Elevated Expression of the Human Mitochondrial Hinge Protein Gene in Cancer

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Abstract

The steady-state level of mitochondrial hinge protein (hinge) RNA is elevated in various cell lines established from human tumors compared to that in normal cells or cells treated with mitogen. Sequences upstream of the hinge gene were cloned and determined. A number of potential protein factor binding DNA elements are present in this region, one of which may possess transcription enhancer-like properties for respiratory chain genes. This element is actually located within the highly repetitive human Alu sequences.

Introduction

The mitochondrial hinge protein of Mr 9000 is a component of complex III in the OXPHOS system (1). This system consists of five complexes, four of which are assembled from protein subunits encoded by nDNA and miDNA genes. nDNA codes for more than 100 of these subunits; miDNA codes for 13. Expression of these genes in response to demands on energy metabolism must in some ways be coordinated so that a proper stoichiometry of the subunits is maintained (2, 3).

In our studies, the technique of subtractive cDNA cloning was used to isolate gene sequences differentially expressed by two human prostate cell lines (4) and sequences differentially expressed between cancerous and normal prostatic cells. Results from the latter study showed that the expression of a subtracted cDNA, pc29, as indicated by the level of its RNA, is elevated in cancerous prostate. pc29 encodes the mitochondrial hinge protein. The hinge protein is nuclear encoded, and its DNA sequence was reported by Ohta et al. (5) 6 years ago. Here, we show that an elevated level of hinge RNA is also detected in cultured cells established from tumors of nonprostatic tissues and not in normal cells. The higher level of hinge RNA in transformed cells corroborates the observation that neoplasia is associated with the induction of OXPHOS genes (6). A corollary to that is the observation that the expression of hinge is lowered in human promyelocytic HL60 cells induced to differentiate in vitro (5).

Given the fact that the expression of the hinge gene responds to the state of cellular differentiation, we set about to isolate sequences upstream of the pc29 gene with the aim of identifying DNA elements that might govern this regulation. Once identified, experiments are carried out to characterize the interaction between them and their binding factors.

Materials and Methods

Subtractive cDNA Libraries. cDNA libraries were generated from an NP and a CP. The normal prostate was obtained from a car accident victim and the cancerous prostate from a patient's resected tumor. The tumor was scored by a Gleason grade of 1 + 2 and composed of well differentiated, diploid prostatic cells. There was no penetration of the glandular capsule and no evidence of metastasis. Subtractive cDNA libraries of NP minus CP and CP minus NP were created as described (4). Subtractive clones representing differentially expressed genes were chosen on the basis of their unequal frequency in the NP and CP libraries. One such clone, pc29, from CP minus NP, was found to encode the human mitochondrial hinge protein. The cdNA was incomplete, and a full-length version was isolated from a prostate cell line library (4) by the PCR method (7). One of the primers used was derived from pc29 coding sequences and the other from placid vector sequences next to the cdNA insert.

Human Cells. Cell lines were obtained from the American Type Culture Collection (Rockville, MD). LoVo, metastatic colon carcinoma, was cultured in Ham's F-12. HeLa, carcinoma of the cervix, was cultured in Eagle's minimum essential medium. MOLT4, T-cell leukemia, was cultured in RPMI 1640. K562, chronic myelogenous leukemia, was cultured in RPMI 1640. PBL were obtained from a male donor. The WBC were concentrated by centrifugation over histopaque (8), washed, and resuspended in PBS. PBL were seeded in RPMI 1640 with 5 μg/ml phytohemagglutinin and cultured for 48 h. Human fibroblasts from amniotic fluids (R. Barathur; generous gifts of Roche Biomedical Laboratories) were cultured in RPMI 1640. All media were supplemented with 10% fetal bovine serum.

