Vol. 185, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 29, 1992 Pages 127-133

IDENTIFICATION OF CELLULAR DIFFERENTIATION-DEPENDENT NUCLEAR FACTORS THAT BIND TO A HUMAN GENE FOR THYMIDYLATE SYNTHASE

Nobuyuki Horie, Ryushi Nozawa* and Keiichi Takeishi[1]

Laboratories of Genetic Engineering and *Microbiology,
School of Food and Nutritional Sciences, University of Shizuoka, Yada, Shizuoka,
Shizuoka 422, Japan

Received April 10, 1992

Summary: Three nuclear factors were identified that interact with sequences in the 5'-upstream region of the human thymidylate synthase gene. Two of these factors interact with a sequence around the initiation codon of the thymidylate synthase gene. The amounts of these two factors changed dramatically as human promyelocytic leukemia HL-60 cells differentiated into macrophage-like cells by the treatment with 1,25-dihydroxyvitamin D_3. The change was closely correlated with the decrease in the amount of thymidylate synthase mRNA during the differentiation. These findings suggest that the specific nuclear factors are involved in the regulation of the expression of human thymidylate synthase gene during the differentiation of HL-60 cells.

Thymidylate synthase (TS, EC 2.1.1.45) catalyzes the conversion of deoxyuridylate to thymidylate. Because this enzyme is essential for regulation of the balanced supply of the four precursors for the normal replication of DNA, the activity of TS is tightly regulated depending on the state of cell proliferation (1-4). The regulated expression of the human TS (hTS) gene has been studied in connection with the structure of the gene (5,6) and the cell-cycle dependence (7). However, the molecular mechanisms of the regulated expression, such as the interaction of regulatory proteins with the hTS gene, remain to be elucidated.

In this study, we identified three nuclear factors that bind to the 5'-upstream region of the hTS gene. One of these factors binds to an octamer sequence upstream from the cap site and the other two interact with a sequence around the initiation codon of the hTS gene. The level of the latter two factors changed dramatically during differentiation of human promyelocytic leukemia HL-60 cells, in which the level of hTS mRNA was reported to decrease rapidly during the differentiation (8). Because the change in the level of the nuclear factors was closely correlated with the decrease in the level of hTS mRNA during the differentiation, it is suggested that these two nuclear factors are

[1]To whom correspondence should be addressed.

0006-291X/92 $4.00

involved in the regulation of the expression of the hTS gene during the differentiation of HL-60 cells.

METHODS

Cell Lines and Culture Conditions

HeLa S3 (sc) cells were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan), and cultured in suspension in ES medium (9) containing 10% fetal calf serum. HL-60 cells were cultured in F12 medium containing 10% fetal calf serum. For induction of differentiation, HL-60 cells (2×10^5 cells per ml) were treated with 10^{-7} M 1,25-dihydroxyvitamin D_3 (VD3, Roussel Pharmaceutical, Paris) for various periods. The medium containing VD3 was renewed every 3 days.

Fragments of DNA for Gel Mobility Shift Analysis

DNA fragments for gel mobility shift assay were prepared from plasmid pHRR68, a subclone of a fragment of the hTS gene (5). DNA fragments were labeled at the 3'-end with [α-^{32}P] dCTP (3000Ci/mmol, NEN) by the Klenow fragment and purified by polyacrylamide gel electrophoresis.

Gel Mobility Shift Analysis

Nuclear extracts were prepared from cultured cells according to Schreiber et. al. (10). Protein concentration was determined with a protein assay kit (Bio-Rad Lab., CA) and standardized with bovine gamma globulin. The extracts typically contained 2-5 mg of protein per ml. The conditions for gel mobility shift assay were as described previously (11).

Methylation Interference Assay

The XbaI-BglI fragment of the hTS gene was labeled at the 5'-end of BglI site with ^{32}P and used for methylation interference assay (12).

RESULTS

Nuclear Factors That Bind to the 5'-Upstream Region of a Human Gene for Thymidylate Synthase

DNA-binding nuclear factors that interact with the upstream region of a hTS gene were surveyed by gel mobility shift analysis using an ScaI-SalI fragment (222 bp), an SalI-XbaI fragment (149 bp) and an XbaI-BglI (216 bp) fragment as probes (see Fig. 1C). Among these probes, the ScaI-SalI fragment and the XbaI-BglI fragment were found to interact with specific nuclear factors (bands a, b and c in Fig. 1A). A very small amount of the nuclear factor for band c was detected in HeLa cells reproducibly but the factor was not detected in Raji cells. On the other hand, the nuclear factors for band a and b were detected in HeLa cells and Raji cells. Therefore, these two factors are very likely to be ubiquitous DNA binding factors specific for the hTS gene. As shown in Fig. 1B, the interaction in the formation of band b occurred specifically with a particular DNA sequence. Specificity of interaction in the formation of bands a and c was also confirmed by competitive gel mobility shift analysis (data not shown).

