UNIVERSITY OF ALBERTA

Regulation of the Na^+/H^+ Exchanger Promoter

BY

WEIDONG YANG

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master in Sciences

in

BIOCHEMISTRY

EDMONTON, ALBERTA

SPRING, 1996

ISBN 0-612-10768-X

Canada

University of Alberta

Library Release Form

Name of Authors:	Weidong Yang
Title of the thesis:	Regulation of the Na^+/H^+ Exchanger Promoter
Degree:	Master in Sciences
Year this Degree Granted:	1996

Signature of Author:
#101, 10710-85Ave., Edmonton, AB
T6E 2K8 Canada

Date submitted to FGSR: November 7, 1995

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Regulation of the Na^+/H^+ Exchanger Promoter submitted by Weidong Yang in partial fulfillment of the requirements for the degree of Master in Sciences.

Larry Fliegel, Associate Professor

Marek, Michalak, Professor

Michael, Schultz, Assistant Professor

Colin, Rasmussen, Assistant Professor

Brian D. Sykes, Professor

Constance L. Chik, Associate Professor

Date the thesis approved by the commttee: October 27, 1995

Abstract

This work has been focused on the regulation of mouse NHE-1 promoter in different cell models. We examined the regulation of the NHE-1 promoter in both neonatal rat cardiomyocytes and rat skeletal muscle cell line--L6 cell. In primary myocardium, external acidosis failed to increase the transcription from the promoter. On the contrary, serum treatment increases the promoter activity. Deletion and mutation of AP2 site abolish the stimulatory effect of serum and cause a dramatic decrease in promoter activity. This finding indicates that the cis-acting putative AP2 site and the presence of serum are important in NHE-1 expression in myocardium while external acidosis had no direct effect on the promoter. NHE activity has been shown to vary between proliferating myocytes and differentiated myotubes. Since NHE-1 is believed to be important in this event we examined the regulation of the NHE-1 in L6 cell. Our studies demonstrate that the basal expression of the NHE-1 is mediated largely by two proximal regions of the gene, one is from bp -155 to -171 and another one is between bp -92 and -125. However, during the process of differentiation more distal regions are involved in the 2-3 fold elevation of expression, suggesting NHE-1 is somehow related to cell differentiation. We have also examined the role of a highly conserved poly (dA:dT) element of the promoter in regulation of NHE-1 expression in L6 cells. Deletion or mutation of this region results in a substantial decrease in activity of the promoter. DNA footprinting and gel retardation show protein(s) binding to the element. Insertion of the poly (dA:dT) into upstream of SV40 promoter increases the activity of the foreign promoter. All the results indicate that the poly (dA:dT) rich region is important in upregulation of NHE-1 expression in L6 cell.

Acknowledgments

I am grateful to Larry for allowing me to study in his laboratory and for guiding me to become familiar with the Na^+/H^+ exchanger. Without his help, this work could not be brought to a conclusion. I would like to thank my lab. follows: Jason, Rob, Huayan, Norma, Le, Pavel and Pierre for many inspiring discussions and technical assistances in my training. Finally, I would like to acknowledge my committee members for their patience and guidance.

TABLE OF CONTENTS

CHAPTER FIVE: *CONCLUSION*

LIST OF FIGURES

LIST OF ABBREVIATIONS

bp	base pairs
BSA	bovine serum albumin
Ca^{2+}	calcium ion
$CaCl_2$	calcium chloride
cDNA	complementary deoxyribonucleic acid
Cl^-/HCO_3^+	chloride bicarbonate
CO_2	carbon dioxide
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
dNTP	deoxynucleoside triphosphate
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetate
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
H^+	hydrogen ion
HCl	hydrochloric acid
HEPES	N-2-hydroxyethypiperazine-N'-2-ethanesulfonic acid
kb	kilobase
KCl	potassium chloride
kDa	kilodalton
DMEM	Dulbecco's Modified Eagle Media
$MgCl_2$	magnesium chloride
mRNA	messenger ribonucleic acid
Na^+	sodium ion
NaCl	sodium chloride
NHE-1	Na^+/H^+ exchanger---isoform #1
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pHi	intracellular pH
TE	Tris-EDTA

Vmax	maximal velocity
MCT	mouse renal cortical tubule cell
P19	mouse embryonal carcinoma cell
L6	rat thigh skeletal muscle cell

Base abbreviations

A	adenine
C	cytosine
G	guanine
T	thymine
U	uracil

CHAPTER ONE

Introduction

Introduction

General Background

The overall cellular homeostasis involves in a number of biological processes. Many of these processes are performed by membrane transport. A very important process is the regulation of intracellular pH (pHi). Under physiological conditions, pHi of most mammalian cells is at about pH 7.2. This comes about as a result of an equilibrium between production of acid and elimination of acid. The major source of acid yield is from metabolites. The acid extruding mechanisms are mainly composed of several membrane H^+ transport systems, including the ATP-dependent H^+ pump, the Na^+/H^+ exchanger, and the Na^+-dependent/-independent Cl^-/HCO^- exchangers (1). Although the contributions of those mechanisms change with cell types, the Na^+/H^+ exchanger (NHE) has been demonstrated to be one of the major mechanisms involved in maintaining pHi in most, if not all, cells (2).

The NHE was first functionally identified in vesicles from rabbit kidney and small intestine brush border membrane (3), and has since been found in virtually all cell types examined (4). It has been generally accepted that, under physiological conditions, NHE uses the energy of the inwardly directed sodium electrochemical gradient to carry out the electroneutral exchange of one intracellular H^+ ion for one extracellular Na^+ ion, or, under abnormal conditions, vice versa (5, 6). NHE has negligible activity at physiological pHi, but as the pHi falls to levels away from neutrality by even less than 1 pH unit, the antiporter is rapidly activated and reaches its maximum transport rate. This type of activation indicates that the NHE is a very sensitive mechanism to maintain pHi, and leads to the hypothesis that there might be a proton sensor at the intracellular face of NHE (7).

The finding that the NHE expressed at the apical face of epithelial cells in kidney and intestine showed lower affinities for the inhibition than the exchangers of non-epithelial cells, indicates that in addition to the ubiquitously expressed amiloride sensitive NHE, namely NHE-1, other isoforms also exist with restricted distribution and functions (8-10). By now, five NHE isoforms (NHE-1 to NHE-5) have been identified in mammalian cells with molecular biology techniques (11-16). All these isoforms possess similar N-terminal hydropathy profile of 10 to 12 transmembrane domains with two highly conserved segments presumably involved in ion transport. These isoforms are allosterically activated by intracellular H^+ on their cytoplasmic (C-terminal) domain which is less homologous

than the transmembrane domain in amino acid sequence among isoforms. All the NHE isoforms regulate intracellular pH in a Na^+-dependent manner, and are inhibited by amiloride and its derivatives with different Ki values (17). Among them, NHE-1 has been most extensively studied. Results have indicated that NHE-1 consists of 815 amino acids, with a molecular weight of about 110 kD. Its N-terminal domain consists of 500 amino acids and there have been reports that there are an amiloride binding site, N-, O-linked glycosylation sites (18), an H^+-modifier site and the ion transport site on this domain. Whereas, the C-terminal domain has been shown to contain a number of phosphorylation sites and the potential proton sensor. It has yet to answer whether the functional unit of NHE-1 is a dimer or an oligomer (19). It is known that NHE-1 is amiloride sensitive, growth factor-activatable, and widely expressed in most type of cells. It is therefore called the housekeeping isoform of NHE. However, the whole scenario of how NHE-1 is regulated is still unclear (20).

It has been believed that in addition to protection against cytoplasmic acidification and regulation of cell volume (21), NHE is also implicated in many physiological and pathophysiological cellular events including fertilization (22), cell cycle control (4), differentiation (23, 24), essential hypertension (25), kidney diseases (22) and hypertrophy (4). It has been suggested that NHE is responsible for an elevation of pHi during proliferation, and the intracellular alkalinization plays an important permissive role in cell growth (4, 26). Moreover, although mitogens like serum can stimulate quiescent cells to undergo proliferation (27), it seems that the stimulation is, at least partially, through activation of NHE. Quiescent fibroblasts which are treated to induce intracellular alkalinization can undergo mitosis, even in the absence of serum (26). In the presence of serum, NHE deficient fibroblasts showed lower rate of DNA synthesis and cell growth in comparison to wild cell types, presumably due to the failure to raise pHi (28). Most interestingly, however, is that phorbol esters, a tumor promoter, increases NHE activity (29) and the inhibition of NHE can hinder the proliferation of tumor cells (30). Taken together, this short list suggests that NHE is important for a number of cellular activities. These above mentioned physiological roles are principally performed by the ubiquitously expressed NHE-1 isoform. In the contrast, other NHE isoforms are specifically expressed at the apical face of epithelia where they participate in more specialized salt transport tasks (31).

Rationale

Abnormal NHE-1 activities have been found to be involved in some pathological processes. This includes cardiovascular diseases including essential hypertension (25), postischaemic myocardial arrhythmias (32), and kidney diseases (22). It has been proposed that during ischemia and reperfusion, cell damage and arrhythmias occur due to a so-called ' pH paradox ' (20). Ischemia results in a dramatic accumulation of protons in the myocardium until the reperfusion phase when NHE-1 is activated. This results in a rapid increase in intracellular Na^+. In turn, the increase in intracellular Na^+ activates the Na^+/Ca^{2+} exchanger which leads to increased intracellular Ca^{2+} and subsequent damage to the myocardium. In addition, it has also been shown that NHE-1 inhibitors, such as amiloride and its derivatives, can alleviate the damage and might be potential antiarrhythmic agents in clinical practices (33).

It has been well known that NHE plays an important role in regulation of pHi in different types of muscle cells. This includes skeletal muscle (34), the myocardium (35) and smooth muscle (36). Considering the wide involvement of NHE-1 in both physiological and pathological processes mentioned above, and its predominant expression in myocardium (12, 37, 38) and skeletal muscle (12, 37), it is of great significance to study the regulation of NHE-1 activity in those tissues.

Studies have shown that NHE-1 activity and/or mRNA levels are increased due to a number of treatments including chronic acid loading, agents causing cellular proliferation and treatments resulting in cellular differentiation (39-42). In intact animals (42) and in renal cell lines (43), both NHE-1 Vmax and mRNA levels have been shown to increase due to chronic metabolic acidosis. This may be an adaptive mechanism by which cells can upregulate NHE-1 in response to chronic acid load (44-46). In a limited number of studies on primary cultures of isolated myocytes, it has been shown that chronic low external pH (pHo) treatment allows for a more rapid recovery from an acute acid load (47). Also, low-flow myocardial ischemia causes an increase in steady state NHE-1 message levels, possibly as a result of decreased intracellular pH (47). In addition it causes an increase in the amount of a smaller isoform of the NHE-1 message (45). Acidosis may act as a specific mechanism to increase NHE-1 message and protein levels through both increased transcription and decreased mRNA degradation. Although there is evidence that growth factors stimulate NHE-1 at transcription level in certain cell types (48), little is known about regulation of expression in the myocardium. Preliminary studies have suggested that

serum might be a factor to act on either regulation of activity or expression of the NHE-1 in cultured myocardial cells (39). Also, it was reported that during retinoic acid induced differentiation of human leukemic cells (HL-60), there is an 8.3-fold increase in NHE-1 transcription (40). More recently colleagues in our laboratory have shown that there is a transient increase in the level of the NHE-1 transcription during retinoic acid induced differentiation of P19 cells (48). This data provides evidence to suggest that NHE-1 plays an important role in cellular differentiation. Increased exchange activity during differentiation may be important for differentiation to occur, at least in some cell types (49, 50). Since this requirement has not been shown to occur universally and the role of the NHE-1 may vary between cell types (51), it is of great interest to explore the requirement in other cell types.

It has been shown that the regulation of NHE-1 varies between proliferating myoblasts and differentiated myotubes (34). This suggests that there are important physiological changes in the protein and its function during the process of differentiation. It is also suggested that the skeletal muscle provides an expected model for studying the regulation of NHE-1 at transcription level during both differentiation and non-differentiation.

Although the mechanism of regulation of NHE-1 is extremely important and the effects of mitogenic stimulation have been well documented at protein level (41, 52, 53), few studies have examined the NHE-1 gene (48, 54-56). Until recently, it has been thought that the regulation of NHE-1 activity is controlled by short-term changes (4, 18). However, there is now strong evidence supporting long-term regulation at the pre-translational level. It has been reported that the steady state levels of NHE-1 mRNA increased under certain conditions such as acidosis (42, 57-59), ischemia (54) and differentiation (23, 60). These findings indicate a strong rationale for the examination of the long-term regulation of NHE-1 at transcriptional level. Since the increased levels of NHE-1 mRNA can tell us little about how the gene is regulated itself, the mouse promoter/enhancer of NHE-1 was examined (55, 56). The availability of the promoter has provided us the opportunity of gaining valuable information about the regulation of NHE-1 at the gene level.

According to studies on human NHE-1 promoter, a number of putative cis-elements were found lying just upstream of the human NHE-1 TATA box (55, 56). Those elements include one cyclic AMP response element, two CAAT boxes, four GC boxes, three AP-1

sites and five CACCC boxes (56). Sequential deletions of the promoter region were performed from a CAT reporter plasmid and the effects on CAT activity analyzed (55). The results indicate that there are elements in the promoter which regulate the human NHE-1 gene and there are different NHE-1 regulatory factors in various tissues (55). In addition, similar results have been obtained through the study of mouse NHE-1 promoter in our laboratory. It has been shown that there are two CAAT boxes, an SP-1 site, a cyclic AMP response element binding site, and an AP-2 like site on the upstream of a TATA box in a 1.1 kb mouse NHE-1 promoter (61). Partial deletion of the 1.1 kb fragment up to a putative AP-2 binding region did not result in significant decreases in the level of promoter directed transcription. However, deletion of the putative AP-2 site resulted in a loss of most of the reporter gene activity in mouse fibroblast (NIH3T3) and human Hep G2 cells (61). The transcription factor AP-2 is one factor that may be important in regulation of the NHE-1 gene (48). Interestingly, the comparison among human (55), rabbit (62) and mouse (61) NHE-1 promoter sequences also shows that there are highly conserved elements which may be important in regulation of NHE-1. It is therefore our belief that the study on the promoter will most likely reveal transcription elements which are involved in the regulation of NHE-1 gene.

Objectives

In our experiments, two cell models, primary myocardium and skeletal muscle, will be employed based on the information discussed above. With primary myocardial cultures, we will attempt to determine if the increased level of NHE-1 mRNA observed during ischemia is due to the increase in promoter activity. By using differentially deleted mouse NHE-1 promoter constructs, we will examine the basal regulation of the gene in this cell type. In particular, we will examine the effects of serum in primary cardiomyocytes and will attempt to localize where any responsive elements are located in the promoter. With L6 cells, a skeletal muscle cell line, conditions for differentiation will be established. In both differentiated and non-differentiated L6 cells, progressively deleted mouse NHE-1 promoter constructs will be tested to determine the locations of potential responsive elements of the promoter, and to observe whether the regulation of NHE-1 promoter activity depends on cellular differentiation or not. We will also examine a highly conserved poly (dA:dT) element. This element is highly conserved in the proximal region of the NHE-1 gene promoter isolated. We will analyze its importance in regulation of the NHE-1 gene.

References

1. Madshus, I. H. (1988) Regulation of intracellular pH in eukaryotic cells. *Biochem. J.* **250,** 1-8

2. Rothstein, A. (1989) The Na/H exchange system in cell pH and volume control. *Rev. Physiol. Biochem. Pharmacol.* **112,** 235-257

3. Murer, H., Hopfer, U., and Kinne, R. (1976) Sodium/proton antiport in brush border-membrane vesicles isolated from rat small intestine and kidney. *Biochem. J.* **154,** 597-604

4. Grinstein, S., Rotin, D., and Mason, M. J. (1989) Na^+/H^+ exchange and growth factor-induced cytosolic pH changes. Role in cellular proliferation. *Biochim. Biophys. Acta* **988,** 73-97

5. Wakabayashi, S., Sardet, C., Fafournoux, P., Counillon, L., Meloche, S., Pages, G., and Pouyssegur, J. (1992) Structure function of the growth factor-activatable Na^+/H^+ exchanger (NHE1). *Rev. Physiol. Biochem. Pharmacol.* **119,** 157-186

6. Aronson, P. S. (1985) Kinetic properties of the plasma membrane Na^+-H^+ exchanger. *Annu. Rev. Physiol.* **47,** 545-560

7. Aronson, P. S., Nee J., and Suhm, M. A. (1982) Modifier role of internal H^+ in activating the Na^+-H^+ exchanger in renal microvillus membrane vesicles. *Nature* **299,** 161-163

8. Haggerty, J. G., Agarwal, N., Reilly, R. F., Adelberg, E. A., and Slayman, C. W. (1988) Pharmacologically different Na/H antiporters on the apical and basolateral surfaces of cultured porcine kidney cells (LLC-PK1). *Proc. Natl. Acad. Sci. USA* **85,** 6797-6801.

9. Foster, E. S., Dujeda, P. K., and Brasitus, T. A. (1986) Na^+-H^+ exchange in rat colonic brush-border membrane vesicles. *Am. J. Physiol.* **250,** G781-G787

10. Ramamoorthy, S., Tiruppathi, C., Nair, C. N., Mehesh, V. B., Leibach, F. H., and Ganapathy, V. (1991) Relative sensitivity to inhibition by cimetidine and clonidine differentiates between the two types of Na^+-H^+ exchangers in cultured cells. *Biochem. J.* **280**, 317-322

11. Sardet, C., Franchi, A., and Pouysségur, J. (1989) Molecular cloning, primary structure, and expression of the human growth factor-activatable Na/H antiporter. *Cell* **56**, 271-280

12. Orlowski, J., Kandasamy, R. A., and Shull, G. E. (1992) Molecular cloning of putative members of the Na/H exchanger gene family. *J. Biol. Chem.* **267**, 9331-9339

13. Tse, C. M, Brant, S. R., Walker, M. S., Pouyssegur, J., and Donowitz, M. (1992) Cloning and sequencing of a rabbit cDNA encoding an intestinal and kidney-specific Na^+/H^+ exchanger isoform (NHE-3). *J. Biol. Chem.* **267**, 9340-9346

14. Tse, M., Levine, S., Yun, C., Brant, S., Counillon, L. T., Pouyssegur, J., and Donowitz, M. (1993) Structure/function studies of the epithelial isoforms of the mammalian Na^+/H^+ exchanger gene family. *J. Membr. Biol.* **135**, 93-108

15. Wang, Z., Orlowski, J., and Shull, G. E. (1993) Primary structure and functional expression of a novel gastrointestinal isoform of the rat Na/H exchanger. *J. Biol. Chem.* **268**, 11925-11928

16. Klanke, C. A., Su, Y. R., Callen, D. F., Wang, Z., Meneton, P., Baird, N., Kandasamy, R. A., Orlowski, J., Otterud, B. E., Leppert, M., Shull, G. E., and Menon, A. G. (1995) Molecular cloning and physical and genetic mapping of a novel human Na^+/H^+ exchanger (NHE5/SLC9A5) to chromosome 16q22.1. *Genomics* **25**, 615-622

17. Counillon, J., and Pouysségur, J. (1995) Structure-function studies and molecular regulation of the growth-factors activatable Na^+/H^+ exchanger (NHE-1). *Cardiovasc. Research* **29**, 147-154

18. Sardet, C., Counillon, L., Franchi, A., and Pouyssegur, J. (1990) Growth factors induce phosphorylation of the Na^+/H^+ antiporter, glycoprotein of 110 kD. *Science* **247,** 723-726

19. Otsu, K., Kinsella, J., Sacktor, B., and Froehlich, J. P. (1989) Transient state kinetic evidence for an oligomer in the mechanism of Na^+-H^+ exchange. *Proc. Natl. Acad. Sci. USA* **86,** 4818-4822

20. Fliegel, L., and Frohlich, O. (1993) The Na^+/H^+ Exchanger: An Update on Structure, Regulation and Cardiac Physiology. *Biochem. J.* **296,** 273-285

21. Boron, W. F., and Boulpaep, E. L. (1983) Intracellular pH regulation in the renal proximal tubule of the salamander. *J. Gen. Physiol.* **81,** 29-52

22. Mahnensmith, R. L., and Aronson, P. S. (1985) The plasma membrane sodium-hydrogen exchanger and its role in physiological and pathophysiological processes. *Circ. Res.* **56,** 773-785

23. Rao, G. N., Sardet, C., Pouyssegur, J., and Berk, B. C. (1992) Na^+/H^+ antiporter gene expression increases during retinoic acid-induced granulocytic differentiation of HL60 cells. *J. Cell. Physiol.* **151,** 361-366

24. Ladoux, A., Cragoe, E. J., Geny, B., Abita, J. P., and Frelin, C. (1987) Differentiation of human promyelocytic HL 60 cells by retinoic acid is accompanied by an increase in the intracellular pH. *J. Biol. Chem.* **262,** 811-816

25. Aviv, A., and Lasker, N. (1992) Defects in membrane transports of ion as possible pathogenic factors in hypertension. *Curr. Opin. Nephrol. Hypertens.* **1,** 68-72

26. Busa, W. B., and Nuccitelli, R. (1984) Metabolic regulation via intracellular pH. *Am. J. Physiol.* **246,** R409-R438

27. Pouyssegur, J., Franchi, A., Kohno, M., L'Allemain, G., and Paris, S. (1986) Na-H exchange and growth control in fibroblasts: A genetic approach. *Current topics in membranes and transport* **26,** 210-219

28. L'Allemain, G., Paris, S., and Pouyssegur, J. (1984) Growth factor action and intracellular pH regulation in fibroblasts. Evidence for a major role of the Na/H antiporter. *J. Biol. Chem* **259**, 5809-5815

29. Burns, C. P., and Rozengurt, E. (1983) Serum, platelet-derived growth factor, vasopressin, and phorbol ester increase intracellular pH in Swiss 3T3 cells. *Biochem. Biophys. Res. Commun.* **116**, 931-938

30. Horvat, B., Taheri, S., and Salihagic, A. (1993) Tumor cell proliferation is abolished by inhibitors of Na/H and HCO3/Cl exchange. *Eur. J. Cancer* **29A**, 132-137

31. Burckhardt, B. C., and Burckhardt, G. (1988) Cellular mechanisms of proximal tubular acidification. *Haussinger D, ed. pH Homeostasis, New York: Academic Press* 233-257

32. Lazdunski, M., Frelin, C., and Vigne, P. (1985) The sodium/hydrogen exchange system in cardiac cells. Its biochemical and pharmacological properties and its role in regulating internal concentrations of sodium and internal pH. *J. Mol. Cell Cardiol.* **17**, 1029-1042

33. Duff, H. J. (1995) Clinical and in vivo antiarrhythmic potential of sodium-hydrogen exchange inhibitors. *Cardiovasc. Research* **29**, 89-93

34. Vigne, P., Frelin, C., and Lazdunski, M. (1985) The Na^+/H^+ antiport is activated by serum & phorbol esters in proliferating myoblasts but not in differentiated myoblasts. *J. Biol. Chem.* **260**, 8008-8013

35. Fliegel, L., and Dyck, J. R. B. (1995) Molecular Biology of the Cardiac Na^+/H^+ Exchanger. *Cardiovasc. Research* **29**, 155-159

36. Grinstein, S., Rotin, D., and Mason, M. J. (1989) Na^+/H^+ exchange and growth factor-induced cytosolic pH changes. Role in cellular proliferation. *Biochim. et Biophys. Acta.* **988**, 73-97

37. Fliegel, L., Dyck, J. R. B., Wang, H., Fong, C., and Haworth, R. S. (1993) Cloning and analysis of the human myocardial Na^+/H^+ exchanger. *Mol. Cell. Biochem.* **125,** 137-143

38. Fliegel, L., Sardet, C., Pouysségur, J., and Barr, A. (1991) Identification of the protein and cDNA of the cardiac Na/H exchanger. *FEBS Lett.* **279,** 25-29

39. Green, R. D., Frelin, C., Vigne, P., and Lazdunski, M. (1986) The activity of the Na/H antiporter in cultured cardiac cells is dependent on the culture conditions used. *FEBS Lett.* **196,** 163-166

40. Rao, G. N., Sardet, C., Pouyssegur, J., and Berk, B. C. (1992) Na^+/H^+ antiporter gene expression increases during retinoic acid-induced granulocytic differentiation of HL60 cells. *J. Cell. Physiol.* **151,** 361-366

41. Horie, S., Moe, O., Miller, R. T., and Alpern, R. J. (1992) Long-term activation of protein kinase c causes chronic Na/H antiporter stimulation in cultured proximal tubule cells. *J. Clin. Invest.* **89,** 365-372

42. Krapf, R., Pearce, D., Lynch, C., Xi, X. P., Reudelhuber, T. L., Pouyssegur, J., and Rector, F. C. Jr. (1991) Expression of rat renal Na/H antiporter mRNA levels in response to respiratory and metabolic acidosis. *J. Clin. Invest.* **87,** 747-751

43. Igarashi, P., Freed, M. I., Ganz, B., and Reilly, R. F. (1992) Effects of chronic metabolic acidosis on Na-H exchangers in LLC-PK1 renal epithelial cells. *Am. J. Physiol.* **263,** F83-F88.

