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Sequence and detailed organization of the human caveolin-1 and -2 genes located near the D7S522 locus (7q31.1)

Methylation of a CpG island in the 5′ promoter region of the caveolin-1 gene in human breast cancer cell lines

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Abstract The CA microsatellite repeat marker, D7S522, is located at the center of a ~1000 kb smallest common deleted region that is lost in many forms of human cancer. It has been proposed that a putative tumor suppressor gene lies in close proximity to D7S522, within this smallest common deleted region. However, the genes located in proximity to D7S522 have remained elusive. Recently, we identified five independent BAC clones (~100–200 kb) containing D7S522 and the human genes encoding caveolins 1 and 2. Here, we present the detailed organization of the caveolin locus and its relationship to D7S522, as deduced using a shot-gun sequencing approach. We derived two adjacent contigs for a total coverage of ~250 kb. Analysis of these contigs reveals that D7S522 is located ~67 kb upstream of the caveolin-2 gene and that the caveolin-2 gene is located ~19 kb upstream of the caveolin-1 gene, providing for the first time a detailed genetic map of this region. Further sequence analysis reveals many interesting features of the caveolin genes; these include the intron-exon boundaries and several previously unrecognized CA repeats that lie within or in close proximity to the caveolin genes. The first and second exons of both caveolin genes are embedded within CpG islands. These results suggest that regulation of caveolin gene expression may be controlled, in part, by methylation of these CpG regions. In support of this notion, we show here that the CGs in the 5′ promoter region of the caveolin-1 gene are functionally methylated in two human breast cancer cell lines (MCF7 and T-47D) that fail to express the caveolin-1 protein. In contrast, the same CGs in cultured normal human mammary epithelial cells (NHMECs) are non-methylated and these cells express high levels of the caveolin-1 protein. Comparison of the human locus with the same locus in the pufferfish *Fugu rubripes* reveals that the overall organization of the caveolin-1/-2 locus is conserved from pufferfish to man. In conclusion, our current studies provide a systematic basis for diagnostically evaluating the potential deletion, mutation, or methylation of the caveolin genes in a variety of human tumors.

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Key words: Caveolin genes; Tumor suppressor locus; Chromosome 7q31.1; Microsatellite marker D7S522; CpG islands

1. Introduction

Deletion of the q31 region of human chromosome 7 has been implicated in the pathogenesis of many different forms of human cancers [1–16]. Such deletions have been detected using LOH (loss of heterozygosity) analysis, by employing specific polymorphic CA repeat microsatellite markers that map to the 7q31.1 region. Among these CA markers, D7S522 is the most informative. Deletions in the 7q31.1 region are normally distributed around the D7S522 locus, thereby defining a smallest common deleted region (SCDR) of ~1000 kb [8,12,14,17]. Interestingly, loss of the D7S522 locus occurs in a wide variety of human epithelial tumors, including human primary breast [6], prostate [8,10], ovarian [18], colon [14], and renal cell carcinomas [11]. In addition, D7S522 spans a common fragile site, FRA7G, that resides at 7q31.1 [11,19]. Given the association of 7q31.1 and D7S522 LOH with carcinogenesis, it has been proposed by numerous laboratories that an as yet unknown tumor suppressor gene resides within the 7q31.1 region. However, no genes have been previously localized to the D7S522 locus.

Recently, we showed that the human genes encoding caveolins 1 and 2 are contained within the same human genomic BAC clones and co-localize to the q31.1 region of human chromosome 7, as observed by FISH analysis [20]. Caveolin family members function as scaffolding proteins to organize and concentrate specific lipids (cholesterol and glyco-sphingolipids) and lipid-modified signaling molecules (Src-like kinases, H-Ras, eNOS and G-proteins) within caveolae membranes [21–23].

CA microsatellite repeat marker analysis of these caveolin genomic clones indicated that they contain the marker D7S522, but not other microsatellite repeat markers tested [20]. The close proximity of caveolins 1 and 2 to the D7S522 locus was confirmed by using a panel of human STS markers that are known to map in the neighborhood of the D7S522 locus [20]. As it has been shown that the caveolin-1 protein possesses transformation suppressor activity [24–27], we proposed that the caveolin-1 gene may represent the candidate tumor suppressor gene at the D7S522 locus on human chromosome 7 [20,28]. However, the detailed organization of the caveolin genes and their physical distances from each other and from D7S522 is unknown.

Here, we present the sequence and detailed organization of the caveolin locus and its relationship to D7S522, as deduced using a shot-gun sequencing approach. Our results provide for

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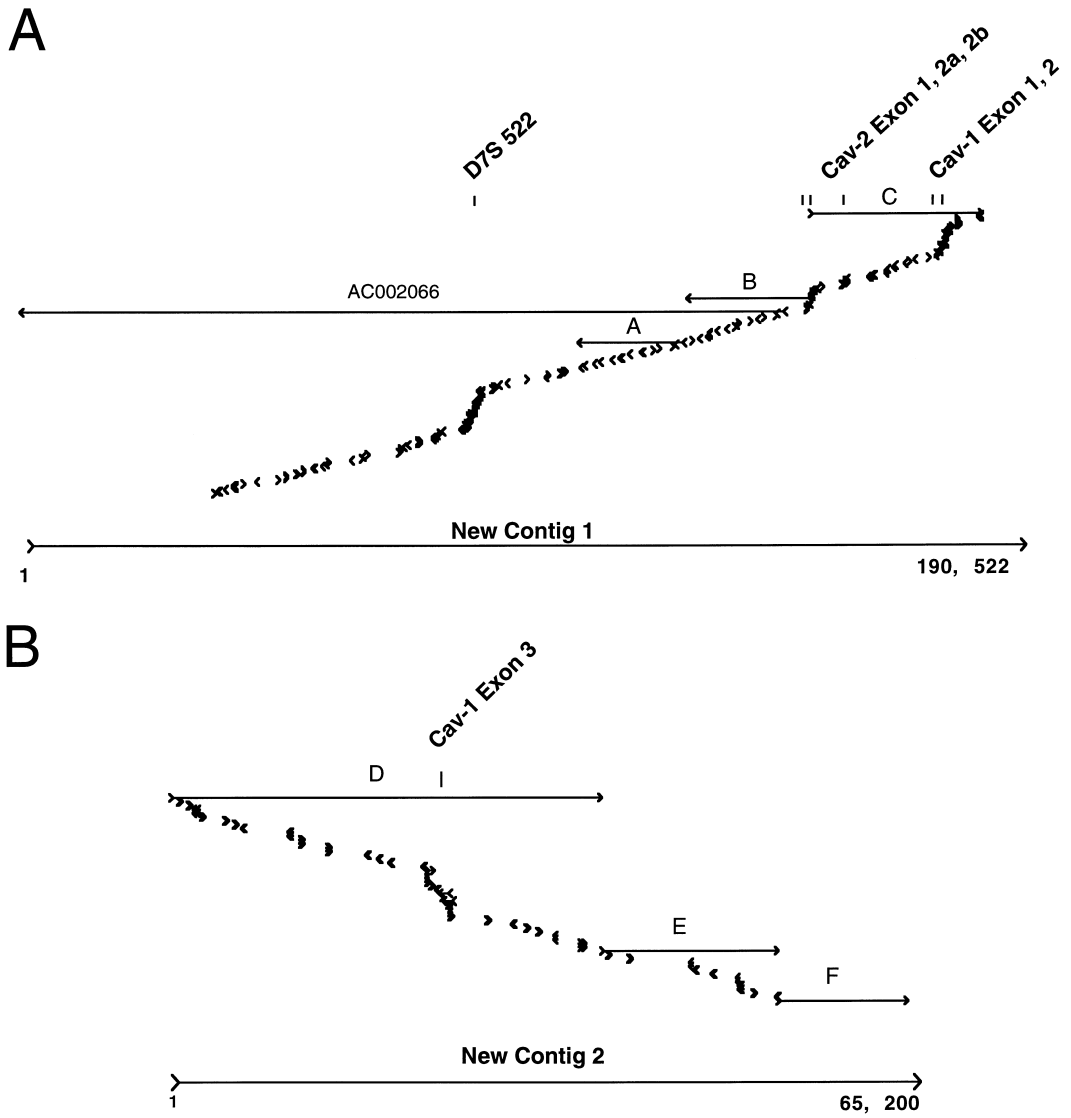

