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Isolation of a novel *ras*-recision gene that is induced by hydrogen peroxide from a mouse osteoblastic cell line, MC3T3-E1

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Abstract **Hydrogen peroxide appears to mediate growth factor actions, and it inhibits DNA synthesis in normal mouse osteoblastic cells (MC3T3-E1) at non-toxic doses. However the sensitivity of cells to H_2O_2 is greatly decreased in their *ras*-transformants. To understand the molecular basis of this sensitivity to H_2O_2, we attempted to identify H_2O_2-inducible cDNA clones from MC3T3 cells by differential screening of cDNA libraries, and one of such genes, named HIC-53, was isolated. The level of HIC-53 mRNA was moderately increased by H_2O_2 as well as by calcium ionophore or dexamethasone, but was not increased by the addition of serum, tumor promoting phorbol ester, or epidermal growth factor. Among mouse organs, HIC-53 mRNA levels were higher in the kidney and lung, but were almost undetectable in the brain, heart, bone, muscle or spleen. In MC3T3 cells transformed with v-Ki-*ras*, the HIC-53 mRNA level was markedly decreased, and effect of H_2O_2 was abolished. Although the biological function of HIC-53 is unknown at present, the predicted amino acid sequence exhibited some similarity with bovine cardiac Na^+/Ca^+ exchanger. The nucleotide sequence of HIC-53 cDNA showed no significant similarity with other known gene sequences.**

Key words: Hydrogen peroxide; *ras*-recision gene

1. Introduction

Active oxygen species are generally toxic to living cells because they modify and damage various biologically important molecules [1–3], but recent evidence indicates that active oxygen species at low doses act as mediators of cellular physiological responses [4–9]. Active oxygen species are produced from cultured cells upon stimulation with mitogens or tumor promoters [10,11], and non-toxic levels of hydrogen peroxide or superoxide anion induced DNA synthesis as well as the expression of the early response gene family [6,12,13]. Scavengers for free radicals inhibited gene expression induced by cytokines [14,15]. Furthermore, hydrogen peroxide generated from cells treated with TGF β1 was found to act as a second messenger for the transcriptional induction of egr-1 gene [8]. In addition to growth-related functions, active oxygen radicals seem to be involved in apoptosis that is prevented by the Bcl-2 gene product [16].

In signal transduction pathways of active oxygen species in mammalian cells, activities of several protein kinases [17] and protein phosphatases [18,19] were found to be regulated through redox-based mechanisms. DNA binding activity of nuclear transcription factors could be a target for redox regulation, and NFκB activity is known to be modulated by reactive oxygen species [4]. The transcriptional capacity of the bacterial OxyR protein is activated by direct oxidation [20], but there has been no direct evidence that mammalian transcription factors are directly regulated by active oxygen species. To elucidate the molecular basis of the actions of these active oxygen species, isolation of genes that are induced by them would give us a better understanding in the mechanisms involved. In this study, we have successfully isolated one such gene that showed differential characteristics in terms of induction profiles when stimulated with H_2O_2.

2. Materials and methods

2.1. Cell culture

Mouse osteoblastic MC3T3-E1 cells were cultured in Dulbecco's modified MEM (DMEM) supplemented with 10% fetal bovine serum in a humidified atmosphere of 5% CO_2/95% air. MC3T3 cells transformed with v-Ki-ras (clones K1, K2, K10) or v-Ha-ras (H2) were cultured similarly as described [21].

2.2. Measurement of DNA synthesis

Cells in logarithmically growing phase were plated in 35-mm dishes at a concentration of 5×10^5 cells/dish, and medium was changed one day later. DNA synthesis was measured by the incorporation of [^{3}H]thymidine (0.1 μCi/ml) after incubating for 12 h into acid-precipitable fractions. Radioactivity was determined in a liquid scintillation counter.

2.3. cDNA libraries and differential screening

Two cDNA libraries were constructed as follows: double-stranded cDNA was synthesized using poly(A)$^+$ RNA obtained from MC3T3 cells treated with 0.2 mM H_2O_2 for 4 h. cDNA library I was constructed by directional cloning using poly(dT) primer linked to synthetic *Xho*I and GAGA sequences using a kit (Strategene, Madison, WI) following the manufacturer's instructions. The cDNA was inserted into the *Eco*RI/*Xho*I site of λZAP. cDNA library II was prepared from double-stranded cDNA synthesized with random primer, and the cDNA fragment was inserted into the *Eco*RI site of λgt10 vector using an *Eco*RI adaptor (a kit from Pharmacia, Uppsala). Differential screening was carried out using ^{32}P-labeled cDNA synthesized with poly(A)$^+$ RNA from untreated MC3T3 cells or cells treated with 0.2 mM H_2O_2 for 4 h as described previously [22].

