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Simultaneous Expression of Salivary and Pancreatic Amylase Genes in Cultured Mouse Hepatoma Cells

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The tissue-specific expression of two types of mouse amylase genes does not overlap in vivo; the Amy-1 locus is transcribed in the parotid gland and the liver, while expression of *Amy-2* is limited to the pancreas. We identified a mouse hepatoma cell line, Hepa 1-6, in which both amylase genes can be simultaneously expressed. *Amy-1* is constitutively active in these cells and is inducible by dexamethasone at the level of mRNA. We demonstrated that the liver-specific promoter of *Amy-1* is utilized by the dexamethasone-treated hepatoma cells, and that glucocorticoid consensus sequences are present upstream of this promoter. *Amy-2* is not detectable constitutively, but can be activated if the cells are cultured in serum-free medium containing dexamethasone. Expression of *Amy-2* in a nonpancreatic cell type has not previously been observed. We speculate that induction of *Amy-1* and activation of *Amy-2* may involve different regulatory mechanisms. Hepa 1-6 cells provide an experimental system for molecular analysis of these events.

The mouse amylase multigene family includes two types of genes, *Amy-1* and *Amy-2*, which are closely related in structure but differ in tissue specificity (8, 23). The Amy-1 locus, which is expressed in the parotid gland and in the liver, is unusual in that two alternative promoters are associated with the same coding sequences (9, 24). The parotid-specific promoter of *Amy-1* is located 7.6 kilobases (kb) upstream from the first codon and the weaker liver promoter is at a position between the parotid promoter and the first codon (30). The liver promoter is also transcribed at a low level in the parotid gland (25). Expression of *Amy-2* appears to be limited to the pancreas in adult animals.

The amylase genes are clustered on mouse chromosome 3, and an intergenic distance of 22 kb between copies of *Amy-1* and *Amy-2* has been observed in two inbred strains (20, 29). The chromosomal arrangement of *Amy-1* and *Amy-2* and their tissue-specific promoters is represented in Fig. 1.

Glucocorticoids appear to function as developmental regulatory signals for *Amy-1* and *Amy-2*. Administration of dexamethasone to pregnant rats induces amylase synthesis in the fetal pancreas several days before its normal appearance (11, 21). In the interval between birth and weaning, dexamethasone induces premature development of adult amylase levels in the rat pancreas and the parotid gland (22, 27). In addition, induction of amylase by dexamethasone has recently been observed in cultured pancreatic acinar cells (15). The effect of dexamethasone on amylase expression in the liver, on the other hand, has not been previously studied. The mechanism of glucocorticoid induction of several mammalian genes appears to be mediated by the binding of the steroid-receptor complex to a consensus sequence located upstream of the cap site (10, 14). It is not known whether related sequences are responsible for the induction of *Amy-1* and *Amy-2*.

Molecular characterization of the hormonal regulation of amylase genes will be facilitated by the availability of inducible cell lines. We have identified a mouse hepatoma cell line, Hepa 1-6, in which both *Amy-1* and *Amy-2* are regulated by the synthetic glucocorticoid dexamethasone. Hepa 1-6 was derived from the previously described Hepa 1 cells which retain several liver-specific phenotypes in vitro (6). In addition to the dexamethasone induction, the effects of other components of the culture medium on amylase expression have been studied.

MATERIALS AND METHODS

Cell culture. Hepa 1-6 was isolated as a subclone of Hepa 1, originally derived from the BW 7756 hepatoma of C57L/J mice (6). The cells were maintained in M/M medium (composed of three parts minimal essential medium and one part Waymouth MAB 87/3) plus 10% fetal bovine serum, 0.5% penicillin, and streptomycin (10,000 U/ml) (GIBCO Laboratories, Grand Island, N.Y.). For some experiments cells were grown in a defined medium which consisted of M/M plus 3×10^{-8} M selenium, with or without 10^{-6} M dexamethasone, or in fully supplemented medium (SuM) containing 50 ng of EGF per ml, 1 μg of insulin per ml (Collaborative Research, Lexington, Mass.), 10 μg of transferrin per ml, 3×10^{-8} M selenium, 10^{-6} M dexamethasone, 50 ng of growth hormone per ml, and 10^{-6} M triiodothyronine. Chemicals were obtained from Sigma Chemical Co., St. Louis, Mo., unless otherwise noted.

Cells were cultivated in tissue culture plastic flasks (Corning Glass Co., Corning, N.Y.), fed every 48 to 72 h, and passaged weekly by trypsinization.

