MOLECULAR AND CELLULAR BIOLOGY, July 1986, p. 2551–2561
0270-7306/86/072551-11$02.00/0

Vol. 6, No. 7

Localization of DNA Sequences Involved in Dexamethasone-Dependent Expression of the Rat α_1-Acid Glycoprotein Gene

HEINZ BAUMANN[1]* AND LYNNE E. MAQUAT[2]

Department of Cell and Tumor Biology[1] and Department of Human Genetics,[2] Roswell Park Memorial Institute, Buffalo, New York 14263

Received 4 February 1986/Accepted 17 April 1986

Synthesis of rat α_1-acid glycoprotein (AGP), one of the major inflammation-induced plasma proteins, is positively regulated by dexamethasone. To define the dexamethasone-responsive genetic element, we isolated and tested AGP gene sequences for the ability to confer glucocorticoid induction to the bacterial chloramphenicol acetyltransferase (CAT) gene in L cells. A 141-base-pair region of the AGP gene, including 120 base pairs of DNA upstream from the start site of transcription and 21 base pairs of the 5′ untranslated region, was sufficient for maximal CAT gene induction by dexamethasone. To localize more precisely the AGP glucocorticoid-responsive element, parts of this 141-base-pair region were inserted 5′ to either an AGP promoter-CAT gene or a human triosephosphate isomerase promoter-CAT gene, both of which lacked a response to the steroid. The AGP gene region between 120 and 42 base pairs upstream from the start site of transcription was found to mediate maximal dexamethasone induction of CAT enzyme levels. This result was unexpected because (i) this region does not contain sequence homologies to known glucocorticoid receptor-binding sites and (ii) those AGP gene regions that lay further upstream and were homologous to other glucocorticoid receptor-binding sites were inactive in the CAT assay. The fact that the AGP gene region mediating dexamethasone regulation was distinct from the transcribed region indicates that glucocorticoids increase AGP gene expression primarily at the transcriptional rather than the posttranscriptional level.

Acute systemic injury in mammals causes a profound change in the hepatic synthesis and circulating concentration of plasma proteins (21, 24). α_1-Acid glycoprotein (AGP), a heavily glycosylated protein with a molecular weight of 41,000 to 45,000 (39), belongs to the subset of plasma proteins whose production is increased severalfold (3, 21, 22). AGP appears to function in modulating the activity of the immune system during the course of an acute-phase reaction (6, 10, 11, 15). In recent years, the regulation of rat AGP has been extensively studied. Several laboratories have shown that (i) rat AGP production is modulated by glucocorticoids and by hepatocyte-stimulating factor (3, 5, 34), (ii) AGP regulation by these hormones is reproducible in rat hepatoma (HTC) cells or in primary cultures of adult rat hepatocytes (2, 22, 42), (iii) the action of glucocorticoids on AGP expression is dependent upon de novo protein synthesis (5, 42), (iv) glucocorticoids and hepatocyte-stimulating factor act synergistically (4, 5), and (v) the modulation of AGP synthesis is the consequence of a corresponding change in the AGP mRNA concentration (34). The inflammation- or hormone-mediated increase in AGP expression has been correlated with an elevated rate of gene transcription in both intact liver (23) and cultured hepatocytes (4). An exception, however, has been reported for the glucocorticoid-stimulated AGP mRNA accumulation in HTC cells, where the regulatory influence of the steroid was attributed to a change in the stability of AGP nuclear transcripts (43).

Recently, the AGP gene has been isolated and sequenced (25, 33). Comparison of AGP gene sequences with the sequences of other glucocorticoid-regulated genes revealed five potential glucocorticoid receptor-binding sites having a precise or near match with the consensus hexamer TGTTCT (25, 33). Two of these sites are located upstream of the transcription start site, +1, as defined by Reinke and Feigelson (33; see Materials and Methods) at positions −438 (25) and −271 (33). The remaining three sites are located downstream of the transcription start site at positions +854, +4014, and +4296 (25). These sites, and especially those 5′ to the gene, have been proposed to be instrumental in mediating glucocorticoid stimulation of AGP gene expression (33).

In this communication, we demonstrate that the putative glucocorticoid receptor-binding sites located at positions −438 and −271 of the AGP gene were not required for the dexamethasone response mediated by mouse fibroblasts. However, a dexamethasone-responsive region was present between positions −120 and −42. Since this region lay outside of the transcribed portion of the gene and could confer dexamethasone induction when placed in *cis* to, e.g., a transcriptionally competent triosephosphate isomerase promoter-chloramphenicol acetyltransferase (CAT) gene, we conclude that dexamethasone enhancement of AGP gene expression is controlled primarily at the level of transcription initiation.

MATERIALS AND METHODS

Isolation of the rat AGP gene. AGP gene sequences were isolated (28) from a genomic library of Sprague-Dawley rat DNA that was partially digested with *Eco*RI and cloned into lambda Charon 4A DNA (a generous gift of J. Bonner, Phytogen, Pasadena, Calif.). The library was screened with an 850-base-pair (bp) *Pst*I fragment of Buffalo rat AGP cDNA derived from the plasmid pIRL-10 (1). This fragment

* Corresponding author.

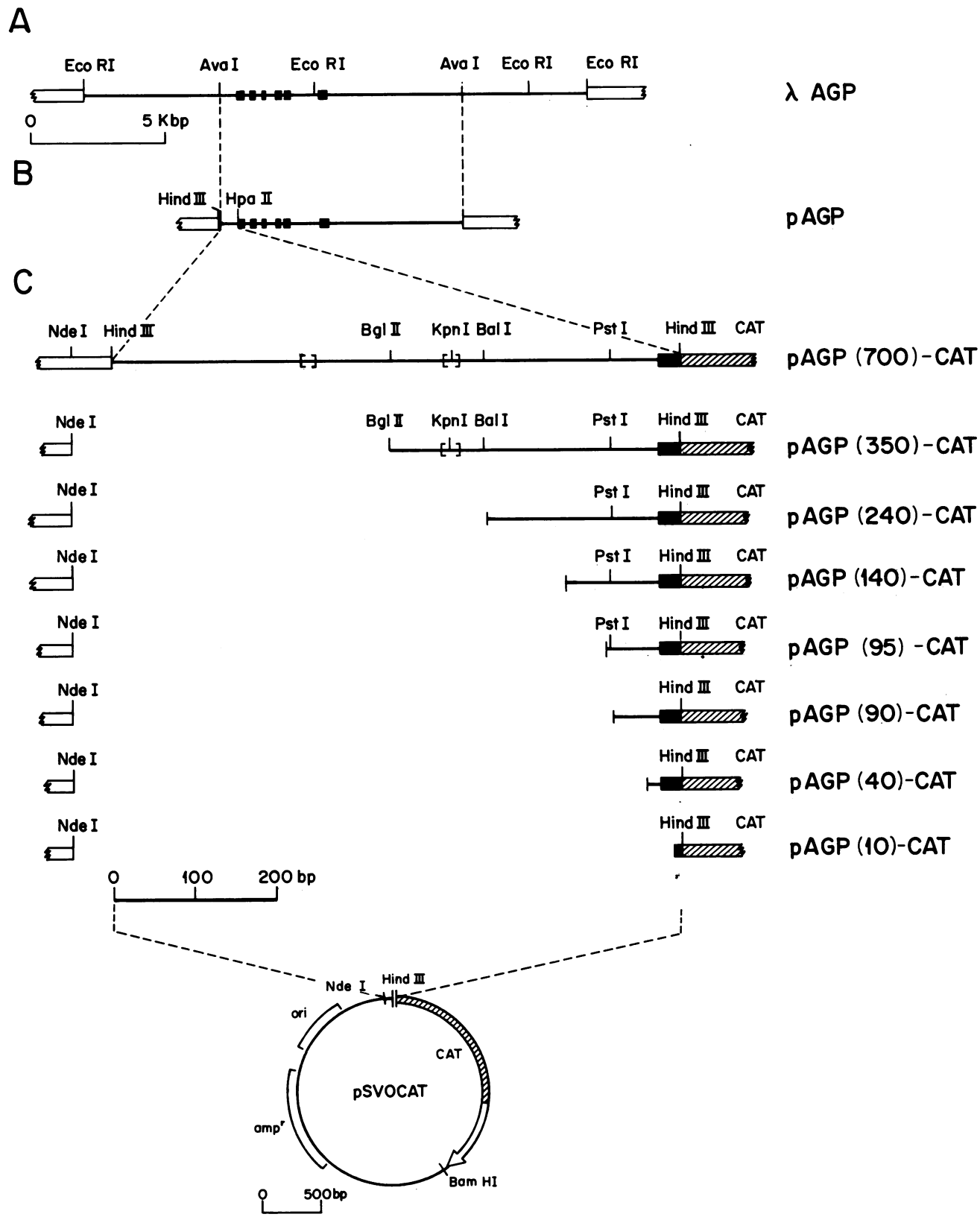

FIG. 1. Structure of recombinant AGP DNAs. A. Organization of rat AGP gene sequences in λAGP. The horizontal line represents 18 kb of rat DNA, solid blocks indicate the six exons of the AGP gene (33), and open boxes indicate λ DNA. B. Organization of rat AGP gene sequences in pAGP. The horizontal line designates the 9-kb *Ava*I fragment derived from λAGP. Open boxes specify pUC13 DNA. C. Structure of the AGP-CAT gene derivatives. The 5′ end of the AGP insert in pAGP was excised with *Hin*dIII (which cleaves within the