Fig. 1. Derivation of contigs 1 and 2. Random sequences from a human BAC clone containing D7S522 and the human genes encoding caveolins 1 and 2 (represented here as < or >) were used to identify, join, and order sequences from two other human BAC clones. One BAC clone RG030H15 (Genbank Accession #AC002066) contained the marker D7S522. The other BAC clone RG011K01 (Genbank Accession #AC006159) contained eight previously unordered contigs, that we termed A through H. A (21.1 kb); B (24.7 kb); C (33.9); D (38.1 kb); E (15.6 kb); F (11.6 kb); G (10.1 kb); and H (10.3 kb). These sequences were combined using the program Sequencer to create two new adjacent contigs representing ~250 kb. A: New contig 1. This ~190 kb contig combines our shot-gun sequencing with sequences from the entire BAC clone RG030H15 (Genbank Accession #AC002066) and contigs A, B, and C from BAC clone RG011K01 (Genbank Accession #AC006159). The positions of D7S522 and the caveolin exons are as indicated. B: New contig 2. This ~65 kb contig combines our shot-gun sequencing with contigs D, E, and F from BAC clone RG011K01 (Genbank Accession #AC006159). However, we were unable to position contigs G and H within our alignments. The position of caveolin-1/exon 3 is as indicated. The sequences of new contigs 1 and 2 have been deposited in Genbank under accession numbers AJ133269 and AF125348.

the first time a detailed genetic map of this important chromosomal region.

2. Materials and methods

2.1. Materials

The cDNAs for human caveolin-1 and -2 were as we described previously [20]. Normal human mammary epithelial cells were obtained from BioWhittaker/Clonetics (Walkersville, MD, USA). MCF7 (HTB-22) and T-47D (HTB-133) cells were obtained from ATCC. CpGenome and CpG WIZ kits were purchased from Oncor/Intergen (Purchase, NY, USA). Anti-caveolin-1 IgG (mAb 2297) were the generous gift of Dr. Roberto Campos-Gonzalez (Transduction Laboratories, Lexington, KY, USA). BAC clones containing the microsatellite marker D7S522 and the genes encoding human caveolin-1 and -2 were as we described previously [20].

2.2. Library construction and shot-gun sequencing approach

We constructed a BAC DNA shot-gun library using high power ultrasound sonication methods. This was accomplished by generating a pool of random sized BAC DNA fragments from a single ~200 kb BAC DNA clone. The fragments were end repaired using various repair enzymes. The fragments were then subsequently blunt end cloned into the pBluescript SK^- vector. More specifically, several samples (~5–15 μg each) of the BAC clone were prepared and brought to a 50 μl volume. Each sample was then sonicated for a specific time interval, i.e. 5 s, 10 s, 15 s, 20 s. Afterwards, ~5–10 μl of

each sonicated sample was analyzed by agarose gel electrophoresis to determine if any shearing occurred. Next, each of the samples was pooled and subjected to blunt end repair with mung bean nuclease. After end repair, the sample was phenol-chloroform extracted and ethanol precipitated. The entire sample was loaded onto a 0.8% SeaKem GTG agarose gel. The gel region in the range of ~1–3 kb was excised and purified. Approximately, 10 μl of this purified sample was then blunt end ligated into the pBluescript SK$^-$ vector using T4 DNA ligase. The ligation mixture was transformed into *E. coli* (DH10B cells) and plated out for growth and subsequent blue/white selection. Clones were robotically picked, ordered into eight 96-well plates, and DNA was prepared for clone end sequencing using vector primers. In addition, the eight 96-well plates were compressed into two 384-well plates and robotically gridded onto filters (see below) that were subsequently hybridized with probes containing D7S522 or the coding regions of caveolins 1 and 2. Positive clones were retrieved from the ordered library (stored as glycerol stocks at −80°C) and subjected to end and internal sequencing. This approach ensured coverage of D7S522 and the coding exons of caveolins 1 and 2. Custom robotic services were provided by Genome Systems, Inc. (St. Louis, MO, USA).

2.3. Gridding of the shot-gun library

The library consisted of eight microtiter dishes in a 96-well format housing a total of 768 bacterial clones suspended in a solution comprised of LB medium containing glycerol to facilitate storage at −80°C. These eight blocks were compressed into two 384-well microtiter dishes to facilitate robot compatibility. The compression consisted of transferring small amounts of bacteria from the first four 96-well blocks into one 384-well block (named plate #1 [384-well]) by means of a 96-pin replicator. Plate #1 [96-well] resides in the A1 quadrant of plate #1 [384-well], plate #2 [96-well] resides in quadrant A2 of plate #1 [384-well], plate #3 [96-well] resides in the B1 quadrant of plate #1 [384-well] and plate #4 resides in the B2 quadrant of plate #1 [384-well]. Plate #2 [384-well] consists of plates 5–8 [96-well] according to the same pattern. After the replication was completed, plates #1 and 2 [384-well] were incubated at 37°C for 16 h to facilitate bacterial growth. These plates were then robotically gridded onto a nylon membrane. This process entails the use of a 384-pin spotting assembly, which is capable of spotting small amounts of bacteria onto the membrane in a precise mathematical grid. The plates are spotted onto the membrane in such a fashion that each bacterial grid, or spot, may be correlated to an exact plate and well location. This is accomplished by double-spotting each plate and well location in a unique pattern and position which effectively eliminates the occurrence of false positives. After gridding was completed the membranes were then lifted onto agar plates and incubated at 37°C for approximately 14 h, then chemically processed to expose, denature, and fix the DNA of the bacteria on the membrane.