44. Akiba, T., Rocco, V. K., and Warnock, D. G. (1987) Parallel adaptation of the rabbit renal cortical sodium/proton antiporter and sodium/bicarbonate cotransporter in metabolic acidosis and alkalosis. *J. Clin. Invest.* **80,** 308-315

45. Dyck, J. R. B., Lopaschuk, G. D., and Fliegel, L. (1992) Identification of a small Na^+/H^+ exchanger-like message in the rabbit myocardium. *FEBS Lett.* **310,** 255-259

46. Moe, O. W., Tejedor, A. Levi , M., Seldin, D. W., Preisig, P. A., and Alpern, R. J. (1991) Dietary NaCl modulates Na^+/H^+ antiporter activity in renal cortical apical membrane vesicles. *Am. J. Physiol.* **260,** F130-137

47. Fliegel, L., Maddaford, T. G., Pierce, G., and Dyck, J. R. B. (1995) Induction of Expression of the Na^+/H^+ Exchanger in the Rat Myocardium. *Cardiovasc. Research* **29,** 203-208

48. Dyck, J. R. B., and Fliegel, L. (1995) Specific activation of the Na^+/H^+ exchanger during neuronal differentiation of embryonal carcinoma cells. *J. Biol. Chem.* **270,** 10420-10427

49. Alvarez, J., Garcia-Sancho, J., Mollinedo, F., and Sanchez, A. (1989) Intracellular Ca^{2+} potentiates Na^+/H^+ exchange and cell differentiation induced by phorbol ester in U937 cells. *Eur. J. Biochem.* **183,** 709-714

50. Hazav, P., Shany, S., Moran, A., and Levy, R. (1989) Involvement of intracellular pH elevation in the effect of 1,25-dihydroxyvitamin D3 on HL60 cells. *Cancer Res.* **49,** 72-75

51. Vairo, G., and Hamilton, J. A. (1993) Evidence against a requirement for Na^+/H^+ exchange activity for proliferation or differentiation of HL 60 leukemic cells. *Cell Growth Diff.* **4,** 461-466

52. Rao, G. N., Sardet, C., Pouyssegur, J., and Berk, B. C. (1990) Differential regulation of Na^+/H^+ antiporter gene expression in vascular smooth muscle cells by hypertrophic and hyperplastic stimuli. *J. Biol. Chem.* **265,** 19393-19396

53. Elsing, C., Reichem, J., Marti, U., and Renner, E. L. (1994) Hepatocellular Na^+/H^+ exchange is activated at transcriptional and posttranslational levels in rat biliary cirrhosis. *Gastroenterology* **107,** 468-478

54. Dyck, J. R. B., Maddaford, T., Pierce, G. N., and Fliegel, L. (1995) Induction of expression of the sodium-hydrogen exchanger in rat myocardium. *Cardiovasc. Research* **29,** 203-208

55. Kolyada, A. Y., Lebedeva, T. V., Johns, C. A., and Madias, N. E. (1994) Proximal regulatory elements and nuclear activities required for transcription of the human Na/H exchanger (NHE-1) gene. *Biochim. Biophys. Acta.* **1217,** 54-64

56. Miller, R. T., Counillon, L., Pages, G., Lifton, R. P., Sardet, C., and Pouyssegur, J. (1991) Structure of the 5'-flanking regulatory region and gene for the human growth factor-activatable Na/H exchanger NHE-1. *J. Biol. Chem.* **266,** 10813-10819

57. Moe, O. W., Miller, R. T., Horie, S., Cano, A., Preisig, P. A., and Alpern, R. J. (1991) Differential regulation of Na/H antiporter by acid in renal epithelial cells and fibroblasts. *J. Clin. Invest.* **88,** 1703-1708

58. Horie, S., Moe, O., Tejedor, A., and Alpern, R. J. (1990) Preincubation in acid medium increases Na/H antiporter activity in cultured renal proximal tubule cells. *Proc. Natl. Acad. Sci. USA* **87,** 4742-4745

59. Horie, S., Moe, O., Yamaji, Y., Cano, A., Miller, R. T., and Alpern, R. J. (1992) Role of protein kinase C and transcription factor AP-1 in the acid-induced increase in Na/H antiporter activity. *Proc. Natl. Acad. Sci. USA* **89,** 5236-5240

60. Rao, G. N., de Roux, N., Sardet, C., Pouyssegur, J., and Berk, B. C. (1991) Na^+/H^+ antiporter gene expression during monocytic differentiation of HL60 cells. *J. Biol. Chem.* **266,** 13485-13488

61. Dyck, J. R. B., Silva, N. L. C. L., and Fliegel, L. (1995) Activation of the Na^+/H^+ exchanger gene by the transcription factor AP-2. *J. Biol. Chem.* **270,** 1375-1381

62. Blaurock, M. C., Reboucas, N. A., Kusnezov, J. L., and Igarashi, P. (1995) Phylogenetically conserved sequences in the promoter of the rabbit sodium-hydrogen exchanger isoform 1 gene (NHE1/SLC9A1). *Biochim. Biophys. Acta* **1262,** 159-163

CHAPTER TWO

Regulation of the NHE-1 Promoter in the Mammalian Myocardium

This version is in press in the Am. J. Physiol.

Weidong Yang, Jason R.B. Dyck, Huayan Wang and Larry Fliegel

Introduction

The amiloride sensitive Na^+/H^+ exchanger (NHE-1) is a ubiquitous transmembrane transporter involved in pH regulation. The protein mediates the exchange of one intracellular H^+ for one extracellular Na^+ when decreases in intracellular pH occur (11). Recently, more than one form of the Na^+/H^+ exchanger has been shown to exist. In the myocardium however, the predominant form is the NHE-1 isoform (6, 23). The presence of this antiporter and its role in regulation of intracellular pH is of special importance to the functioning myocardium. For example, during ischemia, normal resting intracellular pH drops dramatically thereby causing depression of myocardial contractility. The lowered pHi depresses contractility by acting on a number of steps of excitation-contraction coupling (8). Because the myocardial Na^+/H^+ exchanger is activated over a low pH range it is likely to be involved in pH regulation during an acute acid challenge to the heart.

Regulation of Na^+/H^+ exchanger transcription has not been well studied. In tissues such as the kidney, there are adaptive mechanisms by which cells can upregulate NHE-1 in response to chronic acid load. In intact animals and in renal cell lines, both NHE-1 Vmax and mRNA levels have been shown to increase due to chronic metabolic acidosis (1, 5, 16, 19, 22). When a mouse renal cortical tubule cell line (MCT) was treated with acidic medium, an increase in both NHE activity and NHE-1 mRNA level was also observed (21). In primary cultures of isolated myocytes it has been shown that chronic low external pH treatment allows for a more rapid recovery from an acute acid load (9). Also, low-flow myocardial ischemia causes an increase in steady state NHE-1 message levels, possibly as a result of decreased intracellular pH (9). In addition it causes an increase in the amount of a smaller isoform of the NHE-1 message (5). Acidosis may act as a specific mechanism to increase NHE-1 message and protein levels through both increased transcription and decreased mRNA degradation. Other factors that affect regulation of transcription in the myocardium are not well known. Serum is one such factor that has been suggested to act on either regulation of activity or expression of the Na^+/H^+ antiporter in cultured myocardial cells (10).

The purpose of this study was to examine regulation of expression of the Na^+/H^+ antiporter in primary cultures of isolated myocytes and in MCT cell as well. Specifically we examined factors that affect activity of the exchanger and are suspected of affecting regulation of transcription in the myocardium. These factors included acidosis and serum. In addition, we examined basic regulatory factors involved in regulation of transcription of

the Na^+/H^+ exchanger gene in the myocardium. Our results represent the first examination of how the NHE-1 promoter is regulated in the mammalian myocardium.

Material and Methods

Materials

Restriction endonuclease and DNA-modifying enzymes were obtained from Boehringer Mannheim (Laval, Quebec, Canada) and Bethesda Research Laboratories (Gaithersburg, MD, USA). The pBluescript plasmids used for subcloning were from Stratagene (LaJolla, CA, USA). Plasmids pXP-1 and pSV-hGH were gifts from Dr. M. Nemer of the Institute de Recherches Cliniques de Montreal, Montreal, Quebec, Canada. All other chemicals not listed were of analytical grade or molecular biology grade and were purchased from Fisher Scientific (Ottawa, Ont., Canada), Sigma (St. Louis, MO, USA) or BDH (Toronto, Ont., Canada). MCT cells were a generous gift of Dr. R. Alpern (University of Texas Southwestern Medical Center, Dallas, Texas) and were cultured as described earlier (14)

Reporter Plasmid Constructs

pXP-1MP was constructed as described earlier (4). Briefly, a 2.2 kb fragment containing the mouse NHE-1 promoter/exchanger region was cloned from a genomic DNA library and digested with Pst 1 and Sma 1. An intermediate plasmid which contained a Pst 1 site flanked by a Hind III and Sal 1 was used to transfer the Pst-Sma 1.1 kb fragment into pXP-1 directionally. pXP-1 was digested with Hind III and Sal I and the 1.1. kb fragment contains base pairs -1085 to +22 of the NHE-1 promoter. To construct pMP+AP2 plasmid, two oligonucleotides were used as primers for polymerase chain reaction (# 1, ttgg atc CGT GAC ACT TCC TTC CCT and #3, cc ttc gaa GGG TCC CGC GGT AGC GGA) were synthesized to amplify base pairs -125 to +22 of the gene. The PCR product had the restriction enzyme sites Bam HI and Sma 1 generated on either end. This product was digested with Bam HI and Sma 1 and inserted directionally into pXP-1. Similarly, pMP-AP2 plasmid was made using the product of the primers # 3 and #2 (#2, ttgg atc CTG CAC CGC GCG GGC GCT) and the 114 bp product (-92 to +22) was inserted into pXP-1. The mutations in pMP(MUT)AP2 were generated by PCR, using a mismatched oligonucleotide (#1-mut, ttgg atc CGT GAC ACT TCC TTT TTT AAG TAA TAT AAG CCG CTG CAC CG) similar to oligonucleotide #1 above. Mutations in

pMP(MUTb)AP2 were also constructed in a similar fashion using a mismatched oligonucleotide (#3b-mut, ttgg atc CGT GAC ACT TCC TTT TTT AAG CGA CAG AAG CCG CTG C). All plasmids were sequenced to verify proper orientation and fidelity of PCR.

Primary Cultures of Isolated Myocytes

Primary cardiomyocyte cultures were prepared from neonatal rats essentially as we have described earlier (6). Briefly, hearts were removed from 6-day old rats under aseptic conditions and ventricles minced to small size. The tissue was digested with a series of treatments with 0.1% collagenase (Worthington, Biochemical Corp.). To remove nonmyocardial cells selectively a differential attachment procedure was used. Dissociated cells were incubated in Corning T-75 culture flasks at 37°C in a humidified atmosphere (5% CO_2, 95% air) for a 20 minute period. During this time noncardiomyocytes (fibroblasts, endothelial cells, and smooth muscle cells) attach and the majority of the myocytes remain in suspension. Subsequently cardiomyocytes were removed and plated at 1 X 10^5 cells/cm^2. They were maintained in medium containing DMEM/F12 supplemented with 10 µg/ml transferrin, 10 µg/ml insulin, 10 ng/ml selenium, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mg/ml BSA, 5 µg/ml linoleic acid, 3 mM pyruvic acid, 0.1 mM MEM-nonessential amino acids, 10% MEM vitamins, 30 mM HEPES pH 7.4 and with or without 10% fetal bovine sera. In some experiments myocytes were maintained in the same medium at pH 6.9 for 18 hours. Examination of neonatal myocytes kept in culture for up to 2 weeks by light microscopy or immunostaining revealed cultures essentially free of nonmyocardial cells. The myocardiocytes possessed myocyte like morphology and characteristic cross-striations. Actin immunocytochemistry was done by Dr. M. Opas, Dept. of Anatomy, U. of Toronto and myocytes showed the characteristic myocyte like structure.

Transfection and Reporter Assays

Cells were plated onto 35 mm dishes at a density of 1 X 10^5 cells/cm^2. Each dish received 2.0 µg of luciferase reporter plasmid and 2.0 µg of pSV-ß-Galactosidase plasmid or pSV-hGH as an internal control. Myocytes were transiently transfected using the $Ca_3(PO_4)_2$ precipitation technique (2, 7). After transfection, cells were allowed to incubate at 37°C for five hours before being washed with fresh media and left for 36 hr. After 36 hours cells were harvested and the cell lysate was assayed for both luciferase

activity and the ß-Galactosidase activity. The medium was aspirated and the cells washed in 1x PBS-1mM EDTA. The cells were then allowed to incubate in 1 ml of PBS-1mM EDTA for 15 minutes. Cells were then scraped, transferred to a microcentrifuge tube, and pelleted at 10,000 rpm for 30 seconds. The supernatant was removed and the pellet was resuspended in 50 µl of ice cold lysis buffer (Tris 1M pH 7.8, 10% NP40 and 1M DTT) for 15 minutes. The solution was then pelleted at 14,000 rpm for 10 minutes at 4°C. The supernatant was removed and assayed for luciferase and ß-Galactosidase activity. Each luciferase assay contained 30 µl of the cell lysate and 100 µl of the luciferase assay reagent (Tricine 20 mM, $MgCO_3$ 1.07 mM, $MgSO_4$ 2.67 mM, EDTA 0.1 mM, DTT 33.3 mM, luciferin 470 mM, ATP 530 mM, Coenzyme A 270 mM, and BSA 1 ng/ml). The ß-galactosidase assay included 5 µl of cell lysate, 95 µl H_2O and 20 µl of o-Nitrophenyl-ß-D-galactopyranoside, incubated at 37°C for 60 minutes. After 60 minutes, 60 µl of 1M Na_2CO_3 was added to stop the reaction and the optical density was measured at a wavelength of 420 nM. Luciferase activity was assayed with an Berthold Lumat LB 9501/16 luminometer and normalized to ß-Galactosidase for efficiency of transfection. Human growth hormone production from pSV-hGH was measured using a radioimmunoassay kit from Immunocorp. Results are the mean ± SE of 4-6 experiments. Where not shown SE was too small to be displayed. Statistical significance was determined with a Mann-Whitney U test.

DNA Binding Assays

Nuclear extracts were prepared from myocytes or whole hearts according to the procedure of Schreiber (24). The synthetic oligonucleotides of the sequence 5' TTC CTT CCC TGG GCG ACA GGG GCC 3' (MPAP2a) and 5' GGC CCC TGT CGC CCA GGG AAG GAA 3' (MPAP2b) were made corresponding to the mouse promoter AP-2 region. The oligonucleotides were end-labeled with [γ-^{32}P]-ATP using T4 polynucleotide kinase. They were heated to 95°C for 2 minutes and cooled to room temperature overnight for annealing. DNA binding reactions were carried out as follows: [^{32}P]-labeled oligonucleotides (30 000 CPM) were mixed with purified AP-2 (1.4 µg) or myocyte nuclear extract (5 µg) in a binding buffer (4% glycerol, 1.25 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris-HCl, pH 7.5 and 0.05 µg/ml poly dI:dC). The reaction was incubated for 10 minutes at room temperature and size fractionated on 4% polyacrylamide gels. Gels were dried and exposed to X-ray film for 16 hours. In some experiments nuclear extracts were treated to specifically remove AP-2 protein before gel mobility shift assays. Twenty µl of a 10% solution of Protein A was incubated with 4 µg

of AP-2 antibody (IgG, Santa Cruz Biotechnology Inc.) for 4 h at 4°C. After 4 hours the solution was washed 3 times (20mM Tris, pH 7.9, 400mM NaCl, 1mM EDTA, 1mM EGTA, 1mM DTT, and 1mM PMSF), mixed with 5 µl of heart nuclear extracts and incubated at 4°C for 16 hours. The mixture was then spun at 6500 rpm for 2 min. and the supernatants were collected. Control experiments contained equal amounts of irrelevant IgG.

RNA Isolation and Measurement of NHE-1 mRNA Levels by PCR

Total RNA was isolated from ventricular myocytes as described earlier (9). Poly (A^+) RNA was purified with a PolyAtract™ mRNA isolation kit as described by the manufacturer (Promega). For reverse transcriptase PCR Poly (A^+) RNA was isolated as described above and 2 µg was copied with reverse transcriptase from the Invitrogen cDNA cycle™ kit using the procedures described by the manufacturer.

We used a competitive reverse transcriptase PCR assay to measure NHE-1 mRNA levels. The assay is based on the use of a competitive substrate that competes with the reverse transcribed cDNA. The principle is the same as a competitive PCR assay that we have recently described (28). The assay uses competitive PCR to measure relative levels of NHE-1 in comparison to levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primers for the Na^+/H^+ exchanger were NHQ-1 (tacggtACCCTGCTCTTCTGCCTC) and NHQ-2 (gatgcaTGCGGATTCCTCCTCCTT). These primers were specific to NHE-1 and were used to amplify a 649 bp product from reverse transcribed cDNA. To a series of these amplification reactions an increasing amount of competitive template was added. Increasing amounts of competitor result in decreasing amounts of DNA product. The Na^+/H^+ exchanger competitive template was produced by using cDNA from rat mRNA. The primers NHQ-1 and NHQ-2 were used to amplify the 649 bp PCR product which was subcloned into the plasmid pTZ 19 producing the plasmid p16. An internal Eco 47III fragment was removed and the plasmid (Δp16) was religated with a resulting 496 bp insert.

To normalize for any differences in purity of the mRNA sample plus and differences in reverse transcription we used the internal standard of GAPDH. The GAPDH competitive template was produced using a similar procedure. The primers GA3P-1 (tatggatCCTTCATTGACCTCAAC) and GA3P-2 (aatctcgAGTTGTCATGGATGACC) were used to amplify a 396 bp product from rat cDNA. The 396 bp product was subcloned into pTZ 19 producing the plasmid pGA3. The competitive template was made using the

restriction enzyme Nco 1 that removed an internal 155 bp. The resulting plasmid was called ΔpGA3 and amplification of this plasmid with the primers produced a 241 bp product.

PCR reactions contained 1 µl of reverse transcribed cDNA in a total volume of 50 µl containing 50 mM HEPES pH 7.9, 50 mM KCl, 1.5 mM $MgCl_2$, 100 µM each dNTP, 2.5 U of Taq polymerase. Reactions contained 1 µCi of [α-^{32}P] dCTP (3,000 Ci/mmol). Amplification was for 30 cycles under the following conditions: 94°C for 45 sec, 60°C for 45 sec, and 72°C for 60 sec on a Coy TempCycler. Ten-µl portions were resolved on 9% polyacrylamide gels.

For quantification of relative amounts of Na^+/H^+ exchanger, PCR was done with decreasing amounts of competitor DNA. Products of reactions were resolved on 9% gels and the incorporated radioactivity quantified with a model BAS1000 phosphoimager (Fuji Photo Film Co., Ltd.) For illustrations the amount of radioactivity incorporated was shown using autoradiography. For quantification of relative amounts of message, the log of the ratio of CPM in competitor-derived products to target-derived products is plotted versus the log of the number of competitor template molecules. When the target-derived and competitor-derived products have the same number of incorporated counts, the target and competitor DNA templates were present at the same levels, corresponding to a logarithm equal to 0. After measurement the amounts of Na^+/H^+ exchanger were normalized using the amounts of GAPDH. GAPDH showed no consistent pattern of changes corresponding to any treatments of tissues. Because the target and competitor DNA were of different size they could amplify with slightly different efficiency. We therefore compared the kinetics of amplification of the two targets and found no appreciable differences (not shown). Although no differences in amplification were noted, for the present series of experiments we only compared the relative amounts of cDNA in samples and not absolute amounts to the amount of competitor.

Results

To characterize the expression of the Na^+/H^+ exchanger in isolated myocytes we made primary cultures of neonatal rat myocytes essentially as described earlier (6). Myocytes could be kept in culture for several weeks though transfection was 36 hours after preparation. Examination of both stained and unstained cells suggested that cultures were essentially 95% free of other contaminating cell types as described earlier (6).

We have recently isolated and sequenced the NHE-1 mouse promoter (4). Figure 1 shows the NHE-1 promoter in diagrammatic form with the essential features noted. The sequence was reported earlier (4). Five constructs of the promoter were used; pXP-1MP, pMP+AP2, pMP-AP2, pMP(MUT)AP2 and pMP(MUTb)AP2. The plasmid pXP-1MP, pMP+AP2 and pMP-AP2 contain bp's from -1085, -125, -92 to +22 of the NHE-1 promoter. The plasmids pMP(MUT)AP2 and pMP(MUT)AP2 contain several mutations in the AP-2 containing region (**Fig. 1-1**).

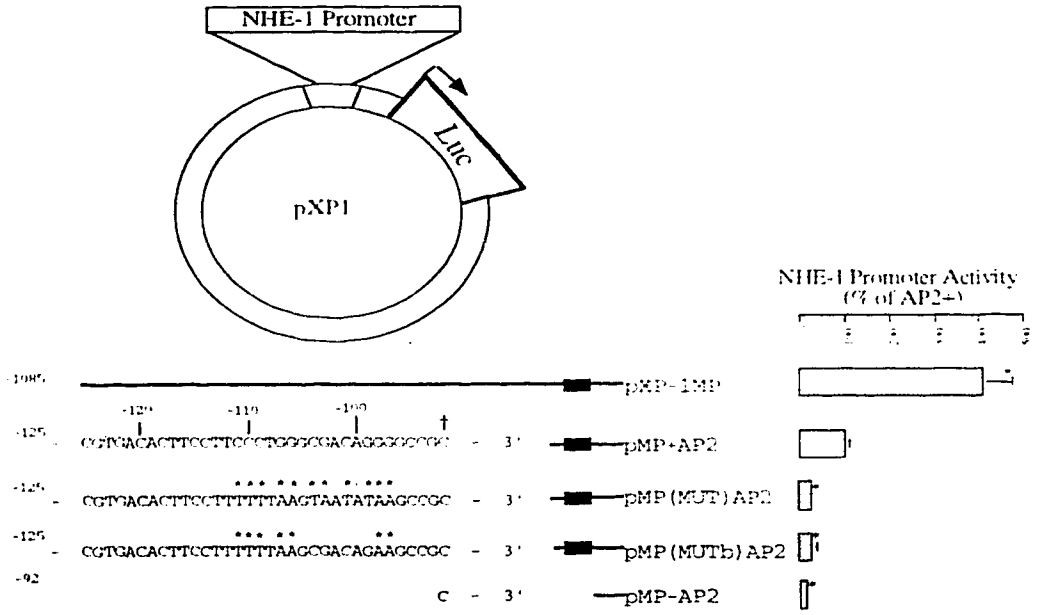

Fig. 1-1. Schematic diagram of constructs used for transfection of neonatal rat cardiomyocytes and relative luciferase activities. pXP-1MP contains the complete insert from -1085 to +22 of the mouse promoter/enhancer region. pMP+AP2 contains the region between -125 and +22 and pMP-AP2 contains the region between -92 and +22. The AP-2 containing sequence is shown. pMP(MUT)AP2 is the same as pMP+AP2 with the nucleotide mutations shown and denoted by the asterisk *. pMP(MUTb)AP2 is a partial mutation of pMP+AP2 with the mutations denoted by the asterisks *. The labeled sequence indicates the region containing the putative AP-2 site is deleted in pMP-AP2. The location of the beginning of the sequence of pMP-AP2 is denoted by †. All plasmids, pXP-1MP, pMP+AP2, pMP-AP2, pMP(MUT)AP2 and pMP(MUTb)AP2 were cotransfected with pSV-ß-Galactosidase as described in the "Materials and Methods". Luciferase values are relative to that of the transfection with pMP+AP2. * $P < 0.01$ vs. values for pMP+AP2.