Protein determinations. Cellular protein in the monolayer was solubilized in 8 M guanidium hydrochloride, 20 mM sodium acetate (pH 7.0). Protein concentrations were determined with the Bio-Rad protein assay kit (Bio-Rad Laboratories, Rockville Centre, N.Y.).

Amylase activity in cell supernatants. Amylase activity was quantitated by a modification of the 3,5-dinitrosalicylate assay as previously described (3). One unit of amylase activity catalyzes the release of 1 μmol of reducing groups per min at 30°C. The specific activity of purified mouse pancreatic amylase assayed by this method is approximately 2,000 U/mg (26).

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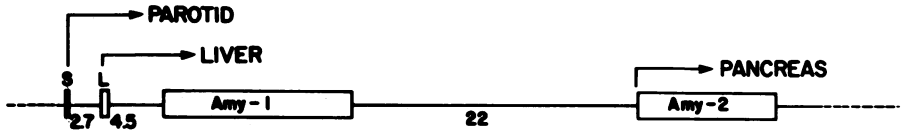

FIG. 1. Organization of the mouse amylase gene region. The locations of the three tissue-specific transcriptional promoters are indicated by the arrows.

To separate AMY-1 and AMY-2, 10 to 40 μl samples of culture medium were subjected to electrophoresis on 7.5% polyacrylamide gels (pH 8.1) for 20 to 24 h at 125 V, and amylase activity was visualized by the starch-iodine staining method as described previously (2). The gels were incubated at 37°C for 30 to 60 min in a 2% solution of potato starch (Sigma no. S-2630). The starch solution was removed by rinsing several times with water, and the gel was stained by the addition of KI/I_2 solution.

Immunotitration. We have previously described the rabbit antisera which were raised against purified amylase from mouse pancreas (3) and mouse salivary gland (12). At high concentrations, these antisera react with both amylases, but at appropriate dilutions, specific immunoprecipitation can be obtained. Culture medium was incubated overnight with twofold dilutions of antiserum, and the immunoprecipitated amylase was removed by centrifugation. The supernatant solution was analyzed by electrophoresis as described above.

Purification of ^{35}S-labeled amylase. Approximately 10^6 cells were cultured for 0 to 36 h in the presence of 100 μCi of ^{35}S-methionine per ml (Amersham Corp., Arlington Heights, Ill.). Amylase was purified from 0.5 ml of culture medium by precipitation with high-molecular-weight glycogen (3). Unlabeled carrier amylase was provided by the addition of 20 μl of a 20% homogenate of dog pancreas. This carrier was chosen because it does not comigrate with either of the mouse amylases. The purified amylase was examined by polyacrylamide gel electrophoresis as described above. The gels were fixed in 10% trichloroacetic acid, treated with En^{3}Hance (New England Nuclear Corp., Boston, Mass.), and dried onto clear dialysis tubing. The x-ray film was exposed at −70°C for 24 to 48 h.

Amylase mRNA levels. *Amy-1* mRNA was detected in polyadenylated [poly(A)$^+$] RNA isolated from cells grown in M/M plus 10% serum plus 10^{-6} M dexamethasone. Total cellular RNA was isolated after cell lysis in 8 M guanidium hydrochloride as described by Chirgwin et al. (5) and separated from protein and DNA by the procedure of Glisin et al. (7). Poly(A)$^+$ RNA was isolated by chromatography on oligodeoxythymidylate cellulose (1). After electrophoresis on formaldehyde (2.2 M)-agarose (2%) gels, the RNA was transferred to nitrocellulose filters (16). Hybridization was carried out at 60°C in 50% formamide with a mixture of two riboprobes derived from the cloned amylase cDNAs pMPa21 and pMSa104 (8). The riboprobes were constructed by isolation of the cDNA inserts after digestion with *Pst*I and cloning into the *Pst*I site of pGEM-1 (Promega Biotech, Madison, Wisc.). Riboprobe transcripts complementary to amylase mRNA were synthesized in the presence of ^{32}P-UTP, using T7 polymerase (Promega) as recommended by the supplier.

DNA sequence analysis. The isolation of a cosmid clone, cSamD4, containing the mouse *Amy-1* gene from strain YBR/Ki has been described previously (29). To locate the liver promoter, the restriction site map of the 5′ flanking region was compared with that of the gene from strain A/J (23). An 0.95-kb *Hin*dIII fragment was isolated and digested with *Eco*RI, which cleaves within the exon containing the 5′ noncoding region of the liver transcript. Fragments were kinase-labeled and sequenced by the method of Maxam and Gilbert (17), using a citrate (A+G) reaction (13).

