“Spot 14” Protein Functions at the Pretranslational Level in the Regulation of Hepatic Metabolism by Thyroid Hormone and Glucose* 

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Suzanne B. Brown, Marybeth Maloney, and William B. Kinlaw‡
From the Department of Medicine, Division of Endocrinology and Metabolism, Dartmouth Medical School, Lebanon, New Hampshire 03756

“Spot 14” protein appears rapidly in nuclei of hepatocytes exposed to glucose and thyroid hormone. Exposure of glucose- and T3-treated hepatocytes to a spot 14 antisense oligonucleotide inhibited induction of mRNAs encoding malic enzyme, ATP citrate-lyase, fatty acid synthase, liver-type pyruvate kinase, phosphoenolpyruvate carboxykinase, and type I deiodinase but not hydroxymethylglutaryl-CoA reductase, cytochrome c, and actin mRNAs. Induction of spot 14, ATP citrate-lyase, and fatty acid synthase polypeptides, but not propionyl-CoA carboxylase and mitochondrial pyruvate carboxylase, was inhibited. Antisense treatment of hepatocytes transfected with a reporter controlled by a glucose- and T3-inducible fragment of the pyruvate kinase gene promoter inhibited reporter activity, as did cotransfection of the reporter and a spot 14 antisense plasmid. Spot 14 protein acts in the induction of mRNAs coding for key lipogenic (malic enzyme, ATP citrate-lyase, fatty acid synthase), glycolytic (pyruvate kinase), and gluconeogenic enzymes (phosphoenolpyruvate carboxykinase), as well as the diet-responsive type I deiodinase, but not those involved in mitochondrial respiration (cytochrome c) or cholesterol synthesis (hydroxymethylglutaryl-CoA reductase). Transfection experiments indicated that these effects are mediated at the transcriptional level. The protein functions in the activation of genes involved in metabolic switching between the fasted and fed states in liver.

Liver, adipose, and lactating mammary tissues synthesize fatty acids for use as fuel. In those tissues lipogenesis is regulated by availability of dietary substrates and circulating hormones that control fuel metabolism. Regulated expression of genes that encode lipogenic enzymes is a major mechanism underlying this response. Spot 14 gene expression has been associated with such tissue-specific control. Spot 14 mRNA is abundant only in lipogenic tissues (1, 2), and its expression changes rapidly in response to stimuli that modulate fatty acid formation, including intake of dietary carbohydrate and polyunsaturated fat and levels of thyroid hormone and glucagon (3–9). Spot 14 protein levels paralleled those of its mRNA (10), and immunohistochemical analysis indicated that it was nuclear in location (11) with a zonal distribution in liver identical to that of the lipogenic enzymes (12).

Multifaceted regulation and nuclear localization of the spot 14 protein and its nuclear location suggested that it could function to control lipogenesis. We recently examined that hypothesis by determining the metabolic effects of antisense-mediated inhibition of spot 14 protein expression in cultured rat hepatocytes (13). Induction of lipid synthesis by glucose and triiodothyronine (T3)1 was inhibited in cells treated with a spot 14 antisense oligonucleotide, and Western analysis indicated that this resulted from diminished accumulation of lipogenic enzymes, including fatty acid synthase and ATP citrate-lyase. Activity of malic enzyme was also reduced, as was expression of malic enzyme mRNA.

We have now addressed the mechanism underlying reduced lipogenesis in hepatocytes treated with the antisense oligonucleotide. Our findings indicate that spot 14 protein participates in the tissue-specific induction of mRNAs encoding several lipogenic enzymes and of other inducible mRNAs involved in metabolic adaptation. Transfection studies further indicated that it functioned to promote pyruvate kinase gene transcription.

MATERIALS AND METHODS

Hepatocyte Culture—Collagenase perfusion of livers from male Sprague-Dawley rats (Charles River, Cambridge, MA) weighing approximately 150 g and maintained on a 12-h photoperiod (lights on at 0700 h) with ad libitum access to normal chow (Ralston Purina, St. Louis, MO) was as described previously (13, 14). Cells were plated in positively charged plastic dishes (143 × 106 cells/cm2) in serum-free modified William’s E medium containing penicillin, streptomycin, 5.5 mM glucose, and no linoleic acid (Life Technologies, Inc.).