3P-labeled cDNA Probes. The techniques used are found in Ref. 9. Cellular RNA was prepared by the LiCl-urea method. Polyadenylated RNA was selected by oligodeoxycytidylic acid-cellulose chromatography. 3P-labeled cdNA was synthesized with reverse transcriptase and oligodeoxythymidylic acid primer. The unincorporated label was removed by chromatography on Sephadex G-50 (Sigma). Fractions containing 3P-cDNA were pooled and used as probes in blot hybridization under conditions of high stringency. Visual examination of the signal intensities was used to estimate the abundance of each cdNA sequence in the cellular RNA. Clones labeled pN designate cdNA isolated from the NP minus CP library; those labeled pC or pcB designate cdNA from the CP minus NP library. Plasmid DNA of the subtracted clones were digested with EcoRI and PstI. Resultant fragments were separated by gel electrophoresis and blotted onto filter paper.

Phage λ Human DNA Library. Ejaculate (10 ml) from a healthy donor was centrifuged in a Beckman JS-7.5 rotor at 3800 rpm for 10 min. The pellet was resuspended in PBS and centrifuged three times, successively, at 3800, 3500, and 3000 rpm. The pellet was at last resuspended in 10 ml PBS, 15% sarkosyl. The suspension was incubated at room temperature for 1 h, rapidly frozen, and stored overnight at −20°C. On the following day, the spermatoctye preparation was thawed and 3.2 ml of STE (60% sucrose, 10 m Tris-HCl (pH 7.5), 1 m EDTA) were added and 2.5 ml of the spermatoctye suspension were layered over a cushion of 6.6 ml STE in each of 6 Beckman SW41 tubes and centrifuged at 14,000 rpm for 1 h. The tubes were drained carefully and the contents were resuspended in 20 ml of 50 m Tris-HCl (pH 7.4), 10 μM EDTA, 10 mM NaCl, 2% sodium dodecyl sulfate, 100 μM βHSC5H5OH and digested with proteinase K overnight at 37°C. DNA was processed as described (9). Thirty μg of sperm DNA were partially digested with 0.3 units of restriction enzyme Sau3AI. DNA molecules from 5 to >20 kilobase pairs were purified from agaroze gel and ligated to λ-DASH/BamHI arms (Stratagene). The library was plated onto 20 150-mm dishes to give 50,000 plaques/plate. Recombinant DNA was isolated from each plate individually by the glycerol gradient method (9).
pC29 Gene Upstream Sequences. PCR was used to detect λ clones containing the hinge protein gene sequences. The two primers were 5'-2 and 2b, located 5' and 3' of the cDNA, respectively, and the template DNA were DNA prepared individually from the plates. Positive plates were identified by the presence of a 400-base pair product. For the isolation of gene upstream sequences, another pair of primers, 2a and LZ-1, were used. Primer 2a is located in the coding region of pC29 while primer LZ-1 (GGGTAAGAGACTCAC-TATAGGCCGAA) is located in the λ vector on one side of the insert sequences. DNA from one of the positive plates (see Fig. 2A, 4) was used in this PCR. The reaction was carried out with the inclusion of gene 32 protein (Pharmacia) (10) under cycling parameters of 94°C for 1 s, 60°C for 1 s, and 72°C for 2 min for 35 cycles (11). A product band of 2.4 kilobase pairs was purified and ligated to the vector pCRII (Invitrogen) (12). The insert DNA in one of the resultant clones was subcloned into plasmid vector pLOT732 (4) for DNA sequence analysis. Sequences of the subclones were determined by the use of sequencing primers OC-2 (TGTTGTGTGGAATTGTG) and VA-3 (GTTTCCCCAGTCACGAC) which flank the cloning site of pLOT732.

