT Activity. Cell extracts were prepared, and CAT assays were performed, following the technique of Gorman et al. (Gorman et al., 1982). The β-galactosidase activity (An et al., 1982) and the proteins (Bradford, 1976) were assayed as described. The β-galactosidase activity was used to correct for variations in transfection efficiency in HepG2 cells. The ratio of CAT activity to β-galactosidase activity was calculated and expressed as the percent of the ratio obtained in cells transfected by the CAT gene driven by the thymidine kinase promoter (arbitrary units).

RESULTS

Nuclear Transcription Experiments. To determine whether the changes in cAspAT mRNA mediated by glucocorticoids, insulin, and cAMP were the result of changes in the cAspAT gene template activity, gene transcription rates were assayed in isolated nuclei using the nuclear run-on transcription assay. Nuclei were isolated 2 h after exposure of the Fao cells either to no hormone or to 100 nM dexamethasone alone or in combination with 100 nM insulin or 0.5 mM 8Br-cAMP. Radiolabeled mRNAs were hybridized either to the cAspAT probe or to other control probes. The results of a typical experiment are shown in Figure 1. Data from three different experiments have been quantified by densitometric scanning. The mean and SE of these experiments, expressed in arbitrary units, were 4 ± 2, 29 ± 10, 67 ± 8, and 18 ± 6, respectively, for cells not treated or for cells treated with dexamethasone, with dexamethasone plus cAMP, or with dexamethasone plus insulin. The transcription rate of the cAspAT gene is very low in control cells. Treatment with dexamethasone increased approximately 7-fold the rate of transcription; the combination of this glucococorticoid with insulin led to approximately a 50% decrease of the rate of transcription found with dexamethasone alone, whereas the combination of dexamethasone with 8Br-cAMP provoked a 2-fold increase in this rate. These data correlate well with the data previously obtained on the cAspAT mRNA level in Fao cells (Barouki et al., 1989).

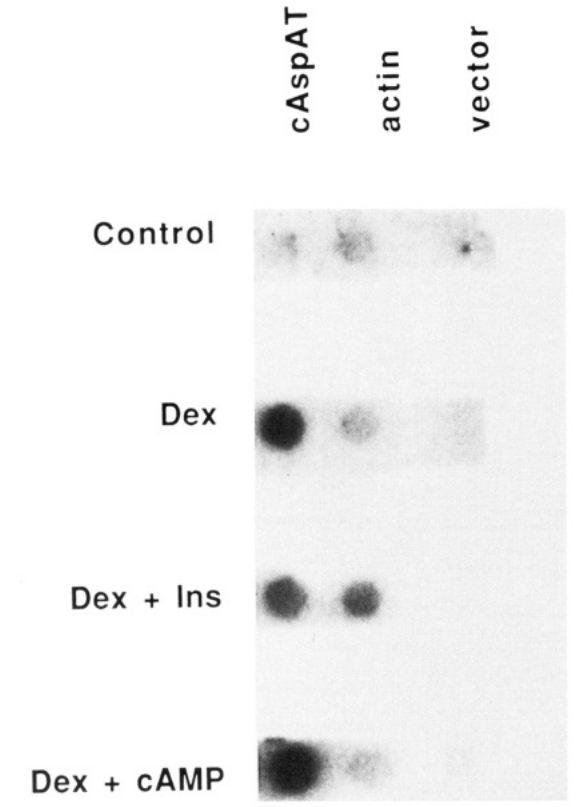


FIGURE 1: Nuclear run-on transcription assay. Nuclear run-ons were performed to compare the cAspAT gene transcription in control Fao cells (control) or cells treated with 100 nM dexamethasone alone (Dex) or in combination with 100 nM insulin (Dex + Ins) or 0.5 mM 8Br-cAMP (Dex + cAMP). The plasmids pSP65 (negative control, vector), pGEM3Z-actin (positive control, actin), and pGEM3cAspAT (cAspAT) were spotted on each membrane. The membranes were then hybridized with labeled RNA from the Fao cells treated as indicated. Quantification of the cAspAT signal was achieved by densitometric analysis.

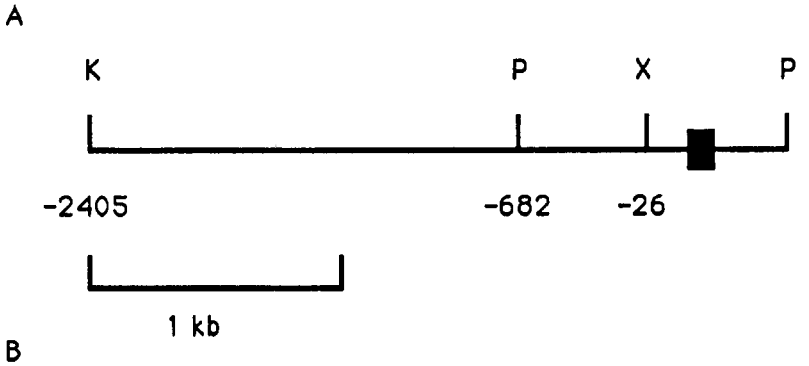
Sequence Analysis of the cAspAT Gene. The isolation of the 5′ region of the gene and the sequence of a 1.1-kb fragment have been previously reported (Pavé-Preux et al., 1990). We have now completed the sequence of the promoter up to −2405 bp (+1 being the A of the first ATG used for translation) (Figure 2). Since glucocorticoids, cAMP, and insulin regulate the enzyme activity and the mRNA level (Barouki et al., 1989), we looked for DNA motifs known to mediate their action. Several sequences showing a close homology to GRE (Beato, 1989) or to GRE hemipalindromes have been reported in the proximal 682 bp of the promoter (Pavé-Preux et al., 1990). Additional sequences were found in the distal part of the promoter. The sequence and the position of all the putative GREs are summarized in Table I. We also searched for the consensus sequence TGACGTCA, the cAMP responsive element (CRE) (Roesler et al., 1988); we were able to locate a 7/8 base match at position −1737/−1731 and a 6/8 base match at position −539/−531. However, both matches included either a gap or an insertion. cAMP may also regulate gene activity through the AP-2 binding site (Roesler et al., 1988). Six sequences with 7 of 8 bases matched for an AP-2 binding site were found within the cAspAT gene (see Table I and Discussion). Recently, several sequences have been reported which can mediate the effects of insulin at the gene level (O'Brien et al., 1990; Nasrin et al., 1990; Keller et al., 1990; Stumpo et al., 1988; Philippe, 1991; Prager et al., 1990; Stanley, 1992). However, no obvious homology was found by computer analysis between the cAspAT promoter and the various sequences reported. One exception was the sequence which mediates insulin action for the phosphoenolpyruvate carboxykinase (PEPCK) gene. At position −1374/−1365 and in the reverse orientation, we identified a sequence identical to the 10-bp sequence described for the PEPCK gene (O'Brien et al., 1991; Table I).

