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REGULATION OF MOUSE ORNITHINE DECARBOXYLASE GENE EXPRESSION IN A MACROPHAGE-LIKE CELL LINE: SYNERGISTIC INDUCTION BY BACTERIAL LIPOPOLYSACCHARIDE AND CAMP

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The ability of the promotor/enhancer region of the mouse ornithine decarboxylase gene to respond to various stimuli was studied. This region was subcloned into multiple fragments and these were inserted in front of the chloramphenicol acetyltransferase gene on an expression vector, pBLCAT3. These ODC/CAT constructs were transfected into a mouse macrophage-like cell line, RAW264. The transfected cells were stimulated by bacterial lipopolysaccharide, 8-bromo cAMP or both followed by analysis of chloramphenicol acetyltransferase activity. Optimal inducible chloramphenicol acetyltransferase expression was obtained when sequences from -90 to +12 (with respect to the transcriptional start site) were tested in cells treated with a combination of lipopolysaccharide and 8-bromo cAMP. A putative cyclic AMP response element located at -48 was altered by site-directed mutagenesis but these alterations did not diminish activity in response to stimulation with lipopolysaccharide and 8-bromo cAMP.

Ornithine decarboxylase (ODC) is a fundamental enzyme for cell growth. Its expression is regulated at multiple levels including; transcription (1), post-transcription (2), translation (3) and post translation (4). We have previously shown that bacterial lipopolysaccharide (LPS) induces the rapid expression of ODC transcription in a mouse macrophage-like cell line, RAW264 (5). This induction by LPS could be enhanced by 8-bromo cAMP (8-br cAMP) while 8-br cAMP alone did not have an inducing effect. The induction and enhancement effects were shown to be, in part, transcriptional. The ODC gene from several different species has been cloned and sequenced (6,7,8, 9,10,11). This sequence information indicates that multiple putative regulation elements exist

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The abbreviations used are: ODC, ornithine decarboxylase; LPS, bacterial lipopolysaccharide; CRE, cAMP responsive element; 8-br cAMP, 8-bromo cAMP; CAT, Chloramphenicol acetyltransferase; bp, base pairs; AP-2, activator protein 2.

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in the 5' flanking region of the gene and these elements are conserved in the ODC genes from different species (7,8,9,11). Although these putative regulatory sequences have been identified, the functions of these elements have not been fully studied.

In this study, we have cloned fragments of the 5' flanking region of the mouse ODC gene 5' to a reporter gene, chloramphenicol acetyl transferase (CAT). The cloned constructs were transfected into the RAW264, a macrophage-like cell line and the effect of LPS and 8-br cAMP stimulation of these cells was determined by assessing CAT activity. We have also utilized oligonucleotide-directed site specific mutagenesis to determine if a specific Cis acting element was responsible for regulation by LPS and cAMP.

MATERIAL AND METHODS

Plasmid Constructions

ODC gene fragments used in this study were derived from a murine ODC genomic clone kindly provided by Dr. P. Coffino (UCSF, San Francisco, CA,). All ODC fragments were subcloned into a CAT expression vector, pBLCAT3 (provided by Dr. B. Luckow, 12). pBLCAT3 contains a promoter free CAT gene ligated to an SV40 intronic fragment and polyadenylation signal with polylinker sequences present both 5' to the CAT gene and 3' to the SV40 intronic fragment. The fragments of the ODC gene used in this study were 1) Bam HI, -1773 to Bam HI, +322; 2) PST I, -1658 to PST I, +12; 3) Hind III, -635 to Pst I, +12; 4) Sma I, -134 to Pst I, +12; 5) Xma III, -90 to Pst I, +12 and 6) Alu I, -63 to Pst I, +12. All of the nucleotide positions are given relative to the transcription start site. These fragments were subcloned into pBLCAT3 5' to the CAT gene. The clones are named pSZN where N is the position of the 5' end of the fragment.

In some studies the inserted DNA was modified using the oligonucleotide-directed, site-specific mutagenesis technique described by Vandeyar et al (13). This was accomplished after subcloning the fragment into M13mp18. All sequences were confirmed by DNA sequencing using modified T7 polymerase (Sequenase, U.S. Biochemicals, Cleveland, OH, 14).

Cell Culture and Transfections

All tissue culture related reagents used were tested for endotoxin contamination by the Limulus amebocyte lysate assay (Sigma Chemical Co., St. Louis, MO). Only those containing less than 0.13 ng/ml endotoxin were used. In some cases, endotoxin contamination was removed by immobilized polymyxin B (Detoxi-Gel, Pierce Chemical Co.).