2.4. RNA extraction and analysis

Total RNA was extracted using the guanidium/hot phenol method [23], and samples of 20 μg of RNA/lane was separated on agarose gel containing 2 M formaldehyde. RNA was transferred onto a nylon membrane (Hybond-N, Amersham), and hybridized with molecular probes. Mouse α-tubulin cDNA (Mα1) was used as a reference to monitor the amounts of RNA in each lane.

2.5. DNA sequence

Nucleotide sequence was determined by dideoxynucleotide chain termination in conjunction with unidirectional deletion [24]. The sequences of both strands were determined.

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Accession Number of the sequence shown in this study is GenBank L43371.

SSDI 0014-5793(95)00957-4

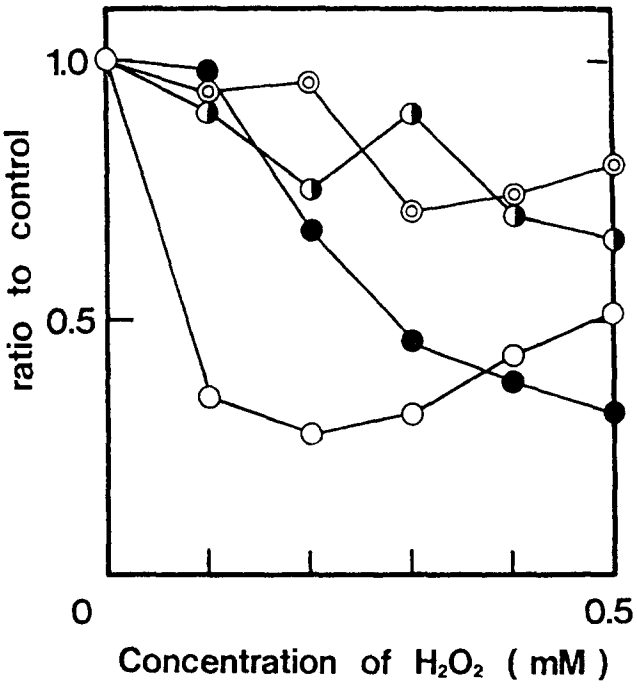

Fig. 1. Sensitivity of DNA synthesis to H_2O_2 in normal and transformed MC3T3 cells. Cells were treated with various concentrations of H_2O_2, and 12 h later, [^{3}H]thymidine was added. Cells were then incubated another 12 h. Acid-insoluble radioactivity was measured. ○, MC3T3; ⊙, ◑; Ki-ras-transformed K1, and K4, respectively; ● Ha-ras-transformed H2.

3. Results and discussion

3.1. Sensitivity of DNA synthesis to H_2O_2 in normal and transformed cells

We have previously shown the involvement of H_2O_2 in negative growth regulation by the action of TGF β1 [7]. Since transformed cells are generally refractory to negative growth regulation, the sensitivity of normal and transformed cells to H_2O_2 was compared. Normal MC3T3-E1 and *ras*-transformed cells in the logarithmic phase were treated with low doses of H_2O_2 for 12 h, and [^{3}H]thymidine incorporation was measured. As shown in Fig. 1, normal cells were more sensitive to H_2O_2 than the transformed cells, and as low as 0.1 mM greatly inhibited DNA synthesis in normal cells. In contrast, DNA synthesis in the *ras*-transformed K1 and K4 cells were much more refractory to H_2O_2. Cell viability as assessed by the dye-exclusion test was more than 95% in every case, but colony forming ability decreased significantly above concentrations of 0.3 mM H_2O_2 in both normal and transformed cells. These results indicate that non-toxic doses of H_2O_2 inhibited DNA synthesis in normal cells, but not in *ras*-transformed cells.

