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Transcriptional stimulation of the δ1-crystallin gene by insulin-like growth factor I and insulin requires DNA cis elements in chicken

(embryonic lens/development/hormone/receptors)

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ABSTRACT **Insulin-like growth factor I (IGF-I) and insulin regulate expression of the endogenous δ1-crystallin gene in embryonic lens cells that express receptors for both peptides. To further analyze the transcriptional component of this hormonal effect, transient transfections of lens cells were prepared with DNA constructs containing deletions of the δ1-crystallin promoter and the chloramphenicol acetyltransferase reporter gene. A 77-nucleotide DNA segment of the δ1-crystallin promoter from nucleotide positions −120 to −43 confers sensitivity to insulin and IGF-I. The hormonal effect is dose-dependent, and maximal stimulation of promoter activity (2- to 2.5-fold induction) is obtained with 10^{-8} M IGF-I and 10^{-7} M insulin. Mobility-shift DNA-binding analysis shows specific binding of nuclear protein(s) to the δ1-crystallin promoter DNA between positions −120 and +23, which appears to be regulated by IGF-I. An SP1-binding motif is involved in this DNA–protein interaction. The bivalent IgG fraction of an anti-insulin receptor antiserum (B-10), known to mimic insulin action in other systems, stimulates promoter activity to the same extent as insulin.**

Insulin-like growth factor I (IGF-I) and insulin regulate the activity of genes involved in cell growth and differentiation (1–3). Although both hormones have been reported to affect transcription, only for insulin is there any information on the DNA cis-acting elements that mediate hormonal action on gene expression (4, 5). The embryonic lens is a suitable developmental system in which to follow the differentiation process of epithelial cells into mitotic-arrested differentiated fiber cells. The epithelial cells have membrane receptors for insulin and, in greater abundance, for IGF-I (6). We (7) and others (8) have shown that insulin and IGF-I, in addition to fetal calf serum (FCS) and vitreous humor, stimulate the terminal differentiation of lens epithelial cells from chicken embryos in early organogenesis. The differentiation state is reflected morphologically in cell elongation and biochemically in increased synthesis of δ-crystallin. Previously we had shown the effect of IGF-I and insulin on transcription of the endogenous gene, although it was not clear whether the hormones induced δ-crystallin gene activity specifically or as part of a more general positive effect on transcription during development. We, therefore, began to analyze the cis-acting DNA sequences that could be important for the specific hormonal and developmental regulation of the δ1-crystallin gene.

Crystallins, the main soluble proteins of the eye lens, are encoded by several gene families the expression of which is highly regulated in ontogeny. δ1-Crystallin is the principal structural protein synthesized in the developing chicken lens; its poly(A)$^+$ RNA is 70–80% of all mRNAs detected *in vivo* (9). The promoter of the δ1-crystallin gene contains positive [between nucleotides (nt) −120 and −43] and negative (between nt −603 and −120) regulatory domains (10). The latter contains three times the negative regulatory element (CCTCTC) postulated by Baniahmad *et al.* (11), present in other genes. The positive domain contains one SP1-binding site from nt −78 to −73 and a CCAAT box from nt −71 to −67 (Fig. 1). It has been shown that the interaction of one or more transcription factors with the SP1 region (G + C-rich box) is required for promoter activity of the δ1-crystallin gene in a heterologous system (12, 13). Quite intriguing, the argininosuccinate lyase gene, an urea cycle enzyme, shares the gene with δ-crystallin. The transcriptional regulation of the enzyme, however, is much less known (9). We have used primary cultures of embryonic lens cells in transient transfection assays with several DNA constructs containing deletions of the δ1-crystallin promoter fused to the chloramphenicol acetyltransferase (CAT) gene. The effect of IGF-I and insulin, as well as the effect of IgG-induced activation of receptors, has been studied. We provide preliminary characterization of nuclear proteins binding specifically to the SP1 DNA motif.

MATERIALS AND METHODS

Materials. Labeled nucleotides and [^{14}C]chloramphenicol were purchased from DuPont/NEN. TLC plates were from Merck, recombinant human IGF-I was from Amgen Biologicals, human insulin was from Eli Lilly, and acetyl CoA and protein A–Sepharose were from Pharmacia. The tissue culture medium (DMEM), FCS, and trypsin were purchased from Biofluids (Rockville, MD); and LB medium was from Digene. All other reagents were molecular biology grade. The oligonucleotide containing one SP1-binding site was purchased from Stratagene.