RESULTS

Constitutive and inducible expression of *Amy-1* in Hepa 1-6 cells. In our initial experiments, Hepa 1-6 cells were cultured in M/M plus 10% serum. When cell extracts and conditioned culture medium were examined on acrylamide gels, both contained low levels of amylase activity. More than 95% of the amylase activity is present in extracellular medium. The electrophoretic mobility of this constitutively expressed and secreted amylase was identical to that of salivary amylase (AMY-1) from C57L/J mice, the strain of origin of the hepatoma line. To determine whether the expression of *Amy-1* was responsive to glucocorticoids, 10^{-6} M dexamethasone was added to the M/M plus serum culture medium for 48 h. A significant increase in amylase production was observed (Fig. 2, lanes 1 and 2). The concentration of amylase in the medium increased more than 30-fold during 48 h of culture in the presence of dexamethasone, from less than 0.04 U of culture medium per ml to 1.4 U/ml.

Induction of *Amy-2* in SuM. To determine the effects of other hormones and growth factors on amylase expression, Hepa 1-6 cells were cultured in SuM, which contains seven growth factors including 10^{-6} M dexamethasone. After six weeks of culture in SuM, the cells were secreting AMY-1 at the fully induced level described above (Fig. 2, lane 3). In addition, a second major amylase isozyme was also present in the medium at levels comparable to that of AMY-1. The electrophoretic mobility of the second isozyme was identical to that of pancreatic amylase (AMY-2) isolated from C57L/J mice. Minor activity bands corresponding to deamidation products of pancreatic amylase were also visible; these accumulated during storage of the samples. No amylase activity was detectable in control samples of SuM (data not shown).

Immunological identification of AMY-1 and AMY-2. To confirm the identity of the two amylase isozymes produced by Hepa 1-6 cells cultured in SuM, the medium was tested with specific antisera raised against purified mouse salivary and pancreatic amylases. Samples of conditioned SuM were incubated with twofold dilutions of antisera (Fig. 3). The antiserum to pancreatic amylase specifically removed the isozyme which comigrates with authentic AMY-2 as well as its deamidation products (Fig. 3A). Likewise, the antiserum to salivary amylase specifically removed the isozyme which comigrates with AMY-1. Thus by two independent criteria, electrophoretic mobility and antigenicity, the amylase proteins secreted by Hepa 1-6 cells can be identified as the products of the *Amy-1* and *Amy-2* genes.

De novo synthesis of AMY-1 and AMY-2. Fully induced Hepa 1-6 cells growing in SuM were labelled with [^{35}S]methionine. After 2 to 4 h, amylase protein was purified from the medium and examined by radioautography (Fig. 4). As

early as 2 h after the addition of [^{35}S]methionine, radiolabeled amylase was detected in the medium, and the amount of label in the secreted amylase increased with time. These results demonstrate that the products of *Amy-1* and *Amy-2* are synthesized from amino acid precursors by the hepatoma cells.

Effects of other growth factors. The effect of each component of SuM on amylase expression was tested. Dexamethasone was required for a high level of expression of both *Amy-1* and *Amy-2*. Fully-induced cells growing in SuM were transferred to medium containing all the components except dexamethasone (Fig. 5). The removal of dexamethasone resulted in the loss of *Amy-2* expression, and the return of *Amy-1* to constitutive levels.

Elimination of four components of SuM (triiodothyronine, epidermal growth factor, transferrin, and growth hormone), singly or in various combinations, did not influence the expression of *Amy-1* or *Amy-2*. Selenium was essential for cell growth in the absence of serum. Thus, the basal medium in which amylase induction was observed contained M/M, 3 $\times$ 10^{-8} M selenium, and 10^{-6} M dexamethasone.

The other component of SuM which influenced amylase secretion was insulin. When insulin was removed from SuM,