Results and Discussion

In our study involving the use of subtractive cDNA cloning to isolate differentially expressed gene sequences between normal and cancerous prostate tissues, a cDNA clone pC29, which encodes the human mitochondrial hinge protein, was identified. The expression of pC29 appeared to be elevated in the cancerous prostate. In this investigation, a number of cultured cell lines derived from human cancers, as well as PBL and fetal fibroblasts, were analyzed for expression of the cloned sequences. A panel of 11 clones that include NP minus CP clones pN80 [mitochondrial ND4 (2, 13)], pN44 [prostate secretory protein of 94 amino acid residues (14)], pN4a; and CP minus NP clones pC29, pCB30, pCB45, pCB47, pCB124, pCB131, pCB135, pCB177 [mitochondrial COIII (2, 13)] was probed with 32P-labeled cDNA generated from polyadenylated RNA of the cancer cell lines, PBL, and fibroblasts (Fig. 1). The plasmid DNA were digested with EcoRI and PstI, and the products were blotted onto filters for hybridization. The hybridization results indicated that some of these prostate-derived cDNA clones (pN80, pC29, pCB45, pCB47, pCB131, and pCB177) are also expressed in nonprostatic cells. Others (pN44, pN4a, pCB30, pCB124, and pCB135) are either not expressed or not detectable in these nonprostatic cells. Notably, the expression of pC29 hinge protein is elevated in every cell line tested as compared to PBL and fibroblasts. It is barely detectable in phytohemagglutinin-stimulated PBL and fibroblasts. Compare its signal intensity to that of pN80, the expression of which does not fluctuate. Increased expression of other respiratory genes such as ANT and ATP synthase β has been observed in SV40-transformed diploid fibroblasts (6).

Previously, it was shown that when leukemic HL60 cells were induced to differentiate in vitro, the level of hinge RNA was reduced 5-fold (5). This down-regulation of an OXPHOS gene as a result of

![Fig. 1. Expression of prostate-derived clones in normal cells and cultured cancer cell lines. Duplicate EcoRI/PstI digests of candidate subtracted clones were hybridized with 32P-labeled cDNA synthesized from polyadenylated RNA isolated from cancer cell lines MOLT4, LoVo, HeLa, and K562 and from mitogen-stimulated PBL and fibroblasts. The insert sizes are around 500 base pairs.](image-url)
differentiation was later observed for ATP synthase β, cytochrome c, and ANT (3). Apparently, cellular transformation is marked by elevated and differentiation by lowered expression of certain genes in the OXPHOS system. An increase in OXPHOS gene expression in transformed cells might be a response to a higher demand for oxidative phosphorylation as that occurs in response to intense muscular (15) or neuronal activities (16). It has long been known that tumor cells operate differently than normal cells in meeting their energy demands. However, whereas normal cells satisfy most of their ATP requirement through oxidative phosphorylation, cancer cells satisfy one-half of theirs through glycolysis. Rapidly growing tumor cells especially show, among others, a decline in the utilization of oxidative phosphorylation, a higher glucose consumption and a lowered mitochondrial content that cannot be merely attributed to a faster rate of cell division (17). Consequently, a down-regulation of OXPHOS genes is perhaps expected. Why, then, do transformed cells show a higher steady-state level of hinge RNA during a shift toward larger dependence on glycolysis for energy production while at the same time no change in the expression of the mitochondrial OXPHOS components pN80 and pCB177 is seen? Does more hinge protein make oxidative phosphorylation less efficient?

In order to study mechanisms that regulate the expression of hinge protein, we isolated its gene upstream sequences. Using PCR, we cloned out hinge sequences from a phage λ human DNA library. First, with a pair of 5’ and 3’ pC29-specific primers (5'-2 and 2b) a 400-base pair PCR product was obtained from human sperm and λ library DNA as templates (Fig. 2A, left, Lanes 3 and 4). The size of this product precludes the presence of introns in the pC29 gene. Next, λ library DNA prepared individually from 20 plates (Fig. 2A, middle, Lanes 1–20), each containing ~50,000 plaques, were used as templates in PCR. The result showed that plates 1, 4, 5, and 15 contained pC29 clones as indicated by the presence of the 400-base pair product.