RAW264 cells, a macrophage-like cell line (15), were maintained in Dulbecco's modification of Eagle's medium (DMEM, Hyclone, Logan, UT) supplemented with 10 ml/liter of Penicillin-Streptomycin (Hybri-max, Sigma Chemical Co., St. Louis, MO) and 5% supplemented calf serum (Hyclone, Logan, UT). Cells were grown at 37°C in 5% CO_2, humidified air.

Cells were transfected using a calcium phosphate co-precipitation procedure (16). Cells were serum starved 12 hours after transfection. CAT assays were performed 36 hours after transfection. Stimulation of LPS (1μg/ml) or 8-br cAMP (0.5mM) or both were added 16 hours before CAT assay.

CAT Assays

CAT assays were performed as described (17). The protein content of each sample was determined by the method of Bradford (18) and the final results were calculated to

represent CAT activity as pM of acetate bound to chloramphenicol / mg of cellular protein / hr (pM/mg/hour).

RESULTS AND DISCUSSION

Six plasmid constructs of ODC/pBLCAT3; pSZ1773, pSZ1658, pSZ635, pSZ134, pSZ90 and pSZ63 (see method section), were transfected into RAW264 cells. CAT activities were determined after stimulation with LPS and 8-br cAMP. The combination of stimuli was used to induce a maximal increase in transcription rate. As shown in figure 1, there was an increase in CAT activity when the shorter constructs, pSZ134 and pSZ90 were compared to constructs containing longer segments of the 5' flanking region (pSZ1773, pSZ1658 and pSZ635). One possible mechanism for this increase in inducible CAT activity is that a repressor or silencer-like element(s) may be present between -134 and -635 of mouse ODC. Losing this element(s) may result in an overall increase in CAT expression. There was also a pronounced decrease in CAT activity when the shorter DNA segment present in pSZ63 was compared to pSZ134 or pSZ90. The cause of the decreased activities is possibly due to the loss of a positive regulating element between -63 and -90 bp of the ODC gene. When the DNA sequence between -134 and the transcription start site was analyzed several putative regulatory elements were identified. These include an AP-2 site at -112 bp, a CCAAT box at -82 bp, a CRE at -48 bp and a TATAA box at -29 bp. All these putative regulating elements are highly conserved among Human, mouse and rat ODC genes. A possible CCAAT box sequence (actual sequence in ODC is CCGAT) is present in the pSZ134 and pSZ90

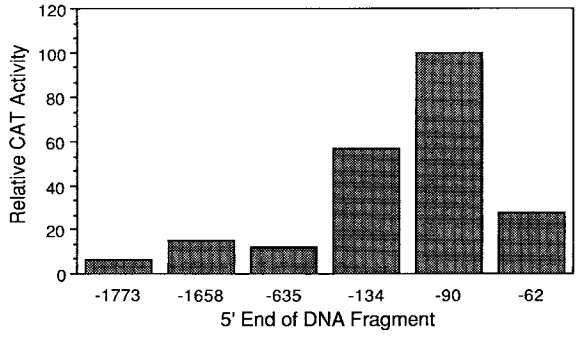

Figure 1. Effects of ODC 5' flanking region fragments on the expression of CAT activity. Fragments of the 5' flanking region of ODC gene were inserted 5' to the CAT gene in the expression vector pBLCAT3. The fragments tested were 1) -1773 to +322; 2) -1658 to +12; 3) -635 to +12; 4) -134 to +12; 5) -90 to +12 and 6) -63 to +12. These constructs were transfected into RAW264 cells. Cells were serum-starved after transfection. Twenty hours after transfection the cells were stimulated with 1μg/ml LPS and 0.5 mM 8-br cAMP. After an additional overnight incubation the cells were lysed and CAT activity was determined. Relative CAT activity is determined by dividing the activity of each clone by the activity of the most active clone pSZ90 which was given an arbitrary value of 100%.

constructs but not in the pSZ63 construct. Loss of this sequence may be the reason for decreased CAT activity of the pSZ63 construct.

Both an AP-2 site and a CRE are potentially responsive to cAMP stimulation, constructs pSZ134, pSZ90 and pSZ63 were studied for their responsiveness to cAMP stimulation. These three constructs were transfected into RAW264 cells, and CAT activities were assayed without any stimulation of the cells or after stimulation with LPS, 8-br cAMP or both LPS and 8-br cAMP. The effects of these stimulations are shown as fold induction of CAT activity relative to the unstimulated cells transfected with the same plasmid. As demonstrated in figure 2, these constructs gave the same pattern of responsiveness. Treatment with either 1μg/ml LPS alone or 0.5 mM 8-br cAMP alone did not cause a reproducibly significant increase in CAT activity. CAT activity was significantly induced by treatment with a combination of LPS and 8-br cAMP. As all three constructs had the same induction pattern by LPS and 8-br cAMP, the element(s) responsive for the induction must reside in the -63 to +12 bp fragment of ODC gene in pSZ63. That excluded the putative AP-2 site at -112 and the putative CCAAT box at -82.

