Biochemical and Biophysical Research Communications **272,** 98–103 (2000)
doi:10.1006/bbrc.2000.2740, available online at http://www.idealibrary.com on IDEAL®

Retinoic Acid Switches Differential Expression of FGF8 Isoforms in LNCaP Cells

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Received April 17, 2000

Retinoic acid (RA) is described as an inhibitor of prostate cancer cell growth. We utilized reverse transcription–polymerase chain reaction (RT–PCR) to analyze expression of different isoforms of fibroblast growth factor 8 (FGF8) in response to RA. Results in the prostate cancer cell line LNCaP show that whereas overall expression levels of FGF8 appear to remain constant, RA addition induces an inversion of the ratio between FGF8a and -b mRNAs. Along with this observed "isoform switch," unexpected expression of retinoic acid receptor alpha was detected. Although preliminary, these data allow one to hypothesize on the existence of a possible link between the morphogenic hormone RA and the regulation of the potent mitogen FGF8.

Key Words: **retinoic acid; fgf8; isoform expression.**

Fibroblast growth factors regulate a wide range of physiological processes such as cell growth and differentiation as well as pathological routes to cancer formation. Expression of fibroblast growth factor 8 (*fgf 8*) has been correlated with murine embryogenesis in gastrulation, in brain and vertebrate limb development [1–5]. In adult tissues the expression pattern of *fgf 8* is mostly restricted in gonadal tissue (testis, ovaries) [6, 7].

Fgf 8 was originally cloned from conditioned medium of testosterone-stimulated androgen-dependent cancer cell line SC-3 derived from mouse mammary carcinoma SC-115 [8]. The role of *fgf 8* in mammary carcinoma was corroborated by its discovery as the third *fgf* gene which acts in synergy with *Wnt-1* on the induction of mammary tumors following infection with mouse mammary tumor virus [6]. Through alternative splicing, the *fgf 8* gene encodes a family of polypeptides with different transforming activities on NIH-3T3 cells. Among other isoforms (FGF8a, -b, -e, and -f), FGF8b has been shown to possess the highest tumorigenic activity in nude mouse [9, 10].

Studies on prostatic cancer tissues show elevated expression of *fgf 8* mRNA in 70% of cases [11]. The polypeptide expressed in prostate cancer has been shown to be the tumorigenic FGF8b isoform [12]. In addition expression of *fgf 8* antisense reduces the growth rate of the human prostatic carcinoma cells (DU145) and inhibits their soft agar clonogenic activity [13].

One of the envisaged therapies for prostate cancer, developed in the recent years is using the ability of retinoic acid (RA) to inhibit proliferation by inducing differentiation and/or apoptosis. RA is in clinical use as an antileukemia agent in acute promyelocytic leukemia, which is linked to the expression of fusion products containing the alpha subtype of retinoic acid receptor (RARα) [14]. RA plays a critical role in many aspects of proliferation and differentiation of a wide spectrum of cell types [15, 16], and several studies have shown the correlation low serum levels of retinol with increased risk for the development of prostate cancer [17, 18]. Albeit RA is recognized as a potential treatment and preventive agent for prostate cancer, the precise underlying mechanism leading to inhibition of tumor formation and metastasis remains unclear up to date.

Studies of the molecular mechanism of action of RA have revealed that they exert their effect by binding to the retinoic acid receptors (RARs α, β, and γ) and retinoic X receptors (RXRs α, β, and γ) which all are ligand-dependent transcriptional regulators belonging to the super-family of nuclear receptors [19, 20]. Recent studies using retinoids and specific ligand-analogues for different RARs and RXRs suggest that the antiproliferative effects of retinoids in prostate cancer can be mediated by the RARs [21], more specifically by their regulatory function of discrete sets of genes [22].

The present report describes the effect of retinoic acid on the regulation of expression of different isoforms of *fgf 8* presumably through the activity of RARα present in LNCaP cells.

