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Cloning of a GADD34-like Gene That Interacts with the Zinc-Finger Transcription Factor Which Binds to the p21^{WAF} Promoter

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A histone deacetylase inhibitor has been shown to induce differentiation of many cancer cells and senescence-like state of human fibroblasts. Previously, our data suggested that the region responsive to trichostatin A (TSA), a specific inhibitor of histone deacetylase, treatment in the p21^{WAF1} promoter is located −100 bp upstream from transcription initiation site and contains a GC-box where both Sp1 and Sp3 are responsible. Here we show that another zinc-finger transcription factor, BFCOL1, which binds to the proximal proα2(I) collagen promoter, could also bind to this GC-box of the p21 promoter. In addition, we cloned a gene whose product interacts with this factor by yeast two-hybrid method. The cloned gene was a variant of GADD34 and lacking one PEST region. We found that this cDNA product decreased the DNA binding activity of BFCOL1 to the GC-rich region of p21 minimal promoter. © 1999 Academic Press

p21^{WAF1} (p21) was first cloned and characterized as an important effector that acts to inhibit cyclin dependent kinase activity in p53-mediated cell cycle arrest induced by DNA damage (1-3). It was found subsequently that the induction of p21 occurs in various differentiation systems p53-independently (4-8). Previously we reported that sodium butyrate induces senescence-like phenotype in NIH3T3 cells and enhances mouse p21 promoter activity. We also concluded that sodium butyrate-induced p21 promoter activity is p53 independent (9).

It has been shown that a GC-rich region in the human p21 promoter, located upstream of the TATA box, acts as an important regulatory element responsive to various agents (10-14). We showed that the region responsive to trichostatin A (TSA), a specific inhibitor of histone deacetylase, treatment in the p21^{WAF1} promoter is located −100 bp upstream from transcription initiation site and contains a GC-box. We also showed that both Sp1 and Sp3 are responsible for TSA-induced transactivation of the murine p21 promoter in NIH3T3 cells (15). Thus this GC-rich region and its *in vivo* binding factors may play a critical role in p21 expression. However, there exist the possibilities that the other transcription factors and/or cofactors could form complex in GC-box and play a role for the induction of p21 promoter. In this paper, we examined this possibility by using the BFCOL1 transcriptional factor (16) which binds to the proximal GC-rich promoter of mouse proα2(I) collagen promoter and further tried to clone the proteins which interact with this factor.

MATERIALS AND METHODS

Cell culture. NIH3T3 were maintained in a 37°C humidified atmosphere containing 5% CO_2 in DMEM supplemented with 10% FCS. BALB/c 3T310(1) cells were p53-deficient mouse fibroblasts cell line, which were kindly donated from Dr. Levine AL (17).

Gel retardation assay. The recombinant protein (products of in vitro transcription and translation) was incubated with 5 fmol ^{32}P-labeled double-stranded oligonucleotide corresponding to the region from −40 to −10 bp from TATA box in the p21 promoter (GC-probe) in a volume of 10 μl. Incubation was carried out at room temperature for 20 min. All binding reactions contained 10 mM Tris-HCl (pH 7.5), 4% glycerol, 50 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT, 1 mM $MgCl_2$, and 0.5 μg poly (dA-dT). Following electrophoresis in a 5% polyacrylamide Tris borate/EDTA (TBE) gel, the gel was dried and subjected to an autoradiography at room temperature. For competition, 1 pmol of unlabeled oligonucleotides were included in the reactions. The oligonucleotides used in these experiments were as follows: GC-probe: 5′-ggttggtcctgcctctgaggggcggggcctgggccgag-3′; mutated GC-probe: 5′-ggttggtcctgcctctgagggttcggggcctgggccgag-3′; consensus Sp1 binding oligo: 5′-attcgatcggggcggggcgagc-3′; mutated Sp1 binding oligo: 5′-attcgatcggttcggggcgagc-3′. The oligonucleotides of the mouse proα2(I) promoter were described previously (16).

