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Hsp60/CHAPERONIN GENE EXPRESSION AND DIFFERENTIATION OF HUMAN COLON ADENOCARCINOMA AND MULTIPOTENT LEUKAEMIC CELLS

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SUMMARY: Elevated mitochondrial gene expression is an early event in the switch from proliferation to differentiation of the human colon adenocarcinoma cell line, HT29, promoted by trehalose replacement of exogenous glucose (1). Here we report the isolation and elevated expression of *hsp60*, the gene encoding chaperonin, a mitochondrial protein required for assembly of mitochondrial and cellular proteins. In contrast to HT29, leukaemic cells (HL60 and K562) neither differentiated nor altered their mitochondrial gene expression after treatment with trehalose. However, differentiation of these cells, as promoted by 12-*O*-tetradecanoylphorbol-13-acetate actually resulted in decreased levels of *hsp60* mRNA expression as well as mitochondrial RNA expression, suggesting significant differences in involvement of mitochondria in the differentiation of these cell lineages.

The switch from proliferation to differentiation of HT29, a human colon adenocarcinoma cell line, can be controlled by use of various inducing agents (1, 2), thus making it possible to determine the early molecular events leading up to expression of goblet-like and absorptive cell phenotypes. An early event in this process which is promoted by trehalose (α-D-glucopyranosyl--α-D-glucopyranoside), and by other inducers such as butyrate and galactose, is the marked increase in levels of mitochondrial RNA encoding various enzyme subunits (1). The possible importance of mitochondrial involvement in HT29 differentiation is further shown here by our finding that *hsp60*/chaperonin (3) mRNA, is also expressed at a high level. Since *hsp60* is important in post-translational assembly, stabilization, and translocation of mitochondrially associated proteins (4), we tested the possible generality of trehalose's use to induce *hsp60*, mitochondrial DNA expression and differentiation of two multipotent leukaemic cells lines, HL60 and K562. These results indicate significant differences in the patterns of *hsp60* and mitochondrial gene expression in these cell lines and HT29.

MATERIALS AND METHODS

Cell culture and treatment: HT-29 cells were grown in glucose-free Dulbecco's modified Eagle's Medium (DMEM, Gibco BRL) with supplements (25 mM glucose, 10 % fetal bovine

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serum (v/v) and 40 μg/ml gentamicin) at 37°C and 8 % CO_2. For nutrient shift experiments approximately 10^7 cells, grown in 80 cm^2 flasks with glucose-containing medium, were subsequently incubated for 6 days in trehalose-containing medium. Cells were harvested following trypsin-EDTA treatment and frozen at -75°C until use. Promyelocytic HL60 (5) and multipotent human haematopoietic K562 (6) cells were grown in RPMI 1640 medium and supplements (10% fetal bovine serum and 100 u/ml penicillin, 100 μg/ml streptomycin and 250 ng/ml Fungizone, BRL) at 37°C and 5 % CO_2. Cells were transferred every 3 days to fresh medium. To induce differentiation of HL60 cells towards adherent macrophage cells (7) and K562 cells towards the megakaryocytic/monocytic cells (8), 5 x 10^6 cells of each type were seeded in 10 ml medium containing 160 nM 12-*O*-tetradecanoylphorbol-13-acetate (TPA, Sigma) and harvested after 48 h.

Construction and screening of cDNA libraries: A cDNA λgt 10 recombinant library was prepared using RNA from 6 day-old HT29 cells grown in trehalose as described earlier (1). For differential screening of this cDNA library, duplicate copies of about 1500 plaques (cDNA clones) per 85 mm agar plate were made using Hybond nylon membrane filters (Amersham). Filters were then hybridized to ^{32}P-labelled cDNA made from RNA of cells treated for 6 days with either glucose (GLU) or trehalose (TRE) in the medium. After hybridization and washes, filters were exposed to Kodak X-Omat film and resultant autoradiographs were compared visually to pick plaques which hybridized differently to the two probes. Inserts of λgt10 clones were amplified *in vitro* with custom λgt 10 primers (New England Biolabs) and AmpliTaq polymerase (Perkin-Elmer) as described elsewhere (9). Inserts were subcloned into the *Sma* I site of the Bluescript vector. DNA sequencing was carried out using Sequenase (USB Corp.). Nucleotide sequences were compared with EMBL\GenBank databases using the FASTA program in the GCG package (Version 6) from the Wisconsin University (10).

