Promoter Methylation and Silencing of the Retinoic Acid Receptor-β Gene in Lung Carcinomas

Arvind K. Virmani, Asha Rathi, Sabine Zöchbauer-Müller, Nicoletta Sacchi, Yasuro Fukuyama, David Bryant, Anirban Maitra, Shashank Heda, Kwun M. Fong, Frederik Thunnissen, John D. Minna, Adi F. Gazdar

Background: **Retinoic acid plays an important role in lung development and differentiation, acting primarily via nuclear receptors encoded by the retinoic acid receptor-β (RARβ) gene. Because receptor isoforms RARβ2 and RARβ4 are repressed in human lung cancers, we investigated whether methylation of their promoter, P2, might lead to silencing of the RARβ gene in human lung tumors and cell lines.** ***Methods:*** **Methylation of the P2 promoter from small-cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC) cell lines and tumor samples was analyzed by the methylation-specific polymerase chain reaction (PCR). Expression of RARβ2 and RARβ4 was analyzed by reverse transcription–PCR. Loss of heterozygosity (LOH) was analyzed by PCR amplification followed by electrophoretic separation of PCR products. Statistical differences were analyzed by Fisher's exact test with continuity correction.** ***Results:*** **The P2 promoter was methylated in 72% (63 of 87) of SCLC and in 41% (52 of 127) of NSCLC tumors and cell lines, and the difference was statistically significant (two-sided *P*<.001). By contrast, in 57 of 58 control samples, we observed only the unmethylated form of the gene. Four tumor cell lines with unmethylated promoter regions expressed both RARβ2 and RARβ4. Four tumor lines with methylated promoter regions lacked expression of these isoforms, but demethylation by exposure to 5-aza-2′-deoxycytidine restored their expression. LOH at chromosome 3p24 was observed in 100% (13 of 13) of SCLC lines and 67% (12 of 18) of NSCLC cell lines, and the difference was statistically significant (two-sided *P* = .028).** ***Conclusions:*** **Methylation of the RARβ P2 promoter is one mechanism that silences RARβ2 and RARβ4 expression in many lung cancers, particularly SCLC. Chemical demethylation is a potential approach to lung cancer therapy. [J Natl Cancer Inst 2000;92:1303–7]**

High frequencies of loss of heterozygosity (LOH) at chromosomal region 3p21–3p24 occur in several tumor types, suggesting the presence of one or more tumor suppressor genes in the short arm of chromosome 3 *(1–5).* Among the genes known to map within this frequently deleted region is the retinoic acid receptor-β (RARβ) gene.

Retinoids, analogues of vitamin A, are needed for normal lung development and differentiation *(6,7).* They can reverse preneoplastic lesions and prevent second primary tumors of the upper aerodigestive tract *(8).* These effects are mediated via nuclear retinoic acid receptors (RARs) and retinoid X receptors (RXRs), which stimulate transcription factors in response to binding of a retinoic acid ligand. Each receptor group includes three subtypes (α, β, and γ). Receptors of the RAR family are differentially expressed during development and in adult life, and there is strong evidence that RARβ plays a central role in growth regulation of epithelial cells and in tumorigenesis *(9–11).*

The human RARβ gene generates multiple isoforms by use of promoters P1 and P2 and alternative splicing *(12,13).* P1 directs the transcription of isoform RARβ1, whereas P2 promotes the transcription of isoforms RARβ2 and RARβ4 *(14).* Isoform RARβ3 is expressed from the P1 promoter in mice, but it is absent in humans. These isoforms have been shown to vary in their ability to trans-activate retinoic acid-responsive promoters. It is thought that the receptors, through diversity in structure and patterns of expression, are able to control different subsets of retinoic acid-responsive genes to achieve the multiple effects of retinoic acid.