Hepatocyte Treatments—Cells were placed in modified William’s E medium without antibiotics 5 h after plating. Some media contained 8 μg/ml Lipofectin (Life Technologies, Inc.) and 4 μM phosphorothioate oligonucleotides (Oligos Etc, Wilsonville, OR). Oligonucleotides employed were S14 (GGCTTGCAGCAGCGGCTG; an antisense sequence in which the 3′ residue corresponds to the G of the translational start codon of the rat spot 14 mRNA sequence) and PPI (GAAGCCGATCCA-CAGGGCC; an antisense sequence in which the 3′ residue corresponds to the G of the start codon of the rat preproinsulin I mRNA, which is not expressed in liver). Neither oligonucleotide displayed sequence similarity to any other mRNA studied. Media were replaced the following morning and again 24 h later with either modified William’s E medium alone or fortified with 27.5 mM glucose and 50 mM NaCl, and oligonucleotides (2 μM, without Lipofectin); cells were harvested 24 h later. We showed previously that the spot 14 antisense oligonucleotide specifically inhibited the induction of triglyceride synthesis under these circumstances and that the effect is dependent upon both the oligonucleotide sequence and the presence of the intended target mRNA (13).

Plasmid Transfection—Sequential introduction of reporter plasmids and oligonucleotides was required because simultaneous application interfered with plasmid transfection. Following 5 h in plating medium, plasmids (3.9 μg each of a plasmid containing base pairs −4316 to +12

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‡ To whom correspondence should be addressed: 714 West Borwell Bldg., 1 Medical Center Dr., Lebanon, NH 03756. Tel.: 603-650-8744; Fax: 603-650-6130; E-mail: William.B.Kinlaw.III@Hitchcock.ORG.

1 The abbreviations used are: T3, triiodothyronine; PK, pyruvate kinase; CAT, chloramphenicol acetyltransferase; RSV, Rous sarcoma virus; LUC, luciferase.
of the rat liver-type pyruvate kinase promoter fused to the chloramphenicol acetyltransferase gene (PK_{4316-CAT} (15), kindly provided by H. Towle, University of Minnesota) and a plasmid containing the firefly luciferase gene fused to the Rous sarcoma virus promoter (RSV-LUC)) were introduced by lipofection (Lipofectin). Media were removed 4 h later and replaced with media containing oligonucleotides as described above. Luciferase activity was quantitated in a liquid scintillation counter. Luciferase activity was determined in a BioOrbit 1251 luminometer (Pharmacia Biotech Inc.). In another experiment, a BglIII-BamHI fragment containing a modified full-length rat spot 14 cDNA (11) was ligated in the antisense orientation into the BglII site of the pCMV4 vector (kindly supplied by D. Russell, University of Southwestern Texas) and cotransfected with the PR_{4316-CAT} reporter (1.5 μg of each/plate) and RSV-LUC (3.0 μg/plate) to allow correction for transfection efficiency.

**Protein and mRNA Analyses**—Proteins and total RNA were extracted with Trizol reagent (Life Technologies, Inc.) and were concentrated in Centricon-3 columns (Amicon, Beverly, MA) for Western analysis. Affinity-purified rabbit anti-glutathione S-transferase-spot 14 fusion protein IgG was employed as reported previously (11). Detection of rat ATP citrate-lyase and fatty acid synthase was as described (12). Pyruvate carboxylase and propionyl-CoA carboxylase enzymes were visualized via their biotin prosthetic groups with a streptavidin-alkaline phosphatase conjugate (12). Integrity of total RNA was assured by molecular weight electrophoretic standards. Panel a was probed with affinity-purified anti-glutathione S-transferase-spot 14 fusion protein IgG (S14). Panels b and c were probed with anti-rat fatty acid synthase (FAS) or anti-rat ATP citrate-lyase (ACL) IgG, respectively. Panel d was probed with a streptavidin-alkaline phosphatase conjugate to reveal the biotin-containing enzymes pyruvate carboxylase (PYR) and propionyl-CoA carboxylase (PROP). Bands at the bottom of lanes in panels a and d are tracking dye (pyronin Y).

