Insulin Response of a Hybrid Amylase/CAT Gene in Transgenic Mice*

(Received for publication, June 6, 1988)

Laurelee Osborn, Michael P. Rosenberg‡, Scott A. Keller, Chao-Nan Ting, and Miriam H. Meisler

From the Department of Human Genetics, University of Michigan, Ann Arbor, Michigan 48109–0618 and the §Mammalian Genetics Laboratory, National Cancer Institute, National Institutes of Health, Frederick, Maryland 21701–1940

Expression of an amylase/CAT hybrid gene was analyzed in transgenic mice. The amylase promoter was derived from a pancreatic amylase gene whose expression is repressed in diabetic animals. Pancreas-specific expression of the amylase/chloramphenicol acetyltransferase (CAT) construct was observed in two independent transgenic lines. Correct initiation of transcription was demonstrated by protection of an antisense riboprobe. To evaluate the insulin dependence of the hybrid gene, diabetes was induced by treatment with streptozotocin. As a result of this treatment, pancreatic CAT activity was reduced to undetectable levels. Subsequent administration of insulin restored CAT activity to normal levels. The abundance of CAT transcripts was also greatly reduced in diabetic pancreas. These studies localize the determinants of pancreas specificity and insulin dependence to the region between -208 and +19 of the mouse pancreatic Amy-2.2 gene. The results are consistent with an effect of insulin on amylase transcription, rather than post-transcriptional regulation of mRNA processing or stability.

Within the past few years, both positive and negative effects of insulin on the abundance of specific messenger RNAs have been demonstrated (reviewed in Ref. 1). One of the largest of these responses is the positive effect of insulin on pancreatic amy-2.2 mRNA. This effect can be demonstrated in vivo by administration of the β cell toxin streptozotocin, which produces insulin-dependent diabetes. In streptozotocin-diabetic rats, amy-2.2 mRNA concentration is reduced from 20% of total pancreatic mRNA down to undetectable levels; the effect can be reversed by subsequent administration of insulin (2). The mouse genome contains two classes of pancreatic amylase genes which differ in their response to insulin (3, 4). Like the rat gene, expression of mouse Amy-2.2 is completely repressed in diabetic animals and is restored to normal levels by treatment with insulin (4, 5). We have used transgenic mice to localize the cis-acting DNA elements required for regulation of Amy-2.2. In previous studies we demonstrated tissue-specific and hormonal regulation of an intact Amy-2.2 gene and a 2.4-kb amylase minigene (4). In addition to 208 nucleotides of the 5′-flanking sequence, the amylase minigene contained the entire coding sequence, the first intron, and 0.3 kb of the 3′-flanking sequence (4).

In order to further localize the insulin-responsive sequences, we have constructed a hybrid gene containing 227 base pairs of amylase sequence fused to the bacterial chloramphenicol acetyltransferase (CAT) coding sequence. Unlike the previous constructs, the transcript of this hybrid gene does not contain amylase-derived intron or coding sequences. The observed in vivo regulation of this construct strongly suggests that insulin influences amylase transcription.

MATERIALS AND METHODS

Transgenic Mice—For construction of the amylase/CAT hybrid gene, the amylase promoter was isolated as a 227-base pair BglII/BglI fragment from the amylase minigene AM-0.2/3.1 (4). This promoter fragment was treated with S1 nuclease to generate blunt ends and cloned into the Smal site of the promoterless vector pSVOCAT (6). The junction region of the construct was sequenced from the synthetic oligonucleotide primer (5′)GGCTTCTTACATGCTGAGTA (3′), complementary to CAT sequences +38 to +54 of the hybrid gene (Fig. 1). The 1.9-kb BglII/BamHI fragment containing the hybrid gene was isolated and microinjected into fertilized mouse eggs as previously described (4). Transgenic mice were identified by Southern blot of DNA isolated from tails at the time of weaning. The blots were probed with a 0.54-kb Real fragment containing the CAT sequences from +163 to +688 of the hybrid gene (Fig. 1). This probe does not hybridize with endogenous mouse sequences.