There is, however, a putative CRE at -48 bp of the ODC gene. The CRE was first found in the rat somatostatin gene (19), and has since been found in a number of cAMP inducible genes (20). Mutations of the core part of the CRE can totally abolish the activity of a CRE (21,22). To test the role of the putative CRE in the induction of CAT activity by LPS and cAMP, oligonucleotide-directed site-specific mutations were made within this element. The consensus CRE sequence is TGACGTCA, while the sequence present in ODC

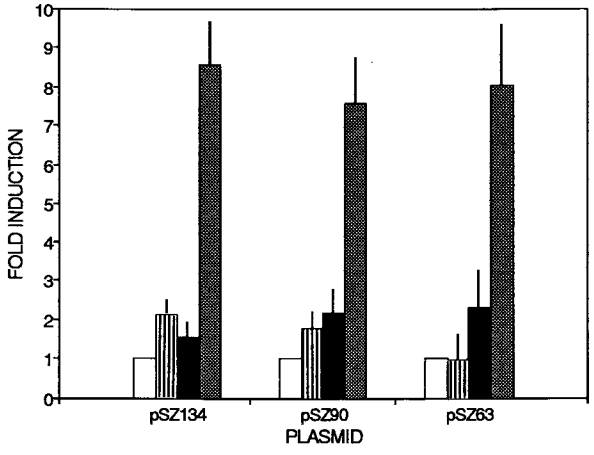

Figure 2. Induction of CAT activity by LPS and 8-br cAMP. pSZ134, pSZ90 and pSZ63 were transfected into RAW264 cells. Twenty hours after transfection the transfected cells were stimulated with LPS, 8-br cAMP or both LPS and 8-br cAMP. After an additional overnight incubation cells were harvested and CAT activity was determined. Conditions tested are no treatment (□); 1 μg/ml LPS (▥); 0.5mM 8-br cAMP (■); or both LPS and 8-br cAMP (▩).

TABLE 1. Effect of Mutations of the Putative CRE on CAT Activity

Clones	Putative CRE Sequence[a]	Fold Induction of CAT activity[b] Mean ± Std (N)
pSZ134	TGACGACG	8.6 ± 1.0 (7)
pSZ90		7.6 ± 1.1 (11)
pSZ63		8.0 ± 1.4 (5)
pSZ134*	AGACGACG	8.8 ± 0.8 (2)
pSZ90*		5.1 ± 1.3 (2)
pSZ63*		16.2 ± 2.0 (2)
pSZ63**	AGATTACG	9.7 ± 2.8 (2)

[a] Sequence of Putative CRE. * refers to the sequence with a single mutation. ** refers to the sequence with 3 mutations.

[b] RAW264 cells were transfected with the ODC-CAT construct by the calcium/phosphate coprecipitation method. After 20 hours the cells were treated with 1 μg/ml LPS and 0.5mM 8-bromo cAMP. 16 hours later the cells were harvested and CAT activity was determined. Fold activity was determined by comparing to the activity observed when similarly transfected cells were not stimulated.

is TGACGACG. As shown in table 1, this sequence was mutated to AGACGACG and AGATTACG. These alterations have previously been shown to eliminate CREB binding activity (21,22). The first mutation AGACGACG was tested in three CAT constructs; pSZ134, pSZ90 and pSZ63. The second mutation, AGATTACG, was only tested in the shorter pSZ63 construct. The induction of CAT activity by LPS and 8-br cAMP of all these mutated constructs was similar to the activity observed in the non-mutated constructs. This indicated the putative CRE of the mouse ODC gene was not involved in the synergistic induction by LPS and 8-br cAMP. It is not known if this sequence will function as a CRE in other cells or after different stimulation.

These results showed that there are multiple regulating elements both of positive and negative nature in the 5'-flanking region of the mouse ODC gene. There is a element(s) in the -63 to +12 bp fragment that is responsive to a synergistic induction by LPS and 8-br cAMP. The putative CRE at -48 bp is not, however, the responsive element.