**RESULTS**

Western blot verified that the treatments resulted in the expected changes in expression of spot 14 protein and three lipogenic enzymes, whereas levels of two other specific enzymes were unaltered (Fig. 1). Maintenance of hepatocytes in 5.5 mM glucose and no T_{3} for 72 h resulted in near-detectable levels of spot 14 protein (panel a, lanes 1 and 2), whereas exposure to 27.5 mM glucose and 50 nM T_{3} for 48 h (lanes 3 and 4) induced expression to a level comparable to that observed in liver from a hyperthyroid, carbohydrate-fed rat (lane 7). In contrast to the effect of the control oligonucleotide (PP1, lane 6), treatment with the spot 14 antisense oligonucleotide markedly inhibited induction of the protein (AS, lane 5).

Western analysis of the same protein preparations using antibodies directed against fatty acid synthase (panel b) or ATP citrate-lyase (panel c) confirmed our previous report of the effect of the treatments (13). High glucose and T_{3} concentrations caused a marked induction of both enzymes, and this was inhibited by transfection of the spot 14, but not the preproinsulin I oligonucleotide.

In contrast to fatty acid synthase and ATP citrate-lyase, levels of mitochondrial pyruvate carboxylase and propionyl-CoA carboxylase were not induced by glucose and T_{3}, and also were not lowered in antisense-treated cells (panel d). We were unable to detect acetyl-CoA carboxylase in the extracts.

To determine whether reduced expression of lipogenic enzymes in spot 14 antisense-treated cells resulted from lowered levels of their mRNAs, we undertook slot-blot analysis of mRNA extracted from the same hepatocytes used for Western analyses (Fig. 2). Each signal was corrected for that observed upon reanalysis with an actin cDNA probe. The mean level of malic enzyme mRNA increased 7-fold after exposure to high glucose and T_{3} concentrations. The spot 14 antisense oligomer induced approximately 14- and 6-fold, respectively. Transfection of a spot 14 antisense oligonucleotide (lane 5), or a control preproinsulin I oligonucleotide (lane 6). Liver protein from a T_{3}-treated, glucose-fed rat was loaded in lane 7.**, molecular weight electrophoretic standards. Panel a was probed with GLUH antisense oligonucleotide (S14). Panels b and c were probed with anti-rat fatty acid synthase (FAS) or anti-rat ATP citrate-lyase (ACL) IgG, respectively. Panel d was probed with a streptavidin-alkaline phosphatase conjugate to reveal the biotin-containing enzymes pyruvate carboxylase (PYR) and propionyl-CoA carboxylase (PROP). Bands at the bottom of lanes in panels a and d are tracking dye (pyronin Y).
on mRNA levels. We employed two different approaches to this question. In the case of the preproinsulin I antisense oligonucleotide (Fig. 3). Mean CAT activity, corrected for the activity of a cotransfected luciferase reporter (RSV-LUC), in cells maintained in 5.5 mM glucose without T3 or any oligonucleotide, was indistinguishable from the background level in untransfected cells. Transfected hepatocytes treated with the control oligonucleotide exhibited a significant (p < 0.05) induction of CAT activity in response to 27.5 mM glucose plus 50 nM T3, whereas cells treated with the antisense oligonucleotide expressed 76% less CAT activity (p < 0.05). In another experiment we cotransfected a construct containing a full-length spot 14 cDNA in the antisense orientation with respect to the cytomegalovirus promoter (SI14-anti) or the nonrecombinant vector (CMV4) with PK1316- 

**DISCUSSION**

We showed previously that induction of long chain fatty acid synthesis and of two major lipogenic enzymes, fatty acid synthase and ATP citrate-lyase, was impaired in hepatocytes treated with the antisense oligonucleotide (13). Levels of malic enzyme activity were also reduced, in conjunction with lowered relative expression of malic enzyme mRNA. These findings were confirmed in the present study. In concert with our previous immunohistochemical demonstration of nuclear localization of spot 14 protein (11), this prompted the proposal that spot 14 could function in the transduction of glucose- and T3-initiated signals for increased lipogenesis at the transcriptional level. The major finding in the current experiments was that the reduced expression of fatty acid synthase and ATP citrate-lyase polypeptides was accompanied by diminished expression of their respective mRNAs.

