Regulation of Lens Fiber Cell Differentiation by Transcription Factor c-Maf

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To elucidate the regulatory mechanisms underlying lens development, we searched for members of the large Maf family, which are expressed in the mouse lens, and found three, c-Maf, MafB, and Nrl. Of these, the earliest factor expressed in the lens was c-Maf. The expression of c-Maf was most prominent in lens fiber cells and persisted throughout lens development. To examine the functional contribution of c-Maf to lens development, we isolated genomic clones encompassing the murine c-maf gene and carried out its targeted disruption. Insertion of the β-galactosidase (lacZ) gene into the c-maf locus allowed visualization of c-Maf accumulation in heterozygous mutant mice by staining for LacZ activity. Homozygous mutant embryos and newborns lacked normal lenses. Histological examination of these mice revealed defective differentiation of lens fiber cells. The expression of crystallin genes was severely impaired in the c-maf-null mutant mouse lens. These results demonstrate that c-Maf is an indispensable regulator of lens differentiation during murine development.

Lens development commences in the 9.5-day-old (e9.5) mouse embryo by invagination of the lens placode to form lens pits on either side of the prospective forebrain (1, 2). Subsequently at e10.5, the lens pit forms a lens vesicle, where embryonic ectodermal cells differentiate into primary lens fiber cells. By e13.0, the primary posterior lens fiber cells grow into the lumen to eventually fill the lens vesicle. The anterior cells of the vesicle become epithelial cells and constitute the lens germinal epithelium; secondary fiber cells then differentiate from the epithelial cells after this stage. This arrangement persists throughout the lifetime of the animal, as new lens fibers are continuously regenerated (3).

Differentiation of the lens involves biosynthesis of a group of fibrous lens-specific proteins called crystallins, which constitute 80–90% of the soluble protein of the lens (4–6). The regulation of the crystallin genes has been characterized extensively (7–10), and an enhancer for the chicken crystallin gene has been identified (11, 12). Biochemical analyses of the core region of this enhancer revealed key interacting transcription factors (13, 14). Of the cis elements identified in the enhancer, the cCE2 sequence, which shares high similarity with the Maf responsive element (MARE1 (15)), is crucial for its transcriptional activity. MARE-related consensus sequences have also been found in the regulatory regions of other lens-specific genes (12).

Recently a new transcription factor, L-Maf, which can interact with the cCE2 enhancer element, was isolated from chicken lens (13). L-Maf is a member of the large Maf oncoprotein/transcription factor family (16–18). The Maf family factors contain a basic leucine zipper domain and bind to MARE either as homodimers or as heterodimers with other basic leucine zipper transcription factors (19). L-Maf regulates the expression of multiple lens-specific genes, and its forced expression can convert primary chick embryonic neural retina cells in culture to a lens fiber cell fate, indicating that vertebrate lens induction and differentiation can be triggered by the ectopic expression of L-Maf (13).

Transcripts encoding other members of the Maf family in lens tissues have been identified (Ref. 20–22) Nrl mRNA was detected in embryonic mouse lens (20), whereas c-Maf and MafB mRNAs were found in both embryonic and adult rat lens (21, 22), suggesting that each of these individual large Maf proteins might play distinct roles during lens development. Thus the vertebrate lens could provide an excellent model system for dissecting both the individual as well as complementary functional roles of large Maf family transcription factors.

To elucidate the functional contributions of Maf family factors to lens development, we set out to identify the large Maf factors specifically expressed in the mouse lens. We found that three large Maf family proteins, c-Maf, MafB, and Nrl, are expressed in the embryonic and adult mouse lens. We therefore documented the expression profile of c-Maf and MafB mRNAs and also performed targeted disruption of the c-maf gene in embryonic stem (ES) cells to generate c-maf germ line mutant mice. Targeted mutation of the c-maf gene results in perinatal lethality in homozygous mutant animals, permitting us to examine the earliest stages of lens development in the embryo. These results demonstrate that c-Maf is essential for normal lens development and that its function cannot be complemented by other large Maf proteins.
Experimental Procedures

Display of RNA Transcripts—Total RNAs were prepared from the lenses of e12.5- e18.5 embryos and adult mice. Degenerate sense (5'-GAGGGATCCATGGA(A/G)TA(C/T)TAGA(C/T)ATG(A/G)CTT(C/T)GA) and antisense (5'-AGG(GA)TTCC(A/G)TA(C/T)ACCC(A/G)CTT(C/T)CT) oligonucleotide primers containing engineered BamHI and EcoRI recognition sites (underlined), respectively, were synthesized. These primers were conserved between c-Maf, MafB, Nrl, and L-Maf. Coupled reverse transcriptase-polymerase chain reaction (RT-PCR) was performed under the following conditions: annealing temperature of 50 °C and elongation temperature of 72 °C for 45 cycles.