Dependence of the Levels of Nuclear Factors on the State of Cell Differentiation

To study the function of the nuclear factors that bind to the 5'-upstream region of the hTS gene, we used induction of differentiation of human promyelocytic leukemia HL-60

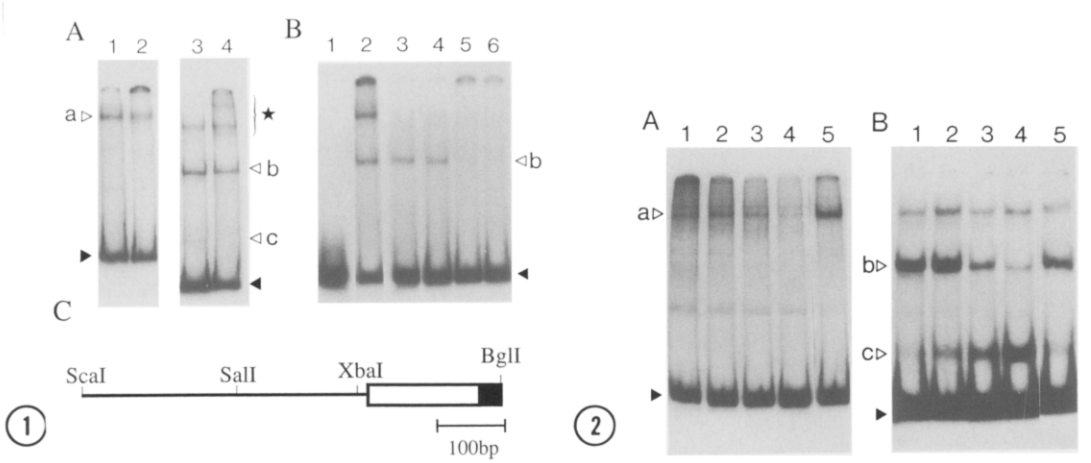

Fig. 1. Nuclear factors that bind to the 5'-upstream region of the hTS gene. A, Gel mobility shift analysis using fragments of DNA from the 5'-upstream region of the hTS gene (see Fig. 1C). An end-labeled 222-bp ScaI-SalI fragment (lanes 1 and 2) and an end-labeled 216-bp XbaI-BglI fragment (lanes 3 and 4) were incubated with nuclear extracts prepared from HeLa cells (lanes 1 and 3) or Raji cells (lanes 2 and 4). Filled triangles indicate bands of free DNA. Open triangles indicate shifted bands of nucleoprotein complexes. Bands in the region with a star represent complexes formed with non-specific DNA binding proteins, as proven by competitive gel mobility shift analysis (see Fig. 1B). B, Competition for binding to nuclear factor by specific and non-specific DNA fragment. An end-labeled XbaI-BglI fragment (0.5 ng) was incubated with nuclear extracts prepared from HeLa cells in the presence of 25 ng (lanes 3 and 5) or 50 ng (lanes 4 and 6) of a competitor DNA fragment. Lane 1, no extracts; lane 2, no competitor; lanes 3 and 4, the ScaI-XbaI fragment as competitor; lanes 5 and 6, the XbaI-BglI fragment as competitor. C, Structure of the 5'-upstream region of the hTS gene. Restriction sites are shown at the top. Open and solid bars represent untranslated and translated regions, respectively.

Fig. 2. Qualitative and quantitative changes in nucleoprotein complexes during the differentiation of HL-60 cells. An end-labeled 371-bp ScaI-XbaI fragment (panel A) and a 216-bp XbaI-BglI fragment (panel B) were incubated with nuclear extracts from undifferentiated HL-60 cells (lane 1), from HL-60 cells treated with VD3 for 2 days (lane 2), 3 days (lane 3), or 6 days(lane 4), or from HeLa cells (lane 5). Open triangles indicate nucleoprotein complexes. Filled triangles point to bands of free DNA.

cells as a model for switching cellular proliferation. HL-60 cells differentiate into macrophage-like cells as a result of treatment with VD3 and their proliferation decreases rapidly during the differentiation (13,14). The level of the hTS mRNA was recently reported to decrease rapidly during the differentiation induced by VD3 (8). To examine whether the level of the nuclear factors change during the differentiation, nuclear extracts were prepared from HL-60 cells treated with VD3 for various periods of time and analyzed by the gel mobility shift assay. As shown in Fig. 2, the levels of the DNA-binding factors varied during differentiation of HL-60 cells induced by VD3.