Primary Cell Culture, Transfection, and Hormonal Treatment. Patches of lens epithelial cells (PLE) from day-6 to day-7 chicken embryos were prepared as described (10) by modifying the procedure of Menko *et al.* (14). Fifteen lenses were used per 35-mm dish. After 48-hr incubation in 2 ml of DMEM/10% FCS at 37°C in 5% CO_2, the cells were washed and transfected by the calcium phosphate-DNA precipitation technique (15) with 10 μg of the plasmid. After being extensively washed the culture was continued without FCS for 24 hr either without (control cells) or with different concentrations of insulin or IGF-I. The cells were then harvested with trypsin and kept frozen in 0.25 M Tris·HCl, pH 7.4, until the CAT assay. When cells were prepared without FCS during the first 48 hr, very few PLE were formed and they did not express transfected DNA.

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Abbreviations: IGF-I, insulin-like growth factor I; PLE, patches of lens epithelial cells; CAT, chloramphenicol acetyltransferase; FCS, fetal calf serum; nt, nucleotide(s).

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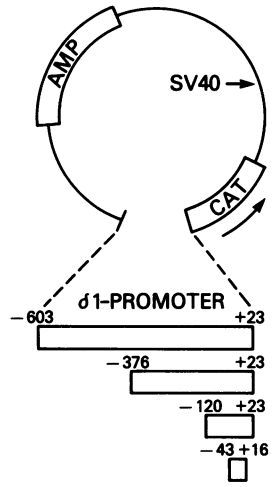

PLASMID	RELATIVE ACTIVITY
TB13	1
TB14	3
CP1	20
TB15	0.8

$^{-120}$ CCAGGGAGAGGGGGCAGAGCTGGGCTGACGAGGGACACCCCGCCCCCAATGGGGCGTGACGAGCTGCCA

GCCCAGGCTCCGGGGCACGTAAAAGCGGGCTGTGAGACCGGAGAGCACGGAGCGACCAGCAGGGCTGA $^{+23}$

FIG. 1. (*Top*) Expression vector and activity of the δ1-crystallin promoter deletions. δ1-Crystallin promoter deletions fused to the CAT gene are schematically shown in the expression vector pSVO-CAT, which contains the ampicillin-resistant gene and polyadenylylation signals of the simian virus 40 early region. The 5′ and 3′ borders of the δ1-crystallin promoter are indicated with the nucleotide position referred to the cap site (+1). Relative CAT activity of each plasmid in 10% FCS, when transfected in PLE of day-11 to day-14 embryos, is indicated (data from ref. 10). SV40, simian virus 40. (*Bottom*) Sequences surrounding the cap site (+1) of the δ-crystallin gene, insert of plasmid pCP1. The SP1-binding site and the CAAT box are underlined. Boldface letters correspond to part of exon 1.

Plasmid Constructs. The hybrid genes used are shown in Fig. 1 (10). Plasmids were grown in LB medium/ampicillin overnight, and their DNA was extracted as described (16). Supercoiled DNA was banded twice by ultracentrifugation in CsCl.

CAT and β-Galactosidase Assays. After homogenization with a glass tissue grinder, the CAT activity of the cell extract was assayed with [^{14}C]chloramphenicol and acetyl coenzyme A (6 mM, final concentration) for 1–3 hr at 37°C, as described (17). The acetylated and nonacetylated forms of [^{14}C]-chloramphenicol were extracted with ethyl acetate and separated by TLC with chloroform/methanol (95:5). The amount of radioactivity associated with the acetylated [^{14}C]chloramphenicol was determined in a scintillation counter, and the result was normalized relative to protein content. In some experiments, transfection efficiency was monitored by cotransfecting pTB1 (10), a plasmid containing the *lacZ* gene under the control of the Rous sarcoma virus long terminal repeat promoter. The β-galactosidase activity in transfected cells was measured.