Cloning of a cofactor proteins with BFCOL1 using the yeast two-hybrid system. The yeast strain Y190 (MATa, leu2-3,112, ura3-52, trp1-901, his3-Δ200, ade2-101, gal4Δgal80Δ URA3 GAL1-lacZ, LYS GAL-HIS3, cyh^r) was purchased from Clontech. The yeast reporter

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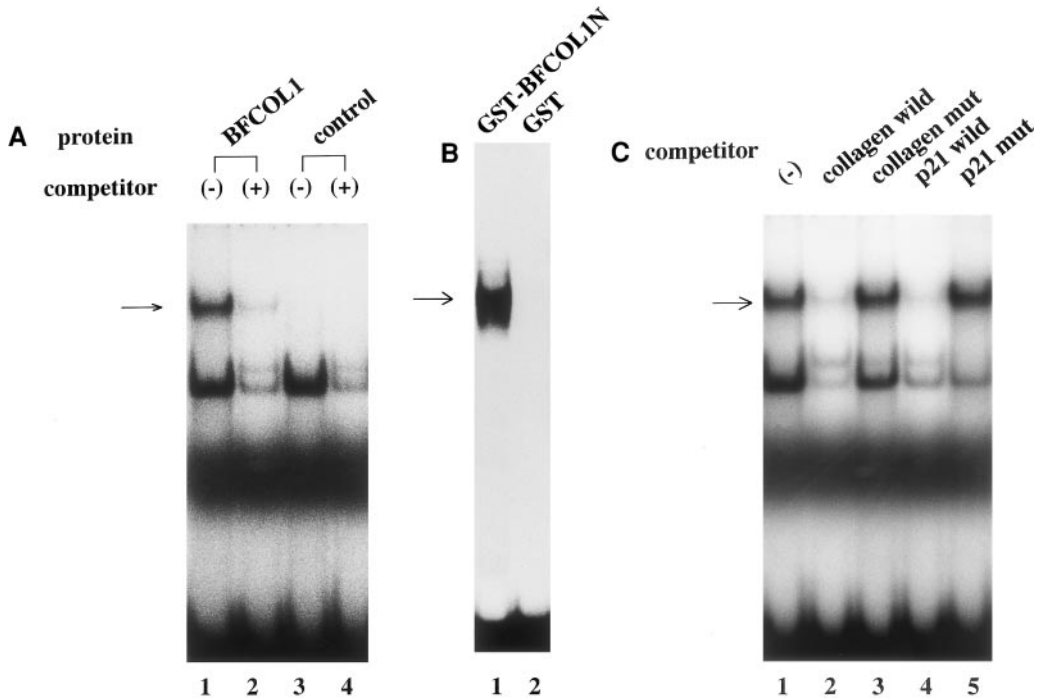

FIG. 1. Gel shift assays. (A) Double-stranded ^{32}P-labeled double-stranded oligonucleotides corresponding to the region from −40 to −10 bp from TATA box in the p21 promoter (GC-probe) were incubated with the polypeptide products of pCITE2C-BFCOL1 or pCITE2C vector alone obtained by in vitro transcription and translation. The used unlabeled competitor oligonucleotide (200 times over labeled oligonucleotide) is indicated at the top of the panel. Reactions were processed as indicated in Materials and Methods. (B) Double-stranded ^{32}P-labeled oligonucleotide were incubated with the GST-BFCOL1N (16) or GST alone. (C) Double-stranded ^{32}P-labeled oligonucleotides were incubated with the polypeptide products of pCITE2C-BFCOL1 obtained by in vitro transcription and translation. The used unlabeled competitor oligonucleotide (200 times over labeled oligonucleotide) is indicated at the top of the panel.