Analysis of the Promoter Region. To identify DNA sequences involved in the expression and in the regulation of the rat cAspAT gene, we prepared deletion fragments of the 5′ upstream region of the promoter, which were fused to the CAT reporter gene. The structures of the various constructs are sketched in Figure 3. Since numerous start sites are found for this gene (Pavé-Preux et al., 1990), we chose the A of the first ATG used for translation as +1. The plasmids were transiently expressed in the hepatoma cell line HepG2. Although not responsive to hormones, this cell line was chosen to test the basal promoter activity of the various constructs because the efficiency of the transfection allows the use of the reference plasmid RSV-β-gal. All the constructs tested displayed similar basal promoter activity (Figure 3). Therefore, the region from −2405 to −682 is apparently not required for the basal promoter activity.

We then tried to determine the responsiveness of the chimeric plasmids to cAMP, insulin, or glucocorticoids. Transient transfections into the hormone-sensitive Fao cell line of plasmids p−2405/−26CAT and p−682/−26CAT were performed. The cells were subsequently treated with either no hormone, 100 nM dexamethasone, 100 nM dexamethasone plus 0.5 mM 8Br-cAMP, or 100 nM dexamethasone plus 10 nM insulin. The results of transient transfections were poorly reproducible. Therefore, we decided to study stable transfectants. Stably transfected Fao cells were obtained by transfecting cells from one or two plates with both a plasmid carrying the gene for resistance to neomycin and a plasmid carrying the CAT gene under the control of a cAspAT promoter fragment. Cells were then selected for their resistance to G-418, as described in Experimental Procedures. Approximately 20–60 colonies were obtained from one original plate. Thus, the transfection efficiency could be estimated to be one to three clones per 10^5 cells. We pooled the surviving cells from one transfection to minimize the variation due to random integration in the genome (Abraham, 1985; Kato et al., 1986; Stief et al., 1989). Indeed, experiments elsewhere (Forest et al., 1990) and in our laboratory have shown that 15–20% of the clones resistant to G-418 express CAT activity. Among these, approximately two-thirds of the clones that we obtained showed regulation by hormones.

cAMP Regulation of the cAspAT Gene Promoter. We have shown that cAMP potentiates the induction of cAspAT gene transcription by glucocorticoids (Figure 1). Fao cells, permanently transfected with either the p−2405/−26CAT or the p−682/−26CAT construct, were treated for 18 h with dexamethasone in the presence or in the absence of 8Br-cAMP. The results of two typical experiments with the chimeric plasmids are shown in Figure 4. For both constructs, 0.5 mM 8Br-cAMP increased about 2–4-fold, on the average, the stimulation of transcription due to dexamethasone. Therefore, cAMP appears to act on the −682/−26-bp fragment.

Insulin Regulation of the cAspAT Gene Promoter. We first carried out experiments on the two constructs p−2405/−26CAT and p−682/−26CAT. For each construct, at least three independent transfections with at least three different plasmid preparations were performed. Cells transfected with the shortest deletion construct (p−682/−26CAT) responded weakly or not at all to 10 nM insulin (Table II). In contrast, Fao cells stably transfected with the longest plasmid responded