Preparation of Nuclear Extracts. Nuclear extracts were prepared from whole lenses of groups of 100–200 chicken embryos from days 6, 7, and 11, according to Dignam *et al.* (18), as modified by Wildeman *et al.* (19). Lenses cleaned from adherent iris were placed in Ham's F-10 medium, the capsule was opened, and the cells were incubated for 1 hr at 37°C (5% CO_2) with or without 10^{-8} M IGF-I. At end of the incubation the cells were washed with phosphate-buffered saline and stored frozen in liquid nitrogen. Untreated lenses were frozen without disruption. The nuclear proteins were extracted in the presence of phenylmethylsulfonyl fluoride and dithiothreitol. A preparation of 175–200 lenses from days 6 or 7 usually yielded 0.25 ml of nuclear extract with an average protein concentration of 1–1.5 mg/ml, determined by the BCA protein assay (Pierce) with albumin as standard.

DNA Fragment for Binding Assay. The 143-base-pair (bp) DNA fragment of the δ1-crystallin promoter spanning from nt −120 to +23 was prepared by digesting the δ1.5 plasmid (20) with Sau96 I/Dde I, separation in 2.5% agarose gel, electroelution, and purification of the insert. For labeling, the cohesive ends were filled with radioactive nucleotides by using the Klenow fragment of DNA polymerase I.

Mobility-Shift DNA Binding Assay. The labeled DNA fragment was incubated for 30 min at 22°C in a total volume of 20 μl containing 8–16 μg of protein from lens nuclear extract in 12 mM Hepes, pH 7.9/12 mM KCl/0.6 mM $MgCl_2$/1.2 mM dithiothreitol/10.2% glycerol containing 2.5 μg of poly-(dI·dC). The DNA–protein complexes were separated from free DNA by electrophoresis with 5% polyacrylamide gels (acrylamide/bisacrylamide ratio 60:1) under nondenaturing conditions (21). The gels were prerun for 2.5 hr in 0.5× TBE (1× TBE is 90 mM Tris borate, pH 7.8/90 mM boric acid/2 mM EDTA). The samples were electrophoresed at 200 V until they entered the gel and then at 120–140 V. Gels were dried under vacuum and autoradiographed.

Preparation of Anti-Receptor IgG. Antibodies were purified from the serum of insulin-resistant patient B-10 (22) by precipitation with ammonium sulfate, acid elution from a protein A–Sepharose column, and dialysis against phosphate-buffered saline (Digene), pH 8. The contaminating insulin that remained in the sample was removed by acidification at 4°C for 20 min and charcoal absorption. We excluded the presence of significant contamination with insulin or IGF-I in this preparation by RIA.

RESULTS

IGF-I and Insulin Stimulate Specifically the Activity of Transfected δ1-Crystallin Promoter into Lens Cells. DNA of the plasmid pCP1 (Fig. 1) was efficiently transfected into PLE from embryos of 6–7 days. All transfections were transient expression experiments in which the hormones were added after DNA transfection. The hormones stimulated δ1-crystallin promoter activity in a dose-dependent manner (Fig. 2). A 2- to 3-fold increase in CAT activity was elicited by 10^{-8} M IGF-I, and no further increase was seen with 10^{-7} M. Insulin at 10^{-9} M and 10^{-8} M was stimulatory, but the variation among experiments was large. Insulin at 10^{-7} M was as potent a stimulus as IGF-I. IGF-I potency is in the range of the stimulus obtained by 10% FCS (data not shown). We did not test other analogs of insulin or IGF-I because previously (7) we found a very low effect of proinsulin and IGF-II on δ-crystallin mRNA accumulation. We had previously shown that, after DNA transfection of this plasmid into cells treated with FCS, CAT RNA initiates at the same site in the promoter of the hybrid gene as does δ1-crystallin RNA in the endogenous gene (10). The time course of the hormonal stimulus (data not shown) indicated that at 24 hr the effect was maximum and no further increase occurred at 48 hr. To test the specificity of the effect upon δ1-crystallin promoter, the plasmid pSV2CAT carrying the simian virus 40 early promoter was transfected under the same conditions and incubated with or without hormones. No effect of insulin or IGF-I either at 10^{-9} M (Table 1) or 10^{-7} M (data not shown) was seen on this control promoter.

