BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **238,** 77–80 (1997)
ARTICLE NO. RC977239

Vitamin D Receptor: No Evidence for Allele-Specific mRNA Stability in Cells Which Are Heterozygous for the Taq I Restriction Enzyme Polymorphism

Walter Verbeek, Adrian F. Gombart, Masaaki Shiohara, Moray Campbell, and H. Phillip Koeffler
Division of Hematology/Oncology, Department of Medicine, Cedars Sinai Medical Center, 8700 Beverly Boulevard, B-208, Los Angeles, California 90048

Received August 4, 1997

Allelic variations of the vitamin D receptor (VDR) gene have been associated with the risk of developing prostate cancer in men and osteoporosis in postmenopausal women. Three RFLP's (TaqI, ApaI, BsmI) define two common haplotypes: BAt and baT. None of these polymorphisms change the translated protein. Since sequence variations in the 3′ UTR of VDR have been linked to the different haplotypes, investigators have proposed that the stability of VDR mRNA is influenced by allelic variations. Indirect evidence suggested that allele T is less stable than allele t. In this study, we used a RT-PCR based approach to compare the stability of the big T and small t allele in normal heterozygous lymphocytes and the heterozygous cell lines NB4 (myeloid leukemia) and PC-3 and DU 145 (prostate cancers). In all three cases, we did not find a significant difference in stability. Interestingly, we consistently observed 30% less RT-PCR product derived from the small t allele mRNA in steady state, a finding which also speaks against a higher stability of the small t allele mRNA. These results indicate a variation in transcriptional regulation rather than mRNA stability between the alleles. We hypothesize that an unknown gene or genes in linkage with the polymorphisms is (are) responsible for the relationship between risk of prostate cancer and VDR polymorphisms. © 1997 Academic Press

Recently, epidemiologic studies correlated allelic variations of the vitamin D receptor (VDR) with the risk of developing prostate cancer in men (1,2). Allelic variations of the VDR have also been linked to the risk of osteoporosis in postmenopausal women (3,4); the latter, however, has been critically questioned (5,6). The alleles of human VDR can be distinguished by restriction enzyme length polymorphisms (RFLPs) found for Bsm I, Apa I and Taq I (7). The presence (b,a or t) or absence (B,A or T) of a restriction site defines the specific allele. Neither of these three polymorphisms alters the predicted amino acid sequence of the translated VDR protein. The Bsm I and Apa I polymorphisms are located in intron 8 of the VDR gene (3,8). Only the Taq I polymorphism is located in the coding region, but leads to a silent codon change in exon 9, with ATT and ATC both coding for isoleucine (codon 352) (9). The RFLP's are highly linked in the population. The Bsm I cleavage site (b) is associated in 97% of individuals with the absence of a Taq I site (T) (3). This linkage disequilibrium results in two common haplotypes: BAt and baT [Figure 1]. Morrison and colleagues found that sequence variations of the 3′untranslated region (UTR) of the VDR are also in linkage with the RFLP's (3). They suggested that variations of the 3′ UTR, e.g. variable length of a poly A sequence, could influence the mRNA stability of the VDR thereby possibly explaining the phenotypic effects. A recent study showed that men homozygous for the t allele have one third the risk of developing prostate cancer compared to men either heterozygous or homozygous for the T allele (1). This finding was confirmed by a second study which associated the length of the poly A sequence in the 3′UTR and the risk for prostate cancer (2). Especially heterozygous individuals carrying one allele with a longer poly A sequence (A18-22; linked to allele T) had a four- to five-fold increased risk. Since allele-specific differences in transcriptional activity or mRNA stability have been implicated as the underlying mechanism, we directly compared the prevalence of VDR mRNA from each allele in normal heterozygous lymphocytes, one promyelocytic leukemia cell line (NB4) and two prostate cancer cell lines (PC-3, DU 145). In addition, we compared the mRNA stability of each VDR allele in these model systems.

MATERIALS AND METHODS

Cell lines. PC-3, DU145 were purchased from ATCC (Gaithersburg, MD). NB4 was kindly provided by Dr. J. Lanotte, Hospital St.

