Increases in mRNA
Concentrations of the \( \alpha \) and \( \beta \) Subunits of Prolyl 4-Hydroxylase Accompany Increased Gene Expression of Type IV Collagen during Differentiation of Mouse F9 Cells*

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Mouse F9 teratocarcinoma stem cells differentiate in monolayer cultures in the presence of retinoic acid, dibutyryl cAMP, and isobutyl methylxanthine. This differentiation is associated with a marked increase in the synthesis rates and mRNA concentrations of basement membrane proteins such as type IV collagen. We report here that the differentiation also involves an increase of up to 30-fold in the concentrations of the mRNAs for the \( \alpha \) and \( \beta \) subunits of prolyl 4-hydroxylase, the enzyme required for the cotranslational and post-translational hydroxylation of proline residues in collagens. The time courses and magnitudes of increases in these two mRNA concentrations were similar to those observed in the same experiments for the mRNA of the \( \alpha \) chain of type IV collagen. In the differentiated F9 cells the concentration of the \( \alpha \) subunit mRNA was about 30% of the \( \beta \) subunit mRNA concentration. Northern blot analyses indicated that the sizes of the \( \alpha \) and \( \beta \) subunit mRNAs in the differentiated mouse F9 cells are similar to those in human skin fibroblasts. The F9 cell differentiation system appears to provide a useful model for studies on the regulation of prolyl 4-hydroxylase synthesis.

Mouse F9 teratocarcinoma stem cells can be induced to differentiate in monolayer cultures in the presence of low concentrations of retinoic acid. The effect of retinoic acid is enhanced by dibutyryl cAMP and isobutyl methylxanthine, but the two compounds alone do not induce this differentiation (1-5). The differentiation is associated with a striking change in cell morphology and a marked increase in the synthesis and secretion of basement membrane proteins such as type IV collagen and laminin (1-3). Increases in the concentrations of the respective mRNAs are also observed, maximal increases being found within 4-5 days after retinoic acid addition (4-7). This F9 cell differentiation system has become an increasingly popular model for studies on regulation of the synthesis of various basement membrane components and other developmentally regulated events (8, 9).

Type IV collagen has characteristically a high content of 4-hydroxyproline, an amino acid found in collagens and in a few other proteins with collagen-like amino acid sequences (10, 11). The 4-hydroxyproline is formed by a cotranslational and post-translational modification catalysed by prolyl 4-hydroxylase (for reviews, see Refs. 10 and 11). This enzyme plays a central role in collagen synthesis, as the 4-hydroxyproline residues formed are essential for the folding of the newly synthesized collagenous polypeptide chains into triple helical molecules. The active prolyl 4-hydroxylase is an \( \alpha \)\( \beta \) tetramer, the \( \alpha \) subunits probably contributing the major part to the catalytic sites (10, 11). Complete cDNA-derived amino acid sequences have recently been determined for both types of subunit of the human (12, 13) and the chick enzyme (14-16). The \( \beta \) subunit has been found to be identical to the enzyme protein disulfide isomerase (12, 17) and a cellular thyroid hormone binding protein (18) and highly similar to a glycosylation site binding protein of oligosaccharyl transferase (19).

Only trace amounts of prolyl 4-hydroxylase activity are found in the undifferentiated F9 cells, this activity being strikingly higher in the differentiated F9 cells (8). The present paper reports that differentiation of the F9 cells is accompanied by marked increases in the concentrations of the mRNAs for both the \( \alpha \) and \( \beta \) subunits of prolyl 4-hydroxylase.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Mouse F9 teratocarcinoma cells (obtained from Dr. Eero Lehtonen, Department of Pathology, University of Helsinki, Helsinki, Finland) were cultured on gelatinized dishes in RPMI 1640 medium (GIBCO) containing 10% Serum Plus medium supplement (KC Biologicals). To induce differentiation, 0.1, 0.5, or 2.5 \( \mu \)M retinoic acid, 0.1, 0.5, or 2.5 \( \mu \)M dibutyryl cyclic AMP, and 0.04, 0.2, or 1.0 \( \mu \)M isobutylmethylxanthine were added to the cells (4-7).