In Situ Hybridization—For whole mount in situ hybridization analysis, e9.5-e11.5 embryos were dissected in phosphate-buffered saline and fixed overnight in 4% paraformaldehyde plus 2 mM EGTA in phosphate-buffered saline at 4 °C. The embryos were then treated with proteinase K for at least 10 min (depending on the embryo stage). After postfixation, the embryos were prehybridized and then hybridized with RNA probes (23). The embryos were subsequently incubated with alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche Molecular Biochemicals). Hybridization signals were visualized using nitalblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as phosphatase.

For cellular resolution in situ analysis, e9.5-e11.5 embryos were fixed in 4% paraformaldehyde in phosphate-buffered saline. Embryonic lens tissue was cut into 16–20-µm frozen sections. In situ hybridization was performed as described previously (24). After hybridization, the sections were processed for immunocytochemistry with anti-digoxigenin antibody, as described above.

Expression of Large Maf Family Members in the Mouse Lens—To identify the large Maf family members that are expressed in the mouse lens, we performed RT-PCR display analysis using degenerate oligonucleotides encoding amino acid sequences that were conserved among the large Maf factors. From both embryonic and adult lens cells, seven PCR products were observed (data not shown). All seven PCR bands were subcloned, and their sequences were determined by examining at least five independent clones corresponding to each band. From the sequence analysis, three of the bands were amplified from c-Maf cDNA, and interestingly, all three c-Maf amplicons were substantially smaller than predicted. We found that each of them was deleted in some portion of a GC-rich sequence present in the c-Maf coding sequence. We presume that this heterogeneity might be the result of an RT or PCR artifact because of deletions caused by formation of hairpin loops in this GC-rich sequence.

RESULTS

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Of the four remaining PCR bands, two were shown to encode MafB and Nrl. Although one of the remaining distinct bands was approximately the expected size for L-Maf, sequence analysis revealed that this band did not contain L-Maf or indeed any Maf-related product (15 independent clones were analyzed). Similarly, the sole remaining band also appeared to be an artifact unrelated to Maf sequence. Thus we were unable to isolate the mouse homologue of chicken L-Maf from lens RNA in this analysis.

c-Maf Expression Starts in Head Ectoderm Destined to Become the Lens Vessel—To determine the temporal and spatial expression profiles of the three large Maf factors we detected in the lens, we performed in situ hybridization analyses on both whole mount and thin-sectioned e9.0 to e14.5 mouse embryos. Two independent RNA probes were prepared from the 5' and 3' end regions of the c-Maf cDNA. From whole-mount in situ analysis, we found that c-Maf mRNA was expressed in the midline of the forebrain (Fig. 1A) and in the eye region (Fig. 1B) of e9.0 embryos. In e10.0 to e10.5 embryos, c-Maf expression in the developing lens progressively intensified, whereas expression from the midline diminished with age. Embryos were also hybridized with sense probes, but we did not detect any substantial signals (data not shown). We performed this whole mount in situ analysis 4 times, and a total of 35 embryos were hybridized with the antisense probes, and 10 embryos were hybridized with the sense probes. The results were quite reproducible.

In the thin section in situ analysis, c-Maf mRNAs were localized to the head ectoderm destined to become the lens vesicle in e10.0 embryos (Fig. 2A). It is of interest to note that c-Maf mRNA was already present in the head ectoderm of e9.0 embryos, before the overlying ectoderm began to invaginate, and was also detected in the lens placode. By e10.5, the lens placode, which now strongly expressed c-Maf, had folded inward to form the vesicle (Fig. 2B). At e11.5, c-Maf continued to be abundantly expressed in the lens. The expression was much stronger in the primary fiber cells (Fig. 2, C (arrow) and D, Immunohistochemistry—Embryos were fixed in ice-cold 10% neutral formalin in phosphate-buffered saline for 2 h, dehydrated with ethanol, embedded in paraffin, and sectioned at a 3-µm thickness. The sections were dewaxed and incubated for 20 min with anti-α-A, αB-, γ-, and γ-crystallin monoclonal antibodies (27). Sections were then incubated with biotin-conjugated goat anti-mouse IgG and avidin-alkaline phosphatase.