3.2. Isolation of H_2O_2-inducible cDNA clone

To get some insights on the molecular mechanism of the effects of H_2O_2, we have been trying to isolate cDNA clones that were induced by H_2O_2 using differential screening. ^{32}P-labeled cDNA probes were prepared from poly(A)$^+$ RNA of untreated and H_2O_2-treated MC3T3 cells. After screening about 2×10^5 independent phage clones of the library I, one candidate clone named HIC-53, which exhibited a stronger signal with the probe synthesized from H_2O_2-treated cells, was isolated. Northern blot analysis indicated that the HIC-53 mRNA level was modelately induced by 0.2 mM H_2O_2, reaching a peak at about 2 h after induction (Fig. 2).

HIC-53 expression was examined in cells stimulated with various agents, and level of HIC-53 mRNA was found to be increased by calcium ionophore A23187 and dexamethasone, but not by the addition of serum, phorbol 12-myristate-13-acetate, or epidermal growth factor (data not shown). It was also not affected by DNA-damaging agents such as mitomycin C or adriamycin (data not shown), and this characteristic is distinct from *gadd* genes whose expression is increased in cells irradiated with γ-ray [25]. DNA damage caused by H_2O_2, thus, may not participate in HIC-53 expression.

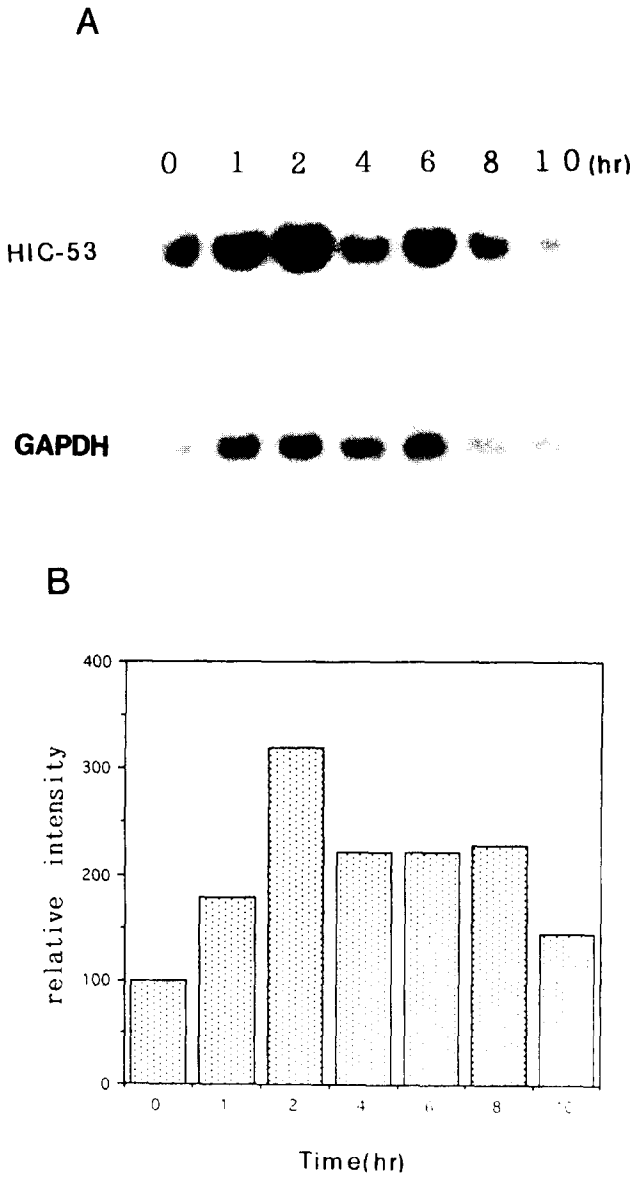

Fig. 2. HIC-53 mRNA levels in H_2O_2-treated cells. (A) MC3T3 cells were treated with 0.2 mM H_2O_2 for various periods of time, and RNA was extracted. Twenty μg/lane of total RNA was run on a gel, transferred to a membrane, and hybridized with ^{32}P-labeled HIC-53 or reference glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA probes. (B) Relative HIC-53 mRNA levels normalized with GAPDH as determined by densitometric scanning of autoradiograms.

Tissue distribution of HIC-53 mRNA was examined using total RNA from mouse organs. The HIC-53 mRNA level was high in the kidney and lung, while it was almost undetectable in brain, bone, muscle or spleen, even though HIC-53 was originally isolated from osteoblastic cells (Fig. 3).