was subcloned into M13mp8 and ^{32}P labeled by extension of the universal primer. DNA isolated from the phage λAGP (Fig. 1A) contained the entire AGP gene and, based on restriction enzyme analysis, was identical to the gene described by Reinke and Feigelson (33). In this study, we used the nucleotide numbering system of Reinke and Feigelson (33) since our sequence data were identical to the data of these authors and differed from the data of Liao et al. (25). Reinke and Feigelson have localized the transcription start site by S1 nuclease mapping to 27 bp downstream of the TATA box and 44 bp upstream of the primary translation initiation codon. In contrast, Liao et al. have localized the transcription start site by primer extension to 33 bp downstream of the TATA box. pAGP was constructed by subcloning a 9-kb *Ava*I fragment containing the entire AGP structural gene plus 700 bp of 5′ flanking DNA and 5,000 bp of 3′ flanking DNA into the *Hin*cII site of pUC13. The *Ava*I sites were filled in with Klenow fragment and dNTPs before ligation (Fig. 1B).

Construction of the CAT gene derivatives. A 700-bp fragment of pAGP extending from positions −678 to +21 (where +1 is defined as the transcription start site [33]) was excised with *Hin*dIII and *Hpa*II. The *Hpa*II site was filled in with Klenow fragment and dNTPs, modified with *Hin*dIII linkers, and inserted into the unique *Hin*dIII site of pSV0CAT (17) (Fig. 1C). The resulting construct and all subsequently derived constructs are designated by the total length of the AGP gene fragment present in the plasmid, in this case, pAGP (700)-CAT. To construct deletions from the distal region of the AGP insert, pAGP(700)-CAT was cleaved within the AGP sequence, modified with *Nde*I linkers, and ligated to the *Nde*I site in pSV0CAT. In this way, all deletion endpoints were joined to the identical plasmid sequences. Briefly, deletions in pAGP(700)-CAT were generated by digestion with *Bgl*II, treatment with Klenow fragment and dNTPs, addition of *Nde*I linkers, digestion with *Nde*I, purification of the larger vector-containing fragment, and ligation to yield pAGP(350)-CAT. The remaining deletions were generated by linearizing pAGP(700)-CAT with *Kpn*I at position −262 of the AGP insert, followed by digestion with nuclease *Bal* 31, addition of *Nde*I linkers, digestion with *Nde*I, and self-ligation of the larger vector-containing fragment. The approximate size of the AGP region in each of the resulting plasmids [pAGP(240)-CAT to pAGP(10)-CAT; Fig. 1C] was initially determined by multiple restriction enzyme analysis and served as a designation for the plasmid. This designation is used throughout this communication. Subsequently, the precise size of the AGP region was established for certain constructs by sequencing by the dideoxy chain termination method (36) and the chemical modification method (29). In all cases, size estimates based on restriction enzyme analysis were within a few base pairs of the precise size.

To obtain deletions of the proximal region of the AGP promoter, pAGP(140)-CAT was linearized at the unique *Hin*dIII site and digested with nuclease *Bal* 31. The ends were modified with *Nde*I linkers and religated. The size and DNA sequence of the *Nde*I-*Nde*I fragment was determined as an indication of the extent of the AGP gene deletion. Two plasmids containing a truncated and nonfunctional AGP promoter region of either 101 bp (corresponding to positions −120 to −19) or 78 bp (corresponding to positions −120 to −42) were tested for transcriptional regulation by dexamethasone.

To determine whether the *Nde*I-*Nde*I 101- or 78-bp AGP fragment contains a glucocorticoid-responsive element, each fragment was inserted upstream of the housekeeping gene promoter for human triosephosphate isomerase (TPI) which had been placed upstream of the CAT gene in pSV0CAT (L. E. Maquat, manuscript in preparation). To maintain proper spacing of the AGP gene elements relative to the TPI transcription start site (8), chimeric constructs were prepared with plasmids pTPI(130)-CAT, pTPI(75)-CAT, and pTPI(42)-CAT (L. E. Maquat, manuscript in preparation) having different lengths of the TPI promoter region. The distal borders of the TPI insert in each plasmid ended precisely 130, 75, and 42 bp upstream of the TPI transcription start site, respectively, and were joined to the *Nde*I site of the CAT vector by an *Nde*I linker. The TPI promoter in the former two plasmids is fully functional, while the promoter in the latter is inactive (L. E. Maquat, manuscript in preparation). AGP gene-derived sequences, i.e., the restriction enzyme fragments *Bgl*II-*Bal*I (−338 to −220, Fig. 1C), *Hin*dIII-*Bal*I (−678 to −220, Fig. 1C), or *Nde*I-*Nde*I (−120 to −19 and −120 to −42) were inserted at the *Nde*I site of the TPI-CAT gene constructs. The orientation of the AGP insert was established by restriction enzyme analysis.

L-cell transformation. Ltk$^-$ mouse fibroblasts generously provided by R. Hughes, Roswell Park Memorial Institute) were used in all transformation experiments. To obtain stable transformants, a mixture of 0.5 μg of pSV2gpt (41), 5 μg of pAGP, and 15 μg of carrier L-cell DNA was introduced into L cells by the calcium phosphate precipitate method (18, 44). Cotransformation with a *gpt* gene rather than a thymidine kinase gene was chosen to preserve the tk$^-$ feature of the resulting transformants for future manipulations. Transformants were selected in medium containing mycophenolic acid (6 μg/ml) and 1.4 mM xanthine (41). Individual clones were isolated and expanded. The number of rat AGP gene copies per transformant was determined by Southern blot analysis of genomic DNA (40). The regulated expression of the genes was assessed by Northern blot analysis of cellular RNA (5) and by quantitation of synthesized and secreted AGP by using both two dimensional polyacrylamide gel electrophoresis (31) and rocket immunoelectrophoresis with monospecific antiserum against rat AGP (21).

Assay for CAT activity. L-cell transformation with CAT gene-containing plasmid DNAs was carried out essentially as described by Lopata et al. (26). This technique has proven to be the most efficient and reproducible means of transforming L cells. To assay CAT enzyme levels, plasmid DNA (20 μg each in duplicate dishes) was combined with 1.5 mg of DEAE-dextran (M_r, 500,000) in 3 ml of Tris-buffered saline. From this mixture, 1.5 ml was added to a subconfluent monolayer of L cells (3×10^6 cells per 10-cm culture dish prepared 20 h before transformation). The cells were incu-

polylinker of pUC13) and *Hpa*II and inserted with *Hin*dIII linkers into the *Hin*dIII site of pSV0CAT to generate pAGP(700)-CAT. Deletions were introduced into the AGP region of pAGP(700)-CAT as described in Materials and Methods. All AGP deletion endpoints were modified with an *Nde*I linker and ligated to the unique *Nde*I site in pSV0CAT. Deletion endpoints were localized by restriction enzyme analysis and, in several cases, by DNA sequencing (see Fig. 7). Solid blocks in all but pAGP(10)-CAT indicate 21 bp of the 5′ untranslated region within the first exon, hatched blocks indicate the CAT gene, open blocks specify vector DNA, and brackets specify sequences having homology to known glucocorticoid receptor-binding sites (25, 33).