**Preparation of Labeled cDNA Probes**—The following cDNA probes were used: PA-49, a 2580-base pair (bp) clone that codes for the \( \alpha \) subunit of human prolyl 4-hydroxylase (13); S-138, a 2427-bp clone that codes for the \( \beta \) subunit of human prolyl 4-hydroxylase (12); HT-21, a 2689-bp clone that codes for the \( \alpha \) chain of human type IV collagen (20); and pHcGAP, a 1200-bp clone that codes for glyceroldehyde-3-phosphate dehydrogenase (American Type Culture Collection, probe 57081). For Northern and slot blot hybridizations, pHcGAP contained in plasmid pBR322 and the insert DNAs of cDNA clones PA-49, S-138, and HT-21 were labeled by nick translation with \(^{32}\)P[dATP, \(^{32}\)P[dCTP, \(^{32}\)P[dGTP, and \(^{32}\)P[dTTP to the same specific radioactivity.

**Acess of mRNAs**—Total RNA was extracted from the cultured F9 cells as described previously (21). For Northern blot analysis the RNAs were electrophoresed in a 0.7% agarose gel containing 2 M formaldehyde and transferred to a nitrocellulose filter (22). To assay steady-state levels of mRNAs, three serial dilutions of denatured total RNA (5-20 \( \mu \)g) were dotted onto nitrocellulose paper using a vacuum manifold (Millipore II, Schleicher and Schuell). The filters were air-dried and baked at 78 °C for 2 h. Identical Northern and slot blot filters were incubated with each labeled cDNA clone (2 ng/ml) in a solution containing 50% (v/v) formamide, 5 × SSC (0.15 M NaCl, 0.015 M sodium citrate, pH 6.8), denatured salmon sperm DNA (0.25 mg/ml), 0.1% SDS, 0.2% polyvinylpyrrolidone, 0.2% Ficoll, and 0.2% bovine serum albumin for 24 h at 41 °C. The filters were washed twice with a solution containing 0.5 × SSC and 0.05% SDS at room temperature and once at 65 °C. The blots were exposed to Kodak X-...
Northern Blot Analysis of mRNAs—The F9 cells are of mouse origin, whereas the cDNA probes used for the \( \alpha \) and \( \beta \) subunit of prolyl 4-hydroxylase and the \( \alpha 1 \) chain of type IV collagen have all been made from human mRNAs. The specificity of the hybridization with these probes was therefore established using Northern blot analyses of total cellular RNA from F9 cells cultured in the presence of 2.5 \( \mu M \) retinoic acid, 2.5 mM dibutyryl cAMP, and 1.0 mM isobutylmethylxanthine for 120 h. The three \( ^{32} \)P-labeled cDNA probes each hybridized to a single mRNA species, the sizes of the three mRNAs being 3.0 and 2.5 kb for the \( \alpha \) and \( \beta \) subunits of prolyl 4-hydroxylase and close to 7 kb for the \( \alpha 1 \) chain of type IV collagen, respectively (Fig. 1). All these sizes are in good agreement with those found with these probes for human RNA (12, 13, 23).