Repression of RARβ occurs in non-small-cell lung cancers (NSCLCs) *(5,15,16)* as well as in other human malignancies. Human lung cancers show reduced expression of RARβ2 and RARβ4 messenger RNAs (mRNAs) and protein *(5,15),* and they exhibit resistance to retinoic acid *(17,18).* RARβ2 plays an important role in suppression of murine lung tumorigenesis *(19)* and inhibits the growth of human lung cancer cells *in vitro (18).* These observations suggest that loss of expression of RARβ, especially that of the RARβ2 isoform, may be associated with lung carcinogenesis. In contrast, RARβ1 is expressed in small-cell lung cancer (SCLC) cell lines *(20).*

Analysis of lung cancer cell lines covering the entire open reading frame and the sequences of the retinoic acid response elements failed to show any mutations *(17).* Aberrant methylation of CpG islands was identified as an epigenetic mechanism for the transcriptional silencing (inactivation) of tumor suppressor genes *(21,22).* We investigated the role of aberrant methyl-

Affiliations of authors: A. K. Virmani, A. Maitra, A. F. Gazdar, Hamon Center for Therapeutic Oncology Research and Department of Pathology, University of Texas Southwestern Medical Center, Dallas; A. Rathi, S. Zöchbauer-Müller, Y. Fukuyama, D. Bryant, S. Heda, Hamon Center for Therapeutic Oncology Research; N. Sacchi, Department of Biology, University of Milan, Italy; K. M. Fong, Department of Thoracic Medicine, The Prince Charles Hospital, Brisbane, Australia; F. Thunnissen, Department of Pathology, Canisius Wilhelmina Hospital, Nijmegen, The Netherlands; J. D. Minna, Hamon Center for Therapeutic Oncology Research and Departments of Pharmacology and Internal Medicine, University of Texas Southwestern Medical Center.

Correspondence to: Adi F. Gazdar, M.D., Hamon Center for Therapeutic Oncology Research, University of Texas Southwestern Medical School, 5323 Harry Hines Blvd., Dallas, TX 75390-8593 (e-mail: gazdar@simmons.swmed.edu).

See "Notes" following "References."

ation of the RARβ gene promoter P2 in human lung cancers and cell lines.

Materials and Methods

Cell Lines and Tumor Samples

All human lung cancer cell lines (66 SCLC lines and 78 NSCLC lines) and B-lymphoblastoid lines (n = 31) were established by us *(23)*. The cells were grown in RPMI-1640 medium (Life Technologies, Inc. [GIBCO BRL], Rockville, MD) supplemented with 5% fetal bovine serum and were incubated in 5% CO_2. Samples of tissue from 49 surgically resected primary NSCLC tumors, along with 24 samples of nonmalignant lung tissue from the same patients, were obtained from the Tumor and Tissue Repository at the Hamon Center for Therapeutic Oncology Research, University of Texas Southwestern Medical Center, Dallas. Formalin-fixed, paraffin-embedded sections of 21 SCLC tumors were obtained from The University of Texas M. D. Anderson Cancer Center, Houston, or from Canisius Wilhelmina Hospital, Nijmegen, The Netherlands. Archival paraffin sections of tumor-negative lymph nodes from 12 of the NSCLC patients were utilized as controls for the paraffin-embedded tumors. Epithelial cells from buccal swabs (n = 12) and peripheral blood lymphocytes (n = 10) were collected from 22 healthy volunteers and served as negative controls for the frozen tissues. Appropriate Institutional Review Board permission was obtained from all participating centers, and written informed consent was obtained from all volunteers before usage of prospectively collected fresh samples. Institutional guidelines do not require written permission for the use of anonymous archival paraffin-embedded specimens.

Genomic DNA was obtained from cell lines, primary tumors, and nonmalignant cells by digestion with 200 μg/mL proteinase K (Life Technologies, Inc.) for 1 day at 37 °C, followed by two extractions with phenol–chloroform (1 : 1) *(24)*. DNA was extracted from paraffin sections after precise laser-capture microdissection of the tumor cells or lymph nodes, as described previously *(25)*.

Methylation-Specific Polymerase Chain Reaction

The methylation-specific polymerase chain reaction (PCR) employs an initial bisulfite reaction to modify the DNA. As a result, all unmethylated cytosines are deaminated and converted to uracils, while 5-methylcytosines remain unaltered. Thus, after bisulfite treatment, alleles that were originally methylated have DNA sequences different from those of their corresponding unmethylated alleles, and these differences can be used to design PCR primers that are specific for methylated or unmethylated alleles.

