Vol. 144, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 14, 1987 Pages 402-408

REGULATED EXPRESSION OF HUMAN ATRIAL NATRIURETIC POLYPEPTIDE GENE IN MOUSE L CELLS

Naoharu IWAI, M.D., Masato MATSUNAGA, M.D.*, Kazuya OGAWA, M.D., Etsuko OHTA, M.D., and Chuichi KAWAI, M.D.

The Third Division, Department of Internal Medicine, Faculty of Medicine, and *the College of Medical Technology, Kyoto University, Kyoto, Japan

Received March 2, 1987

Summary : To investigate whether the human atrial natriuretic polypeptide (hANP) gene is responsive to glucocorticoid, we co-introduced the hANP gene (with SV40 enhancer) with HSV-tk gene into mouse tk^- L cells. The transformants with hANP gene with SV40 enhancer expressed hANP specific RNAs. The administration of 1 μM dexamethasone reduced the expressed hANP specific RNAs, especially those that had a physiological initiation site. These results suggest that the hANP gene is really a glucocorticoid responsive gene and may be negatively regulated by glucocorticoid.

Atrial natriuretic polypeptide (ANP), existing mainly in mammalian atria, is a potent natriuretic, diuretic and vasorelaxant polypeptide, which is thought to play key roles in cardiovascular and body fluid homeostasis(1,2,3,4).

The cDNAs and genes for ANP of several mammalian species have been cloned(5,6,7,8,9,10,11), and the expression of human ANP (hANP) in yeast cells has also been reported(12).

Human ANP gene was reported to have a putative glucocorticoid receptor binding site in its IVS2(9). However, whether hANP gene is regulated by glucocorticoid has not been clarified because of difficulties in cultivating hANP producing cells. In order to investigate whether the hANP gene is a glucocorticoid responsive gene, we co-introduced it with herpes simplex virus thymidine kinase gene (HSV-tk) into mouse L cells which are reported to have functional glucocorticoid receptors(13), and examined the effects of glucocorticoid on the transformed cells.

Materials and Method

Human ANP gene and the construction of an expression plasmid

The cloning of hANP gene and the construction of an expression plasmid designated as pSVE-hANP have already been reported (in press in Biochem. Biophys. Res. Commun. 1987). Briefly, a 3.5 kb BamHI-BamHI fragment of hANP gene and the Simian Virus 40 (SV40) Hind III C fragment were subcloned

0006-291X/87 $1.50

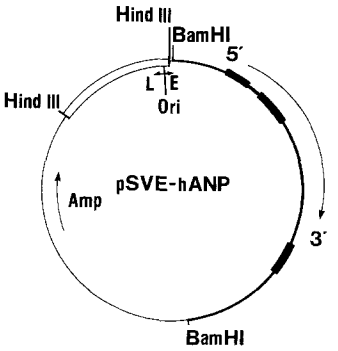

Figure 1. A 3.5 kb BamHI-BamHI fragment of hANP gene is oriented under the control of the SV40 early promoter. E, Ori, L denote the SV40 early promoter, the SV40 replication origin, and the SV40 late promoter, respectively.

to pUC 18 plasmid in a way that hANP gene sequences were under the control of SV40 early promoter (Fig. 1).

Transfection procedures

Mouse tk^- L cells were transfected with 1 μg pX-1, which contains HSV-tk gene sequences (provided by Dr. Mishina, Kyoto Univ.) and 20 μg hANP plasmid DNA per 10^6 cells, as described by Wigler et al(14).

Cell culture

Mouse tk^- L cells were maintained in Dulbecco's modified Eagle's medium supplemented with heat inactivated 10% fetal bovine serum (Gibco). Mouse tk^+ transformants were selected and maintained in this medium with 10% fetal bovine serum, 15 μg/ml hypoxanthine, 1 μg/ml aminopterin and 5 μg/ml thymidine (HAT medium)(14). 1 μM dexamethasone was administered to the transformants for 48 hr.

S_1 nuclease mapping

Total RNA was isolated as described by Chirgwin et al(15). S_1 nuclease mapping was performed according to Sharp et al(16). The RsaI-RsaI 416-nucleotide fragment isolated from hANP gene was end labelled with T_4 polynucleokinase, and cleaved with DraI. Twenty micrograms of total RNA was incubated with this end labelled DraI-RsaI 316-nucleotide fragment for 3 hr at 42 °C in 80% formamide, 0.5 M NaCl, 1 mM EDTA and 50 mM pipes, pH 6.4.

S_1 nuclease digestion was performed at 40 °C for 30 minutes with 0.5 unit S_1 nuclease (Bethesda Research Laboratory) per 1 μg total RNA. Protected DNA-RNA hybrids were denatured and electrophoresed on 7M urea/5% polyacrylamide gel.