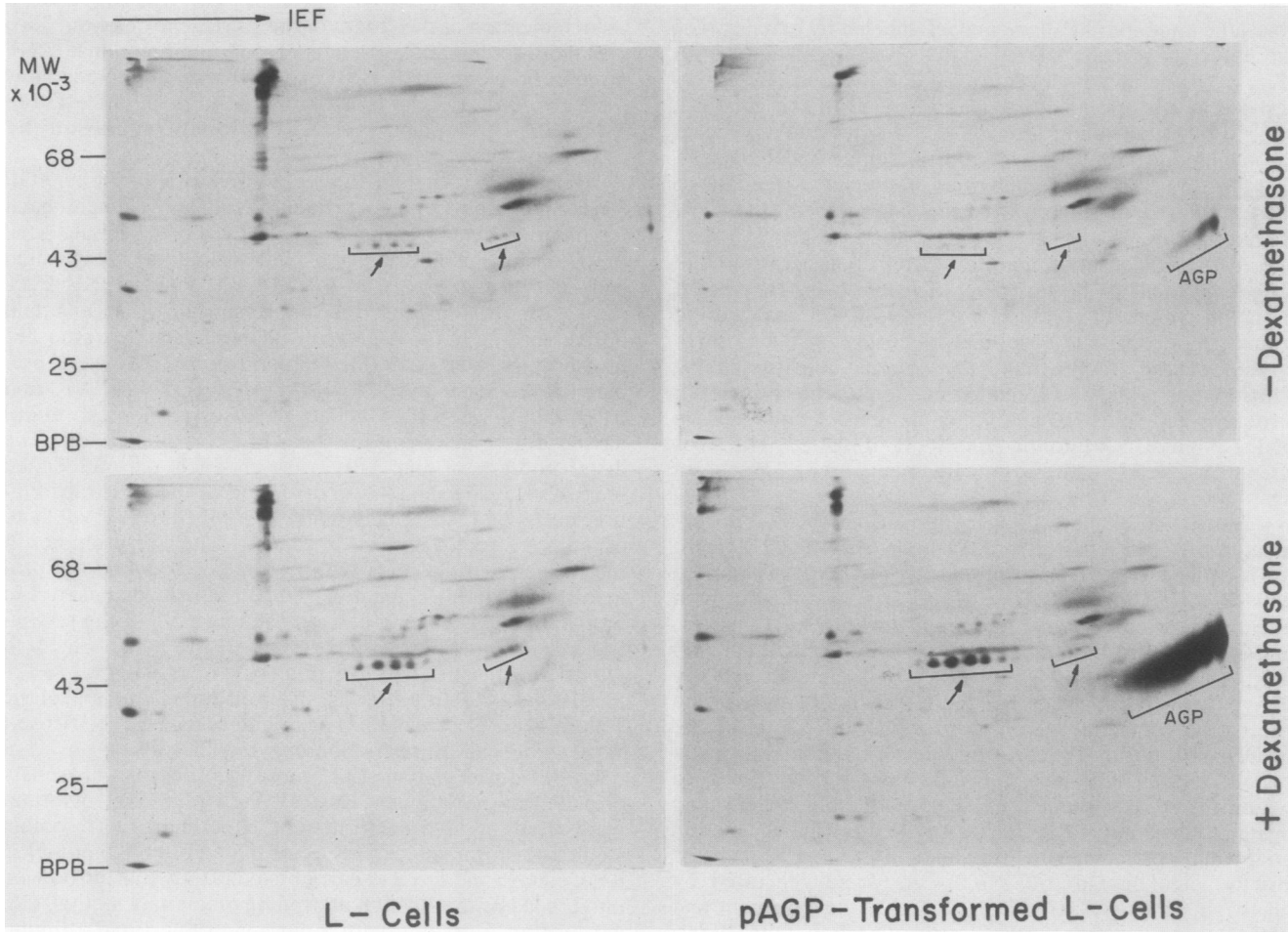

FIG. 2. Expression of rat AGP in L cells. Confluent monolayers of nontransformed L cells and a clonal cell line of pAGP-transformed L cells containing 10 integrated AGP genes per genome were cultured for 24 h in the absence or presence of 1 μM dexamethasone. The medium was aspirated and replaced with fresh medium containing [^{35}S]methionine (100 μCi/ml per 10-cm^2 monolayer). After 6 h, 50-μl aliquots of culture medium containing 180,000 to 270,000 acid-insoluble cpm were subjected to two-dimensional polyacrylamide gel electrophoresis (31). The fluorographic image (7) of the gel after 24 h of exposure is reproduced. Arrows indicate those secretory glycoproteins of the L cells that are increased by dexamethasone. The spot marked with AGP represents rat AGP as identified by immunoprecipitation.

bated with occasional shaking for 40 min at 37°C. A 3-ml portion of serum-free Dulbecco modified Eagle medium (DMEM) containing 0.1 mM chloroquine (27) was added, and the incubation was continued for 80 min. The cells were treated with 10% dimethyl sulfoxide in serum-free DMEM for 2 min at room temperature. After an overnight incubation (18 to 20 h) in DMEM supplemented with 10% fetal calf serum, one of each pair of duplicate plates received 1 μM dexamethasone. After an additional 24 h in culture, the monolayers were washed, scraped off the dishes, and homogenized by ultrasonication. Enzyme assays, including thin-layer chromatography of substrate and products, were performed as described previously (17). The enzyme activity was expressed as the percent conversion of [^{14}C]chloramphenicol into the acetylated forms per hour per milligram of cell protein. In all cases in which the effect of dexamethasone on CAT expression was quantitated, the reaction conditions were selected such that the measured activity fell within the linear range of the assay. In each experimental series, pSV2CAT and pSV0CAT were included as references for the upper and lower limits of CAT enzyme activity in the particular transformation.

In initial experiments (see Fig. 4), one dish of L cells was transformed with each of the CAT plasmids. After 20 h in culture, the treated cells were trypsinized and divided equally between two dishes. One dish was maintained without dexamethasone and one dish was maintained with dexamethasone for 24 h. This regimen was designed to ensure that identical cell cultures were compared. However, we found that the CAT activities from duplicate cell cultures, when transformed in parallel with identical DNA-DEAE solutions, did not differ significantly from each other. Therefore, the simpler protocol of establishing duplicate cultures of transformed cells was adopted for assaying the activities of CAT constructs (see Fig. 5 and 6).

RESULTS

The cloned rat AGP gene contains the element for glucocorticoid-dependent expression. The rat AGP gene present in λAGP DNA is indistinguishable from the AGP gene described by Reinke and Feigelson, as determined by restriction enzyme analysis and partial DNA sequencing (33). To ensure that λAGP also contained the element responsible for

glucocorticoid-dependent expression, L cells were cotransformed with pSV2gpt and λAGP DNA. Twelve clones that were able to grow in the presence of mycophenolic acid and xanthine were tested for AGP expression. All clones showed dexamethasone-stimulated AGP synthesis (data not shown). Although the elevated levels of AGP production were, without exception, below that of rat hepatoma (2) or liver cells (4, 5), the hormone specificity and kinetics of induction were identical to those described for hepatic cells (2, 33, 34, 42). We conclude from these experiments that the fully functional AGP gene was isolated.

As an initial step towards identifying the steroid-sensitive DNA regulatory elements, sequences 5′ to the transcribed portion of the AGP gene were assayed for the ability to confer a dexamethasone response. The 9-kb *Ava*I fragment of λAGP DNA, containing the entire AGP gene-coding region plus 700 bp of 5′ flanking DNA and 5,000 bp of 3′ flanking DNA, was subcloned into pUC13 (Fig. 1B). The resulting plasmid, pAGP, was tested for proper AGP gene regulation in L cells as described above. For each of the cloned transformants, dexamethasone caused a massive stimulation of rat AGP synthesis and secretion (Fig. 2). Induction of rat AGP gene expression did not impair the glucocorticoid stimulation of the endogenous L-cell secretory glycoproteins. The rate of AGP synthesis, determined by quantitative rocket immunoelectrophoresis, surpassed by severalfold that of dexamethasone-treated rat liver or primary rat hepatocytes. The relative change in AGP production was reflected by equally elevated AGP mRNA levels (Fig. 3). Furthermore, the amount of AGP mRNA per transformed L cell after dexamethasone treatment was directly correlated to the number of AGP genes per cell (data not shown). For instance, Southern blot analysis revealed the presence of 10 AGP gene copies per cell for the cell line used in the experiments described in the legends to Fig. 2 and 3. Other cell lines contained fewer genes (i.e., 5) and more genes (i.e., 15 to 25) and also produced proportionally lower or higher quantities of AGP mRNA and protein. This close correlation between the gene number, the amount of mRNA, and the rate of protein synthesis suggests that each integrated AGP gene is functional and regulated by dexamethasone in the transformed cells.