DNA was treated with sodium bisulfite as described previously *(26)*. Briefly, 1 μg of DNA was denatured by incubation with 0.2 *M* NaOH for 10 minutes at 37 °C. Aliquots of 10 m*M* hydroquinone (30 μL) (Sigma Chemical Co., St. Louis, MO) and 3 *M* sodium bisulfite (pH 5.0, 520 μL) (Sigma Chemical Co.) were added, and the solution was incubated at 50 °C for 16 hours. Treated DNA was purified by use of a Wizard DNA Purification System (Promega Corp., Madison, WI). Modified DNA was stored at −70 °C until used.

Amplification of bisulfite-modified DNA for RARβ gene promoter P2 was performed by PCR as described by Côté et al. *(27)* with primers that were specific for either methylated or unmethylated RARβ sequences. Primers used to amplify the methylated RARβ gene were 5′-TCGAGAACGCGAGCGATTCG-3′ (sense) and 5′-GACCAATCCAACCGAAACGA-3′ (antisense). Primers used to amplify the unmethylated RARβ gene were 5′-TTGAGAATGTGAGTGATTTGA-3′ (sense) and 5′-AACCAATCCAACCAAAACAA-3′ (antisense). Normal lymphocyte DNA was treated with *Sss*I DNA methyltransferase (New England Biolabs, Inc., Beverly, MA), subjected to bisulfite modification, and used as a positive-control DNA for each PCR reaction *(28)*. Negative control samples without DNA were included for each set of PCR. PCR of DNA from nonmalignant tissues and samples from healthy volunteers served as negative (unmethylated) controls. PCR products were analyzed on 2% agarose gels containing ethidium bromide (Life Technologies, Inc.). Conversion of all unmethylated Cs to Ts was confirmed by sequencing eight individual PCR products.

Reverse Transcription-PCR Analysis

Four tumor cell lines in which RARβ P2 promoter had been identified as being methylated were incubated in culture medium with and without 5-aza-2′-deoxycytidine (Aza-CdR) at a concentration of 2 μg/mL for 6 days, with medium changes on days 1, 3, and 5 *(29)*. Cells were harvested at the end of the 6th day for extraction of mRNA with a polyadenylic acid tail [poly(A) RNA]. Reverse transcription (RT) was performed on poly(A) RNA with the SuperScript II First-Strand Synthesis System (Life Technologies, Inc.), with the use of RARβ2-gene-specific reverse primer *(29)*. One microliter of the RT reaction mixture was used as template for PCR with primer set 1 *(29)* to produce a 256-base-pair (bp) RARβ2 gene product. A separate PCR was performed on the same RT product by use of primer set 2, which consists of forward primer 110-FP *(30)* and the above-described RT reverse primer, to amplify RARβ2 (623-bp product) and RARβ4 (264-bp product) simultaneously. One-step RT–PCR (Life Technologies, Inc.) was performed with primers for glyceraldehyde-3-phosphate dehydrogenase, a housekeeping gene (sense primer: 5′-ACAGTCCATGCCATCACTGCC-3′; antisense primer: 5′-GCCTGCTCCACCACCTTCTTG-3′), to confirm the integrities of the poly(A) RNAs. PCR products were analyzed on 2% agarose gels.

Analysis of LOH

Fourteen polymorphic microsatellite markers (*see* Table 2) that are located in chromosome region 3p24 and flank the RARβ gene *(31)* were selected for LOH analysis. DNA from 13 SCLC and 18 NSCLC tumor cell lines and their corresponding B-lymphoblastoid lines (constitutional DNA) were analyzed as described previously *(23)*. Briefly, 20 ng of genomic DNA was amplified by PCR in the presence of [α-^{32}P]cytidine 5′-triphosphate by use of the microsatellite markers. The PCR products were separated by electrophoresis in 6% polyacrylamide gels containing 7 *M* urea and were visualized by autoradiography. Markers that identified two bands of different size but of similar intensity in the lane having normal DNA were termed "informative" (i.e., heterozygous). Markers that gave only a single major band in the normal DNA lane were termed "noninformative." LOH was defined as a loss of a band corresponding to an allele present in informative cases.

Statistical Analysis

Statistical differences between groups were examined by use of Fisher's exact test with continuity correction. All *P* values are two-sided. Values of $P<.05$ were considered to be statistically significant.