RNA blot-hybridization analysis

RNA was denatured with 1 M glyoxal/50%(V/V) dimethyl sulfoxide, electrophoresed on a 1.2% agarose gel, and transferred to a Biodyne A membrane (Pall). Hybridization and washing were carried out according to procedures described by Thomas et al(17). Other DNA and RNA manipulations were by standard techniques described by Maniatis et al (Molecular Cloning; T. Maniatis, E.F. Fritsch and J. Sambrook, Cold Spring Harbor Laboratory 1982).

Results

The transformed L cells with pSVE-hANP were subcloned and the two of these clones, designated as Clone 1 and Clone 2, were analyzed.

Fig. 2 shows the RNA blot-hybridization analysis of the transcripts of the transformed L cells and the total RNA from human atria. Both Clones 1

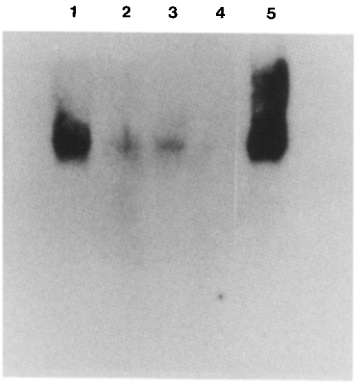

Figure 2. Ten micrograms of total RNA from the transformed L cells and human atria was size-fractionated on a 1.2% agarose gel, blotted to a Biodyne A membrane, and hybridized to the same probe used in S_1 nuclease mapping analysis. RNAs analyzed are as follows; lane 1, total RNA of Clone 2 control; lane 2, total RNA of Clone 2 with 1 μM dexamethasone; lane 3, total RNA of Clone 1 control; lane 4, total RNA of Clone 1 with 1 μM dexamethasone; lane 5, total RNA of human atria.

and 2 expressed hANP specific RNAs and the length of which were almost identical to that of hANP mRNA in human atria (Fig. 2 lane 1, 3, 5). The administration of 1 μM dexamethasone for 48 hr markedly reduced the expressed hANP specific RNAs in both Clones 1 and 2 (Fig. 2 lane 1, 2 and lane 3, 4).

To confirm these results and to exclude a possibility that the expressed hANP specific RNAs were all cryptic, S1 nuclease mapping analysis in the promoter region of the hANP gene was performed (Fig. 3).

Both the transcripts of Clones 1 and 2 protected a fragment of 185 nucleotides, mapping in a region completely identical to the initiation site of hANP mRNA in human atria (designated as major site) (Fig. 3 lane A, C, F). The two extra fragments were also protected by the transcripts of Clone 2. The fragment of about 140 nucleotides, which was also protected by hANP mRNA, may represent the existence of another physiological, even if minor, initiation site (designated as minor site). The other fragment of about 100 nucleotides, which was negligibly protected by hANP mRNA, may be an artificial transcript or a transcript of a more minor initiation site (designated as cryptic site) induced by SV40 enhancer or by the integration sites of the hANP genes.

The reduction of the expressed hANP specific RNAs in both Clones 1 and 2 by dexamethasone was also confirmed by the S_1 nuclease mapping analysis. Of the expressed hANP specific RNA, those that had probable initiation site identical to the major site of hANP mRNA were reduced to 1/2 and to 1/4 in Clone 2 and in Clone 1 respectively (Fig. 3 A, B and C, D). The