plasmid was constructed as follows. SacI-SalI fragment of pAS2-1 vector (Clontech) containing the GAL4 binding domain was inserted into the SacI-SalI site of pRS305HIS (16, 18) to generate pRS305HISpAS. SmaI-NsiI fragment of pPC86BFCOL1 (16) was subcloned into the SmaI-PstI site of pGBT9 vector (Clontech) to make pGBT9-BFCOL1N. The SmaI-NaeI fragment of pGBT9-BFCOL1N was subcloned in frame into the SmaI site of pRS305HISpAS vector to make pRS305HISpAS-BFCOL1N that contained the LEU2 gene as selectable marker. After digestion with ClaI, this vector was used for transformation. Yeast transformation was performed by the polyethylene glycol/lithium acetate method (19). Plasmid integration in the genome of yeast strains was confirmed by Southern blot analysis using a ^{32}P-labeled SmaI-NsiI fragment of pPC86BFCOL1. Cells were then plated on a minimal synthetic dextrose plate without histidine to verify background HIS3 gene activity. One of the yeast strains that had minimal HIS3 gene activity was selected as the strain for the transformation after the initial selection. The yeast strain in which pRS305HISpAS-BFCOL1N was integrated was used for cDNA library transformation. cDNA library constructed in pPC86 vector were same as previously described (16). Ten μg cDNA plasmid from the library was transformed into the yeast strain harboring the reporter plasmid integrated into the genome and plated onto plates including 5 mM 3-amino-1,2,4-triazole (3-AT), but lacking leucine, tryptophan, histidine. Transformation efficiency was about $1 \times 10^5/\mu$g cDNA plasmid. Colonies were picked after 3-5 days. Plasmid cDNAs were extracted and used for retransformation either into the same yeast strain or the yeast strain into which pRS305HISpAS plasmid instead of pRS305HISpAS-BFCOL1N plasmid had been integrated.

DNA sequencing. DNA sequencing was carried out using a Pharmacia ALFDNA sequencer with M13 universal and reverse sequence primers.

Expression of cloned cDNA by in vitro transcription and translation. A polypeptide corresponding to the full length GADD34-like gene was generated using the TNT-coupled reticulocyte lysate system (Promega). The EcoRI-NotI fragment of pPC86 GADD34 was inserted into the EcoRI-NotI site of the pcDNA3.1HISA vector (Invitrogen). ^{35}S-labeled polypeptide products were analyzed by 8% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. These were exposed and analyzed by Fuji BAS1500 image analyzer.

RNA isolation and Northern blot analysis. RNA isolation and Northern blot analysis were carried out as previously described (16), except that the RNA was transferred to Genescreen plus membrane (NEN Life Science) and labeling was performed by random labeling kit (Takara, Kyoto Japan). The membranes were then autoradiographed at −80°C using Fuji RX film. GAPDH was used as an internal control.

RESULTS

1. The Zinc-Finger Transcription Factor BFCOL1 Could Bind to the p21 Promoter

First we carried out the gel retardation assay with the in vitro transcription and translation BFCOL1 product. In Fig. 1A, we could see two bands when we

used the BFCOL1 translation product (lane 1). However, we also detected the lower band when we used the in vitro product which the control plasmid was translated (lane 3), suggesting that the upper band is the BFCOL1 specific band. The upper band is completely competed with the self unlabeled oligonucleotide (lane 2). We confirmed this result with the GST fusion protein, GST-BFCOL1N, which contains the N-terminal zinc finger domain of BFCOL1 protein (Fig. 1B, lane 1). As shown in Fig. 1C, the upper band was clearly competed with wild type p21 minimal promoter oligonucleotide (lane 4) and the wild type oligonucleotide between −180 to 136 bp of the proα2(I) collagen promoter (16) (lane 2). However, this band was not competed with the mutated p21 oligonucleotide which abolish the Sp1 binding (lane 5) nor the mutated collagen oligonucleotide which abolish BFCOL1 binding (16) (lane 3). These results confirmed that recombinant BFCOL1 protein binds to the p21 minimal promoter sequence specifically.

2. Cloning of cDNA for Polypeptide That Interacts with BFCOL1 Transcription Factor

We then performed the yeast two hybrid system to clone the protein which interact with BFCOL1 transcription factor. We used as bait the cDNA of the N-terminal half domain of BFCOL1 gene that contains the zinc finger DNA binding domain. We screened the mouse embryonic fibroblast cDNA library primed with oligo-dT (16). In the yeast strain that was used for selection, the plasmid pRS305HISpASBFCOL1N was integrated into the genome. In this plasmid, SmaI-NsiI fragment of BFCOL1 was fused to the GAL4 binding domain. By screening three million independent colonies, initial 30 histidine-positive colonies were picked. Among them, one cDNA plasmid gave positive upon retransformation of the parental strain. This cDNA could specifically activate the HIS3 gene and β-galactosidase gene of the yeast strain containing the pRS305HISpASBFCOL1N plasmid without activating the HIS3 or β-galactosidase gene of the strain with the pRS305HISpAS control plasmid. The sequence analysis showed that this cDNA almost correspond the 577 to 2270 nucleotide of MyD116 cDNA (20), also named GADD34 (21). However it lacks exact one PEST region (22) and contains the extra one amino acid at position of 1249 of MyD116 (Fig. 2A). Fig. 2B shows the schematic representation of GADD34 (MyD116) and the cDNA we got. Here we call our cDNA as GADD34 for convenience.