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-2405 GGTACCACCC ACAATGGGCG AGCCACTCCA CCCTCCCATG ATCACTAATG AATATAATGT CCTACAGGTG GATCTTATGG
      TGGCATTCTC TCAACTGAGA TCCTTCCTCT CATTTGACTC CAGCTTGTGT CAAGTTGACA TAAAACTACT ATCCAGCAAC
-2245 CCATCACACA GGTTTGACTG TGGAGGTTTT TAGGCTGACA GCAGTCAATA TGTATTTATT TGGAGGCTGG GGCGATGACT
      CGTCCGGTAA AAGTGCTAAG TGCTTGCTGT ACAAACCTCA GGATCTGAGC TTGGAACCCC AGCACACATG TGGAGGCCGA
-2085 GAGAGAGAGA GAGAGAGAAA GTGTGCTTGT ATCTTCCGTG CTGGAGAGCA GAGACCCGCA AGTCTCTGAA GCCCACTGAC
      CTGAGAGTCT AGTAGAACCT GAGGTCCAGG TTCAGTGAGA GACCCTGTCT CAAATAATAA GGTGGAAAGC AACTGAGGAA
-1925 GTCACCTGAT GTTCACCTTT GGCCCCTACT CCAGATGCAG ACGTGTGCAA ATCCGGCCCA CCCCCCCAAT ATGTGCTGCT
      GTGTACCTAA CTGAACGGAG CACATGCGCA CACACAGAGA AATATTTATT TGTTCTTAGC CAAACCGACA GTGTTTAGGT
-1765 AACCCAGGGT TGCTTTTCTC CCTGGGAATG ACGCAGAGAG TGACCATGAG CATTGTTGCC TAACGCTGTG AAGACCACAC
      TTCTAAATTA AATTCTAGGA AGCCTAGGGT GCAGAGTTGG CCCTCGGGCT GTGAGTTTTG GACGCACTGA AAGCGCTCGA
-1605 CCCTGGTTGT TGGACCTTCA CAATTAGCTT GTGACTGTTA GGGGAAAACA TTCATGAAAC GCGATGGGAG GTTTCCTTAA
      AAAGACAAGG GTGATGCTTT TGAAAAGAAA AAGACAGAAA ACCTTAGCAA GCACTGCCCA AAGTTTGATG GCTTATGTTT
-1445 ACCCTCTGAA TGGGAAACTC ATGTGAAGAT TTATCATGTC ACCCAAAGGA AGCCTCGATG CTGTCTTCTC ACAAAACACC
      AAGGAACTGG CTGCTTCTAG CATTGGTTGA ACATCCAGTG TAAGAAACGG AGGAAGGTTT CCCAGATCCC AGGCTATCTA
-1285 TGAGGGGGTG TGGAGCCTGT GTCATAACAA AGCTAGTATT TAGAGAGCAC TTACTGTGTG CGACTAAACA CTTTATGTGT
      CTTAACGCAC CTAATCCCCA CAACAACCCG GAGAAAACAT TATTATCTCC ATCTCACAGA GCAGGAACAG GCCTGCCCCA
-1125 AGTAAGTCAG TGGCAGCCAG CATCTATACC TAACTTGCCT GGCTCCAGAG TCAGGCTCCT ACTCATCAGG ATACTTTCTA
      AAATTGTAAC GTCGGCAACA AAACGCCCCA ACACACAGAG AGACGTTTAA CCAGGCGGAG TATTAAGAGA AGGTTTTATG
-965  GACGGCTGAC AGATGTGAAT CTTCAAGGTC AACAGGTGGA GAAAGGATCT GGACAGAAAT AGAATCTCAG GGGAAGAAGC
      AGCCTGACCT GACAAAAGGC AGCAGCAGTT TGGGGATCTG CCAATCGATG ACAGCTTGCG TGGGAGGAAC ACTGCACAGG
-805  GAGGCCTCAC TAGATCTTTG AAGGTCTTTT AGGGCTAAGG CTTCCTCCGC CCCGGTTACC TACCGGGGTG GGGGCGGGGA
      TGGTAAAATT CTCAAACTAG CCTGTGCTCA TCCCTTTACC TTCTGCAGGG TCTCTCCTGC CAGCGATCAA GGTAGACAAC
-645  ACCCCCAGGT TTCTTTCTGC GGAAGCACTG AGTGGAGATG GAGAAGGTCA CCCGCGATCG GGTACAGCGT GTCCAGGCGG
      ACCACTAGAG TCCAGTGTCG GGGACTTGGC TGTCAGCCCG GCCTGGCTAA CGCAGGAACG TGCGCAGGAA CGGAGCCTGG
-485  CGGGGACAGC CACCTGCTCA GCTGGTACAG AAAGACCTGT TCTCTACTAT TCCCGAGGGC GGGCCACCGC CCCAGGTCTC
      CAGGACCCAG AAGGTCAGGA GGCTGTCCTC TAGGGACTCC CGCCACCCGC ATCATCCTCA TCACCCTAAG GAATAAAAGC
-325  CTCAGCGATT GGAACACGCT ATGCCAATCA TCCTAGTCTT TGCCCACGCC TCCTCCGGTG TCCACTGCAT TGGTTGCATC
      ATACAAGCCT CCGATAAGAT CCTCCTCTTG AATTGGCTAA TAGAGCCTTG TCCCGCCCAA CCCTTCTTTG TCGGAAGGTT
-165  GTGATTGGTT AAGCGCGTTG CCAAGCTCCG GGCGCGGCTC GGTCATTGGA AGCCGCGGGC TGCCCCGCCC GCCGGTGAAG
      GTGAGAGTCT CCTCCAGTCA CAGGTCCCCG CACCGCACGG AGCAGCTCCC AGCTCGTGCT CGAGGTCCCC TCAGATTCCA
                +1
-5    TCGCGATG
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FIGURE 2: 5′ region of the cAspAT gene. (A) Restriction map of the *Kpn*I–*Pst*I fragment. The first exon is boxed (P, P*st*I; K, K*pn*I; X, X*ho*I). (B) Sequence of the proximal 2405 bp of the promoter. The GRE-like sequences are underlined. The IRS sequence is underlined by an arrow. +1 corresponds to the A of the first ATG used for translation.

to insulin (Table II). They displayed a concentration-dependent inhibition by insulin of the CAT expression stimulated by insulin of the CAT expression stimulated by glucocorticoids (Figure 5). The IC_{50} was about 20 pM, and the maximal inhibition of 70% was obtained with 10–50 nM insulin. In these cells, in accordance with the data for the endogenous enzyme activity, insulin alone had no significant effect on the basal level of the promoter activity. To ensure that insulin was acting through its specific receptor, the effect of insulin growth factor I (IGF-I) on the glucocorticoid stimulation of the CAT activity was tested. Indeed, insulin is known to bind to the IGF-I receptor, and IGF-I binds to

