Tandemly Reiterated Negative Enhancer-like Elements Regulate Transcription of a Human Gene for the Large Subunit of Calcium-dependent Protease*

Akiko Hata, Shigeo Ohno‡, Yoshiko Akita, and Koichi Suzuki

From the Department of Molecular Biology, Tokyo Metropolitan Institute of Medical Science, Honkomagome, Bunkyo-ku, Tokyo 113, Japan

Calcium-dependent protease (CANP, Calpain) is an intracellular protease involved in essential cellular functions mediated by calcium. To understand the mechanism regulating the expression of CANP at the transcriptional level, we isolated a human gene for the large subunit of mCANP (CANP mL) and analyzed its 5'-region. The transcription initiation sites were mapped to multiple positions (~142 to -103, A of initiation ATG as +1). The upstream region lacks typical promoter elements such as TATA and CAAT boxes and is characterized by its high GC content (~300 to -20, 70% GC content). Functional analyses of the 5'-region by a transient expression assay on HeLa cells revealed that the region (~202 to -80) has a promoter activity. The upstream half of the promoter region (~202 to -130) acts as an upstream promoter element in an orientation-independent manner. Upstream of the promoter region are tandemly reiterated multiple regulatory regions (~2.5k to -690, ~690 to -460, -460 to -260, and -260 to -202), each of which negatively regulates the CANP mL gene promoter as well as heterologous promoters in an orientation-independent manner. The presence of a cellular factor(s) mediating the action of these positive (promoter) and negative regulatory elements was suggested by an in vivo competition assay. The negative regulation of transcription mediated by these reiterated cis-acting elements and trans-acting factor(s) may play an essential role in the expression of the CANP mL gene.

Calcium ions regulate various cellular functions as messengers of extracellular stimuli through interaction with various calcium-binding proteins. Calcium-dependent protease (CANP, Calpain) is an intracellular protease requiring calcium for catalytic activity, and is one such calcium-binding protein (1). CANP hydrolyzes proteins of limited classes including extracellular matrix. They share identical S subunits, and thus calcium requirements exist. Both are heterodimers composed of L (large, catalytic, 80 kDa) and S (small, regulatory, 30 kDa) subunits (1). They have revealed primary structures of three human CANP proteins (μL, mL, and S) (2). Recent molecular cloning experiments have revealed primary structures of three human CANP proteins (μL, mL, and S) (3–6). They are encoded by distinct genes on distinct human chromosomes, although sequence homologies demonstrate their evolutionary relationship (6).

Knowledge of the mechanism underlying the regulation of genes for ubiquitously expressed intracellular proteins (housekeeping proteins) is quite limited compared with that of genes which are expressed in a tissue-specific manner. One of the reasons is that these genes are usually expressed at a low level making it difficult to isolate cDNA clones and analyze the gene expression.

In this paper, we describe analyses of the promoter region of a human CANP gene (CANP mL) and reveal the presence of a tandemly reiterated array of cis-acting negative regulatory elements upstream of positive regulatory (promoter) elements.

**EXPERIMENTAL PROCEDURES**

**Screening of the Library**—Charon 4A human genomic library (provided by Dr. Y. Sakaki) was constructed essentially as described (7) using high molecular weight DNA from human lymph node partially digested with HaellI and Alul. The hybridization probe used for the screening was a 0.35-kilobase ApaI fragment (probe A1) of the human CANP mL cDNA (p21-5), corresponding to the most N-terminal 355 bp (4). The cDNA was labeled with [α-32P]dCTP (~3000 Ci/mmol, Du Pont-New England Nuclear) by a multiprime DNA labeling system (Amersham Corp.). Hybridization was carried out according to the standard method (7).

**DNA Sequencing**—The insert of the clone was further dissected to generate smaller overlapping subclones in the plasmid Bluescript KS or pUC18. DNA sequencing was performed by the dyeoxy chain termination method on denatured plasmid DNA (8).

**St Nuclease Mapping**—Total RNA from human spleen was extracted by the guanidinium thiocyanate-cesium chloride method essentially as described before (9). Poly(A)* RNA was prepared by

* This investigation has been supported in part by research grants from the Ministry of Education, Science and Culture and the Ministry of Health and Welfare, Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) J04700.