To examine which regions of the NHE-1 promoter are important in activity of the promoter in isolated myocytes, neonatal rat myocytes were transfected with the various constructs of the promoter. The results are shown in **Fig. 1-1**. Transfection with the plasmid pXP-1MP resulted in the highest level of luciferase activity. Reduction of the NHE-1 containing region up to the AP-2 containing region reduced the activity 75%. A

further reduction in the size of the promoter to remove the AP-2 containing site resulted in almost complete reduction in activity of the promoter. Similarly, mutation of several of the residues of this region also resulted in a dramatic loss of luciferase activity produced from this construct eliminating over 70% of the activity remaining in the AP2+ construct (**Fig. 1-1**).

To discern whether the transcription factor AP-2 could be important in regulation of the NHE-1 promoter in the myocardium we used gel mobility shift experiments. The results are shown in **Fig. 1-2.** At least one protein from nuclear extracts of the intact rat myocardium bound to a synthetic oligonucleotide containing the AP-2 consensus sequence (**Fig. 1-2a** lane 6). Protein from HeLa cell nuclear extracts also bound to this oligonucleotide (lane 2). In addition, purified AP-2 protein bound to the synthetic oligonucleotide (lane 7). Because the intact myocardium contains a number of cell types we prepared nuclear extracts from neonatal myocyte cultures that were essentially free of other cells. Nuclear extracts from these cells also contained at least one protein that was able to bind to the synthetic oligonucleotide containing the AP-2 binding sequence (**Fig. 1-2b**).

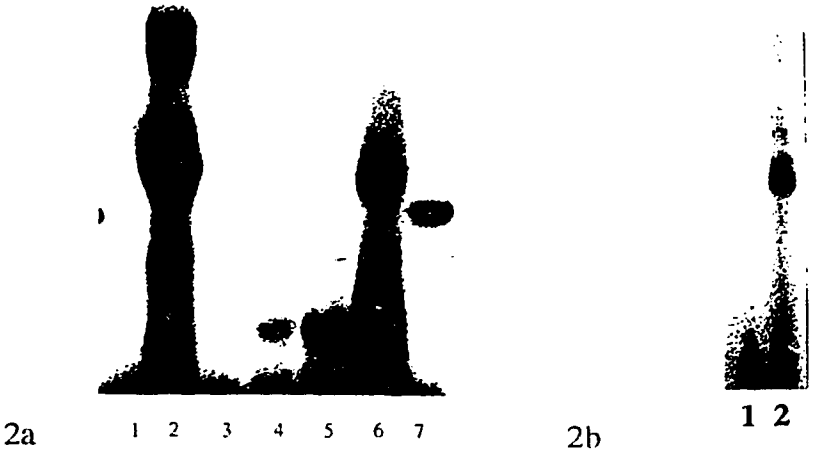

Fig. 1-2. DNA-mobility shift binding assay of the AP-2 containing site. Nuclear extracts were prepared from tissues or cells as described in "Experimental Procedures" and used for mobility shift binding assay with MPAP2a,b. The labeled oligonucleotide MPAP2a,b containing the AP-2 consensus sequence (positions -117 to -94) was incubated with purified human AP-2 protein or nuclear extracts for 10 minutes at room temperature. The binding mixtures were analyzed by electrophoresis on 4% polyacrylamide gels. **Fig. 1-2a,** lane 1, oligonucleotide alone; lane 2, nuclear extract of HeLa cells (5 µg), lanes 3-6 nuclear extract from intact rat myocardium 0.8, 1.6, 3.2 and 5.6 µg respectively. The reaction mixture of lane 7 contains 1.4 µg of purified AP-2 protein. **Fig. 1-2b,** Gel mobility shift binding assay using 5 µg of nuclear extracts from isolated rat myocytes. Lane 1 = control, lane 2 contained 5 µg of nuclear extracts from isolated rat myocytes in addition to the synthetic oligonucleotides.

To confirm that the binding of the myocardial nuclear extract was specific we used competition experiments. The results are shown in **Fig. 1-3a.** Increasing amounts of cold competitor DNA reduced the amount of binding by the myocardial nuclear extract. Non-competitor synthetic oligonucleotide did not reduce the binding to AP-2 binding sequence. To confirm that the protein involved was AP-2 we used DNA mobility shift binding of nuclear extracts that were depleted of AP-2 by immunoprecipitation with anti-AP-2 antibody. Lane 1 (**Fig. 1-3b**) shows control nuclear extracts extract with 4 μg of irrelevant IgG. Lane 2 shows the greatly reduced mobility shift of nuclear extracts that were extracted with anti-AP-2 antibody. These results show a direct involvement of the AP-2 transcription factor in the mobility shift.

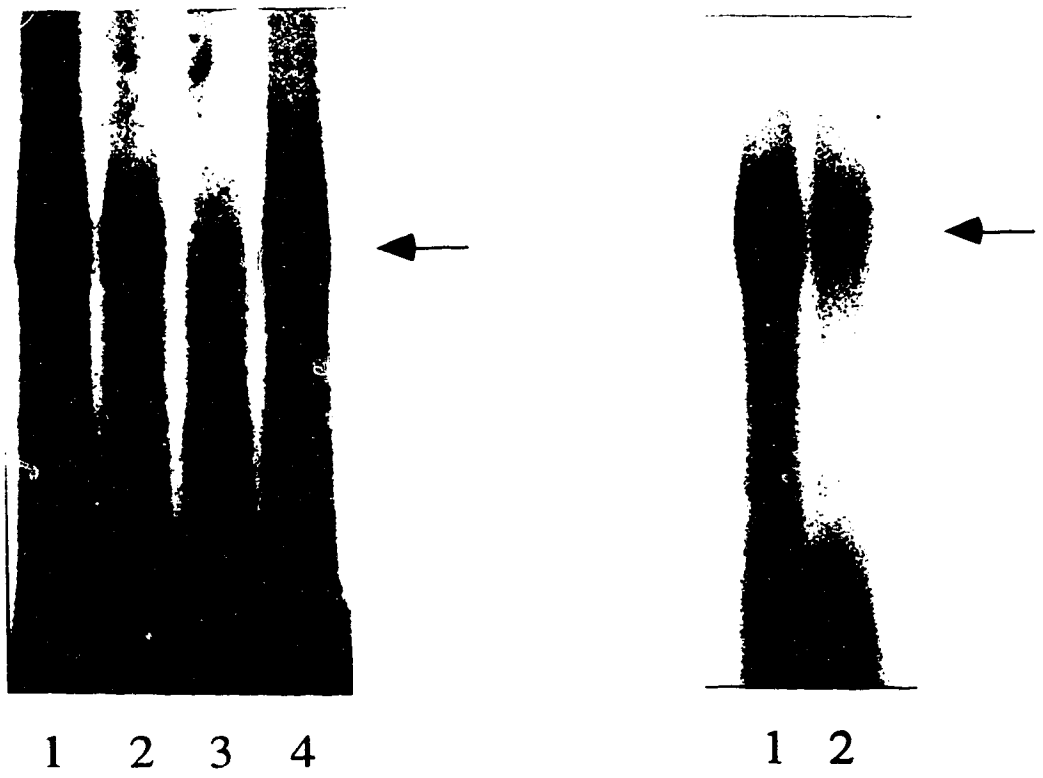

Fig. 1-3. DNA-mobility shift binding assay of the AP-2 containing site with rat heart nuclear extracts. Nuclear extracts and mobility shifts were prepared as described for **Fig. 1-2. Fig. 1-3a,** competition with unlabeled MPAP2ab. Lane 1 labeled MPAP2ab. Lanes 2 and 3 competition with 10 and 100-fold excess of unlabeled MPAP2ab. Lane 4, competition with 100-fold excess of a nonspecific competitor SP-1. **Fig. 1-3b** DNA mobility shift binding assay with cardiac nuclear extracts that were extracted via immunoprecipitation with 4 μg of irrelevant IgG (lane 1) or with 4 μg of anti-AP-2 antibody (lane 2).

Using the plasmid pXP-1MP we examined the regulation of expression of the NHE-1 promoter. The effect of external pH on activity of the promoter in primary cultures of isolated myocytes and in MCT cells is shown in **Fig. 1-4.** There was no difference between the treated and untreated cells. The results show that reduction of the pH of the external media does not increase activity of the NHE-1 promoter.

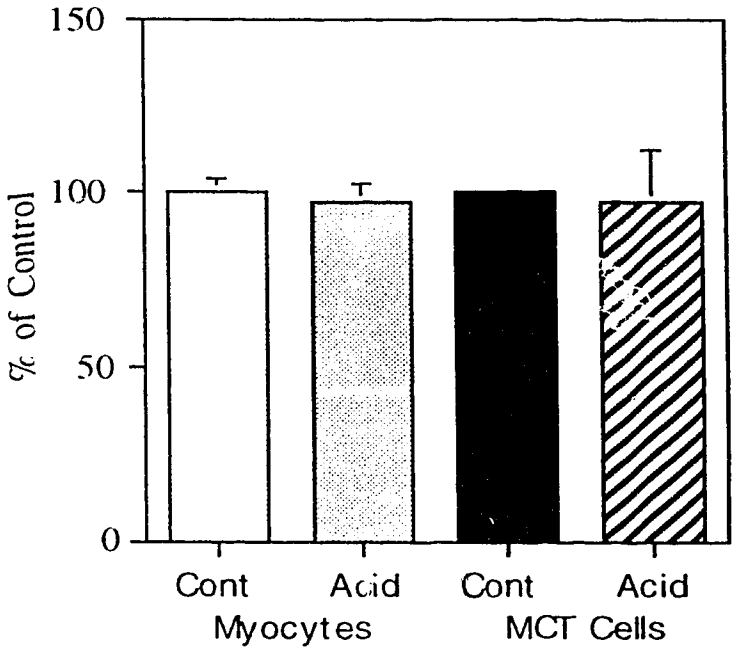

Fig. 1-4. Effect of low external pH on the activity of the NHE-1 promoter in primary cultures of neonatal rat myocytes and in MCT cells. Cardiomyocytes and MCT cells were transfected with pXP-1MP and pSV-ß-Galactosidase as described in the "Material and Methods". Luciferase values are corrected for efficiency of transfection and are normalized to the values of controls. Experimental cells were treated with low external pH as described in "Materials and Methods".

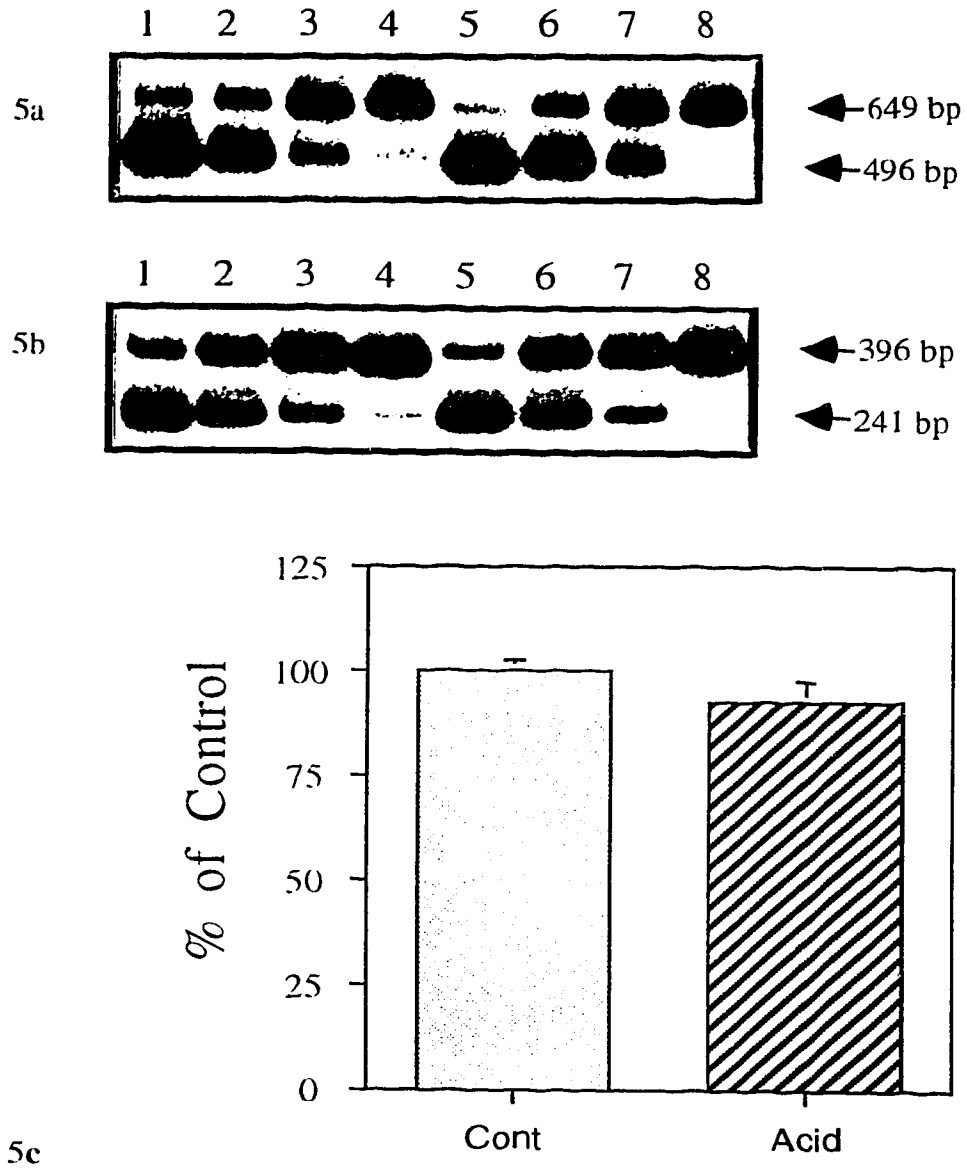

Fig. 1-5. Effect of low external pH on the relative amounts of NHE-1 mRNA in primary cultures of neonatal rat myocytes. Cardiomyocytes were prepared and mRNA measured by competitive PCR as described in the "Material and Methods". **Fig. 1-5a, 1-5b,** determination of NHE-1 and GAPDH levels of control and acidosis treated cardiomyocytes. Competitive PCR was prepared and samples were separated by electrophoresis on 9% acrylamide gels. Autoradiography was used for the purpose of illustration of typical results. **Fig 1-5a** illustrates the determination of mRNA levels of NHE-1. Lanes 1-4 are from pH 6.9 treated myocytes, and lanes 5-8 are from control (pH 7.4) myocytes. The amount of competitor was 46, 4.6, 0.46, 0.046, 46, 4.6, 0.46, 0.046, amol in lanes 1-8, respectively. **Fig. 1-5b** shows the determination of the levels of GAPDH. Lanes 1-4 are from pH 6.9 treated myocytes, and lanes 5-8 are from control (pH 7.4) myocytes. The amount of competitor was 4790, 479, 47.9, 4.79, 4790, 479, 47.9, 4.79, amol in lanes 1-8, respectively. **Fig. 1-5c**, summary of 4 independent

determinations of relative NHE-1 mRNA levels of control and acidosis treated cardiomyocytes.

To determine whether acidosis could cause an elevation of myocyte mRNA levels we used a competitive reverse transcription PCR assay. The assay is essentially similar to one that we have described earlier (28). **Fig. 1-5a** and **1-5b** demonstrate the effectiveness of the assay. With decreasing amounts of NHE-1 competitor the amount of 496 bp PCR product declined. Also, as the amount of competitor decreased the amount of 649 bp PCR product from the NHE-1 cDNA increased. Lanes 1-4 and lanes 5-8 (**Fig. 1-5a**) show the results of control and acidosis treated myocytes respectively. A similar PCR assay was used to measure GAPDH levels (**Fig. 1-5b**). Similarly, with decreasing amounts of competitor the amount of 241 bp PCR product decreased. In addition, the amount of GAPDH (396 bp) PCR product increased. Lanes 1-4 and lanes 5-8 (**Fig. 1-5b**) show the results of control and acidosis treated myocytes respectively. The summary of the corrected results of 4 independent determinations is shown in **Fig. 1-5c**. There was no significant difference in the mRNA levels between control and acidosis treated myocytes.

We also examined the effect of the presence or absence of sera on the activity of the NHE-1 promoter in isolated myocytes. Cells were transfected with pXP-1MP, pMP+AP2, pMP-AP2 and pMP(MUTb)AP2 constructs as described above. Sera (10%) was then introduced to the experimental groups immediately after transfection and cells were harvested after 36 hours. The results are shown in **Fig. 1-6.** When compared to controls, cells treated with serum showed an increase in NHE-1 promoter activity for all constructs. As expected the largest activity of the promoter was with the pXP-1MP construct. The largest effect of serum was also shown with the full sized promoter. A similar result was shown with pMP+AP2. The addition of sera resulted in a substantial increase in activity of the promoter in isolated myocytes. However, the absolute level of the increase could not account for the increase induced by sera with the pXP-1MP transfections. Thus the effects of serum must include elements found in the pXP-1MP construct in addition to elements in the pMP+AP2 plasmid. In the case of both pMP-AP2 and pMP(MUTb)AP2, serum also resulted in small increases in activity of the promoter. However the changes were small compared with those increases seen with the other constructs.

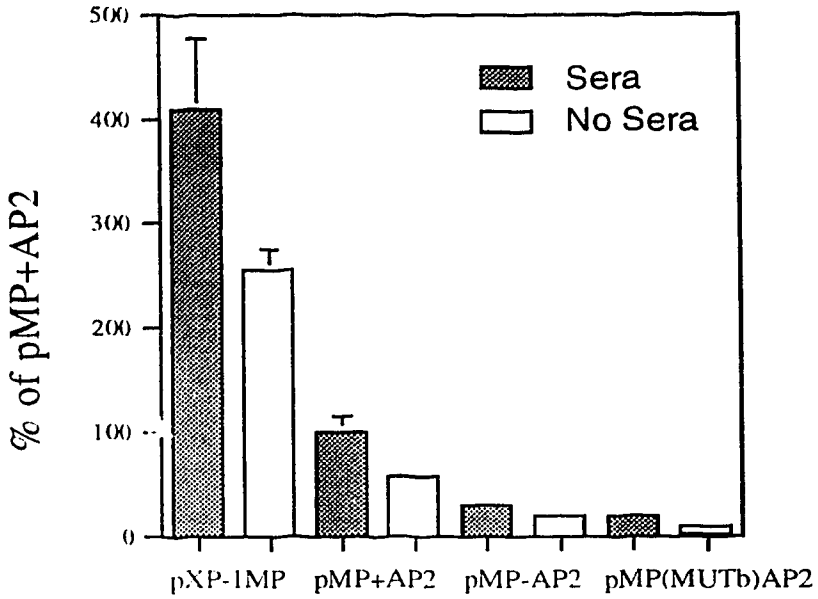

Fig. 1-6. Effect of the presence of 10% sera on the activity of the NHE-1 promoter in primary cultures of neonatal rat myocytes. Cardiomyocytes were prepared as described for **Fig. 1-4.** The plasmids pXP-1MP, pMP+AP2, pMP-AP2 and pMP(MUTb)AP2 were used for transfection as described in **Fig. 1-1.** One day after transfection was complete the cells were incubated in media in the presence or absence of 10% fetal calf sera for 18 hours. Results are expressed as percentage of activity of pMP+AP2 in the presence of sera. Where not shown SE were too small to be displayed.

Discussion

The Na^+/H^+ exchanger is responsible for removal of intracellular protons from the myocardium. Several studies have shown that there is increased expression of the message and increased activity of the protein in response to acidosis (1, 16, 17, 19, 22). We have earlier shown (9) that treatment of primary cultures of isolated myocytes with low external pH results in increased ability of these cells to recover from an acute acid load. The mechanism by which this occurs is not yet clear but in some tissue types it has been suggested to involve protein kinase C and possibly the transcription factor AP-1 (12, 14). Regulation of expression of the NHE-1 has not been studied in detail but the human and the mouse NHE-1 gene have been isolated (4, 20). The transcription factors AP-1 and AP-2 have been implicated in regulation of the gene in fibroblasts (4, 20). More recently some more distal elements have been suggested to be involved in regulating transcription including CREB, AP3, NFY and C/EBP proteins (18). Regulation of expression and activity of the Na^+/H^+ exchanger is known to vary between cell types (21, 26). There has

however, been no examination of the regulation of the NHE-1 promoter in the myocardium. We undertook this study to examine factors controlling the regulation of expression in the mammalian myocardium. Specifically we examined the various regions of the promoter that are important in promoter activity of the gene and several factors thought to be important in NHE-1 expression.

We have recently shown that acidosis induces increased ability of isolated myocytes to recover from an acid load. In addition, ischemia has been shown to induce increases in NHE-1 message levels in the intact isolated perfused heart (9). To study possible causes of these increases we examined promoter activity in isolated myocytes and utilized reporter plasmid transfections. External acidosis did not increase activity of the NHE-1 promoter. **Fig. 1-4** shows that the activity of the NHE-1 promoter construct pXP-1MP was not increased in either isolated myocytes or MCT cells. MCT cells treated with low external pH have been shown to have increased NHE-1 message and activity after treatment with medium of low external pH (13, 21). However, this treatment did not result in activation of the NHE-1 promoter in either cell type. There are several possible explanations for this apparent inconsistency. In the MCT cells increased activity and message of NHE-1 could be due to increased RNA stability. RNA stability has been shown to be a mechanism by which acidosis can cause mRNA levels to rise and thereby result in increased protein and activity. This occurs in the case of phosphoenolpyruvate carboxykinase where both increased transcription and increased RNA stability occur in response to acidosis (15). However, in isolated myocytes our results have shown that this treatment does not increase the level of NHE-1 message (**Fig. 1-5**). This suggests that there must be a change in either regulation of Na^+/H^+ exchanger activity or recruitment of intracellular stores of protein. It has been suggested that acidosis can cause a mobilization of intracellular stores of the Na^+/H^+ exchanger (25). An alternative explanation is that some of the increased rate of recovery we observed earlier is due to activity of other related proteins or other isoforms of the antiporter. Future experiments are necessary to determine if this is the case.

We examined which elements of the NHE-1 promoter are important in basal expression in isolated myocytes. Previous experiments had suggested that the most proximal 130 bp from the first start site of transcription are important in expression in fibroblast cells (4). In addition, comparison of the mouse Na/H exchanger gene (4) with the human gene (20) suggests that this is the only major region of the promoter that is highly conserved between species (not shown). We thus deleted the promoter up to the region that contained the AP-2 site. The results showed that deletion of these more distal

regions of the gene resulted in a 75% loss of activity of the gene. Contained within the deleted region there are putative sites for binding of the transcription factors HGRE, CBF, and several CCAAT box binding proteins including CREB and NFY. It has been suggested that some of these elements are important in other cell types (18). This confirms that some of these elements could also be responsible for regulation of expression in the myocardium, despite the fact that regulation of the protein varies from one cell type to another (21, 27).

We examined the most proximal site likely involved in the regulation of expression of the protein. Our earlier studies have suggested that this site may be important in other tissues (4). We tested the effect of removal or mutation of the site that contained an AP-2 consensus sequence. Removal of the AP-2 containing site eliminated activity of the promoter (**Fig. 1-1**). To prove that the effect was due to omission of the site and not simple shortening of the promoter we mutated several of the residues of pMP+AP2 to form a plasmid pMP(MUT)AP2, that is of the same length as pMP+AP2. This plasmid also directed greatly reduced luciferase activity, comparable to that of pMP-AP2. We tested the effect of a more conservative mutation of the AP2 site. The results with this construct (pMP(MUTb)AP2) showed a reduction of activity of the NHE-1 promoter that was equivalent to that of pMP(MUT)AP2. These results strongly suggest that the transcription factor AP-2 plays an important role in directing expression of the Na^+/H^+ exchanger gene in the myocardium. To confirm the involvement of this transcription factor we constructed a synthetic oligonucleotide that contained the AP-2 sequence. Gel mobility shift assay (**Fig. 1-2**) suggested that nuclear extracts from hearts contained AP-2 protein or an AP-2 like protein that bound to the synthetic oligonucleotide with this sequence. A protein from nuclear extract of isolated myocytes also bound to the same sequence. This confirmed that this transcription factor or a closely related one is present in cardiomyocytes (**Fig. 1-2b**). An interesting feature of the results was that purified AP-2 protein bound to the same synthetic oligonucleotide but produced a smaller shift than seen with protein bound from nuclear extracts. The reason for this difference in mobility is not known. It could be that the endogenous AP-2 or "AP-2 like" protein in nuclear extracts is phosphorylated or glycosylated to a different degree in comparison to the purified protein. Alternatively, a second or a different protein could be associated with this region of the gene.