2.4. Western blot analysis

Equal amounts of protein were loaded on an SDS-PAGE gel (12% acrylamide) and transferred to nitrocellulose. After transfer, nitrocellulose sheets were stained with Ponceau S to visualize protein bands and subjected to immunoblotting. For immunoblotting, incubation conditions were as described by the manufacturer (Amersham), except we supplemented our blocking solution with both 1% BSA and 1% non-fat dry milk (Carnation).

2.5. Methylation analysis

Bisulfite treatment of purified genomic DNA was used to convert non-methylated cytosines to uracil (U), as per the manufacturer's instructions (Oncor/Intergen). Each chemically treated DNA sample was then amplified by PCR and directly sequenced using the same primers used for amplification. As a consequence, non-methylated cytosines will appear as thymidines (T) after PCR and DNA sequencing. Under these conditions, methylated cytosines (5-methylcytosine) are resistant to modification and remain as cytosines.

The primer pairs used for PCR amplification were as follows: forward A, 5′-TGTGTATTTTGtAAAATATGGtTAAttTG-3′; reverse A, 5′-CCATCTCTaCCTTAAAACACAT-3′ yielding an ~350 bp fragment, as predicted.

Note that bases in lower case were changed from C to t (forward primer) or from G to a (reverse primer) to reflect the predicted effects of bisulfite treatment on non-methylated cytosines.

3. Results

3.1. Detailed organization of the human caveolin-1 and -2 genes located near the D7S522 locus (7q31.1)

Recently, we identified five independent BAC clones containing the microsatellite marker D7S522 and the genes encoding human caveolin-1 and -2 [20]. In order to determine the detailed organization of the caveolin genes and their physical distances from each other and from D7S522, we undertook a shot-gun sequencing approach.

One of these five clones (clone #1) was physically sheared to produce random DNA fragments of ~1–3 kb, and employed to construct a library using the vector pBluescript SK$^-$. Over 700 random clones were picked, assigned an ID number, and sequenced at the 5′ end. In addition, these clones were robotically gridded onto filters which were then probed by hybridization with either D7S522 or coding regions of the caveolin genes (Cav-1/exons 1, 2, and 3; Cav-2/exons 1 and 2). Approximately six positive clones were obtained for each of these regions and were then subjected to more extensive sequencing using internal primers. This second approach was taken to ensure coverage of the regions in which we were most interested.

These individual sequences were assembled into greater than 50 contigs which were used to perform database searches. Through this approach, we identified the sequences of two human BAC clones that had been deposited. One BAC clone RG030H15 (Genbank Accession #AC002066) contained the marker D7S522, but not the caveolin genes. The

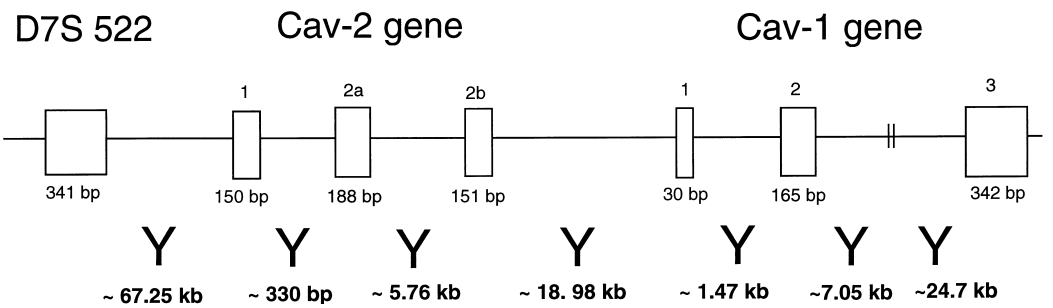

Fig. 2. Detailed organization of the human caveolin-1/-2 locus and its relationship to D7S522. Based on contigs 1 and 2 shown in Fig. 1, the organization of the caveolin-1 and caveolin-2 genes was deduced. The size of each exon and the distance between them is as indicated. Note that the marker D7S522 is located ~67 kb upstream of the caveolin-2 gene and that the caveolin-2 gene is located ~19 kb upstream of the caveolin-1 gene.

Table 1
Intron-exon boundaries for the human caveolin-1 and -2 genes

Name	Upstream Sequence (40 bp)	Downstream Sequence (40 bp)
Cav-1		
Exon 1	GGGAAACCTCCTCACAGTTTTCATCCAGCCACGGGCCAGC	GTAGGCATCCGTGGGGGGGCGCCGGCTCGGGCGTGCGGGG
Exon 2	GCCCTCCCCGTCCTGGCCGTCCGCCCTCCGCCCTCTGCAG	GTAAGCCAAGGCGACCAACAGGGAAGGGCTGGGACAGCT
Exon 3	TTCTATTCTGTGCTCATGTTGTGTCACTTCTTCCTTTTAG	ATGACATTTCAAGGATAGAAGTATACCTGATTTTTTTTCC
Cav-2		
Exon 1	ACCGCCCAGCCGGCTGCAGCGGCCGCGCACCAAGGCTGCG	GTGAAGCCCGGGGTGGGCGGGCCCAAGTCCCCGCTGAGGC
Exon 2a	TTGGCTGACAGTTCGGGGTCCCTGCGTCCTGTCTCCTCAG	GTGAGACGGGGCACACCGGGTGGACCGGCTTTCTGAAACA
Exon 2b	ACAGCAACAATCCTCACACATCTCTCTTCCTTCCTTCCAG	ATACTTGGACCCCAGGTCTGGAGATTGGGATACTGTAATA

The DNA sequence upstream or downstream of a given exon is as indicated above.

other BAC clone RG011K01 (Genbank Accession #AC006159) contained eight unordered contigs, some of which encode regions of the caveolin genes. However, this second BAC clone apparently lacked the marker D7S522.