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0006-291X/00 $35.00

MATERIALS AND METHODS

Cell lines and culture. The LNCaP human prostatic cancer cell line (A. Brinkmann, Erasmus University, Rotterdam, Holland) was routinely maintained in RPMI 1640 medium respectively supplemented with 10% FBS, 1% glutamine and 1% penicillin/streptomycin. Media and normal supplement were from Life Technologies (Paisley, Scotland). The CCT FBS supplement was obtained from Hyclon (Logan, U.S.A.). RA was purchased from Sigma Chemicals (St. Louis, MO).

Competitive RT–PCR. Cells were cultured for 2 days at a density of 10^6 cells per 10-cm plate in CCT FBS prior to induction by 200 nM RA. Total cellular RNA was extracted and purified using the Qiagen RNA-purification kit according to the manufacturer's instruction. Complementary DNA was synthesized from 1 μg of total cellular RNA using 10 pmol oligo(dT)12-18, 2 mM dNTPs and 5 units of superscript enzyme in a final volume of 20 μL. First, annealing reaction without the enzyme was carried out at 72°C for 2 min and cooled on ice, then the reverse transcription reaction was performed at 42°C for 1.5 h. Five microliters of the cDNA reaction was directly mixed to a 100-μL PCR containing 2.5 units of the *Taq* polymerase, 2 mM dNTPs and 100 pmol of the appropriate primer pair in the buffer supplied by the vendor. For normalization of mRNA, sense and antisense primers for HPRT gene were used that generated a 163-bp DNA fragment. The PCR parameters were as follows: denaturation at 92°C for 30 s., annealing at 55°C for 1 min and extension at 70°C for 1 min. for 30 cycles. Primers used for FGF8 amplification were VB1 (5′-AGCTGCCTGCTGTTGCACTT-3′) and VB2 (5′-AGCGCTGTGTAGTTGTTCTC-3′), derived from sequences of exons 1A and 3. PCR parameters were the same as for HPRT amplification with the number of cycles reduced to 25. Two microliters of this first reaction was then analyzed by nested amplification using either pairs or triplets (primer competition) of primers FT1 (5′-CCAGCACAATCTCCGTGAAG-3′), FT2 (5′-CCTCATCCGGACCTACCAAC-3′), FT-3 (5′-TTGCACTTGCTGGTCCTCTG-3′), Fa (5′-CAAGCCCAGCATGT-3′), and Fb (5′-CAAGCCCCAGGTAAC-3′) (Fig. 1) and the following parameters: denaturation at 92°C for 30 s., annealing at 53°C for 1 min and elongation at 70°C for 1 min for 30 cycles. Amplified DNA was analyzed by electrophoresis on 2% agarose gels containing 0.2 mg/L of ethidium bromide and visualized by UV light.

Control RT–PCR were performed using the sense and antisense primer system for HPRT (Promega). Negative controls for RT–PCR were also done, in which all components except cDNA were present.

Nuclear extract and immunoblot analysis. LNCaP cells were harvested in PBS, pelleted by centrifugation, and nuclear extract was prepared as follows: after one freeze-thaw cycle, cells were resuspended in 500 μL of buffer A (20 mM Tris–HCl, pH 8, 1 mM $MgCl_2$, 20 mM KCl, 20 mM $NaMoO_4$, 1 mM DTT, 0.3 mM PMSF) and nuclei were collected by centrifugation (5 min. at 5000 rpm). Pellet was resuspended in 500 μL of buffer B (20 mM Tris–HCl, pH 8, 1.5 mM $MgCl_2$, 600 mM KCl, 20 mM $NaMoO_4$, 0.5 mM DTT, 0.3 mM PMSF, 25% glycerol) and homogenized using a Ultraturrax. The nuclear homogenate was centrifuged at 31,000*g* for 1 h. Supernatant was aliquoted and stored at −70°C. Protein concentration was determined by colorimetry (Bio-Rad) following the manufacturer's instruction and a standard protein solution (2 mg/mL).

For immunoblots analysis, 10 μg of nuclear extract was analyzed by SDS page (10% acrylamide) and analyzed with specific RARs antibodies (RARα SA-157, Biomol; RARβ SA-158, Biomol; RARγ sc-550, Santa Cruz) according to the manufacturer's instruction.