Results

Methylation-specific PCR was performed on bisulfite-modified control and tumor DNA samples by simultaneous use of primers for the methylated and unmethylated forms of the RARβ gene promoter P2. As detailed in Table 1, 72% of (63 of 87) of SCLC and 41% (52 of 127) of NSCLC samples showed

Table 1. Incidence of methylation of the RARβ gene promoter P2 in lung tumors, tumor cell lines, and control tissues

Sample*	No.	No. of methylated samples (%)†
SCLC		
Paraffin-embedded tumors	21	13 (62)
Cell lines	66	50 (76)
All SCLC samples	87	63 (72)
NSCLC		
Frozen tumors	49	21 (43)
Cell lines	78	31 (40)
All NSCLC samples	127	52 (41)
Total lung cancer samples	214	115 (54)
Nonmalignant tissues		
Peripheral blood lymphocytes‡	10	0 (0)
Epithelial cells from buccal swabs‡	12	0 (0)
Paraffin-embedded tissues	12	1 (8)
Frozen lung tissues	24	0 (0)
Total nonmalignant samples	58	1 (1.7)

*SCLC = small-cell lung cancer; NSCLC = non-small-cell lung cancer.

†The differences in methylation incidences between SCLC and NSCLC cell lines were statistically significant for cell lines (two-sided $P<.001$, Fisher's exact test) and for all samples (two-sided $P<.001$) but not for tumor tissues (two-sided $P = .19$)

‡From healthy volunteers.

methylation of RARβ gene promoter P2. The difference in incidence of methylation of all 87 SCLC samples compared with all 127 NSCLC samples was statistically significant ($P<.001$). The incidences of RARβ promoter gene methylation between adenocarcinomas (26 of 71 [37%]) and squamous cell carcinomas (13 of 24 [54%]), the major subtypes of NSCLCs, were not statistically significantly different.

Tumor cell lines are pure tumor populations that are free of nonmalignant cells, and 95% (137 of 144) of the tumor cell lines that we examined were homozygous for either methylated or unmethylated RARβ promoter gene sequences. Five percent (seven of 144) were heterozygous and showed amplification of both methylated and unmethylated RARβ promoter gene sequences. Sample gels are shown in Fig. 1, A.

In tumor samples, most of which consist of mixtures of tumor cells and nonmalignant cells, either the band that corresponds to unmethylated RARβ only or the bands that correspond to both methylated and unmethylated RARβ were present. In 57 of 58 control samples, we observed only the unmethylated form of the gene (Table 1). Only one of the microdissected samples from a histologically normal lymph node that was used as control for paraffin-fixed samples had both methylated and unmethylated DNA. The presence of unmethylated RARβ promoter sequences in all control tissues confirmed the integrity of the DNA in these samples.

Four each of methylation-positive and methylation-negative cell line mRNAs were selected for RARβ2 and RARβ4 gene expression studies. NSCLC cell lines HCC44, HCC193, and HCC515 and SCLC cell line H209, in which the RARβ promoter was unmethylated, contained both RARβ2 and RARβ4 transcripts (data not shown). SCLC cell line H1607 and NSCLC cell lines HCC15, H2087, and HCC1171, in which the RARβ promoters were methylated, lacked both transcripts (Fig 1, B). After demethylation with Aza-CdR, these four cell lines were positive for both transcripts (Fig.1, B), although the intensity of the bands varied in the different cell lines.

We analyzed a panel of 31 SCLC or NSCLC tumor cell lines that were paired with corresponding B-lymphoblastoid cell lines (Table 2) for LOH at or around the RARβ gene by use of 14 polymorphic markers. We found LOH in at least one of the markers in all 13 SCLC cell lines (100%) and in 67% (12 of 18) of the NSCLC cell lines. Marker D3S1567 had a low informative rate (i.e., was seldom heterozygous) but showed 100% loss in both SCLC and NSCLC cell lines. Markers D3S1583, D3S2336, D3S2335, and D3S2337, which are centromeric to D3S1567, showed statistically significant differences in the incidences of LOH in SCLC compared with NSCLC cell lines. Although the precise location of the RARβ gene relative to these loci *(31)* is not known, the region bounded by these markers may be within or close to the gene. While there was no apparent relationship between LOH for any specific marker and methylation status, LOH at one or more markers was present in 89% (16 of 18) of the tumor lines in which the RARβ gene promoter was methylated.

