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CLUSTERING OF UNDERMETHYLATED CCGG AND GCGC SEQUENCES IN THE 5' REGION OF THE Ha-RAS-1 ONCOGENE OF HUMAN LEUKEMIC K562 CELLS

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SUMMARY: The methylation state of the CCGG and GCGC sites of the Ha-ras-1 oncogene was analysed in the human leukemic K562 cell line, which was found to actively transcribe this gene. The results obtained demonstrate that the Ha-ras-1 oncogene is extensively methylated in both exonic, intronic, VTR and 3' untranslated portions, while undermethylations are present in a CG-rich island localized upstream of exon 1, near putative transcription initiation signals. Treatment of K562 cells with 5-azacytidine induces undermethylation of the Ha-ras-1 oncogene without major differences in the accumulation of Ha-ras-1 mRNA trascripts.

Differences in the degree of the overall DNA methylation are consistently detectable when normal cells are compared to neoplastic cells (1-4). In addition, changes in the pattern of DNA methylation have been recently found in genes which are hypothesized to be closely involved in the tumorigenic process, such as the genes of the ras family (5-8). Differential methylation of the Ha-ras-1 oncogene during mouse skin tumor progression has been reported (6). Accordingly Feinberg and Vogelstein have shown that the human Ha-ras-1 oncogene is undermethylated to various extents in lung and colon carcinomas (7-8). These reports, however, did not clarify whether Ha-ras-1 gene undermethylation is related to transcription or is simply caused by a non-specific hypomethylation of the DNA of the neoplastic cells.

The aim of the present study was to determine (a) whether different regions of the Ha-ras-1 gene might retain a differential level of methylation and (b) how this feature, if detected, could be correlated with gene expression.

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MATERIALS AND METHODS

Cell lines and culture conditions. The human leukemia K562(S)(9) and the melanoma Colo 38 (10) cell lines were from Dr. Livia Cioè (Istituto di Virologia, Università di Roma, Italy) and from Dr. Patrizio Giacomini (Laboratorio di Immunologia, Ist. Regina Elena, Roma), respectively. K562(5-azaCR) cells were obtained in our laboratory by culturing for at least 48 days K562(S) cells in the presence of 12.5 uM 5-azacytidine (11). Standard conditions for cell growth were α-medium (GIBCO), 50 mg/l streptomycin, 300 mg/l penicillin, 10-15% fetal calf serum (GIBCO) in 5% CO_2, 80% humidity.

Cytoplasmic dot hybridization. Dot hybridization analysis of cellular cytoplasmic preparations was performed as described by White and Bancroft (12). After cell lysis, 50 ul of post-mitochondrial supernatant were added to 30 ul of 20 x SSC (1 x SSC = 0.15 M NaCl, 0.015 M trisodium citrate) and 20 ul of 37% (w/w) formaldehyde (Fisher), incubated at 60°C for 15 min, suitably diluted in 15 x SSC and applied on a nitrocellulose sheet (11). Pre-hybridization and hybridizations with nick-translated DNA plasmids Pras.1^{tm} (Oncor) and pXCR7, carrying respectively v-Ha-ras-1 and ribosomal rRNA sequences, were performed for 16 hours as previously reported (11).

RNA extraction and Northern analysis. Total cellular RNA was isolated by a modification of the guanidine hydrochloride method, according to Adams et al. (13). 10 ug of total cellular RNA were electrophoresed in a 1.4% formaldehyde-agarose gel and then blotted onto nitrocellulose paper (14). Hybridization was carried out as for dot blot analysis.