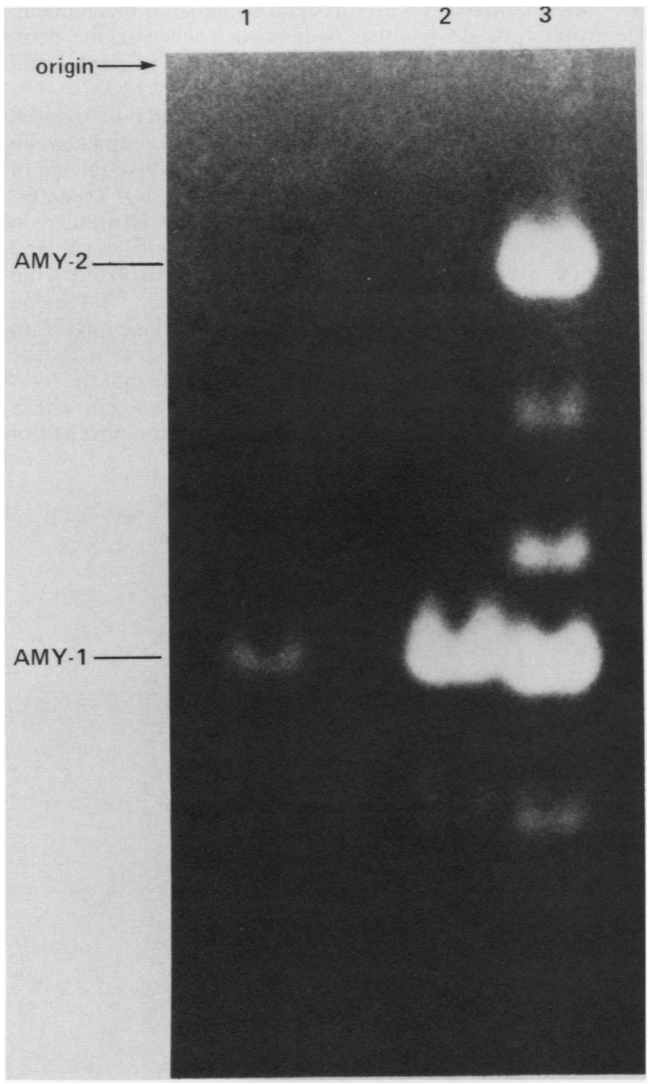

FIG. 2. Independent induction of AMY-1 and AMY-2. Hepa 1-6 cells were cultured in various media (see the text). Samples (20 μl) of culture supernatants were analyzed by electrophoresis, and the gels were stained for amylase activity. Lanes: 1, Hepa 1-6 in M/M + serum without dexamethasone; Hepa 1-6 in M/M + serum with 10^{-6} M dexamethasone; 3, Hepa 1-6 in SuM.

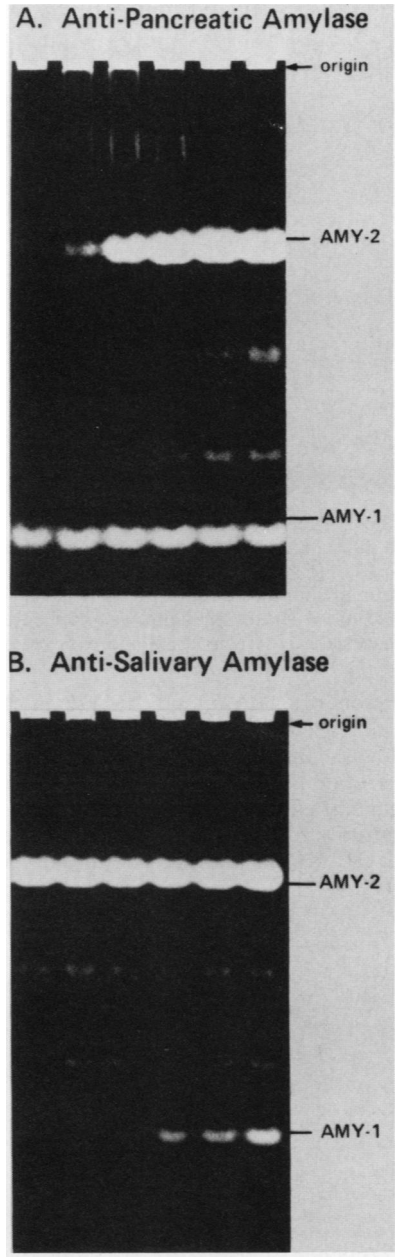

FIG. 3. Immunotitration of amylase secreted by Hepa 1-6 cells. Cells were cultured in SuM. Samples (20 μl) of culture medium were treated either with an antiserum raised against mouse pancreatic amylase (A) or with an antiserum raised against mouse salivary amylase (B). The amount of antiserum was serially diluted, 1:2, from left to right across the gel. After the immunoprecipitated amylase was removed by centrifugation, the supernatant was subjected to electrophoresis. Gels were stained for amylase activity. (A) The volume of anti-pancreatic amylase serum varied from 1 μl (at left) to 32 nl (at right). (B) The volume of anti-salivary amylase varied from 8 nl (at left) to 0.5 nl; the sample at the extreme right was incubated without antiserum.