‡ To whom correspondence should be addressed.

The abbreviations used are: CANP, calcium-activated neutral protease; bp, base pair(s); Pipes, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid; CAT, chloramphenicol transferase; Hepes, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid; IFN, interferon.

6404
chromatography on an oligo(dT)-cellulose column. Hybridization mixture (3 ml, 400 mM NaCl, 40 mM Pipes, pH 6.4, 1 mM EDTA) was added to 10 µg of poly(A)* RNA and dried. The resulting sample was dissolved in 12 µl of a 71% (v/v) formamide solution containing 10,000 cpm of a 5'-end-labeled S1 probe. The S1 probe was a PuuII-RsaI fragment (−460 to −20) of the CANP mL gene. End labeling was performed by nicking a dephosphorylated fragment with [y-³²P]ATP and T4 polynucleotide kinase (Takara Shuzo). After 12-16 h of hybridization at 70 °C, the sample was diluted with 190 µl of ice-cold buffer (250 mM NaCl, 30 mM sodium acetate, pH 4.5, 10 mM ZnCl₂, 10 µg/ml sonicated salmon sperm DNA) and digested with S1 nuclease (Sankyo) for 50 min at 30 °C. Reaction products were dissolved in a formamide-dye solution (250 mM NaCl, 30 mM Pipes, pH 6.4, 1 mM EDTA, 0.5% xylene cyanol FF, 0.5% bromphenol blue), heated at 90 °C for 1 min, and electrophoresed on an 8% polyacrylamide-urea denaturing gel. Protected bands were compared with a Maxam and Gilbert sequencing ladder (10) of a standard 32P-labeled oligonucleotide in 20 µl of a hybridization solution (40 mM Pipes, pH 6.4, 1 mM EDTA, 400 mM NaCl, 50% (v/v) formamide) for 12-18 h at 37 °C. The DNA-RNA hybrid was then collected by ethanol precipitation and dissolved in a reverse transcriptase buffer (50 mM Tris-HCl, pH 8.3, at 42 °C, 100 mM KCl, 10 mM MgCl₂, 20 mM β-mercaptoethanol), primed with oligo(dT) cellulose (Takara Shuzo), and hybridized at 37 °C in a 5% fetal calf serum (Flow Laboratories, Inc.), 100 IU/ml penicillin, 100 mg/ml streptomycin (Flow Laboratories, Inc.), 200 mM sodium acetate, 1 mM EDTA, 400 mM NaCl, and 50% (v/v) formamide solution, heated to 90 °C for 1 min, and analyzed by a 5% polyacrylamide-urea denaturing gel.

**Plasmid Constructions**—All constructions were carried out according to the standard recombinant DNA technique (7). As shown in Fig. 1, the plasmid pSV2CAT contains the simian virus 40 (SV40) early promoter/enhancer sequence (11) located immediately upstream of the CAT gene followed by the SV40 splice and polyadenylation sequences. The plasmid pKSCAT was constructed by inserting a PuuII-HindIII fragment (−522 to +76) from Bluescript KS into the HindIII-AccI site of pSV2CAT. The plasmid pTkCAT contains the herpes simplex virus thymidine kinase gene promoter including the TATA box and an upstream promoter region (−105 to +56) (12) fused upstream of the CAT gene and the polyadenylation sequence from pSV2CAT. The plasmid pIFNCAT was constructed by inserting a human interferon β gene promoter region (−55 to +19) (13) into the EcoRV site of pKSCAT. The p-2.5 kb fragment extending from −2.5 kilobase pair to −20 of the CANP gene was inserted into the Smal site of pKSCAT. This construction served as the starting material for subsequent constructions of deletion mutants. 5'-Deletion mutants (p-482/-20CAT, p-220/-20CAT, and p-20/-20CAT, and p-160/-20CAT) were produced by digestion with restriction endonucleases PuuI, AluI, SmaI, and SacII, respectively (cf. Fig. 4). 3'-End deletion mutants (p-202/-20CAT and p-202/-130CAT) were then produced by digestion with ApuI and HindIII, respectively. Proper restriction fragments located between each restriction site and −20 of the CAT gene were inserted into pKSCAT, pSV2CAT, ptKCAT, and pIFNCAT, and Bluescript KS (cf. Fig. 5 legend). The β-galactosidase expression vector, pCH110, driven by the SV40 early promoter was described by Hall et al. (14). Constructions of competitor plasmids (PC-1-5) and test plasmids (PT-1-2) used for the competition assay are illustrated in Fig. 6A. A Real (−2.5k)-PuuI (−690) fragment, PuuII fragment (−690 to −460), PuuI (−460)-AluI (−260) fragment, AluI (−460)-SmaI (−220) fragment, and SmaI (−220)-HindIII (−130) fragment were inserted into SmaI-digested Bluescript KS to produce p-1 to 5, respectively. PT-1 and PT-2 are the same as p-260/-20CAT and p-202/-20CAT, respectively. Functions of all constructions were confirmed by DNA sequence analyses.