3. In Vitro Transcription and Translation Analysis

To confirm that the cDNA is precisely translated, we did in vitro transcription and translation analysis. As shown in Fig. 3, we could clearly see an approximately 80 kD protein, suggesting that full length cDNA is correctly translated.

4. The Effect of GADD34 on BFCOL1 Binding

We then asked if GADD34 has an effect on the binding of BFCOL1 to p21 promoter. We performed gel shift assay using reticulocyte transcription and translation product. As shown in Fig. 4, the addition of the GADD34 product to the reaction inhibited the specific binding in dose dependent manner. As GADD34 was cloned as bait the zinc finger domain of BFCOL1 which bind to the DNA by the yeast two hybrid analysis, GADD34 product might influence the binding of BFCOL1 on its binding

5. Northern Blot Analysis

p21 has been reported to be induced p53 dependent and independent manner. We tried to confirm the induction pattern of GADD34 mRNA with methyl methanesulfonate (MMS) treatment, because GADD34 was reported to be induced p53 independent manner. Both NIH3T3 and 10(1) cells were treated with 100μg MMS, total RNAs were extracted after the indicated time and subjected to Northern blot analysis. As shown in Fig. 5, GADD34 was increased after 2 hours and the induction continued until 8 hours in both NIH3T3 and 10(1) cells, suggesting that GADD34 is induced p53 independently in the fibroblast-lineage cell lines.

DISCUSSION

Here we showed that BFCOL1 binds to the GC-rich region of the p21 minimal promoter. In the recent our work (15), we reported that one of the histone deacetylase inhibitors, TSA, can induce histone hyperacetylation and p21 expression effectively in NIH3T3 cells; the minimal region of the mouse p21 promoter, containing from −60 bp to +40 bp relative to the TATA box, is essential and sufficient for induction of p21 in NIH3T3 cells by TSA. We report also that a GC-box in this region is critical for both basal and TSA-induced promoter activity, and that Sp1 and Sp3 are the functional activators of this GC-box. However, we noticed that transfection of antisense Sp1 or Sp3 was more effective in reducing TSA-induced p21 minimal promoter activity than basal activity. The different efficiencies of antisense Sp1/Sp3 to basal and TSA-induced activities raised the possibility that other proteins in addition to Sp1 and Sp3 may also bind to the GC-box of the p21 promoter under basal transcription condition, although Sp1 and Sp3 may be the main factors responsible for TSA sensitivity. In addition, the human p21 promoter has 6 Sp1 binding sites near the TATA box, and they function cooperatively or individually under different conditions. That is, butyrate sensitivity requires Sp1-3 site in conjunction with Sp1-5 site and

