cylglycerol by such a mechanism could implicate protein kinase C as a possible transducing pathway. Or, mobilization of intracellular Ca^{2+} by inositol triphosphate could point to Ca^{2+}-calmodulin–dependent protein (CaM) kinases as the transducing factors that couple dopamine stimulation of D_1 receptors to the activation of steroid receptors. CaM kinases phosphorylate and activate the cAMP response element binding–protein (*33*).

Demonstration of "cross-talk" between membrane-associated receptors and intracellular steroid hormone receptors may be of biomedical significance. Dual activation of receptors might occur in situ in brain cells. It may also be possible to activate mutant forms of steroid receptors that exist in diseases (*34*). Finally, the finding that dopamine has direct access to the genome via this family of transcription factors may aid in understanding learning and memory processes.

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Site-Specific Cleavage of Human Chromosome 4 Mediated by Triple-Helix Formation

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Direct physical isolation of specific DNA segments from the human genome is a necessary goal in human genetics. For testing whether triple-helix mediated enzymatic cleavage can liberate a specific segment of a human chromosome, the tip of human chromosome 4, which contains the entire candidate region for the Huntington's disease gene, was chosen as a target. A 16-base pyrimidine oligodeoxyribonucleotide was able to locate a 16-base pair purine target site within more than 10 gigabase pairs of genomic DNA and mediate the exact enzymatic cleavage at that site in more than 80 percent yield. The recognition motif is sufficiently generalizable that most cosmids should contain a sequence targetable by triple-helix formation. This method may facilitate the orchestrated dissection of human chromosomes from normal and affected individuals into megabase sized fragments and facilitate the isolation of candidate gene loci.

PYRIMIDINE OLIGODEOXYRIBONUCLEOTIDES bind in the major groove of DNA parallel to the purine Watson-Crick strand through formation of specific Hoogsteen hydrogen bonds to the purine Watson-Crick base (*1–6*). Specificity is derived from thymine (T) recognition of adenine · thymine (AT) base pairs (TAT triplet); and N3-protonated cytosine (C^+) recognition of guanine · cytosine (GC) base pairs (C + GC triplet) (*1–8*). The generalizability of triple-helix formation has been extended beyondpurine tracts to mixed

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sequences containing all four base pairs by the identification of novel base triplets (*9*), alternate strand triple-helix formation (*10*), and other triple-helix motifs (*11*). By combining oligonucleotide-directed recognition with enzymatic cleavage, near quantitative cleavage at a single target site within megabase (Mb) DNA has been achieved (*12*). In this technique, specific triple-helix formation protects a single overlapping methylase site from modification (*12, 13*). After triple-helix disruption, restriction enzyme digestion produced cleavage in 95% yield at a single site within the *Saccharomyces cerevisiae* genome (14 Mb) (*12*).

In a formal sense, a binding site size of at least 16 bp afforded by the triple helix motif is sufficient to target millions of different sites, each occurring only once in gigabase DNA (*14*). To test whether triple helix–mediated enzymatic cleavage is capable of site-specific cleavage at an endogenous sequence on a human chromosome, we targeted the tip of the short arm of human chromosome 4 (>200 Mb), which contains the Huntington's disease (HD) gene. The HD gene is located within the telomeric band 4p16.3, and a large number of cloned DNA sequences have been genetically and physically mapped to the region (*15–16*). Genetic evidence supports a location of the HD gene within the approximately 4 Mb of DNA between the genetic marker D4S10 (G8) (*15*) and the telomere of chromosome 4p (*17, 18*). Thus, a single cleavage at D4S10 would liberate a large, resolvable fragment that contains the entire HD candidate region.