Changes in Prolyl 4-Hydroxylase mRNA Concentrations in F9 Cells Treated with Retinoic Acid, Dibutyryl CAMP, and Isobutylmethylxanthine for 120 h—F9 cells were cultured with varying concentrations of retinoic acid, dibutyryl cAMP, and isobutylmethylxanthine for 120 h. The total cellular RNA was then isolated and analyzed using slot blot hybridization with \( ^{32} \)P-labeled cDNA probes. When concentrations of the differentiation-inducing compounds were 2.5 \( \mu M \) for retinoic acid, 2.5 mM for dibutyryl cAMP, and 1.0 mM for isobutylmethylxanthine a marked increase was found in the mRNA concentrations of both the \( \alpha \) subunit and the \( \beta \) subunit of the enzyme (Fig. 2). Exact quantification of the magnitudes of increases in these two mRNA concentrations is difficult, because the mRNA concentrations for both types of subunit of the enzyme in the undifferentiated stem cells were found to be very low, at the borderline of detection (Fig. 2). In the experiment shown in Fig. 2 the increase was about 50-fold. When concentrations of all three differentiation-inducing compounds were reduced to \( \frac{1}{2} \), the magnitudes of increases in both mRNA concentrations were identical to those obtained with the concentrations given above (Fig. 2), whereas decreasing the concentrations to \( \frac{1}{2} \times \frac{1}{2} \) produced smaller increases (not shown). A marked increase was also found in the mRNA concentration for the \( \alpha 1 \) chain of type IV collagen (Fig. 2), up to about 50-fold.

In some experiments, however, the magnitudes of the increases in the two prolyl 4-hydroxylase mRNA concentrations and the type IV collagen \( \alpha 1 \) chain mRNA concentration were considerably smaller than those shown in Fig. 2, only 5–10-fold. This was partly due to the known tendency of the F9 cells to occasionally spontaneously differentiate even without addition of retinoic acid, producing slightly higher concentrations of the mRNA for the \( \alpha 1 \) chain of type IV collagen (e.g. Ref. 24) and also the mRNAs for the two types of subunit of prolyl 4-hydroxylase than completely undifferentiated stem cells. This was seen as the presence of slightly elevated amounts of hybridizable material even in the absence of the inducers. The magnitudes of increases in the mRNA concentrations in the differentiated F9 cells in such experiments were correspondingly lower than in the experiments in which the stem cells were completely undifferentiated.

Time Course of Increases in the mRNA Concentrations for the Two Types of Subunit of Prolyl 4-Hydroxylase and the \( \alpha 1 \)
Chain of Type IV Collagen in Differentiating F9 Cells—F9 cells were induced to differentiate by treatment with 2.5 \( \mu \)M retinoic acid, 2.5 mM dibutyryl CAMP, and 1.0 mM isobutylmethylxanthine, and the total cellular RNA was isolated at 0, 24, 48, 72, 96, and 120 h. Concentrations of the various mRNAs were quantified by the slot blot hybridization technique. A marked increase in the concentrations of the mRNAs for both the \( \alpha \) and \( \beta \) subunits of prolyl 4-hydroxylase (Fig. 3) and the \( \alpha1 \) chain of type IV collagen (Fig. 4) was seen already after induction for 48 h, maximal increase being found in 72–120 h (Figs. 3 and 4). In order to verify that the increases seen in these mRNA concentrations were not due to nonspecific effects or artifacts, we also measured the mRNA concentration for glyceraldehyde-3-phosphate dehydrogenase (Fig. 4). No changes were seen in this mRNA concentration.

Ratio of Concentration of the Two Prolyl 4-Hydroxylase mRNAs—The specific activities of the \( \alpha \) and \( \beta \) subunit cDNA probes were similar. Assuming that the two probes had similar affinities for the respective mRNAs and correcting for the fact that the length of the \( \beta \) subunit cDNA was 94% of the length of the \( \alpha \) subunit cDNA, it can be calculated that, in the differentiated F9 cells, the ratio of the \( \alpha \) subunit to \( \beta \) subunit mRNA concentration was 0.34 ± 0.09 (mean ± S.D., \( n = 4 \)). The ratio in the undifferentiated F9 cells could not be determined accurately due to the very low mRNA concentrations. Nevertheless, the ratio could be determined in the spontaneously partially differentiated F9 cells (see above) in which the levels of the two prolyl 4-hydroxylase mRNAs were only about 5–10% of those in the differentiated F9 cells. The ratio in four such cultures was 0.40 ± 0.06 (mean ± S.D.). Thus the ratio of the \( \alpha \) subunit to \( \beta \) subunit mRNA in the undifferentiated F9 cells may be similar to that seen in the differentiated F9 cells.