DNA from one of the positive plates (plate 4) was then used to isolate sequences upstream of the hinge gene by PCR with primers 2a (nucleotides 144–169 of the coding region) and LZ-1 (λ vector sequences on one side of the site of insertion). Two products, 2.4 and 0.6 kilobase pairs, were obtained (Fig. 2A, right, Lane 2). The 0.6-kilobase pair fragment resulted likely from nonspecific priming since its DNA sequence next to the primer did not match pC29 sequences. Nonspecific products were also obtained when primer 2a was paired with a λ vector primer located on the other side of the site of insertion, opposite of LZ-1. The 2.4-kilobase pair fragment was subcloned and sequenced. A restriction map of this DNA is shown in Fig. 2B. Subclones of this DNA are drawn below the map. Sequences contiguous with primer 2a match exactly those in the hinge cDNA (Fig. 3). The sequences diverge beyond ~31, at which the cDNA ends (5). The transcription start of this gene has yet to be located experimentally; position +1 is thus placed at the first nucleotide of the initiating ATG codon.

Upstream of the hinge coding domain, a number of protein factor binding DNA elements are found, many of which match loosely to their respective consensus sequences (18). A prominent feature of the hinge upstream region is a collection of six vestigial human Alu repeats (19) (Fig. 3). Such a clustering of Alu was described in another respiratory gene, ATP synthase β (20). Alu repeats are transposon-like elements dispersed in the human genome, with as many as 500,000 copies. In the hinge DNA, the first Alu is found at position ~49, within 10 nucleotides of the probable transcription start. As such, a TATA box is not found, and transcription start may be positioned by initiator sequences as is common for housekeeping genes (21). Interestingly,

![Fig. 2. Isolation of hinge gene upstream sequences by PCR. A. PCR amplification products from a human DNA library. Left: Lane 1, λ HindIII size markers; Lane 2, β globin-specific primer pair on total λ library DNA; Lane 3, pC29-specific primer pair, 5'-2 and 2b, on sperm DNA; Lane 4, pC29-specific primer pair on total λ library DNA. Center, pC29-specific primer pair (5'-2 and 2b) on DNA from 20 plates (Lanes 1–20) separately. Right, pC29 primer 2a and vector primer LZ-1 on λ DNA from plate 4 to isolate gene upstream sequences. Arrowhead, correct product of 2.4 kilobase pairs. B. restriction map of the 2.4-kilobase pair PCR product. Subclones are shown below the map. OC-2 and VA-3 are sequencing primers used in the sequence analysis of the subclones.](image-url)
Fig. 3. DNA sequence upstream of hinge. Various primer sequences used in PCR are shown with arrowheads to indicate their orientation. Position +1 is placed at the first nucleotide residue of the initiator codon. Upstream sequences end at −2199; downstream sequences end at +169. Sequences beyond +169 (present in pC29) are included. Alu repeats I through VI, enh and enh' homologies are noted.
an AT-rich segment (TAAAAATACAAAAATT) of the Alu sequence is situated 20 nucleotides away from the area of the transcription start. One wonders whether it can function as a substitute TATA box.

More significantly, a sequence element (TAGAGAGtgg-GTTTTCACCA) in the second upstream Alu (at positions −346 to −364) contains a match of 16 of 19 nucleotide residues (mismatches are in lowercase letters, Fig. 3 and 4A) to an element first described by Kagawa et al. (3, 22) as a transcription enhancer peculiar to genes of the respiratory chain. It is found in the genes for ATP synthase β (3, 23), cytochrome c1 (24), ANT2 (25), and perhaps others (26, 27). It is an enhancer because of its orientation-independent positive effect on gene activity, and its ability to influence to a distance either upstream or downstream of the gene. Its similarity to Alu was not discussed however (3). We suggest here that this enhancer element may evolve directly from Alu sequences. From a comparison of the sequences, we propose an OXPHOS enhancer (enh) consensus of (TAGAGANNNG-GTTTTCACCA) with the conserved subdomains in italics. The enh homology in the consensus Alu (TAGAGAGGGGTGTTTCACCGCgg) contains a mismatch in the last position (Fig. 4A). This mismatch and those at other positions of any Alu element may abolish this enhancer activity to many Alu. Three more enh homologies are present on the negative DNA strand in Alu I, IV, and V. Whereas the enh sequences of Alu I and II match well to the enh consensus, those of the distal IV and V have two and four mismatches respectively in the conserved subdomains (Fig. 4B). Whether the Alu element in the hinge gene can enhance transcription still remains to be established. Of the enh sequences, those of the hinge, ATP synthase β, and cytochrome c1 genes are more alike than the others (Fig. 4B). Their dissimilarity may account for some differences in their regulation. For example, expression of ANT2 is stimulated by mitogen (28), but hinge apparently is not (compare Fig. 1, PBL cDNA).