0006-291X/97 $25.00

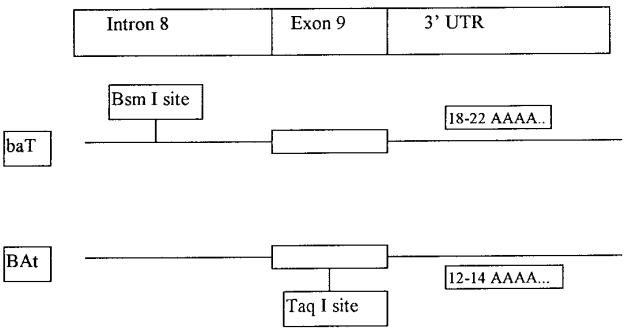

FIG. 1. Schematic drawing of the two common haplotypes of the VDR gene: BAt and baT. Lower case letters indicate the presence of a restriction enzyme site (b, BsmI, a, ApaI, t, TaqI). Linkage occurs between a short poly A sequence (12-14n) in the 3′UTR and the BAt allele, as well as a long poly A (18-22n) sequence and the baT allele.

Louis, Paris, France. Normal donor lymphocytes were isolated by Ficoll-Hypaque density centrifugation from their peripheral blood; they were depleted of adherent cells and expanded in culture with 10 units/ml interleukin 2 for 7 days. DNA and RNA were isolated to identify individuals who were heterozygous for the Taq I polymorphism.

Bsm I restriction enzyme polymorphism. DNA was isolated and used as a template for amplification by the polymerase chain reaction with the following primer pair: 5′ CAACCAAGACTACAAGTACCG-CGTCAGTGA 3′ and 5′ AACCAGCGGGAAGAGGTCAAGGG 3′.

PCR was performed with 35 cycles: 95°C 40 sec, 55°C 35 sec, 72°C 40 sec. The PCR product was digested with Bsm I; fragments were separated by gel electrophoresis through 1.5% agarose. The gel was stained with ethidium bromide, and the bands were visualized by a polaroid picture under UV light.

Taq I restriction enzyme polymorphism. RNA was isolated from 5×10^6 cells. Briefly, cells were harvested in 1 ml Trizol reagent (Gibco, BRL) and transferred to an Eppendorf tube. After chloroform extraction, the RNA was precipitated with isopropanol. After centrifugation, the RNA pellet was washed once with 80 % ethanol and air-dried. The pellet was redissolved in 50 ul of DEPC-treated water. RNA was then exposed to DNAse I for 30 minutes at 37°C followed by a phenol/chloroform extraction, ethanol precipitation and resuspension in DEPC water (Message Clean Kit, Genhunter Cooperation). Five ug of RNA was used for MMLV reverse transcription (RT). RT was performed with 10 uM random p(N6) oligonucleotide primers (Pharmacia) , 40 units RNAse inhibitor (Promega), 2 mM DTT, 500 uM dNTP's in first strand buffer (Gibco, BRL) with 400 units M-MLV at 37° C for 1 hour in a total volume of 50 ul. 1 ul cDNA was used for PCR. The primer set was designed around the polymorphic Taq I site separated by intron 8. Sense primer, AGTCCTTCACCATGGACGACATGT; antisense primer, TGCCAAACACTTCGAGCACAA.

RT-PCR was performed either at 35 cycles for detection on ethidium bromide stained agarose gels or at 25-27 cycles for different templates in the linear range of PCR. RT-PCR resulted in a single product of 440 bp. 10 ul of the PCR product was digested with Taq I for 1 hour at 65°C. Digested and undigested products were run on a 2% agarose gel stained by ethidium bromide.

mRNA stability studies. The prostate cancer cell line PC3 was grown in 10 cm culture dishes in DMEM plus 10% fetal bovine serum (FBS) to semiconfluency. For each time-point, one culture was treated with 5 ug/ml actinomycin D. RNA was extracted at times 0, 30 minutes, 1, 2, 3,4, 6, 8 and 12 hour intervals after addition of actinomycin D. Briefly, culture medium was removed completely and 2 ml of Trizol reagent was added to the cells. The solution was pipetted up and down, then equally distributed in two Eppendorf tubes. RNA isolation was performed as described above. The promyelocytic leukemia cell line NB-4 was grown at a cell density of 0.5×10^6/ml in 10 ml liquid cultures. Actinomycin D-treatment was performed as described above; to harvest cells, they were spun-down, dissolved in 1 ml of Trizol reagent and RNA was isolated according to the manufacturer's protocol. mRNA stability study of normal lymphocytes: Lymphocytes of an individual previously shown to be heterozygous for the Taq I polymorphism were isolated and expanded with 10 units/ml IL-2 for 7 days. Actinomycin D treatment and RNA isolation were performed as described above.