In order to resolve this conflict, we compiled all of our sequences and the sequences of these two BAC clones and used the program Sequencer to align them. The results of this analysis are shown in Fig. 1. Through this approach we were able to order six of the eight contigs from clone RG011K01 and to connect them with clone RG030H15. In most cases, our sequencing results allowed us to connect the unordered contigs from clone RG011K01 with each other as well. This alignment produced two new adjacent contigs (~190 kb and ~65 kb), for an overall coverage of

Table 2
Nine novel CA*n* microsatellite repeats located in close proximity to the human caveolin genes

Name	Location
CAV-CA1	~ 1 kb upstream of Cav-2/ exon 1

cgaaaccagcctgaccaacatggggaaaccccgtctctgctaaaaatacaaaattagccag
gcgtggtggtgcatgcctgtaatcccagctactcgggaggctgaggcaggagaatcgcttg
aacccgggaggcagaggttgcgatgagccgagatcgtgccattgcactccagcctgggcaa
caagagcgaaactctgc**cacacacacacaaacacacacacacacacacacacacacg**cggtg
tagtttaggaagtaaaaaaaaaaaaaaaaaaaaaaaatcagatctcccctcacacctcagatc
tgaaggcacaaactctagggccagggcgttcgcctacccaactccacatgcacttgcaggt
cacctagcactcaggtacctagcactcaggtacattgtggctccttacctctcacgacagc
agcaacaacgtt

CAV-CA2	between Cav-2/ exon 2b and Cav-1/ exon 1

ctgaggcaggagaatggcgtgaacccaggaggcggagcttgcagtgagccaagatcgcgcc
actgcactccagcctgggtgacagagcgagactccgtctcaaaaaaaaaaaaaaaaaaaac
aaagactatttttttttttttctgaggaaaagaaatcctatgtggggttatcctaagcaac
aatcttatttgactagg**cacacaca**aa**cacaca**aaaaactgtgaccatcagtatgttcaaac
aagggaaaatggcaaatagaagattgtaaaacaattttcctggaagaactttggcaatgat
aagcattaatctggggcagatcagatgtagaccaggctctatgatgtgtgatgagctaggg
atctattgatttccatgattttacggttcttagagttgaagaaatattc

CAV-CA3	between Cav-2/ exon 2b and Cav-1/ exon 1

agttagctgagttgcttgaaggcaaaaccagcagtgcagaaataaaccccattctccagta
tgcacaaaagctgatgttgcaattaaagatttgccctggaattagtgaaaaaacaaactca
tcatatgattacaagaaagtaaatttctgtatgaagagcagatcacttatgtgtatgttca
ccacatgaacct**aca**tc**cacacacacacacacacacacacacacacacacacata**gaaa**cac
acaca**cc**c**caca**gagtctttaaagacggactttattttatgctgtaaacctagactttggg
aggctgaagtggagggattgcctgagtccaggagttcgaaaccagcctggcaaaaatcctg
tctctaccaaaaatatataaaattagctgggtctggtggcacacacctgtagtcccagctac
tagggaggctgaggatcacctgagcctgg

CAV-CA4	between Cav-1/ exon 2 and exon 3

cgagaagcaagtgtacgacgcgcacaccaaggagatcgacctggtcaaccgcgaccctaaa
cacctcaacgatgacgtggtcaaggtaagccaaggcgaccaacagggaagggctgggacag
ctctcctctggcagttagcccgtgcatccttctttagcattgccgtgtacgcacaccccac
cccgccccctacacgcg**cacacacacacacacacaca**gagttttgtgggtttgatgtgtgg
gagctcccgcagtcggcagaaacgttacatctccctccttccccatctcccccaatagttag
ttcagctgaaattcagctaaagtgagttttgtagaagttcctataactacactttatcct
agcaaatgagcctattgacctcagcaacagacggcccatactccttgggacggt

CAV-CA5	between Cav-1/ exon 2 and exon 3

gcctgcctcaaaaaaaaaaaaaaaaaaaagtgttaggtgacatgagagaagatcttccaagt
aataagagtggctaatcccaggaatgtgtcaccagaggttattttgtaatagtcgtgtgtt
aaattccttatttgtctatataacttctcaaatccttctgcctctacagttatagtttaac
tggcgcataacagcctt**cacacaca**gcctcataattaaacatagacatacatatgaacac
tttcccctatgccagcaggatacttggtttgtttaggggcaaagaggaattgatgtggcgt
tgtttcaatcagtggttgaaaatgcaagtggtaaacattgaaaaatagaacactgcaaaag
gcatgcattgtatataccaaaaggtcagcatgaagcattatct

CAV-CA6	between Cav-1/ exon 2 and exon 3

ttacgccagataataacacattcagaattctcctttccctgggagattttatcaggttagt
gttcttgtaaacaggagaaagagaaaaatataacttagtaaatagcagtattcactaattc
attcatttattcaacaaatattaatttactacctactacattccagggagcttagagtcta
gtatcagaaataataac**cacacacacaca**ta**cacacaca**ctacattaaataaggatgt
gataggctagatgaaataaataaataaataaaaggtccaggtgagaaaagaaggtgggggc
tagaaagaagtcattgaagaaaaaacatttaggttaaaacattatgaataacttagagtga
gccaagtgcagagtgctgaaggagtgctccaggcaaaatcaacagcaaatggggagtcc

CAV-CA7	between Cav-1/ exon 2 and exon 3

ctgtttgaatgagacgcttgcataaacagcacaaggagtttggggtggggttaaagagaat
ggtgtggtatagggagagctgaatgaggaactgagagagcaaaatcctgtgtttggttcaa
tcactgattacaacctccctgaggctcggtctcctaatctgtaaaatgggggaaataata
cctgccttgcaggtcct**cacacaca**gggcatgatgtgaatccactgaggcatatagcact
gtgtaacatgagttattgctattccaaggcccgtaaaaggctcttgccttggaatatatct
gccacaccaatgcctgcagtccattaatgacacataaaggacactggagataacgatgtcc
cttgttctatgcatccctcccacccatgccagaaaagaaaaca

CAV-CA8	between Cav-1/ exon 2 and exon 3

aacacaggcatctacagtacaatccaggcctcctgttttcagcaggggctgcaagagcact
gcagccttttccccagaggtgtcagtttggcccagtaaagattgcccctgagaaaacacat
gggcaattagagcaaagttcctatgttctggtaacatttaattgtgctatttctcaacctc
ctctg**ca**cc**cacaca**ct**cacacaca**c**aca**tttattccactgacttcaaaggaagctcaac
gtgttaaaaatatgtgtgggaacaaagaagggagtttgaaattggtctaaactctgtataa
ctgggtttgacacgtacattaggatttacaagtatgtatttaatctttttttaaaaaaag
cgtttacatagggttcagaatatgacaataaatcaacatttctatt

CAV-CA9	~ 11. 3 kb down-stream of Cav-1/ exon 3

ttaccagattctatcagttccttcttcaaaatatatcttgcttttgcaccccctgcctcgat
tccactgccacaacctcagtatgagcagccatatatttaaatcctggtctactttaggcagt
ctacctgtttccacccttaaccccacccaactcatttggccactttgctctacagcaacac
atattttcacagaggta**cacacaca**ta**cacacacaca**gaacttaaaatcctctagtatt
ttctcactgcattcagtaaaatgcattcataaaatcacatctctttaccaaggcctacaaa
gttatctgattcagctcctgcctatctctccaatctaattgcataccacaacctcctcatg
atctgtgctctagacacactgatgttccttcagtcccaagaacatgctatggtccc