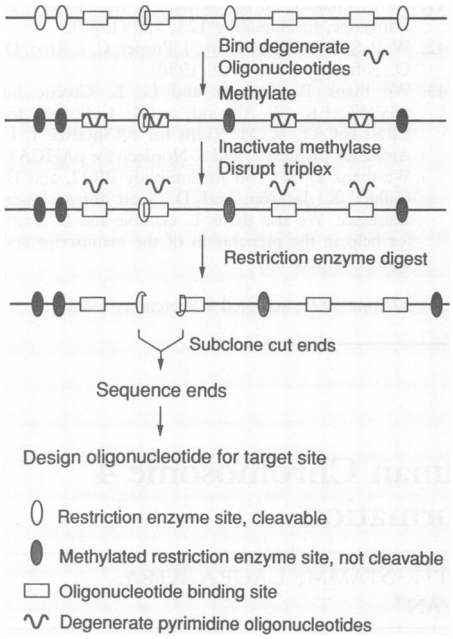

Fig. 1. General scheme for identification of endogenous cosmid sequences targetable by triple-helix mediated enzymatic cleavage with the use of a pool of partially degenerate oligonucleotides. Linearized cosmid DNA was equilibrated with a degenerate pyrimidine oligonucleotide containing a nondegenerate end specific for a methylase site. The Alu I oligonucleotide used to identify this target site had the sequence, 5′-Y_{16} TC-3′, where Y represents a nucleotide ratio of 60% ^{Br}U and 40% ^{Me}C. The DNA was methylated to completion, making all methylation sites not bound by the oligonucleotide mixture resistant to subsequent restriction enzyme digestion. The methylase was removed by phenol extraction, the DNA was precipitated with ethanol, and the triple helix was disrupted by resuspension in a restriction enzyme buffer at high pH. The appearance of a cleavage product upon restriction enzyme digestion demonstrated that a site bound by a fraction of the oligonucleotide mixture overlaps a methylation site. Subcloning of the cut ends and sequencing revealed the site identity. With this sequence information and the rules for oligonucleotide directed recognition of duplex DNA (*1–6, 9, 10*), an oligonucleotide specific for the target site was designed.

The triple helix–recognition motif is sufficiently general that each cosmid should contain at least one endogenous site suitable for enzymatic cleavage (*19*). For cosmid clones that have not been sequenced, a strategy was developed whereby a pool of partially degenerate pyrimidine oligonucleotides was used to identify target sites (Fig. 1). Pyrimidine oligonucleotides were designed with two domains; a degenerate 16-nucleotide (nt) segment capable of binding thousands of purine-rich sequences, and a short specific 2- to 3-nt domain to overlap a methylase–restriction enzyme binding site. The degenerate oligonucleotides were synthesized with 5-methylcytosine (^{Me}C) and 5-bromouracil (^{Br}U) (*4*). These analogs were chosen because ^{Me}C substituted oligonucleotides have higher binding affinities and ^{Br}U substitutions result in slightly reduced binding specificity (*4, 12*). Sequences containing high G content or contiguous G tracts are not efficiently targeted by the pyrimidine triplex motif near neutral pH. Because oligonucleotides in the population specific for such sequences would be less likely to bind, a nucleotide ratio of 60% ^{Br}U and 40% ^{Me}C at each degenerate position was used to minimize the oligonucleotide concentration specific for G-rich sequences. This resulted in a pool of 65,536 different oligodeoxyribonucleotide sequences unequally represented in the population (*20*).

In an effort to identify potential target sites within D4S10, cosmid clone 8C10I5, which contains most of the D4S10 locus (*15*), was screened with five partially degenerate pyrimidine oligonucleotides in combination with five methylase-restriction enzyme sets (Fig. 1). While no detectable cleavage was observed with Eco RI, Msp I, Hae III, or Taq I, a degenerate oligonucleotide specific for Alu I blocked a single Alu I methylation site in 8C10I5. DNA sequencing immediately adjacent to the cleavage site identified the target as a 16-bp purine sequence that overlapped the purine half sites of Alu I methylase (5′-AGCT-3′) and Sst I (5′-GAGCTC-3′), a restriction enzyme sensitive to Alu I methylation (Fig. 2). The ability of a target-specific oligonucleotide to effectively bind and fully protect this site was optimized on