Methylation of the Ha-ras-1 oncogene. The pattern of methylation was studied by Southern blotting of DNA previously digested with the methyl-sensitive restriction enzymes HpaII and HhaI, which cut the sequences CCGG and GCGC only if unmethylated. Procedures for the isolation of DNA and cleavage with restriction endonucleases were as described by Maniatis et al. (14). MspI, HpaII, HhaI, BamHI and XbaI restriction enzymes were purchased from Boehringer-Mannheim (Boehringer Italia, Milan, Italy). To ensure complete digestion, a sample of DNA was removed after addition of the enzyme and DNA was added (DNA: cell line DNA = 1:3). Only after full digestion of DNA, DNA samples were isolated, Southern blotted (15), and hybridized with the genomic T24-C2 Ha-ras-1 specific probe (16).

Computer-assisted analysis of CG display and CG suppression. The IBM-PC compatible program "CG-SUP-I" (S.Volinia, R.Gambari, manuscript in preparation) was developed in our laboratory. CG suppression was evaluated according to Tykocinski et al.(17). The Gene Sequence Data Bank (GenBank®) was from BBN Laboratory (Cambridge, M., USA).

RESULTS

Differential methylation of the Ha-ras-1 oncogene in human tumor cell lines. Fig.1A shows the localization of MspI/HpaII (M) and HhaI (H) sites of the 6.4 Kb BamHI (B1-B2) fragment carrying the Ha-ras-1 oncogene (18). Fig.1B indicates that this oncogene is differentially methylated in K562 erythroleukemia cells as compared to Colo 38 melanoma cells. Both HpaII and HpaII-BamHI generated Ha-ras-1 specific hybridization patterns are indeed, in Colo 38 DNA, superimposable with that generated by MspI, suggesting that extensive undermethylations of this gene are present in Colo 38 melanoma

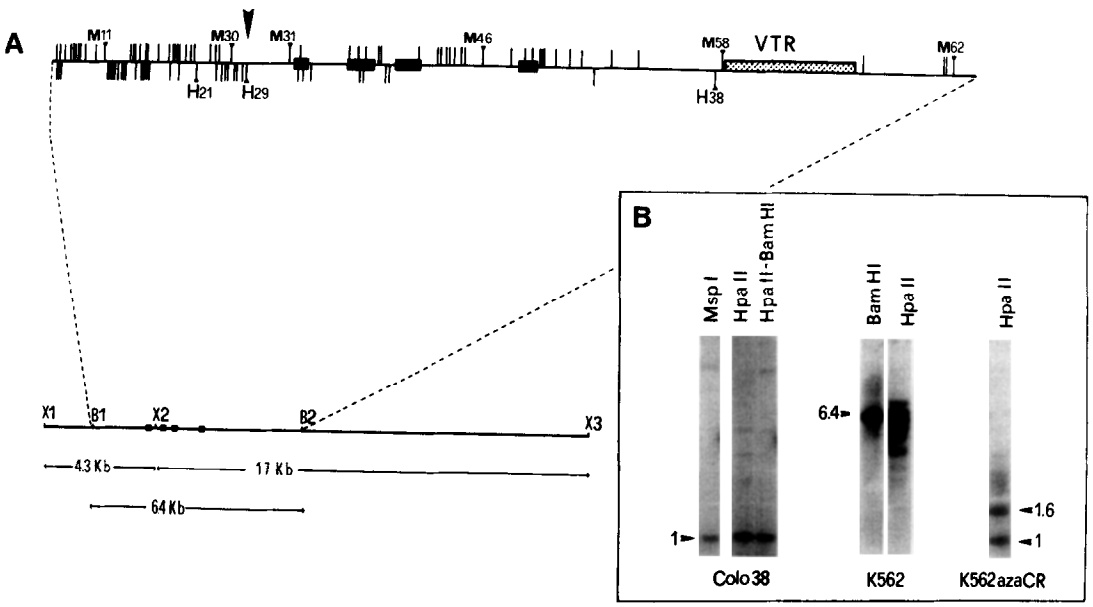

Fig.1. A: Display of CCGG (M1-M62) and GCGC (H1-H38) sequences throughout the BamHI DNA fragment containing the human Ha-ras-1 oncogene. ■ = exons; ▼ = putative promoter region; □ = VTR segment; X1, X2, X3 = XbaI sites; B1, B2 = BamHI sites.
B: Methylation state of CCGG sequences of the Ha-ras-1 oncogene of the erythromyeloid K562 and the melanoma Colo 38 cell lines. The DNA isolated from K562, K562(5-azaCR) and Colo 38 cells was digested with the indicated restriction enzymes, Southern blotted and hybridized with the T24-C3 probe. The sizes (Kb) of the obtained Ha-ras-1 specific fragments are indicated.