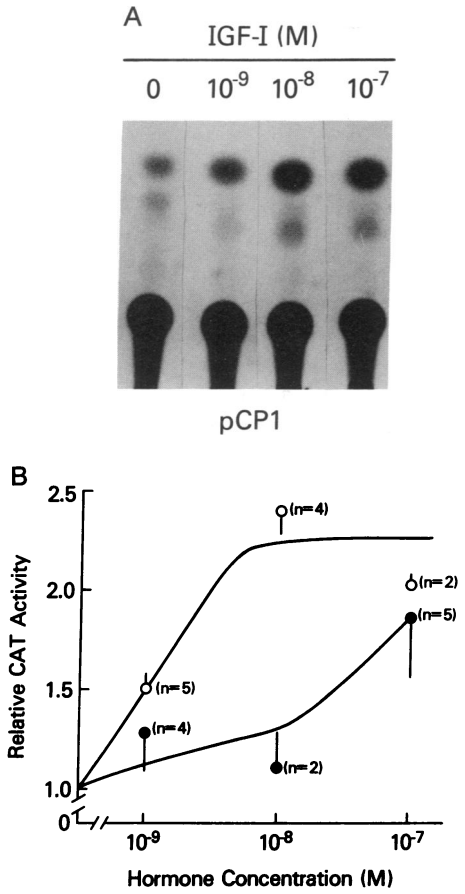

FIG. 2. Induction of pCP1 by insulin and IGF-I is dose dependent. (*A*) PLE from day-6 to -7 chicken embryos were incubated at 37°C (5% CO_2) in DMEM medium/10% FCS. After 24–30 hr, fresh medium without FCS was added, and the cells were transiently transfected by the calcium phosphate procedure with 10 μg of the plasmid pCP1 for 6–8 hr in DMEM medium alone. After being washed, the PLE were incubated for an additional 24 hr with IGF-I or insulin and harvested; CAT activity was then determined. Acetylated and nonacetylated chloramphenicol in the cell lysate were separated by TLC, and an autoradiogram was generated. The film was exposed for 16 hr, and a representative autoradiogram of the effect of IGF-I is shown. (*B*) Transfected cells were incubated as described for *A*. Relative CAT activity is the value of [^{14}C]acetylated chloramphenicol determined by counting the radioactivity in TLC plates and normalizing the results to protein content. The stimulation of CAT activity in cultures treated with insulin (●) or IGF-I (○) is given in an arbitrary scale relative to the level in untreated cultures (1.0). n, Number of independent experiments done at each hormonal concentration. SE is indicated.

Hormonal Stimulus Depends upon a DNA Cis-Acting Region of δ1-Crystallin Promoter. To determine whether the hormonal effect observed required a defined region of the promoter, other deletion mutants (Fig. 1) were transfected and the cultures were treated with IGF-I or insulin. For each stimulation, the control was the unstimulated CAT activity of the particular construct DNA transfected (Table 1). No effect of low concentration (10^{-9} M) of either IGF-I or insulin was seen with pTB13 and pTB15. pTB14 had a marginal stimulatory response to insulin (10^{-9} M) (Table 1). Further experiments were done cotransfecting either pCP1, the most responsive construct at low hormonal dose, or pTB15, and the β-galactosidase gene to control for transfection efficiency. Again, pCP1 responded to IGF-I (10^{-8} M), whereas pTB15 activity was even lower in the IGF-I-treated cells than in their corresponding control (Table 2 and Fig. 3). Thus, removal of sequences from nt −603 to −120 rendered the promoter more sensitive to insulin and IGF-I until deletions reached nt −43 when hormonal responsiveness was lost. It should be noted that in our evaluation of the activity of these promoter deletions reported earlier (10) the transfected cells were from older embryos (day 11) and were cultured in the continuous presence of FCS. Thus, the activities reported in Fig. 1, which differ over a 20-fold range, most likely reflect the extreme responses under multiple hormonal stimuli.