To confirm that the binding of the transcription factor AP-2 was specific we used competition analysis. Unlabelled synthetic oligonucleotide effectively reduced the binding while non-competitor did not reduce the mobility shift (**Fig. 1-3a**). We also used

immunoprecipitation with an anti-AP-2 antibody to specifically reduce the amount of AP-2 protein present in nuclear extracts from the heart. This greatly reduce the mobility shift **(Fig. 1-3b)** and confirmed the involvement of the AP-2 protein in binding to the synthetic oligonucleotide.

We also examined the contribution of sera to the basal activity of the NHE-1 promoter. It has been shown earlier that culture conditions such as the presence or absence of serum affect the activity of the Na^+/H^+ antiporter in the myocardium (10). Serum and various mitogens are also known to cause short term activation of the Na^+/H^+ exchanger in various tissues (26, 27). However, long-term regulation of the gene has not been well studied. We noted that the promoter contains whole or partial consensus sequences for serum response elements and for a number of transcription factors activated by serum including AP-1, AP-2, Pea3 and several others. We tested the effect of serum on the level of NHE-1 expression and found that it did stimulate transcription from the promoter **(Fig. 1-6)**. This occurred with both the 1.1. kb fragment of the promoter and the AP-2 containing construct. This demonstrates for the first time, that the level of NHE-1 transcription is responsive to external stimuli in this cell type. Which of the transcription factors in the sequence is responsible for the increase in transcription is not known. It is clear however, that one or more elements of serum stimulates activity of the NHE-1 promoter in the myocardium. Also, the AP-2 containing construct was responsive to stimulation by serum suggesting that regulation by this or a related transcription factor is important in NHE-1 expression. Other plasmids with the AP-2 site deleted or mutated, also showed slight activation by serum. However, the amount of activity of the promoter was small relative to that of the 1.1 kb and AP-2 containing fragments of the promoter.

Conclusion

Overall, our results represent the first examination of regulation of NHE-1 gene expression in the isolated myocardial cell. We have shown that the proximal 130 bp are important in basal expression and that more distal elements of the gene also play an important role in regulation of expression. There was no effect of external acidosis on transcription of the NHE-1 promoter and no effect on mRNA levels. In contrast serum was able to stimulate expression of the NHE-1 promoter in the myocardial cell. The exact transcription factors that are involved and their specific binding sites in the gene will be the subject of future experiments.

References

1. Akiba, T., V. K. Rocco, and D. G. Warnock. (1987) Parallel adaptation of the rabbit renal cortical sodium/proton antiporter and sodium/bicarbonate cotransporter in metabolic acidosis and alkalosis. *J. Clin. Invest.* **80,** 308-315.

2. Argentin, S., Y. L. Sun, I. Lihrmann, T. J. Schmidt, J. Drouin, and M. Nemer. (1991) Distal cis-acting promoter sequences mediate glucocorticoid stimulation of cardiac atrial natriuretic factor gene transcription. *J. Biol. Chem.* **266,** 23315-23322.

3. Chaillte, J. R., and W. F. Boron. (1985) Intracellular calibration of a pH-sensitive dye in isolated perfused salamander proximal tubules. *J. Gen. Physiol.* **86,** 765-795.

4. Dyck, J. R. B., and L. Fliegel. (1995) Activation of the Na^+/H^+ exchanger promoter by the transcription factor AP-2. *J. Biol. Chem.* **269,** 30734-30739,

5. Dyck, J. R. B., G. D. Lopaschuk, and L. Fliegel. (1992) Identification of a small Na^+/H^+ exchanger-like message in the rabbit myocardium. *FEBS Lett.* **310,** 255-259.

6. Fliegel, L., J. R. B. Dyck, H. Wang, C. Fong, and R. S. Haworth. (1993) Complete nucleotide sequence of the human myocardial Na^+/H^+ exchanger. *Mol. Cell. Biochem.* **125,** 137-143.

7. Fliegel, L., and J. R. B. Dyck. (1994) Molecular biology of the cardiac Na^+/H^+ exchanger. *Cardiovasc. Research* **29,** 155-159.

8. Fliegel, L., and O. Frohlich. (1993) The Na^+/H^+ exchanger: An update on structure, regulation and cardiac physiology. *Biochem. J.* **296,** 273-285.

9. Fliegel, L., T. G. Maddaford, G. Pierce, and J. R. B. Dyck. (1994) Induction of expression of the Na^+/H^+ exchanger in the rat myocardium. *Cardiovasc. Research* **29,** 203-208.

10. Green, R. D., Frelin C. Vigne P. and Lazdunski M. (1986) The activity of the Na/H antiporter in cultured cardiac cells is dependent on the culture conditions used. *FEBS Lett.* **196,** 163-166.

11. Grinstein, S., and A. Rothstein. (1986) Mechanisms of regulation of the Na/H exchanger. *J. Mem. Biol.* **90,** 1-12.

12. Horie, S., O. Moe, R. T. Miller, and R. J. Alpern. (1992) Long term activation of protein kinase C causes chronic Na/H antiporter stimulation in cultured proximal tubule cells. *J. Clin. Invest.* **89,** 365-372.

13. Horie, S., O. Moe, A. Tejedor, and R. J. Alpern. (1990) Preincubation in acid medium increases Na/H antiporter activity in cultured renal proximal tubule cells. *Proc. Natl. Acad. Sci. USA* **87,** 4742-4745.

14. Horie, S., O. Moe, Y. Yamaji, A. Cano, R. T. Miller, and R. J. Alpern. (1992) Role of protein kinase C and transcription factor AP-1 in the acid-induced increase in Na/H antiporter activity. *Proc. Natl. Acad. Sci. U.S.A* **89,** 5236-5240.

15. Hwang, J. J., and N. P. Curthoys. (1991) Effect of acute alterations in acid-base balance on rat renal glutaminase and phosphoenolpyruvate carboxykinase gene expression. *J. Biol. Chem.* **266,** 9392-9396.

16. Igarashi, P., M. I. Freed, B. Ganz, and R. F. Reilly. (1992) Effects of chronic metabolic acidosis on Na-H exchangers in LLC-PK1 renal epithelial cells. *Am. J. Physiol.* **263,** F83-F88.

17. Kinsella, J. L., T. Cujdik, and B. Sacktor. (1984) Na^+-H^+ exchange in isolated renal brush-border membrane vesicles in response to metabolic acidosis. *J. Biol. Chem.* **259,** 13224-13227.

18. Kolyada, A. Y., T. V. Lebedeva, C. A. Johns, and N. E. Madias. (1994) Proximal regulatory elements and nuclear activities required for transcription of the human Na/H exchanger (NHE-1) gene. *Biochim. Biophys. Acta* **1217,** 54-64.

19. Krapf, R., D. Pearce, C. Lynch, X. P. Xi, T. L. Reudelhuber, J. Pouyssegur, and F. C. Rector Jr. (1991) Expression of rat renal Na/H antiporter mRNA levels in response to respiratory and metabolic acidosis. *J. Clin. Invest.* **87,** 747-751.

20. Miller, R. T., L. Counillon, G. Pages, R. P. Lifton, C. Sardet, and J. Pouyssegur. (1991) Structure of the 5'-flanking regulatory region and gene for the human growth factor-activatable Na/H exchanger NHE-1. *J. Biol. Chem.* **266,** 10813-10819.

21. Moe, O. W., R. T. Miller, S. Horie, A. Cano, P. A. Preisig, and R. J. Alpern. (1991) Differential regulation of Na/H antiporter by acid in renal epithelial cells and fibroblasts. *J. Clin. Invest* **88,** 1703-1708.

22. Moe, O. W., A. Tejedor, M. Levi, D. W. Seldin, P. A. Preisig, and R. J. Alpern. (1991) Dietary NaCl modulates Na(+)-H+ antiporter activity in renal cortical apical membrane vesicles. *Am. J. Physiol* **260,** F130-137.

23. Orlowski, J., R. A. Kandasamy, and G. E. Shull. (1992) Molecular cloning of putative members of the Na/H exchanger gene family. cDNA cloning, deduced amino acid sequence, and mRNA tissue expression of the rat Na/H exchanger NHE-1 and two structurally related proteins. *J. Biol. Chem.* **267,** 9331-9339.

24. Schreiber, E., P. Matthias, M.M. Muller, and W. Schaffner. (1989) Rapid detection of octamer binding proteins with miniextracts, prepared from a small number of cells. *Nucleic Acids Res.* **17,** 6419-6423.

25. Soleimani, M., G.L. Bizal, T.D. McKinney, and Y.J. Hattabaugh. (1992) Effect of in vitro metabolic acidosis on luminal Na^+/H^+ exchange and basolateral Na^+ - $O3^-$ cotransport in rabbit kidney proximal tubules. *J. Clin. Invest.* **90,** 211-218.

26. Takaichi, K., D. Wang, D. F. Balkovetz, and D. G. Warnock. (1992) Cloning, sequencing, and expression of Na-H antiporter cDNAs from human tissues. *Am. J. Physiol.* **262,** C1069-C1076.

27. Wakabayashi, S., C. Sardet, P. Fafournoux, L. Counillon, S. Meloche, G. Pages, and J. Pouyssegur. (1992) Structure function of the growth factor-activatable Na^+/H^+ exchanger (NHE1). *Rev. Physiol. Biochem. Pharmacol* **119**, 157-186.

28. Wang, H., L. Fliegel, C. E. Cass, A. Penn, M. Michalak, J. H. Weiner, and B. D. Lemire. (1994) Quantification of mitochondrial DNA in heteroplasmic skin fibroblast cells with competitive PCR. *Biotechniques* **17**, 76-82.

Regulation of NHE-1 Expression in L6 Muscle Cells

This version has been submitted to the Journal Biochim. Biophys. Acta

Weidong Yang, Jason R. B. Dyck and Larry Fliegel

Introduction

The Na^+/H^+ exchanger is a mammalian plasma membrane protein that exchanges intracellular protons for extracellular sodium. It is involved in pH regulation (1), control of cell volume and is stimulated by growth factors (2). Several isoforms of the protein have been identified (NHE-1 to NHE-5). The NHE-1 isoform is the most widely distributed type and is present in most, if not all, mammalian cells (3). Although the mechanism of regulation of protein levels is extremely important, few studies have examined the NHE-1 gene (4-7). The transcription factor AP-2 is one element that may be important in regulation of the NHE-1 gene (4).

Studies have shown that NHE-1 mRNA levels are increased due to a number of treatments including chronic acid loading and treatments causing cellular differentiation (8-10). For example during retinoic acid induced differentiation of human leukemic cells (HL-60), there is an 8.3-fold increase in NHE-1 transcription (8). More recently we have shown that there is a transient increase in the level of NHE-1 transcription during retinoic acid induced differentiation of P19 cells (4). This data provides evidence to suggest that Na^+/H^+ exchanger plays an important role in cellular differentiation. Increased antiporter activity during differentiation may be important for differentiation to occur, at least in some cell types (11, 12). However, this requirement has not been shown to occur universally and the role of the Na^+/H^+ exchanger may vary between cell types (13).

The Na^+/H^+ exchanger also plays an important role in regulation of intracellular pH in different types of muscle cells. This includes skeletal muscle (14), the myocardium (15) and smooth muscle (16). In skeletal muscle the regulation of the Na^+/H^+ antiporter has been shown to vary between proliferating myoblasts and differentiated myotubes (14). This suggests that there are important physiological changes in the protein and its function during the process of differentiation. However, to date there have been no studies on the regulation of the Na^+/H^+ exchanger gene during this process. The purpose of this study was to examine regulation of the NHE-1 gene during the process of muscle differentiation. Because of the important role of the protein in this and other muscle tissues we also examined basal regulation of the promoter activity in this tissue. Our study investigated whether the NHE-1 gene is activated during the differentiation process in muscle cells, similar to what occurs in other cell types.

Materials and Methods

Materials

Restriction endonuclease and DNA-modifying enzymes were obtained from Boehringer Mannheim (Laval, Quebec, Canada) and Bethesda Research Laboratories (Gaithersburg, MD, USA). The pBluescript plasmids used for subcloning were from Stratagene (LaJolla, CA, USA). Plasmid pXP-1 was a gift from Dr. M. Nemer of the Institute de Recherches Cliniques de Montreal, Montreal, Quebec, Canada. All other chemicals not listed were of analytical grade or molecular biology grade and were purchased from Fisher Scientific (Ottawa, On, Canada), Sigma (St. Louis, MO, USA) or BDH (Toronto, Ontario, Canada).

Reporter Plasmid Constructs

A 9 kb genomic clone of the mouse Na^+/H^+ exchanger was isolated by screening of a mouse lambda Gem-11, genomic DNA library (Promega, Madison, Wisconsin, USA). The probes were a fragment of the rabbit cardiac Na^+/H^+ exchanger (NHE-1) cDNA clone (1-691 bp, 15) and a fragment of the 5' untranslated and coding region (1-900) of the human Na^+/H^+ exchanger as described earlier (17). The plasmid pXP-8.5MP was constructed by removing a 13 kb positive fragment of southern blot from lambda Gem-11. Excision was initially with Eco R I and Sst II and the resulting fragment was inserted into the corresponding sites of Bluescript KS-. This clone was then cut with Sst II producing a mouse fragment vector plus insert. This was blunt ended using Klenow and then was digested with Xho I which digested only the vector immediately adjacent to the 5' end of the insert. The resulting large fragment was subcloned into Xho I - Sma I digested pXP1. Digestion of the insert with Sst II resulted in removal of a 700 bp fragment which was downstream of the start site and in the 5' untranslated region.

From the plasmid pXP-8.5MP three other related plasmids were generated (**Fig. 1**). The plasmid pXP-5.3MP was made by removal of a 3.2 kb Bam HI - Bgl II fragment from the 5' end of pXP-8.5MP. The plasmid pXP-5.0MP was made by removal of a 3.5 kb Bam HI - Bam HI piece from the 5' end of pXP-8.5MP. The plasmid pXP-3.8MP was made by removal of a 4.7 kb Sst I - SstI fragment internal to the plasmid pXP-8.5MP.

pXP-1.1MP was constructed in the following way as described earlier (17). Briefly, a 2.2 kb fragment containing the mouse NHE-1 promoter/exchanger region was cloned from a genomic DNA library and digested with Pst 1 and Sma 1. An intermediate plasmid that contained a Pst 1 site flanked by a Hind III and Sal 1 was used to transfer the Pst-Sma 1.1 kb fragment into pXP-1 directionally. pXP-1 was digested with Hind III and Sal I and the 1.1. kb fragment contains base pairs base pairs -1085 to +22 of the NHE-1 promoter. To construct pMP+AP2 plasmid, two oligonucleotides were used as primers for polymerase chain reaction (# 1, ttgg atc CGT GAC ACT TCC TTC CCT and #3, cc ttc gaa GGG TCC CGC GGT AGC GGA) were synthesized to amplify base pairs -125 to +22 of the gene. The PCR product had the restriction enzyme sites Bam HI and Sma 1 generated on either end. This product was digested with Bam HI and Sma 1 and inserted directionally into pXP-1. Similarly, pMP-AP2 plasmid was made using the product of the primers # 3 and #2 (#2, ttgg atc CTG CAC CGC GCG GGC GCT) and the 114 bp product (-92 to +22) was inserted into pXP-1. All plasmids were sequenced to verify proper orientation and fidelity of PCR.

The plasmid pXP-0.2MP was made by modification of pXP-1.1MP. The insert was excised with Sma I and Hind III and digested with Rsa I. This resulted in the production of a 0.2 kb Rsa I - Hind III fragment that was subcloned into the Sma I - Hind III site of pXP1 to form pXP-0.2MP. The plasmids pXP-0.9MP and pXP-0.5MP were generated using unidirectional exonuclease treatment of the 5' end of the insert of pXP-1.1MP.

Growth and Maintenance of Cell Lines

L6 cells were propagated in DMEM supplemented with 10% Fetal Bovine Serum. To examine the effects of differentiation on the activity of the promoter, cells were treated essentially as described earlier (18). Briefly, cells were split into 35 mm dishes and allowed to attach and grown in medium with 10% FBS. When the cells were approximately 80% confluent they were split into 2 groups. One group was grown in medium with 20% FBS (Non-differentiation medium) and the other was grown in medium with 1% FBS (Differentiation medium). Incubation lasts about 6 days for achieving full differentiation of cell. In some experiments differentiation was induced by increasing the calcium concentration in Ca^{2+} free differentiation medium to 9 μM under the same conditions. Cells were fixed with gluteraldehyde and stained with hematoxylin and Eosin where indicated.

Transfection and Reporter Assays

Cells were plated onto 35 mm dishes at a density of 1 X 10^5 cells/cm^2. Each dish received 2 µg of luciferase reporter plasmid and 2.0 µg of pSV-ß-galactosidase as an internal control. L6 cells were transiently transfected using the $CaPO_4$ precipitation technique (17). After transfection, cells were allowed to incubate at 37°C for five hours before being washed with fresh media and left for 36 hr. After 36 hours, cells were harvested and the cell lysate was assayed for luciferase activity and ß-galactosidase activity. The medium was aspirated and the cells washed in 1x PBS-1mM EDTA. The cells were then allowed to sit in 1 ml of PBS-1mM EDTA for 15 minutes. Cells were then scraped, transferred to a microcentrifuge tube, and pelleted at 10,000 rpm for 30 seconds. The supernatant was removed and the pellet was resuspended in 50 µl of ice cold lysis buffer (Tris 1M pH 7.8, 10% NP40 and 1M DTT) for 15 minutes. The solution was then pelleted at 14,000 rpm for 10 minutes at 4°C. The supernatant was removed and assayed for luciferase and ß-Galactosidase activity. Each luciferase assay contained 30 µl of the cell lysate and 100 µl of the luciferase assay reagent (Tricine 20 mM, $MgCO_3$ 1.07 mM, $MgSO_4$ 2.67 mM, EDTA 0.1 mM, DTT 33.3 mM, luciferin 470 mM, ATP 530 mM, Coenzyme A 270 mM, and BSA 1 ng/ml). The ß-galactosidase assay included 5 µl of cell lysate, 95 µl H20 and 20 µl of o-Nitrophenyl-ß-D-galactopyranoside, incubated at 37°C for 60 minutes. After 60 minutes, 60 µl of 1M Na_2CO_3 was added to stop the reaction and the optical density was measured at a wavelength of 420. Luciferase activity was assayed with a Berthold Lumat LB 9501/16 luminometer and normalized to ß-galactosidase for efficiency of transfection.

Stable transfections also used the $CaPO_4$ precipitation technique. Twenty-four hours after transfection the medium was changed to selection medium (10% FBS with 400 µg/ml G418). After 2-3 weeks of growth visible foci were selected, transferred to 96 well plates and grown in selection medium until cells were confluent. Cells were then transferred to 100 mm dishes and luciferase activity was measured as described above. To confirm that inserts were not disrupted we amplified the inserts of the stable cell lines using PCR. For stable lines with pXP-1.1MP the 5' primer was 5' CTC TTT AAA CCA GAC AGA CAG ACA G 3' (MSE) which was directed against the 5' end of the 1.1 kb insert. The 3' primer was 5' TTG GCG TCT TCC ATG GTA CCA ACA GTA CCG G 3' (Luc) which was directed against the beginning of luciferase in the inverse direction. For pMP+AP2 the primers flanking the insert were Luc and 5' TTG GAT CCG TGA CAC TTC CTT CCC T 3' (JD1) which was directed against the 5' end of the insert. When measuring luciferase

activity of stable transfectants, any differences in cell number were corrected by monitoring protein concentration. Results are the mean ± SE of 4-6 experiments. Where not visible SE were too small to be shown. Significance was evaluated using a student's t-test.

RNA Isolation and Measurement of NHE-1 mRNA Levels by PCR

Total RNA was isolated from L6 cells as described earlier (17). Poly (A^+) RNA was purified with a PolyAtract™ mRNA isolation kit as described by the manufacturer (Promega). For reverse transcriptase PCR Poly (A^+) RNA was isolated as described above and 2 µg was copied with reverse transcriptase from the Invitrogen cDNA cycle™ kit using the procedures described by the manufacturer.

We used a competitive reverse transcriptase PCR assay to measure NHE-1 mRNA levels. The assay is based on the use of a competitive substrate that competes with the reverse transcribed cDNA. The principle is the same as a competitive PCR assay that we have recently described (19) and was recently described in detail elsewhere (20). The relative levels of NHE-1 are compared to levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primers for the Na^+/H^+ exchanger were NHQ-1 (tacggtACCCTGCTCTTCTGCCTC) and NHQ-2 (gatgcaTGCGGATCTCCTCCTCCTT). The primers are specific to NHE-1 and amplify a 649 bp product from reverse transcribed cDNA. Increasing amount of competitive template is added to a series of these amplification reactions. This results in decreasing amounts of DNA product. The Na^+/H^+ exchanger competitive template was produced by using cDNA from rat mRNA. The primers NHQ-1 and NHQ-2 were used to amplify the 649 bp PCR product which was subcloned into the plasmid pTZ 19 producing the plasmid p16. An internal Eco 47III fragment was removed and the plasmid (Δp16) was religated with a resulting 496 insert.

Differences in purity of the mRNA sample and in reverse transcription were normalized using the internal standard of GAPDH. The GAPDH competitive template was produced using a similar procedure. The primers GA3P-1 (tatggatCCTTCATTGACCTCAAC) and GA3P-2 (aatctcgAGTTGTCATGGATGACC) were used to amplify a 396 bp product from rat cDNA. The 396 bp product was subcloned into pTZ 19 producing the plasmid pGA3. The competitive template was made using the restriction enzyme Nco I that removed an internal 155 bp. Amplification of the resulting plasmid (ΔpGA3) with the primers produced a 241 bp product.

PCR reactions contained 1 µl of reverse transcribed cDNA in a total volume of 50 µl containing 50 mM HEPES pH 7.9, 50 mM KCl, 1.5 mM $MgCl_2$, 100 µM each dNTP, 2.5 U of Taq polymerase. Reactions also contained 1 µCi of [α-^{32}P] dCTP (3,000 Ci/mmol). Amplification for 30 cycles was under the following conditions: 94 °C for 45 sec, 60 °C for 45 sec, and 72 °C for 60 sec on a Coy TempCycler. Ten-µl samples were resolved on 9% polyacrylamide gels.

For quantification of relative amounts of Na^+/H^+ exchanger, PCR was done with decreasing amounts of competitor DNA. Products of reactions were resolved on 9% gels and the incorporated radioactivity quantified using a model BAS1000 phosphoimager (Fuji Photo Film Co., Ltd.). Illustrations were shown using autoradiography. For quantification of relative amounts of message, the log of the ratio of CPM in competitor-derived products to target-derived products was plotted versus the log of the number of competitor template molecules. When the target-derived and competitor-derived products had the same number of incorporated counts, the target and competitor DNA templates were present in equal amounts, corresponding to a logarithm equal to 0. After measurement the amounts of Na^+/H^+ exchanger were normalized using the amounts of GAPDH. GAPDH did not show any consistent pattern of changes corresponding to any treatments of tissues.

To check if the kinetics of amplification of DNA is independent of the size of template, we did a comparison and regressional analysis of the rate of amplification between NHE-1 and ΔNHE-1 DNA. We adjusted the starting ratio of NHE-1 to ΔNHE-1 DNA template to 0.1, 1, 10, 100 respectively in 4 individual PCR reactions. After PCR, the ratio of amplified NHE-1 to ΔNHE-1 DNA was measured. The starting ratio was converted to log scale (X) and compared with the log of the final ratio (Y). The assumption was that if the regressional coefficient is close to 1 with $P<0.05$, it will indicate that the kinetics of amplification of DNA is not significantly affected by the size of template in our study. Although no differences in amplification were noted, for the present series of experiments we only compared the relative amounts of cDNA in samples to the amount of competitor, not absolute amounts of NHE-1.

Results

Fig. 2-1 shows a schematic diagram of the constructs used for transfection of L6 muscle cells. The sequence of the proximal 1.1 kb was reported earlier (17) and the 5' end of the plasmids pXP-1.1MP, pXP-0.9MP, pXP-0.5MP, pXP-0.2MP, pXP-0.18MP,

pXP+AP2 and pXP-AP2 is illustrated in **Fig. 2-1b.** Digestion with exonuclease resulted in the production of the plasmids pXP-0.9MP, pXP-0.5MP which had terminated at nucleotides -808 and -515 of the promoter. Other reporter constructs were made using PCR or restriction enzyme digestion. They terminated at bp's -171, -155, -125 and -92 respectively for pXP-0.2MP, pXP-0.18MP, pXP+AP2 and pXP-AP2.