**Cell Culture and DNA Transfection**—HeLa cells were grown in Eagle's minimal essential medium medium (Nissui) supplemented with 10% FCS (Flow Laboratories, Inc.), 100 IU/ml penicillin, 100 µg/ml streptomycin (Flow Laboratories, Inc.), 5% dialyzed calf serum, and 1% glutamine (GIBCO), and 0.2% NaHCO₃. All cultures were maintained at 37 °C in 5% CO₂. DNA transfections were done essentially as described (15) with some modifications. Approximately 10⁶ cells were plated onto 10-cm culture dishes 24 h before transfection with a change of medium 4 h before addition of DNA precipitate. A DNA-CaCl₂ mixture (15 µg of plasmid DNA in 0.5 ml of 240 mM CaCl₂) was added dropwise to 0.51 ml of a transfection mixture (4 mM Hepes, 269 mM NaCl, 1.4 mM Na₂HPO₄, 1.4 mM NaHPO₄, adjusted to pH 6.95) with vortexing. After 30 min at room temperature, the precipitates were applied to the cells and incubated at 37 °C. Four hours later the cells were treated with a glycerol solution (15% (v/v) glycerol in 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄ adjusted to pH 7.4) for 1 min and incubated for 4 h at 37 °C. Each transfection experiment was repeated at least four times with two different plasmid preparations. To obtain reproducible results, all plasmids were prepared by alkaline lysis and banding by ultracentrifugation on a cesium chloride-ethidium bromide equilibrium gradient.

**Assay for CAT Activity**—Cells incubated for 48 h after transfection were harvested and disrupted by freeze-thawing. Protein concentrations of extracts were determined with the method previously described (16). In order to confirm the reproducibility of transfection efficiency, we cotransfected the plasmid pCH110 coding for β-galactosidase as an internal reference plasmid and determined β-galactosidase activity of each sample. β-Galactosidase activity was determined spectrophotometrically at 420 nm using o-nitrophenyl-β-D-galactoside (Wako Purechemicals) as a substrate (17). CAT assay was performed on protein equivalents, because protein concentrations changed parallel to β-galactosidase activity. Cell extract equivalent to 15-90 µg of protein was incubated for 30 min at 37 °C in an assay mixture (final volume 150 µl) containing 42 µl of D-threo-dichloroacetlyl-1-14C)chlorophenicol (54 µCi/mmol, Amersham Corp.), 0.5 mM acetyl coenzyme A (Sigma), and 0.47 µM Tris-Cl (pH 7.8). The reactions were terminated with 1 ml of ethyl acetate. The extracted chlorophenicol was dried and dissolved in 10 µl of ethyl acetate. The acetylated chlorophenicol was separated from the nonacylated form by thin-layer chromatography (HPTLC, Silica Gel 60, Merck) developed in chloroform:methanol (95:5, v/v).
RESULTS

Isolation and Sequencing of the 5'-Region of the Human CANP Gene—A cDNA fragment of human CANP mL (4) containing a 144-bp 5'-flanking and a 211-bp protein-coding sequence as a probe, a genomic clone, M1-3, was isolated from a human genomic library. Restriction mapping and Southern hybridization analysis of M1-3 using the cDNA fragment as a probe identified an exon-containing region, and the nucleotide sequence of this region with its 5'-flanking sequence (1154 bp in total) was determined (Fig. 2). The cDNA sequence from -131 to +236 (of the initiation codon as +1) exists in this region in a complete match, identifying the location of the putative first exon. The presence of an intron at +236 was also shown for the chicken CANP L gene (19).