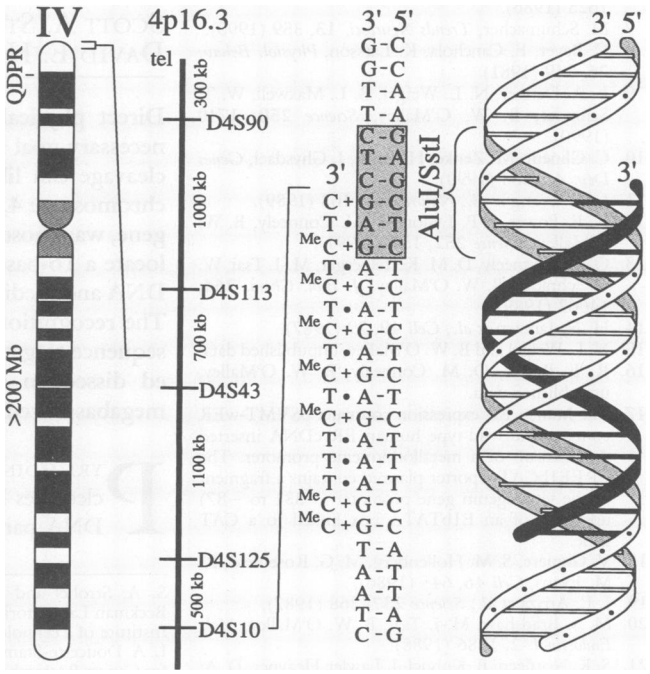

Fig. 2. (**Left**) Schematic diagram of human chromosome 4 showing the cytogenetic location of the QDPR locus at 4p15.3. (**Left-Center**) A physical map of 4p16.3 to 4p16ter showing the location and distance between the loci described. On the basis of pulsed field gel mapping, the size estimate from D4S10 to the telomere is approximately 4 Mb (*17*). The distances indicated between the loci are approximations from the physical map (*17*). (**Right-Center**) The sequence and relative orientation of the Alu I–Sst I target site identified by the degenerate pyrimidine oligonucleotide search. The site is recognized by a 16-base ^{Me}CT oligonucleotide with ^{Me}C + GC and TAT base triplets (Alu-16). (**Right**) Schematic diagram of the triple-helix complex. The pyrimidine oligonucleotide is bound in the major groove, parallel to the purine strand of the DNA duplex and covers the purine half of the Alu I methylation site.

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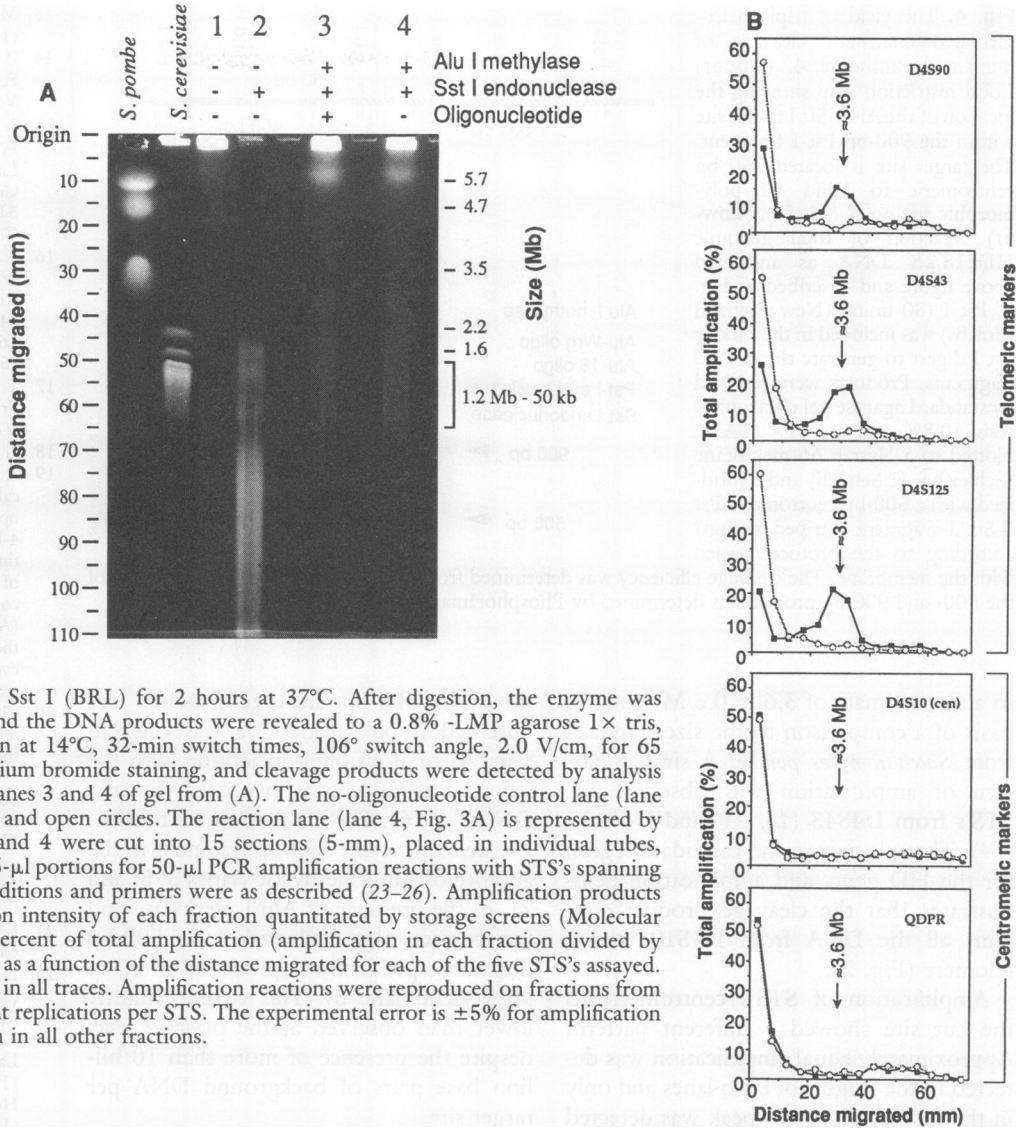