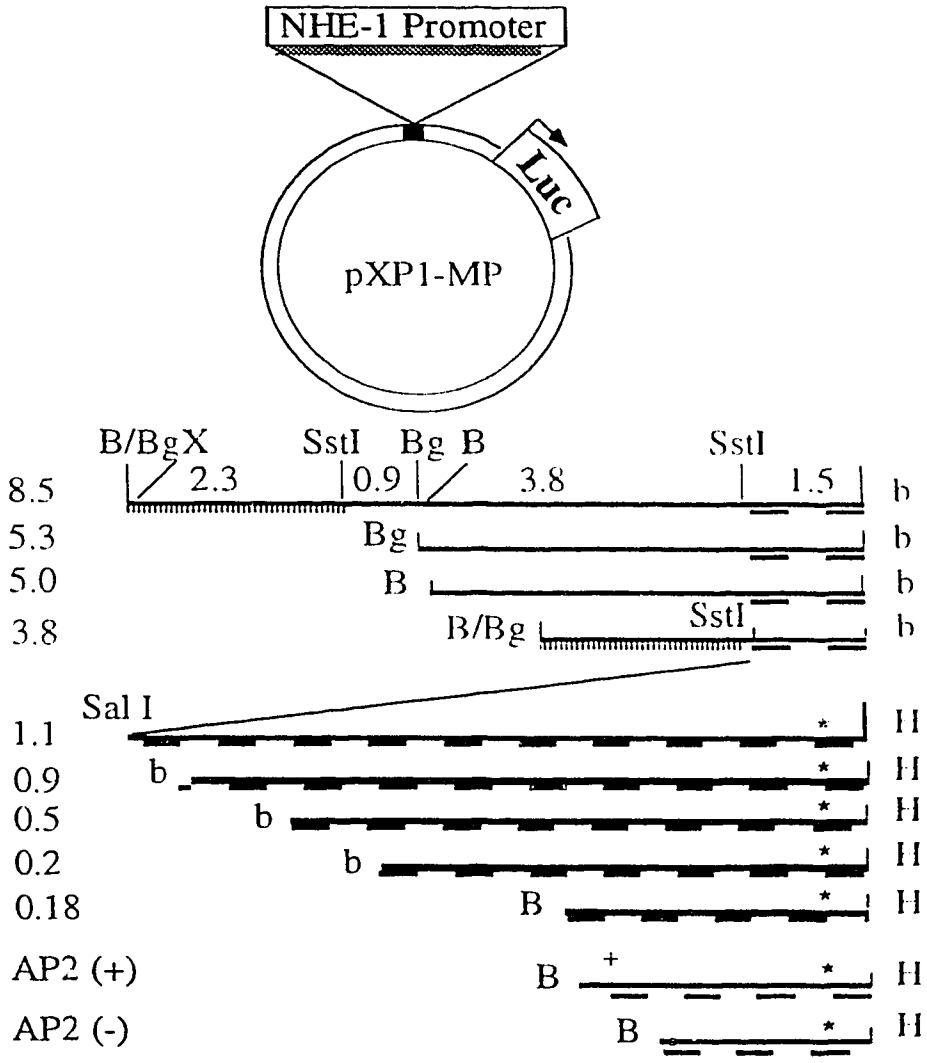

Fig. 1a

Fig. 2-1. Schematic diagram of constructs used for transfection of L6 muscle cells. (a) The plasmids pXP-8.5MP, pXP-5.3MP, pXP-5.0MP, pXP3.8MP, pXP-1.1MP, pXP-0.9MP, pXP-0.5MP, pXP-0.2MP, pXP-0.18MP, pMP+AP2 and pMP-AP2 were constructed as described in the "Experimental Procedures". pXP-8.5MP was made from a 9 kb genomic clone of the mouse Na^+/H^+ exchanger. pXP-5.3MP and pXP5.0MP were derived from pXP-8.5MP by removal of 5' end restriction fragments. pXP3.8MP was made by removal of an internal restriction fragment. Other plasmids represent a progressive set of deletions made by treatment with exonuclease or subcloning smaller appropriate fragments generated via PCR. Abbreviations; AP2+, plasmid with 147 bp of the

NHE-1 promoter containing the AP2+ site; AP2-, plasmid with 114 bp of the NHE-1 promoter not containing the AP2+ site; B, Bam HI; Bg, Bgl II; b, blunt end cloning; H, Hind III; kb, Kilobase; Luc, Luciferase; Poly, Polylinker; X, Xho I; +, approximate position of the AP2+ containing sequence; *, approximate position of the TATA sequence. Insert sizes are not drawn to scale. (b) Nucleotide sequence of the proximal 1.1 kb of the NHE-1 promoter-enhancer region. The 5' ends of the plasmids pXP-0.9MP, pXP-0.5MP, pXP-0.2MP, pXP-0.18MP, pMP+AP2 and pMP-AP2 are indicated by the symbols 0.9, 0.5, 0.2, 0.18, AP2+ and AP2- respectively. pXP-1.1MP encompasses the entire sequence known. The start sites of transcription and the position of the AP-2 site are indicated.

```
                |  10     |  20     |  30     |  40     |  50     |  60
-1085  CAGATAATAA AAATCCTTCT TTTCTCTTTA AACCAGACAG ACAGACAGAC AGATAGACAG  -1026
-1025  ACAGACAAAA ACAATACCCA GCCTCTTACT TTTTGGTTGT TTCACTACTG TTATGAGTTG   -966
 -965  AAACACGCCC TTTCAAAAGA AGCTGAAGTC CTACCCCTGC CCACCCCTCA GTGCCCGAGA   -906
 -905  ATGTGGCTTT ATTTAGATGA GGGGTTTTGT TGTTGTTGTT GTTGTTTTAA ATCTCTTTGA   -846
                                                  0.9
 -845  GACAGGGTCT CCCTACTGAC CTCAGCCTGG TCTAGAACTC ACTTTGTTAC ACAAGCTGAC   -786
 -785  GTTTAGTTAA AAATTCCTCT TTCTTTAGTT TCCCAAATGC TGGAATTACA GTTTCCCACC   -726
 -725  GTTTGGTGGT CAGATTGGGT TTTTGAAGTT GATCAAGGTA AGGTACGCCA TTAGGGCCAA   -666
 -665  TTCCTAATTC AATTTGCTTG GAGTCCTTTT AAAGGGGGAA ATTTGGACCT AAAGACGGAT   -606
 -605  ACACATAAGC TGGGGTTATG CCGCCACAGT CAAGGGAAGC CAATACATTG CCATCAAAGC   -546
                                      0.5
 -545  ACCTGTAGGT GGGATGCAGG AACGGATTTC AGCTCACACG GTCCTCAGCA CAGGTCAATG   -486
 -485  CTTGGGAACA TACAGAGTGC AGATTTCTAG CCCATTTCAT TCATTCATTT AACTTTATTT   -426
 -425  TGTAACACTG GGCATAGGAA CCAATGGTAG ACAAGCACTC TACCTCTGAA CCACGATCCC   -366
 -365  ATCCCTTTCA TTCTAAACAT TTCTCATAGA TCACCTCATT TAACCATCTT GTTATCCTTT   -306
 -305  TGTTATAGCT ATCCATCTCC ACCTTACAGT TGAGAAAGCT GTGAAACAGA GAGGCTAAAT   -246
 -245  AACTTGTTCC AAAGTCACAT GCTAACAAGA AAACACTTTG TAGATAGTAC AGTATGTCAC   -186
                      0.2                 0.18
 -185  TCGTGTTAGA TTGTACTTTT TTTTTTTTTT TCCAATTTAG GTCTCGGCTT CCTCTTCTTA   -126
       AP2+                    AP-2                AP2-
 -125  CGTGACACTT CCTTCCCTGG GCGACAGGGG CCGCTGCACC GCGCGGGCGC TGACAGGTCT    -66
                                                  TATA
  -65  CTCTAGCTTG CTGCGCCTGC TACTCGTTGG TGCCTATAAA TGGCTGCGCT GTGCTCAGCG     -6
       *  *
   -5  AGGCATCAGT CCGCTACCGC GGGACCC                                       +22
                |  10     |  20     |  30     |  40     |  50     |  60
```

Fig 1b

One of the major events during skeletal muscle differentiation is fusion of mononucleated myoblast precursor cells into elongated multinucleated cells. L6 cells have been used as a model of this differentiation (14). To examine this property in L6 cells we

reduced the serum concentration which we and others (14) have used earlier to induce this phenomenon. The results are shown in **Fig. 2-2.** When the serum concentration was reduced over a period of 6 days the majority of the cells changed from mononucleated precursor cells to a population of multinucleated cells (**Fig. 2-2a-c**). In contrast, when the serum concentration remained high, no such change occurred and cells remained mononucleated (**Fig. 2-2d-f**).

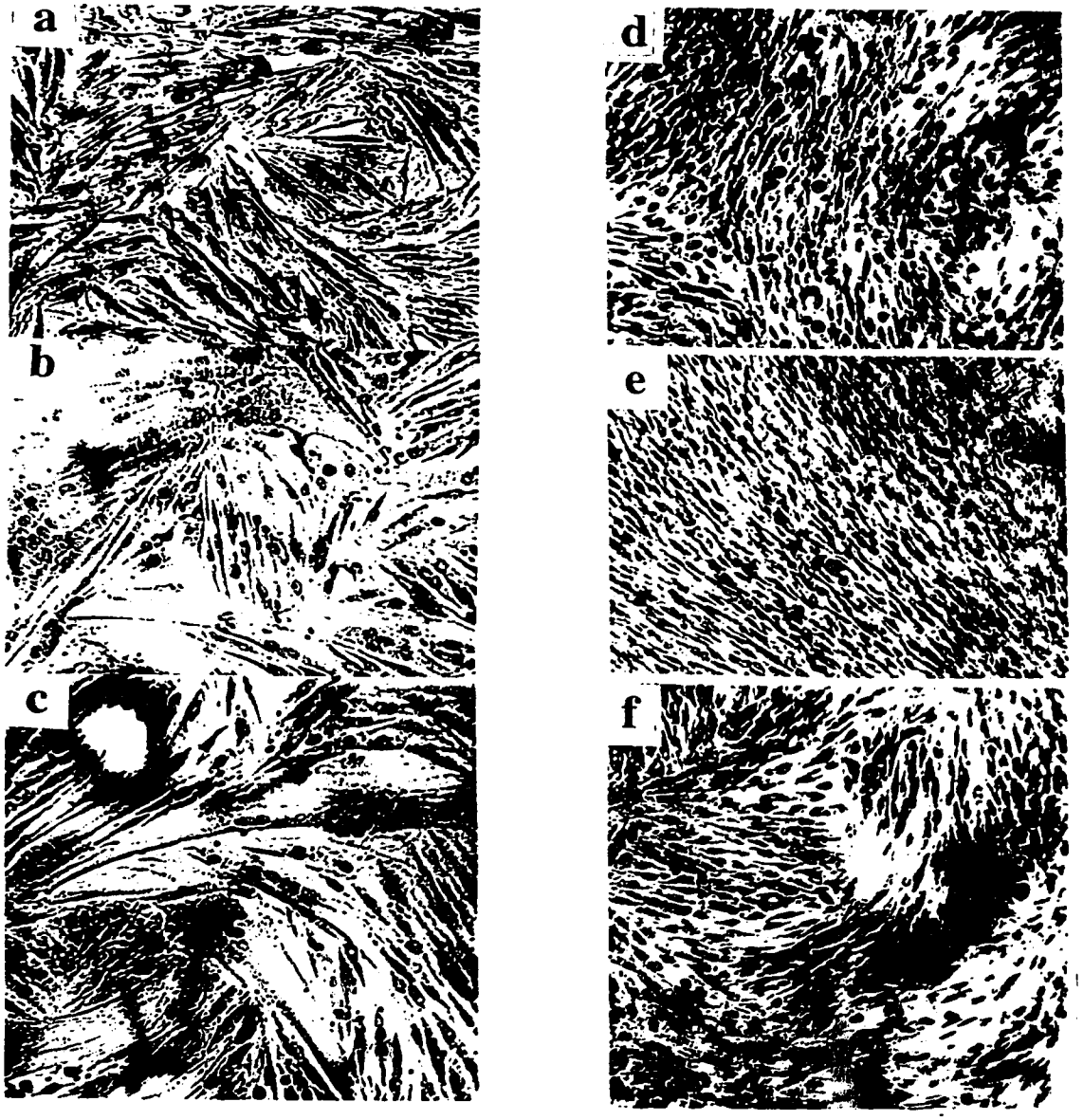

Fig. 2-2. L6 muscle cells grown in culture and fixed and stained as described in "Materials and Methods". At day 0 the medium was changed to one which contained either 1% serum (A-C) or 20% serum (D-F). Photographs illustrate their differentiation into myotubes. Times are 48, 96 and 168 hours after day 0 for samples A and D, B and E, and C and F respectively.

We examined the NHE-1 mRNA levels in L6 cells at varying stages of differentiation. Quantitative competitive PCR was used. Typical results with this procedure are shown in 3a and 3b. With decreasing amounts of NHE-1 competitor the amount of 496 bp PCR product declined. Also, as the amount of competitor decreased the amount of 649 bp PCR product from the NHE-1 cDNA increased. A similar PCR assay was used to measure GAPDH levels (**Fig. 2-3b**). Similarly, with decreasing amounts of competitor the amount of 241 bp PCR product decreased. In addition, the amount of GAPDH (396 bp) PCR product increased. The summary of the corrected results of 5 independent determinations is shown in **Fig. 2-3c.** The results show that L6 cells treated with differentiation media for 6 days express approximately 2.5 to 3-fold the amount of NHE-1 message in comparison to untreated cells and cells treated for 3 days.

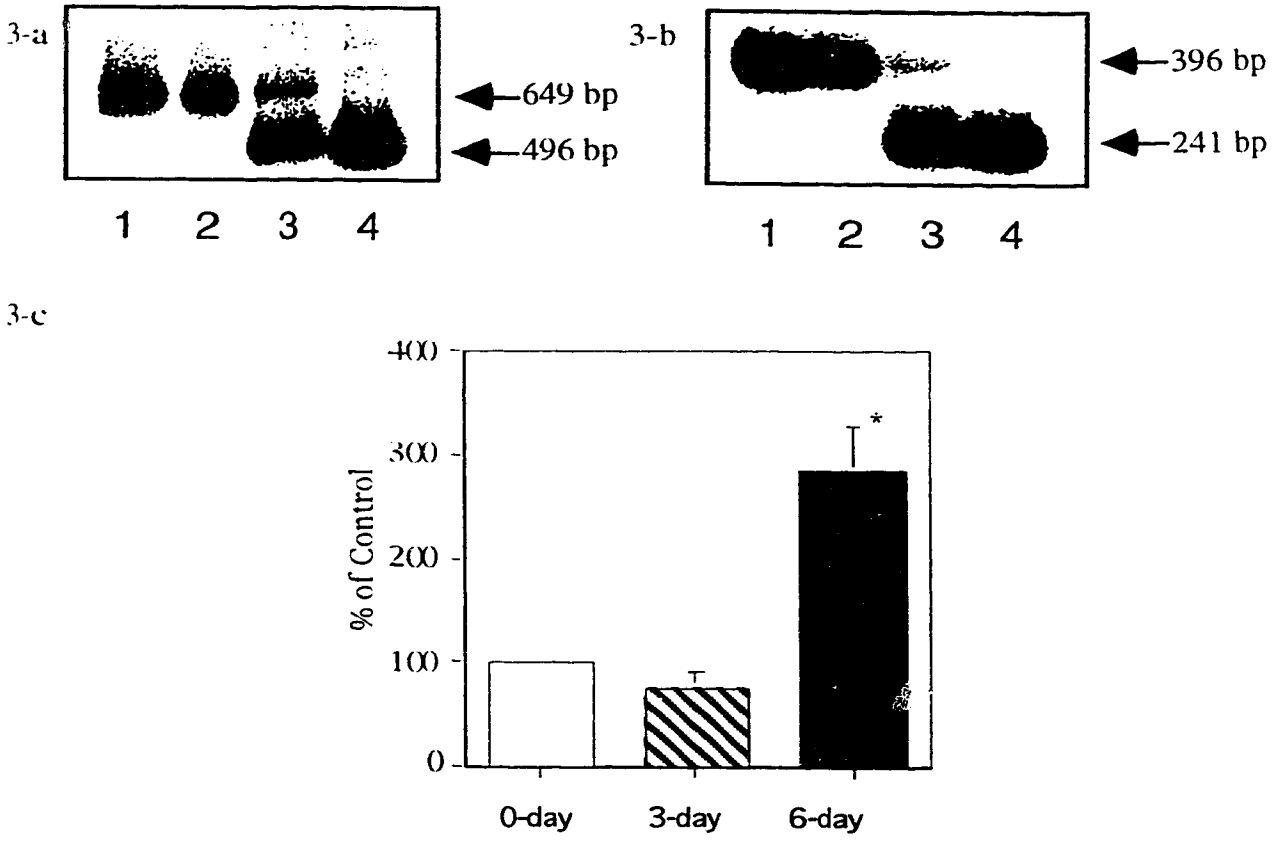

Fig. 2-3. mRNA levels of the Na^+/H^+ exchanger in L6 muscle cells. L6 muscle cells grown in culture and NHE-1 mRNA levels measured using a quantitative competitive PCR assay as described in "Materials and Methods". **Fig. 2-3a, 2-3b,** determination of NHE-1 and GAPDH levels of L6 cells. Competitive PCR was prepared and samples were separated by electrophoresis on 9% acrylamide gels. Autoradiography was used for the purpose of illustration of typical results. **Fig 2-3a** illustrates the determination of mRNA levels of NHE-1. The amount of competitor was 10, 1, 0.1, 0.01pg in lanes 1-4,

respectively. **Fig. 2-3b** shows the determination of the levels of GAPDH. The amount of competitor was 1000, 100, 10, 1 pg in lanes 1-4, respectively. **Fig. 2-3c**, summary of 5 independent determinations of relative NHE-1 mRNA levels of L6 cells prior to differentiation, after 3 days and after 6 days of differentiation. * Significantly different from 0 day and 3 day values at $P < 0.001$.

We have previously isolated the NHE-1 promoter (17). A series of promoter constructs was made from this promoter to examine the relative activity of the promoter in L6 myoblast cells. Transient transfectants were used to compare the activity of the promoter in these constructs. The results are shown in **Fig. 2-4.** Mean relative luciferase values for the pXP-1.1MP construct were 217,000. Deletion of the promoter up to and including the AP2 site (-92) resulted in almost total elimination of activity. Inclusion of the AP2 site (-125) resulted in a significant increase in activity of the promoter. Inclusion of base pairs -155 to -126 in the pXP-0.18MP plasmid resulted in a small insignificant increase in the activity of the promoter. However inclusion of 16 more base pairs including a poly T rich region, resulted in a large, significant increase in activity of the promoter. Inclusion of up to the entire 8.5 kb region of the NHE-1 promoter did not result in further significant increases in activity of the promoter. In fact, both pXP-5.3MP and pXP-5.0MP resulted in less activity of the promoter, however the changes were small and not statistically significant.

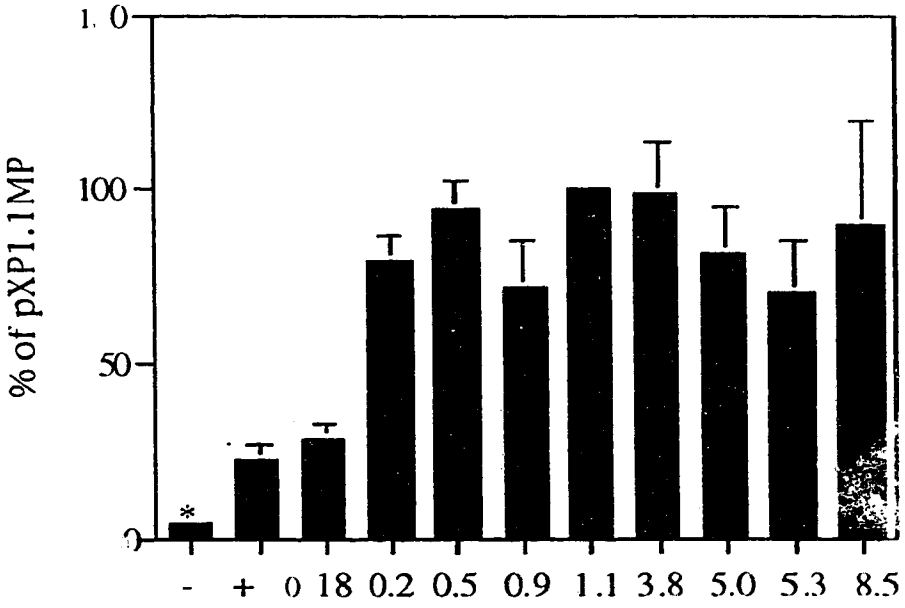

Fig. 2-4. Activity of the NHE-1 promoter in L6 muscle cells. L6 muscle cells were transiently transfected with the plasmids pXP-8.5MP, pXP-5.3MP, pXP-5.0MP, pXP-3.8MP, pXP-1.1MP, pXP-0.9MP, pXP-0.5MP, pXP-0.2MP, pXP-0.18MP, pMP+AP2 and pMP-AP2 as described in the "Materials and Methods". The relative activity of the constructs was compared to that of the pMP+AP2 plasmid. * Significantly different from

the plasmids pXP-0.18MP, pMP+AP2 at $P < 0.01$. $^{+}$ Significantly different from the plasmid pXP-0.2MP and all other larger plasmid constructs at $P < 0.01$.

To examine the effect of differentiation on the activity of the NHE-1 promoter we constructed a series of stable cells lines containing the NHE-1 promoter constructs. We then examined the activity of the cells during the process of differentiation which was induced by reduction of serum concentration to 1%. **Fig. 2-5** shows the comparison of the activity of the NHE-1 promoter constructs during differentiation. In most cases the activity of the promoter was similar during the 2-, 4- and 6- day periods following serum withdrawal. There was a general trend for the activity of the promoter to be increased during differentiation. This was most apparent in the larger constructs, particularly in pXP-1.1MP. In this case the activity during the process of differentiation was more than 2-fold greater than in control cells at day 4 and day 6. This effect was significantly greater in the construct pXP-1.1MP in comparison to pXP-0.2MP or pXP-0.18MP.

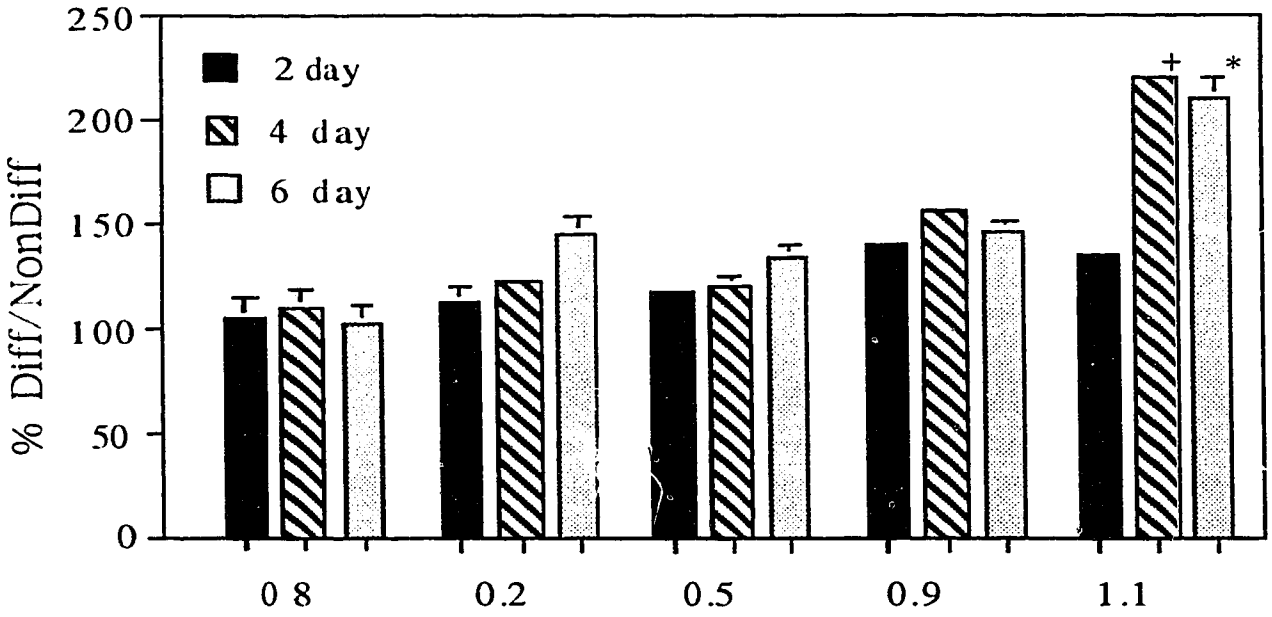

Fig. 2-5. Relative activity of the NHE-1 promoter in L6 cells during differentiation. L6 muscle cells grown in culture were stably transfected with the plasmids pXP-1.1MP (1.1 kb), pXP-0.9MP (0.9 kb), pXP-0.5MP (0.5 kb), pXP-0.2MP (0.2 kb), pXP-0.18MP (0.18 kb) as described in the "Materials and Methods". Cells were then induced to differentiate by reduction in the serum concentration to 1%. The relative activity of the promoter is shown in comparison to cells not induced by reduction of serum concentration. Each group was sampled at 2 days, 4 days and 6 days after serum reduction. $^{*, +}$ Significantly different from the value at 0 days at $P < 0.05$, or $P < 0.01$.