A) **CpG Island containing Cav-2/ exon 1**

CGtaaaaaagaagggaatgggtaagcagggtgatgtctgggagaggaggggctccatagccatgaga
gtcaactctgtaacaccctatagggttacaacactgcccttcatatactgaggtagcagcagggaaact
ttttaattattagaaatattgaactttgcctcccaccccaaacatttttctcattcagttcctgttcttt
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gaatctttcaagacagtctggttcacaagaaaattaaaaggtggaggtCGggCGCGgtggctcaCG
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gcctgtaatcccagctactCGggaggctgaggcaggagaatCGcttgaaccCGggaggcagaggtt
gCGatgagcCGagatCGtgccattgcactccagcctgggcaacaagagCGaaactctgccacaca
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actccacatgcacttgcaggtcacctagcactcaggtacctagcactcaggtacattgtggctcctac
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tgtgttagaattttatgtgaaattaacatttaattctcaCGgacaccccgaaacagatgccacagccc
ccattttgccaaCGaggcagctgaggttcccagaggctcaataccagcaccatgagcCGcagcaCG
caaggcaaacacagcCGgagtgatgcacatacctgcttCGcaccccatgCGcctaaccacaaggtt
cctccctccaggaaggcCGttgtcttccctgggaCGacttgccagctctgaggcatgacagtaCGg
gcccccagaagggtgaccaggaggccctcctCGtcccagctgcCGgCGtcCGctccactgcaggg
ccCGggctgtgactCGtgggaCGgttccctgCGcccCGgggaggtgggCGgggaggggCGg
CGgggCGgggCGggctCGggaCGCGggctgggagctggagcccacagCGtgaagCGtcCGcC
GccCGggcctCGcagggctaggCGaggCGaggggggCGgggCGggCGctaCGggaagggga
ggcCGCGCGgacCGggagcCGcacCGcccagcCGgctgcagCGgcCGCGcaccaaggctgCG

**atggggctggagaCGgagaaggCGgaCGtacagctcttcatggaCGaCGactcctaca
gccaccacagCGgcctCGagtaCGcCGacccCGagaagttCGCGgactCGgaccagg
acCGggatccccacCGgctcaactCGcatctcaag**

gtgaagccCGgggttCGggctcccaagtcccCGctgaggcCGggaagtgCGggCGcccctcagc
ccCGccctaaccCGtcccaccattgctacCGggttggcccCGcaggtctgaggatCGcaccctc
ccCGgtaccacCGtcaccagggCGccCGCGtagcaggaattcttagcaggtttcCGtgCGccac
CGtgaccctaagaaagaggCGgatcctggcaCGtctttcctctgcttcccCGcccaagtCGctCG
gtttcCGgtgtcaagttgctgaagttCGggttctgcttctgtctcctagctgg

B) **CpG Island containing Cav-2/ exon 2a**

gttgattaaacaaaaggaagccccCGggctgaggttagaagcagggggCGgcCGgcctaCGaaa
gaagCGcCGaCGggagcaaCGgccagCGgtgagggcCGgaccaagctggatgggctgagaCGa
gagCGagtacagtctcatgacagattctacagcacacagCGctgagtaCGCGacccagaagttgC
GactCGacaggaCGgatccaCGgctaactgcatctcagtgaagcCGgggCGgCGccaaagtcc
CGCGaggCGggaggtCGgCGccctcagccagcctaacCGtccaccattgctacCGgtCGgccC
GcaggtCGagacCGcaccttccCGgtcccacCGtcaccagcCGccCGgtagccagaatcttagc
cagttcctggCGccccaCGtgaccctagagaagagCGgaCGccctggcaCGtccttccctcctgct
tccccCGcccaaagCGctccCGgttccCGgggCGtcaggttggctgacagttCGgggtccctgC
Gtcctgtctcctcag

**ctgggcttCGaggatgtgatCGcagagcCGgtgactaCGcactcctttgacaaagtgtg
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gcacatctg**

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agatccctgagttggcacataagatttgggattcccaattaactaaaactaatcatctaacctctatgt
cagttttctcactttcaaaactgtgaaaaactgagtgacCG

C) **CpG Island containing Cav-1/ exon 1 and 2**

CGctatcacatttttttttaagtctagtcaattagaaaagtcaaatctttcctcacagccaagcacatta
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agcaaatttacaaaaccaaaaacaatgtatgcctcttggtttcttcatctatggacacctgcactt

Fig. 3. CpG islands are located in close proximity to the first and second exons of the human caveolin genes. Three CpG islands containing (A) Cav-2/exon 1, (B) Cav-2/exon 2a, and (C) Cav-1/exon 1 and 2 are shown. The positions of CGs are indicated by boldface uppercase letters that are underlined. Exon sequences are denoted by boldface lowercase letters and are set apart from adjacent upstream and downstream sequences.