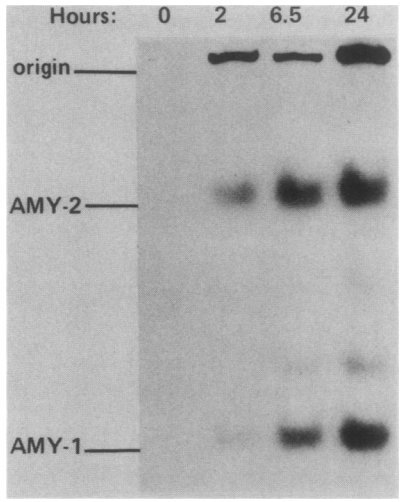

FIG. 4. De novo synthesis of AMY-1 and AMY-2 by Hepa 1-6 cells. At time 0, fully induced cells were transferred into fresh SuM containing 100 μCi of [^{35}S]methionine per ml. At the indicated times, samples of culture medium were removed for analysis. The amylase purified from 0.5 ml of culture medium was applied to each well. After electrophoresis, the gel was dried and exposed to x-ray film.

the concentration of AMY-1 and AMY-2 in the medium consistently increased by approximately two- to threefold (data not shown). Insulin has previously been found to reduce the secretion of other proteins by cultured cells, and may have a general effect on synthesis or export of proteins at the concentration used in these experiments (G. J. Darlington, J. H. Kelly, and G. J. Buffone, unpublished observations).

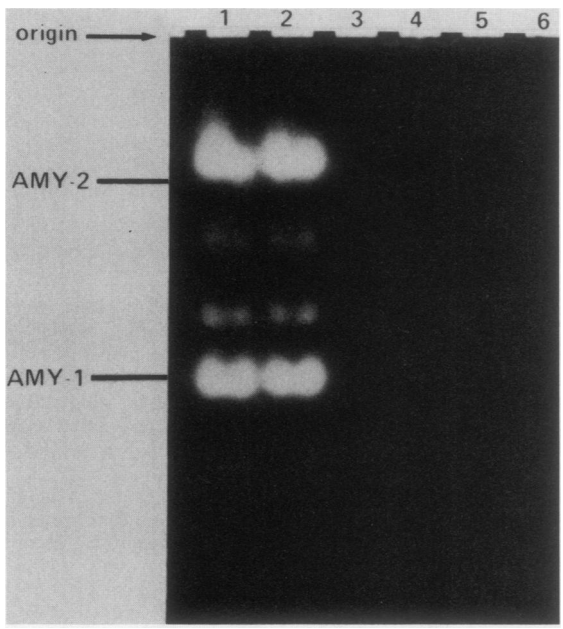

FIG. 5. Dependence of *Amy-1* and *Amy-2* on dexamethasone. Cells were cultured in SuM for 5 weeks and then in SuM lacking dexamethasone for 9 days. Lanes: 1 and 2, supernatant medium for fully induced cells growing in SuM; 3 and 4, unconditioned SuM before culture; 5 and 6, supernatant medium from cells grown in SuM minus dexamethasone.

Inhibition of *Amy-2* expression by serum. The experiments described above demonstrated that dexamethasone induces the synthesis of AMY-1 and AMY-2 when cells were cultured in SuM. We had noted that AMY-2 levels were below detection in the presence of fetal bovine serum and therefore tested the possibility that serum had an inhibitory effect on *Amy-2* expression in the following way. Fetal bovine serum (10%) was added to fully induced cells growing in SuM (Fig. 6). Medium collected 3 days after the addition of serum contained AMY-2, but in the interval between 3 and 6 days, AMY-2 was not secreted into the medium. During continued culture for 3 weeks in SuM plus serum, we did not observe reexpression of *Amy-2*. Throughout this period, *AMY-1*, was synthesized at induced levels.

Concentration dependence of induction by dexamethasone. The concentration dependence of amylase induction was examined in cells grown in M/M plus selenium and dexamethasone for 8 days (Fig. 7). The low effective concentration of dexamethasone, with half-maximal induction at 10^{-8} M, is consistent with a receptor-mediated mechanism. Electrophoretic gel analysis to monitor each isozyme demonstrated that the concentration dependence of *Amy-1* and *Amy-2* was equivalent (data not shown).