cells. The HpaII restriction enzyme, indeed, cuts the sequence CCGG only when unmethylated, while MspI cuts both CCGG and C^{me}CGG sequences (14). By contrast, the Ha-ras-1 oncogene is strongly methylated in K562 cells, as suggested by the presence of HpaII-generated fragments even larger than 6.4 Kb (Fig.1B). Only after treatment of K562 cells with the powerful demethylating agent 5-azacytidine (25 uM) for at least 48 days the Ha-ras-1 oncogene becomes almost completely unmethylated (Fig.1B).

CCGG and GCGC sites of the Ha-ras-1 oncogene of K562 cells: localization of a undermethylated region in the 5' portion of the gene. When the restriction enzyme HpaII is used in combination with BamHI the size of the BamHI 6.4 Kb Ha-ras-1 specific fragment is lowered to 5.2 Kb (Fig.2), suggesting that unmethylated CCGG sites are present within the BamHI fragment localized either at its 3' or 5' end. It should be noted (Fig.1A) that few MspI/HpaII sites (identified as M57-M62) are present in the 3' region of the

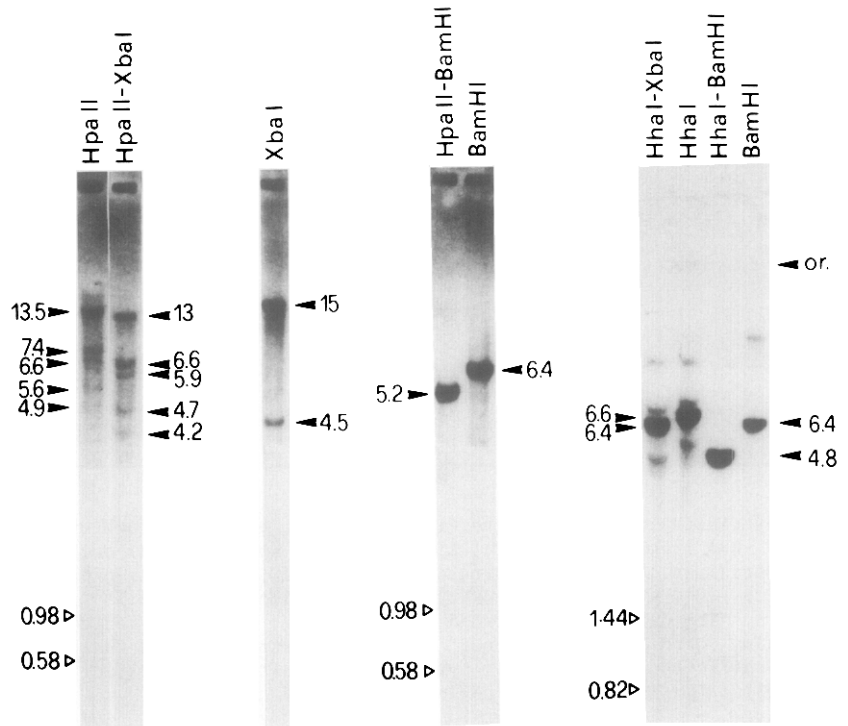

Fig.2. Pattern of methylation of CCGG and GCGC sites of the Ha-ras-1 oncogene of K562 cells. DNA was digested with the indicated restriction enzymes, Southern blotted and hybridized with the T24-C3 probe. Black arrows indicate the sizes (Kb) of the Ha-ras-1 specific fragments. Open arrows (▷) indicate the position of DNA fragments which would be generated by HpaII and HhaI in the presence of unmethylated M57-M60 or H36-H38 sites (see Fig.1A).