RT-PCR Analysis of Crystallin Gene Expression—The heads of e16.5 embryos were dissected, and total RNA was extracted individually. After genotyping, 1 µg of the total RNA was used for cDNA synthesis using Super Script II™ reverse transcriptase (Life Technologies, Inc.) and random hexamer primers. Primer sets and PCR conditions were as described previously (28).
shows a sense probe control) than in epithelial cells and was also prominent in the neural tube (Fig. 2, E (arrowheads) and F, shows a sense probe control).

The expression of MafB and Nrl was also examined by in situ analyses. MafB mRNA was found in lens epithelial cells in e10.5 to e14.5 embryos but not in the lens fiber cells (Fig. 2, G and H, and data not shown). On the other hand we found that Nrl mRNA was not expressed during the early stages of the lens development (Ref. 20 and data not shown). In summary, these data show that c-Maf and MafB are expressed in the developing lens with distinct distribution profiles, suggesting that they may play important but distinct roles in lens differentiation.

**Cloning and Structural Analysis of c-maf Gene**—To enable the analysis of c-maf mutant mice, we screened a 129/SVJ mouse genomic DNA library using a mouse c-Maf cDNA probe. Of 16 clones recovered, 6 were found to encode the entire ORF of the c-maf gene. Restriction enzyme site mapping and sequence analysis indicated that the c-maf ORF is uninterrupted by introns (Fig. 3 A).

To determine the transcription start site, we performed 5'-RACE analysis using e12.5 mouse embryonic RNA. Two cDNA species were recovered; one category of RACE clones was 144 base pairs longer than the second and extended the 5' end of the known cDNA sequence (marked by an asterisk) by 15 base pairs (Fig. 3B). We therefore designated this site as the transcription initiation site. When compared with the genomic sequence, these RACE clones showed no evidence for a distinct first exon, again suggesting that the c-maf gene is composed of but a single exon. A MARE motif was identified at -47 to -38, immediately 3' to the putative TATA box, in the proximal promoter region. We also performed Southern blot analysis on high molecular weight DNA, and restriction enzyme mapping showed only the expected genomic fragment sizes, suggesting that the uninterrupted locus we had cloned was not a pseudogene and that c-maf exists as a single copy gene in the mouse genome (data not shown).

**Gene Targeting of the c-maf Locus**—To disrupt c-maf in ES cells, a targeting construct was prepared to replace virtually the entire gene ORF with the lacZ and neo genes (Fig. 3 C). ES cells were electroporated with the targeting construct, and neomycin-resistant cells were selected in G418-containing medium. Of 360 G418-resistant clones screened, 11 clones had undergone homologous recombination at the c-maf locus. Five independent clones were injected into blastocysts, and male chimeras were generated that transmitted the c-maf mutation to their offspring. Genotyping of progeny was performed by PCR and Southern blot hybridization. Both methods clearly identified homologous recombinants in the c-maf locus (Fig. 3, D and E).

**Expression of the LacZ Gene in Heterozygous Mutant Mice**—Staged embryos and adult tissues from c-maf heterozygous mutant mice were stained for LacZ activity. LacZ expression was strongly detected in the lens, kidney, and brain (data not shown).
shown). In the developing lens, LacZ expression was first detected in the lens primordium of head ectoderm at e9.5 and was restricted to the lens vesicle by e10.5 (Fig. 4, A and B), which was in good agreement with the in situ hybridization results.

By e12.5, LacZ expression was confined to the primary lens fiber cell (Fig. 4C). Signal was also detected in optic nerve (Fig. 4C). LacZ expression was also extensive in the lens fiber cells of e14.5 embryos (Fig. 4D). Importantly, the reporter gene was expressed exclusively in lens fiber cells, but not lens epithelial cells, in the adult (Fig. 4, E and F). Under higher magnification, LacZ staining in the adult lens was exclusive to secondary lens fiber cells (Fig. 4F). These data correlate well with the results from the in situ analysis (above). The data suggested that c-Maf may play an important role in lens fiber cell development.