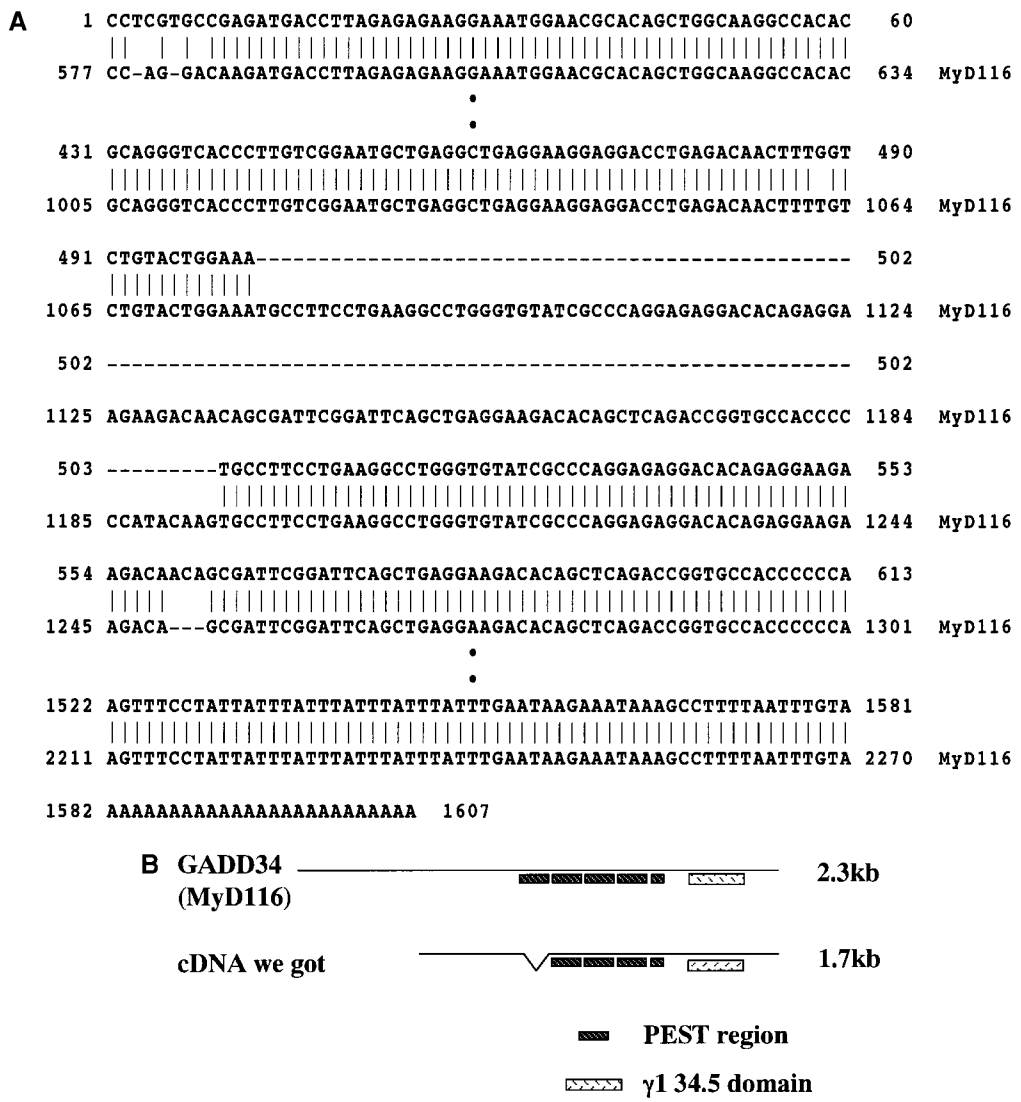

FIG. 2. (A) Comparison of the nucleotide sequence of the cDNA which we got by yeast two hybrid method and the MyD116 cDNA. Numbers correspond to the DNA sequence. Identities between them are represented with vertical bars. (B) Schematic representation of the cDNA that was cloned. REST regions and the γ1 34.5 domain (24) is indicated.

Sp1-6 site, with Sp1-3 site being critical (14). The GC-rich region of the human p21 promoter was also demonstrated to be the responsive element for many agents, such as phorbol esters, Ca^{2+}, TGF-β and butyrate (10-12, 14) the proteins responsible for transcriptional activation though this region do not always to be identical.

These data strongly suggest the transcriptional factors other than Sp1 family are involved in the transcriptional regulation of these promoters. We hypothesized that the transcription factor which have zinc-finger motifs could be the candidates of such promoters. We tested if one zinc-finger transcription factor, BFCOL1 (16), because this cDNA was cloned by yeast one hybrid analysis in the GC-rich proximal regions of the mouse proα2(I) collagen promoter and mRNA of this factor was detected in NIH3T3 cells which we used in the experiments. We confirmed that this transcription factor could bind to the p21 minimal promoter by gel shift analysis. In addition, the binding was completely competed with both p21 and collagen oligonucleotide. However, as the binding was not competed with the oligonucleotides which were introduced mutations, we confirmed that BFCOL1 binds to the p21 minimal promoter sequence specifically.