We have demonstrated that the 5'-flanking region of ODC has both negative and positive effects on transcriptional regulation. Using DNA constructs that contain this region we did not detect the inducibility by LPS alone as we have previously demonstrated using transcription runoff assays. This may mean the element(s) responsible for LPS induction resides in a portion of the gene other than the 5'-flanking region of ODC. It has been reported that responsiveness to phorbol ester stimulation is located in the coding region of

the ODC gene (23). In our previous studies, phorbol ester and LPS had similar induction characteristics on ODC transcription (5). It is possible that element responsive to LPS induction also resides in the coding region.

We have demonstrated a unique synergistic stimulation with the combination of 8-br cAMP and LPS. Further analysis will be required to determine the nature of this synergistic stimulation. Recent reports have indicated that Trans acting transcriptional regulatory factors from different signal pathways could interact. c-Jun and glucocorticoid receptor (24,25) were shown to interact in a manner that inhibited DNA binding. Although we demonstrated synergistic activation instead of mutual inhibition, it is possible that some similar mechanism is involved. Future studies will be directed towards understand the mechanism of the synergistic activation of ODC.

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REFERENCES

1. Katz, A. and Kahana, C., (1987) Mol. Cell. Biol., **7**, 2641-2643.
2. Berger, F.G., Loose, D., Meisner, H. and Watson, G., (1986) Biochemistry, **25**, 1170-1175.
3. White, M.W., Kameji, T., Pegg, A.E. and Morris, D.R., (1987) Eur. J. Biochem. **170**, 87-92.
4. van Daalen Wetters, T., Macrae, M., Brabant, M., Sittler, A. and Coffino, P., (1989) Mol. Cell. Biol.,**9**, 5484-5490.
5. Shurtleff, S.A., McElwain, C.M., and Taffet, S.M., (1988) J. Cell. Physiol., **134**, 453-459.
6. Katz, A., and Kahana, C., (1988) J. Biol. Chem. **263**,7604-7609.
7. Coffino, P., and Chen, E.L., (1988) Nucleic Acids Res., **16**, 2731-2732.
8. Eisenberg, L.M., and Jänne, O.A., (1989) Nucleic Acids Res., **17**, 2359.
9. Wen, L., Huang, J.K., and Blackshear, P.J., (1989) J. Biol. Chem., **264**, 9016-9021.
10. Brabant, M., McConlogue, L., van Daalen Wetters, T. and Coffino. P., (1988) Proc. Natl. Acad. Sci. USA, **85**, 2200-2204.
11. Fitzgerald, M.C., and Flanagan, M.A., (1989) DNA, **8**, 623-634.
12. Luckow, B., and Schütz, G., (1987) Nucleic Acids Res., **15**, 5490.
13. Vandeyar, M.A., Weiner, M.P., Hutton, C.J. and Batt, C.A. (1988) Gene **65**, 129-133.
14. Tabor, S. and Richardson, C.C. (1989) J. Biol. Chem. **264**, 6447-6458.

15. Raschke, W.C., Baird, S., Ralph, P., and Nakoinz, I. (1978) Cell **15**, 261-267.

16. Segal, S. (1986) In Davis, L.G., Dibner, M.D. and Battey, J.F. (eds), Basic Methods in Molecular Biology. Elsevier Science Publishing Co., Inc. pp.286-300.

17. Neumann, J.R., Morency, C.A., and Russian, K.O. (1987) Biotechniques **5**, 444-448.

18. Bradford, M. (1976) Anal. Biochem. **72**, 248-254.

19. Montminy, M.R., Sevarino, K.A., Wagner, J.A., Mandel, G., and Goodman, R.H., (1986) Proc. Natl. Acad. Sci. USA **83**, 6682-6686.

20. Roesler, W.J., Vandenbark, G.R. and Hanson, R.W. (1988) J. Biol. Chem. **263**, 9063-9066.

21. Deutsch, P.J., Hoeffler, J.P., Jameson, J.L., Lin, J.C. and Habener, J.F. (1988) J. Biol. Chem. **263**, 18466-18472.

22. Philippe, J., and Missotten, M. (1990) J. Biol. Chem. **265**, 1465-1469.

23. van Daalen Wetters, T., Brabant, M., and Coffino, P. (1989) Nucleic Acid Res. **17**, 9843-9860.

24. Yang-Yen, H. F., Chambard, J. C., Sun, Y. L., Smeal, T., Schmidt, T.J., Drouin, J. and Karin, M. (1990) Cell **62**, 1205-1215.

25. Schüle, R., Rangarajan, P., Kliewer, S., Ransone, L.J., Bolado, J., Yang, N., Verma, I.M. and Evans, R.M. (1990) Cell **62**, 1217-1226.