Glucocorticoid-dependent regulation of AGP gene expression is mediated by elements in the 5′ flanking region. Having demonstrated that pAGP contains all of the information necessary for glucocorticoid regulation in L cells, it was important to map more precisely the glucocorticoid-responsive element. Sequence analysis has indicated that the gene contains several potential glucocorticoid receptor-binding sites both inside and outside of the translated region (25, 33). Two sites have been localized upstream of the gene at positions −438 and −271. The latter coincides with the *Kpn*I site at position −262. To determine whether either of these sites functions in regulating the AGP gene, a *Hin*dIII-*Hpa*II fragment that contains the entire 5′ flanking region, including the TATA box (located at positions −27 to −21), the transcription initiation site, and 21 bp of the 5′ untranslated region, was excised from pAGP and inserted into pSV0CAT upstream of the CAT gene, yielding pAGP(700)-CAT (Fig. 1C). As shown by others (26), a test of promoter activity with a hybrid promoter-CAT gene can be conveniently performed in L cells (Fig. 4A). This promoter assay is highly sensitive, as demonstrated by the difference in enzyme activity measured for cells transformed with pSV2CAT DNA and the enhancerless and promoterless pSV0CAT DNA. When pAGP(700)-CAT DNA was introduced into L cells, a 30- to 100-fold increase in CAT activity was measured in the presence of dexamethasone relative to that measured in the absence of dexamethasone, suggesting that the 700-bp 5′ flanking region conferred glucocorticoid-dependent regulation to the indicator gene (Fig. 4B). The basal level of CAT enzyme activity derived from pAGP(700)-CAT in L cells not treated with dexamethasone was substantially lower than the level of CAT enzyme activity in L cells transformed with pSV0CAT. L cells transformed with pAGP(700–CAT reverse did not express any detectable CAT activity either with or without dexamethasone treatment (Fig. 4B), indicating that the glucocorticoid inducibility of CAT activity was dependent upon the orientation of the

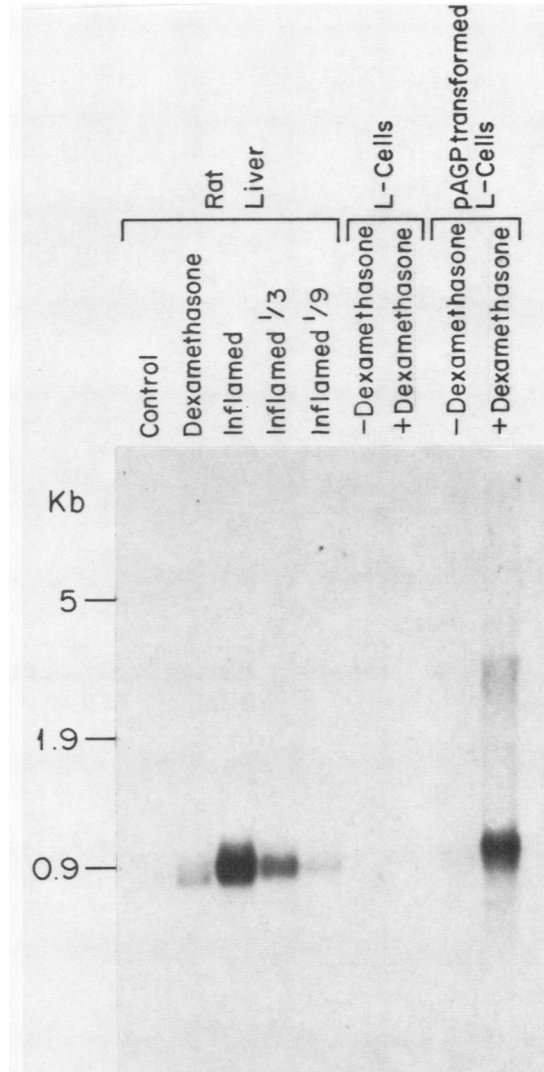

FIG. 3. Effect of dexamethasone on AGP mRNA levels. Total cellular RNA was extracted (16) from normal L cells and pAGP-transformed L cells (the same clonal cell line as described in Fig. 2) that had been cultured for 24 h with or without 1 μM dexamethasone. For comparison, RNA of control liver and of liver 24 h after dexamethasone treatment or turpentine-induced inflammation (2) was also analyzed. RNA (15 μg, or 1/3 and 1/9 dilutions of 15 μg where noted) was electrophoresed in a 1.5% agarose gel in the presence of formaldehyde, transferred to nitrocellulose, and hybridized to nick translated pIRL-10 DNA containing 850 bp of rat AGP cDNA. The nitrocellulose was exposured for 3 h to X-ray film.