oncogene which, if unmethylated, would generate in both HpaII and BamHI-HpaII digests fragments of 0.58 Kb (M57-M58), 0.98 Kb (M58-M59) and 0.58 Kb (M59-M60). Such fragments were never observed in three independent experiments (Fig.2, open arrows and data not shown). In addition Fig.2 shows that in HpaII/XbaI double digests all the HpaII generated Ha-ras-1 fragments are lowered in molecular weight by about 0.7-0.9 Kb.

Taken together these results strongly indicate a methylated state of all the CCGG sequences between the 3' BamHI site (B2) and a MspI/HpaII site which is located about 1200 nucleotides downstream of the 5' BamHI (B1) site and about 700 nucleotides upstream of the X2 site, near putative transcription initiation signals (18). In agreement with these data Fig.2 also suggests that the HhaI sites located between the X2 site and the 3' BamHI(B2) site are methylated, while unmethylated GCGC sites are located between the 5'

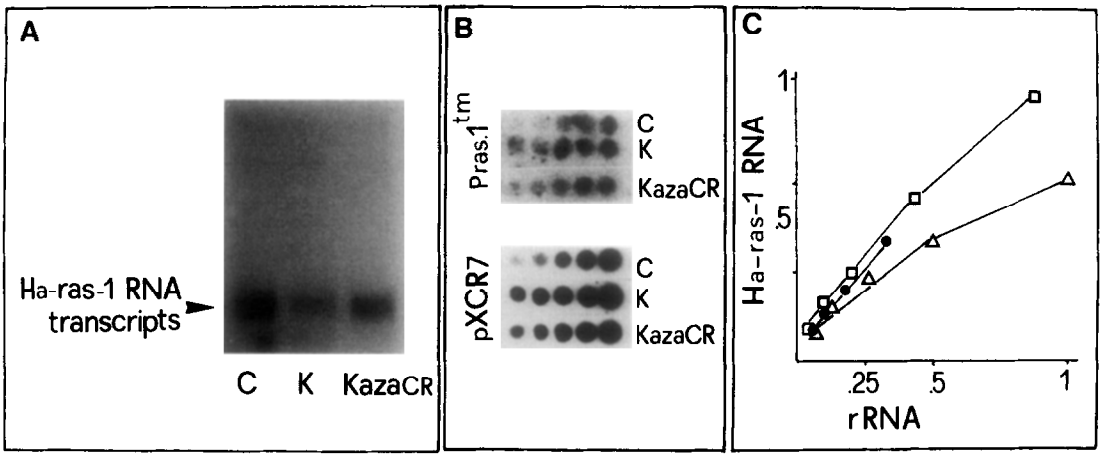

Fig.3. A: Hybridization between the Pras.1^{tm} probe and Northern blotted cytoplasmic RNA from Colo 38 (C), K562 (K) and K562(5-azaCR) cells. B,C: Dot-spot hybridization between formaldehyde-treated cytoplasmic preparations from Colo 38 (C), K562 (K) and K562(5-azaCR) cells and Pras.1^{tm} and pXCR7 probes. The dilutions performed (1, 1/2, 1/4 ...) are indicated. The autoradiograms were scanned through a spectrophotometer and the relative Ha-ras-1 specific and rRNA specific hybridization units were related. ● = K562 cells; □ = K562(5-azaCR) cells; △ = Colo 38 cells.