Table I: Comparison of the cAspAT Sequence with Consensus Sequences

element	consensus	cAspAT sequence	homology	location
GRE	GGTACAnnnTGTTCT	GGTACAnnnnnnnnTGTTCT	12/12	–462/–442
		GGTACAnnnTGTCCA	10/12	–585/–571
		GTCACCnnnTGTTCA	8/12	–1925/–1910
CRE	TGACGTCA	TGGCTGTCA	6/8	–539/–531
		TGACG-CA	7/8	–1737/–1731
AP-2	CCCCAGGC	CCCCAGGT	7/8	–643/–636
		CCCCAGGT	7/8	–416/–409
		CCC-AGGC	7/8	–1298/–1292
AP-2 (inverse)	GCCTGGGG	GCCTGGCG	7/8	–491/–484
		GCCTAGGG	7/8	–1664/–1658
		GC-TGGGG	7/8	–2180/–2174
IRS PEPCK	TGGTGTTTTG	TGGTGTTTTG	10/10	–1374/–1365 inv
		TGATGCTTTTG	8/10	–1514/–1504
		TG-AGTTTTG	8/10	–1634/–1626
IRE GADPH	AACTTTCCCGCCTCTAGCCGAAAG	not found[a]		
IRE amylase	TTGCGTGAGAGTTTCTAAAA	not found		
IRE glucagon	CACGCCTGACTGA	not found		
IRE GH	ATGGCCTGCGC	not found		
IRE prolactin	TCTTAATGACGGAAATAGATG	not found		
SRE	GATGTCCATATTAGGA	not found		

[a] Not found means that no homology better than 75% was found.

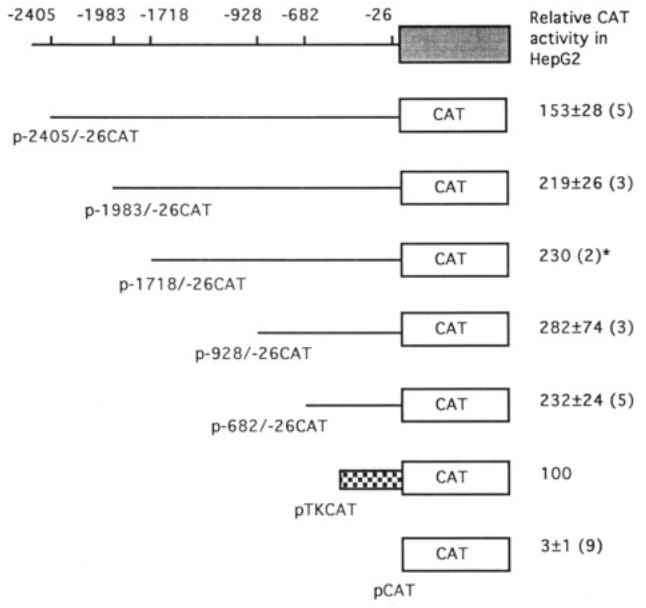

FIGURE 3: Promoter deletions. CAT activities of chimeric plasmids, containing deletions of the cAspAT promoter, were measured in the HepG2 cell line. The size of the fragment tested is indicated in the name of the plasmid. The relative CAT activities (mean ± SE with the number of separate experiments indicated in parentheses) are normalized to that of the pTKCAT plasmid, in which the transcription of the CAT gene is under the control of the TK promoter (100%). The value of 230 for the p–1718/–26CAT (*) is the mean of two values, 175 and 284. The pCAT plasmid corresponds to the vector plasmid without any insert. The checkered box corresponds to the TK promoter; the gray region corresponds to the first exon of the cAspAT gene.

the insulin receptor, although with different affinities (Ullrich et al., 1986). Insulin or IGF-I, alone, had no effect on the basal activity at 1 or 10 nM. As expected, insulin inhibited the glucocorticoid stimulation of the promoter activity (69 and 78% inhibition at 1 and 10 nM, respectively), whereas IGF-I had no effect at 1 nM (122% of the dexamethasone-stimulated activity) and provoked only a 34% inhibition at 10 nM (Figure 6).

Since the –2405/–682-bp fragment is necessary for insulin inhibition, and because a 10/10 base pair homology exists between the cAspAT gene (at position –1374/–1365) and the IRS (insulin responsive sequence) from the PEPCK gene, which was found to mediate the insulin action on the heterologous TK promoter (O'Brien et al., 1991), we decided to mutate this element in the cAspAT gene. We chose to use the mutation described to abolish the insulin inhibitory action on the TK promoter (O'Brien et al., 1991). While generating this mutant (Mut 10) by the PCR technique, we also obtained an additional mutant with three altered bases (Mut 11). The nucleotide sequences of the wild-type promoter and of the mutants are shown in Table III. Both mutants were stably transfected into the Fao cell line. Their ability to mediate insulin action was found to be similar. As shown in Table II, dexamethasone elicited the same induction (6–9-fold on average) for both the mutant and the wild-type p–2405/–26CAT constructs. Insulin, at 10 nM, inhibited this stimulation with the same potency in either the wild-type plasmid (41% inhibition on average) or the mutants (51% inhibition on average). Thus, although the IRS sequences of the cAspAT and PEPCK genes are identical, and in spite of similar hormonal regulation, the IRS sequence is not critical for the insulin action on the cAspAT gene.

Several other deletion plasmids in the region –2405/–682 bp (Figure 3) further defined the site of action of insulin on the cAspAT gene. Dexamethasone stimulates the CAT activity in all the constructs (Table II); however, the induction was lower for the three shortest plasmids (3–5-fold on average) than for the two longest ones (6–9-fold on average). A weak inhibition by insulin was also obtained with the three shortest deletions (5% inhibition on average), in contrast to the 30–51% inhibition found with the longest ones. These results indicate that the –1983/–1718-bp fragment is required for insulin action, and they confirm that the IRS-like element located at position –1374/–1365 is not in the fragment of the cAspAT promoter required for the insulin effect.