Allele-specific mRNA levels. For semiquantitative experiments, the linear range of RT-PCR was determined by template-dilution experiments. Detection at low cycle numbers was achieved by direct incorporation of ^{32}P-dCTP during the RT-PCR reaction. The product was digested by Taq I; fragments were separated on a nondenaturing polyacrylamide gel. The dried gel was exposed to an X-Omat film for autoradiography. Alternatively, amplification was performed by cold PCR; fragments were separated by agarose gel electrophoresis and detected after Southern blot hybridization to a ^{32}P-labeled VDR probe (440 bp fragment flanking Taq I site).

Southern blot hybridization. Taq I digested RT-PCR fragments were separated by electrophoresis on a 1.5% agarose gel in TBE buffer at 50 volts for 1.5 hours. The gel was then incubated in 400 mM NaOH for 30 minutes and subsequently blotted by alkaline transfer to a Hybond N + Nylon membrane as described by the manufacturer (Amersham). The membrane was briefly rinsed with $4 \times$ SSC and air dried.

VDR probe. The 440 bp VDR fragment was amplified by PCR from cDNA derived from normal lymphocytes with a tt alleotype, gel purified and digested with Taq I. The probe was labeled by random primed alpha ^{32}P dCTP incorporation before hybridization. The intensity of bands was quantified with an Ambis radio imaging system.

RESULTS

The genotype of Bsm I and Taq I restriction enzyme length polymorphisms in the VDR gene was determined for a variety of solid tumor and hematopoietic cell lines as well as normal lymphocytes from healthy donors. The prostate cancer cell lines (PC-3, DU145), and the hematopoietic cell line (NB4) were found to be heterozygous for Bb and Tt (Figure 2). Normal lymphocytes of genotype (tt) served as a control for complete Taq I digestion. The relative prevalence of each VDR mRNA was determined by semiquantitative RT-PCR of a VDR mRNA fragment flanking the Taq I site of these heterozygous cell lines. The amplified ^{32}P dCTP labeled RT-PCR product was digested by Taq I and separated on a polyacrylamide gel.

For increased sensitivity at lower PCR cycle numbers, Taq I digested PCR products were blotted and probed with the same ^{32}P labeled, Taq I-digested VDR fragment. In three repeated experiments, quantification of band intensities gave a distribution of T:t of 1.5:1 in normal and cell line NB-4, and 1.5:1 - 2:1 in PC3 and DU145 (Figure 3).

To test the hypothesis that the mRNA stability of the big T and little t allele differ, we performed mRNA stability studies in normal lymphocytes and cell lines NB4, PC3 (data not shown). At each time-point, we

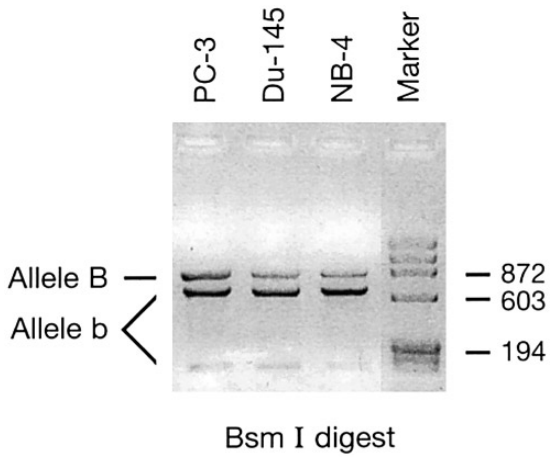

FIG. 2. A VDR fragment including the BsmI RFLP was amplified from DNA of cell lines PC-3, DU-145, and NB4. The BsmI digested fragments were separated by agarose gel electrophoresis and stained with ethidium bromide showing the heterozygous genotype of all three cell lines. The right lane contains molecular weight markers (marker).

compared the abundance of the two RT-PCR products, derived from either T or t. The ratio of the two RT-PCR products did not significantly change over time, although a decrease in mRNA could be documented when amplification conditions were adjusted to the linear range of PCR. This indicated a similar mRNA stability for both alleles in the cell lines. Figure 4 shows that the mRNA stability experiments of normal lymphocytes and NB4 cells gave almost identical results. Although RT-PCR is not an appropriate method to measure an exact mRNA half life, we estimated a short half-life of approximately 1.5 hours.