Fig. 3. (**A**) Triple-helix mediated enzymatic cleavage of human chromosome 4 with reagents indicated above figure. The DNA was prepared from somatic cell hybrid HD113.2B in low melting point (LMP) agarose at ≈100 μg/ml by standard procedures (*27*). Approximately 80 μl of agarose embedded genomic DNA (≈8 μg) was equilibrated in triple-helix–Alu I methylase buffer (50 mM NaCl, 1 mM spermine-4HCl, 50 mM tris-HCl, 10 mM EDTA, 1 mM β-mercaptoethanol, pH 7.0) and incubated for 24 hours at room temperature with 5 μM Alu–16 target-specific oligonucleotide. *S*-Adenosylmethionine (SAM) (160 μM) and bovine serum albumin (BSA) (100 μg/ml) were added, and the DNA was methylated to completion with 25 units of Alu I methylase (New England Biolabs) at room temperature for 8 hours. Methylase inactivation and triplex disruption were as described (*3*). The DNA was reequilibrated in Sst I restriction enzyme buffer (50 mM NaCl, 50 mM tris-HCl, pH 7.9, and 10 mM $MgCl_2$) and cut to completion with 20 units of Sst I (BRL) for 2 hours at 37°C. After digestion, the enzyme was inactivated by heat for 10 min at 55°C and the DNA products were revealed to a 0.8% -LMP agarose 1× tris, acetate, EDTA (TAE) pulsed-field gel run at 14°C, 32-min switch times, 106° switch angle, 2.0 V/cm, for 65 hours. Reactions were visualized by ethidium bromide staining, and cleavage products were detected by analysis with the PCR (*23*). (**B**) PCR analysis of lanes 3 and 4 of gel from (A). The no-oligonucleotide control lane (lane 3, Fig. 3A) is represented with gray lines and open circles. The reaction lane (lane 4, Fig. 3A) is represented by black lines with filled squares. Lanes 3 and 4 were cut into 15 sections (5-mm), placed in individual tubes, denatured at 96°C, vortexed, and used in 5-μl portions for 50-μl PCR amplification reactions with STS's spanning the 4p16.3 region. The amplification conditions and primers were as described (*23–26*). Amplification products were blot hybridized and the amplification intensity of each fraction quantitated by storage screens (Molecular Dynamics 400S PhosphorImager). The percent of total amplification (amplification in each fraction divided by total amplification in the lane) was plotted as a function of the distance migrated for each of the five STS's assayed. The lane origin is the first point to the left in all traces. Amplification reactions were reproduced on fractions from four different gels, resulting in two to eight replications per STS. The experimental error is ±5% for amplification at each origin, and ±2% for amplification in all other fractions.

plasmid (6.6 kb) and yeast genomic DNA (14 Mb), resulting in more than 98% and 90% cleavage yields at the target site, respectively. No secondary cleavage was detected at other Sst I sites in either substrate. Thus, after triple-helix formation and exhaustive Alu I methylation, the megabase genomic DNA could be cut with Sst I to reveal only the site bound by the oligonucleotide (Fig. 2).