Table 1. Stimulation of δ1-crystallin/CAT constructs after hormonal addition

Construct	Stimulation, -fold	
	Insulin (10^{-9} M)	IGF-I (10^{-9} M)
pTB13	0.8 (0.6–0.9)	0.8 (0.7–0.9)
pTB14	1.4 (1.0–1.7)	1.1 (0.9–1.6)
pCP1	1.3 (1.0–1.6)	1.5 (1.1–2.0)*
pTB15	0.7 (0.6–0.7)	0.6 (0.4–0.7)
pSV2CAT	0.8 (0.8–0.9)	0.9 (0.8–0.9)

Construct DNAs were transfected into PLE, and response to the hormones was evaluated. Data are expressed as relative CAT activity normalized per protein in arbitrary units with respect to untreated cultures. In each case the control (nonstimulated CAT activity of each construct) was valued at 1. Data represent the means of two to five experiments with the range in parenthesis.
*Statistically significant, $P < 0.05$. Note that the stimulatory effect on pCP1 is much lower with 10^{-9} M than with 10^{-8} or 10^{-7} M (Fig. 2*B*).

Specific Binding of Lens Nuclear Proteins to the δ1-Crystallin Promoter. Because the region of DNA between nt −120 and −43 appeared necessary for hormonal sensitivity of the δ1-crystallin promoter, we began to explore whether we could identify nuclear factors that mediate IGF-I and/or insulin action. Nuclear extracts from chicken embryo whole lens, incubated with or without IGF-I, were tested for their ability to bind a 143-bp fragment (nt −120 to +23, insert of pCP1) from the δ1-crystallin promoter. Two major retarded bands were recognized in gel-shift assays. These bands were specifically abolished by competition with excess nonradioactive pCP1-insert DNA (Fig. 4*A*). The signal of the predominant band in the nuclear extract from cells treated with IGF-I increased, compared with untreated cells (Fig. 4*A*) in three independent cultures and nuclear preparations.

In some experiments additional retarded bands became apparent after IGF-I treatment. The appearance of the retarded complexes was protein concentration-dependent (data not shown), was not inhibited by excess single-stranded DNA or unrelated DNA (Fig. 4*B*), and was absent when binding

Table 2. Effect of IGF-I on CAT conversion relative to β-galactosidase

Construct	CAT converted, %		Induction, -fold	Mean ± SE
	Control	IGF-I (10^{-8} M)		
pCP1				
Exp 1	19	40	2.1	
Exp 2	22	48	2.2	2.4 ± 0.3
Exp 3	3	9	3.0	
pTB15				
Exp 1	21	12	0.6	
Exp 2	15	3	0.2	0.4 ± 0.1
Exp 3	7	4	0.5	

Value given for each experiment is a mean result of duplicate dishes for control and treated cells. The percent of CAT converted is corrected by protein content and β-galactosidase activity. The three experiments (Exp) were done in parallel for the two plasmids. Note that these values are percent of converted CAT, while Fig. 3 represents the same experiments calculated as cpm of acetylated chloramphenicol normalized by protein content and β-galactosidase activity.

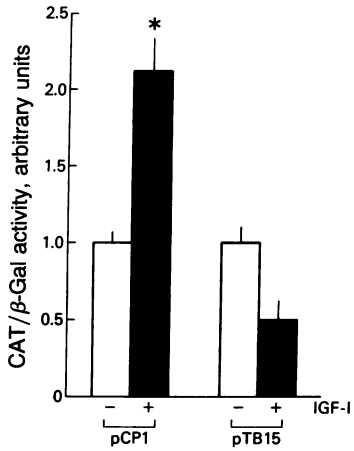

FIG. 3. Relative CAT activity of pCP1 and pTB15 constructs. PLE were cotransfected with a vector containing the β-galactosidase gene and either pCP1 or pTB15. Each group was incubated for 24 hr with or without 10^{-8} M IGF-I, and the CAT and β-galactosidase activities were determined, as described. The relative CAT activity of the treated cells is shown with respect to controls after being normalized per protein content and β-galactosidase activity. Each bar represents the mean value of three different experiments with duplicate points. *, $P < 0.05$.