**c-maf Homozygous Mutant Mice Lack Normal Lens Structure—c-maf heterozygous mutant mice, derived from two different ES cell clones and maintained in either C57BL/6J × 129/SV or ICR × 129/SV hybrid genetic backgrounds, were intercrossed. 106 embryos, newborns, and one-month-old mice were collected from 17 litters (Table I). Five c-maf homozygous null mutant newborns were recovered. Although this was lower than the expected Mendelian ratio, it indicated that c-maf homozygous mutants could complete gestation. However, no homozygous mutant mice were found among the 1-month postnatal group, indicating that the lack of c-maf resulted in complete postnatal lethality. The etiology of this peri- and postnatal lethality remains to be elucidated.

Examination of c-maf homozygous mutant newborn mice revealed that they lacked normal lens structures (Fig. 5A; B is a wild type control). From microscopic histological examination, we failed to detect normal elongation of the lens fiber cells in the c-maf (−/−) lens (Fig. 5C), which were properly formed in wild type littermates (Fig. 5D).

To better define the time lens malformation in c-maf mutant animals was first apparent, we examined e11.5 and e16.5 embryos. The lens structure in e16.5 wild type embryos was visible after formaldehyde fixation, but in c-maf (−/−) embryos, no normal lens structure was evident (Fig. 5E; F shows a wild type littermate), and there was no elongation of lens fiber cells (Fig. 5G, H shows wild type). However, in e11.5 embryos, the lens vesicle was beginning to form, indicating that lens development seems to progress normally despite the absence of the c-Maf protein (data not shown). In other eye structures, the retinal

**FIG. 3. Structure and targeted disruption of c-maf gene.** A, restriction enzyme map of c-maf gene. B, sequence of the c-maf’ proximal promoter and upstream region. The 5’ end of the reported c-Maf cDNA sequence is indicated by an asterisk. The transcription start site is shown as +1, and the rest of the sequence is numbered from this site. C, schematic representations of the wild type allele, targeting vector, and mutant allele are shown. The solid box in the wild type allele represents the coding sequence. Restriction enzyme sites are EcoRI; S, SalI; M, MluI; H, HindIII; N, Ncol; X, XhoI. The positions of wild type and mutant allele-specific 5’ primers and the common 3’ primer used in the genotyping analysis are indicated. D, genotyping of four mutant ES cell clones heterozygous for c-maf gene targeting by both Southern blotting and PCR. The selected clones are 44 (lane 1), 144 (lane 2), 153 (lane 3), and 194 (lane 4). E, an F2 litter was genotyped by both Southern hybridization and PCR. This litter had 2 wild types (lanes 4 and 7), 1 homozygous mutant (lane 1), and 4 heterozygous mutants (lanes 2, 3, 5, and 6) neonatal pups. WT, wild type allele; hbp, kilobase pairs; MT, mutant allele.

**FIG. 4. LacZ reporter gene expression during lens development of heterozygous c-maf mutant mice.** LacZ expression (arrowhead) is detected at e9.5 in the head ectoderm region where the optic cup (arrow) contacts (A). LacZ activity is also detected in the lens fiber cells of e10.5 to e14.5 c-maf heterozygous mutant embryos (B–D) and in the lens of 9-week-old adult mouse (E). Higher magnification of the adult lens shows that the epithelial cell layer exhibits no LacZ staining (F, arrow). Fi, lens fiber cells; LV, lens vesicle; Re, retina.
layer or pigment epithelium may be affected to some extent, but further analysis will be necessary to determine whether this is caused directly by c-Maf deficiency or as a secondary consequence of impaired lens formation (Fig. 5G).

### Table I

| Stage | Litters | Genotypes | Total |
|-------|---------|------------|-------|
| e12.5 | 2       | +/-        | 13    |
| e16.5 | 2       | +/-        | 15    |
| Newborn| 9      | +/-        | 50    |
| 1 month | 4    | +/-        | 28    |

The c-maf mating are for two different ES clones, all bred into either the C57BL/6J or ICR background. Their embryos, newborns, and one-month-old mice were genotyped by the same PCR method as the ES cell line analysis.