CAT Assays—Tissues were homogenized in 1–3 ml of 0.25 M Tris, pH 7.8. Homogenates were heated at 65 °C for 10 min and centrifuged to remove denatured protein. The concentration of soluble protein in the supernatant was determined using the Bio-Rad protein assay. CAT activity was assayed essentially as described by Gorman et al. (6), using 100 μg of soluble protein, 0.2 μCi of [3H]chloramphenicol (Du Pont-New England Nuclear), and 0.33 mg of acetyl-CoA in a total volume of 150 μl. Reactions were incubated for 3 h at 37 °C. TLC bands corresponding to monoacetylated chloramphenicol were counted in a liquid scintillation counter. Each sample was assayed in duplicate. Data were corrected for the presence of co-migrating radioactivity in the [3H]chloramphenicol substrate. Assays were demonstrated to be within linear range for incubation time and enzyme concentration. Standards containing 50 milliunits of purified CAT (Sigma) were included with each set of assays, generating approximately 30,000 cpm in monoacetylated chloramphenicol. Values obtained from tissues were converted to milliunits by comparison with this standard. (One unit of purified CAT from Sigma catalyzes the acetylation of 1 nmol of chloramphenicol/min at pH 7.8 and 25 °C.) CAT activity was stable during storage of homogenates for several weeks at −70 °C.

Streptozotocin and Insulin Treatment—Streptozotocin was administered by intraperitoneal injection of 40 mg/kg on 6 successive days. Insulin was administered to diabetic animals by implantation of an Alzet osmotic pump which released 0.75 unit of insulin/day for a period of 2 weeks (4).

Ribonuclease Protection Assay—In order to determine the start site for transcription, the 484-nucleotide fragment which extends from

* This research was supported by United States Public Health Service Grant GM24872 (to M. H. M.), by a Pilot Project from the Michigan Diabetes Research and Training Center (P50 DK 20572), and by the Gastrointestinal Hormone Research Core Center (P50 DK 34933). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by United States Public Health Service Training Grant in Developmental Biology, T32 HD 07247.

§ Supported by the National Cancer Institute, DHHA, under contract NO 1-CO-23909 with Bionetics Research, Inc.

1 The abbreviations used are: kb, kilobase(s); CAT, chloramphenicol acetyltransferase.
the BgII site at –208 to the EcoRI site at +276 of the hybrid gene (Fig. 1) was cloned into the vector pGEM-1 (Promega Biotec, Madison, WI) after linearization by digestion with BamHI and EcoRI. The resulting clone was designated pACP1. A single-stranded uniformly labeled 531-nucleotide RNA probe was generated by digestion of pACP1 with HindIII followed by transcription with T7 polymerase in the presence of [32P]UTP, according to the procedure recommended by Promega for synthesis of a high specific activity probe. Total pancreatic RNA was isolated, and its integrity was evaluated as previously described (4). The ribonuclease protection assay was carried out according to the method of Melton et al. (7).

RESULTS

Production of Transgenic Lines Carrying the Amylase/CAT Hybrid Gene—The hybrid gene contains Amy–22 sequences from –208 to +19 fused to the Escherichia coli CAT structural gene (Fig. 1). The 1.9-kb BgII/BamHI fragment carrying the amylase promoter and CAT coding sequences was isolated and microinjected into fertilized mouse eggs to generate transgenic mice. Three independent animals carrying the injected DNA were identified, and two transgenic lines, designated tg730 and tg1217, have been established. Genomic DNA from mice of both lines contains a 1.9-kb EcoRI fragment which hybridizes with a probe containing CAT sequences (Fig. 2). Since the injected fragment is 1.9 kb in length and contains a single EcoRI site, the hybridization pattern indicates that a tandem array of gene copies has been integrated. There are approximately 100 copies of the hybrid gene/haploid genome in line tg730, and approximately 200 copies in line tg1217, as estimated by comparison of hybridization intensity with standards. Founder animals were crossed with C57BL/6J mice to produce transgenic lines which have been maintained for several generations by repeated crossing with C57BL/6J mice. Both transgenic lines appear to contain a single insertion site, as indicated by transmission of the hybridizing fragment to 50% of offspring.