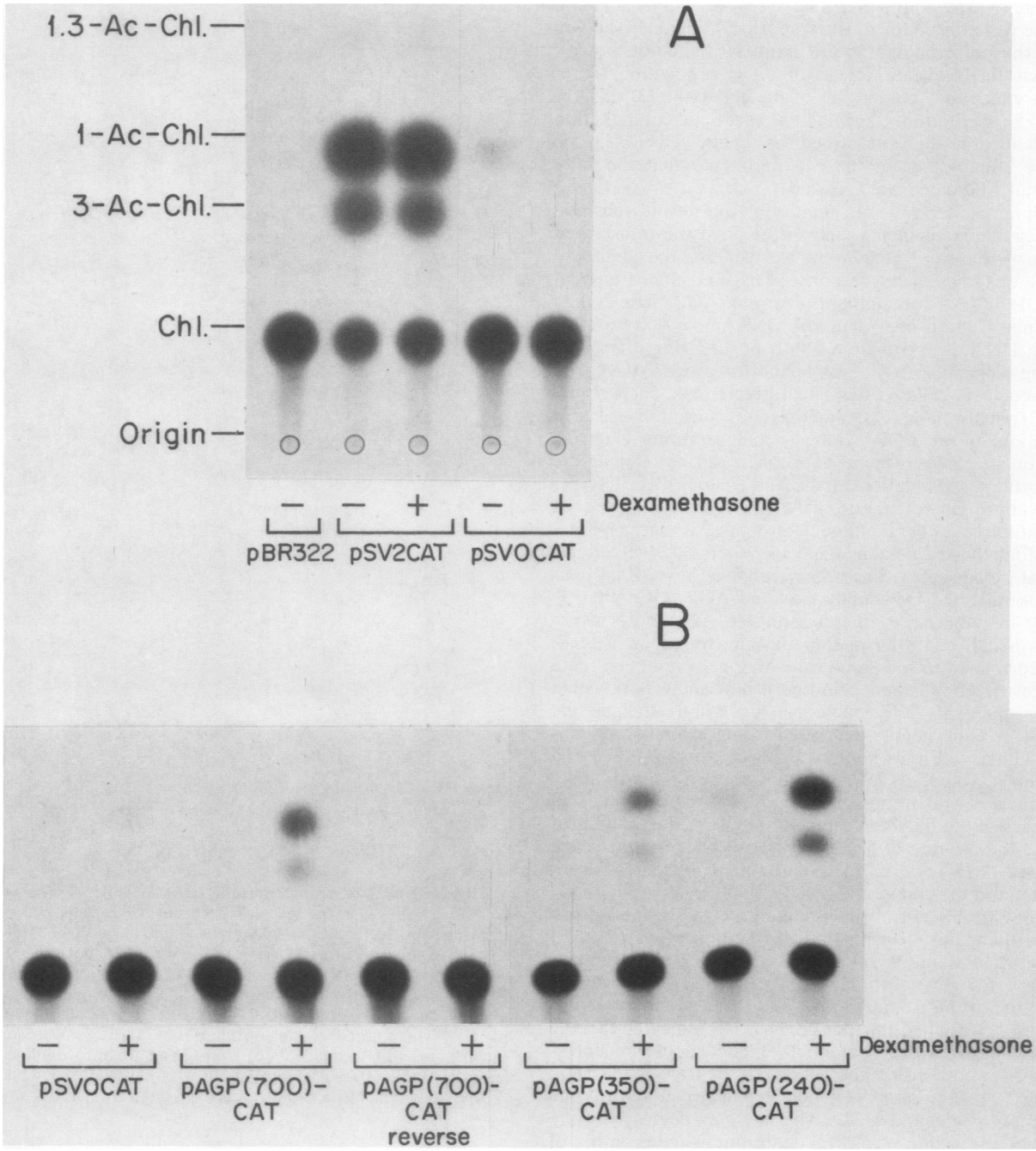

FIG. 4. Expression of CAT activity in transformed L cells. (A) To determine the sensitivity of the assay and the influence of dexamethasone, 10 μg of plasmid DNA was introduced into L cells (3×10^6 per 10-cm dish). After 18 h, the cells were trypsinized and divided between two dishes, and one was maintained without dexamethasone and one was maintained with dexamethasone. After 24 h, the cells were washed, scraped, and homogenized by ultrasonication in 100 μl of 0.25 mM Tris hydrochloride (pH 7.8). After microcentrifugation, 25 μl of cell extract containing 180 to 250 μg of protein was added to 80 μl of a reaction mixture composed of 0.75 M Tris hydrochloride (pH 7.8), 0.6 mM acetyl coenzyme A, and 0.4 μCi of [^{14}C]chloramphenicol (Amersham) (17, 26). The reaction was stopped after a 2-h incubation at 37°C by extraction with 1 ml of ethyl acetate. Chloramphenicol (Chl) and its acetylated forms (3-Ac-Chl, 1-Ac-Chl, and 1,3-Ac-Chl) were separated by thin-layer chromatography (17) and exposed to X-ray film for 20 h. (B) Dexamethasone-regulated expression of AGP-CAT gene fusions was assayed in L cells by transformation as described for panel A. pAGP(700)-CAT reverse refers to a construct in which the 700-bp AGP gene fragment was inserted in the reverse orientation relative to pAGP(700)-CAT; i.e., transcription of the 700-bp fragment and that of the CAT gene are divergent.

AGP insert. From these results, we conclude that the 700-bp AGP gene fragment contained a functional promoter as well as sequences that determine glucocorticoid-induced levels of AGP gene expression. The extent to which the promoter and glucocorticoid-responsive element overlapped could not be deduced from these experiments (see below).

Basal level and glucocorticoid-stimulated transcription are controlled by 140 bp immediately upstream of the AGP gene transcription start site. To assess the distal boundary of the element that is essential for glucocorticoid regulation, CAT plasmid DNAs harboring progressive deletions from the distal end of the 700-bp AGP insert were introduced into L cells (Fig. 1C). For each deletion, the CAT activity in transformed cells grown in the absence or presence of dexamethasone was quantitated to determine the levels of basal and stimulated CAT gene expression (see Fig. 4B, 5, and 6A). Stepwise elimination of the two sequences sharing homology with known glucocorticoid receptor-binding sites did not significantly affect dexamethasone stimulation of CAT expression [compare pAGP(350)-CAT and pAGP(240)-CAT with pAGP(700)-CAT in Fig. 4B]. Of all the deletions tested, pAGP(160)-CAT consistently showed the highest inducibility (Fig. 5). With the removal of 30 bp from pAGP(140)-CAT, yielding pAGP(110)-CAT, a drastic reduction of glucocorticoid regulation occurred. Although CAT expression was consistently elevated by dexamethasone, the magnitude of stimulation did not exceed onefold above basal level. More extensive deletions (concomitant with the loss of the *PstI* site at position −64) to generate pAGP(90)-CAT resulted in a level of CAT gene expression that was completely refractory to dexamethasone (Fig. 5 and 6A). Similarly, no glucocorticoid regulation was detected with the more truncated pAGP-CAT DNAs. Only when the AGP insert was reduced to 40 bp was the basal level of CAT gene expression restored to levels approximating those of pSV0CAT (Fig. 5 and 6A). Possibly, sequences within the 140 bp immediately upstream of the AGP transcription start site are not only responsible for glucocorticoid regulation but also for maintaining a low level of expression in the absence of the steroid.

To confirm the results obtained with the deletions indicating that the region upstream of the *PstI* site at −64, and especially the predicted glucocorticoid receptor-binding sites at positions −438 and −271, are not involved in hormone regulation, AGP gene sequences extending from positions −678 to −220 (*Hin*dIII to *Bal*I) or from −338 to −220 (*Bgl*II to *Bal*I) (Fig. 1B) were placed in both orientations upstream of a human triosephosphate isomerase promoter. Previous studies of this housekeeping gene promoter have shown that pTPI(130)-CAT contains all of the sequences required for maximal CAT enzyme production (L. E. Maquat, manuscript in preparation; see Fig. 7B for the sequence). None of these AGP-TPI-CAT constructs showed any glucocorticoid regulation of CAT gene expression (data not shown). Moreover, the level of CAT gene expression directed by the TPI promoter, normally between 4- and 10-fold above that of pSV0CAT, was not affected by the AGP gene insertions. Therefore, the putative regulatory sequences at positions −438 and −271 were not active in the glucocorticoid response mediated by L cells.

Of all the deletions tested, a single construct, pAGP(210)-CAT, did not conform with the findings presented above. The particular construct consistently produced a basal level of CAT expression that was as high as the dexamethasone-induced level (Fig. 6B). At the present time, we have no explanation for this result. It is possible that juxtapositioning

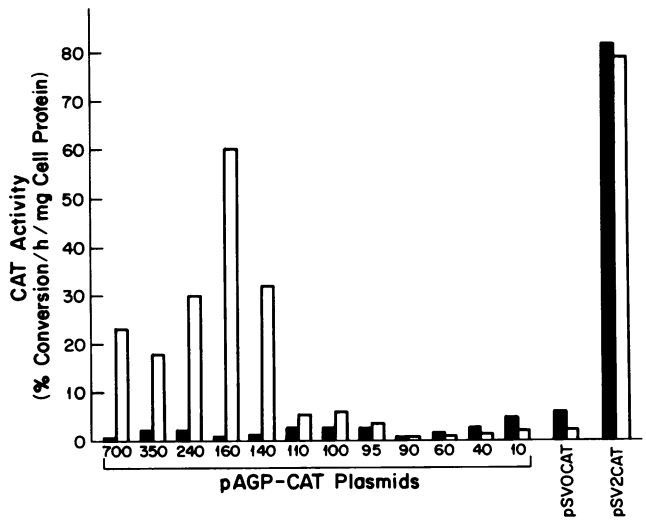

FIG. 5. Determination of the distal end of the glucocorticoid regulatory element. In three to four separate experiments, duplicate cultures of L cells were transformed with the indicated plasmid DNA (see Fig. 1C for representative structures) and assayed for CAT activity after treatment with (□) or without (■) dexamethasone as described in Materials and Methods and in the legend to Fig. 6. CAT activities were calculated as the percent conversion of substrate per hour per milligram of cell protein. Except for homogenates of cells transformed with pSV2CAT DNA, the CAT enzyme activity increased in a linear fashion with increasing reaction times (from 30 min to 4 h) and increasing concentrations of cell homogenates (from 100 to 750 μg of protein). The data are averaged from the different experiments.

pSV0CAT sequences and sequences at the 210-bp AGP insert creates a structural change that either interferes with a hypothetical suppression of transcription in the absence of dexamethasone or generates a site that is responsive to a transcriptional activator.

The glucocorticoid regulatory element lies between positions −120 and −42. The results from the transformation experiments have localized the glucocorticoid regulatory element to a region between positions −140 and +21. The ability of sequences upstream of the AGP gene to regulate CAT gene expression suggests that the glucocorticoid response is at the transcriptional level, in agreement with measurements of AGP gene transcription rates in rat liver nuclei (23) and primary cultures of rat hepatocytes (4). However, based on nuclear runoff data, Vannice et al. (43) have proposed that glucocorticoid regulation of AGP expression in rat hepatoma (HTC) cells is managed solely by changes in the posttranscriptional stability of newly synthesized RNA. Since the pAGP-CAT constructs contain 21 bp of the AGP gene 5′ untranslated region, it is conceivable that this segment by itself can confer glucocorticoid-dependent stability to the AGP-CAT transcripts. The data presented above do not differentiate between this hypothesis and a model envoking a transcriptional response to the steroid.

