BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **226,** 222–225 (1996)
ARTICLE NO. 1336

Two Nuclear Proteins Bind to the Promoter P3 Region of the Human Parathyroid Hormone-Related Peptide Gene

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Received July 25, 1996

We have used a 5′ regulatory sequence fragment of the human parathyroid hormone-related peptide (PTHrP) gene to identify nuclear DNA-binding proteins (DBP), using South-Western analysis. The PTHrP 549 bp DNA fragment was amplified from a human genomic DNA, and composed of 5′ non-coding exon Ic, the intervening intron and 5′ non-coding exon II. The DNA fragment bound very specifically to a 70 kDa and a 65 kD protein from the nuclear extract of human hepatocyte, HepG2, and keratinocyte, SVK-14, cell lines. This is the first evidence of a physical binding between nuclear protein and the human PTHrP gene. The 70 kDa and 65 kDa nuclear proteins may have a role in the regulation of human PTHrP gene expression.

Parathyroid hormone-related peptide is a potent calcitropic agent that has been implicated to cause hypercalcaemia in the humoral hypercalcaemia of malignancy (HHM) syndrome. It was originally isolated and characterised from tumours of patients with HHM. Structurally, it is related to parathyroid hormone (PTH) in 8 of the first 13 amino-terminal residues, and thus shares similar biological activities with PTH through a common PTHrP/PTH receptor (1). Additionally, it also possesses unique properties including relaxation of vascular, gastrointestinal and other smooth muscles, placental calcium transport, inhibition of myometrial contraction, and cell growth and differentiation (2).

The human PTHrP gene spans more than 19 kb of genomic DNA. It consists of 9 exons in which four are non-coding at the amino terminus and five are coding (3,4). In addition, it also contains two TATA- (P1 and P3) and one GC-rich (P2) promoters that together with preferential splicing will determine the size of the mRNA transcript. In the normal human and tumourous cells and tissues, all three promoters are used to initiate PTHrP mRNA transcription in a cell- or tissue-specific manner (5,6). Apart from the human heart tissues which use P3 only (7), and human amnion which uses P2 in the transcriptional initiation to produce a PTHrP mRNA encoding PTHrP(1-139) (8). In one study, P3 is reported to have the strongest transcriptional activity (6). The reason for this is unknown but could be caused by the factors that bind to P3 neighbouring regulatory sequences to influence the promoter activity. Several regions lying upstream of the P3 promoter are able to exert influences on a reporter chloramphenicol acetyltransferase gene (5), indicating such a mechanism may exist to regulate PTHrP gene expression. However, this mechanism requires the binding of DBP to DBP-consensus sequence. No such PTHrP DBP has yet been identified. Therefore, this study was set out to identify PTHrP DBP. A human PTHrP DNA fragment containing P3 and its promixal/distal sequences

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0006-291X/96 $18.00

was used in the South-Western analysis on human cell nuclear extracts. Our data presented show two distinct nuclear proteins bound specifically to the human PTHrP DNA fragment.

MATERIALS AND METHODS

Amplification of a human PTHrP DNA fragment was done by polymerase chain reaction (PCR) from human genomic DNA using specific PTHrP primers (position of forward primer is 1413-1432: 5′-TTTCAACTCGCC-TCCAACCT-3′ and position of reverse primer is 1934-1953: 5′-GCAACCGGCTACTCCAACTG-3′) as described (3). In a 100 μl reaction contained 100 ng of template, 10 μl of 10× Taq DNA polymerase buffer (Boehringer Mannheim), 0.25 μM of each primer, 200 μM of each deoxynucleotide triphosphate (Pharmacia), 2.5U of Taq DNA polymerase (Boehringer Mannheim) and overlaid with 100 μl of light oil. Amplification was performed in an automated thermal cycler (Perkin-Elmer Cetus) as follow: 1 cycle at 94°C for 10 minutes; followed by 30 cycles at 94°C for 1 minute, 60°C for 1 minute and 72°C for 1 minute. The PCR product (2 μl) was cloned into TA vector as described by the manufacturer (Invitrogen). The sequence of PCR insert was determined bidirectionally using Sequenase kit (United States Biotechnology).

PTHrP PCR insert or cDNA insert from pCIS2.BRF1.1 (Dr. W. Wood, Genentech Inc., USA) was labelled with [a-^{32}P]dCTP (Amersham) using oligonucleotide labelling kit (Pharmacia) and purified using NICK^{TM} column (Pharmacia). Oligonucleotides used as positive control contained TEF/2 protein motif C5 consensus sequence and as negative control contained yeast GCN4 recognition sequence from the Affinity Screening Kit (British Biotechnology). Oligonucleotides were 5′-end labelled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Pharmacia) and purified as above.

The transformed human keratinocyte (SVK-14) and human hepatocyte (HepG2) cells were cultured to confluence as described (9,10). In the positive control experiment, some SVK-14 cells were treated with an overdose of insulin concentration, 600-fold (K+) and not (K−) for 12 hours. The nuclear extract was then prepared from 1×10^6 cells using the method described (11) and its concentration was determined as described (12). 15 μg of nuclear extract was separated on a 15% polyacrylamide gel under a non-denaturing or denaturing condition, electroblotted onto Hybond-C+ extra membrane (Amersham), incubated with labelled ^{32}P-probe and washed as described (13) in the cold room. The membrane was exposed to Kodak XAR-5 X-ray films and autoradiographed at −70°C with intensifying screens. The experiment was carried out under different incubation conditions: (1) single and double-stranded ^{32}P-labelled PTHrP PCR or cDNA probe, (2) presence and absence of DNA competitor poly(dI-dC).poly(dI-dC) (Pharmacia), and (3) presence and absence of yeast total RNA (Boehringer Mannheim).