Amy-1 is transcribed from the liver promoter in induced hepatoma cells. A 100-base-pair difference in length between the amylase mRNA of liver and parotid is a consequence of the different length of their noncoding exons (24). To determine which of the two promoters of *Amy-1* is utilized in Hepa 1-6, mRNA from cells cultured in MM with serum and dexamethasone was compared with mRNA isolated from mouse parotid and liver. Hybridization with ^{32}P-labeled amylase cDNA revealed a single band of amylase mRNA in dexamethasone-induced cells which comigrated with the liver mRNA (Fig. 8 lanes 4 and 6). We conclude that the liver promoter is used for transcription of *Amy-1* in these dexamethasone-induced cells. Furthermore, the absence of

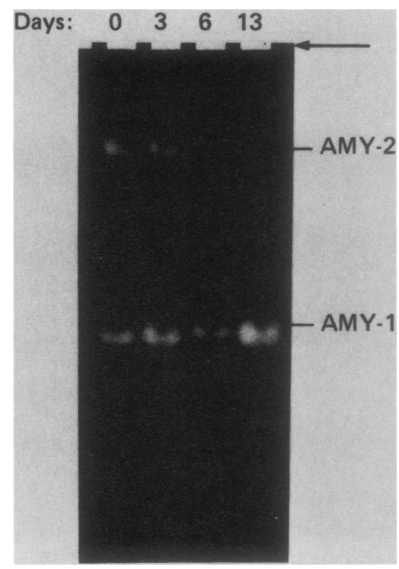

FIG. 6. Inhibition of AMY-2 expression by serum. Hepa 1-6 cells which had been cultured for 13 days in SuM were fully induced for AMY-1 and AMY-2 when 10% fetal calf serum was added to the medium on day 0. Samples of medium were taken for analysis after 3, 6, and 13 days with refeeding of the cells 72 h before sampling.

Amy-2 mRNA in this sample suggests that serum reduces this gene product by lowering its abundance.

In noninduced Hepa 1-6 cells, amylase mRNA could not be detected (Fig. 8, lane 5). In contrast, a mouse albumin cDNA probe hybridized to a comparable extent with the induced and uninduced samples on this filter, demonstrating the presence of equal quantities of RNA. The lack of detectable amylase mRNA in uninduced cells indicates that the abundance of the *Amy-1* mRNA increases after exposure to dexamethasone.

A dexamethasone consensus sequence upstream of the Amy-1 liver promoter. A 17-base-pair consensus sequence which is essential for glucocorticoid induction has been identified in the 5′ flanking region of several inducible genes (14). To determine whether the liver promoter of *Amy-1* includes a related sequence, a fragment containing the promoter was subcloned from a cosmid clone containing the mouse *Amy-1* gene. The sequence of a 627-bp fragment, including 374 bp of the 5′ flanking region of the liver promoter, is presented in Fig. 9A. One region with strong homology to the glucocorticoid consensus sequence begins at −207, and a second sequence is present in reverse orientation beginning at −309 (Fig. 9A). Both the location and the degree of homology of these sites are within the range observed for functional glucocorticoid sites (Fig. 9B). While it seems likely that these sites are involved in the induction of *Amy-1* by dexamethasone, functional tests will be required for confirmation.

The glucocorticoid site at −207 has the potential for secondary structure in which the consensus sequence occupies the stem of a stem-loop structure (Fig. 9C). This stem is stabilized by complementary pairing of 14 to 17 base pairs. Such a structure might facilitate binding of the glucocorticoid receptor protein.

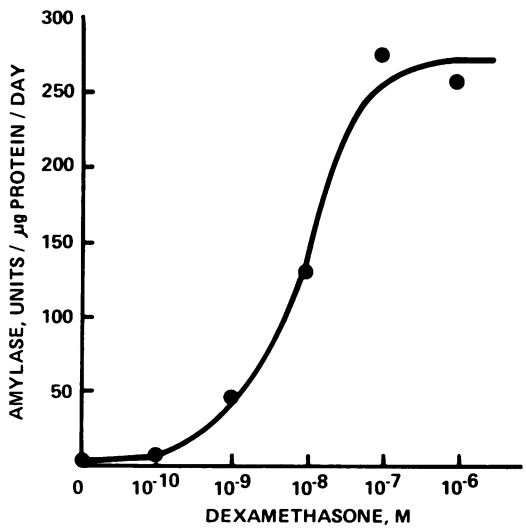

FIG. 7. Dependence of Amy-1 induction on dexamethasone concentration. Cells were cultured in M/M with selenium and the indicated concentration of dexamethasone, 10^{-6} to 10^{-10}M. Samples of medium were taken 8 days after the addition of dexamethasone with refeeding 48 h before sampling. Amylase activity in the supernatant and cellular protein were measured as described in the text. At this point in the induction, AMY-2 is barely detectable in the medium (Fig. 6), and the activity values represent predominantly AMY-1.