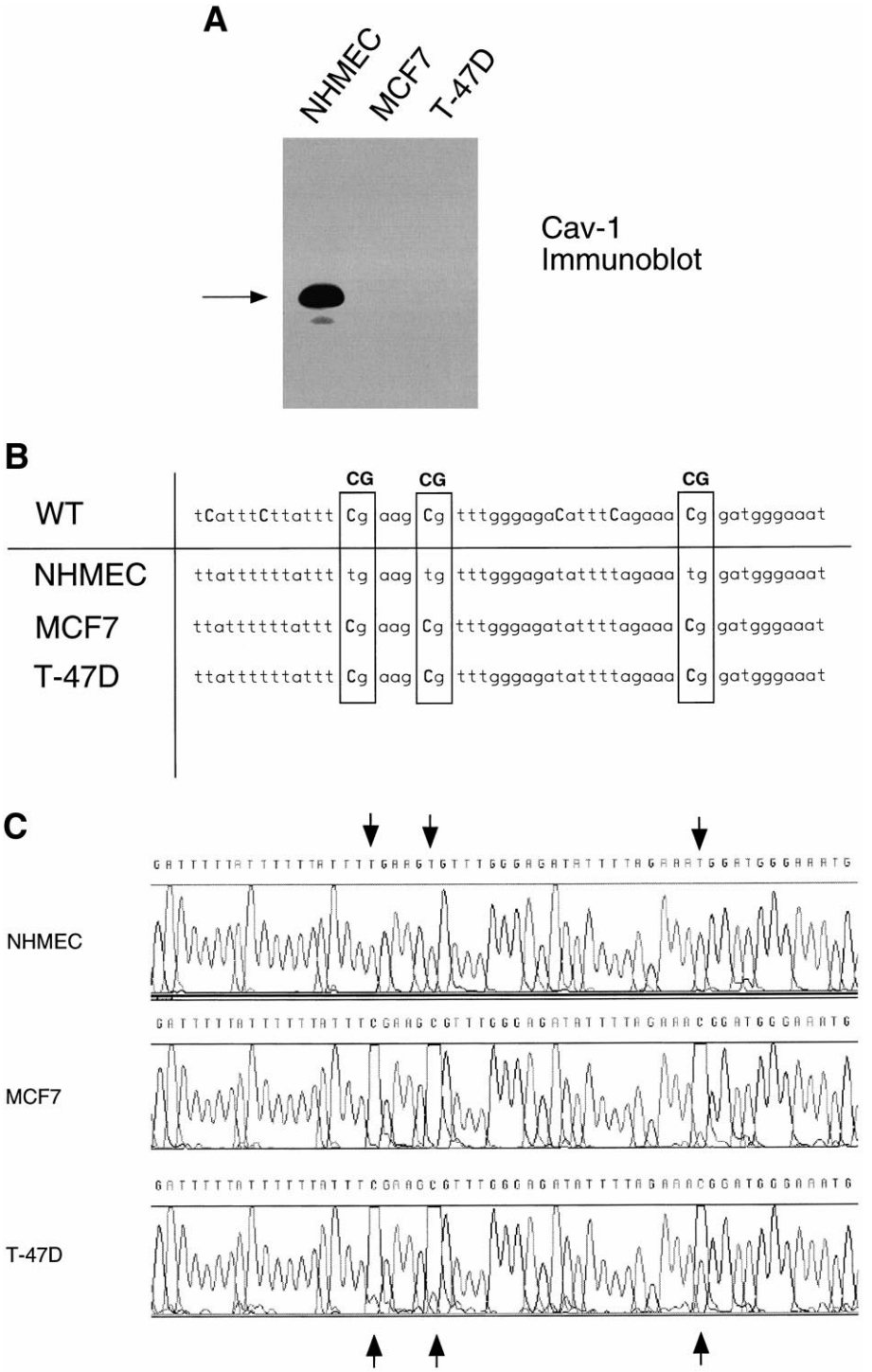

Fig. 4. Methylation of CpG islands in the 5′ promoter region of the caveolin-1 gene in breast cancer cell lines that fail to express caveolin-1. Panel A: Western blot analysis. Using a caveolin-1-specific monoclonal antibody probe (mAb 2297), we analyzed the expression of the caveolin-1 protein in normal human mammary epithelial cells (NHMECs) and two well-studied human breast cancer cell lines (MCF7 and T-47D) by immunoblotting. Note that caveolin-1 expression is dramatically down-regulated in these breast cancer-derived cell lines, as compared with the normal human control. Each lane contains equal amounts of total protein. B and C: DNA methylation analysis. Panel B: Genomic DNA was prepared from each cell type and treated with excess bisulfite to chemically modify non-methylated cytosines (C) so that they appear as thymidine (t) after PCR and DNA sequencing. Methylated cytosines are unaffected by bisulfite treatment and remain as cytosines (C). The unmodified wild-type (WT) sequence is shown above, and the DNA sequences of chemically modified DNA from a given cell type are shown below. Cytosines are shown in bold uppercase letters; methylated cytosines are boxed and are as indicated (CG in boldface). Note that certain cytosines (C) in the 5′ promoter region of the caveolin-1 gene are non-methylated in NHMECs and are converted to thymidines (t); the same cytosines (C) in MCF7 and T-47D cells were clearly methylated and remain as cytosines (C). Panel C: Chromatogram represented schematically in panel B.

~250 kb across this region. Unfortunately, our sequencing efforts to directly join contig 1 and contig 2 were unsuccessful; this may be due to the large distance between them. The sequences of these two new contigs have been deposited in Genbank under accession numbers AJ133269 and AF125348.

Contig 1 contains D7S522, the caveolin-2 gene (exons 1, 2a, and 2b), and a portion of the caveolin-1 gene (exons 1 and 2). Contig 2, located downstream of contig 1, contains a portion of the caveolin-1 gene (exon 3). These two contigs were assembled into a single region shown in Fig. 2. Note that D7S522 is located ~67 kb upstream of the caveolin-2 gene and that the caveolin-2 gene is located ~19 kb upstream of the caveolin-1 gene. In addition, it is interesting to note that the caveolin-2 gene contains a previously undetected intron that divides exon 2 into two separate exons. Hence, we have termed these exons 2a and 2b. These two exons (2a and 2b) are joined to form a single exon in caveolin-1 and caveolin-3, suggesting that caveolin-2 may be the precursor for caveolins 1 and 3 through gene duplication.

3.2. Analysis of the sequences surrounding the exons of the human caveolin-1 and caveolin-2 genes

3.2.1. Intron-exon boundaries. Based on the sequences compiled in contigs 1 and 2, we deduced the intron-exon boundaries of the caveolin genes. Sequences upstream and downstream of a given exon are listed in Table 1. These sequences conform well to the known consensus for donor and acceptor splice junctions. In addition, these sequences will be extremely useful in searching for mutations within the adjacent coding regions of caveolin-1 and caveolin-2.

3.2.2. CA microsatellite repeats. Through sequence analysis of contigs 1 and 2, we identified nine CA microsatellite repeats that are present either in close proximity to or within the caveolin genes, termed CAV-CA1 through 9. These sequences are detailed in Table 2. Note that one is located ~1 kb upstream of Cav 2/exon 1, two are located between Cav-2/exon 2b and Cav-1/exon 1, five are located between Cav-1/exon 2 and 3, and one is located ~11.3 kb downstream of Cav-1/exon 3. As almost all CA repeats are thought to be polymorphic, these caveolin-proximal CA repeats will be useful in LOH (loss of heterozygosity) analysis to determine whether a given region of the caveolin genes is deleted in human tumors. However, we have not yet checked if these particular novel CA repeats are indeed polymorphic.

3.2.3. CpG islands. Hypermethylation of cytosine-guanine-rich areas, termed CpG islands, in the 5′ promoter region or the first exon of a given gene has been suggested as a potential mechanism for down-regulating the expression of tumor suppressor genes in certain human tumors, i.e. gene silencing [29,30]. Thus, we examined contigs 1 and 2 for CG-rich regions. Interestingly, three CG-rich regions were identified and they are all located in close proximity to the caveolin genes (Fig. 3). More specifically, exons 1 and 2a of caveolin-2 (Fig. 3A, B) and exons 1 and 2 of caveolin-1 (Fig. 3C) are embedded within these CpG islands. Thus, our studies identify potential sites for methylation that may regulate caveolin gene expression.

3.3. Methylation of CpG islands in the 5′ promoter region of the caveolin-1 gene in breast cancer cell lines that fail to express caveolin-1

In order to evaluate whether DNA methylation correlates with down-regulation of caveolin-1 gene expression, we next experimentally determined if any of the CGs in the 5′ region of the caveolin-1 gene were functionally methylated.