**Crystallin Gene Expression Is Disrupted in the c-Maf-null Mouse Lens**—One plausible explanation for the lens malformation in c-maf knockout mice is that c-Maf is necessary for the expression of crystallin genes during lens fiber cell development. To address this question, we examined the expression of crystallin genes using anti-mouse αA-, αB-, β-, and γ-crystallin monoclonal antibodies (27).

In the lens of c-maf mutant heterozygous newborns, these antibodies clearly detected the respective crystallins; the signals were specifically restricted to lens (Fig. 6). In contrast, the anti-crystallin antibody staining was markedly reduced in the lens of c-Maf-null newborns. In the c-Maf-null newborns, there was a layer of primary lens fiber cells despite the absence of a typical lens structure. An important finding here is that the primary lens fiber cells were immunoreactive to the anti-αB- and β-crystallin antibodies (Fig. 6, B and C, arrows). Also, when higher concentration of anti-αA-crystallin antibody was employed, positive signals were detected (data not shown). Thus, although the α- and β-crystallin genes are under the positive regulation of c-Maf, they can be weakly transcripted without c-Maf. However, the expression of γ-crystallin, known to be specifically expressed in lens fiber cells, was not detected in the c-Maf-null mouse lens, even at the highest concentrations of antibody used (Fig. 6D). Thus, γ-crystallin gene transcription is under strict c-Maf regulatory control.

Enucleated anti-crystallin immunoreactive cells were also found in the lens cavity of c-Maf-deficient newborns. These cells are strongly immunoreactive with the αB-crystallin antibody (arrowheads in Fig. 6B) and may represent primary lens fiber cells whose maturation was arrested by the c-Maf deficiency.

To detect crystallin expression more sensitively, we also performed RT-PCR analysis. Total RNA was extracted from the heads of e16.5 embryos, and mRNA for each class of crystallin was amplified using specific PCR primer pairs (28). We found that the expression of all crystallin genes was significantly reduced, and in particular, the expression of γ-crystallin mRNA apparently disappeared (Fig. 7). In summary, these results thus demonstrate that c-Maf positively regulates the expression of crystallin genes during lens fiber cell differentiation.

**DISCUSSION**

From the RT-PCR, in situ hybridization, and LacZ staining analyses, we showed here that the c-Maf transcription factor is expressed specifically in lens fiber cells during embryonic lens development. This expression persists throughout gestation and continues into adulthood. Targeted disruption of c-maf resulted in a specific block in lens fiber cell differentiation, consequently leaving the lens cavity empty. These results dem-

**FIG. 5.** c-maf-deficient mice lack normal lens structure. Homozygous c-maf mutant newborns lack a normal lens structure (A, arrow; B shows wild type). c-Maf deficiency prevents elongation of posterior lens fiber cells in c-maf homozygous mutant (C) but not in wild type (D) animals. A normal lens structure is not visible in the e16.5 c-maf-homozygous mutant embryo (E, arrow), whereas the wild type e16.5 embryo has a visible lens structure (F). In the e16.5 c-maf null mutant embryo (G), unlike its wild type littermate (H), the lens fiber cells are not elongated, and consequently, the lens cavity is empty. Sections were stained with hematoxylin and eosin. Original magnification of the sections are ×200 (C, G, and H) or ×100 (D).

**FIG. 6.** Expression of crystallins in the lens of c-maf heterozygous and homozygous mutant mice. Immunohistochemical analysis was performed using monoclonal antibodies that recognize specifically mouse αA-, αB-, β-, and γ-crystallins (A to D). E shows a negative control using bovine serum albumin instead of a primary antibody. Antibodies against αA-, αB-, and β-crystallins were used at 100-fold dilution, whereas neat anti-γ-crystallin antibody was used. The arrows in B and C indicate signals in primary lens fiber cells. Note that in the c-maf (-/-) lens, degenerating cells are present (B, arrowhead). Fl, lens fiber cells; E, lens epithelial cells.
onstrate that c-Maf is required for proper lens development.