**DISCUSSION**

In most cells and tissues the \( \beta \) subunit of prolyl 4-hydroxylase is produced in 10–100-fold excess over the \( \alpha \) subunit and enters a pool of the multifunctional \( \beta \) subunit before being incorporated into the prolyl 4-hydroxylase tetramer (10, 25). Regulation of the amounts of the active enzyme tetramer thus mainly occurs through regulation of the amounts of the \( \alpha \) subunit (10, 25). Nevertheless, in some situations the levels of the \( \beta \) subunit are also regulated. For example, the concentrations of both the \( \alpha \) and \( \beta \) subunits are low in cultured transformed cells when compared with nontransformed cells (26). The present study provides another example of a situation in which the synthesis rates of both the \( \alpha \) and \( \beta \) subunits are altered markedly.

The undifferentiated F9 stem cells were found to contain only trace amounts of the mRNAs for the two types of prolyl 4-hydroxylase subunit. Differentiation of these cells was associated with an increase in these two mRNAs of up to 50-fold, the time course and magnitude of this increase being similar to those observed for the mRNA of the \( \alpha1 \) chain of type IV collagen. The ratio of the \( \alpha \) subunit to \( \beta \) subunit mRNA concentration in the slightly and fully differentiation-induced F9 cells was found to be about 0.3–0.4, which is markedly higher than the ratio of 0.1 determined with the same cDNA probes in cultured human skin fibroblasts. The latter ratio agrees well with the ratio of the \( \alpha \) subunit to \( \beta \) subunit concentration in cultured fibroblasts (25), and it thus seems likely that the excess of the \( \beta \) subunit synthesis in the undifferentiated and differentiated F9 cells may be much lower than found for most other cells.

The increase in the mRNA concentration for the \( \alpha1 \) chain of type IV collagen in the differentiated F9 cells has been demonstrated to be largely due to an increased rate of transcription of the respective gene (27). Nevertheless, collagen mRNA concentrations are also regulated at the level of mRNA stability (28), and an increased mRNA stability has been suggested to contribute to a significant part of the increase in the type IV collagen mRNA concentrations during F9 cell differentiation (6). This suggestion is based on the finding that the levels of stimulation of transcription of the type IV collagen \( \alpha1 \) gene in differentiation-induced F9 cells are not sufficient to account for the accumulated concentration of the corresponding mRNA observed (6). The present data do not indicate whether the increases in the prolyl 4-hydroxylase mRNA concentrations likewise are due to more than one mechanism. The availability of both cDNA (12–16) and genomic clones (29, 30) for the two types of subunit of prolyl 4-hydroxylase now provides tools for detailed studies on the mechanisms involved in the regulation of the synthesis of this enzyme and on the mechanisms by which the synthesis of

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prolyl 4-hydroxylase is coordinated with changes in the rate of collagen synthesis. The F9 cell differentiation system appears to provide a highly useful model for these investigations.