BamHI(B1) site and a HhaI site located about 1600 nucleotides downstream of this BamHI site and about 300 nucleotides upstream of the X2 site. The major evidence supporting this conclusion is that 1.44 Kb (H36-H37) and 0.82 Kb (H37-H38) fragments were never detected after digestion with the methyl-sensitive HhaI restriction enzyme (cuts the sequence GCGC only when unmethylated). Therefore we conclude (a) that the H36, H37 and H38 sites are methylated and (b) that the 4.8 Kb fragment detected after HhaI-BamHI double digestion is generated by unmethylated GCGC sequences localized in the 5' region of the Ha-ras-1 oncogene. Accordingly, the sizes of the Ha-ras-1 fragments generated by XbaI-HhaI double digestion are all smaller of about 0.2 Kb with respect to the HhaI fragments (Fig.2).

Expression of the Ha-ras-1 oncogene in Colo 38 and K562 cells. Fig.3A shows that the use of the v-ras SstI-PstI 0.7 Kb probe (P.ras1^{tm}) allows, after Northern blotting analysis, the identification of Ha-ras-1 specific RNA transcripts both in melanoma Colo 38 and erythroleukemic K562 cells. In order to quantitate the level of Ha-ras-1 RNA sequences with respect to ribosomal rRNA, hybridization was performed between dot spotted cytoplasms and P-ras-1^{tm} and pXCR7 probes. The results obtained (Fig.3B

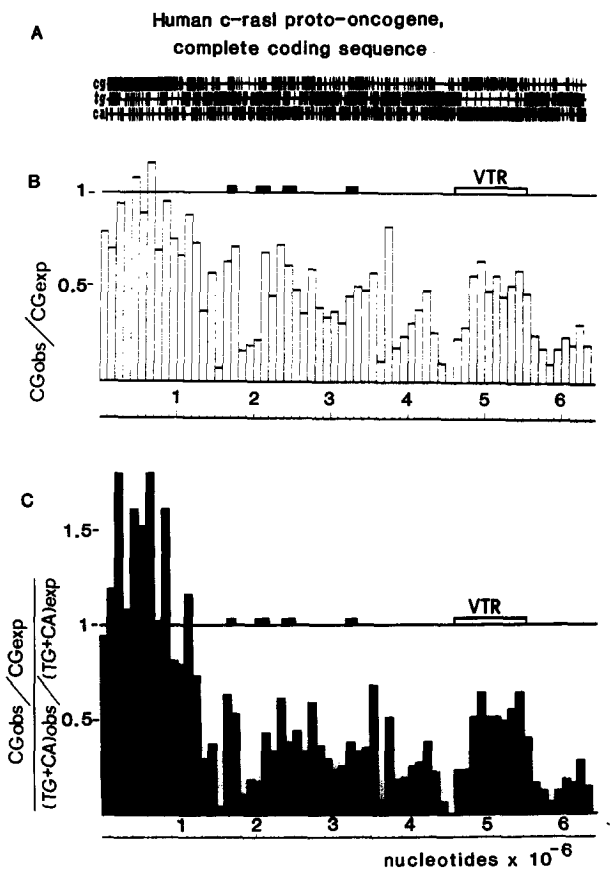

Fig.4. A: Display of CG, TG, and CA dinucleotides throughout the human Ha-ras-1 oncogene sequence published by Capon et al. (18).
B,C: CG observed / CG expected frequencies (B), related with CA and TG frequencies (C).

and C) (a) confirm that the Ha-ras-1 oncogene is expressed in both K562 and Colo 38 cells and (b) suggest that 5-azaCR, which is able to induce dramatic changes in the methylation pattern of the Ha-ras-1 oncogene (see Fig.1B), has only minor effects on the steady state levels of Ha-ras-1 RNA transcripts.