To determine the proximal boundary of the AGP glucocorticoid-responsive element and, in particular, whether the 5′ untranslated region of the AGP gene is essential for glucocorticoid regulation, progressive deletions were introduced into the proximal portion of the AGP insert in pAGP(140)-CAT. This plasmid DNA was cleaved at the AGP-CAT gene junction by *Hin*dIII and then incubated with nuclease *Bal* 31. Two fragments that had 39 and 62 bp

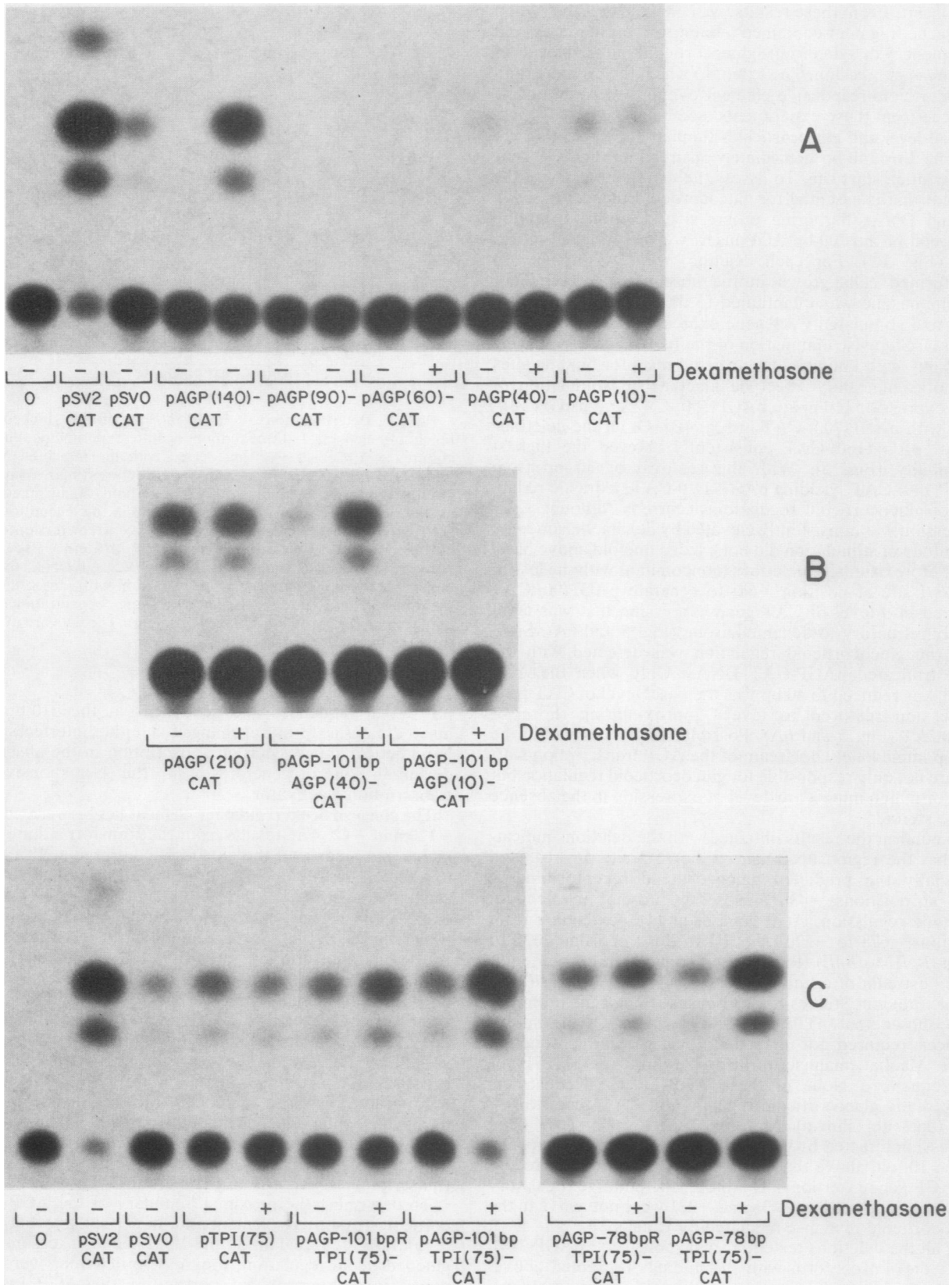

FIG. 6. Effect of distal and proximal deletions on the glucocorticoid-regulated expression of pAGP-CAT plasmids. L-cell transformations in duplicate and in three separate experiments and CAT enzyme assays were performed as described in the legend to Fig. 5. Representative results of these experiments are reproduced. (A) With progressive distal deletions within the AGP-derived insert of pAGP-CAT, the

removed from the AGP gene insert (and contained sequences −120 to −19 and −120 to −42, respectively) were chosen for further study (Fig. 7). Since the larger (101-bp) fragment still included the AGP gene TATA box, it was recombined with pAGP(40)-CAT and pAGP(10)-CAT, which lack this box. The first construct was used to assess the extent to which restoration of both steroid regulation and promotion takes place. The latter construct was used to determine whether the 101-bp fragment still contains a fully functional promoter. Insertion of the 101-bp fragment in the correct orientation upstream of the AGP sequence in pAGP(40)-CAT regenerated a level of glucocorticoid-regulated CAT gene expression that was comparable to the level found for the parental pAGP(140)-CAT construct (Fig. 6B). In the absence of dexamethasone, the basal level of the 101-bp insertion construct was 10-fold higher than that of pAGP(140)-CAT (Fig. 6A). The cause of this increase in basal level is unknown but must reflect the difference in the promoter region which includes one *NdeI* linker sequence. A reverse orientation of the 101-bp fragment in pAGP(40)-CAT failed to exert any effect on the indicator gene, and CAT gene expression was identical to the expression of unmodified pAGP(40)-CAT DNA (see Fig. 6A). Addition of the 101-bp fragment in the correct orientation upstream of the AGP sequence in pAGP(10)-CAT showed a very reduced but still detectable glucocorticoid-mediated induction of CAT activity (Fig. 6B). It is likely that the internal sequence spanning positions −20 to +10, which is missing from the latter construct, is needed to form a fully functional promoter.

The functionality of the 101-bp fragment was next assayed independently of an AGP promoter. First, the fragment was inserted upstream of the TPI region of pTPI(42)-CAT, a plasmid that contains a nonfunctional TPI gene promoter. Assay of this construct in L cells showed no regulation and no promotion of CAT gene transcription above the pSV0CAT level with or without dexamethasone (data not shown). By using pTPI(75)-CAT, a plasmid with a functional TPI promoter, as a recipient for the 101-bp fragment, glucocorticoid-regulated CAT expression was reconstructed (Fig. 6C). The only functional difference between this chimeric construct and the parental pAGP(140)-CAT construct was the absence of a reduction in the basal level of CAT gene expression. The basal level was essentially the same as that observed for pTPI(75)-CAT. The glucocorticoid regulation mediated by the 101-bp fragment in pTPI(75)-CAT was partly dependent on fragment orientation. In reverse orientation, the 101-bp fragment still conferred regulation to the CAT gene; however, the magnitude of stimulation was found in three independent measurements to be only 2-fold as compared with 5- to 10-fold in constructs with the correct orientation (Fig. 6C).

To assess whether the TATA box that is still present in the 101-bp fragment is necessary for glucocorticoid regulation, the more truncated 78-bp fragment containing the sequence −120 to −42 (Fig. 7) was inserted in both orientations upstream of the TPI region of pTPI(75)-CAT. Assay of these constructs yielded essentially the same qualitative and quantitative results as were obtained with the 101-bp fragment (Fig. 6C).

In summary, results from the distal and proximal AGP gene deletion studies (Fig. 5 and 6) localized the sequence through which glucocorticoid enhances expression of the AGP gene to the region between positions −120 to −42.