We examined if the rate of amplification for NHE-1 DNA is different from that for the smaller ΔNHE-1 DNA. We compared the amount of initial template to that of PCR amplified NHE-1 and ΔNHE-1 DNA (**Fig. 2-6**). The results of the analysis showed that the regressional coefficient R is 0.983 with statistical significance (P=0.016<0.05). This indicates that there is no appreciable difference in the kinetics of amplification between the two DNA fragments. The quantitative PCR employed in this study is therefore a reliable method reflecting the levels of mRNA for NHE-1.

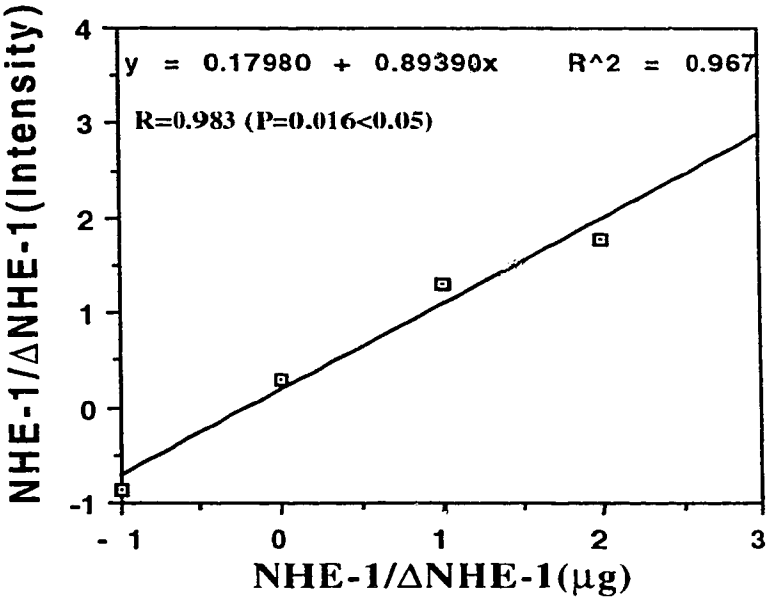

Fig. 2-6 Regressional analysis of amplification kinetics of NHE-1 and ΔNHE-1 in quantitative PCR. Specific amounts of NHE-1 and ΔNHE-1 DNA were added to 4 reactions. After PCR the ratio of the products was calculated and compared to the starting DNA. The regressional coefficient was calculated between the two ratios using Statview®SE+Graphics. P<0.05 is set as the significant level.

We used calcium deprivation to examine the specificity of effects on promoter activity. Reduction of medium calcium has been shown to inhibit the process of differentiation even after reduction of serum concentrations (24). L6 cells stably transfected with the plasmid pXP-0.2MP were subjected to reduced serum for 6 days in the presence or absence of reduced calcium (9 μM). After 6 days with normal calcium concentration the activity of the promoter rose almost 50% in comparison with that of day zero and the cells fused. In addition, by day 6, the promoter activity under reduced calcium is about 47% of

that under normal calcium. The cells also remained mononucleated and did not have the appearance of elongated multinucleated cells (**data not shown**).

Discussion

The rat L6 myoblast cell line has been used to analyze mechanisms whereby growth factors regulate cell growth and differentiation. In high serum concentrations these myoblasts grow rapidly and do not express muscle phenotypes. However, in low serum concentrations the cells fuse to form multinucleated myotubes that express many muscle specific proteins (14). Our initial studies characterized basal regulation of the NHE-1 promoter in rapidly growing myoblast L6 cells. We found that inclusion of the initial 0.2 kb of the promoter resulted in full activity of the gene (**Fig. 2-4**). Larger constructs of the gene did not result in much greater activity of the promoter. It was notable that there was a 3-4-fold increase in activity of the promoter when comparing the activity of the pMP+AP2 plasmids with the pXP-0.2MP construct. Most of this increase in activity can be attributed to a pyrimidine rich region of the promoter since the plasmid pXP-0.18 was only slightly elevated in activity in comparison to the pMP+AP2 plasmid (**Fig. 2-1b**). It was surprising that there was only small increases in activity of the promoter with further increases in the promoter size. We (4, 17) and others (6) have shown earlier that some more distal elements may have a significant role in NHE-1 expression in fibroblasts, P19 cells, HepG2 and in vascular smooth muscle cells.

DNA footprinting experiments by Molyada et al. (6) suggested that there are 4 regions of the human NHE-1 promoter (A-D) that may be important in NHE-1 expression. They noted that one region of the human promoter, element D, was significant in expression in HepG2 and smooth muscle cells. However element D is not conserved in the mouse promoter in contrast to the pyrimidine rich (poly T rich) region (**Fig. 2-1b,** bp -169 to -155). Element D does not seem to be important in basal expression in this cell type. The plasmid pXP-0.2MP does not contain the region corresponding to element D while the plasmid pXP-0.5MP does. However the plasmid pXP-0.5MP had only slightly elevated activity in comparison to pXP-0.2MP (**Fig. 2-4**). These results suggest that this region of the promoter does not play a significant role in the mouse gene, at least in this cell type. Another region which they identified in their study (6) was region C. This corresponded to bp -126 to -146 of the gene and was also not conserved in the mouse promoter. We also found that inclusion of this region of the gene did not result in significantly increased activity of the promoter. There was no significant difference in the activity of plasmid pMP+AP2 and pXP-0.18MP. Thus this region does not appear to play a significant role in basal expression of the promoter in this model. Two other regions noted in this study were, A and B. Region B corresponded to the AP2 containing site

which we have examined earlier (17). Removal of this site caused a dramatic decrease in the level of activity of the promoter. Region A was more proximal to the start site than region B. However further analysis of the more proximal region was not practical due to the already very low levels of luciferase.

Overall, the results of this experiment show that there are significant differences in regulation of the NHE-1 promoter in this cell type. It appears that two critical elements in regulation of basal activity of the promoter are the AP2 containing region between base pairs -125 and -92 and the pyrimidine (poly T) rich region between base pairs -155 and -171. We have earlier shown that the transcription factor AP-2 plays an important role in basal expression in fibroblasts and P19 cells (5). It appears as though in this cell type it is also important although not to as great an extent. The importance of the pyrimidine rich region is a novel finding. This region is highly conserved in the mouse and human genes suggesting it may have an important function. The exact role of this region in expression is not yet elucidated. Future studies will examine the importance of this region.

The Na^+/H^+ exchanger is involved in cell growth and differentiation and in pH regulation in muscle cells. During cellular proliferation the exchanger elevates intracellular pH and this pH change has been shown to play an important permissive role in growth in some cell types (22). Cell differentiation however, results in even more drastic alterations in growth patterns. In several models of differentiation both the activity and amount of the Na^+/H^+ exchanger is elevated. For example in HL60 cells, the activity of the Na^+/H^+ exchanger increases immediately prior to differentiation into granulocyte-like cells. In addition there is an 18-fold increase in NHE-1 mRNA levels as well as a 7-fold increase in protein levels (8, 23). We have also recently shown that the activity of the Na^+/H^+ exchanger is dramatically increased during retinoic acid induced differentiation of P19 cells (4). We therefore used rat L6 muscle cells to examine the activity of the promoter in this model. Initially we examined mRNA levels of the myoblasts during various stages of differentiation. We used a quantitative competitive PCR to assay the samples. The results are show in **Fig. 2-3.** mRNA levels increase in the final stages of differentiation and we also found that the activity of the promoter was elevated in comparison to cells not grown in medium which causes differentiation (**Fig. 2-5**). This was more pronounced in the stable transfectants with the larger regions of the promoter. The maximal effect was with the plasmid pXP-1.1MP that increased luciferase activity up to 2-fold 4-6 days after serum removal. The level of stimulation by the process of differentiation is not high. We found up to 10-fold increases in NHE-1 promoter activity during differentiation of P19 cells (4).

In HL60 cells effects of similar magnitude were seen on mRNA levels (8, 23). In addition in P19 cells the activity of the promoter increased transiently during transfection and then declined, which suggested a role for the protein that is related to the differentiation process itself. In this case, the elevation of activity of the promoter seemed to be more related to the change in phenotype of the cell. Both mRNA levels and the activity of the promoter did not rise rapidly or to a very high degree. It may be that in this instance the role of the Na^+/H^+ exchanger in skeletal muscle differentiation is not as significant as in P19 or HL60 cells. It should be noted that response of the promoter to removal of serum is atypical of other cells. We have examined the effects of removal and reintroduction of serum on fibroblasts and isolated cardiomyocytes. In both cases removal of serum resulted in decreased activity of the promoter from the plasmid pXP-1.1MP (20). It may be that the removal of serum and the variety of growth factors it contains, partially masks any effects of differentiation. It is of interest that slightly larger effects occurred with the larger plasmid pXP-1.1MP. This suggests that some other elements may become more crucial to expression of NHE-1 during the process of differentiation.

Conclusion

Overall, our results have examined regulation of expression of the NHE-1 promoter in rat L6 cells. We have shown that there are two regions critical for basal expression in this cell type. One is from bp -155 to -171. A second more proximal region is between bp -92 and -125. Muscle differentiation causes a small stimulation of activity of the more proximal regions of the promoter. However, some elements more distal in the promoter mediated larger 2-fold increases in activity during muscle differentiation. Future studies will examine the precise processes mediating the regulation of the gene in this cell type.

References

1. Boron, W. F., and Boulpaep, E. L. (1983) Intracellular pH regulation in the renal proximal tubule of the salamander. *J. Gen. Physiol.* **81,** 29-52

2. Grinstein, S., Clarke, C. A., and Rothstein, A. (1983) Activation of Na^+/H^+ exchange in lymphocytes by osmotically induced volume changes and by cytoplasmic acidification. *J. Gen. Physiol.* **82,** 619-638

3. Pouyssegur, J. (1985) The growth factor-activatable Na^+/H^+ exchange system: a genetic approach. *Trends Biochem. Sci.* **10,** 453-455

4. Dyck, J. R. B., and Fliegel, L. (1995) Specific activation of the Na^+/H^+ exchanger during neuronal differentiation of embryonal carcinoma cells. *J. Biol. Chem.* **270,** 10420-10427

5. Dyck, J. R. B., Maddaford, T., Pierce, G. N., and Fliegel, L. (1995) Induction of expression of the sodium-hydrogen exchanger in rat myocardium. *Cardiovasc. Research* **29,** 203-208

6. Kolyada, A. Y., Lebedeva, T. V., Johns, C. A., and Madias, N. E. (1994) Proximal regulatory elements and nuclear activities required for transcription of the human Na/H exchanger (NHE-1) gene. *Biochim. Biophys. Acta.* **1217,** 54-64

7. Miller, R. T., Counillon, L., Pages, G., Lifton, R. P., Sardet, C., and Pouyssegur, J. (1991) Structure of the 5'-flanking regulatory region and gene for the human growth factor-activatable Na/H exchanger NHE-1. *J. Biol. Chem* **266,** 10813-10819

8. Rao, G. N., Sardet, C., Pouyssegur, J., and Berk, B. C. (1992) Na^+/H^+ antiporter gene expression increases during retinoic acid-induced granulocytic differentiation of HL60 cells. *J. Cell. Physiol.* **151,** 361-366

9. Horie, S., Moe, O., Miller, R. T., and Alpern, R. J. (1992) Long-term activation of protein kinase C causes chronic Na/H antiporter stimulation in cultured proximal tubule cells. *J. Clin. Invest.* **89,** 365-372

10. Krapf, R., Pearce, D., Lynch, C., Xi, X. P., Reudelhuber, T. L., Pouyssegur, J., and Rector, F. C. Jr. (1991) Expression of rat renal Na/H antiporter mRNA levels in response to respiratory and metabolic acidosis. *J. Clin. Invest.* **87,** 747-751

11. Alvarez, J., Garcia-Sancho, J., Mollinedo, F., and Sanchez, A. (1989) Intracellular $Ca2^+$ potentiates Na^+/H^+ exchange and cell differentiation induced by phorbol ester in U937 cells. *Eur. J. Biochem.* **183,** 709-714

12. Hazav, P., Shany, S., Moran, A., and Levy, R. (1989) Involvement of intracellular pH elevation in the effect of 1,25-dihydroxyvitamin D3 on HL60 cells. *Cancer Res.* **49,** 72-75

13. Vairo, G., and Hamilton, J. A. (1993) Evidence against a requirement for Na^+/H^+ exchange activity for proliferation or differentiation of HL 60 leukemic cells. *Cell Growth Diff.* **4,** 461-466

14. Vigne, P., Frelin, C., and Lazdunski, M. (1985) The Na^+/H^+ antiport is activated by serum & phorbol esters in proliferating myoblasts but not in differentiated myoblasts. *J. Biol. Chem.* **260,** 8008-8013

15. Fliegel, L., and Dyck, J. R. B. (1995) Molecular Biology of the Cardiac Na^+/H^+ Exchanger. *Cardiovasc. Research* **29,** 155-159

16. Grinstein, S., Rotin, D., and Mason, M. J. (1989) Na^+/H^+ exchange and growth factor-induced cytosolic pH changes. Role in cellular proliferation. *Biochim. et Biophys. Acta.* **988,** 73-970

17. Dyck, J. R. B., Silva, N. L. C. L., and Fliegel, L. (1995) Activation of the Na^+/H^+ exchanger gene by the transcription factor AP-2. *J. Biol. Chem.* **270,** 1375-1381

18. Altenberg, G. A., Aristomuno, P. C., Amorena, C. E., and Taquini, A. C. (1989) Amiloride prevents the metabolic acidosis of a KCl load in nephrectomized rats. *Clin.Sci.* **76,** 649-652

19. Wang, H., Fliegel, L., Cass, C. E., Penn, A., Michalak, M., Weiner, J. H., and B. D. Lemire (1994) Quantification of mitochondrial DNA in heteroplasmic skin fibroblast cells with competitive PCR. *Biotechniques* **17,** 76-82

20. Yang, W., Dyck, J. R. B., Wang, H., and Fliegel, L. (1995) Regulation of the NHE-1 promoter in the mammalian myocardium. **In Press in Am. J. Physiol.,**

21. Mankus, R., Schwartz, J. H., and Alexander, E. A. (1993) Acidification adaptation in cultured inner medullary collecting duct cells. *Am. J. Physiol.* **264,** F765-F769

22. Grinstein, S., Rotin, D., and Mason, M. J. (1989) Na^+/H^+ exchange and growth factor-induced cytosolic pH changes. Role in cellular proliferation. *Biochim. Biophys. Acta* **988,** 73-97

23. Ladoux, A., Miglierina, R., Krawice, I., Cragoe, E. J., Abita, J. P., and Frelin, C. (1988) Single-cell analysis of the intracelluation of U937 human leukemic cells. *Eur. J. Biochem.* **175,** 455-460

24. Shainberg, A., Yagil, G., and Yaffe, D. (1969) Control of myogenesis in vitro by Ca^{2+} concentration in nutritional medium. *Exptl. Cell Res.* **58,** 163-167

CHAPTER FOUR

Regulation of Na+/H+ Exchanger Gene Expression: Role of a Novel Poly (dA:dT) Element in Regulation of the NHE-1 Promoter

A version of this chapter has been submitted to J. Biol. Chem.

Weidong Yang and Larry Fliegel

Introduction

The Na/H exchanger is a mammalian plasma membrane protein that removes one intracellular proton in exchange for an extracellular sodium. It is involved in pH regulation (1), control of cell volume and is stimulated by growth factors (2). Several isoforms of the protein have been identified (NHE-1 to NHE-5). The NHE-1 isoform is the most widely distributed type and is present in most, if not all, mammalian cells (3). Studies have shown that Na^+/H^+ exchanger mRNA levels are increased due to a number of treatments including chronic acid loading and treatments causing cellular differentiation (4-6). For example, during retinoic acid induced differentiation of human leukemic cells (HL-60), there is an 8.3-fold increase in NHE-1 transcription (4). More recently we have shown that during retinoic acid induced differentiation of P19 cells there is a transient increase in the level of NHE-1 transcription (7). Increased antiporter activity during differentiation may be important for differentiation to occur, at least in some cells types (8, 9). However, this requirement has not been shown to occur universally and the role of the Na^+/H^+ exchanger may vary between cell types (10, 11).

There have been few studies on regulation of the NHE-1 isoform of the Na^+/H^+ exchanger. Miller and coworkers (12), were the first group to isolate the upstream region of the human NHE-1 gene. They identified the intron-exon boundaries, the start sites of transcription and have provided the sequence of the 5' untranslated region along with approximately 1.3 kb of the promoter/enhancer region (12). Recently, another group has identified regions of the promoter that can bind nuclear proteins (13). We have examined the mouse NHE-1 gene (14). A 1.1 kb fragment upstream of the 5'-untranslated region along was isolated and characterized. We identified the transcription factor AP-2 or an AP-2 like protein as being involved in the regulation of the mouse NHE-1 gene (7, 14). This transcription factor is involved in regulation of the gene during cellular differentiation of P19 cells. We have also recently shown that serum stimulates expression of the promoter in some cell types (15). Another group has recently isolated the rabbit NHE-1 promoter (16). The sequence of the clone was similar to the human sequence and 708 proximal bp exhibited orientation-dependent activity.

Clearly a number of regions of the NHE-1 promoter contribute to the basal expression of the gene. We and others (7, 13-15) have shown that a stepwise reduction in the size of the NHE-1 promoter results in a gradual reduction in activity of the gene. This effect varies between cell types(7, 13, 15). Besides this evidence, several regions of the

gene have been suggested to be involved by DNA footprinting experiments (13, 14). We have noted the presence of a highly conserved "poly (dA:dT)" rich region in the NHE-1 promoter. In this study we examine the function of this region in L6 cells. The results suggest that this conserved region plays an important role in regulation of NHE-1 expression.

Materials and Methods

Materials

Restriction endonuclease and DNA-modifying enzymes were obtained from Boehringer Mannheim (Laval, Quebec, Canada) and Bethesda Research Laboratories (Gaithersburg, MD, USA). The pBluescript plasmids used for subcloning were from Stratagene (LaJolla, CA, USA). Plasmid pXP-1 was a gift from Dr. M. Nemer of the Institut de Recherches Cliniques de Montreal, Montreal, Quebec, Canada. Other chemicals were of analytical grade or molecular biology grade and were purchased from Fisher Scientific (Ottawa, On, Canada), Sigma (St. Louis, MO, USA) or BDH (Toronto, Ontario, Canada).

Reporter Plasmid Constructs

The isolation and characterization of the Na^+/H^+ exchanger promoter was described earlier. pXP-1.1MP was constructed as described earlier (14). To construct pMP+AP2 plasmid, two oligonucleotides were used as primers for polymerase chain reaction (# 1, ttgg atc CGT GAC ACT TCC TTC CCT and #3, cc ttc gaa GGG TCC CGC GGT AGC GGA) were synthesized to amplify base pairs -125 to +22 of the gene. The PCR product had the restriction enzyme sites Bam HI and Sma I generated on either end. This product was digested with Bam HI and Sma I and inserted directionally into pXP-1. Similarly, pMP-AP2 plasmid was made using the product of the primers # 3 and #2 (#2, ttgg atc CTG CAC CGC GCG GGC GCT) and the 114 bp product (-92 to +22) was inserted into pXP-1. The plasmid pXP-0.2MP was made by modification of pXP-1.1MP. The insert was excised with Sma I and Hind III and digested with Rsa I. This resulted in the production of a 0.2 kb Rsa I - Hind III fragment that was subcloned into the Sma I - Hind III site of pXP1 to form pXP-0.2MP. All plasmids were sequenced to verify proper orientation and fidelity of PCR. The plasmid pXP-0.18MP was made using the primer (#4 ccc gga TCC AAT TTA GGT CTC GGC TTC C) with the primer (#3, cc ttc gaa GGG TCC CGC GGT AGC

GGA). The PCR product had restriction enzyme sites of Bam H I and Hind III and was inserted directionally into pXP-1.

To construct pXP-Tn-SV40, the oligonucleotides (poly T) 5' GAT CCT GTA CTT TTT TTT TTT TTT TCC AAT TCG A 3' and (poly A) 5' GAT CTC GAA TTG GAA AAA AAA AAA AAA AGT ACA G 3' were annealed, ligated together and then digested with restriction enzymes Bam HI and Bgl II. The product was size fractionated on a 9 % acrylamide gel, and the ligated product of the oligonucleotides was purified and ligated into the Bam HI site of pBluescript-SK⁻ (Stratagene, LaJolla, CA, USA). The resulting plasmid (pBS-Tn) was digested with Eco R I and Hind III. The SV40 promoter was digested from pCAT-promoter plasmid (Promega) using Eco RI and Hind III and was ligated into the corresponding sites of digested pBluescript SK- construct. The total Tn - SV40 promoter fragment was removed from pBluescript SK- with the restriction enzymes Sst I and Hind III. The plasmid pXP-1 was digested with Hind III and Sst I and ligated with the Sst I to Hind III fragment. The resulting plasmid pXP-Tn-SV40 contained one copy of the poly T site of the mouse NHE-1 promoter located 5' to the SV40 promoter. The final construct was sequenced to verify proper orientation and fidelity of PCR. The plasmid pSVLuc contained the SV40 promoter inserted into pXP1 and was without the poly T rich insert. It was constructed as reported earlier (7).

To construct the plasmid pmut-0.2MP we used PCR. The primer mutPolyT 5' cac gga tcc ACT gTT Tgc ggT Tgg TTC CAA TTT AGG TCT CGG CTT CCT CTT C 3' was used with the primer #3, cc ttc gaa GGG TCC CGC GGT AGC GGA to construct a mutated form of the 0.2 kb insert. (Mutations within the poly (dA:dT) containing region are indicated by lower case letters.) The PCR product was cloned into the plasmid pXP1 as described above and the sequence was verified to confirm the presence of insert and the mutations.

Growth and Maintenance of Cell Lines

L6 cells were normally propagated in DMEM supplemented with 10% Fetal Bovine Serum essentially as described earlier (17). Cells were split into 35 mm dishes and allowed to attach and grown in medium with 10% FBS.

Transfection and Reporter Assays

Cells were plated onto 35 mm dishes at a density of 1 X 10^5 cells/cm^2. Each dish received 2.0 µg of luciferase reporter plasmid and 2.0 µg of pSV-ß-Galactosidase as an internal control. L6 cells were transiently transfected using the $CaPO_4$ precipitation technique (14). After transfection, cells were allowed to incubate at 37°C for five hours before being washed with fresh media and left for 36 hr. After 36 hours, the cells were harvested and the cell lysate was assayed for luciferase activity and ß-Galactosidase activity. The medium was aspirated and the cells washed in 1x PBS-1mM EDTA. The cells were then allowed to sit in 1 ml of PBS-1mM EDTA for 15 minutes. Cells were then scraped, transferred to a microcentrifuge tube, and pelleted at 14,000 rpm for 5 seconds. The supernatant was removed and the pellet was resuspended in 50 µl of ice cold lysis buffer (Tris 100 mM pH 7.8, 10% NP40 and 1 mM DTT) for 15 minutes. The solution was then pelleted at 14,000 rpm for 10 minutes at 4°C. The supernatant was removed and assayed for luciferase and ß-Galactosidase activity. Each luciferase assay contained 30 µl of the cell lysate and 100 µl of the luciferase assay reagent (Tricine 20 mM, $MgCO_3$ 1.07 mM, $MgSO_4$ 2.67 mM, EDTA 0.1 mM, DTT 33.3 mM, luciferin 470 µM, ATP 530 µM, Coenzyme A 270 µM, and BSA 1 ng/ml). The ß-galactosidase assay included 20 µl of cell lysate, 80 µl H_2O and 20 µl of o-Nitrophenyl-ß-D-galactopyranoside, incubated at 37°C for 60 minutes. After 60 minutes, 60 µl of 1M Na_2CO_3 was added to stop the reaction and the optical density was measured at a wavelength of 420. Luciferase activity was assayed with an LKB luminometer and normalized to ß-Galactosidase for efficiency of transfection.