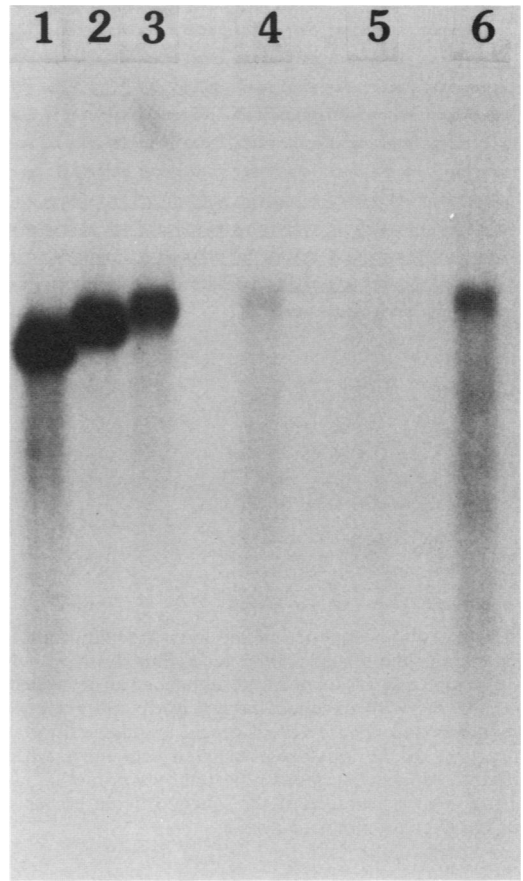

FIG. 8. Amylase mRNA in induced hepatoma cells corresponds in size to the liver transcript. Hepa 1-6 was cultured in MM with 10% serum and 10^{-6} M dexamethasone to specifically induce the expression of *Amy-1*. Electrophoresis was carried out in the presence of formaldehyde as described in the text. The cRNA hybridization probe was from pGEM-1 constructs carrying amylase cDNA sequences. Lanes: 1, μg of cellular RNA from pancreas; 2, 1 μg of cellular RNA from parotid; 3, 2 μg of poly(A)$^+$ RNA from liver; 4, 10 μg of poly(A)$^+$ RNA from Hepa 1-6 cells grown in dexamethasone; 5, 10 μg of poly(A)$^+$ RNA for Hepa 1-6 cells grown without dexamethasone; 6, 20 μg of poly(A)$^+$ RNA from Hepa 1-6 cells grown in dexamethasone.

DISCUSSION

We have demonstrated that the liver promoter of *Amy-1* is transcribed in Hepa 1-6 cells and that its expression is induced by dexamethasone. The glucocorticoid response of this promoter has not previously been described. The response of the liver promoter to glucocorticoids is typical of other steroid-inducible genes with respect to dependence on hormone concentration. We also observed an increase in the abundance of *Amy-1* mRNA after treatment with dexamethasone; however, because the amylase mRNA could not be detected in uninduced cells, the magnitude of the effect could not be estimated. Confirmation of the induction of *Amy-1* mRNA and also of *Amy-2* mRNA has recently been obtained by nuclease-protection assays (L. Samuelson, unpublished data). One or both of the glucocorticoid consensus sequences which we have identified upstream of this promoter may be involved in induction of amylase mRNA. The potential stem-loop structure of one of these sequences is unusual; it will be of great interest to test the functional role

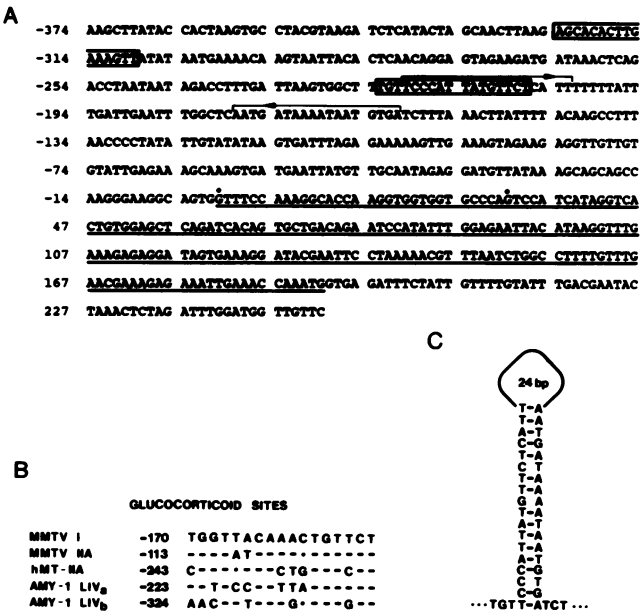

FIG. 9. Nucleotide sequence of the liver-specific promoter and noncoding exon of the mouse *Amy-1* gene from strain YBR/Ki. (A) The 627-bp fragment was isolated and sequenced as described in the text. Two regions with excellent homology to the glucocorticoid consensus are boxed. The inverted repeat is indicated by arrows. (B) Comparison of the amylase-associated sequences with other glucocorticoid responsive regions reported by Karin et al. (14). (C) Potential secondary structure of the glucocorticoid consensus sequence of the liver promoter of *Amy-1*.