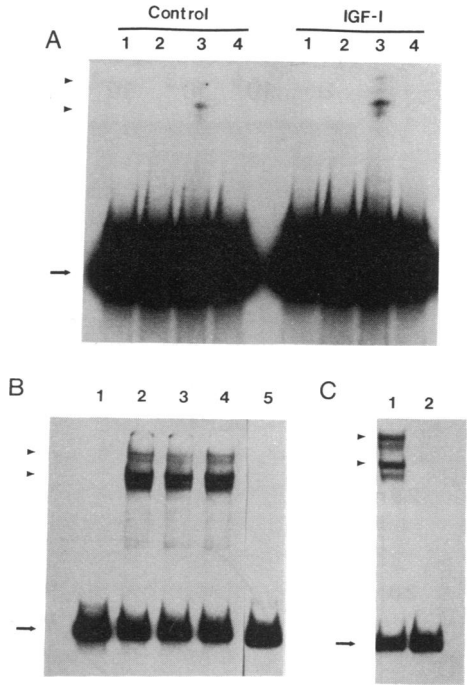

FIG. 4. Mobility-shift DNA-binding assay reveals specific protein binding to δ1-crystallin promoter, increased by IGF-I. (*A*) The 143-bp DNA fragment corresponding to the insert of pCP1, was purified and labeled. Nuclear extracts were prepared from whole lenses incubated for 1 hr either with medium alone (control) or with 10^{-8} M IGF-I. Binding of this fragment to the proteins from nuclear extracts was for 30 min at 22°C. Electrophoresis under nondenaturing conditions was in a 5% polyacrylamide slab gel. The lagging DNA/protein(s) complex(s) (arrowheads) were separated from free DNA (arrow). Lanes: 1, no protein extract added; 2, 8 μg of protein added; 3, 16 μg of protein added; 4, binding was done with 16 μg of protein and excess (500-fold) of the nonradioactive DNA fragment. As little as 10-fold excess nonradioactive DNA competed for the signal in other experiments. (*B*) Nuclear extracts from fresh-frozen 11-day-old embryo lenses (15 μg per lane) were incubated under the same conditions as above. The labeled 143-bp fragment was incubated without any protein (lane 1), 20 μg of protein (lane 2), protein plus excess M13 single-stranded DNA (lane 3), protein and excess 123-bp fragment of M_r marker ladder (lane 4), and protein and proteinase K (lane 5). Note that no inhibition of binding occurs with unrelated DNAs. (*C*) The DNA–protein complexes obtained with 20 μg of nuclear extract (11-day-old embryo lenses) (lane 1) were not seen when incubation was done with excess (100×) double-stranded oligonucleotide containing one SP1 site (lane 2). The difference in sharpness of the retarded bands among *A–C* is probably because degradation of nuclear proteins is more intense when lens cells are cultured (*A*) than when tissue is frozen immediately after dissection (*B* and *C*).

was done with proteinase K, indicating that the nuclear proteins were essential to shift mobility of the labeled DNA in the gel (Fig. 4*B*). The retarded complexes were completely inhibited by a double-stranded oligonucleotide containing the SP1-binding motif (Fig. 4*C*).

Anti-Insulin Receptor Antibody Mimics Insulin Action on Transcription. Previously, we had shown the stimulatory effect of a polyclonal anti-insulin receptor antibody (B-10) on the steady-state levels of δ-crystallin mRNA (7). To determine whether this bivalent IgG could stimulate transcription of the transfected δ1-crystallin promoter, lens cells were treated with antibody alone under the same conditions described for the hormonal treatment. The effect of antibody B-10 was in the same range of that elicited by 10^{-7} M insulin (Fig. 5), indicating that the activation of receptor(s) is sufficient for inducing transcription of the δ1-crystallin transfected promoter.

DISCUSSION

IGF-I and insulin stimulate the differentiation of embryonic lens cells in primary cultures (8). δ-Crystallin mRNA, a biochemical marker of this differentiation process, increases after treatment with the two hormones, at least in part, through stimulation of transcription (7). We show here that DNA sequences located between nt −120 and −43 of the δ1-crystallin promoter are required for IGF-I and insulin activation of transcription. The activity of the δ1-crystallin promoter and not other promoter transfected under the same conditions (pSV2CAT) is stimulated by IGF-I and insulin. Thus, the transcriptional effect of the hormones is promoter-specific. Within the δ1-crystallin promoter there are specific sequences that mediate the IGF-I and insulin responses. A segment of the δ1-crystallin promoter (nt −120 to −43), known to contain positive regulatory sequences (10), confers responsiveness to the hormones. Sequences between nt −376 and −120 allow low hormonal responsiveness. By contrast, inclusion of nt −603 to −376 directed basal expression of the gene without significant change after IGF-I or insulin treatment. The deletion construct that lacks the SP1 binding and CCAAT regions (pTB15) had the lowest activity of all upon hormonal treatment, even below the basal level of expression (Fig. 3). Whether the presence of the intronic enhancer described in the δ1-crystallin gene (23) in an expression vector will render the nonresponsive deletions sensitive to insulin and IGF-I remains for investigation. Although insulin