An S1 nuclease mapping experiment using a genomic DNA fragment (−460 to −20) 5'-end-labeled at position −20 identified multiple signals (between positions −137 and −103) corresponding to the 5'-end of the exon (Fig. 3A and 2B, closed triangles). The fact that the cDNA starts at around −130 and that no splicing acceptor sequence (AG) is seen in the near upstream region of the 5'-ends indicates that the exon is actually the first exon and that transcription starts from multiple sites. This is supported by functional analysis showing the presence of promoter activity just upstream of the cluster of the putative transcription initiation sites.

The upstream region of the transcription initiation sites does not contain a TATA-like sequence and is extremely rich in GC residues (−300 to −20, 76% GC content). These structural features may explain the presence of multiple transcription initiation sites.

Deletion Analysis of the Promoter Region—To identify DNA sequences involved in expression of the human CANP mL gene, a series of 5'- and 3'-deletion fragments of the 5'-region of the CANP gene was ligated to the 5'-upstream region of the bacterial CAT gene in a CAT expression plasmid, pKSCAT (Fig. 1). These CAT constructs were tested for promoter activity by transfection into HeLa cells. Fig. 4 shows structures of various deletion mutants and their relative promoter activities determined by the transient expression assay. The longest construct, p-2.5k/-20CAT, showed relatively low promoter activity. However, as the deletion proceeds to −202 (p-460/-20CAT, p-260/-20CAT, and p-202/-20CAT), the levels of CAT expression gradually increased. Complete removal of a −2.5 kilobase pair to −202 region (p-202/-20CAT) resulted in a 13-fold increase in CAT expression. This indicates that a −2.5 kilobase pair to −202 region negatively regulates the promoter activity of the downstream region (−202 to −20).

Primer extension analysis of the CAT poly(A)+ RNA obtained from cells transfected with p-202/-20CAT demonstrated that the 5'-end of the CAT mRNA locates −142 and −141 (Fig. 3B and Fig. 2B, open triangles). These positions are essentially the same as those determined for the wild type.

FIG. 2. Restriction map and nucleotide sequence of the human CANP mL gene 5'-flanking region. A, restriction map of the genomic clone M1-3 is shown. The first exon (Exon 1) is indicated by a closed box. Restriction endonuclease cleavage sites are: E, EcoRI; H, HindIII; S, Smal; B, BanHI; P, Poul; R, RsaI. B, nucleotide sequence of the human CANP mL gene 5'-flanking region (1154 bp). The sequence extends to 719 nucleotides upstream of the ATG initiation codon. Numbering of the sequence starts with A of the ATG initiation codon as number +1. Major transcription initiation sites are shown by triangles. Closed and open triangles indicate transcription initiation sites determined by S1 nuclease mapping analysis and the primer extension method, respectively. Underlined are a β-globin promoter element and the consensus sequences for transcription factors Sp1 and AP-1 binding sites. Arrows show characteristic 15-bp direct repeats in the P1 region. Short vertical lines indicate the borders of transcription regulatory elements. Double lines indicate an 8-bp common sequence existing in the N1, N2, and N3 regions. P1 and P2 are upstream and downstream promoter elements, and N1 to N4 are negative elements.
Functional Dissection of the Promoter Region—To further characterize the promoter region (−202 to −80), various chimeric promoters were constructed, and their activities were analyzed by means of CAT expression (Fig. 5). When fused upstream of the TK promoter (−105 to +60) which contains both upstream (CAAT box) and downstream (TATA box) promoter elements (pTKCAT), the CANP promoter region (−202 to −80) did not produce a significant increase in the TK promoter activity (p-202/-80TKCAT) (Fig. 5A). This was confirmed by another CAT assay using a smaller amount of cell extract (equivalent to 54 μg of protein). The relative CAT activity of the CANP promoter to the TK promoter was 1.14 (71.2 and 62.5% acetylation for p-202/-80TKCAT and pTKCAT, respectively). However, when fused upstream of the IFN promoter sequence (−55 to +19), which contains the TATA box and the transcription initiation site (pIFNCAT), the CANP promoter region (−202 to −80) showed a marked increase in CAT expression (p-202/-80IFNCAT) (Fig. 5A). A similar effect was also shown using half of the promoter region, P2, in an orientation-independent manner (p-202/-130IFNCAT, p-130/-202IFNCAT). This indicates that the region from −202 to −130 (P2) contains upstream promoter element(s), whereas the region from −130 to −80 (P1) contains downstream element(s).