of these sequences by transfection of modified genes into Hepa 1-6 cells.

A portion of the *Amy-1* liver promoter from mouse strain A/J has also been sequenced (30). Only 4 differences in 176-nucleotides are present in the sequence available for comparison between the two strains. The reported A/J sequence did not extend into the region of the glucocorticoid consensus sequences.

The expression of *Amy-2* in hepatoma cells was unexpected, since this gene is not known to be active in liver in vivo. The evidence that *Amy-2* is not active in liver includes the failure to isolate transcripts from mouse liver cDNA (9), the identical thermal stability of mRNA from the adult rat liver and parotid gland, which differ from pancreatic mRNA stability (11), and the failure to detect *Amy-2* transcripts in liver RNA by nuclease protection experiments (L. Samuelson, unpublished data). It remains possible that *Amy-2* may be expressed transiently during liver development. If so, its expression in hepatoma cells may be likened to the expression of the fetal liver proteins alpha-fetoprotein and aldolase A in these cells (7). Alternatively, the production of *Amy-2* may represent an ectopic expression related to the transformed character of the hepatoma line.

The expression of *Amy-2* in Hepa 1-6 differs from the expression of *Amy-1* in several respects (Table 1). Constitutive expression of *Amy-2* in the absence of dexamethasone cannot be detected. *Amy-2* expression requires the presence of the hormone. In addition, there appears to be a differential effect of serum, which inhibits the expression of *Amy-2*. High levels of *Amy-2* were only observed in the absence of serum. The phenomenon of *Amy-2* induction in Hepa 1-6 cells may reflect the activation of a previously silent gene and could provide a model for investigation of the reorganization of inactive chromatin. Our observations demonstrate that there is no intrinsic barrier to expression of *Amy-1* and *Amy-2* in the same cells.

TABLE 1. Comparison of *Amy-1* (salivary amylase) and *Amy-2* (pancreatic amylase) expression in liver cells

Expression in:	*Amy-1*	*Amy-2*
Adult liver	Present	None
Hepa 1-6 cells in MM+		
serum	Constitutive	None
serum + dexamethasone (10^{-8} M)	Induced	None
selenium	Constitutive	None
selenium + dexamethasone (10^{-8} M)	Induced	Induced
Effect of serum on expression	No effect	Inhibition

The constitutive expression of *Amy-1* provides an additional example of retention of a liver-specific phenotype by Hepa 1-6 cells. Four other glucocorticoid-sensitive genes have been examined in the mouse hepatoma cells with varying results. Hepa derivatives including Hepa 1-6 are not inducible for tyrosine aminotransferase (6; unpublished data). Metallothionein was induced by only twofold (18). Meyer et al. (19) reported a minimal stimulation by hydrocortisone of glycerol-3-phosphate dehydrogenase for one subline of Hepa but no increase for a second subline. However, Brown and Papaconstantinou (4) described the synergistic stimulation of albumin secretion by hydrocortisone and $N^6,8^2$-dibutyryl cyclic AMP. The basis for the variability in response to glucocorticoids in Hepa cells is not known, although subline differences may account for some of the variation. It is also worthwhile noting that most of the previous studies cited were carried out with cells cultured in serum, which may reduce the induction of other genes in addition to *Amy-2*.

Investigation of the mode of action of steroids in a variety of cell types has revealed alternative mechanisms to transcriptional activation as the molecular basis of induction of mRNA. For example, the rate of transcription of the alpha-1 acid glycoprotein gene is not increased by the inducer dexamethasone; this induction appears to involve an effect on mRNA processing or stabilization (28). The induction of the amylases by dexamethasone in Hepa 1-6 cells provides an experimental system for the determination of the mechanisms of elevation of these gene products. The Hepa 1-6 cells also provide a useful and unique cell system for the expression of transfected amylase genes to identify sequences of importance in dexamethasone response and tissue-specific expression.

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