Quantitative RNA analysis: Total RNA was extracted from cells (10^8 cells/sample) by the guanidium isothiocyanate/CsCl centrifugation method (11). RNA concentrations were estimated by O.D. 260 nm and standardized against content of elongation factor EF1α mRNA (1). Each sample of total RNA (12 μg) was dissolved in 20 μl of 44% formamide, 20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, 6 % formaldehyde, 0.25% bromophenol blue, 0.25% xylene cyanol FF pH 7.0 and heated 10 min at 70 °C before fractionating in a 1% agarose gel with 20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA pH 7.0 and 2 % formaldehyde. After electrophoresis at 2 V/cm for 18 hours the RNA was electrotransferred (Hoeffer Sci. Instruments) to Nytran membranes in 40 mM Tris-acetate, 1 mM EDTA at 4°C for one hour at 15 V, followed by three hours at 45 V. Prehybridizations were done in 6 x SSC, 5 x Denhardt's solution, 0.5% SDS and 100 μg/ml denatured salmon sperm DNA for 2 hours. Hybridizations were for 18 hours in the same solution with 5 x 10^6 - 10^7 cpm/ml of random primed probe. Hybridized membranes were washed for 15 min at 23°C using 2 x SSC, 0.2 % SDS and twice for 30 min at 65°C in 0.2 x SSC, 0.2 % SDS before exposing to Kodak X-Omat films. Membranes were reused for EF1α RNA measurements after washing for 30 min at 95°C in 0.2 x SSC, 0.2 % SDS. Autoradiographs were analyzed using a computing scanning densitometer (Model 300A, Molecular Dynamics) with software (ImageQuant version 3.0).

RESULTS

Isolation and identification of hsp60 cDNA clone: We have been using differential hybridization as a means for obtaining cDNA clones which encode abundant, preferentially expressed mRNA obtained from HT29 cells induced to differentiate in the presence of exogenously added trehalose (1). So far the majority of cDNA clones of this category have been found to encode mitochondrial RNA sequences (ref. 1 and Lu, X., MacManus, J.P. and Seligy, V.L., Manuscript in preparation). The clone DP201 which was eventually shown to encode *hsp60* sequence, was one of four candidate cDNAs detected by this hybridization method as well as not having mitochondrial DNA sequence. However, as shown in Fig.1 (lanes a, a') only DP201

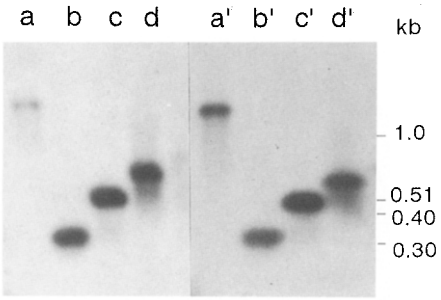

Fig. 1. **Autoradiogram of *in vitro* amplified cDNA inserts from λgt10 cDNA clones selected by differential hybridization.** See Methods for details. Products of individual cDNA clones were electrophoresed, blotted and hybridized to ^{32}P-labelled cDNA made from RNA of cells grown in glucose (left panel, lanes **a** - **d**) or trehalose (right panel, **a'**- **d'**). Lanes **a** and **a'** contain cDNA from DP201 (*hsp60*). Position of 1 kb ladder size markers (BRL) are indicated on right.