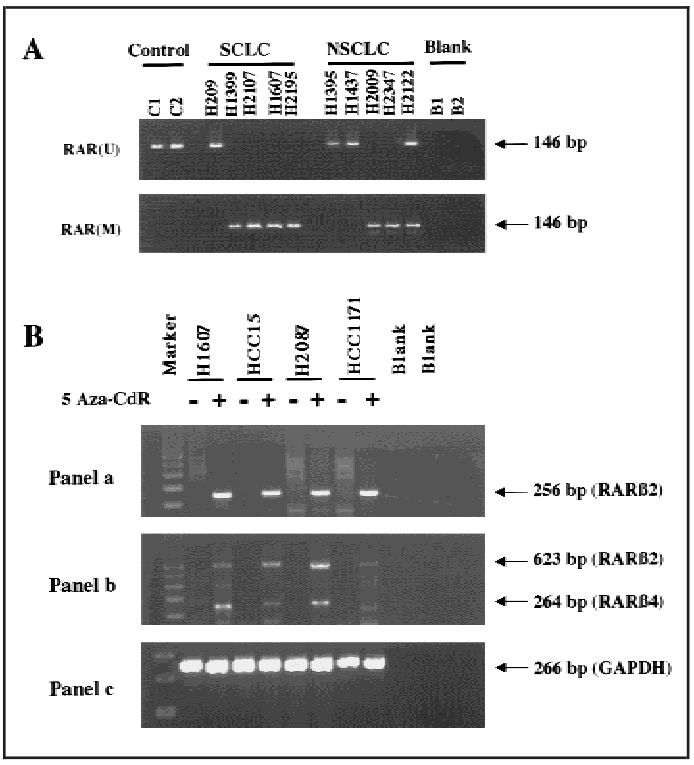

Fig. 1. Methylation of the retinoic acid receptor-β (RARβ) P2 promoter and expression of RARβ isoforms in lung cancers. **A**) Examples of methylation-specific polymerase chain reaction (PCR) analysis of the RARβ P2 promoter region. DNA from five small-cell lung cancer (SCLC) cell lines (H209, H1399, H2107, H1607, and H2195) and five non-small-cell lung cancer (NSCLC) cell lines (H1395, H1437, H2009, H2347, and H2122) was treated with bisulfite and amplified by PCR, as described in the text, by use of primers specific for unmethylated or methylated DNA. The resulting products were analyzed by gel electrophoresis. A visible product in the **RAR(U)** gel indicates that the RARβ gene is unmethylated in the corresponding DNA; a visible product in the **RAR(M)** gel indicates that the gene is methylated. Product in both gels indicates that both methylated and unmethylated forms of the gene are present. **Lanes C1** and **C2** show the products of PCR analysis of identically treated DNA from normal lymphocytes and buccal epithelial cells, respectively. **Lanes B1** and **B2** are negative PCR controls (no template DNA). **B**) Reverse transcription (RT)–PCR analysis of expression of RARβ2. Tumor cell lines H1607, HCC15, H2087, and HCC1171 were incubated with (**+ lanes**) or without (**– lanes**) 5-aza-2′-deoxycytidine (5 Aza-CdR) for 6 days. Their messenger RNA (mRNA) was extracted, and complementary DNAs were produced by RT for use as templates for PCR analysis, as described in the text. The resulting products were analyzed by gel electrophoresis. **Panel a** shows the results obtained by use of primer set 1, which produces a 256-base-pair (bp) product that indicates that the transcript of RARβ2 was present (i.e., the gene was expressed). **Panel b** shows the results obtained by use of primer set 2, which produces 623-bp and 264-bp products that indicate that the transcripts of RARβ2 and RARβ4, respectively, were present (i.e., the genes were expressed). **Panel c** shows the results obtained after one-step RT–PCR analysis with primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a housekeeping gene that produces a 266-bp product (to demonstrate the integrities of the extracted mRNAs). Size markers are shown in the **marker lane** of each gel. **Blank lanes** are RT control (no enzyme added) and PCR control (no RT template added).