Electrophoretic mobility shift assay (EMSA). Oligonucleotides DR2 *crbpI* sens (5′-AGCTTGTAGGTCAAAAGGTCAGAC-3′) and DR2 *crbpI* anti (5′-AGCTGTCTGACCTTTTGACCTACA-3′) corresponding to the retinoic acid responsive element RARE DR2 sequence from the *crbpI* promoter with additional *Hin*dIII cohesive ends were radiolabeled and annealed using the following conditions: 800 pmol of each oligonucleotide were mixed in a PCR tube in the presence of 5 units of *Taq* DNA polymerase and 125 μCi of [α-^{32}P]dATP in a 100-μL final volume. Annealing and radiolabeling were performed in a thermocycler with the following parameters: denaturation at 94°C for 1 min, annealing at 60°C for 1 min, radiolabeling at 68°C for 10 min and cooling to 4°C at 0.1°C/s. Competitor probe RARE crpbI ARE-PAL (ARE PAL sens 5′-AGCTTAGAACAGCATGTTCTG-3′ and ARE PAL anti 5′-GATCCAGAACATGCTGTTCTA-3′) were prepared using the same conditions except that 1 μL 1 mM dATP was used in place of [α-^{32}P]dATP. The oligonucleotide concentration was adjusted to 1 pmol/μL. EMSA reactions (30 μL) contained 2 pmol of radiolabeled oligonucleotide, and LNCaP nuclear extract (1 μg) in the presence of varying amounts of competitor oligonucleotides (1, 2, 4, 8, and 16 pmol), 1 μg poly(dI–dC), 0.1% Triton X-100, 1 mM DTT, 100 mM Tris–HCl, pH 8.0, and 50 mM KCl. After 1 h, mixtures were subjected to electrophoresis through a 7% polyacrylamide gel containing 0.1% Triton X-100 and 1× Tris borate buffer (0.2 M borate and 0.045 M Tris) for 1 h at 130 V. Gels were subsequently dried and exposed overnight on X-ray films (Kodak X-AR).

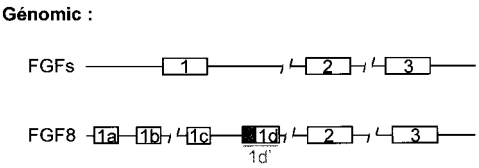

FIG. 1. Schematic representation of the genomic organization of the *fgf* gene family (top) and of *fgf8* gene (bottom).

RESULTS AND DISCUSSION

Template-Competition Study of Isoform Switch

Alternative splicing of the *fgf8* primary transcript leads to a pattern of expression, which is the most complex among the members of *fgf* gene family [23]. Whereas other fgfs have a single exon 1 (Fig. 1, top), the corresponding sequence in *fgf8* consists of four different exons (exon 1a, -b, -c, and -d) which are combined during splicing to yield the various isoforms of FGF8 (Fig. 1, bottom). Moreover, alternative splicing allows two possibilities for exon 1 d (1d or 1d′). The different FGF8 isoforms (FGF8a, -b, -e, and -f) contain identical 5′- and 3′-termini, but the splicing between the exon 1b (or 1c for FGF8e and -f) and exon 1d (or 1d′ for FGF8 b and -f) is different (Fig. 2A). Hence, this alternative splicing of primary mRNA results in the production of four mRNAs different in length, the expression of which can be analyzed after reverse transcription and amplification.

Although PCR has proven to be useful in amplifying specific mRNAs (especially those present at a low copy number), the absolute quantification of the amount of discrete mRNAs present in the starting material can sometimes be difficult. The splicing characteristics of FGF8 were such that we opted for an adaptation of an elegant technique described by Gilliland [24]. This method uses the competition of an identical set of primers for different templates. One main advantage of such an approach over other attempts for quantitative