3.3. Levels of HIC-53 mRNA in transformed cells

We have previously isolated a set of TGF β1-inducible cDNA clones, and found that among 6 clones isolated, mRNA levels of two of them (TSC-36 and -160 identical to *rrg* [26]) were decreased significantly [27]. Since H_2O_2 seems to be released from TGFβ1-treated cells and participates in the signal transduction pathways of TGFβ1, at least in part [8], we next examined the level of HIC-53 in *ras*-transformed cells. Total RNAs from normal MC3T3 cells or *ras*-transformed K1, K4, and H2 cells in their pre-confluent states were used to analyze HIC-53 mRNA levels. The results shown in Fig. 4 clearly show that basal level of HIC-53 was markedly decreased in the transformed cells, and that was not induced by H_2O_2.

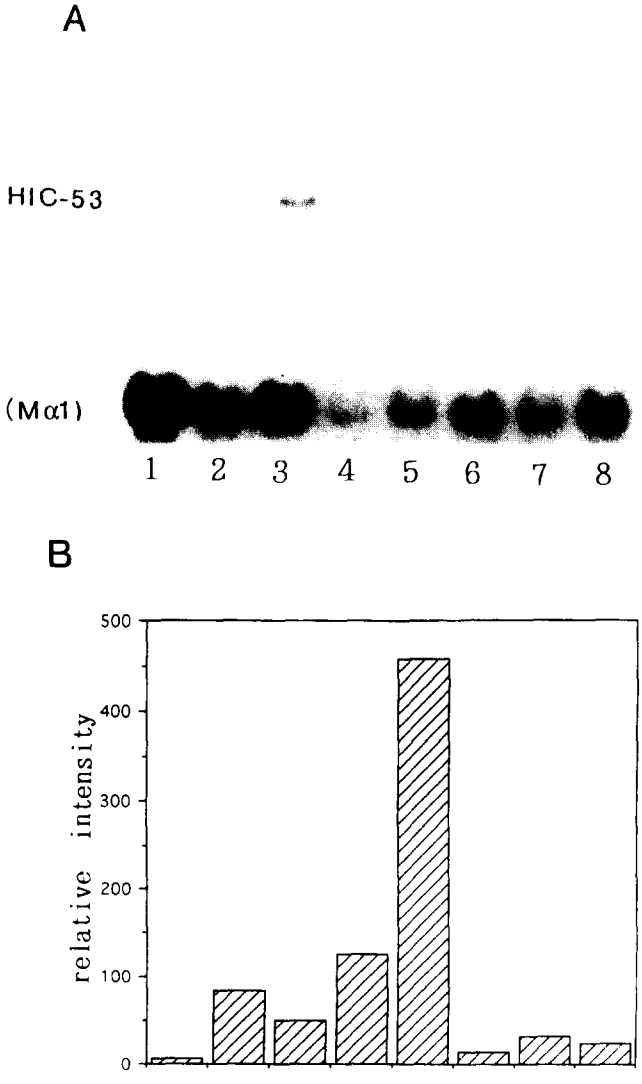

Fig. 3. Tissue distribution of HIC-53 mRNA in mouse organs. (A) Total RNA was extracted from various mouse organs, and 20 μg/lane of RNA was run on a gel. (B) Relative HIC-53 mRNA levels normalized with Mα1 (mouse α tubulin) as determined by densitometric scanning of autoradiograms. 1 = brain; 2 = heart; 3 = lung; 4 = liver; 5 = kidney; 6 = bone; 7 = muscle; 8 = spleen.

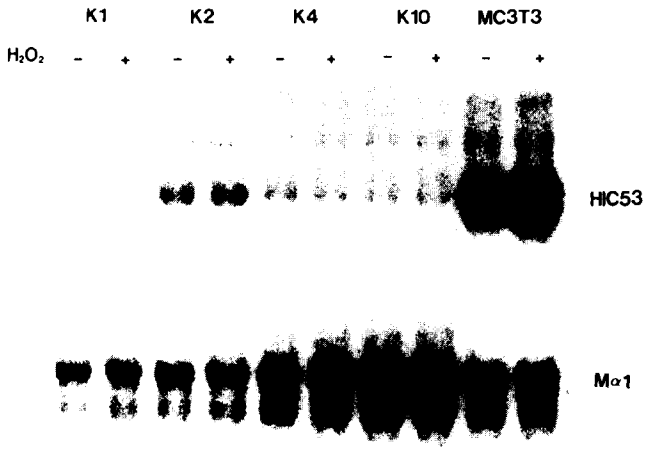

Fig. 4. HIC-53 mRNA levels in normal and transformed MC3T3 cells. Cells were either untreated or treated with 0.2 mM H_2O_2 for 4 h, and total RNA was extracted. Levels of HIC-53 mRNA were determined as described in Fig. 3.