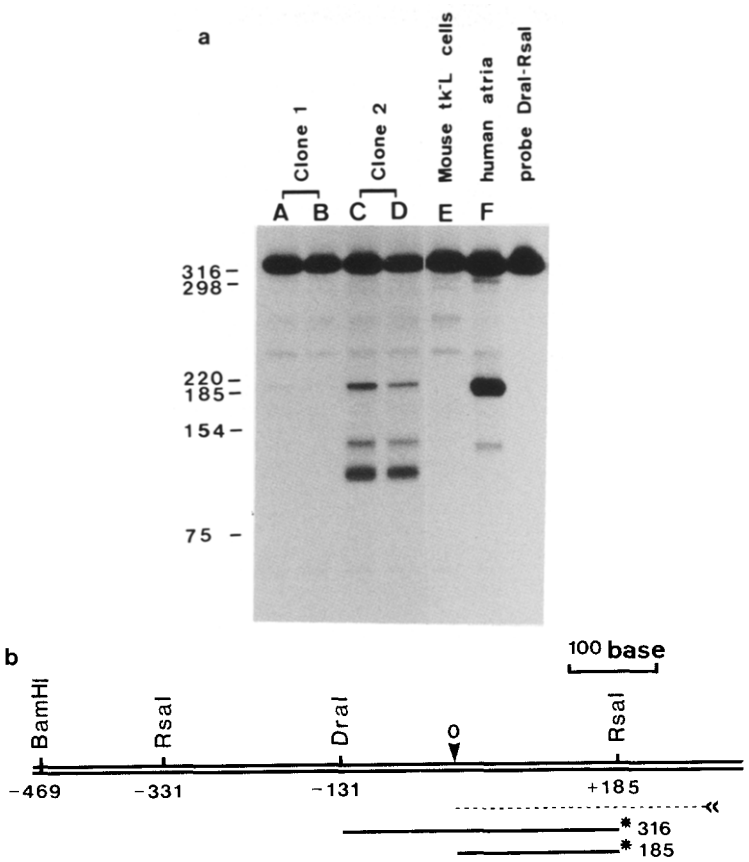

Figure 3. S_1 nuclease mapping analysis. (a) S_1 nuclease mapping with the use of DraI-RsaI 316-nucleotide fragment derived from pBR-hANP. The sizes of the protected fragments and size markers are given in nucleotides on the side of the antoradiogram. RNAs analyzed are as follows: lane A, total RNA of Clone 1 control; lane B, total RNA of Clone 1 with 1 μM dexamethasone; lane C, total RNA of Clone 2 control; lane D, total RNA of Clone 2 with 1 μM dexamethasone; lane E, total RNA of mouse tk^- L cells; lane F, total RNA of human atria. (b) The probe used and the predicted S_1 nuclease resistant DNA fragment. The upper double line indicates hANP gene sequences. The dotted line indicates hANP mRNA and the single line the probe used. The asterisk represents the site of ^{32}P labelling. The arrow indicates the major initiation site.

Table 1

Quantification of Human ANP specific RNA

Initiation site	Human Atria	Clone 1			Clone 2		
		D(-)*	D(+)†	% D(+)/D(-)	D(-)*	D(+)†	% D(+)/D(-)
Major	100	2.2	0.53	24.1%	22.8	11.4	50.0%
Minor	11.5	n.d.	n.d.	--	16.8	11.8	70.2%
Cryptic	(±)	n.d.	n.d.	--	41.3	33.2	80.4%

Each value was determined from the Fig. 2 autoradiogram by the densitometric assay.

D(-)* no administration of dexamethasone
D(+)† administration of 1 μM dexamethasone
n.d. not detected

transcripts from the minor and the cryptic site detected in Clone 2 were less affected by dexamethasone (Fig. 3 C, D)(Table 1).

Discussion

We have shown that the hANP gene with SV40 enhancer, when introduced to mouse L cells, is expressed and is negatively regulated by dexamethasone.

Heterologous cells, mouse L cells in this case, may have a second messenger system that differs from the systems of human ANP producing cells. However, the effects of glucocorticoid seem to be exerted by the interaction between the steroid hormone-receptor complexes and the consensus sequences in responsive genes(18). Also it was reported that some of the glucocorticoid responsive genes, such as human growth hormone gene(19), rat growth hormone gene (with and without SV40 enhancer)(20), and rat α_{2u} globulin gene(21), when introduced to mouse L cells, were all regulated similarly and positively by glucocorticoid as are in the cells natively expressing them. Thus, the present results obtained in mouse L cells suggest that the hANP gene in vivo is negatively regulated by glucocorticoid.

The cryptic RNA, and the transcripts from the probable minor initiation site were expressed in a large amount in Clone 2. However, in Clone 1, only the transcripts from the major site were detected. This difference may be ascribed to the difference of the integration sites of pSVE-hANPs between Clones 1 and 2. And also some other cryptic RNA may exist which were not detected by the probe used in S_1 experiment, for the decrease in the expressed RNA levels observed by blotting analysis seemed to be much greater than that detected by S_1 analysis. Rat growth hormone gene lacking 5' flanking sequences was reported to be regulated by glucocorticoid in mouse L cells as in the cells natively expressing it(20). So, for the aim of whether hANP gene is really glucocorticoid responsive, we may not be particular about the initiation site of the expressed RNA.

It remains to be determined whether the hormonal reduction of the expressed hANP specific RNA is due to control of transcription or to reduced stability of the expressed hANP specific RNA, though the former is more probable.

During the preparation of this paper, Gardner et al reported that rat ANP mRNA in rat hearts was increased by dexamethasone in an in vivo study(22). On the other hand, Tonolo et al. reported(23) plasma atrial natriuretic peptide concentration was negatively correlated with blood pressure in rats treated with low dose subcutaneous infusions of dexamethasone. Our results may support the latter one.

Further studies are required to understand the regulation of the hANP gene.

Acknowledgement

We are indebted to Miss Hiroko Kimura for preparing the manuscript.