The intensity of the band that corresponded to nucleoprotein complex containing the ScaI-XbaI DNA fragment (band a in Fig. 2A) was weakened during the cellular differentiation, but the mobility of the complex did not change. By contrast, the pattern of the complexes that contained the XbaI-BglI DNA fragment changed dramatically. The amount of the major complex formed with nuclear extracts from untreated HL-60 cells (band b in Fig. 2B) decreased during the differentiation, whereas the amount of the

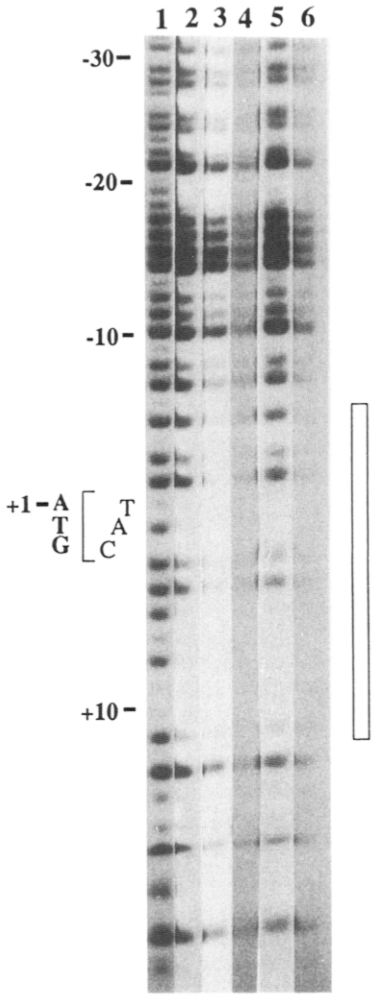

Fig. 3. Methylation interference experiment with a fragment of DNA that corresponds to the region around the initiation codon of the hTS gene. The partially methylated fragment was incubated with nuclear extracts from HeLa cells or from HL-60 cells that had been treated with VD3 for 3 days. Bands of nucleoprotein complexes and free DNA were excised from preparative gels and the positions of methylated guanine residues were analyzed after piperidine treatment. Lane 1, a control purine ladder (A+G); lanes 2 and 5, a guanine ladder generated from free DNA; lane 3, DNA from the complex c obtained with extracts of HL-60 cells (see Fig. 2B); lane 4, DNA from the complex b obtained with extracts of HL-60 cells; lane 6, DNA from the complex b obtained with extracts of HeLa cells. Nucleotide numbers and the position of initiation codon are shown on the left side. An open bar on the right represents the interacting region of the DNA fragment.

minor complex with high mobility (band c in Fig. 2B) increased markedly during the differentiation.

Nucleotide Sequences Involved in the Formation of Nucleoprotein Complexes

To examine the nucleotide sequence that interacts with DNA binding factors at high resolution, we used methylation interference analysis (12). The nucleoprotein complex obtained with nuclear extracts of HeLa cells and the two types of the complexes obtained with those of differentiated HL-60 cells were analyzed (see Fig. 3A). For

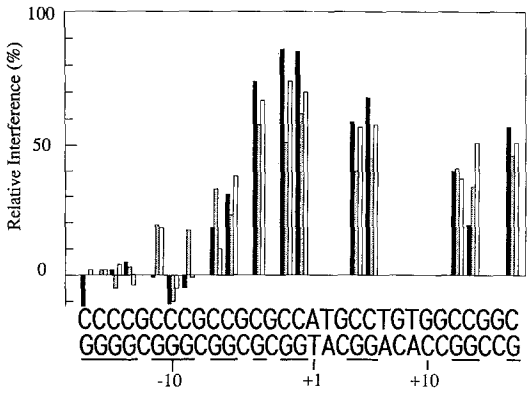

Fig. 4. Quantitative analysis of methylation interference experiment. The relative reductions of each guanine band in the ladders were calculated from densitometric scanning of sequence ladders obtained in the methylation interference experiment. The autoradiograph was scanned with UltroScan XL (Pharmacia LKB Biotechnology AB, Uppsala, Sweden). The density of each band was normalized and the ratio of reduction of the intensity of a band from a nucleoprotein complex to that of the corresponding band from free DNA was calculated. Values on the ordinate show relative interference by a particular guanine residue in the formation of the complex when that guanine residue was methylated. Dotted and filled bars represent data for complexes b and c, respectively, obtained with nuclear extracts from differentiated HL-60 cells, and open bars represent data for complex b obtained with extracts of HeLa cells (see Fig. 2B). The nucleotide sequence and the nucleotide positions, numbered from the initiation codon, are shown at the bottom.

quantitative analysis, the intensity of each band in the sequencing gel was measured and the ratio of reduction of the intensity for each guanine band caused by methylation was calculated. As shown in Fig. 4, the three patterns of methylation interference were essentially the same among these complexes and the intensities of guanine residues in the region from -4 to +11 (numbered from the adenine residue in the initiation codon of the hTS gene) diminished in the DNA from the nucleoprotein complexes as compared to those in control DNA. These observations indicate that both types of nucleoprotein complex shared the same nucleotide sequence including the initiation codon of the hTS gene.