In contrast to the c-maf-deficient mice, the Elo (eye lens obsolescence) mutant mouse suffers from inviability of central lens fiber cells. The affected and malformed central fiber cells necrosed and completely collapsed, leaving the lens vesicle partially open (29). In the Elo mutant mouse, there is a frame-shift mutation in one of the crystallin genes, γE-crystallin (30). Interestingly, a MARE motif that is similar to the cCE2 element has also been found in the lens-specific regulatory region of each γ-crystallin gene (12, 28), suggesting that γ-crystallin genes are the target genes of c-Maf. However, lens fiber cell differentiation in the c-maf-deficient mouse is arrested before central fiber cells are formed, thus precluding the observation of an Elo phenotype in the c-maf mutants.

The expression of γ-crystallins could not be detected by immunohistochemistry or by RT-PCR in c-Maf-deficient newborns or e16.5 mutant embryos, respectively, further supporting the notion that c-Maf is an important transcriptional regulator of the γ-crystallin genes. In addition, the expression of aa-, αb-, and β-crystallins were found to be down-regulated in the c-Maf-deficient mice. Similar observations were recently targeted the disruption sox-1 mutation (28). Although Sox-1 is indispensable for mouse lens fiber cell differentiation, crystallins are expressed in the sox-1 (−/−) lens. Careful comparison of the c-maf- and sox-1-deficient mouse lenses revealed that the c-maf defect results in a more profound defect in crystallin gene expression than does the sox-1 deficiency. In addition, αa-and αb-crystallin gene-targeted mutations do not lead to a severe defect in lens formation (6). These observations raise the question as to whether the decrease or absence of crystallin gene expression is the main cause of lens malformation in the c-Maf-deficient mouse.

From the analysis of crystallin gene expression presented here, it is probable that other lens-specific genes that are also under the regulatory influence of c-Maf and the consequent lack of this gene product(s) may be responsible for the differentiation block in lens fiber cells in c-Maf-deficient mice.

Two other large Maf family factors, MafB and Nrl, are also expressed in the mouse lens (Ref. 20 and this study), therefore prompting the question as to how Maf family transcription factors individually or cooperatively execute their roles during vertebrate lens formation. An important finding here is that MafB mRNA is expressed exclusively in lens epithelial cells, whereas Nrl is expressed widely in the lens but at a much later stage of lens development than c-Maf and MafB. Compared with the other two large Maf proteins, c-Maf is unique in that its expression is restricted to lens fiber cells after the formation of the lens structure. The complementary distribution of c-Maf and MafB in the lens fiber and epithelial cells, respectively, is intriguing and suggests that each large Maf factor has a unique role in vertebrate lens development.

A natural MafB mouse mutant, kreisler (kr), has previously been described that lacks normal rhombomere formation and inner ear structure (31). However, no phenotype has been reported in the eyes or lens of the kr mouse. It was shown recently that the kr mutant mouse is not a MafB-null mutant, as MafB activity persists in many functional aspects (32). Therefore, the role of MafB in the differentiation and function of lens epithelial cells remains to be clarified.

Another large Maf factor, L-Maf, was isolated from the chicken and was shown to be a key regulator of aa-crystallin gene transcription in the lens (13). Because MARE motifs are found in the regulatory regions of many lens-specific genes, Maf family transcription factor are probably required for multiple aspects of normal lens development. In the chicken, L-Maf appears to be the prime target of a lens induction signal emanating from the optic vesicle (13). Although we did not detect a murine homologue of L-Maf in our experiments, the existence of four distinct large Maf factors has already been demonstrated in Xenopus. Therefore the existence of a murine L-Maf gene is plausible. The differential functional contributions of c-Maf, L-Maf, and other large Maf transcription factors to vertebrate lens formation are yet to be fully elucidated.

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FIG. 7. RT-PCR analysis of crystallin gene expression in the lens of c-maf-heterozygous and homozygous mutant mice. mRNA for each class of crystallin was amplified using specific PCR primer pairs (28), and total RNA was extracted from the heads of e16.5 embryos. A set of primers that amplifies hypoxanthine phosphoribosyl-transferase (HPRT (28)) was used as an internal control. To identify γE and γF, the PCR products amplified with same set of primers were digested with Bgl II (γE, 149 and 103 base pairs; γF, 252 base pairs).
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