Negative Regulatory Elements Act on Heterologous Promoters in an Orientation-independent Manner—Analysis of the 5′-deletion mutants of the CANP-CAT fusion constructs shown in Fig. 4 suggested the presence of a control element(s) which negatively regulates the downstream promoter (P1 + P2). We next examined whether the upstream sequence acts on heterologous promoters. As shown in Fig. 5B, when inserted upstream of the TK promoter, each of the upstream regions (N4, N3, and N1) repressed TK promoter activity (p-2.5k/-690TKCAT, p-690/-460TKCAT, and p-260/-292TKCAT). Interestingly, each of the upstream regions acts in an orientation-independent manner (p-690/-2.5kTKCAT, p-460/-690TKCAT, and p-202/-280TKCAT). The degree of repression, however, depends on the region inserted. Further, N3 also repressed the activity of the SV40 early promoter/enhancer (p-690/-460SVCAT, p-460/-690SVCAT) in an orientation-independent manner (Fig. 5C). These results clearly indicate that the CANP gene contains at least three (N4, N3, and N1) negative regulatory elements which independently repress promoters of different origins in an orientation-independent manner. Moreover, N2 was also identified as a negative element as described below.

Competition of Trans-acting Cellular Factors That Bind to CANP Gene—As a step toward identifying cellular factors that mediate the effects of the promoter and negative regulatory elements on transcription of the CANP gene, in vivo competition experiments were carried out. A constant amount of a test plasmid containing positive or negative elements (pT-1, p-260/-20CAT, pT-2, p-202/-20CAT) fused upstream of the CAT gene was cotransfected into HeLa cells with increasing amounts of competitor DNA (pC-1 to 5) (Fig. 6A). Plasmid vector Bluescript KS lacking both the CAT gene and the CANP gene was used to normalize the amount of DNA transfected into the cells. If cellular factors mediate the function of a cis-acting element, CAT expression of the test plasmid would be increased or decreased in the presence of competitor plasmid.

First we examined whether additional copies of the CANP positively controlled region decreased its own activity. When the test plasmid pT-2 (p-202/-20CAT), containing the positive element of the CANP gene, was transfected into cells with competitor plasmid pC-5, a decrease in CAT activity

CANP gene using S1 nucleases (Fig. 2B). Slight differences in these results may reflect a difference in downstream sequences or detection methods.

Deletion of −202 to −160 resulted in a drastic decrease in CAT expression (Fig. 4, A and B, closed circles) indicating that this region is involved in promoter function. The 3′-deletion of −80 to −20 (p-202/-80CAT) (Fig. 4, A and B, open circles) indicates that this region is not required for promoter function. However, removal of −130 to −80 (p-202/-130CAT) resulted in a 70% decrease in promoter activity indicating that this region is required for full promoter activity. These results demonstrate that the −202 to −80 region is a promoter region for the CANP mL gene.
with an increasing amount of competitor was observed (Fig. 6B). At a 17-fold excess of competing positive element, the level of CAT activity decreased to 30% of the initial level. Control experiments using the plasmid containing the upstream fragment (–260 to –202) caused no decrease in CAT expression (Fig. 6D, open bars). These results strongly suggest the presence of a limiting amount of cellular factor(s) interacting directly or indirectly with the promoter element, P2, and that the factor(s) is essential for promoter activity.

We next examined the effect of cellular factor(s) acting on the negative elements N4 to N1. The test plasmid pT-1 (p-260/-20CAT), containing the promoter region and negative element (N1), was transfected into cells with competitor plasmid pC-4. Increasing amounts of pC-4 resulted in an increase in CAT activity (Fig. 6C). At a 17-fold excess of competitor plasmid, the level of CAT activity of pT-1 increased to 440% of the initial level. These results suggest that the binding of one or more cellular factors present in a limiting amount in HeLa cells is essential for the function of the negative element (N1) of the CANP gene.