Under optimized conditions, triple-helix mediated enzymatic cleavage was used to target human chromosome 4 in DNA prepared from HD113.2B, a somatic cell hybrid containing only human chromosome 4 in a mouse background (*21*) (Fig. 3A). Analysis of the reaction products within the range of 1 to 6 Mb by pulsed-field gel electrophoresis (*22*), revealed that most of the DNA did not migrate from the origin and remained intact (lanes 1, 3, and 4). When methylation was omitted and the DNA was digested with Sst I, no DNA remained at the origin, a result consistent with complete digestion (lane 2). Reactions in the presence (lane 3) or absence (lane 4) of oligonucleotide appeared identical by ethidium bromide staining. Because the maximum yield of the telomeric segment of chromosome 4 was 2 ng (from ~8 μg of total DNA), any cleavage product would be undetectable with ethidium bromide staining, thus requiring a more sensitive detection system.

Southern blot hybridization revealed a discrete product approximately 3.5 Mb in size. To improve the sensitivity of detection, a PCR (polymerase chain reaction) based assay to detect small quantities of specific megabase DNA segments was applied to the gel products shown in Fig. 3A (Fig. 3B). The full length of lanes 3 (with oligonucleotide) and 4 (without oligonucleotide) were cut into 5-mm sections perpendicular to the length of the gel and placed into individual tubes. Each fraction was analyzed for genomic DNA content by the PCR with the use of primer sets specific for sequence tagged sites (STS) known to be either telomeric or centromeric to the target site. Centromeric primers would be expected to only amplify the gel fraction containing the origin because the main portion of the chromosome would be too large to migrate into the gel. Telomeric markers, however, would amplify both the origin and any fractions containing the telomeric product expected from restriction mapping to be about 4 Mb in size (*17, 18*).

Amplification of D4S90 (*23*), the most telomeric 4p marker (Fig. 2) (*16, 17*), was detected in the origin of both lanes (Fig. 3B). The amplification signal was weaker in the origin of lane 3, consistent with chromosomal cleavage. Significant amplification within lane 3 was also observed in fractions 6 and 7, while the control lane containing no oligonucleotide remained baseline in all other fractions (lane 4). Fractions 6 and 7 correspond to a migration distance of 25 to 35 mm and correlate

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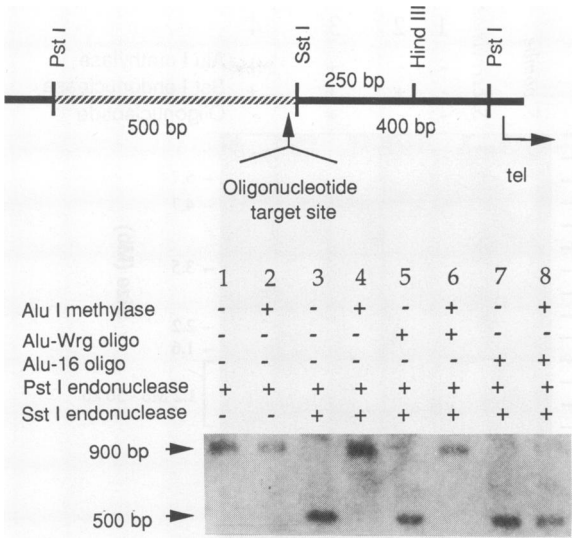