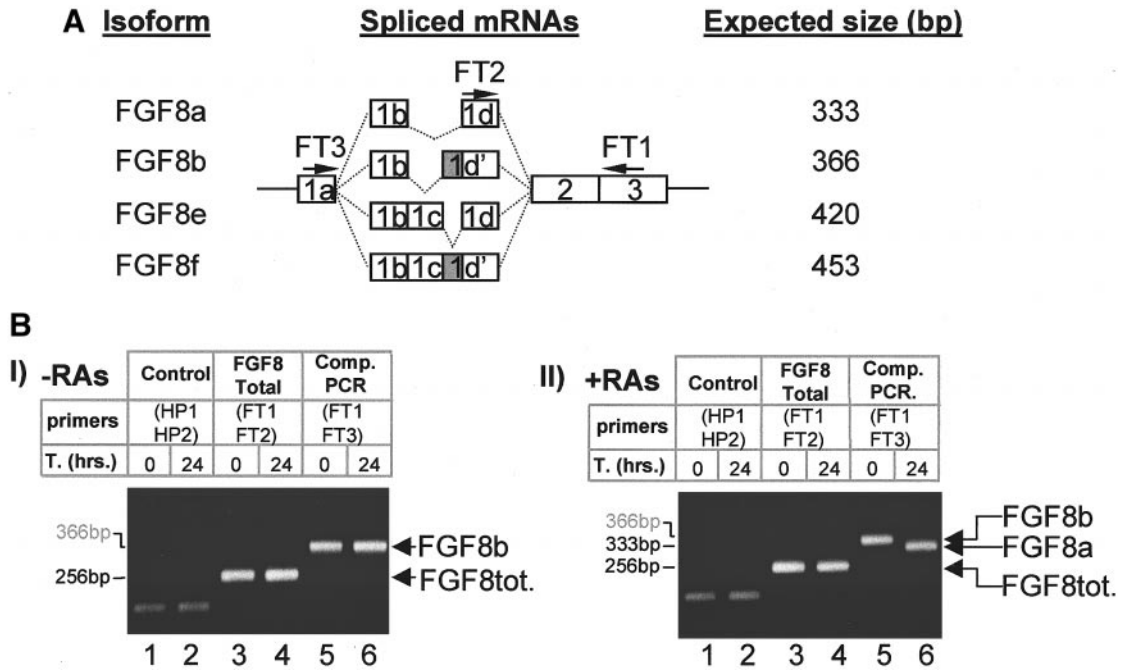

FIG. 2. RT–PCR analysis of fgf8 expression in LNCaP cells. (A) Alternative splicing of the primary mRNA of the *fgf 8* gene. The four cDNA corresponding to the isoforms of human FGF8a, b, e, and f are shown. Position and orientation of primers FT1, FT2 and FT3 are depicted as arrows. Calculated size of specific products is indicated to the right of each isoform. (B) Agarose gel analysis of RT–PCR products from RNA of LNCaP cells after 24 h of culture in absence (panel I) or presence (panel II) of retinoic acid (RA). Controls RT–PCR (HPRT, see Materials and Methods) are shown in lanes 1 and 2.

RT–PCR is that the read-out comes as a ratio between two RNA species out of one experiment, which eliminates variables inherent to the sensitivity of PCR experiments.

In a first "template-competition" study, primer pairs were designed as follows (also depicted in Fig. 1C). The primer pair {FT1/FT2} was used as a general amplification system for the generation of a 256-bp fragment common to the mRNAs of all FGF8 isoforms. The used primer pair {FT1/FT3} was designed for 'template-competition' analysis of differential expression of the FGF8 isoforms; i.e., oligonucleotides were corresponding to sequences common to the four mRNA species but located on both sides of the alternatively spliced region. Therefore, the size of the generated PCR product depends directly on the type of FGF8 isoform: 333, 366, 420, and 453 bp for FGF8a, -b, -e, and -f, respectively. As more than one PCR product is generated, the competition in the reaction for making a second product is exponentially increasing which renders this latter undetectable when a threshold ratio between the starting amounts of the two mRNAs templates is passed. This phenomenon is expected to be even more pronounced in our case where nested PCR (hence double amplification) was used. Figure 2B depicts the results of total FGF8 expression and a template competition-PCR for the detection of FGF8 isoforms with or without retinoic acid induction (panels II and I, respectively). Intensities of bands in panel I show that no significant difference occurs between total expression levels of *fgf 8* mRNAs after 24 h of culture (lanes 3 and 4). Template competitive-PCR (primers {FT1/FT3}) (Fig. 2, panel I), only shows a band corresponding to the size of FGF8b (366 bp, panel I, lanes 5 and 6). Panel II compares the results of the same experiments before and after 24 h of exposure to RA. The template competition reaction (primers {FT1-FT3}) demonstrates that the predominance of FGF8b at time zero switches within 24 h to the expression of FGF8a product as the major isoform (lane 5 versus lane 6). Note that FGF8 expression remains stable during this time experiment ({FT1–FT2}; lanes 3 and 4).