As a first step toward this goal, we analyzed the expression of the caveolin-1 protein in normal human mammary epithelial cells (NHMECs) as compared with caveolin-1 expression

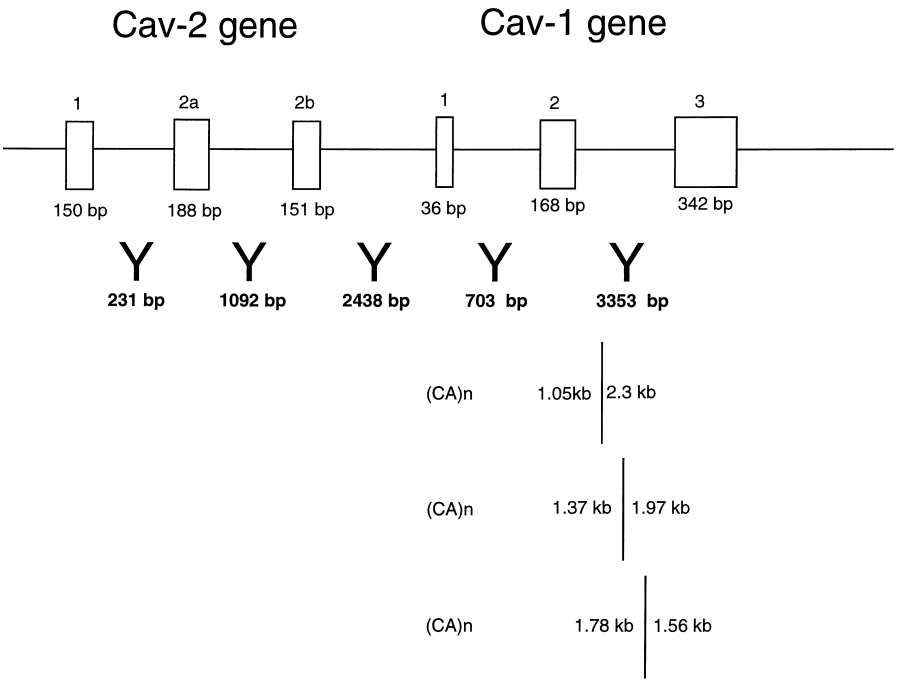

Fig. 5. Detailed organization of the caveolin-1/-2 locus in the pufferfish *Fugu rubripes*. This diagram is based on the sequence of an ~10 kb genomic clone from *Fugu rubripes* (Genbank Accession #AJ010316) that contains the genes encoding caveolins 1 and 2. The size of each exon and the distance between them is as indicated. Note that the sizes of the exons and their organization are highly conserved from pufferfish to man (compare with Fig. 2). The positions of three CA repeats located between caveolin-1/exon 2 and 3 are also indicated.

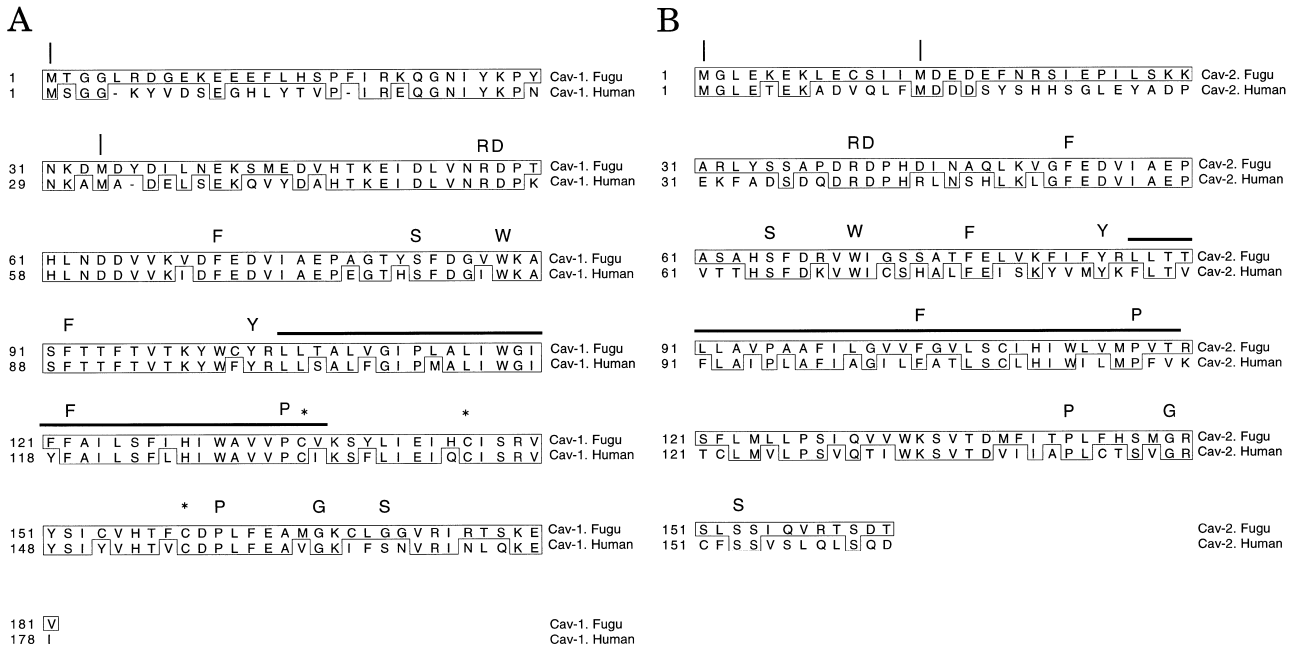

Fig. 6. Protein sequence alignments of caveolins 1 and 2 from pufferfish and man. A: Caveolin-1. Sequences identical to pufferfish caveolin-1 are boxed. B: Caveolin-2. Sequences identical to pufferfish caveolin-2 are boxed. The positions of two alternate start sites (thin vertical line), the membrane spanning domain (thick overline), three conserved cysteines that are site of palmitoylation in mammalian caveolin-1 (asterisks (*)), and the 12 residues that are invariant from *C. elegans* to man (uppercase letters above the sequence) are as indicated. Note that the sequences for caveolin-1 are more closely related (~74% identical) than the sequences for caveolin-2 (~49% identical).

in two well-studied human breast cancer cell lines (MCF7 and T-47D). Fig. 4A shows that caveolin-1 expression is dramatically down-regulated in these breast cancer-derived cell lines, as compared with the normal human control by Western blot analysis.