DISCUSSION

The first conclusion of this study is that the regulation of cAspAT by glucocorticoids, cAMP, and insulin occurs at the transcriptional level. This was clearly established using run-on experiments in Fao cells. Furthermore, in transfection experiments of promoter fragments coupled to the CAT gene, it was clear that a 2405-bp fragment contained elements

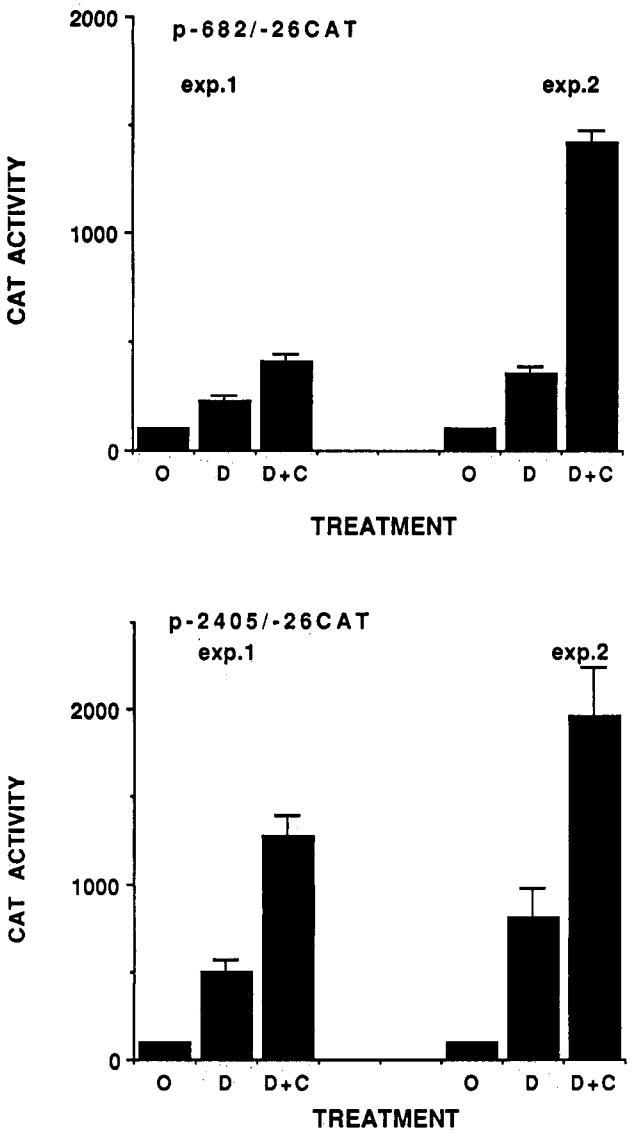

FIGURE 4: Effect of cAMP on cAspAT promoter fragments. CAT assays were performed on Fao cells permanently transfected with two different preparations of either the p–682/–26CAT or the p–2405/–26CAT plasmid. In these experiments cells were treated for 18 h with no drug (O), 100 nM dexamethasone (D), or 100 nM dexamethasone and 0.5 mM 8Br-cAMP (D+C). The CAT activity, expressed as the percent of the activity of control cells, is the mean ± SE of two or three determinations.

permitting regulation by the three effectors. There was a good correlation between the magnitudes of the hormonal responses in both assays, suggesting that the 2405-bp fragment included all the elements necessary for the hormonal effects. This fragment was thus the starting material to further map the various response elements.

We initially attempted to use transient transfection of Fao cells with deleted promoter fragments. The results of these experiments were poorly reproducible, particularly for mapping the insulin responsive fragment. Only stable transfections led to reproducible effects. The difficulties encountered with transient transfections are due, in part, to the poor transfectability of the Fao cells, an observation shared by different laboratories (Forest et al., 1990; Ott et al., 1984; Thayer et al., 1990). Alternatively, the chromatin structure could be critical for the hormonal regulation of the gene. It is possible that a functional structure is achieved only when the transfected DNA is integrated into the cellular genomic DNA, as postulated by O'Brien and co-workers (O'Brien et al., 1991).

Table II: Effect of Glucocorticoids and Insulin on cAspAT Promoter Fragments Stably Transfected in Fao Cells[a]

plasmid	*n*	*x*-fold stimulation by Dex	*x*-fold stimulation by Dex + insulin
p–2405/–26CAT	2	4.3	2.1 (–51%)
	10	10.9	6.3 (–42%)
	1	2.9	1.9 (–34%)
	1	9.7	7.6 (–21%)
	1	4.8	3.5 (–26%)
	5	3.9	2.2 (–44%)
	2	10.0	3.7 (–53%)
	1	5.7	2.1 (–53%)
p–2405/–26MUT11CAT	7	12.1	5.6 (–54%)
p–2405/–26MUT10CAT	5	5.6	3.6 (–35%)
	1	6.6	2.4 (–63%)
	1	9.3	4.7 (–50%)
p–1983/–26CAT	6	4.2	2.5 (–41%)
	8	11.4	8.2 (–28%)
	1	5.3	4.5 (–15%)
	1	7.7	4.6 (–40%)
	1	6.6	5.0 (–24%)
p–1718/–26CAT	5	4.1	4.0 (–3%)
	9	4.6	4.0 (–13%)
	1	2.7	2.9 (+8%)
	1	5.6	5.8 (+5%)
	1	4.4	4.5 (+4%)
p–928/–26CAT	5	3.8	3.7 (–3%)
	6	4.2	5.2 (+23%)
	1	5.6	5.1 (–8%)
	1	8.0	5.5 (–31%)
p–682/–26CAT	2	2.3	1.7 (–26%)
	4	2.1	2.5 (+17%)
	1	4.0	3.8 (–4%)

[a] Each line represents a separate transfection experiment; *n* represents the number of separate determinations. In these experiments, cells either were not treated or were treated for 24 h with 100 nM dexamethasone alone or in combination with 10 nM insulin. The number in parentheses indicates the % variation caused by insulin (either inhibition (–) or stimulation (+)) of the response of the cells to dexamethasone.