Next we hypothesized that cofactors of the transcription factors are involved in the complex binding to the p21 promoter. We successfully cloned one cDNA, which nucleotide sequence was very similar to that of GADD34 (MyD116) gene (20). Although the sequence of the cDNA we got was different from the sequence of the GADD34, we could confirm that the sequence was

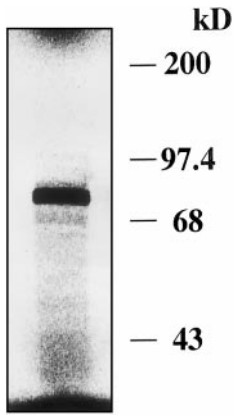

FIG. 3. Synthesis of polypeptide from the cDNA by in vitro transcription and translation. pcDNA3.1HISA GADD34 plasmid was incubated with rabbit reticulocyte lysate and ^{35}S-methionine. In addition, T7 RNA polymerase was used. Polypeptide product was electrophoresed on a 8% polyacrylamide SDS gel. Molecular mass standards are indicated on the right.

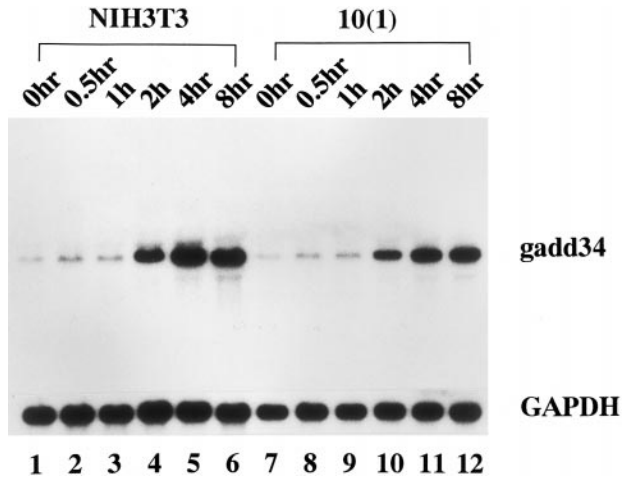

FIG. 5. Northern blot analysis. Total RNA was extracted from NIH3T3 cell and 10(1) cells treated with 100 μg MMS and Northern blot analysis was performed with ^{32}P-labeled GADD34 cDNA probe. GAPDH probe was used as an internal RNA-loading control. Incubation time with MMS is indicated at the top of the panel.

correctly translated (Fig. 3). In addition, the protein product of the cDNA has the effect on the binding of the zinc-finger transcription factor (Fig. 4). It might bind to the zinc-finger DNA binding domain and inhibit the DNA binding. Interestingly, the mRNA of GADD34 was dramatically induced by DNA damaging drug treatment p53 independently (Fig. 5). It has been reported that GADD34 was also induced by other DNA damaging stimuli (23). From our present study and the preliminary data that BFCOL1 suppress proα2(I) collagen promoter activity (16), it will be suggested that one of the zinc finger proteins, BFCOL1, binds to the GC-rich region in p21 minimal promoter, competes to Sp1 family transcription factor and suppress p21 promoter activity. The DNA damaging stress induces GADD34 (Fig. 5). It will be suggested that GADD34 induced p21 promoter activity by decreasing the binding capacity of BFCOL1 to GC-rich region of p21 minimal promoter. We are currently investigating these possibilities by transfection experiments.

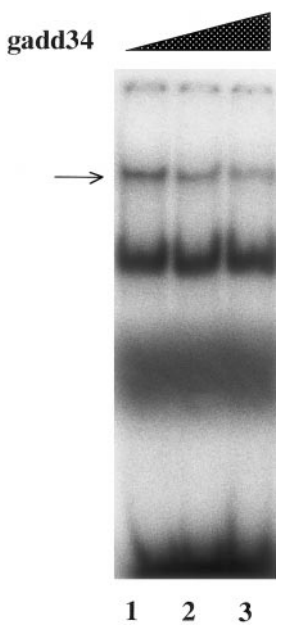

FIG. 4. Gel shift assays. Double-stranded ^{32}P-labeled oligonucleotides corresponding to the region from −40 to −10 bp from TATA box in the p21 promoter (GC-probe) were incubated with the combination of the polypeptide products of BFCOL1 and GADD34 obtained by in vitro transcription and translation. The volume of GADD34 used for the reaction is illustrated at the top of the panel. Densities of upper bands are as follows: lane 1, 8296; lane 2, 5206, lane 3, 3838. Reactions were processed as indicated in Materials and Methods.

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