In another capacity, the Alu repeats may serve as modular regulatory elements. Nuclear OXPHOS genes scattered in the genome may be coordinately regulated by Alu sequences which are themselves more alike than the others (Fig. 4A). Their dissimilarity may point to an Alu element in the hinge gene can enhance transcription still remains to be established. Of the enh sequences, those of the hinge, ATP synthase β, and cytochrome c1 genes are more alike than the others (Fig. 4B). Their dissimilarity may account for some differences in their regulation. For example, expression of ANT2 is stimulated by mitogen (28), but hinge apparently is not (compare Fig. 1, PBL cDNA).

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The isolation of the hinge gene now allows us to investigate its regulation under various physiological conditions, particularly with regard to differentiation and transformation. The possible role of Alu-like sequences in gene regulation in other animals has recently been documented (34). In our case, the interaction between the putative enhancer factor and its target DNA sequence will be examined by methods which can pinpoint the DNA contact residues, define the precise sequence specificity, and yield information about the physical properties, such as molecular weight, of the factor.

HUMAN MITOCHONDRIAL HINGE PROTEIN GENE

**A**

<table>
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<tr>
<td>hinge</td>
<td>TTTTTTTGTTTTTTTAGAGAGGGGTGTTTCACCGCgg</td>
</tr>
<tr>
<td>ATP syn β</td>
<td>TTTTTTTGTTTTTTTAGAGAGGGGTGTTTCACCGCgg</td>
</tr>
<tr>
<td>cyto c1</td>
<td>TTTTTTTGTTTTTTTAGAGAGGGGTGTTTCACCGCgg</td>
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<tr>
<td>consensus</td>
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</tr>
<tr>
<td>ANT2</td>
<td>TTTTTTTGTTTTTTTAGAGAGGGGTGTTTCACCGCgg</td>
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<tr>
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<tr>
<td>QB (−)</td>
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</tr>
<tr>
<td>c-myc</td>
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</tr>
<tr>
<td>Mt4</td>
<td>TTTTTTTGTTTTTTTAGAGAGGGGTGTTTCACCGCgg</td>
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**B**

<table>
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<tr>
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<th>Position</th>
<th>Strand</th>
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<th>Match to consensus</th>
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<td>−</td>
<td>TAGAGATGGGGGTGTTTCACCA</td>
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<tr>
<td>Alu II</td>
<td>−346</td>
<td>+</td>
<td>TAGAGATGGGGGTGTTTCACCA</td>
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<tr>
<td>Alu IV</td>
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<td>−</td>
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<td>17/19</td>
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<tr>
<td>Alu V</td>
<td>−1850</td>
<td>−</td>
<td>THaAGACGGGGGTGTTTCACCA</td>
<td>15/19</td>
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**C**

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Fig. 4. Enhancer elements and Alu. A, comparison of enhancer sequences found in different OXPHOS genes and consensus Alu. −, the sequence is found on the negative strand. B, comparison of the four enhancer homologies found in the Alu elements upstream of hinge. Lowercase letters, mismatched bases. C, cartoon depicting the Alu, enh, and other sequence motifs.
Acknowledgments

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References


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