To examine whether the trans-acting factor(s) which interacts with the negative element (N1, –260 to –202) is common to other negative elements (N4, N3, and N2) located upstream of the positive element (P1 + P2), we transfected the test plasmid pT-1 with each of the competitor plasmids pC-1, -2, -3, or -5. Surprisingly, all the competitor plasmids containing N2, N3, and N4 increased the CAT activity to 400% of the initial level, equivalent to that observed with N1 (Fig. 6D, striped bars). On the other hand, pC-5, which contains the P2 region, did not increase the CAT activity. These results suggest that all four negative elements recognize the same or similar cellular factor(s).

**DISCUSSION**

In this study, we isolated and characterized the upstream region of the human CANP mL gene. Functional analysis of the CANP mL gene upstream region by means of a transient expression assay on HeLa cells using CAT constructs identified four negative regulatory regions (N4, N3, N2, and N1) tandemly reiterated just upstream of the promoter region (P2 and P1).

The promoter region (P2 + P1; P2, –202 to –130; P1, –130 to –80) is extremely rich in GC residues, lacks a TATA box, and contains multiple transcription initiation sites which cluster between –142 and –103. These are structural features common to many genes of housekeeping proteins such as hypoxanthine phosphoribosyltransferase (20, 21), adenosine deaminase (22), and hydroxymethylglutaryl coenzyme A reductase (23). The gene for the small subunit of CANP (CANP S) also shares similar structural characteristics in its 5'-flanking region (24). Although the CANP promoter region does not contain typical promoter motifs such as TATA and CAAT boxes, it possesses a promoter activity whose strength is comparable to that of the TK gene. The P2 region alone exerts full promoter activity when fused upstream of the TATA box of IFN promoter in either orientation (p-202/-130IFNCAT, p-130/-202IFNCAT), although the P2 region by itself is not sufficient for full promoter activity (p-202/-130CAT). Thus, P2 seems to correspond functionally to an orientation-independent upstream promoter element found in several genes (25). In fact, P2 contains a sequence known to act as an upstream promoter element in, for example, the β-globin gene (CCACACCCCG, starting at –166) (26) and consensus sequence for Spl recognition (GGGGGCCGGG, starting at –155) (27).
Multiple Negative Regulatory Elements of Human CANP Gene

Competition experiments indicate that the promoter activity of P2 is mediated by cellular factor(s) specifically interacting with the P2 sequence. Cellular factors such as Sp1 and a protein in HeLa cells which interacts with the β-globin upstream element are candidates for mediating the promoter function of the P2 region. It should be noted that, whatever the factors are, the amount of the molecule regulating the promoter function of P2 is limited in cells.

In contrast to the P2 region, the P1 region of the CANP mL promoter may correspond functionally to the downstream promoter element which defines the direction and location of transcription initiation. The IFN gene promoter containing the TATA box can be substituted for the P1 region without any reduction in promoter activity (p-202/-80CAT versus p-202/-130IFNCAT). A characteristic sequence in the P1 region, a direct repeat of a 15-bp sequence CGCT/CCGCAGC/TGGCG/CG, may be involved in the function of P1. The presence of a sequence conserved in the AP-1 binding sites in the P1-P2 junction of the CANP mL gene (TGAATCA, starting at -132) suggests the involvement of AP-1 in the regulation of CANP mL gene transcription. A potential AP-1 binding sequence (TGAGTCA, starting at -108) is also seen in the corresponding region of the CANP S gene. Since CANP irreversibly activates protein kinase C (1), it would be quite interesting to examine whether the CANP mL gene (and S gene) responds to 12-O-tetradecanoylphorbol-13-acetate via a protein kinase C-mediated process.

One of the most intriguing results obtained from the functional analysis of the CANP gene is the elucidation of multiple negative regulatory elements. Serial deletions of these negative regulatory regions (N4, N3, N2, and N1) result in a gradual increase in the promoter activity (Fig. 4B). At least three of these negative elements (N1, N3, and N4) also repress heterologous promoter activity, such as the TK gene, when inserted just upstream of the promoter in either orientation. Because the negative action of N1 disappears in the presence of an excess amount of these negative regions, the negative action of N1 is mediated by cellular factor(s), and all four negative elements recognize the same factor(s).

The presence of a common sequence GGC/GCCGTC/G in regions N1, N3, and N4 is not yet been determined) may explain the notion that the cellular trans-acting factor(s) interacting with these negative elements are identical or similar.