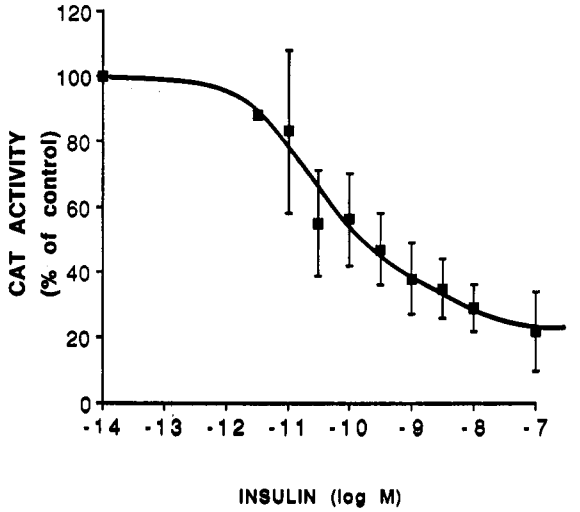

FIGURE 5: Effect of insulin on the cAspAT promoter activity. Fao cells permanently transfected with the p–2405/–26CAT plasmid were treated for 24 h with 100 nM dexamethasone and increasing concentrations of insulin. The CAT activity in the presence of dexamethasone alone represents 100% activity. Since insulin alone was ineffective, 0% represents the CAT activity in the cells not treated. Maximal inhibition (70%) was obtained in the presence of 10 nM insulin. Each value represents the mean ± SE of five separate experiments except for 30 pM (four experiments) and 100 nM (2 experiments).

Although we initially studied individual clones that were stably transfected, we later shifted to the study of pools of clones resulting from a transfection experiment. Using the latter approach, our goal was to minimize the variation due to random

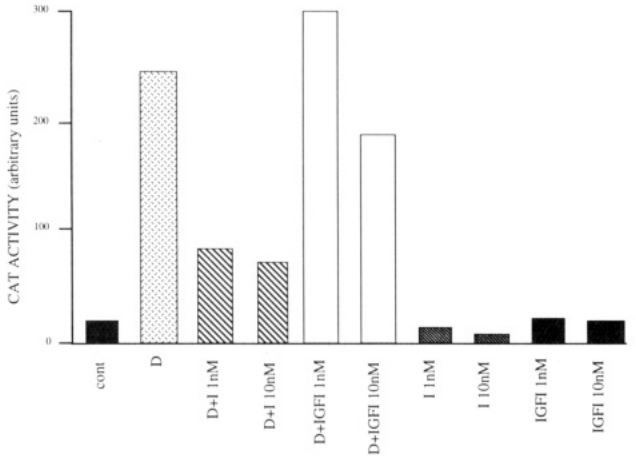

FIGURE 6: Comparison of the effects of insulin and IGF-I on cAspAT promoter activity. Fao cells permanently transfected with the p–2405/–26CAT plasmid were either not treated (cont) or treated with various effectors: insulin (I), 1 or 10 nM; IGF-I (IGFI), 1 or 10 nM; dexamethasone, 100 nM alone (D) or in the presence of 1 nM insulin (D+I 1 nM), 10 nM insulin (D+I 10 nM), 1 nM IGF-I (D+IGFI 1 nM), or 10 nM IGF-I (D+IGFI 10 nM). The CAT activity, expressed in arbitrary units, represents the mean of two separate experiments. The ranges were respectively 5, 149, 10, 40, 182, 179, 12, 8, 7, and 6 for the various treatments above.

Table III: Sequences of the Mutated IRS Elements[a]

IRS element	sequence
wild type	5′-AGCCAGTTCCTTGGTGTTTTGTGAGAAGAC-3′
mutant 10	5′-AGCCAGTTCCTTGGTGGGGGGTTGAGAAGAC-3′
mutant 11	5′-AGCTAGTTCCTTGGTGGGGGTTAAGACGAC-3′

[a] Two mutants of the cAspAT gene promoter sequence between positions –1357 and –1380 were generated. The sequences shown correspond to the antisense strand; thus, the 5′ and the 3′ in the table correspond to positions –1354 and –1383 of the promoter, respectively. The mutated bases are underlined.

integration in the genome. Each assay represents the average of tens of individual clones. Obviously, this approach using permanently transfected cells is much more time consuming than the one using transient transfections, but it may represent the only approach, other than the use of transgenic mice, when integration into genomic DNA is required for gene regulation to occur.

Insulin inhibits dexamethasone-stimulated transcription of the cAspAT gene. The maximal inhibition observed for the p–2405/–26CAT construct was 53%. This agrees with the data obtained previously for the endogenous gene in Fao cells. Indeed, insulin decreased the cAspAT mRNA signal by 50% in the presence of dexamethasone, and the maximal effect on the activity was 70–85% (Barouki et al., 1989). Complete inhibition was never observed. In the present study, we ascertained that insulin was acting through its own receptor by comparing the potencies of insulin and IGF-I.

During the last few years, numerous sequences have been proposed as mediating the effect of insulin on gene transcription. They have been identified in the genes encoding PEPCK, glyceraldehyde-3-phosphate dehydrogenase, amylase, c-*fos*, growth hormone, glucagon and prolactin (Table I; O'Brien et al., 1990; Nasrin et al., 1990; Keller et al., 1990; Stumpo et al., 1988; Philippe, 1991; Prager et al., 1990; Stanley, 1992). In the cAspAT gene, we identified a sequence which is identical to the sequence reported for the PEPCK gene. We mutated this sequence following the base modification described by O'Brien et al. (O'Brien et al., 1991), which was shown to destroy the action of insulin on the heterologous TK promoter. Mutation of the IRS element in the cAspAT gene promoter did not abolish the inhibitory action of the hormone. Therefore, although this 10-bp sequence is found with various degrees of homology in many genes regulated by insulin (O'Brien & Granner, 1991), it does not alone mediate the action of insulin on the cAspAT gene promoter. Additional deletion experiments revealed that the inhibition by insulin is lost when the fragment –1983/–1718 bp is removed. This fragment does not contain any sequence homologous to the IRS.