Primer-Competition Study

Since we only observed the expression of isoforms FGF8a and -b, we further analyzed the dynamics of the switch between these two mRNA species. We performed a primer-competition study designed to be specific for FGF8a and b isoforms. The experimental scheme is summarized in Fig. 3A. The oligonucleotide sequence of primer {Fa} is complementary to the splicing junction between exons 1b and 1d. Hence, primer pair {FT1/Fa} produces a 300-bp fragment, specifically representing the amplification of FGF8a. Similarly, primer pair {FT1/Fb} is designed as a specific amplification system for FGF8b. Sequence of primer {Fb} corresponding to the splicing junction between exons 1b–

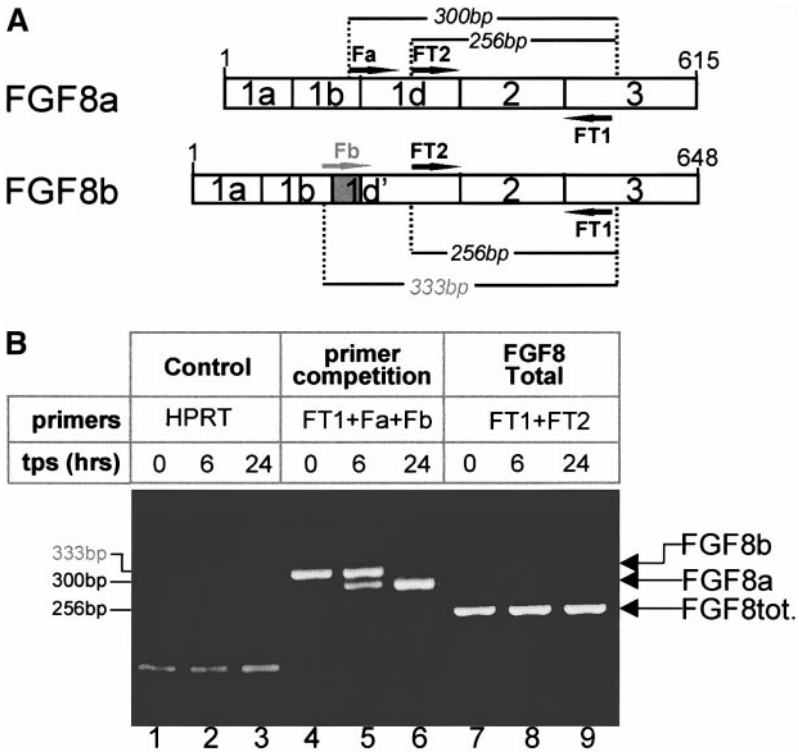

FIG. 3. Primer competition study of fgf8a and b isoform expression. (A) Schematic representation of fgf8 isoforms FGF8a and -b. size of specific PCR product are indicated. Arrows depict position and orientation of primers. (B) Agarose gel analysis of RT–PCR products with specific primer pairs for isoforms FGF8a and -b following RA induction at indicated time. Other details are as in Fig. 2.

1d′, and in this case, the generated PCR product has an expected size of 333 bp.

The primer-competition experiment was set up as follows: the primer triplet {FT1-Fa-Fb} was incorporated into the PCR so that the two specific primer pairs FT1 + Fa and FT1 + Fb could compete for the production of their respective isoforms. When the three primers were simultaneously present in the reaction (Fig. 3B; lanes 4, 5, and 6), one can observe a switch occurring between $t = 0$ h, where only FGF8b is detected; $t =$ 6 h where both isoforms are present; and $t = 24$ h, where FGF8a appears as the predominant isoform. Using the same RNA sample, the PCR using a primer pair common to all isoforms {FT1–FT2} showed consistency in the levels of total FGF8 (lanes 7 to 9). This confirmed the occurrence of a "switch" in the expression of the two isoforms: i.e., FGF8b mRNA levels decreases while FGF8a becomes detected as the dominantly expressed species.