DISCUSSION

A rat genomic sequence encoding the entire AGP gene has been isolated and tested for glucocorticoid-responsive elements in L cells. L cells were chosen because of their high transformation efficiency and because of their glucocorticoid responsiveness. Indeed, L cells and hepatic cells produced the same kinetics and specificity of dexamethasone-induced rat AGP gene expression (33). The dexamethasone-sensitive rat hepatoma cells (HTC or H-35 cells) were highly inefficient in incorporating DNA by either the DEAE-dextran method (26) or the calcium phosphate method (18) and were, therefore, not suitable for testing regulatory gene elements.

A single glucocorticoid-regulating sequence was found to lie between positions −120 and −42 of the AGP gene (Fig. 7). This sequence may represent a new type of glucocorticoid-responsive element because it does not share homology with the bona fide dexamethasone receptor-binding sites of other genes (9, 13, 19, 30, 32, 37, 38). Surprisingly, sequences at positions −431 and −272 that contain homologies to known steroid receptor-binding sites were inactive in L cells. We have to stress, however, that in the absence of corresponding studies in hepatic cells, we cannot rule out the possibility that these sites are operative in hepatocytes but nonfunctional in L cells. The putative steroid receptor-binding sites lying downstream of the transcription start site at positions +854, +4014, and +4296 (25) may also contribute to hepatic AGP gene regulation. These sequences have yet to be tested for function in either L cells or in hepatic cells.

The regulatory element of the AGP gene is similar to the element of other steroid-sensitive genes (9, 19, 37) in that it enhanced transcription and acted independently of orientation (Fig. 6C). However, the AGP element differed from all other characterized dexamethasone receptor-binding sites not only in sequence but also in its very close proximity to the promoter region. It is premature to speculate about the mechanistic involvement of the −120 to −42 region in steroid regulation without first determining whether the dexamethasone receptor interacts with this AGP gene element and if AGP gene regulation involves the same cellular factors as other glucocorticoid-sensitive genes. Consequently, we will make a major effort to establish (i) what nuclear factors, including a glucocorticoid receptor, interact with this regulatory sequence of the AGP gene, (ii) how these factors assemble to ensure enhanced transcription of the AGP gene, and (iii) how glucocorticoid-mediated AGP

glucocorticoid regulation of CAT gene expression is lost, and the basal level typical of pSV0CAT is restored. (B) The expression of CAT activity in L cells transformed with pAGP(210)CAT is as high in the absence as in the presence of dexamethasone. When the 101-bp AGP gene fragment (−120 to −19) was placed upstream and in the same transcriptional orientation as the transcriptionally inactive pAGP(40)-CAT construct, promoter activity and glucocorticoid regulation are fully restored. The same fragment when placed upstream of pAGP(10)-CAT is unable to regenerate promoter activity although glucocorticoid stimulation of CAT expression is detectable. (C) The 101- and 78-bp AGP gene fragments, when placed upstream of and in the same transcriptional orientation as the TPI promoter in pTPI(75)-CAT, confer full glucocorticoid inducibility to the CAT gene. The fragments in reverse orientation (lanes pAGP-101 bp R-TPI(75)-CAT and pAGP-78 bp R-TPI(75)-CAT) confer glucocorticoid inducibility, but to a lower extent.

A. AGP

```
AGCCCAAAGCTGGCTTGAGGGAACATTTTGTGCAAGACATTTCCCAAGTGCTGGTGAGATTGTGCCACAGCTCTGCAGCC
-140      -130      -120      -110      -100      -90       -80       -70 PstI

CCTGGCGACGCCCATGCCCTTCCCACACCTTGTTATAAAAGTCACTGCACTCTCCAGCCACCAGTTAGCTCTTCCTGGGCC
-60       -50       -40       -30       -20       -10       +1        +10       +20
```

B. TPI

```
GGCCGGGGCGGCGGCAGGAGGGCGGGCGGGGGGCAGGGCTCCGGGGGACTGGGCGGGCCATGGCGGAGGA
-130      -120      -110      -100      -90       -80       -70

CGGCGAGGAGGCGGAGTTCCACTTCGCGGCGCTCTATATAAGTGGGCAGTGGCCGACTGCGCG
-60       -50       -40       -30       -20       -10       +1
```

C.

Construct	Distal/Proximal Endpoints of AGP Gene Inserts	Distal/Proximal Endpoints of TPI Gene Inserts
pAGP(160)-CAT	-140/+21	-
pAGP(140)-CAT	-120/+21	-
pAGP(90)-CAT	-64/+21	-
pAGP-101 bp TPI(75)-CAT	-120/-19	-75/+3
pAGP-78 bp TPI(75)-CAT	-120/-42	-75/+3

FIG. 7. (A) Nucleotide sequence of the 5′ flanking region of the AGP gene containing the glucocorticoid regulatory element. The TATA box is enframed, the diagnostic *Pst*I site at position −64 is underlined, and the minimal sequence required for glucocorticoid regulation represented by the 78-bp AGP insert is overlined. Nucleotides are numbered relative to the transcription start site, +1, as defined by Reinke and Feigelson (33). Our sequence data are identical to the data of those authors; however, ambiguities observed with both the dideoxy chain termination and chemical modification methods prevented conclusive assessment of positions −57 to −55. (B) Nucleotide sequence of the 5′ flanking region of the TPI gene present in the AGP-TPI-CAT constructs. The TATA box is enframed. Nucleotides are numbered relative to the transcription start site, +1 (8). (C) Distal and proximal endpoints of the AGP gene inserts in the indicated constructs are listed. End points are numbered as in panels A and B and were determined by sequencing each construct directly.

gene regulation compares with the regulation of other glucocorticoid-responsive genes, e.g., the mouse mammary tumor virus (9, 32, 37, 38), metallothionein (19), and rat fibrinogen genes (14).

Our results with AGP-CAT constructs tested in L cells suggest that the first 50 bp upstream of the AGP gene were responsible for maintaining a level of transcription that is well below that of the promoterless pSV0CAT element in the absence of dexamethasone. Since the glucocorticoid regulatory element and the promoter of the AGP gene are inseparable in our assay, it may be that glucocorticoid regulation of the AGP gene is achieved not only by enhancing transcription initiation but also by relieving an inhibition of transcription. If the low level of transcription is lost, e.g., through mutation, a phenotype of AGP gene expression would be obtained as described for HTC cells (15). Whether HTC cells are able to increase the characteristically high level of AGP gene transcription upon exposure to dexamethasone is not certain due to the semiquantitative nature of the transcription assay used by Vannice et al. (15).

AGP gene regulation in the liver during the acute-phase response is influenced not only by corticosteroids but also by other inflammatory factors (2), such as the hepatocyte-stimulating factor (35). The experiments reported here shed some light on the regulatory mechanisms controlled by steroids. Future studies will provide information on the mechanism of AGP induction by hepatocyte stimulating factor and how AGP gene expression is coordinated with the expression of genes that encode other acute-phase plasma proteins (12, 14).

ACKNOWLEDGMENTS

We thank Ira O. Daar for his excellent instructions of molecular technologies to H.B.; Robert Hughes, Jr., for his advice on establishing stably transformed cell lines; Gerald P. Jahreis, James R. Krug, and Wendy Jo Rickard for technical assistance; John Yates for comments on the manuscript; and Marcia Held for secretarial work. We also thank P. Feigelson, Columbia University, N.Y., for encouraging H.B. to make a full commitment to this project.

This work was supported by Public Health Service grants CA26122 and AM/GM33938 from the National Institutes of Health. Both authors are recipients of an Established Investigator Award from the American Heart Association.

LITERATURE CITED

1. **Baumann, H., and F. G. Berger.** 1985. Genetics and evolution of the acute phase proteins in mice. Mol. Gen. Genet. **201**:505–512.
2. **Baumann, H., C. L. Firestone, T. L. Burgess, K. W. Gross, K. R. Yamamoto, and W. A. Held.** 1983. Dexamethasone regulation of α_1-acid glycoprotein and other acute phase reactants in rat liver and hepatoma cells. J. Biol. Chem. **258**:563–570.
3. **Baumann, H., and W. A. Held.** 1981. Biosynthesis and hormone-regulated expression of secretory glycoproteins in rat liver and hepatoma cells. Effect of glucocorticoids and inflammation. J. Biol. Chem. **256**:10145–10155.
4. **Baumann, H., R. E. Hill, D. N. Sauder, and G. P. Jahreis.** 1986. Regulation of major acute phase plasma proteins by hepatocyte-stimulating factors of human squamous carcinoma cells. J. Cell Biol. **102**:370–383.
5. **Baumann, H., G. P. Jahreis, D. N. Sauder, and A. Koj.** 1984. Human keratinocytes and monocytes release factors which regulate the synthesis of major acute phase plasma proteins in hepatic cells from man, rat and mouse. J. Biol. Chem. **259**:7331–7342.