Expression of three mRNAs encoding inducible enzymes not directly involved in lipogenesis, pyruvate kinase, phosphoenolpyruvate carboxykinase, and type I deiodinase, was also inhibited by antisense treatment. Hepatic pyruvate kinase and type I deiodinase mRNAs are known to increase during refeeding in vivo, as is the case for the lipogenic enzymes, whereas phosphoenolpyruvate carboxykinase mRNA declines in that circumstance. Pyruvate kinase induction is mediated at the transcriptional level by a signal initiated by glucose metabolism (15). The signal is transduced via a major late transcription-like...
factor, hepatic nuclear factor-4 (24), and an accessory factor acting in cis with a CACGTG motif (25). We speculate that spot 14 protein could be involved, directly or indirectly, in modulating the interaction of one or more of these factors with the pyruvate kinase promoter.

In contrast to pyruvate kinase, phosphoenolpyruvate carboxykinase gene transcription is induced by glucagon and cAMP analogs with constant glucocorticoid (10 nM dexamethasone) and insulin (0.01 unit/ml) concentrations. The culture conditions therefore do not reproduce the reduced cellular cAMP and rising insulin levels associated with refeeding in vivo. This provides an explanation for the apparent paradox of simultaneous induction of pyruvate kinase and phosphoenolpyruvate carboxykinase in T3- and glucose-treated cells. The observed 1.7-fold induction in phosphoenolpyruvate carboxykinase mRNA may be attributed to T3, and this effect was abrogated by antisense treatment. In view of the effect on antisense treatment on pyruvate kinase mRNA expression, it appears that spot 14 protein plays a role in both T3- and glucose-mediated signaling pathways, although we did not address this issue in the current experiments.

The mechanism underlying the regulation of the deiodinase is less well characterized, although it occurs at the pretranslational level. Deiodinase mRNA in liver declines during fasting, experimental diabetes mellitus, and hypothyroidism and is induced by T3 treatment (30). Our data show that T3 and glucose act to stimulate deiodinase expression directly at the hepatocellular level and that spot 14 protein functions in this response.

T3 administration causes accumulation of some, but not all, mitochondrial polypeptides in liver. The observation that cytochrome c mRNA was induced, whereas pyruvate carboxylase polypeptide was not, is consistent with previous reports of uncoordinated regulation by T3 of mitochondrial components encoded in the nuclear genome (31). The lack of any effect of the spot 14 antisense oligonucleotide on either pyruvate carboxylase polypeptide or cytochrome c mRNA expression indicates that the protein does not participate in the regulation of mitochondrial function by carbohydrate or T3.

Because of considerations discussed in the Introduction, we focused previously on the fatty acid synthetic pathway as the locus of spot 14 protein function (13). Reduced expression of genes encoding enzymes involved in lipogenesis, glycolysis, and T3 production in antisense-treated hepatocytes in the current studies suggests a broader role for the protein in metabolic adaptation. At least in the case of pyruvate kinase, the protein functions at the level of transcription. Immunohistochemical studies from our laboratory showed that the induction of both spot 14 protein and lipogenic enzymes by T3 in rat liver is limited to the perivenous zone of the hepatic lobule (12), whereas others have demonstrated uniform distribution of hepatic nuclear T3 receptors (32). The current findings prompt the hypothesis that spot 14 could serve as a tissue- or zone-specific adaptor between generic transcription factors and their potential target genes.