DISCUSSION

The essential sequences for the transcription of the hTS gene are located in the region downstream from the ScaI site (12), which lies 557 bp upstream from the adenine residue of the initiation codon. The sequence analysis revealed that an octamer sequence is present in the region mentioned above, but there are no sequences homologous to a CAAT box, TATA box, or GC box sequence. In the mouse TS gene, the binding site of Sp1 is essential for the normal level of transcription of the gene (15). However, the sequence in this region exhibits no homology to that in the corresponding region of the human TS gene. In the present study, we demonstrated that three kinds of nuclear factor found in human cells interacted with the 5'-terminal region of the hTS gene. One of these factors corresponding the band a in Fig. 1A,

named NF-TS1, bound to an octamer sequence upstream from the cap site of the hTS gene (unpublished results), and the other two factors, named NF-TS2 for the band b and NF-TS3 for the band c in Fig. 1A, interacted with the sequence around the initiation codon of the hTS gene (Fig. 4).

The amount of NF-TS2 decreased during the cellular differentiation, while the amount of NF-TS3 increased markedly (Fig. 2B). On the other hand, both of the factors shared the same binding site that included the initiation codon of the hTS gene. Therefore, NF-TS2 is likely to convert into NF-TS3 during the differentiation of HL-60 cells. Because TS is essential for cellular proliferation and because the rate of proliferation of HL-60 cells decreases during the differentiation, it is suggested that NF-TS2 and NF-TS3 modulate the expression of the hTS gene in association with the cellular differentiation. NF-TS2 was also found as a major band in the analysis of nuclear extracts from HeLa cells and Raji cells. Because TS activity is much higher in proliferating HeLa, Raji and HL-60 cells than in differentiated HL-60 cells, NF-TS2 complex is probably important for adequate expression of hTS gene. In fact, the change in the amount of TS mRNA during the differentiation of HL-60 cells (8) appears to be closely correlated with the change in the levels of NF-TS2 observed in this study. Although precise mechanism of the conversion of these two complexes remains to be elucidated, dissociation of accessory proteins or specific degradation of functional DNA-protein complex possibly occurs during the differentiation of HL-60 cells, which results in reduced level of expression of the hTS gene.

ACKNOWLEDGMENTS

This work was supported in part by Grants-in-Aid for Cancer Research and for Scientific Research from the Ministry of Education, Science and Culture of Japan and by a Grant-in-Aid from the TERUMO LIFE SCIENCE FOUNDATION.

REFERENCES

1. Maley, F. and Maley, G. F. (1960) J. Biol. Chem., **235**, 2968-2970.
2. Haurani, F. I., Kardinal. C. G. and Biermann, W. A. (1978) J. Cell Physiol., **95**, 49-56.
3. Hartmann, K.-U. and Heidelberger, C. (1961) J. Biol. Chem., **236**, 3006-3013.
4. Lochshin, A., Moran, R. G. and Danenberg, P. V. (1979) Proc. Natl. Acad. Sci. USA, **76**, 750-754.
5. Takeishi, K., Ayusawa, D., Kaneda, S., Shimizu, K. and Seno, T. (1989) J. Biochem. (Tokyo), **106**, 575-583.
6. Kaneda, S., Nalbantoglu, J., Takeishi, K., Shimizu, K., Gotoh, O., Seno, T., and Ayusawa, D. (1990) J. Biol. Chem., **265**, 20277- 20284.
7. Ayusawa, D., Shimizu, K., Koyama, H., Kaneda, S., Takeishi, K., and Seno, T. (1986) J. Mol. Biol., **190**, 559-567.
8. Kuruto, R., Nozawa, R. Takeishi, K., Arai, K., Yokota, T. and Takasaki, Y. (1990) J. Biochem., **108**, 650-653.
9. Koyama, H. and Kodama, H. (1982) Cancer Res., **42**, 4210-4214.

10. Schreiber, E., Matthias, P., Müller, M. M. and Schaffner, W. (1989) Nucleic Acids Res., **17**, 6419.
11. Knight, G. B., Gudas, J. M. and Pardee, A. (1987) Proc. Natl. Acad. Sci. USA, **84**, 8350-8354.
12. Sen, R. and Baltimore, D. (1986) Cell, **46**, 705-716.
13. Collins, S. J. (1987) Blood, **70**, 1233-1244.
14. Mihara, K., Cao, X. R. Yen, A., Chandler, S, Driscoll, B., Murphree, A. L. T'Ang, A. and Fung, Y. K. T. (1989) Science, **246**, 1300-1303.
15. Deng, T., Li, Y., Jolliff, K. and Johnson, L. F. (1989) Mol. Cell. Biol., **9**, 4079-4082.