exhibits a significant quantitative difference when hybridized with cDNA made from RNA of trehalose treated cells. The restriction map (Fig.2) and nucleotide sequence (data not shown) of this 2.2 kb cDNA, when searched for identity in GenBank/EMBL databases established a 99.7% homology with the human chaperonin (*hsp60*) cDNA sequence reported by Jindal and co-workers (3). Furthermore, the size of the mRNA (Fig. 3A) which was probed using radiolabelled DP201 cDNA is also in good agreement with the expected 2.2 kb *hsp60* transcript. Quantitation of the amount of radiolabelled DP201 cDNA that hybridized to this mRNA in total RNA from glucose and trehalose grown cells indicated a 4 to 5 fold increase in *hsp60* mRNA in trehalose grown cells (Fig. 3B). The increased expression of *hsp60* is consistent with the

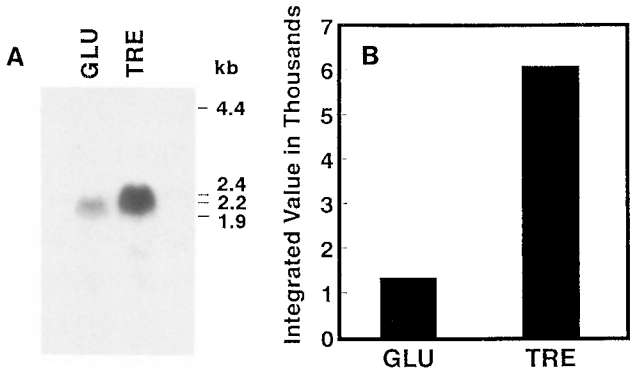

Fig. 2. **Estimated size and expression level of HT29 derived *hsp60* mRNA. A**, Autoradiograms of DP201 cDNA hybridized to electrophoretically fractionated total RNA from cells grown in glucose (GLU) or trehalose (TRE) for 6 days. **B**, Quantitation of autoradiograms were done using a laser densitometer. Increase in *hsp60* mRNA is estimated to be ≥ 400%.

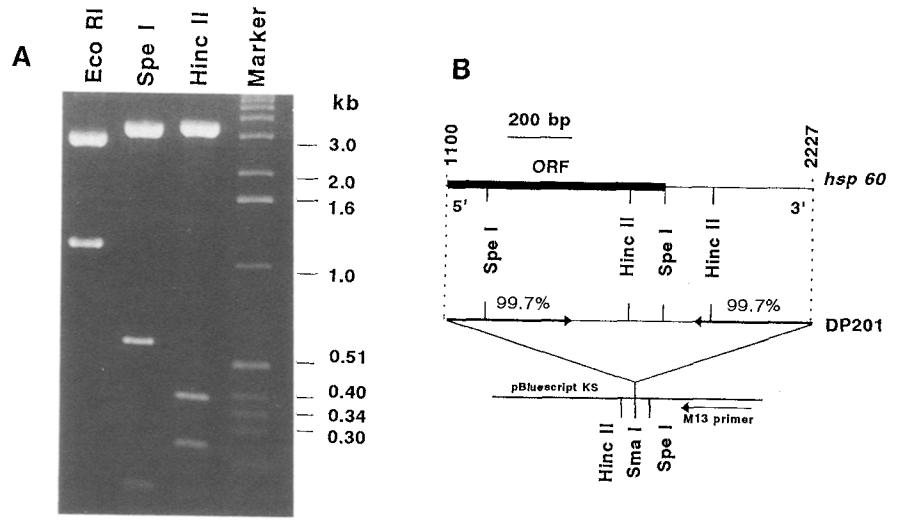

Fig. 3. **Identification of cDNA clone DP201 as human hsp60/chaperonin. A**, Ethidium bromide stained restriction fragments of DP201 separated in 1% agarose. **B**, Matching restriction site map of *hsp60*, chaperonin cDNA published by Jindal et al. (3) showing extent of sequence overlap and identity (99.7%). *Hsp60* sequence (Accession No. M22382) was retrieved from EMBL/GenBank. Bold arrows located at either end of DP201 show extent of sequence obtained and matched. DP201 cDNA contains the *hsp60* open reading frame (ORF, codon 15 to 573) and the 461 nt 3' UTR.

increase in mitochondrial RNA expression which also occurs during the switch from HT29 proliferation to expression of the differentiation phenotype (1).