The experimental set up that was used in the primer competition study allowed the observation of another characteristic of the RA-induced isoform switch. The results of the RT–PCR performed 6 h after RA induction, shows presence of bands corresponding to both isoforms FGF8a and -b (lane 5). In turn, this means that the process resulting in the isoform switch is initiated earlier than 6 h after induction.

Expression of Retinoic Acid Receptors in LNCaP Cells

To preliminary investigate possible actors responsible for this unique RA-induced phenomenon in LNCaP cells, we next analyzed the expression of several variants of RARs by immunoblot. Nuclear extract was prepared and analyzed using antibodies specific for each subtype (RARα, -β, and γ) after denaturing electrophoresis. The obtained immunoblots (Fig. 4A) revealed the presence of retinoic acid receptor RARα (lane 1) whereas subtypes RARβ and RARγ were not detected (lanes 2 and 3). The ability of the same nuclear extract to form nucleoprotein complex was analyzed by electromobility shift experiment using a radiolabeled oligonucleotide corresponding to the sequence of the retinoic acid responsive element of *crbp I* gene (5′-gtAGGTCAaaAGGTCAga-3′; [25]). A retarded complex was observed after coincubation of LNCaP nuclear extract and radiolabeled-DR2 CRBPI oligonucleotide (Fig. 4B, lane 2, panels I and II). Specificity of the protein–DNA interaction was then assessed by competition with increasing concentrations of unlabeled DR2 CRBPI or with an unspecific oligonucleotide corresponding to the consensus androgen-responsive element (ARE; 5′-AGAACAgcaTGTTCT-3′; panel II). Intensity of retarded band only decreased in presence of competing amount of DR2 CRBPI (compare lanes 3 to

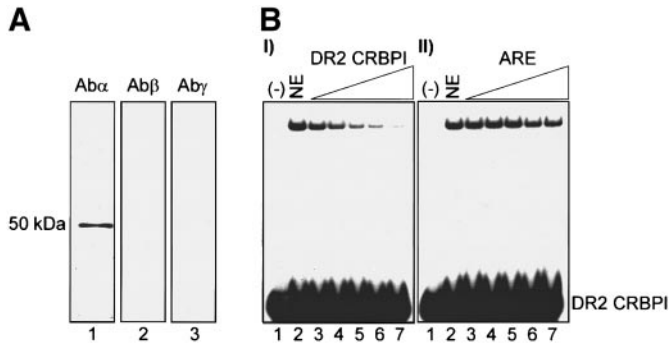

FIG. 4. Expression of retinoic acid receptors in LNCaP cells. (A) Immunoblot detection of retinoic acid receptors (RARα, β and γ) in LNCaP cells nuclear extract. (B) EMSA analysis of retinoic acid receptor in LNCaP nuclear extract. Analysis of band shift specificity by competition with increasing amounts of unlabeled specific (DR2 CRBPI, panel I) and unspecific (ARE, panel II) oligonucleotides.

7 from panels I and II) demonstrating the specificity of the observed nucleoprotein complex.

Among retinoic acid receptors, RARγ has been described as having a major impact in the normal development of the prostate in mouse [26]. Moreover, expression of RARs isoforms in human prostate is different between normal and cancer status: whereas RARγ is the predominantly detected subtype in normal prostate tissues, prostate cancer tissues show expression of RARα [27]. This is in agreement with our observations in LNCaP cells.

Altogether, our results show that addition of retinoic acid has a profound effect on the differential expression of two isoforms of fgf8 in a cell line expressing RARα. It will be interesting to study further the mechanisms underlying this phenomenon, possibly at the transcriptional level. Indeed, it is already tempting to hypothesize that retinoic acid receptor(s) could exert their transcriptional regulator function on the promoter of *fgf8* gene. This would in turn provide valuable insights into the possible link between a morphogen and a growth factor both involved in proliferation/differentiation status of cells.

ACKNOWLEDGMENTS

We are grateful to Dr. A. Brinckmann for generously providing LNCaP cells. We thank Drs. J-M. Egly, T. Klimkait, and S. Ferrari for critical reading of the manuscript.

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