CpG display within the human Ha-ras-1 oncogene. Fig.4A shows that CpG dinucleotides are clustered in the 5' portion of the human Ha-ras-1 oncogene. In addition Fig.4B, 4C and Table I also show that the (CG)obs/(CG)exp ratios are significantly higher in the 5' portion of the gene (nucleotides 0-500, 501-1000) than in the exonic and the intronic regions. These data identify a CG-rich island localized upstream of the Ha-ras-1 exon 1 and are in good agreement with the results showing that this region is undermethylated (Fig.2). Indeed, the unmethylated CG dinucleotides retain a lower probability,

TABLE I

Human Ha-ras-1 oncogene: frequency of CG, TG, and CA dinucleotides

Fragment= (nucleotides)	Gene region	observed/expected ratios[b] CG	TG	CA	CG/(TG+CA)
0-500	5'	0.898	1.06	0.88	0.46
501-1000	5'	0.887	0.77	1.35	0.42
1001-1664	5'	0.528	1.21	1.33	0.2
1665-1774	Exon 1	0.832	1.62	1.02	0.32
1775-2042	Intron	0.228	1.49	1.29	0.082
2043-2220	Exon 2	0.551	1.35	1.7	0.18
2221-2374	Intron	0.555	1.42	1.63	0.18
2375-2533	Exon 3	0.716	1.66	1.13	0.26
2534-3231	Intron	0.37	1.28	1.4	0.14
3232-3350	Exon 4	0.472	2.02	1	0.16
3351-6453	Intron-VTR-3'	0.322	1.27	1.4	0.12

[a] The Ha-ras-1 nucleotide sequence was derived from Capon et al.(18).
[b] Obs/exp dinucleotide ratios were calculated according to Tykocinski et al. (17).

with respect to methylated CpG dinucleotide, to be converted into TG or CA following deamination of the cytosine (19). Accordingly, Table I and Fig.4C show that where CG dinucleotides are less frequent (for instance in introns and in exons 2, 3 and 4) the (TG+CA) frequencies are higher than those expected.

DISCUSSION

The major conclusion gathered from the results presented in this paper is that only undermethylation(s) restricted to the 5' portion of the Ha-ras-1 oncogene of human leukemic K562 cells might be required for its expression. This region displays potential transcription initiation signals, including a potential Goldberg-Hogness box (18), which precedes a GGTAACCT sequence which resembles the consensus sequence that appears 70-80 bp upstream of the RNA start of most of the eukaryotic promoters (18).

In addition our results indicate that transcription of the Ha-ras-1 oncogene can take place even in the presence of full methylations of the CCGG and GCGC sites of the coding regions, as well as of the introns and of the 3' non-coding portion of the gene. These results are of interest for at least two reasons. First, they clearly indicate different roles for CG methylation within the BamHI DNA fragment carrying the Ha-ras-1 oncogene.

Second, since undermethylation of the 3' portion of the Ha-ras-1 oncogene does not appear to be required for gene expression, the unmethylated state of the Ha-ras-1 gene often observed in tumor tissues, should simply be considered as a marker of the overall methylation state of the tumor cells analysed, rather than as a specific signal of transcriptional activation. From this point of view the experiment reported in this paper should encourage further investigations to determine whether in other tumor cell lines or in human primary tumor tissues the Ha-ras-1 oncogene is "organized" as in K562 cells. The computer-assisted analysis of CpG distribution throughout the human Ha-ras-1 oncogene deserves some comments as well. As pointed out by different groups (17,19), the ^{m}CG dinucleotide retains an higher capability, with respect to unmethylated CG, to be converted to TG or CA following deamination of the methyl-cytosine. Therefore, CG-rich islands are usually highly conserved and unmethylated (19). In agreement, the portion of the Ha-ras-1 oncogene which displays the highest concentration of CG sequences corresponds to the DNA region which is unmethylated in K562 cells (see Fig.1 and Fig.2). We, therefore, propose that undermethylation of the 5' portion of the Ha-ras-1 oncogene might be a general molecular feature of eukaryotic cells. Ha-ras-1 molecular probes specific for this 5' region should be considered in order to prove or disprove this hypothesis.

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