Effects of trehalose and TPA on leukaemic cell differentiation: To determine whether trehalose can effect differentiation of cell types other than of colonic origin, similar experiments were carried out using K562 (6), a multipotent haematopoietic cell line and HL60 (5), a promyelocytic cell line. Both cell lines respond to a number of compounds, including the phorbol ester, TPA, which induce a cohort of differentiation phenotypes (7, 8). Incubation of these cells with medium containing no sugar other than 25 mM trehalose for up to 6 days, produced no major changes in either their growth pattern or their morphologies. Yields of total RNA from 2-day and 6-day cultures of these cells, containing either glucose or trehalose were also comparable. When probed with ND4, the mitochondrial gene encoding NADH dehydrogenase subunit 4 (1), the ND4 levels which are normally increased by 8 fold in HT29 cells after 2 to 3 days of treatment with trehalose (1), remained fairly constant in either cell line relative to elongation factor EF1α mRNA content (Fig.4A). Since no changes in either *hsp60* transcript size or levels of expression were observed (relative to glucose cultures), these results demonstrate that trehalose does not have an adverse affect on these cells and may act selectively on colonic cells. In contrast to these results are those obtained by treating HL60 and K562 cells with TPA which promotes changes in growth rate and morphology of these cells. As

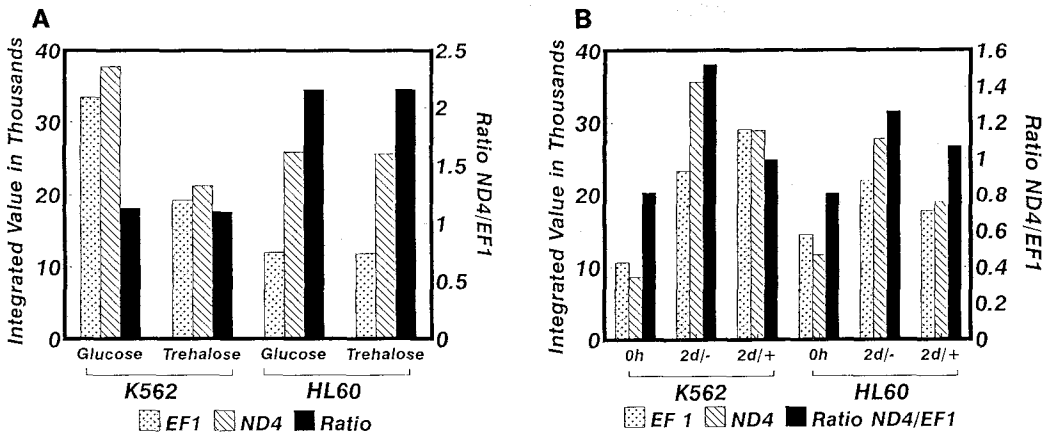

Fig. 4. **Expression of NADH dehydrogenase subunit 4 (ND4) mitochondrial RNA in leukaemic cells after various treatments. A**, Quantitation of autoradiograms resulting from hybridizations of total RNA from K562 and HL60 cells incubated in 25 mM glucose or trehalose containing medium. First probe was cDNA clone encoding ND4; second hybridization was done using EF1α cDNA (1). **B**, Similar quantitation as in **A**, but using total RNA from K562 and HL60 cells at 0 hours and 2 days of no TPA (2d/-) and 160 nM TPA (2d/+). See Methods and ref.1 for details.

shown in Fig. 4B, ND4 RNA expression decreased approximately 20% after 2 days exposure to TPA, and *hsp60* mRNA expression was decreased by at least 60% (Fig. 5 A and B). The decrease in *hsp60* expression is consistent with the observed drop in ND4 RNA expression and unlike the results from HT29 differentiation studies.