FIG. 5. Effect of positive or negative elements on expression of heterologous promoters. Constructions of various CAT plasmids are illustrated on the left. Promoter activity of each construct was determined by means of CAT expression on HeLa cells. CAT expression is represented as percent acetylation as described under "Experimental Procedures" by using pKSCAT and pSV2CAT as a negative and a positive control, respectively. Results shown in A and B were done in parallel. Relative activities were calculated taking the expression of pTKCAT (A, B) or pSV2CAT (C) as 1.0. Cell extracts equivalent to 90 μg (A, B) or 15 μg of protein (C) were used for the CAT assay. Arrows indicate the directions and locations of CANP gene fragments. Open boxes show heterologous promoters: TK, thymidine kinase gene promoter; IFN, interferon-β gene promoter; and SV40, simian virus 40 gene early promoter, respectively. *, †, ‡, § indicate CAAT box, TATA box, 72-bp repeated sequence in IFN, and 21-bp repeated sequence in SV40 early promoter, respectively. Closed boxes show the CAT gene.

| Transfected DNA | % Acetylation | Relative Activity |
|----------------|--------------|------------------|
| p-202/-80TKCAT | 96.1         | 1.04             |
| p-202/-80CAT  | 63.0         | 0.68             |
| p-202/-80IFNCAT | 10.2        | 0.11             |
| p-202/-130IFNCAT | 68.6        | 0.75             |
| p-130/-202IFNCAT | 78.3        | 0.85             |
| p-202/-260TKCAT | 73.1         | 0.79             |
| p-202/-260CAT  | 18.8         | 0.20             |
| p-2.5k/-690TKCAT | 4.9          | 0.05             |
| p-690/-2.5kCAT | 4.6          | 0.05             |
| p-690/-460TKCAT | 0.2          | <0.01            |
| p-460/-690TKCAT | 37.0         | 0.40             |
| p-460/-202TKCAT | 73.9         | 0.80             |
| p-690/-460SVCAT | 72.9         | 1.00             |
| p-460/-690SVCAT | 48.5         | 0.67             |
| p-460/-690SVCAT | 38.8         | 0.53             |
The presence of cis-acting elements which negatively regulate promoter activity has been reported for several genes including rat α-fetoprotein (28), rat insulin 1 (29), rat growth hormone (30), human IFN-α (31), and human apolipoprotein CIII gene (32), although their precise nature, including the presence of a trans-acting factor, is unknown in most cases. It is conceivable that the number of the reiterations will control the level of the regulationary action depending on the concentration of the trans-acting factor. This may explain the difference in the gene expression level between different types of cells where the concentration of the trans-acting factor is different. Interestingly, the negative elements of the CANP mRN gene share several features with positive regulatory elements of inducible genes such as the metallothionein gene. Both regulatory sequences are composed of tandemly reiterated multiple elements, each element acting in heterologous promoters in an orientation-independent manner, whose regulatory function is mediated by trans-acting factors which exist in limited amounts.

Taking into consideration an ubiquitous distribution of CANP among tissues of higher animals, expression of the CANP gene may be explained by the presence of rather general promoter elements, whose activity is mediated mainly by an ubiquitous trans-acting factor such as Sp1, and tandemly reiterated negative regulatory elements, whose activities are mediated by a ubiquitous trans-acting factor whose concentration is cell type-specific. Negative regulatory elements may also be responsible for the feedback regulation of genes where the concentration of the gene product must be strictly maintained. This kind of regulation may be required for genes whose products form a heteromorphic structure with other gene products. The presence of stretches of conserved sequences in corresponding regions of the large and small subunits of human CANP genes (-360 to -60, 50% sequence homology including potential Sp1 and AP-1 recognition sequences, data not shown) suggests that expression of the genes coding for the two CANP subunits is co-regulated at the level of transcription and that their regulation is mediated by the same factor(s) acting on the negative regulatory elements. Future experiments on the cellular factor(s) acting on the CANP gene negative regulatory elements will explore these issues and eventually lead to the elucidation of a general mechanism regulating the expression of intracellular housekeeping genes.