3.4. Nucleotide sequence of HIC-53 cDNA

The original cDNA clone of HIC-53 isolated by differential screening of library I contained a fragment of about 700 bp fragment. This cDNA fragment was then used to screen library II, which was prepared using random primer to isolate the full-length cDNA. Several overlapping positive clones were isolated after extensive screening, and the sequence obtained from these clones is shown in Fig. 5. The determined nucleotide sequence of the constructed cDNA has about 1400 bases, but this size is smaller than that of its mRNA (about 2000 bases by Northern blot). We screened the cDNA libraries I and II extensively, but were unable to obtain any longer 5'-end of the HIC-53 cDNA, possibly due to unknown higher order structures of mRNA or for technical problems.

A search of the GenBank Data Base (release 73) revealed no significant similarity of the nucleotide sequence of HIC-53 with known gene sequences. Even this sequence of the isolated HIC-53 cDNA contains no initiation codon, but we predicted that the longest open reading frame as shown in Fig. 5A might share some similarity with bovine cardiac Na^+/Ca^{2+} exchanger [28] based on homology search of NBRF data base. The region that showed similarity includes the putative transmembrane domain (Fig. 5B), suggesting that HIC-53 may encode a membrane protein, but further study is required to verify this point.

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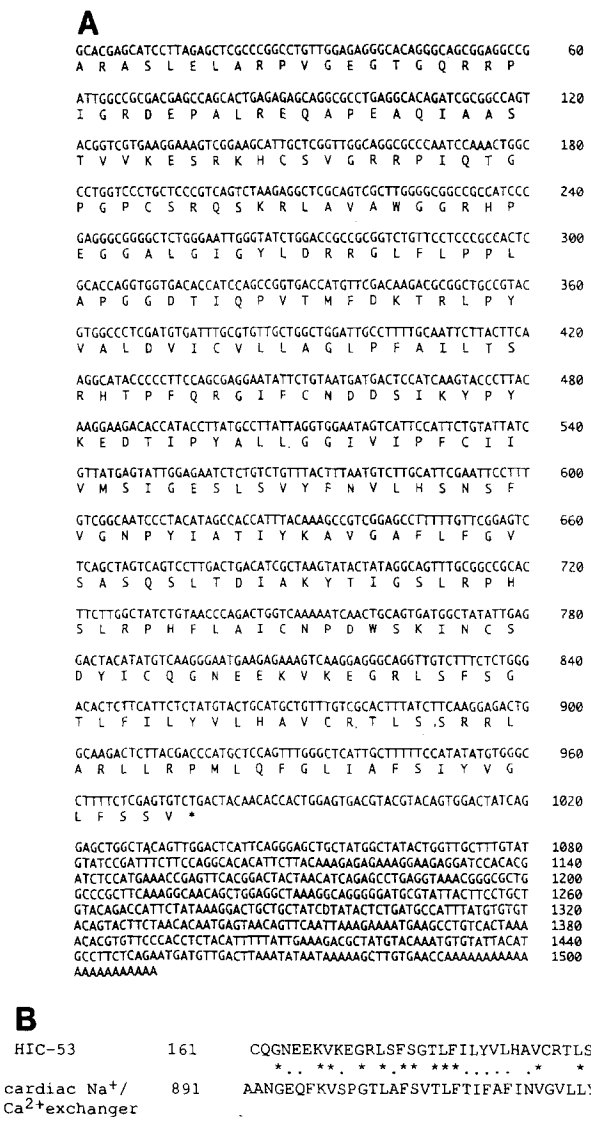

Fig. 5. Nucleotide sequence of HIC-53 cDNA. (A) Nucleotide sequence and putative open reading frame. (B) The region that showed similarity with cadiac Na^+/Ca^{2+} exchanger [28].

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