REFERENCES

1. Jump, D., and Oppenheimer, J. (1985) Endocrinology 117, 2259–2266
2. Freake, H. C., and Oppenheimer, J. H. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 9070–9074
3. Jump, D. B., Bell, A., and Santiago, V. (1990) J. Biol. Chem. 265, 3474–3478
4. Jump, D. B., Clarke, S. D., MacDougald, O., and Thelen, A. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 8454–8458
5. Jump, D. B., Narayan, P., Towle, H., and Oppenheimer, J. H. (1984) J. Biol. Chem. 259, 2789–2797
6. Mariash, C. N., Kaiser, F. E., Schwartz, H. L., Towle, H. C., and Oppenheimer, J. H. (1981) J. Clin. Invest. 65, 1126–1134
7. Carr, E. F., Bingham, C., Oppenheimer, J. H., Kistner, C., and Mariash, C. N. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 974–978
8. Kinlaw, W. B., Schwartz, H. L., Towle, H. C., and Oppenheimer, J. H. (1986) J. Clin. Invest. 78, 1091–1098
9. Kinlaw, W. B., Schwartz, H. L., Hablin, P. S., Mariash, C. N., and Oppenheimer, J. H. (1988) Endocrinology 123, 2255–2260
10. Kinlaw, W. B., Leng, N. C., and Oppenheimer, J. H. (1989) J. Biol. Chem. 264, 19779–19783
11. Kinlaw, W. B., Tron, P., and Friedmann, A. S. (1992) Endocrinology 131, 3120–3122
12. Kinlaw, W. B., Tron, P., and Witters, L. A. (1993) Endocrinology 133, 645–650
13. Kinlaw, W. B., Church, J. L., Harmon, J., and Mariash, C. N. (1995) J. Biol. Chem. 270, 16615–16618
14. Mariash, C., Jump, D., and Oppenheimer, J. (1984) Biochem. Biophys. Res. Commun. 123, 1122–1129
15. Thompson, K. S., and Towle, H. C. (1991) J. Biol. Chem. 266, 8679–8682
16. Levi, A., Juanita, D. E., and Paterson, B. (1985) Science 229, 393–395
17. Strait, K. A., Kinlaw, W. B., Mariash, C. N., and Oppenheimer, J. H. (1989) J. Biol. Chem. 264, 19784–19789
18. Paulauskis, J. D., and Sul, H. S. (1989) J. Biol. Chem. 264, 574–577
19. Elshourbagy, N. A., Near, J. C., Kmetz, P. J., Sathe, G. M., Southan, C., Strickler, J. E., Gross, M., Young, J. F., Wells, T. N. C., and Groot, P. M. E. (1990) J. Biol. Chem. 265, 1430–1435
20. Berry, M., Banu, L., and Larsen, P. (1991) Nature 349, 438–440
21. Bosch, F., Hatzoglou, M., Park, E. A., and Hansen, R. W. (1990) J. Biol. Chem. 265, 13677–13682
22. Scarpulla, R. C., Agne, K. M., and Wu, R. (1981) J. Biol. Chem. 256, 6480–6490
23. Wilkinson, L., Hill, M., and Yang, E. (1992) SYSTAT: Statistics, Version 5, 2nd Ed., SYSTAT, Inc. Evanston, IL
24. Liu, Z., and Towle, H. (1995) Biochem. J. 308, 105–111
25. Shih, H.-M., Liu, Z., and Towle, H. C. (1995) J. Biol. Chem. 270, 21991–21997
26. Lamers, W., Hanson, R., and Meisner, H. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 5137–5141
27. Salavert, A., and Iynedjian, P. B. (1983) J. Biol. Chem. 258, 13404–13412
28. Giralt, M., Park, E. A., Gurney, A. L., Liu, J., Kakimi, P., and Hanson, R. W. (1991) J. Biol. Chem. 266, 21991–21996
29. Granzer, D., Andreone, T., Kazuyuki, S., and Beale, E. (1983) Nature 305, 549–551
30. O’Mara, B., Dittrich, W., Lauterio, T., and St. Germain, D. (1993) Endocrinology 133, 1715–1723
31. Luzakova, K., and Nelsen, B. (1992) Eur. J. Biochem. 207, 247–251
32. Tagami, T., Nakamura, H., Sasaki, S., Mori, T., Yoshioka, H., Yoshida, H., and Imura, H. (1990) Endocrinology 127, 1727–1734