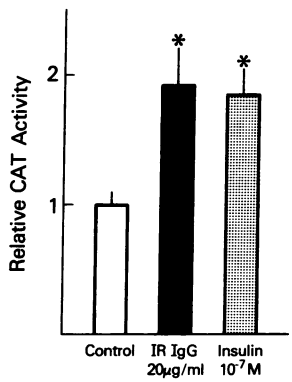

FIG. 5. Anti-insulin receptor (IR) IgG stimulates transcription of δ1-crystallin promoter as well as insulin. PLE transiently transfected with pCP1 were cultured for 24 hr without additions (control) or with either anti-insulin receptor IgG at 20 μg/ml or insulin at 10^{-7} M. Values are the mean ± SEM of four experiments. *, $P < 0.05$ with respect to control.

and/or IGF-I regulate transcription of a variety of genes—e.g., growth hormone (1, 2), c-*myc* (3), thyroglobulin (24), protein p33 (25), and others (26), little is known about the DNA elements responsive to the hormones. A few promoters of genes known to be regulated by insulin—e.g., amylase (27), phospho*enol*pyruvate carboxykinase (28), and the best studied, c-*fos* (29–31)—share a common sequence that resembles the serum-response element (32) and is not readily found in the δ1-crystallin promoter. In the glyceraldehyde-3-phosphate dehydrogenase promoter the shorter element of the two DNA sequences that appear to mediate the insulin response and interacts with a protein distinct from SP1 (M. Alexander-Bridges, personal communication) (CCCGCCTC) has 7 of 8 nt common with nt −78 to −71 (CCCGCCCC) of the δ1-crystallin promoter. As has been elegantly shown for genes inducible by transforming growth factor β, only a few base pairs of a promoter could render it sensitive to a growth factor (33). We have detected nuclear proteins that bind specifically to the −120 to +23 δ1-crystallin promoter region and to the SP1 motif within it. Two major bands are demonstrated in mobility-shift assays (Fig. 4 *B* and *C*). When the lens cells were cultured with IGF-I, an increase in the signal of the DNA–protein complexes occurred (Fig. 4*A*). Further work will elucidate whether the stimulated protein maps exclusively to the DNA SP1 site or includes protein(s) binding to the CCAAT box.

We have recently characterized by HPLC an IGF-I immunoactivity from the vitreous humor of chicken embryos (H. Robcis, T. Caldes, J.A., and F.d.P., unpublished observation). It is likely that endogenous, perhaps locally produced, IGF-I regulates lens cell differentiation *in vivo*. The effects of the hormones appear to be receptor-mediated. The effective IGF-I concentration is suggestive of an action through IGF-I receptors (6), the most abundant in these cells. The high concentration of insulin required is more consistent with insulin acting also through the IGF-I receptor and not through the insulin receptor. However, the promoter regions used in our study contain only part of the known regulatory sequences found *in vivo*. Thus, as we suggested for the regulation of expression of the endogenous gene (7), the insulin receptor may, indeed, be mediating the action of insulin on δ-crystallin expression *in vivo*, without conflict with the transfection results. We can only provide indirect evidence of the involvement of receptors in this system. Others have used stimulatory antibodies against the insulin receptor (34–38). We found that an antibody that preferentially recognizes the insulin receptor (B-10) (39) mimicked the effect of insulin, not only on δ-crystallin mRNA steady-state levels (7) but on transcription of the transfected construct promoter as well. c-*fos* activation of transcription by insulin has been shown to require intact insulin receptors (29). Whether in addition to activating their receptors insulin and IGF-I can have direct intracellular effects remains a matter of controversy (40). In lens epithelial cells it is intriguing that IGF-I is rapidly internalized and can be found in the nucleus (A. Peralta Soler, J.A., R. Smith, F.d.P., and L. Jarett, unpublished work).

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