DISCUSSION

Epidemiological evidence which associates the BB tt allelotypes of VDR with an increased risk for osteoporosis in postmenopausal women and a decreased risk for the development of prostate cancer in men requires a biological explanation for these phenomena. The role of 1,25 dihydroxyvitamin D_3 in bone metabolism (calcium mobilization) and its antiproliferative activity on a variety of cell types including prostate cancer are at least compatible with a direct involvement of polymorphic variations of the VDR (10-13). Since none of the polymorphisms alter the predicted amino acid sequence of the VDR protein, the regulation of expression of VDR is a potential discriminating feature. Morrison and colleagues found that variations of the 3′UTR of VDR were linked to the Bsm I and Taq I RFLP's. In minigene reporter constructs, in which the 3′ UTR of alleles BAt and baT were fused 3′ to a luciferase reporter gene, luciferase activity was 30-40% lower when the bAT construct was transfected in COS cells (3). They, therefore, proposed that differences in mRNA stability (T mRNA being less stable than t mRNA) may be responsible for phenotypic differences (14,15).

To keep all influences equal for both VDR mRNAs transcribed either from the T or t allele, we chose a heterozygous system (Tt) as opposed to the comparison of two (tt vs. TT) homozygous cell lines. We consistently found approximately 30% less RT-PCR product derived from the t than the T allele in normal lymphocytes, the hematopoietic cell line NB4 and the prostate cancer cell lines PC-3 and DU-145. We could not demonstrate a difference in VDR mRNA stability between the two alleles in either the cell lines or the normal lymphocytes.

In conclusion, we have no evidence that the bAT allele mRNA is less stable or less expressed than the Bat allele mRNA. On the contrary, our data are consistent with a slightly favored transcription of the baT allele in heterozygous individuals. Studies have shown that circulating levels of 1,25 dihydroxyvitamin D_3 are significantly higher in individuals homozygous for the BAt allele (BB individuals: [Mean ± SD] 134±42 pM; bb: 99±40 pM) (3,16). Possibly, higher concentrations of a circulating ligand are associated in a given population with a lower baseline receptor mRNA expression which is mediated by allelic variations in the receptor gene in order to ensure calcium homeostasis. This might counterbalance the concentration-dependent effect of 1,25 dihydroxyvitamin D_3 to increase the VDR number/cell by increasing mRNA stability or prolonging protein half-life (17-21). An unknown gene in linkage with the locus for the VDR may be responsible for the correlation of specific VDR alleles and the risk of prostate cancer.

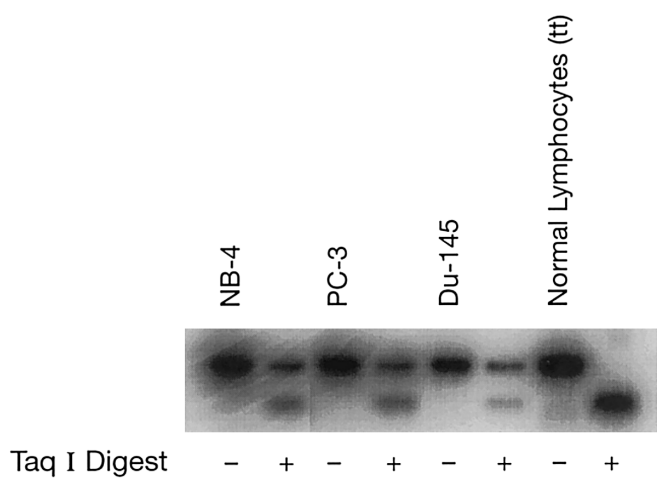

FIG. 3. Southern blot of undigested (−) and Taq I digested (+) VDR RT-PCR products of the heterozygous cell lines NB-4, PC-3, and DU145. The upper bands represent allele T, the lower bands allele t. The right two lanes demonstrate the complete digest of a homozygous tt lymphocyte control sample. Radio imaging shows a consistent imbalance in the prevalence of alleles T and t. The ratio of T:t is 1.5:1 in NB-4 and 1.5 - 2:1 in PC-3 and DU-145.