DISCUSSION

RARβ may be the member of the RAR receptor family that is primarily responsible for mediating the effects of retinoic acid *(32)*. Reduced expression of RARβ has been reported in lung cancer *(33)* and in other solid tumors *(29)*. Geradts et al. *(17)* reported loss of RARβ expression in 75% of SCLC tumor lines and in 53% of NSCLC lines. Xu et al. *(15)* observed reduced expression of RARβ2 in NSCLC tumors. A marked decrease in the expression of RARβ, as well as a high frequency of LOH at 3p24, was also observed in non-neoplastic lesions *(5)*. Only two reports addressed expression of RARβ4. Sommer et al. *(30)* demonstrated that the ratio of RARβ4 to RARβ2 is elevated in breast tumor cell lines. In mice that carry an RARβ4-like trans-

Table 2. Loss of heterozygosity (LOH) at chromosome locus 3p24 in small-cell lung cancer (SCLC) and in non-small-cell lung cancer (NSCLC) cell lines

Marker*	No. of LOH/No. of informative cases (% LOH)		
	SCLC (n = 13)	NSCLC (n = 18)	P†
D3S1599	7/7 (100)	3/9 (33)	**.011**
D3S3659	7/8 (88)	6/11 (55)	.18
D3S3598	7/7 (100)	7/11 (64)	.12
D3S3700	7/8 (88)	7/9 (78)	1.00
D3S1567	6/6 (100)	4/4 (100)	—
D3S1583	5/5 (100)	3/9 (33)	**.031**
D3S2336	8/9 (89)	5/17 (29)	**.011**
D3S2335	9/9 (100)	6/12 (50)	**.019**
D3S2337	9/10 (90)	6/15 (40)	**.018**
D3S1266	10/10 (100)	5/8 (63)	.069
D3S1283	8/10 (80)	7/12 (58)	.38
D3S1609	5/5 (100)	6/10 (60)	.23
D3S3547	8/8 (100)	4/8 (50)	.077
D3S3527	7/8 (88)	7/12 (58)	.33
Any	13/13 (100)	12/18 (67)	**.028**

*While their precise order is controversial, the markers are arranged, as best as we could determine, in order from telomeric (D3S1599) to centromeric (D3S3527).

†Differences in incidence of LOH between SCLC and NSCLC cell lines were analyzed by use of Fisher's exact test. All *P* values are two-sided. Statistically significant values are printed in **boldface.**

gene, RARβ4-like expression showed tissue-specific variation *(34)* and was reduced in lung tissue. These data support the hypothesis that one or more isoforms of the RARβ gene may exert tumor-suppressive effects.

Aberrant methylation of the RARβ promoter gene has been observed previously in breast and colon cancers *(29,35,36).* Our observations demonstrate a high frequency of aberrant DNA methylation of the RARβ P2 promoter gene in lung cancers, particularly in SCLC. In the eight cell lines that we tested, there was complete concordance between aberrant methylation of the P2 promoter and silencing of both RARβ2 and RARβ4 transcripts. Furthermore, treatment with Aza-CdR restored transcript expression, indicating that methylation is one of the mechanisms responsible for loss of expression. Allelic losses at or around 3p24, the chromosomal location of RARβ, were more frequent in SCLC (100%) than in NSCLC (67%). The high frequency of LOH at 3p24, combined with the presence of only methylated sequences in most cell lines, fulfills the criteria for Knudson's two-hit hypothesis for tumor suppressor gene inactivation *(37).* While strong circumstantial evidence exists for the role of inactivation of the RARβ gene in lung cancer pathogenesis, the possibility that other genes at 3p are responsible for or contribute to lung cancer pathogenesis must be considered.

Several genes are known to be inactivated in lung cancers by aberrant methylation *(38).* The frequencies of aberrant methylation of the RARβ gene reported herein are among the highest for any gene described to date in lung cancers *(38).* Ayoub et al. *(39)* have reported frequent repression of both RARβ2 and RARβ4 in the bronchial epithelium of smokers. Their finding and those of other investigators *(33,40)* suggest that alteration in RAR expression is an early event in lung cancer pathogenesis. Tumor cells may release their DNA into the circulation, which would allow detection of aberrant methylation in DNA from the sera of lung cancer patients *(38).* Chemical reversal of methylation-related gene silencing (an epigenetic phenomenon) is a potential therapeutic approach *(41).* Our findings indicate that aberrant methylation of the RARβ gene is a frequent abnormality in lung cancers and may have clinical applications for risk assessment, for diagnosis, and for novel therapeutic approaches.

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NOTES

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