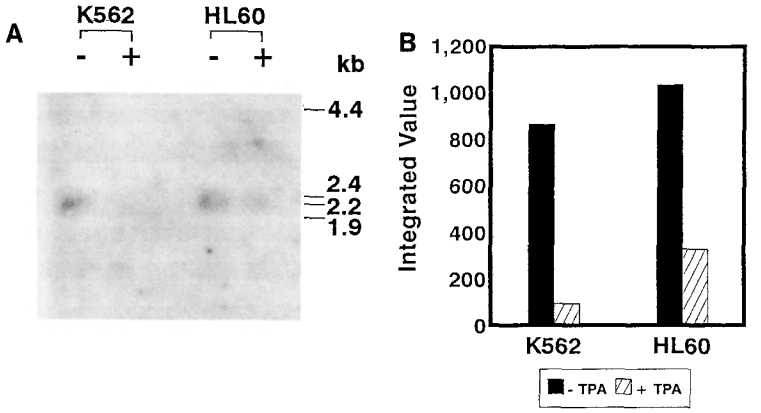

Fig. 5. ***hsp60* mRNA expression levels in TPA treated multipotent leukaemic cells.** RNA was isolated from K562 and HL60 cells after 6 days of culture in absence (-) or presence of TPA (+). RNA was separated as described in Fig.2 and probed with DP201 cDNA. After autoradiography of hybridization filters (**A**), autoradiograms were quantitated (**B**).

DISCUSSION

Since mitochondria play a vital, multifunctional role in cell homeostasis, measurment of its activity, as reflected by quantitative changes in specific mitochondrial RNA levels should prove useful for appraising metabolic changes required for the alternation between cell proliferation and differentiation. The discovery that HT29 adenocarcinoma cells exhibit significant, upward expression of its mitochondrial DNA in response to several known inducers of goblet and enterocyte-like differentiation indicates the possible involvement of regulated, mitochondrial activity in the process of colonic cell differentiation (1). The present finding that the *hsp60* gene expression is also elevated in differentiated HT29 cells is consistent with the former observations and the proposed function of its product. *Hsp60* is a matrix associated, mitochondrial homolog of the chaperonin family (12, 13, 14) whose demand may be expected to increase if mitochondrial expression is required for differentiation-dependent alteration of HT29 metabolism and cytoarchitecture. However, since *hsp60* is essential for cell survival, and is expressed in response to heat shock-inducing temperatures as well as to a variety of physiological and metabolic stress-related conditions (4), it is not known if elevated *hsp60* expression is in HT29 cells which are committed to a specific differentiation pathway such as goblet-like cells(1) or in cells which have undergone severe metabolic stress leading to death. In yeast, glucose represses expression of *hsp60*, like many other nuclear genes which encode mitochondrially associated proteins (15), and replacement of exogenous glucose with galactose results in a 4 to 5-fold increase in cellular *hsp60* concentration within several hours (13). In HT29, removal of exogenous glucose (no sugar) triggers a marked decrease in growth as well as an increase in morphological differentiation (16) and mitochondrial-DNA expression (1). However, expression levels of mitochondrial DNA and goblet-like cells are even further increased when glucose is substituted with galactose, butyrate and trehalose (in order of increased effect) (1). At least part of this increased response may be linked to the use of alternate substrates (1, 17) It may be useful to study the expression of other heat-shock and glucose response genes when HT29 is triggered to differentiate by various inducers.

It is possible that tumour cells derived from different cell lineages do not have the same metabolic requirements for maintenance of their proliferation and differentiation states. In the present study trehalose was found to have little or no effect on HL60 and K562 cell growth, differentiation and mitochondrial DNA expression. These results suggest that this sugar does not unduly stress these cells. However, exposure of these cells to TPA, which induces their differentiation, results in a decrease in both *hsp60* mRNA and mitochondrial RNA (ND4) levels. The reason for this drop in *hsp60* expression is not known, but the same observation in HL60 was made recently by Shakoori et al. (18). They also found that *hsp60* mRNA expression was less in differentiated osteoblasts. Their studies further indicate lineage-specific differences in expression patterns of other heat shock proteins such as *hsp70* and *hsp27*, and suggest that differences in *hsp60* expression are consistent with the respective levels of metabolic activities

required by these cells for their functions. Further understanding of *hsp60* involvement in proliferation and differentiation may be possible through the analysis of *hsp60* promoter interactions with regulatory proteins that occur in HT29 and HL60 cells, respectively.

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