DNA Binding Assays

Nuclear extracts were prepared from L6 cells as reported by Schreiber et al. (18). The synthetic oligonucleotides of the sequence 5' gat cGT ACT TTT TTT TTT TTT TTC C 3' (Poly T 5') and 5' gat cGG AAA AAA AAA AAA AAA GTA C 3' (Poly T 3') were made which correspond to the pyrimidine rich region of the mouse Na^+/H^+ exchanger promoter (base pairs -173 to -153). The mutated synthetic oligonucleotides of 5' gat cGT ACT gTT Tgc ggT Tgg TTC T 3' (mut. Poly T 5') and 5' gat cGG AAc cAA ccg cAA AcA GTA C 3' (mut. Poly T 3') were also made which correspond to the same region. The oligonucleotides were end-labeled with [γ-^{32}P]-ATP using T4 polynucleotide kinase. They were heated to 95°C for 2 minutes and cooled to room temperature overnight for annealing. DNA binding reactions were for 10 minutes at room temperature and contained: 30,000 - 40,000 CPM of [γ-^{32}P]-labeled oligonucleotides mixed with L6 nuclear extract (5 µg) in a

binding buffer (5% glycerol, 1.0 mM EDTA, 20 mM Tris-HCl, pH 8.0 and 0.02 - 2,500 µg/ml poly dI:dC). Binding assays were in a volume of 10 µl. Some assays contained cold competitor at concentrations from 9.2 ng/ml to 612.7 µg/ml. After electrophoresis on 6% polyacrylamide gels the gels were dried and exposed to X-ray film for 16 hours at -70°C.

DNase I Footprinting

The probes for DNase I footprinting were isolated from the plasmid pXP-0.2MP. Using the restriction enzymes Bam H I and Hind III the insert was removed and inserted into pBluescript that had been digested with the same enzymes. For 5' endlabeling the resulting plasmid was cut with Xba I and the 3' recessed end was filled with the Klenow fragment of *E. coli* DNA polymerase I in a reaction containing [α-^{32}P]-dCTP. The DNA was then cut with Hind III and gel purified using a 9% acrylamide gel. The resulting 248 bp fragment was used for footprinting. For a 3' end label the pBluescript plasmid containing the insert was initially digested with Sal I. The protruding 5' end was filled in using a reaction mixture that contained [α-^{32}P]-dCTP, and the Klenow fragment of *E. coli* DNA polymerase I. For DNase I footprinting the 251 bp fragment or the 248 bp fragment (about 30 000 CPM) were incubated with 5-40 µg of L6 nuclear extracts at room temperature for 25 minutes and then treated with DNase I for 10 seconds to 2 min. The reaction was terminated by phenol/chloroform extraction. After precipitation the sample was resuspended in 3 µl of 10 mM Tris (pH 7.4) 1 mM EDTA plus 2 µl of running buffer (95% formamide-dye, 20 mM EDTA, 0.05% Bromophenol Blue, 0.05 % Xylene Cyanol) and electrophoresed on a 6% acrylamide/ 7M urea sequencing gel. DNA sequence ladder was synthesized using the template DNA (M13mp18) provided by the manufacturer (United States Biochemical Corporation) and run on the same DNA sequencing gel.

Results

We have earlier cloned and sequenced the NHE-1 promoter. We noticed that progressive deletion of the promoter resulted in reduced activity of the reporter in a number of cell types (7, 14). Recently, the human (12), mouse (14) and rabbit (16) NHE-1 isoforms of the promoter have been isolated and cloned. A comparison of the homology of these clones showed that only some short proximal regions were highly conserved. One of these regions is illustrated in **Fig. 3-1.** It consists predominantly of a stretch of unbroken thymidine base pairs. In the rabbit gene it is interrupted by a single adenine residue.

```
-189   TGATTCTGGA CTTTGCTTTT TTTTTATTTT TTTATTTTTT TTGCCATCTC   140 Rabbit
       *   *   * **   * * ***** ***** **** *   * ***         * *
-185   TCGTGTTAGA TTGTACTTTT TTTTTTTTTT TCCAATTTAG CTCTCGGCTT   136 Mouse
       **     **      * ****** ********** *     ***
-191   TCTGTATATT CAGGACTTTT TTTTTTTTTT TTTTTTTTGT CATCTCTGAC   142 human
```

Fig. 3-1. Alignment of "poly (dA:dT)" containing regions of the human (12), mouse (14) and rabbit (16), NHE-1 promoters. The alignment was made using the DNA analysis program MacVector™. Numbering is based on the original publications and begins from first start site of transcription. * indicates identity with the mouse NHE-1 promoter.

To examine the role of the poly (dA:dT) region in activity of the NHE-1 promoter we constructed the vectors shown in **Fig. 3-2.** pXP-1.1MP contained the entire sequence of the mouse NHE-1 promoter. pXP-0.2MP contained up to bp -171 of the mouse promoter and pXP-0.18MP contained up to bp -155 of the NHE-1 promoter. To examine the relative activities of these constructs we used transient transfections of L6 cells. The results are shown in **Fig. 3-2.** There was a slight reduction in activity of the NHE-1 promoter when comparing pXP-1.1MP to pXP-0.2MP. However this was not significant. In contrast the removal of bp 171 to 156 resulted in a large, significant loss of activity of the promoter.

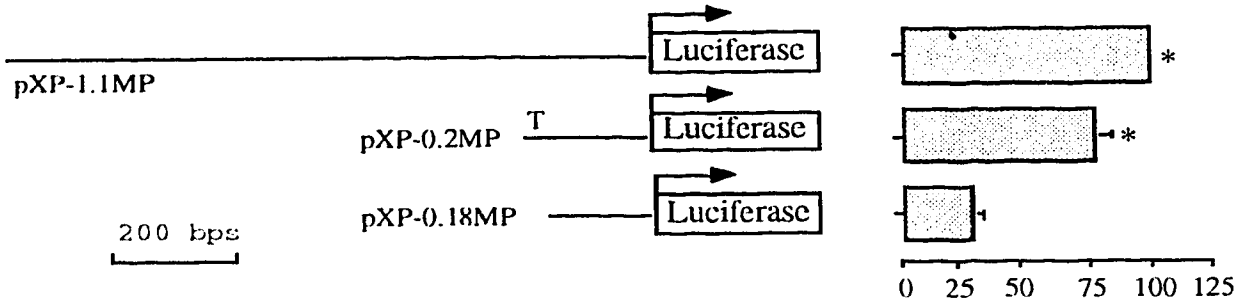

Fig. 3-2. Activity of the NHE-1 promoter in L6 muscle cells. L6 muscle cells were transiently transfected with the plasmids pXP-1.1MP, pXP-0.2MP and pXP-0.18MP as described in the "Materials and Methods". The relative activity of the constructs was compared to that of the pXP-1.1MP plasmid. * Significantly different from the plasmids pXP-0.18MP at $P < 0.001$. The poly (dA:dT) containing region is indicated by "T".

We used DNase I footprinting analysis to confirm if this region of the gene could interact with a protein or proteins of nuclear extracts from L6 cells. The results are shown in **Fig. 3-3. Fig. 3-3a** shows an examination of DNase footprinting of the promoter fragment that was labeled on the 5' end before digestion. Lane 1 shows the DNase I treated sample without nuclear extract. Lanes 2, 3 and 4 show the effect of 5, 10 and 20 µg of nuclear extract respectively. There is clearly a protected region between approximately bp -181 to -154 of the promoter. This region contains the poly (dA:dT) site which is from bp -169 to -155. **Fig. 3-3b** shows an examination of the same region of the promoter with labeling of the 3' end of the fragment. Lane 1 shows the treatment without nuclear extract, lanes 2-6 in the presence of 5, 10, 20, 30 and 40 µg's respectively. Again there is a protected region corresponding to the base pairs containing the poly T site.

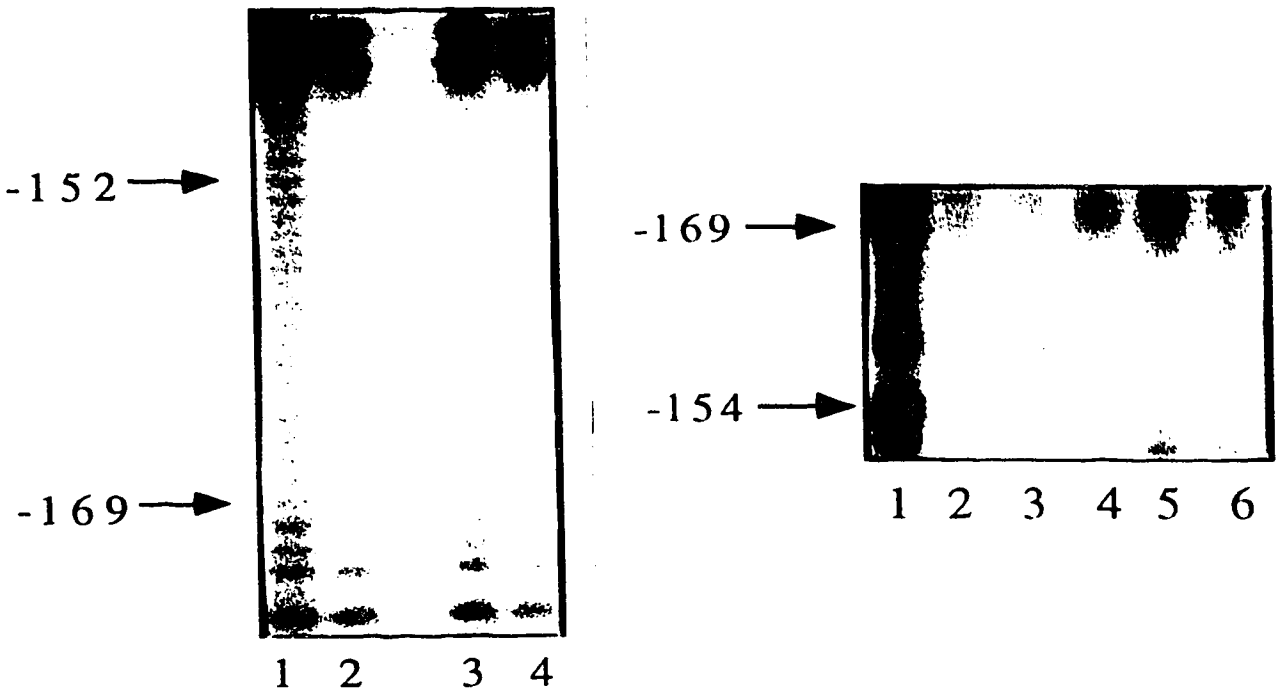

Fig. 3-3. DNase I footprinting analysis of the mouse NHE-1 promoter. DNase I footprint analysis was performed with the mouse promoter using bp -171 to +22 of the promoter fragment labeled on either strand at the 5' or 3' end. A, footprint analysis of the fragment with labeling on the 5' end. Lane 1 the naked DNA fragment was treated with DNase I (0.04 units for 60 s at room temperature). Lanes 2-4, the fragments were incubated with DNase I (1 unit for 60 s at room temperature) in the presence of increasing amount of L6 nuclear extracts, 5, 10 and 20 μg respectively. B, footprint analysis of the fragment with labeling on the 3' end. Lane 1 the naked DNA fragment was treated with DNase I (1 units for 60 s at room temperature). Lanes 2-6, the fragments were incubated with DNase I (1 units for 60 s at room temperature) in the presence of increasing amount of L6 nuclear extracts, 5, 10, 20, 30 and 40 μg respectively.

To confirm that a protein component or components of the nuclear extract could bind to the poly (dA:dT) containing region we used DNA mobility shift binding assay and competition analysis. The results are shown in **Fig. 3-4.** Competition was with unlabelled poly (dA:dT) oligonucleotides or with commercially obtained poly dI·dC. Lanes 2-7 show that increasing amounts of unlabelled poly (dA:dT) containing oligonucleotide reduces the amount of shifted DNA. Lanes 8-14 show the effect of increasing amounts of nonspecific sequence competitor. There was no effect of the relatively large amounts of non-competitor on the amount of principal band of shifted DNA. There was a reduction in the amount of some larger shifted complexes that may represent non-specific interactions with the poly T oligonucleotide.

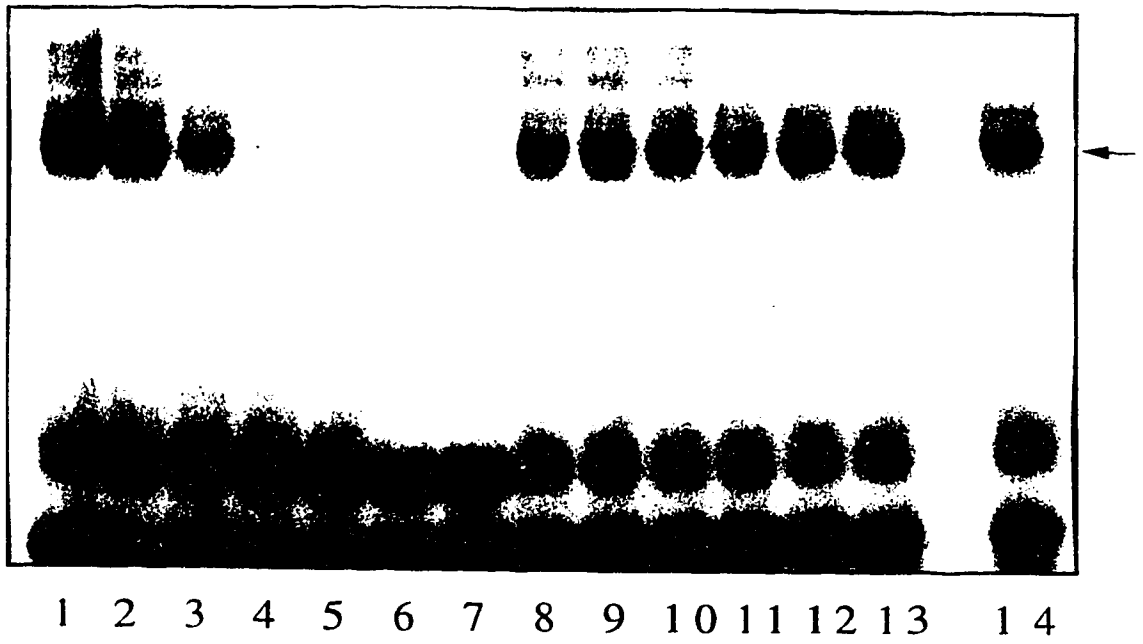

Fig. 3-4. DNA-mobility shift binding assay and competition analysis of the mouse NHE-1 poly (dA:dT) site. The labeled oligonucleotides corresponding to the positions -173 to -153 were incubated with nuclear extracts from L6 cells for 10 min at room temperature. The binding mixtures were analyzed by electrophoresis on 6% polyacrylamide gels as described in the "Experimental Procedures". Lane 1, nuclear extract alone (5 ug) added to the binding reaction. Lane 7 no nuclear extract added. Lanes 2 - 6 reaction mixture containing nuclear extract and increasing amount of unlabelled competitor oligonucleotide, 0.919, 15.3, 61.3, 613, 6,127 ng respectively. Lanes 8-14 reaction mixture containing nuclear extract and increasing amount of nonspecific sequence competitor poly dI:dC 0.2, 1.0, 3.3, 10, 60, 250 and 2,500 ng respectively.

We examined the effects of the poly (dA:dT) site from the NHE-1 gene when inserted into another promoter. The plasmid pXP-Tn-SV40 contained the oligonucleotides -174 to -149 of the mouse NHE-1 promoter. Mean relative luciferase values were over 18,000 for the control cells transfected with pSVLuc. The results showed that insertion of the poly (dA:dT) containing site resulted in a doubling of the luciferase activity from the SV40 promoter (**Fig. 3-5**).

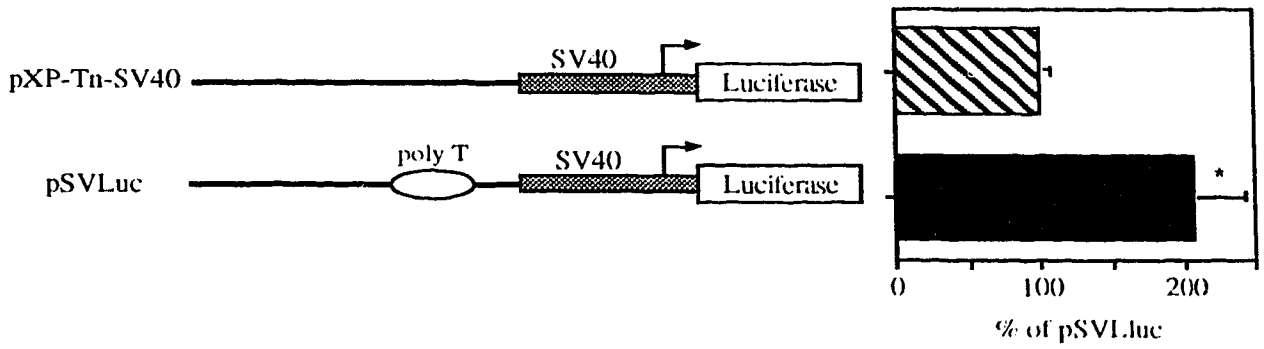

Fig. 3-5. Effects of insertion of the poly (dA:dT) rich region into the SV40 promoter. The poly (dA:dT) rich region (nucleotides -169 to -155) was inserted into the SV40 promoter as described in the "Experimental Procedures". The plasmids pXP-Tn-SV40 and pSVLuc were transiently transfected into L6 cells. Corrected luciferase values are shown. * Significantly different from the plasmid pSVLuc at $P < 0.05$.

To confirm the specific sequence of the poly (dA:dT) region was important we inserted several mutations into this region. The effect on NHE-1 promoter activity is shown in **Fig. 3-6.** The mutated form of the poly (dA:dT) region had the same activity as the deleted promoter. These results show that the effects of deletion of this region on activity of the gene are not due to simple reduction in the size of the promoter.

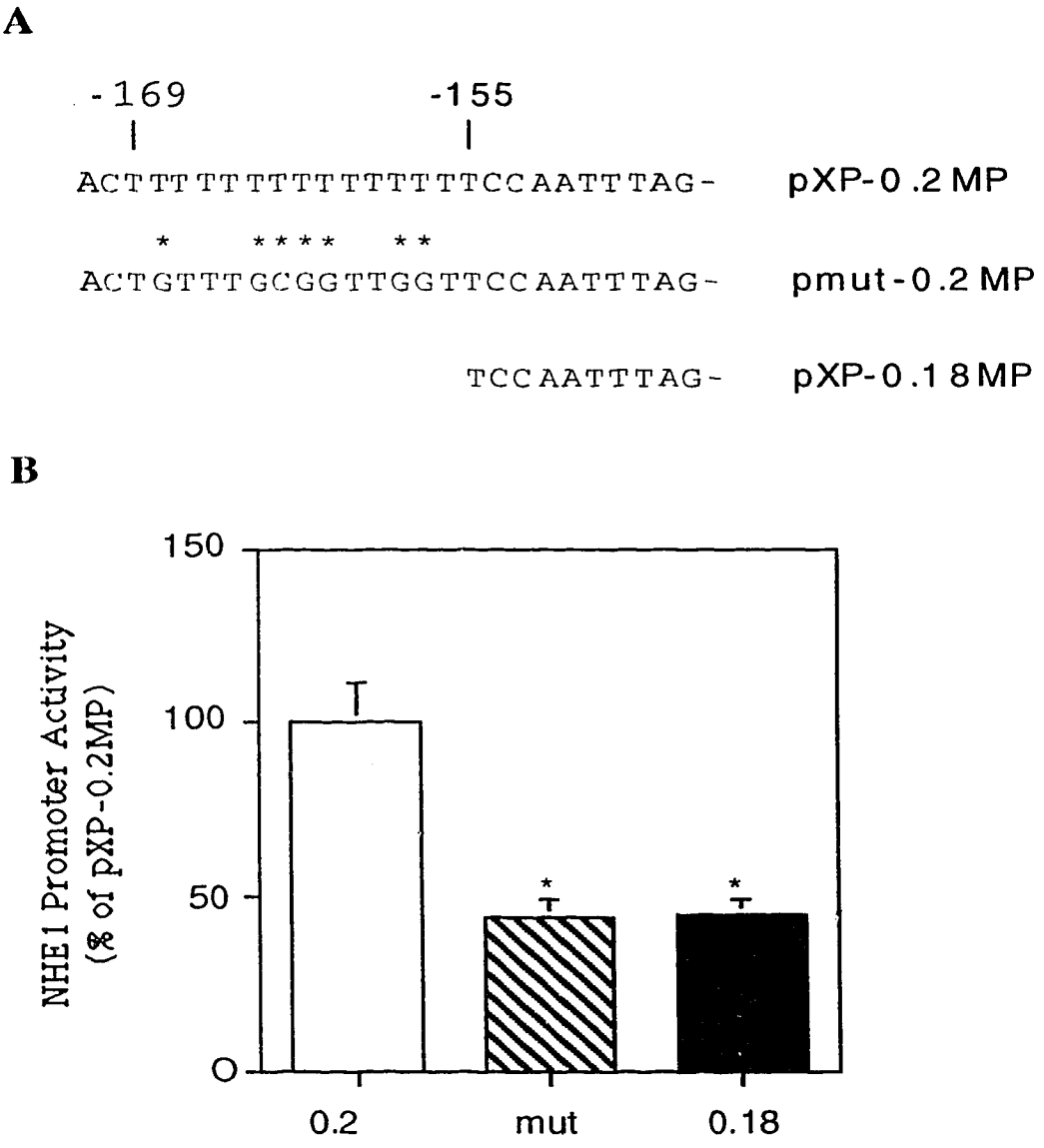

Fig. 3-6. Effect of disruption of the poly (dA:dT) site on activity of the NHE-1 promoter. A comparison was made of the wild type promoter with an intact poly (dA:dT) site, to the promoter with the poly (dA:dT) site deleted or mutated. A, comparison of the relevant regions of the promoter. The numbers correspond to the positions of the nucleotides relative to the first transcription initiation site. B, The levels of luciferase activity are shown for L6 cells transiently transfected with the plasmids pXP-0.2MP (0.2), pmut-0.2MP (mut) and pXP-0.18MP (0.18). Results are shown relative to the values

obtained for pXP-0.2MP. * Significantly different from the plasmid pXP-0.2MP at P <0.01.

To further compare the specificity of the binding between the poly (dA:dT) region and protein(s) in L6 cell nuclear extract, we used DNA mobility shift binding assay with both wild type and mutated version of the synthetic poly (dA:dT). The results are shown in **Fig. 3-7.** Lanes 1-4 are results for the wild type poly (dA:dT). Lanes 5-8 are for the mutated poly (dA:dT). Wild type poly (dA:dT) shows similar shift pattern to what we observed earlier (**Fig. 3-4**). However, the mutated poly (dA:dT) element failed to cause the same shift, in either presence or absence of the specific competitor.