Acknowledgments—We wish to thank Drs. Yoshiyuki Sakaki, Akihiko Okude, Tadatsugu Taniguchi, and Hiroshi Kawasaki for providing the human genomic library, pTKCAT, IFN-β plasmid, and synthetic oligonucleotide, respectively.

REFERENCES

1. Suzuki, K. (1987) Trends Biochem. Sci. 120, 103-105
2. Kawasaki, H., Imajoh, S., Kawashima, S., Hayashi, H., and Suzuki, K. (1986) J. Biochem. (Tokyo) 99, 1525-1532
3. Aoki, K., Imajoh, S., Ohno, S., Emori, Y., Koike, M., Kosaki, G., and Suzuki, K. (1986) FEBS Lett. 205, 313-317
4. Imajoh, S., Aoki, K., Ohno, S., Emori, Y., Kawasaki, H., and Suzuki, K. (1988) Biochemistry 27, 8122-8128
5. Ohno, S., Emori, Y., and Suzuki, K. (1986) Nucleic Acids Res. 14, 5559
6. Ohno, S., Emori, Y., Sugihara, H., Imajoh, S., and Suzuki, K. (1987) Methods Enzymol. 139, 363-379
7. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
8. Hattori, M., and Sakaki, Y. (1986) Anal. Biochem. 152, 232-238
9. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., and Rutter, W. J. (1979) Biochemistry 18, 5294-5299
10. Maxam, A. M., and Gilbert, W. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 560-564
11. Benoist, C., and Chambon, P. (1981) Nature 290, 304-310
12. McKnight, S. L., Kingsbury, R. C., Spence, A., and Smith, M. (1984) Cell 37, 253-262
13. Fujita, T., Ohno, S., Yasumitsu, H., and Taniguchi, T. (1985) Cell 41, 489-496
14. Hall, C. V., Jacob, P. E., Ringold, G. M., and Lee, F. (1983) J. Mol. Appl. Genet. 2, 101-104
Multiple Negative Regulatory Elements of Human CANP Gene

15. Graham, F., and Van der Eb, A. (1973) Virology 52, 456–467
16. Gorman, C. M., Moffat, L. F., and Howard, B. H. (1982) Mol. Cell. Biol. 2, 1044–1053
17. Miller, J. H. (1972) in Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
18. Sleigh, M. J. (1986) Anal. Biochem. 156, 251–256
19. Emori, Y., Ohno, S., Tobita, M., and Suzuki, K. (1986) FEBS Lett. 194, 249–252
20. Melton, D. W., Konecki, D. S., Brennand, J., and Caskey, C. T. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 2147–2151
21. Melton, D. W., McEwan, C., McKie, A. B., and Reid, A. M. (1986) Cell 44, 319–328
22. Valerio, D., Duyvesteyn, M. G. C., Dekker, B. M. M., Weeda, G., Berkvens, T. M., Van der Eb, A. J., Van Ormandt, H., and Van der Eb, A. J. (1985) EMBO J. 4, 437–443
23. Reynolds, G. A., Basu, S. K., Osborne, T. F., Chin, D. J., Gill, G., Brown, M. S., Goldstein, J. L., and Luskey, K. L. (1984) Cell 38, 275–288
24. Miyake, S., Emori, Y., and Suzuki, K. (1986) Nucleic Acids Res. 14, 8805–8817
25. Maniatis, T., Goodbourn, S., and Fischer, J. A. (1987) Science 236, 1237–1245
26. Jones, K. A., Kadonaga, J. T., Rosenfeld, P. J., Kelly, T. J., and Tjian, R. (1987) Cell 48, 79–89
27. Dynan, W. S., and Tjian, R. (1983) Cell 35, 79–87
28. Muglia, L., and Rothman-Denes, L. B. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 7653–7657
29. Laimins, L., Holmgren-Konig, M., and Khoury, G. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 3151–3155
30. Wight, P. A., Crew, M. D., and Spindler, S. R. (1987) J. Biol. Chem. 262, 5659–5663
31. Goodburn, S., Burstein, H., and Maniatis, T. (1986) Cell 45, 601–610
32. Reue, K., Leff, T., and Breslow, J. L. (1988) J. Biol. Chem. 263, 6857–6864
33. Lee, W., Haslinger, A., Karin, M., and Tjian, R. (1987) Nature 325, 368–372