RESULTS

PCR analysis yielded an expected size of 549 bp DNA fragment of the human PTHrP gene which consists of exon Ic, the intervening intron and exon II (abbreviated as $PCR_{Ic\text{-}II}$). $PCR_{Ic\text{-}II}$ contains many DBP-consensus sequences and a TATA-promoter P3 in the intron (3). The sequence of $PCR_{Ic\text{-}II}$ was confirmed by bidirectional sequencing. Fig. 1 shows that $PCR_{Ic\text{-}II}$ bound to two bands of approximately 70 kDa and 65 kDa in both keratinocyte treated with a non-physiological dose of insulin (K+) and control (K−), and HepG2 cells (C). The signal of a 70 kDa band was more intense compared to a 65 kDa band. In the positive control, an oligonucleotide containing TEF/2 protein motif C5 consensus sequence was used and bound to the expected molecular weight nuclear protein of 60 kDa in all K+, K− and HepG2 cells (B). The signal in K+ cells was more intense than in K− cells because insulin was known to induce 60 kDa nuclear protein expression in the SVK14 cell line (unpublished data). Note that the expression of 70 kDa and 65 kDa proteins was independent on insulin. In the negative control, oligonucleotides (A/B) containing yeast GCN4 recognition sequence or a human PTHrP cDNA containing coding exons were unable to bind to any nuclear protein in both keratinocyte and hepatoma cells (A). $PCR_{Ic\text{-}II}$ bound to the same nuclear proteins under denatured or non-denatured conditions, and in the presence or absence of both DNA and RNA competitors.

DISCUSSION

DNA-binding proteins play an important role in the regulation of gene expression. In the human PTHrP gene, there are numerous DBP-consensus sequences and responsive elements such as 1,25-dihydroxyvitamin D (14-16), fetal calf serum, growth factor and epidermal growth factor (17), oestrogen and glucocorticoid (16), and cis-acting elements (5,6), that have been implicated to affect

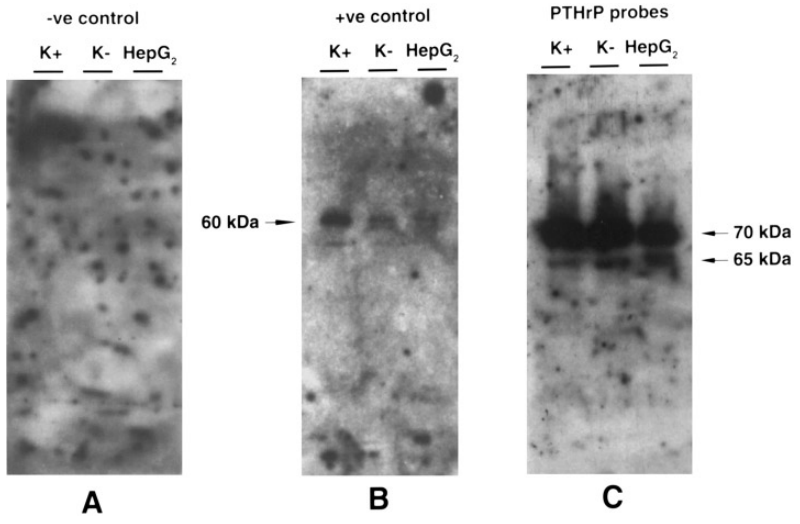

FIG. 1. South-Western analysis of cell nuclear extract with DNA probes. (**A**) Negative control using GCN4 oligonucleotides A/B or human PTHrP cDNA insert as probes, (**B**) positive control using TEF/2 protein motif C5 consensus sequence oligonucleotides as probe, and (**C**) using single or double-stranded $PCR_{Ic\text{-}II}$ as probe. Nuclear extract was from human keratinocyte SKV14 cells pretreated with insulin (K+) or without (K−), and from human hepatocyte HepG2 cells.

transcriptional activity of the human PTHrP gene. To further understand the steps involve in the regulation of PTHrP gene expression, this study concentrated on a promoter P3 region of the human PTHrP gene. This promoter has a 30-fold higher transcriptional activity than P1 (6), and is located in the intron between exon Ic and exon II. Using an amplified human PTHrP DNA fragment spanning between exon Ic and exon II ($PCR_{Ic\text{-}II}$), our data show that two nuclear proteins of 70 kDa and 65 kDa specifically bound to $PCR_{Ic\text{-}II}$, in two human cell lines. One cell line HepG2 is known to express and secrete immunoreactive PTHrP (10) whereas the other SVK-14 has not yet been studied. But human keratinocytes are well documented to express and secrete immunoreactive PTHrP (18-21). The amount of 70 kDa and 65 kDa nuclear proteins appeared to be independent on insulin, compared to a positive control 60 kDa nuclear protein which was bound by TEF/2 protein motif C5 consensus sequence oligonucleotide in keratinocyte cells. A oligonucleotide containing yeast GCN4 recognition sequence did not bind to any nuclear protein in either cell line, and neither the human PTHrP cDNA containing the coding exons III and IV. These data strongly support the authenticity of an interaction between 70 kDa and 65 kDa nuclear proteins and human PTHrP 5′ regulatory sequence. It is most likely that the 70 kDa and 65 kDa nuclear proteins are distinct because they were both detected under non-denatured and denatured conditions. However, the identity and functional property of these nuclear proteins on PTHrP transcriptional activity are unknown, and will remain unknown until the proteins are isolated. Thus, the next phase of investigation would be to isolate these nuclear proteins in order to study their potential role in regulating the human PTHrP gene expression.

ACKNOWLEDGMENTS

Thuan D. Bui was a Wellcome Trust Ph.D. scholar. We would like to thank Dr. W. Wood for the gift of plasmid pCIS2.BRF1.1.

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