Pancreas-specific Expression of the Hybrid Gene—Expression of the hybrid gene was detected by assay of CAT activity in homogenates of various tissues. In mice of lines tg730 and tg1217, CAT activity was readily detected in pancreatic homogenates. The pancreatic CAT activity was 151 ± 22 milliunits/mg protein (mean ± S.E., n = 13) in line tg730 and 51 ± 18 milliunits/mg protein (n = 5) in line tg1217. In both lines, the CAT activity in pancreas was more than 100-fold greater than in the other tissues which were assayed: salivary gland, liver, spleen, heart, lung, muscle, testis, brain, kidney, intestine, and thymus. None of these tissues contained measurable CAT activity (data not shown).

Loss of Expression of the Hybrid Gene in Diabetic Mice—To test the insulin dependence of pancreatic CAT expression, diabetes was induced in lines tg730 and tg1217 by treatment with streptozotocin. Diabetic animals with urinary glucose concentrations above 5 g/100 ml were identified using Chemstrip UGK (Boehringer Mannheim). The level of pancreatic CAT activity in these animals was reduced more than 100-fold from untreated levels (Table I). The magnitude of this reduction is similar to that of the intact Amy–22 gene (4).

Response to Treatment with Insulin—Two diabetic mice of line tg730 were treated with insulin by implantation of an osmotic pump which released 0.75 units of insulin/day for a period of 2 weeks. This insulin treatment restored pancreatic
Correct Initiation of Transcription of the Hybrid Gene—
Total cellular RNA was isolated from the pancreas of transgenic mice and hybridized with a uniformly labeled riboprobe which spanned the start site for transcription from the amylase promoter (Fig. 4). Transcripts which were correctly initiated from the amylase promoter at nucleotide +1 would be expected to protect a 276-nucleotide fragment of this riboprobe. The major protected fragment in RNA from untreated pancreas corresponded in length to this predicted fragment (Fig. 4, lanes 1 and 2). This result demonstrates that the amylase promoter directs expression of the amylase/CAT hybrid gene. There is no evidence of initiation of transcription at positions upstream of the amylase start site for transcription.

Pancreatic RNA from three diabetic mice of line tg730 was also examined (Fig. 4, lanes 3–5). The level of transcripts in diabetic pancreas was significantly lower than that in the untreated animals.

**DISCUSSION**

The amylase/CAT hybrid gene contains sufficient amylase sequence to determine pancreas-specific expression. Previous studies have localized pancreatic enhancer sequences in the rat elastase I gene (8) and the rat chymotrypsin and amylase genes (9). Similar sequences are present in all of the pancreas-specific genes which have been sequenced to date, including both human and rodent genes (10). In the construct studied here, related sequences are located between nucleotides −160 and −120. At least two pancreas-specific nuclear proteins with affinity for this sequence can be detected in gel retardation assays.2

We have demonstrated that the 227-nucleotide fragment of the pancreatic amylase gene transfers insulin response to the bacterial CAT gene. This amylase fragment contains 208 nucleotides of 5′-flanking sequence and 19 nucleotides of transcribed leader sequence. It is unlikely that the transcribed leader is involved in the insulin response, because the same sequence is present in the gene Amy-2.1, which is not insulin-dependent (4, 5). The response to insulin therefore appears to be determined by sequences upstream of the transcribed region of the amylase gene, indicating that the mechanism of regulation is transcriptional.

While this work was in progress, studies of three other insulin-responsive promoters have appeared. The region of the rat phosphoenolpyruvate carboxykinase gene between −600 and +62 was found to confer repression by insulin in transient assays of a CAT fusion gene (11). A CAT fusion gene containing human glyceraldehyde-3-phosphate dehydrogenase sequences between −487 and +20 was induced by insulin in stably transfected cells and in transient assays (12). Similar results were observed for a murine c-fos hybrid gene containing sequences −356 to +109 (13). The metabolic reg-

---

2 G. Howard, P. R. Keller, T. M. Johnson, and M. H. Meisler, manuscript in preparation.
ulation of a phosphoenolpyruvate carboxykinase/growth hormone construct in transgenic mice was also indicative of negative regulation by insulin (14). Computer comparison of these insulin-responsive promoter regions revealed two common elements (Table II). However, the functional significance of these shared sequences will require experimental evaluation.

