Analysis of the Promoter of Mutated Human Whey Acidic Protein (WAP) Gene

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Abstract. Although whey acidic protein (WAP) has been identified in the milk of a range of species, it has been predicted that WAP is not secreted into human milk as a result of critical point mutations within the coding region. In the present study, we first investigated computationally the promoter region of mutated human WAP genes by comparing with those of other known WAP genes. Computational database analyses showed that the human WAP promoter region was highly conserved, as in other species with milk WAP. Next, we evaluated the activity of the human WAP promoter (2.6 kb) using a reporter gene assay. MCF-7 cells were stably transfected with the hWAP/hGH (human growth hormone) fusion gene, cultured on Matrigel, and treated with lactogenic hormones. Radioimmunoassay detected hGH in the culture medium, indicating that the human WAP promoter was responsible for the lactogenic hormones. The human WAP promoter was significantly more active in MCF-7 cells than the mouse WAP promoter (2.4 kb). The present results provide us with important information on the molecular evolution of milk protein genes.

Key words: Database analyses, Promoter assay, Whey acidic protein (WAP)

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the milk of various species of monotremes and marsupials, and the results of previous studies on WAP function, it has been postulated that synthesis and secretion of WAP is widespread in many species that have been subject to a wide range of evolutionary selection pressures. However, Rival-Gervier et al. [23] predicted that human WAP has critical point mutations within the coding region by comparing the pig WAP gene and human WAP gene. Thus, the expression of WAP gene varies among species. From the point of view of the molecular evolution of milk protein genes, it is interesting to know whether the mutated human WAP gene is transcribed or not. It is extremely difficult to obtain fresh mammary tissue from pregnant or lactating women. Therefore, in the present study, we computationally analyzed the promoter region of human WAP gene by comparison with functional WAP genes, and experimentally assayed the promoter activity of human WAP gene using human mammary tumor MCF-7 cells.

Materials and Methods

Database analyses

Regulatory elements of the 5' region (7 kb) of the putative human WAP gene were computationally analyzed. The binding site of STAT5 was predicted by TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCHJ.html). NF-1 (nuclear factor 1), GRE (glucocorticoid receptor binding element), and HSSA (DNase I hypersensitive site-A) and HSSB were predicted with reference to the sequences of the 5' regulatory elements of mouse [1], rat [2], and rabbit [4] WAP genes.

Human WAP promoter assay

Mouse WAP promoter (2.4 kb)/hGH plasmid (mWAP/hGH), as described previously [24], was used as a positive control for the functional WAP promoter. The human WAP promoter (2.6 kb) fragment was amplified using 5'-CAA-TTG-GGG-TGA-ATC-TGA-CGG-C-3' as the forward primer and 5'-GGA-TCC-GGT-GGC-GGG-TGG-CAG-GCA-GG-3' as reverse primer. A 2.6 kb segment of the WAP promoter was inserted into the pBR327-hGH vector (hWAP/hGH) and amplified. To transfect into MCF-7 cells, hWAP/hGH and mWAP/hGH vectors were linearized by digesting with Eco RI, respectively.

MCF-7 cells derived from a human mammary tumor were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum. For establishment of stable transfected cells, the hWAP/hGH or mWAP/hGH plasmids were transfected using of Profection Mammalian Transfection System Calcium Phosphate (Promega, Madison, WI, USA) according to the manufacturer’s instructions. Cells demonstrating stable expression of plasmids were selected by culture passage for 10 days in a complete medium containing G418 (700 µg/ml).

To induce expression from the WAP promoter, cells were cultured in induction medium containing 5 µg/ml insulin, 5 µg/ml prolactin, and 10⁻⁶ M dexamethasone on 6 cm-diameter dishes that were pre-coated with 0.3 mg Matrigel (Sigma, St. Louis, MO, USA).

The hWAP/hGH clonal- or mWAP/hGH clonal-MCF-7 cells were cultured in the induction medium for 4 days, and then total RNA was extracted from these cells using TRIZOL reagent (Life Technologies, Lockville, MD, USA). One microgram of total RNA was denatured and reverse-transcribed into cDNA using SuperScript III (Invitrogen, Carlsbed, CA, USA) in a reaction volume of 20 µl, according to the manufacturer’s protocol. Real-time quantitative RT-PCR was conducted using a LightCycler System (Roche, Basel, Switzerland) according to the manufacturer’s instructions. The PCR products were then sequenced and confirmed to be the appropriate transcript. The human GAPDH transcripts (primers: 5’-TGG-TAT-CGT-GGA-AGG-ACT-CAT-GAC-3’, forward; 5’-ATG-CCA-GTG-AGC-TTC-CGG-TTC-AGC-3’, reverse; Tm=58C) were used as an endogenous control. The human GH gene-specific primers (primers: 5’-TGG-TAT-CGT-GGA-AGG-ACT-CAT-GAC-3’, forward; 5’-ATG-CCA-GTG-AGC-TTC-CGG-TTC-AGC-3’, reverse; Tm=58C) were designed as described by Ozturk-Winder et al. [25]. The PCR reactions consisted of 30 (hGH) or 24 (GAPDH) cycles of 1 min at 94 C, 1 min at 58 C, and 1 min at 72 C. The size of each PCR product was confirmed by electrophoresis using 1% agarose gels in TAE buffer. The relative expression ratio of hGH was calculated as the number of copies of hGH versus the number of copies of the internal control, hGAPDH.