References

1) Maack, T., Camargo, M.J.F., Kleinert, H.D., Laragh, J.H., and Atlas, S.A. (1985) Atrial natriuretic factor: structure and functional properties. Kidney Int. 27, 607-615.
2) Cantin, M., and Genest, J. (1985) The heart and the atrial natriuretic factor. Endocrine Reviews 6, 107-127.
3) Needleman, P., Adams, S.P., Cole, B.R., Currie, M.G., Geller, D.M., Michener, M.L., Saper, C.B., Schwartz, D., and Standaert, D.G. (1985) Atriopeptins as cardiac hormones. Hypertension 7, 469-482.
4) Ballermann, B.J., and Brenner, B.M. (1985) Biologically active atrial peptides. J. Clin. Invest. 76, 2041-2048.
5) Nakayama, K., Ohkubo, H., Hirose, T., Inayama, S., and Nakanishi, S. (1984) mRNA sequence for human cardiodilatin-atrial natriuretic factor precursor and regulation of precursor mRNA in rat atria. Nature 310, 699-701.
6) Seidman, C.E., Duby, A.D., Choi, E., Graham, R.M., Haber, E., Homcy, C., Smith, J.A., and Seidman, J.G. (1984) The structure of rat preproatrial natriuretic factor as defined by a complementary DNA clone. Science 225, 324-326.
7) Nemer, M., Chamberland, M., Sirois, D., Argentin, S., Drouin, J., Dixon, R.A.F., Zivin, R.A., and Condra, J.H. (1984) Gene structure of human cardiac hormone precursor, pronatriodilatin. Nature 312, 654-656.
8) Greenberg, B.D., Bencen, G.H., Seilhamer, J.J., Lewicki, J.A., and Fiddes, J.C. (1984) Nucleotide sequence of the gene encoding human atrial natriuretic factor precursor. Nature 312, 656-658.
9) Seidman, C.E., Bloch, K.D., Klein, K.A., Smith, J.A., and Seidman, J.G. (1984) Nucleotide sequences of the human and mouse atrial natriuretic factor genes. Science 226, 1206-1209.
10) Oikawa, S., Imai, M., Inuzuka, C., Tawaragi, Y., Nakazato, H., and Matsuo, H. (1985) Structure of dog and rabbit precursors of atrial natriuretic polypeptides deduced from nucleotide sequence of cloned cDNA. Biochem. Biophys. Res. Commun. 132, 892-899.
11) Vlasuk, G.P., Miller, J., Bencen, G.H., and Lewicki, J.A. (1986) Structure and analysis of the bovine atrial natriuretic peptide precursor gene. Biochem. Biophys. Res. Commun. 136, 396-403.
12) Vlasuk, G.P., Bencen, G.H., Scarborough, R.M., Tsai, P.K., Whang, J.L., Maack T, Camargo, M.J.F., Kirsher, S.W., and Abraham, J.A. (1986) Expression and secretion of biologically active human atrial natriuretic peptide in Saccharomyces cerevisiae. J. Biol. Chem. 261, 4789-4796.
13) Lippman, M.E., and Thompson, E.B. (1974) Steroid receptors and the mechanism of the specificity of glucocorticoid responsiveness of somatic cell hybrids between hepatoma tissue culture cells and mouse fibroblasts. J. Biol. Chem. 249, 2483-2488.
14) Wigler, M., Silverstein, S., Lee, L.S., Pellicer, A., Cheng, Y.C., and Axel, R. (1977) Transfer of purified herpes virus thymidine kinase gene to cultured mouse cells. Cell 11, 223-232.
15) Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J., and Rutter, W.J. (1979) Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18, 5294-5299.
16) Berk, A.J., and Sharp, P.A. (1977) Sizing and mapping of early adenorvirus mRNAs by gel electrophoresis of S1 endonuclease digested hybrids. Cell 12, 721-732.

17) Thomas, P.S. (1980) Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc. Natl. Acad. Sci. USA 77, 5201-5205.
18) Yamamoto, K.R. (1985) Steroid receptor regulated transcription of specific genes and gene networks. Ann. Rev. Genet. 19, 209-252.
19) Robins, D.M., Paek, I., Seeburg, P.H., and Axel, R. (1982) Regulated expression of human growth hormone genes in mouse cells. Cell 29, 623-631.
20) Birnbaum, M.J., and Baxter, J.D. (1986) Glucocorticoids regulate the expression of a rat growth hormone gene lacking 5' Flanking sequences. J. Biol. Chem. 261, 291-297.
21) Kurtz, D.T. (1981) Hormonal inducibility of rat α_2u globulin genes in transfected mouse cells. Nature 291, 629-631.
22) Gardner, D.G., Hane, S., Trachewsky, D., Schenk, D., and Baxter, J.D. (1986) Atrial natriuretic peptide mRNA is regulated by glucocoricoids in vivo. Biochem. Biophys. Res. Commun. 139, 1047-1054.
23) Tonolo, T., Fraser, R., Connell, J.M.C., and Kenyon, C.J. (1986) Glucocorticoid-induced hypertension in rats. J. Hypertension 4, 789.