We next examined the pattern of caveolin-1 gene methylation in normal human mammary epithelial cells (NHMECs) and the corresponding breast cancer cell lines (MCF7 and T-47D). Genomic DNA prepared from each cell type was chemically treated with excess bisulfite to chemically modify non-methylated cytosines (C) so that they will later appear as thymidine (T) after PCR and DNA sequencing. Methylated cytosines are unaffected by bisulfite treatment and remain as cytosines (C) after PCR and DNA sequencing. Using this approach, we observed that certain CGs in the 5′ promoter region of the caveolin-1 gene are non-methylated in NHMECs that express the caveolin-1 protein (Fig. 4B, C). In striking contrast, the same CGs in MCF7 and T-47D cells were clearly methylated and these cells fail to express caveolin-1 (Fig. 4B, C). These observations are consistent with the idea that methylation of the caveolin-1 gene in transformed cells may lead to a loss of caveolin-1 expression via gene silencing.

3.4. The overall organization of the caveolin-1/-2 locus is conserved from pufferfish to man

Through database searches, we identified a ~10 kb genomic clone from *Fugu rubripes* (pufferfish; Genbank Accession #AJ010316) that contains the genes encoding caveolins 1 and 2. The overall organization of this locus is shown in Fig. 5. Thus, we compared this locus to the corresponding locus we deduced in humans.

Note that the sizes of the exons and their organization are highly conserved from pufferfish to man. However, in the pufferfish, the intervening sequences are much smaller. For example, the human locus encoding caveolins 1 and 2 is >60 kb, while the corresponding region in pufferfish is ~9 kb. This is consistent with the observation that the *Fugu rubripes* genome is approximately 7.5 times smaller than that of mammals (primarily due to reduced intron size) [31], but with a similar number of genes (9 kb × 7.5 = 67.5 kb).

We also compared the protein sequences of caveolins 1 and 2 from Fugu and man and these alignments are shown in Fig. 6. Note that caveolins from Fugu and man show a high degree of sequence conservation. Caveolin-1 from Fugu is ~84% similar and ~74% identical to caveolin-1 from man, while caveolin-2 from Fugu is ~69% similar and ~49% identical to caveolin-2 from man. These results indicate that caveolin-2 can tolerate a higher mutation rate than caveolin-1, despite the fact that these two genes are located adjacently within the genome.

4. Discussion

Caveolae are 50–100 nm invaginations of the plasma membrane [32]. It has been proposed that caveolae participate in trafficking and signal transduction processes [21–23]. Caveolin, a 21–24 kDa integral membrane protein, is a principal component of caveolae membranes in vivo [33–37]. Caveolin is only the first member of a new gene family; as a consequence, caveolin has been re-termed caveolin-1 [38]. Caveolin-1 is most highly expressed in differentiated cell types such as epithelial cells, endothelial cells, adipocytes and fibroblasts (reviewed in [21,39]). Caveolin-2 shows the same tissue distribution as caveolin-1 [40], while the expression of caveolin-3 is restricted to muscle cell types [41–43].

It has been proposed that caveolin family members function as scaffolding proteins [44] to organize and concentrate specific lipids (cholesterol and glyco-sphingolipids; [45–47]) and

lipid-modified signaling molecules (Src-like kinases, H-Ras, eNOS and G-proteins; [45,48–52]) within caveolae membranes. In support of this idea, caveolin-1 binding can functionally suppress the GTPase activity of hetero-trimeric G-proteins and inhibit the kinase activity of Src family tyrosine kinases, EGF receptor kinases, protein kinase C isoforms, and components of the p42/44 MAP kinase cascade (MEK and ERK) through a common caveolin domain, termed the *caveolin-scaffolding domain* [26,48–50,53–56].

Several independent lines of evidence are consistent with the idea that caveolin-1 functions as a tumor suppressor gene: (i) caveolin-1 mRNA and protein expression are down-regulated during cell transformation of cultured NIH 3T3 cells, in transgenic mouse models of breast cancer, and in cell lines derived from human breast cancers [24–27,57]; (ii) recombinant expression of caveolin-1 in transformed NIH 3T3 cells or cell lines derived from human breast cancers can suppress their transformed phenotype, such as anchorage-independent growth in soft agar [25,27]; and (iii) selective down-regulation of caveolin-1 expression using an anti-sense approach is sufficient to promote anchorage-independent growth in soft agar, drive tumor formation in nude mice, and hyperactivate the p42/44 MAP kinase cascade in otherwise normal NIH 3T3 cells [58].

Independently, recent genetic evidence shows that the caveolin-1 and -2 genes are co-localized to a suspected tumor suppressor locus in mice (6-A2) and humans (7q31.1/D7S522) [20,28,59]. In addition, the human caveolin-1 and -2 genes map at a maximum distance of ~100–200 kb from D7S522 [20]. This region (D7S522) is the center of the smallest common deleted region (SCDR), which harbors a presumed tumor suppressor gene [8,12,14,17]. However, the detailed organization of the caveolin genes and their exact physical distances from the D7S522 locus and from each other has remained unknown.

Here, through a shot-gun sequencing approach, we derived two adjacent contigs (~190 and ~65 kb) that contain both D7S522 and the caveolin genes, for a total coverage of ~250 kb across this genomic region. Detailed analysis of these contigs shows that D7S522 is located ~67 kb upstream of the caveolin-2 gene and that the caveolin-2 gene is located ~19 kb upstream of the caveolin-1 gene. As such, the human genes encoding caveolins 1 and 2 are the closest known genes in the immediate vicinity of the D7S522 locus. Sequence analysis of this region reveals (i) the intron-exon boundaries of the caveolin genes, (ii) previously unrecognized CA repeats that lie within or close to the caveolin genes, and (iii) that the first and second exons of both caveolin genes are embedded within CpG islands, i.e. potential sites for regulating gene expression by methylation. This critical genetic information will allow us and others to systematically evaluate whether the human caveolin genes are potentially deleted, mutated, or silenced by methylation during human tumor formation or metastasis.

In order to examine if caveolin-proximal CpG islands are functionally methylated in vivo, we analyzed the methylation status of a CpG island in the 5′ promoter region of the caveolin-1 gene in a variety of cultured cells. We observed that this CpG region is non-methylated in cultured normal human mammary epithelial cells (NHMECs) that express high levels of the caveolin-1 protein product. In contrast, we found that this CpG region is clearly methylated in two highly transformed human breast cancer cell lines (MCF7 and T-47D) that fail to express the caveolin-1 protein. In this regard, it is interesting to note that recombinant expression of caveolin-1 in T-47D cells is sufficient to revert their transformed phenotype, preventing their anchorage-independent growth in soft agar [27]. These findings are consistent with the general observation that methylation of CpG islands in the 5′ promoter region of a given tumor suppressor gene (p16, p15, VHL and E-cadherin) strictly correlates with down-regulation of their expression [29,30]. This type of gene silencing highlights an important alternative mechanism for inactivation of tumor suppressor genes, i.e. one that does not require point mutation or deletion of a specific gene [29,30].

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