The analysis of the data indicates that the induction of the CAT gene by glucocorticoids varies with the length of the construct. Indeed, dexamethasone is less potent in stimulating transcription of the CAT gene when the fragment –1983/–1718 bp is removed (Table II). This could signify that, although palindromic GRE-like elements have been identified in the proximal promoter, between positions –585 and –382 (Pavé-Preux et al., 1990), additional elements, in the distal –1983/–1718-bp fragment, are required for full induction by glucocorticoids. In particular, one element at position –1925/–1910 (GTCACCtgaTGTTCA) displays a strong homology to the consensus GRE. Thus, the distal fragment at position –1983/–1718 is necessary for maximal glucocorticoid regulation of the cAspAT promoter and for the inhibitory action of insulin. Insulin, apparently, has no effect on the proximal glucocorticoid responsive elements. Our observations account for the physiologal effects of insulin on cAspAT. First, this hormone inhibits glucocorticoid induction of enzyme levels but has no effect on the basal enzyme level. Second, the inhibition is only partial and does not prevent completely the glucocorticoid effect. Our current working hypothesis is that the distal region of the promoter includes an insulin responsive element which counteracts the distal glucocorticoid stimulatory effect.

One consequence of the presence of the two regulatory regions in the cAspAT promoter is the separation of the positive cAMP effect from the negative insulin effect. We have shown here that cAMP acts on a proximal –682/–26-bp fragment of the promoter. Indeed, cAMP, in combination with glucocorticoids, produced a 3-fold increase of the signal compared to that obtained with dexamethasone alone. This is very similar to the 2–3-fold increase found for the endogenous cAspAT gene (Barouki et al., 1989). In contrast, cAMP alone was unable to stimulate the cAspAT promoter activity. Recently, Rangarajan and co-workers demonstrated that protein kinase A could modulate the glucocorticoid receptor function in the absence of the cAMP response element binding protein in F9 embryonal carcinoma cells (Rangarajan et al., 1992). It was suggested that protein kinase A could enhance the binding of the glucocorticoid receptor to its target via a phosphorylation/dephosphorylation mechanism. It is possible that cAMP promotes an increased binding of the glucocorticoid receptor to the cAspAT promoter via a similar mechanism. In contrast, insulin required a distal –1983/–1718-bp fragment. Both fragments were necessary for the maximal glucocorticoid effect. There are other examples of functional distal GREs. For example, two GREs have been identified at about –2.5 kb in the tyrosine aminotransferase gene (Jantzen et al., 1987). Furthermore, two widely separated GREs can interact. In the case of the rat tryptophan oxygenase gene, a proximal GRE at –440 bp is responsible for a 2-fold increase by glucocorticoids, whereas the distal one at –1200 bp leads to a 3.5-fold induction (Danesch et al., 1987).

Direct interaction between elements responsive to different hormones or transcription factors have been reported. In the case of the PEPCK gene promoter, glucocorticoids act through a glucocorticoid responsive unit (GRU) comprising two glucocorticoid receptor binding sites and two accessory factor binding sites (Forest et al., 1990; Imai et al., 1990). In this system, insulin acts on a fragment located in the region which binds the accessory factor 2 (O'Brien, 1990). Similarly, a consensus AP-1 site is present within the response element to vitamin D and retinoic acid in the human osteocalcin gene (Schüle et al., 1990a). The ovalbumin gene is regulated by estrogen via a complex with c-*fos*/c-*jun* (Gaub et al., 1990). An interaction between the glucocorticoid receptor and the c-*jun* protooncogene has been described as being responsible for the inhibitory effect of glucocorticoids on the transcription of the collagenase gene (Jonat et al., 1990; Yang-Yen et al., 1990; Schüle et al., 1990b). Also, interactions between the glucocorticoid receptor, c-*jun*, and c-*fos* on a composite GRE lead either to positive or to negative regulation of the mouse proliferin gene (Diamond et al., 1990). Work is now in progress to define more precisely the regions in the cAspAT promoter involved in the insulin and the distal glucocorticoid responses.

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REFERENCES

Abraham, I. (1985) in *DNA-mediated gene transfer* (Gottesman, M. M., Ed.) pp 181–210, John Wiley & Sons, New York.

Alonso, S., Minty, A., Bourlet, Y., & Buckingham, M. (1986) *J. Mol. Evol. 23*, 11–22.

An, G., Hidaka, K., & Siminovitch, L. (1982) *Mol. Cell. Biol. 2*, 1628–1632.

Antras, J., Lasnier, F., & Pairault, J. (1991) *J. Biol. Chem. 266*, 1157–1161.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Smith, J. A., Seidman, J. G., & Struhl, K. (1987) in *Current Protocols in Molecular Biology*, John Wiley & Sons, New York.

Barouki, R., Pavé-Preux, M., Bousquet-Lemercier, B., Pol, S., Bouguet, J., & Hanoune, J. (1989) *Eur. J. Biochem. 186*, 79–85.

Beato, M. (1989) *Cell 56*, 335–344.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Christen, P., Graf-Hausner, U., Bossa, F., & Doonan, S. (1985) in *Transaminases* (Christen, P., & Metzler, D. E., Eds.) pp 173–185, John Wiley & Sons, New York.

Cooper, A. J. L., & Meister, A. (1985) in *Transaminases* (Christen, P., & Metzler, D. E., Eds.) pp 534–563, John Wiley & Sons, New York.