6. **Bennett, M., and K. Schmid.** 1980. Immunosuppression by human plasma α_1-acid glycoproteins: importance of the carbohydrate moiety. Proc. Natl. Acad. Sci. USA **77**:6109–6113.
7. **Bonner, W. M., and R. A. Laskey.** 1974. A film detection for tritium-labeled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. **46**:83–88.
8. **Brown, J. R., I. O. Daar, J. R. Krug, and L. E. Maquat.** 1985. Characterization of the functional gene and several processed pseudogenes in the human triosephosphate isomerase gene family. Mol. Cell. Biol. **5**:1694–1706.
9. **Chandler, V. L., B. A. Maler, and K. R. Yamamoto.** 1983. DNA sequences bound specifically by glucocorticoid receptor in vitro render a heterologous promoter hormone responsive in vivo. Cell **33**:489–499.
10. **Cheresh, P. A., D. H. Haynes, and J. A. Distasio.** 1984. Interaction of an acute phase reactant, α_1-acid glycoprotein (orosomucoid) with the lymphoid cell surface: a model for non-specific immune suppression. Immunology **51**:541–548.
11. **Chiu, K. M., R. F. Mortenson, A. P. Osmand, and H. Gewurz.** 1977. Interactions of α_1-acid glycoprotein with the immune system. Immunology **32**:997–1005.
12. **Crabtree, G. R., and J. A. Kant.** 1982. Coordinate accumulation of the mRNAs for the α, β, and γ chains of rat fibrinogen following defibrination. J. Biol. Chem. **257**:7277–7279.
13. **Davidson, E. H., H. T. Jacobs, and R. J. Britten.** 1983. Very short repeats and coordinate induction of genes. Nature (London) **301**:468–470.
14. **Fowlkes, D. M., N. T. Mullis, C. M. Coureau, and G. R. Crabtree.** 1984. Potential basis for the regulation of the coordinately expressed fibrinogen genes: homology in the 5′ flanking regions. Proc. Natl. Acad. Sci. USA **81**:2313–2316.
15. **Friedman, J. M.** 1983. Control of malaria virulence by α_1-acid glycoprotein (orosomucoid), an acute-phase (inflammatory) reactant. Proc. Natl. Acad. Sci. USA **80**:5421–542.
16. **Glisin, V., R. Crkvenjakov, and C. Byus.** 1974. Ribonucleic acid isolation by cesium chloride centrifugation. Biochemistry **13**:2633–2637.
17. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2**:1044–1051.
18. **Graham, F. L., and A. J. Van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52**:456–461.
19. **Karin, M., A. Haslinger, H. Holtgreve, R. I. Richards, P. Krauter, H. M. Westphal, and M. Beato.** 1984. Characterization of DNA sequences through which cadmium and glucocorticoid hormones induce human metallothionein-II_A gene. Nature (London) **308**:513–519.
20. **Koj, A.** 1974. Acute phase reactants, p. 73–125. *In* A. C. Allison (ed.), Structure and function of plasma proteins, vol. 1. Plenum Publishing Corp., New York.
21. **Koj, A., A. Dubin, H. Kasperczyk, J. Beretta, and A. H. Gordon.** 1982. Changes in the blood level and affinity to concanavalin A of rat plasma glycoproteins during acute inflammation and hepatoma growth. Biochem. J. **206**:545–553.
22. **Koj, A., J. Gauldie, E. Regoeczi, D. N. Sauder, and A. D. Sweeney.** 1984. The acute phase response of cultured rat hepatocytes. Biochem. J. **224**:505–514.
23. **Kulkarni, A. B., R. Reinke, and P. Feigelson.** 1985. Acute phase mediators and glucocorticoids elevate α_1-acid glycoprotein gene transcription. J. Biol. Chem. **260**:15386–15389.
24. **Kushner, I.** 1982. The phenomenon of the acute phase response. Ann. N.Y. Acad. Sci. **389**:39–48.
25. **Liao, Y.-C. J., J. M. Taylor, J. L. Vannice, G. A. Clawson, and E. A. Smuckler.** 1985. Structure of the rat α_1-acid glycoprotein gene. Mol. Cell. Biol. **5**:3634–3639.
26. **Lopata, M. A., D. W. Cleveland, and B. Sollner-Webb.** 1984. High level transient expression of a chloramphenicol acetyl transferase gene by DEAE-dextran mediated DNA transfection coupled with a dimethylsulfoxide or glycerol shock treatment. Nucleic Acids Res. **12**:5707–5717.
27. **Luthman, H., and G. Magnusson.** 1983. High efficiency polyoma DNA transfection of chloroquine treated cells. Nucleic Acids Res. **5**:1295–1308.
28. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
29. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65**:499–560.
30. **Moore, D. D., A. R. Marks, D. I. Buckley, G. Kapler, F. Payvar, and H. Goodman.** 1985. The first intron of the human growth hormone gene contains a binding site for glucocorticoid receptor. Proc. Natl. Acad. Sci. USA **82**:699–702.
31. **O'Farrell, P. H.** 1975. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. **250**:4007–4021.
32. **Ponta, H., N. Kennedy, P. Skroch, N. E. Hynes, and B. Groner.** 1985. Hormonal response region in the mouse mammary tumor virus long terminal repeat can be dissociated from the proviral promoter and has enhancer properties. Proc. Natl. Acad. Sci. USA **82**:1020–1024.
33. **Reinke, R., and P. Feigelson.** 1985. Rat α_1-acid glycoprotein gene sequence and regulation by glucocorticoids in transfected L-cells. J. Biol. Chem. **260**:4397–4403.
34. **Ricca, G. A., R. W. Hamilton, J. W. McLean, A. Conn, J. E. Kalinyak, and J. M. Taylor.** 1981. Rat α_1-acid glycoprotein messenger RNA. J. Biol. Chem. **256**:10362–10368.
35. **Ritchie, D. G., and G. M. Fuller.** 1983. Hepatocyte-stimulating factor: a monocyte-derived acute-phase regulatory protein. Ann. N.Y. Acad. Sci. **408**:490–502.
36. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
37. **Scheidereit, C., and M. Beato.** 1984. Contact between hormone receptor and DNA double helix within a glucocorticoid regulatory element of mouse mammary tumor virus. Proc. Natl. Acad. Sci. USA **81**:3029–3033.
38. **Scheidereit, C., S. Geisse, H. M. Westphal, and M. Beato.** 1983. The glucocorticoid receptor binds to defined nucleotide sequences near the promoter of mouse mammary tumor virus. Nature (London) **304**:749–752.
39. **Schmid, K.** 1975. α_1-acid glycoprotein, p. 184–228. *In* F. Putnam (ed.), The plasma proteins: structure, function and genetic control, vol. 1. Academic Press, Inc., New York.
40. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
41. **Southern, P. J., and P. Berg.** 1982. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. J. Mol. Appl. Genet. **1**:327–341.
42. **Vannice, J. L., G. M. Ringold, J. W. McLean, and J. M. Taylor.** 1983. Induction of the acute-phase reactant, α_1-acid glycoprotein, by glucocorticoids in rat hepatoma cells. DNA **2**:205–212.
43. **Vannice, J. L., J. M. Taylor, and G. M. Ringold.** 1984. Glucocorticoid-mediated induction of α_1-acid glycoprotein: evidence for hormone-regulated RNA processing. Proc. Natl. Acad. Sci. USA **81**:4241–4245.
44. **Wigler, M., S. Silverstein, L. S. Lee, A. Pellicer, Y. C. Cheng, and R. Axel.** 1977. Transfer of purified herpes virus thymidine kinase gene to cultured mouse cells. Cell **11**:223–232.