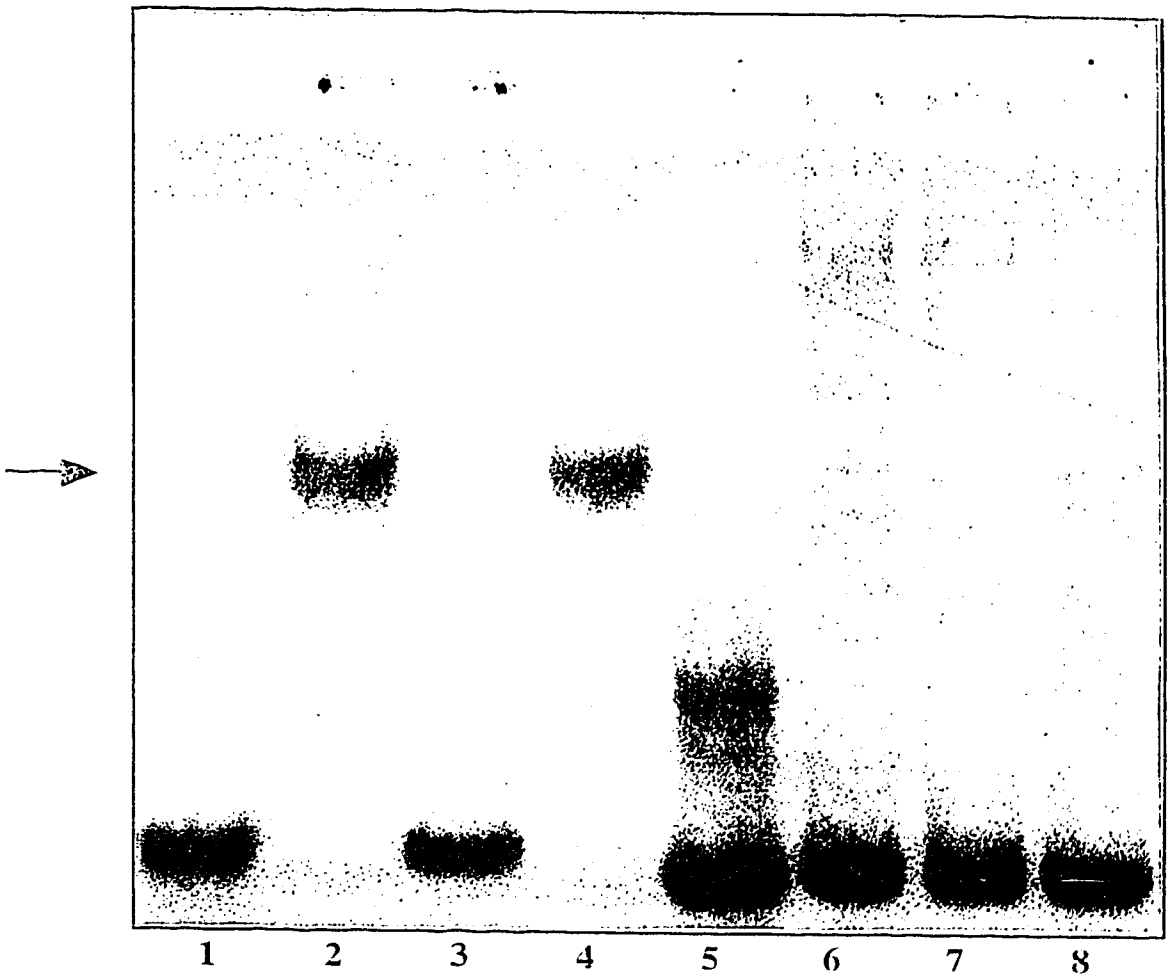

Fig. 3-7. DNA-mobility shift binding assay of the wild type and mutated poly (dA:dT). The assay was carried out as described in " Materials and Methods". Lane 1, labled poly (dA:dT) alone in the binding reaction. Lanes 2-4, 5 μg of nuclear extract added with no unlabled specific competitor (Lane 2), with 6.1μg of unlabled specific competitor (Lane 3), and with 2.5 μg of unlabled non-specific competitor (Lane 4). Lane 5, labled mutated poly (dA:dT) alone in the binding reaction. Lanes 6-8, 5 μg of nuclear extract added with no

unlabled specific competitor (Lane 6), with 6.1µg of unlabled specific competitor (Lane 7), and with 2.5 µg of unlabled non-specific competitor (Lane 8).

Discussion

Previous studies have found that there are several regions of potential importance in the NHE-1 promoter (7, 13-15). We have earlier shown that the transcription factor AP-2 is important in NHE-1 expression, especially during the process of cellular differentiation (7, 14). However our, and others' results (7, 13, 15) suggest that other regions of the gene are also important in NHE-1 expression. We had earlier (14) noted that essential features of the AP-2 containing region were conserved between different species. We therefore examined the different NHE-1 promoters of various species for regions of homology. We noticed the presence of a highly conserved region (**Fig. 3-1**) consisting predominantly of poly (dA:dT). To examine the importance of this region in L6 cells we constructed a series of plasmids. One contained the entire 1.1 kb mouse promoter, a second was constructed to include just passed the poly (dA:dT) containing region and a third did not contain the poly (dA:dT) region. Deletion of most of the promoter up to the poly (dA:dT) resulted in only a moderate loss of activity of 20-25%. However, removal of the poly (dA:dT) region resulted in a loss of approximately half of the activity of the promoter. These results suggested that this region of the NHE-1 gene is important for basal expression of the NHE-1 gene.

A variety of experiments suggest that the poly (dA:dT) element is important in NHE-1 expression. DNase I footprinting (**Fig. 3-3**) suggested that a protein or proteins of L6 cells nuclear extract does bind to this region. In addition, gel mobility shift analysis also suggested that this region of the gene does bind to a protein or proteins contained in the nuclear extracts. The binding was specific in that it was removed by competitor oligonucleotide but high concentrations of non-competitor had no effect. We also examined whether the poly (dA:dT) region could activate a foreign promoter. We inserted it into the SV40 promoter. The results (**Fig. 3-5**) showed that this poly (dA:dT) site could activate this promoter. Because the two plasmids, pXP-Tn-SV40 and pSVLuc, differed only in the presence of the poly (dA:dT) region, it was clear that this region plays a significant role in regulation of NHE-1 expression. Finally, to confirm that the sequence of the poly (dA:dT) region is important in stimulating expression of the NHE-1 promoter, we mutated several residues of the poly (dA:dT) sequence. The effect was to eliminate totally the stimulatory consequences of inclusion of this sequence, and the shifted band (**Fig. 3-4. & Fig. 3-7.**) as well.

Although we have noted the presence of poly (dA:dT) elements in a number of genes (not shown) there have been few studies on such elements. It has been noted that the actin gene of Dictyostelium contains one such elements that is important in regulation of this gene. In this case the element was 45 consecutive residues and functioned to promote the level of expression of the promoter (19). However in this study the region was not analyzed in detail and they did not examine binding of nuclear proteins to this region. Several studies have examined a related element with a poly (dA-dT) like structure. Such an element was also examined in the actin gene of Dictyostelium (19). Removal of the A/T element resulted in a 12-fold reduction in activity of the promoter. Similarly, deletion of the A/T element of the rice actin gene (20) reduced activity of this promoter. No specific protein was examined in this study also. However, one study (21) has noted that the Lux R protein is responsible for binding to a poly (dA-dT) region of the *Vibrio harveyi* Lux gene. The Lux R protein was a single polypeptide of Mr. 23,000 and bound to (A + T) rich regions of the luxC gene. Whether this protein is similar to what was binding to the poly (dA:dT) region of the NHE-1 promoter is not known at this time. However, it may be unlikely because the poly (dA-dT) region was markedly different from the homo poly (dA:dT) region of the NHE-1 gene. In addition it was found that while poly (dA-dT) was able to compete out Lux DNA binding in gel retardation assays, poly (dA)-poly (dT) was not. These results suggest that the protein involved with the NHE-1 gene is likely quite different from the lux-specific DNA-binding protein.

One possibility is that the poly (dA:dT) element functions by alteration of the structure of the promoter. It has recently been shown (22) that this type of structure can modulate expression of the yeast his3 promoter (22). Insertion of this element resulted in stimulation of activity of the promoter. The effect varied inversely with activity of the promoter. A more active promoter resulted in less stimulation by the element. Datin, a protein isolated from yeast and known as a poly (dA:dT) specific binding factor, was identified as a potential protein mediator of the effect however Datin functioned to inhibit expression. Analysis of the accessibility of the chromatin structure suggested that the site functions to improve the accessibility of the promoter. It was suggested that this could be due to destabilization of nucleosomes covering this region. While the analysis of this structure in yeast has been demonstrated, it is not yet known if the same sequence plays a similar role in humans. There are many long poly (dA:dT) tracts in a variety of genes which have this potential. Our results examined one such tract in the Na^+/H^+ exchanger promoter. They show for the first time, that one such tract is capable of modulating the

expression of a mammalian promoter. Future experiments are necessary to determine if the mechanism is analogous to that described in the yeast promoter (22).

References

1. Boron, W. F., and Boulpaep, E. L. (1983) Intracellular pH regulation in the renal proximal tubule of the salamander. *J. Gen. Physiol.* **81,** 29-52

2. Grinstein, S., Clarke, C. A., and Rothstein, A. (1983) Activation of Na^+ exchange in lymphocytes by osmotically induced volume changes a cytoplasmic acidification. *J. Gen. Physiol.* **82,** 619-638

3. Pouyssegur, J. (1985) The growth factor-activatable Na^+/H^+ exchange system: a genetic approach. *Trends Biochem. Sci.* **10,** 453-455

4. Rao, G. N., Sardet, C., Pouyssegur, J., and Berk, B. C. (1992) Na^+/H^+ antiporter gene expression increases during retinoic acid-induced granulocytic differentiation of HL60 cells. *J. Cell. Physiol.* **151,** 361-366

5. Horie, S., Moe, O., Miller, R. T., and Alpern, R. J. (1992) Long-term activation of protein kinase C causes chronic Na/H antiporter stimulation in cultured proximal tubule cells. *J. Clin. Invest.* **89,** 365-372

6. Krapf, R., Pearce, D., Lynch, C., Xi, X. P., Reudelhuber, T. L., Pouyssegur, J., and Rector, F. C. Jr. (1991) Expression of rat renal Na/H antiporter mRNA levels in response to respiratory and metabolic acidosis. *J. Clin. Invest.* **87,** 747-751

7. Dyck, J. R. B., and Fliegel, L. (1995) Specific activation of the Na^+/H^+ exchanger during neuronal differentiation of embryonal carcinoma cells. *J. Biol. Chem.* **270,** 10420-10427

8. Alvarez, J., Garcia-Sancho, J., Mollinedo, F., and Sanchez, A. (1989) Intracellular Ca2+ potentiates Na^+/H^+ exchange and cell differentiation induced by phorbol ester in U937 cells. *Eur. J. Biochem.* **183,** 709-714

9. Hazav, P., Shany, S., Moran, A., and Levy, R. (1989) Involvement of intracellular pH elevation in the effect of 1,25-dihydroxyvitamin D3 on HL60 cells. *Cancer Res.* **49,** 72-75

10. Vairo, G., and Hamilton, J. A. (1993) Evidence against a requirement for Na^+/H^+ exchange activity for proliferation or differentiation of HL 60 leukemic cells. *Cell Growth Diff.* **4,** 461-466

11. Ladoux, A., Cragoe, E. J., Geny, B., Abita, J. P., and Frelin, C. (1987) Differentiation of human promyelocytic HL 60 cells by retinoic acid is accompanied by an increase in the intracellular pH. *J. Biol. Chem.* **262,** 811-816

12. Miller, R. T., Counillon, L., Pages, G., Lifton, R. P., Sardet, C., and Pouyssegur, J. (1991) Structure of the 5'-flanking regulatory region and gene for the human growth factor-activatable Na/H exchanger NHE-1. *J. Biol. Chem* **266,** 10813-10819

13. Kolyada, A. Y., Lebedeva, T. V., Johns, C. A., and Madias, N. E. (1994) Proximal regulatory elements and nuclear activities required for transcription of the human Na/H exchanger (NHE-1) gene. *Biochim. Biophys. Acta.* **1217,** 54-64

14. Dyck, J. R. B., Silva, N. L. C. L., and Fliegel, L. (1995) Activation of the Na^+/H^+ exchanger gene by the transcription factor AP-2. *J. Biol. Chem.* **270,** 1375-1381

15. Yang, W., Dyck, J. R. B., Wang, H., and Fliegel, L. (1995) Regulation of the NHE-1 promoter in the mammalian myocardium. **In Press in Am. J. Physiol.,**

16. Blaurock, N. C., Reboucas, N. A., Kusnezov, J. L., and Igarashi, P. (1995) Phylogenetically conserved sequences in the promoter of the rabbit sodium-hydrogen exchanger isoform 1 gene (NHE1 SLC9A1). *Biochim. Biophys. Acta* **1262,** 159-163

17. Altenberg, G. A., Aristomuno, P. C., Amorena, C. E., and Taquini, A. C. (1989) Amiloride prevents the metabolic acidosis of a KCl load in nephrectomized rats. *Clin.Sci.* **76,** 649-652

18. Schreiber, E., Matthias, P., Muller, M. M., and Schaffner, W. (1989) Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. *Nucleic Acids Res.* **17**, 6419

19. Hori, R., and Firtel, R. A. (1994) Identification and characterization of multiple A/T-rich cis-acting elements that control expression from Dictyostelium actin promoters: the dictyostelium actin upstream activating sequence confers growth phase expression and has enhancer-like properties. *Nucl. Acids Res.* **22**, 5099-5111

20. Wang, Y., Zhang, W., Cao, J., McElroy, D., and Wu, R. (1992) Characterization of cis-acting elements regulating transcription from the promoter of a constitutively active rice actin gene. *Mol. Cell. Biol.* **12**, 3399-3406

21. Swartzman, E., and Meighen, E. A. (1993) Purification and characterization of a poly (dA-dT) lux-specific DNA-binding protein from Vibrio harveyi and identification as LuxR. *J. Biol. Chem.* **268**, 16701-16716

22. Iyer, V., and Struhl, K. (1995) Poly (dA:dT), a ubiquitous promoter element that stimulates transcription via its intrinsic DNA structure. *EMBO J.* **14**, 2570-2579

Conclusion

Conclusion

Cellular processes depend upon normal homeostasis. The level of intracellular pH (pHi), for example, affects cell proliferation, differentiation and in myocytes, contractility (1- 5). It has been shown that NHE-1, the major isoform of the Na^+/H^+ exchanger, is the principal regulator of pHi in most cell types (6). In addition to regulation by posttranslational level reported by others, our studies demonstrate that NHE-1 is also controlled at transcriptional level. In primary myocardium, the mouse NHE-1 promoter can be upregulated by serum but not by chronic acidosis. In non-differentiated skeletal muscle cell, we have found that there are two regions [AP2 site & poly (dA:dT)] of the promoter responsible for the basal regulation of NHE-1 gene expression. In addition, the same promoter shows increased activity in response to differentiation. However, neither of these two regions seems to be responsible for the increased activity in differentiated cells. More distal region of the promoter is most likely to be responsible for the increased activity in differentiated L6 cells. The data support the idea that gene expression of NHE-1, like modification of NHE-1 protein, is indeed an important part responsible for the regulation of its activity.

It has been reported that some cells are able to upregulate mRNA levels of the NHE so as to adapt to the chronic acid stimulation (7-9). In primary myocardium, we did not observe this adaptation of mRNA levels. In a previous study, increased NHE-1 mRNA was observed in this laboratory when rat hearts were subject to low-flow ischemia. The same result was not obtained when the hearts were subject to much more severe ischemia (10). As the mechanism involved in the increase in NHE-1 message in cardiomyocytes during ischemia has yet to be determined, it was suggested that severe ischemia may be detrimental to cells and thus abolish the adaptation observed under milder conditions (10). Although this is a reasonable explanation, more work has to be done before any conclusion can be made.

One element which was found to regulate NHE-1 expression was serum. Serum functioned to upregulate the activity of NHE-1 promoter in primary cardiomyocytes. It has been well known that NHE-1 activity is upregulated by mitogens in many cell types (11). Our data first shows that the NHE-1 promoter is also regulated in similar manner in primary cardiomyocytes. Considering the role NHE-1 plays in heart ischemia, it is of great significance to explore the mechanism governing the expression of NHE-1 gene in the intact myocardium in future.

L6 skeletal muscle cells can undergo differentiation at low concentrations of serum (12). Interestingly, it has also been shown that the regulation of the NHE varies between proliferating myoblasts and the differentiated myotubes, suggesting the important physiological changes in the NHE-1 protein and its function during differentiation (13). Studies with other cell types have again demonstrated that NHE-1 mRNA level increases when cells are undergoing differentiation (14-16). It has been suggested that the increased NHE activity may be important for differentiation to occur, at least in some cell types (17, 18). However, to date there have been no studies on the regulation of NHE-1 gene, the dominant isoform in skeletal muscle, during the process of differentiation. It was our belief that this study of muscle cell would provide valuable information for better understanding the role of NHE-1 in the differentiation process.

In our study on L6 cells, we have found that regulation of the NHE-1 promoter is different between differentiated and non-differentiated cells. In non-differentiated muscle cells, there are two elements of the promoter important for the regulation of NHE-1 expression level. One is the AP2 site containing region between base pairs -92 and -125 which has been previously shown to be critical in myocardium and other cells (19). Another one is the poly (dA:dT) region between base pairs -155 and 171 which is a novel finding.

In L6 cells we found that Poly (dA:dT) containing promoter (0.2kb) gives comparable activity to 1.1 kb promoter. Removal of the poly (dA:dT) or its mutation leads to less than half the activity of the promoter. DNA footprinting and DNA bandshift show protein(s) of L6 cell nuclear extract specifically bind to the poly (dA:dT) element. Mutated poly (dA:dT) fails to bind to the DNA binding protein(s). Insertion of the element upstream of the SV40 promoter demonstrates that the poly (dA:dT) can enhance the activity of a foreign promoter. This study suggests that the intrinsic poly (dA:dT) may be an important element involved in the regulation of the NHE-1 promoter activity. There have been few studies which directly examine the poly (dA:dT) element. One study in yeast indicated that a poly (dA:dT) itself can regulate promoter activity (20). Binding of the poly (dA:dT) to a known transcription factor Datin resulted in an inhibition of promoter activity (20). There is also a study indicating that the transcriptional factor(s) may be involved in the process (21). Our findings suggest that the poly (dA:dT) element is important in regulation of NHE-1 expression in non-differentiated L6 cells and that the regulation may be mediated by a transcriptional factor(s). Future studies may isolate and characterize the factor(s) involved.

It is well known that myoblast differentiation is programmed by members of the MyoD family and accompanied by transcriptional activation of a large set of muscle-specific genes (22). During differentiation, the muscle cell undergoes a dramatic change in morphology and function, from single nucleus myoblast to multinucleated myotubes (23). Although there is no any direct evidence indicating that cellular homeostasis like regulation of pHi plays a role in the differentiation, it is possible that pHi regulation is important for this process. It will be of great interest to observe how differentiation is related to the pHi regulation and regulation of NHE-1 gene expression.

Our data showed that there is a 2-3 fold increase in NHE-1 mRNA level when comparing differentiated and non-differentiated L6 cells. However, when stable transfectants of the cells undergo differentiation, we observed that there is an about 1.7 fold increase in activity of a 1.1 kb reporter plasmid. It is possible that some more distal element can account for at least part of the discrepancy between NHE-1 promoter activity and message levels. Also, the increased NHE-1 mRNA level may be a combination of an increase in promoter activity and a decrease in mRNA degradation. The element responsible for the increased promoter activity in differentiated L6 cells is most likely located somewhere upstream of poly (dA:dT) region (p.46, **Fig. 2-5**). An interesting extention of our current study is to purify the possible transcriptional factor(s) and to study the mechanism by which the factor(s) regulates promoter activity through interaction with poly (dA:dT). It is only then that we can better understand the possible interrelationships between the transcriptional factor(s) and the major pHi regulator: NHE-1.

References

1. Rao, G. N., Sardet, C., Pouyssegur, J., and Berk, B. C. (1992) Na^+/H^+ antiporter gene expression increases during retinoic acid-induced granulocytic differentiation of HL60 cells. *J. Cell. Physiol.* **151,** 361-366

2. Grinstein, S , Rotin, D., and Mason, M. J. (1989) Na^+/H^+ exchange and growth factor-induced cytosolic pH changes. Role in cellular proliferation. *Biochim. Biophys. Acta* **988,** 73-97

3. Ladoux, A., Cragoe, E. J., Geny, B., Abita, J. P., and Frelin, C. (1987) Differentiation of human promyelocytic HL 60 cells by retinoic acid is accompanied by an increase in the intracellular ph. *J. Biol. Chem.* **262,** 811-816

4. Jeffrey, F. M. H., Mallow, C. R., and Radda, G. K. (1987) Influence of intracellular acidosis on contractile function in the working rat heart. *Am. J. Physiol.* **253,** H1499-H1505

5. Katz, A. M., and Hech, H. H. (1969) The early "pump" failure of the ischemic heart. *Am. J. Med.* **47,** 497-502

6. Sardet, C., Franchi, A., and Pouysségur, J. (1989) Molecular cloning, primary structure, and expression of the human growth factor-activatable Na/H antiporter. *Cell* **56,** 271-280.

7. Akiba, T., Rocco, V. K., and Warnock, D. G. (1987) Parallel adaptation of the rabbit renal cortical sodium/proton antiporter and sodium/bicarbonate cotransporter in metabolic acidosis and alkalosis. *J. Clin. Invest.* **80,** 308-315

8. Dyck, J. R. B., Lopaschuk, G. D., and Fliegel, L. (1992) Identification of a small Na^+/H^+ exchanger-like message in the rabbit myocardium. *FEBS Lett.* **310,** 255-259

9. Moe, O. W., Tejedor, A. Levi , M., Seldin, D. W., Preisig, P. A., and Alpern, R. J. (1991) Dietary NaCl modulates Na^+/H^+ antiporter activity in renal cortical apical membrane vesicles. *Am. J. Physiol.* **260,** F130-137

10. Dyck, J. R. B., Maddaford, T., Pierce, G. N., and Fliegel, L. (1995) Induction of expression of the sodium-hydrogen exchanger in rat myocardium. *Cardiovasc. Research* **29,** 203-208

11. Dyck, J. R. B., and Fliegel, L. (1995) Specific activation of the Na^+/H^+ exchanger during neuronal differentiation of embryonal carcinoma cells. *J. Biol. Chem.* **270,** 10420-10427

12. Yaffe, D. (1968) Retention of differentiation potentialities during prolonged cultivation of myogenic cells. *Proc. Natl. Acad. Sci. USA* **61,** 477-483

13. Vigne, P., Frelin, C., and Lazdunski, M. (1985) The Na+/H+ antiport is activated by serum & phorbol esters in proliferating myoblasts but not in differentiated myoblasts. *J. Biol. Chem.* **260,** 8008-8013

14. Rao, G. N., Sardet, C., Pouyssegur, J., and Berk, B. C. (1992) Na+/H+ antiporter gene expression increases during retinoic acid-induced granulocytic differentiation of HL60 cells. *J. Cell. Physiol.* **151,** 361-366

15. Horie, S., Moe, O., Miller, R. T., and Alpern, R. J. (1992) Long-term activation of protein kinase c causes chronic Na/H antiporter stimulation in cultured proximal tubule cells. *J. Clin. Invest.* **89,** 365-372

16. Krapf, R., Pearce, D., Lynch, C., Xi, X. P., Reudelhuber, T. L., Pouyssegur, J., and Rector, F. C. Jr. (1991) Expression of rat renal Na/H antiporter mRNA levels in response to respiratory and metabolic acidosis. *J. Clin. Invest.* **87,** 747-751

17. Alvarez, J., Garcia-Sancho, J., Mollinedo, F., and Sanchez, A. (1989) Intracellular $Ca2^+$ potentiates Na^+/H^+ exchange and cell differentiation induced by phorbol ester in U937 cells. *Eur. J. Biochem.* **183,** 709-714

18. Hazav, P., Shany, S., Moran, A., and Levy, R. (1989) Involvement of intracellular pH elevation in the effect of 1,25-dihydroxyvitamin D3 on HL60 cells. *Cancer Res.* **49,** 72-75

19. Yang, W., Dyck, J. R. B., Wang, H., and Fliegel, L. (1995) Regulation of the NHE-1 promoter in the mammalian myocardium. **In Press in Am. J. Physiol.**

20. Iyer, V., and Struhl, K. (1995) Poly (dA:dT), a ubiquitous promoter element that stimulates transcription via its intrinsic DNA structure. *EMBO J.* **14,** 2570-2579

21. Hori, R., and Firtel, R. A. (1994) Identification and characterization of multiple A/T-rich cis-acting elements that control expression from Dictyostelium actin promoters: the dictyostelium actin upstream activating sequence confers grwoth phase expression and has enhancer-like properties. *Nucl. Acid. Res.* **22,** 5099-5111

22. Wright, W. E., Sassoon, D. A., and Lin, V. K. (1989) Myogenin, a factor regulating myogenesis, has a domain homologous to MyoD. *Cell* **56,** 607-617

23. Edmondson, D. G., and Olson, E. N. (1989) A gene with homology to the myc similarity region of MyoD1 is expressed during myogenesis and is sufficient to activate the muscle differentiation program. *Genes & Development* **3,** 628-640