Fig. 4. The yield of triple helix–mediated enzymatic cleavage of human chromosome 4. (**Upper**) Local restriction map showing the location of the Alu I-Sst I target site within the 900-bp Pst I fragment. The target site is located 250 bp centromeric to Hind III polymorphic site no. 1 (*15, 25*). (**Lower**) Reaction of total genomic HD113.2B DNA as indicated above figure and described in Fig. 5. Pst I (80 units) (New England Biolabs) was included in the 2-hour Sst I digest to generate the desired fragments. Products were resolved by standard agarose gel electrophoresis (0.8% agarose, 1× TAE), blotted to a Nytran 66 membrane (Schleicher & Schuell) and hybridized with a 500-bp centromeric Pst I–Sst I fragment (striped region) according to the protocol issued with the membrane. The cleavage efficiency was determined from the relative radioactive intensities of the 500- and 900-bp products as determined by PhosphorImager analysis.

to a size estimate of 3.6 ± 0.3 Mb, on the basis of a comparison to the size standard from *Saccharomyces pombe*. A similar pattern of amplification was observed for STS's from D4S43 (*16, 23*) and D4S125 (*24*). These loci span the candidate region for the HD gene, and amplification demonstrates that the cleavage product contains all the DNA from D4S10 to the telomere (Fig. 2).

Amplification of STS's centromeric to the cut site showed a different pattern. Approximately equal amplification was detected in the origins of both lanes and only in the first fraction. No peak was detected with a D4S10 STS located 12 kb centromeric to the cut site (*25*), nor was a peak present with a QDPR STS located in 4p15.3 (*26*), one cytogenetic band centromeric to D4S10 (Fig. 2). This result demonstrated that these proximal loci are not found in the 3.6-Mb cleavage product, and indicated that contamination was not contributing to the peaks seen with the telomeric STS's.

The cleavage efficiency at the target site was determined directly by DNA (Southern) blotting. The Sst I target site is found near the center of a 900-bp Pst I fragment (Fig. 4, lanes 1 to 3). After Pst I digestion, cleavage at the Sst I site can be readily detected and quantitated by Southern blot hybridization with the 500-bp centromeric fragment (Fig. 4). The ratio of the number of counts at 500 bp to the total at both the 500- and 900-bp sites is the cleavage efficiency. To determine the yield of cleavage at the Sst I site in human chromosome 4, we performed reactions as described in the legend of Fig. 3A except that Pst I was included in the restriction enzyme digestion. No 500-bp cleavage product was observed if oligonucleotide was omitted (lane 4) or if an oligonucleotide with the incorrect sequence was incubated with the DNA (lane 6). The DNA was quantitatively converted to the 500-bp fragment in the absence of Alu I methylase (lanes 3, 5, and 7). In the presence of Alu I methylase and the correct oligonucleotide, the 500-bp diagnostic product was produced in 80 to 90% yield (lane 8). This is only slightly lower than observed at the plasmid level despite the presence of more than 10 billion base pairs of background DNA per target site.

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19. A sequence suitable for triple-helix formation was calculated to occur approximately once every 2000 bp (*2*). A target sequence that also overlaps a single 4-bp methylase would occur approximately 64 times less frequently, or every 120,000 bp. The use of five commercially available 4-bp methylases compatible with the enzymatic cleavage technique (Alu I, dam, Hae III, Hpa II, and Taq I) increases the frequency of a targetable site to about one every 24,000 bp, less than the average insert size of a cosmid clone.
20. The ^{Br}U and ^{Mc}C nucleotide ratio at each position resulted in an oligonucleotide pool in which 85% of the molecules have an overall base ratio for ^{Br}U to ^{Mc}C of 50% or higher. At 10 μM total oligonucleotide, the $(^{Br}U)_{16}$ sequence had the highest concentration at 3 nM, and the $(^{Mc}C)_{16}$ the lowest at 4 pM. The oligonucleotide composition with the highest total concentration (2 μM total, 0.25 nM in each sequence) were sequences with overall base content $(^{Br}U)_{10}(^{Mc}C)_6$.
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24. Because a nonpolymorphic PCR product was desired for D4S125, primers to the 3′ side of the variable terminal repeat were selected from the sequence published by B. Richards, G. T. Horn, J. J. Merrill, K. W. Klinger, *Genomics* **9**, 235 (1991). The primers were 5′-CTCTGGTCTGAGGTGCT-GAC-3′ and 5′-CGGCAGCTGAGGAGGTGCCT-3′. Reactions were performed at 28 cycles, 94°C, 1 min; 60°C, 1 min; and 72°C, 1 min; and standard buffer conditions as described (*23*).
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