Danesch, U., Gloss, B., Schmid, W., Schüle, R., & Renkawitz, R. (1987) *EMBO J. 6*, 625–630.

Deschatrette, J., & Weiss, M. C. (1974) *Biochimie 56*, 1603–1611.

Diamond, M. I., Miner, J. N., Yoshinaga, S. K., & Yamamoto, K. R. (1990) *Science 249*, 1266–1272.

Edlund, T., Walker, M. D., Barr, P. J., & Rutter, W. J. (1985) *Science 230*, 912–916.

Forest, C. D., O'Brien, R. M., Lucas, P. C., Magnuson, M. A., & Granner, D. K. (1990) *Mol. Endocrinol. 4*, 1302–1310.

Gaub, M.-P., Bellard, M., Scheuer, I., Chambon, P., & Sassone-Corsi, P. (1990) *Cell 63*, 1267–1276.

Gorman, C. M., Merlino, G. T., Willingham, M. C., Pastan, I., & Howard, B. H. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 6777–6781.

Horio, Y., Tanaka, T., Taketoshi, M., Uno, T., & Wada, H. (1988) *J. Biochem. 103*, 805–808.

Imai, E., Stromstedt, P. E., Quinn, P. G., Carlstedt-Duke, J., Gustafsson, J. A., & Granner, D. K. (1990) *Mol. Cell. Biol. 10*, 4712–4719.

Jantzen, H. M., Strähle, U., Gloss, B., Stewart, F., Schmid, W., Boshart, M., Miksicek, R., & Schütz, G. (1987) *Cell 49*, 29–38.

Jonat, C., Rahmsdorf, H. J., Park, K. K., Cato, A. C. B., Gebel, S., Ponta, H., & Herrlich, P. (1990) *Cell 62*, 1189–1204.

Kato, S., Anderson, R. A., & Camerini-Otero, R. D. (1986) *Mol. Cell. Biol. 6*, 1787–1795.

Katunuma, N., Okada, M., & Nishii, Y. (1966) *Adv. Enzyme Regul. 4*, 317–335.

Keller, S. A., Rosenberg, M. P., Johnson, T. M., Howard, G., & Meisler, M. H. (1990) *Genes Dev. 4*, 1316–1321.

Knowles, B. B., Howe, C. D., & Aden, D. P. (1980) *Science 209*, 497–499.

Nasrin, N., Ercolani, L., Denaro, M., Kong, F., Kang, I., & Alexander, M. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 5273–5277.

O'Brien, R. M., & Granner, D. K. (1991) *Biochem. J. 278*, 609–619.

O'Brien, R. M., Lucas, P. C., Forest, C. D., Magnuson, M. A., & Granner, D. K. (1990) *Science 249*, 533–537.

O'Brien, R. M., Bonovitch, M. T., Forest, C. D., & Granner, D. K. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 6580–6584.

Ott, M. O., Sperling, L., Herbomel, P., Yaniv, M., & Weiss, M. C. (1984) *EMBO. J. 3*, 2505–2510.

Pavé-Preux, M., Ferry, N., Bouguet, J., Hanoune, J., & Barouki, R. (1988) *J. Biol. Chem. 263*, 17459–17466.

Pavé-Preux, M., Aggerbeck, M., Veyssier, C., Bousquet-Lemercier, B., Hanoune, J., & Barouki, R. (1990) *J. Biol. Chem. 265*, 4444–4448.

Philippe, J. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 7224–7227.

Prager, D., Gebremedhin, S., & Melmed, S. (1990) *J. Clin. Invest. 85*, 1680–1685.

Rangarajan, P. N., Umesono, K., & Evans, R. M. (1992) *Mol. Endocrinol. 6*, 1451–1457.

Roesler, W. J., Vandenbark, G. R., & Hanson, R. W. (1988) *J. Biol. Chem. 263*, 9063–9066.

Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463–5467.

Schüle, R., Umesono, K., Mangelsdorf, D. J., Bolado, J., Pike, J. W., & Evans, R. M. (1990a) *Cell 61*, 497–504.

Schüle, R., Rangarajan, P., Kliewer, S., Ransome, L. J., Bolado, J., Yang, N., Verma, I. M., & Evans, R. M. (1990b) *Cell 62*, 1217–1226.

Southern, P. J., & Berg, P. (1982) *J. Mol. Appl. Genet. 1*, 327–341.

Stanley, F. M. (1992) *J. Biol. Chem. 267*, 16719–16726.

Stief, A., Winter, D. M., Stratling, W. H., & Sippel, A. E. (1989) *Nature 341*, 343–345.

Stumpo, D. J., Stewart, T. N., Gilman, M. Z., & Blackshear, P. J. (1988) *J. Biol. Chem. 263*, 1611–1614.

Thayer, M. J., Lugo, T. G., Leach, R. J., & Fournier, R. E. K. (1990) *Mol. Cell. Biol. 10*, 2660–2668.

Ullrich, A., Gray, A., Tam, W., Yang-Feng, T., Tsubokawa, M., Collins, C., Henzel, W., Le Bon, T., Kathuria, S., Chen, E., Jacobs, S., Francke, U., Ramachandran, J., & Fujita-Yamaguchi, Y. (1986) *EMBO J. 5*, 2503–2512.

Vaulont, S., Munnich, A., Marie, J., Reach, G., Pichard, A.-L., Simon, M.-P., Besmond, C., Barbry, P., & Kahn, A. (1984) *Biochem. Biophys. Res. Commun. 125*, 135–141.

Wigler, M., Pellicer, A., Silverstein, S., Axel, R., Urlaub, G., & Chasin, L. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 1373–1376.

Yang-Yen, H.-F., Chambard, J.-C., Sun, Y.-L., Smeal, T., Schmidt, T. J., Drouin, J., & Karin, M. (1990) *Cell 62*, 1205–1215.