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REFERENCES
1. Strickland, S., Smith, K. K., and Marotti, K. R. (1980) Cell 27, 347-355
2. Hogan, B. L. M. (1980) Dev. Biol. 76, 275-285
3. Cooper, A. R., Taylor, A., and Hogan, B. L. M. (1983) Dev. Biol. 99, 510-516
4. Kurkinen, M., Barlow, D. P., Helfman, D. M., Williams, J. G., and Hogan, L. M. (1983) Nucleic Acids Res. 11, 6199-6209
5. Wang, S.-Y., and Gudas, L. J. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 5880-5884
6. Grover, A., Edwards, S. A., Bourdon, M., and Adamson, E. D. (1987) Differentiation 36, 138-144
7. Kleinman, H. K., Ebihara, J., Killen, P. D., Sasaki, M., Cannon, F. B., Yamada, Y., and Martin, G. R. (1987) Dev. Biol. 122, 373-378
8. Roguska, M. A., and Gudas, L. J. (1985) J. Biol. Chem. 260, 7681-7687
9. Wang, S.-Y., and Gudas, L. J. (1988) J. Cell. Physiol. 136, 305-311
10. Kivirikko, K. I., Myllylä, R., and Pihlajaniemi, T. (1989) FASEB J. 3, 1699-1707
11. Kivirikko, K. I., Helaakoski, T., Vuori, K., Tasanen, K., Parkkonen, T., Myllylä, R., and Pihlajaniemi, T. (1990) Ann. N. Y. Acad. Sci. 680, 132-142
12. Pihlajaniemi, T., Helaakoski, T., Tasanen, K., Myllylä, R., Huhtala, M.-L., Koivu, J., and Kivirikko, K. I. (1987) EMBO J. 6, 643-649
13. Helaakoski, T., Vuori, K., Myllylä, R., Kivirikko, K. I., and Pihlajaniemi, T. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 4392-4396
14. Bassuk, J. A., Kao, W. W.-Y., Herzer, P., Kederaha, N. L., Seyer, J., De Martino, J. A., Daugherty, B. L., Mark, G. E., and Berg, R. A. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 7382-7386
15. Parkkonen, T., Kivirikko, K. I., and Pihlajaniemi, T. (1988) Biochem. J. 256, 1005-1011
16. Kao, W. W.-Y., Nakazawa, M., Aida, T., Everson, W. V., Kao, C. W.-C., Seyer, J. M., and Hughes, S. H. (1988) Connect. Tissue Res. 18, 157-174
17. Koivu, J., Myllylä, R., Helaakoski, T., Pihlajaniemi, T., Tasanen, K., and Kivirikko, K. I. (1987) J. Biol. Chem. 262, 6447-6449
18. Cheng, S.-Y., Gong, Q.-H., Parkinson, C., Robinson, E. A., Appella, E., Merlino, G. T., and Paclan, I. (1987) J. Biol. Chem. 262, 11221-11227
19. Geetha-Habib, M., Noiva, R., Kaplan, H. A., and Lennarz, W. J. (1986) Cell 44, 1063-1063
20. Pihlajaniemi, T., Tryggvason, K., Myers, J. C., Kurkinen, M., Lebo, R., Cheung, M.-C., Prockop, D. J., and Boyd, C. D. (1985) J. Biol. Chem. 260, 7681-7687
21. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, p. 196, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
22. Thomas, P. S. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 5201-5205
23. Myers, J. C., Brinker, J. M., Kefalides, N. A., Rosenbloom, J., Wang, S.-Y., and Gudas, L. (1986) Nucleic Acids Res. 14, 4499-4517
24. Smith, B. D. and Baldwin, C. T. (1989) Biochem. J. 255, 85-89
25. Kivirikko, K. I., and Myllylä, R. (1980) in The Enzymology of Posttranslational Modification of Proteins (Freedman, R. B., and Hawkins, H. C., eds) pp. 53-104, Academic Press, London
26. Myllylä, R., Koivu, J., Pihlajaniemi, T., and Kivirikko, K. I. (1983) Eur. J. Biochem. 134, 7-11
27. Wang, S.-Y., La Rosa, G. J., and Gudas, L. J. (1985) Dev. Biol. 107, 75-86
28. Hamalainen, L., Oikarinen, J., and Kivirikko, K. I. (1985) J. Biol. Chem. 260, 720-725
29. Tasanen, K., Parkkonen, T., Chow, L. T., Kivirikko, K. I., and Pihlajaniemi, T. (1983) J. Biol. Chem. 258, 16218-16224
30. Helaakoski, T., Vuori, K., Parkkonen, T., Myllylä, R., Chow, L. T., Kivirikko, K. I., and Pihlajaniemi, T. (1990) Ann. N. Y. Acad. Sci. 680, 473-476