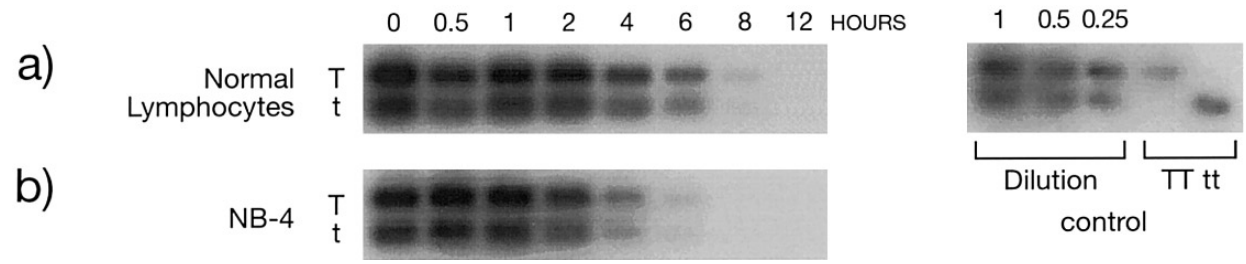

FIG. 4. Allele specific mRNA stability studies. RNA was isolated at different time intervals after addition of actinomycin D to normal lymphocytes as well as cell lines NB-4. RNA from each time point was used for cDNA synthesis. PCR was performed with primers flanking the Taq I site. The PCR product was digested with Taq I; the resulting fragments were separated on a 1.5% agarose gel and detected by Southern blot. The ratio between the alleles was determined at each time point by Ambis radio imaging. None of the experiments showed a change of the allelic ratio over time.

ACKNOWLEDGMENTS

This paper was supported by NIH Grants CA 43277, CA 42710, CA 70675, and CA 26038 and Deutsche Forschungsgemeinschaft.

REFERENCES

1. Taylor, J. A., Hirvonen, A., Watson, M., Pittman, G., Mohler, J. L., and Bell, D. A. (1996) *Cancer Res.* **56,** 4108–4110.
2. Ingles, S. A., Ross, R. K., Yu, M. C., Irvine, R. A., La Pera, G., Haile, R. W., and Coetzee, G. A. (1997) *J. Natl. Cancer Institute* **89,** 6–10.
3. Morrison, N. A., Cheng, Qi J., Tokita, A., Kelly, P., Crofts, L., Nguyen, T. V., Sambrook, P. N., and Eisman, J. A. (1994) *Nature* **367,** 284–287.
4. Eismann, J. A. (1995) *J. of Bone and Mineral Res.* **9,** 1289–1293.
5. Peacock, M. (1995) *Journal of Bone and Mineral Research* **10,** 1294–1296.
6. Garnero, P., Borel, O., Sornay-Rendu, E., and Delmas, P. D. (1995) *J. of Bone and Mineral Res.* **10,** 1283–1288.
7. Hustmyer, F. G., DeLuca, H. F., and Peacock, M. (1993) *Human Molecular Genetics* **2,** 487.
8. Baker, A. R., Donnell, D. P., Hughes, M., Crisp, T. M., Mangelsdorf, D. J., Haussler, M. R., Pike, J. W., Shine, J., and O'Malley, B. W. (1988) *Proc Natl Acad Sci* **85,** 3294–3298.
9. Farrow, S. (1994) *Lancet* **343,** 1242.
10. Konety, B. R., Schwartz, G. G., Acierno, J. S., Becich, J., and Getzenberg, R. H. (1996) *Cell Growth and Differentiation* **7,** 1563–1570.
11. Sokowronski, R. J., Peehl, D. M., and Feldman, D. (1993) *Endocrinology* **132,** 1952–1960.
12. Hedlund, T. E., Moffatt, K. A., and Miller, G. J. (1996) *J Steroid Biochem and Mol Biol* **58,** 277–288.
13. Christakos, S., Raval-Pandya, M., Wernyj, R. P., and Yang, W. (1996) *Biochemical Journal* **313,** 361–371.
14. Corde, E. H., Guess, H. A., Hulka, B. S., Friedman, G. D., Sadler, M., Vollmer, R. T., Lobaugh, B., Drezner, M. K., Vogelman, J. H., and Orentreich, N. (1993) *Cancer Epidemiology, Biomarkers and Prevention* **2,** 467–472.
15. Beelmann, C. A., and Parker, R. (1995) *Cell* **81,** 179–183.
16. Sachs, A. B. (1993) *Cell* **74,** 413–421.
17. Mahonen, A., and Maenpaa, P. A. (1994) *Biochem Biophys. Res Comm.* **205,** 1179–1186.
18. Arbour, N. C., Prahl, J. M., and DeLuca, H. F. (1993) *Molecular Endocrinology* **7,** 1307–1312.
19. Schwartz, G. G., and Hulka, B. S. (1990) *Anticancer Research* **10,** 1307–1311.
20. Hanchete, C. L., and Schwartz, G. G. (1992) *Cancer* **70,** 2861–2869.
21. Morrison, N. A., Yeoman, R., Kelly, P. J., and Eisman, J. A. (1992) *Proc Natl. Acad Sci* **89,** 6665–6669.