To investigate the expression of the WAP/hGH
transgene in MCF-7 cells, the cells were cultured in induction medium for 2 days, and then 4 ml of medium was harvested. The concentration of hGH was measured using an RIA kit (Daiichi Radioisotope Lab., Tokyo, Japan) according to the manufacturer's instructions.

**Statistical analysis**

The data were analyzed by analysis of variance using the Student's t-test. Values of p<0.05 were considered to indicate statistical significance.

**Results and Discussion**

We investigated whether or not the human WAP gene transcribed messenger RNA. First, we computationally compared the human WAP promoter with the promoter sequences of several other known WAP genes. Figure 1 provides a comparison of the 5' upstream regulatory elements of WAP promoters in humans and the other species examined: rabbits, mice, and rats. A putative TATA box was found at the expected position, i.e., –76 to –57 bp from the translation start point. This TATA box completely coincided with that found in the WAP gene of rodents, rabbits, and pigs, and did not appear to be typical TATA consensus sequences, but a modified sequence (TTTAAA).

Two HSSs (DNase I hypersensitive sites) were predicted to be located at –6.8 kb (HSS-A) and –0.65 kb (HSS-B). The human WAP promoter has STAT5-binding sites (TTCNNNGAA) that are essential for reaction specifically with prolactin and for expression of WAP in a tissue- and stage-specific fashion. However, in comparison with rodent WAPs, the nuclear factor I (NF-I)-binding sites (half-site: TGGCA; palindromic site: TGGCA) were not present.
TTGGCN5GCCAA) and glucocorticoid receptor (GR)-binding sites (GREs) were not highly conserved in human WAP. Other transcriptional sites in human WAP for reaction with lactogenic hormones were not conserved in the WAPs of other species, so we could not predict the homology score.

To investigate whether the human WAP promoter is actually active, we performed a reporter gene assay using human growth hormone gene (hGH) as a reporter gene (Fig. 2A). Real-time PCR of mRNA extracted from hWAP/hGH clonal cells showed that human WAP was activated by lactogenic hormones in the presence of Matrigel (B and C in Fig. 2). Furthermore, hGH was detected in the culture medium used for hWAP/hGH clonal MCF-7 cell culture, and the concentration of hGH produced by these cells was approximately 2.5 times higher than that by mouseWAP/hGH clonal cells (Fig. 2D).

Putative human WAP, which is a WAP pseudogene, loses its translation ability due to several unique nucleotide modifications, including a mutation in the translation start codon, a point deletion of the splicing site in the second intron, the presence of a stop codon in the third exon, and the insertion of extra-Ts in the poly A signal [23].

In a previous study, we found that enforced expression of the mouse WAP transgene inhibited proliferation of MCF-7 cells derived from a human mammary tumor [26]. We also found that activity of the WAP promoter in mWAP/hGH transgenic females was easily examined by measuring plasma hGH [27]. In the present study, we combined these methods, using MCF-7 cells and the WAP/hGH fusion construct to examine the promoter activity of the putative human WAP gene. The results of our experiments demonstrated that the exogenous human WAP promoter was activated by treatment with Matrigel and lactogenic hormones in the human mammary tumor MCF-7 cells in the same way as the same promoter of the mouse WAP gene. Its activity was somewhat higher than that of the mouse WAP promoter, although this may have been due to the difference in length of the nucleotides between the mouse (2.4 kb) and human (2.6 kb) promoters and/or a difference in species-specificity between human cells and human gene sequences.

β-lactoglobulin is another example of a gene that is expressed in the milk of ruminants, pigs, and rabbits, but that has not been detected in the milk of rodents and primates [28]. Rabbits, pigs, brushtail possum, and the tammar wallaby produce WAP and β-lactoglobulin [5, 7]. However, rodents produce WAP, but not β-lactoglobulin, whereas ruminants produce β-lactoglobulin, but not WAP. Our computational analyses showed that the WAP genes of ruminants were mutated (data not shown). Interestingly, humans are unusual because they synthesize neither β-lactoglobulin nor WAP in milk [23]. However, the present results showed that the activity of human WAP promoter was still alive, speculating that human WAP may be transcribed in the mammary gland. To understand the molecular evolution of several whey proteins in milk, it is important to clarify whether WAP and/or β-lactoglobulin are present in the milk of mammalian species. Because there is no similarity in protein composition and structure between WAP and β-lactoglobulin, it is not likely that these milk proteins play a compensatory role in relation to one of the proteins in eutherians and marsupials.

In future studies, it would be of interest to investigate the biological meaning of the presence or absence of WAP expression in the mammary glands of different species.

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