It has been suggested that the repression of pancreatic amylase production in diabetic animals may serve the physiological function of preventing conversion of dietary carbohydrate into absorbable sugars. The magnitude of this effect is one of the largest known effects of insulin on messenger RNA concentration (19). Expression in transgenic mice provides a useful experimental system for identification of the sequences involved in this response.

Acknowledgments—We thank Deborah Swing, National Cancer Institute Frederick Cancer Research Facility, BRI Basic Research Program, for assistance with microinjection, Martha Haviland for the cloning of plasmid pACP1, Paul Keller for isolation of pancreatic RNA, and Lisa Campeau for expert manuscript preparation.

REFERENCES

1. Granner, D. K., Sasaki, K. & Chu, D. (1986) Ann. N. Y. Acad. Sci. 478, 175–190
2. Korc, M., Owerbach, D., Quinto, C. & Rutter, W. J. (1981) Science 213, 351–353
3. Gumucio, D. L., Wiebauer, K., Dranginis, A., Samuelson, L. C., Treisman, L. O., Caldwell, R. M., Antonucci, T. K. & Meisler, M. H. (1985) J. Biol. Chem. 260, 13483–13489
4. Osborn, L., Rosenberg, M. P., Keller, S. A. & Meisler, M. H. (1987) Mol. Cell. Biol. 7, 326–334
5. Dranginis, A., Morley, M., Nesbitt, M., Rosenblum, B. B. & Meisler, M. H. (1984) J. Biol. Chem. 259, 12216–12219
6. Gorman, C. M., Moffat, L. F. & Howard, B. H. (1982) Mol. Cell. Biol. 2, 1044–1051
7. Melton, D. A., Krieg, P. A., Rebagliati, M. R., Maniatis, T., Zinn, K. & Green, M. R. (1984) Nucleic Acids Res. 12, 7035–7056
8. Hammer, R. E., Swift, G. H., Ornitz, D. M., Quaife, C. J., Palmiter, R. D., Brinster, R. L. & MacDonald, R. J. (1987) Mol. Cell. Biol. 7, 2966–2967
9. Boulet, A. M., Erwin, C. R. & Rutter, W. J. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 3599–3603
10. Gumucio, D. L., Wiebauer, K., Caldwell, R. M., Samuelson, L. C. & Meisler, M. H. (1988) Mol. Cell. Biol. 8, 1197–1205
11. Magnuson, M. A., Quinn, P. G. & Granner, D. A. (1987) J. Biol. Chem. 262, 14917–14920
12. Alexander, M. C., Lomanio, M., Nasrin, N. & Ramaika, C. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 5092–5096
13. Stumpo, D. J., Stewart, T. N., Gilman, M. Z. & Blackshear, P. J. (1988) J. Biol. Chem. 263, 1611–1614
14. McGrane, M. M., Devente, J., Yun, J., Bloom, J., Parks, E., Wynshaw-Boris, A., Wagner, T., Rottman, P. M. & Hanson, R. W. (1988) J. Biol. Chem. 263, 11443–11451
15. Wynshaw-Boris, A., Lugo, T. G., Short, J. M., Fournier, R. E. K. & Hanson, R. W. (1984) J. Biol. Chem. 259, 12161–12169
16. Beale, E. G., Chrapkiewicz, N. B., Schohle, H. A., Metz, R. J., Quick, D. P., Noble, R. L., Donelson, J. E., Biemann, K. & Granner, D. K. (1985) J. Biol. Chem. 260, 10748–10760
17. Treisman, R. (1985) Cell 42, 889–902
18. Gilman, M. Z., Wilson, R. & Weinberg, R. A. (1986) Mol. Cell. Biol. 6, 4505–4516
19. Meisler, M. H. & Howard, G. (1989) Annu. Rev. Physiol. 51, in press