Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 1531–1535, March 1983
Biochemistry

Use of synthetic oligonucleotide probes complementary to genes for human HLA-DR α and β as extension primers for the isolation of 5′-specific genomic clones

(major histocompatibility complex/synthetic oligonucleotide extension/genomic cloning)

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Contributed by Hugh O. McDevitt, December 10, 1982

ABSTRACT We have synthesized 175-nucleotide-long probes for the DNA of human histocompatibility antigens HLA-DR α and β by extending on poly(A)$^+$mRNA from B-cell lines with short synthetic deoxyribonucleotide primers complementary to the predicted nucleotide sequence of the NH_2 terminus of both polypeptides. The synthesis of the probe for the α-chain DNA was a two-step process starting with 11-mers which were extended by dideoxynucleotide chain termination experiments to a 20-mer of predicted sequence. The synthesized 20-mer was then used to generate a 175-nucleotide cDNA probe which was shown to encode the appropriate amino acids for the α chain and was used to select a human genomic DNA clone containing the coding sequences for HLA-DR α. For the β polypeptide an 18-mer homologous to the NH_2-terminal sequence of a cDNA clone from another B-cell line was used to extend on poly(A)$^+$mRNA isolated from a B-cell line. Preliminary sequence analysis of a 175-base-long extension product indicates a match of the cDNA sequence to the published sequence of a clone for HLA-DR β. Information from these extension experiments helps to establish the sensitivity and specificity of the primer extension method.

Human histocompatibility genes, *HLA-D*, are equivalent to murine *Ia* or "immune response" genes (*H-2* I) (for review, see ref. 1). Their protein products, called HLA-DR, are expressed on the surfaces of B lymphocytes and macrophages as noncovalently linked dimers, one called α (P34, heavy chain) and the other called β (P29, light chain) (2). The *H-2 I* region gene products play an important role in antigen presentation to T lymphocytes (3) and in interactions between T helper cells and B lymphocytes (4).

To achieve better understanding of the structure and function of HLA-DR, cloning of the genes encoding HLA-DR was undertaken. HLA-DR-specific mRNAs are not abundant in expressor cells (5), and conventional antisera or monoclonal antibodies do not recognize dissociated α or β subunits or those synthesized *in vitro* (6). Therefore, to isolate these genes, synthetic oligonucleotides, corresponding to limited known and conserved NH_2-terminal amino acid sequence information, were constructed for the α and β subunits. These were then extended on mRNAs from expressor cells to give longer cDNA products which then provided sufficient specificity for direct screening of genomic libraries.

The characterization of extended products for HLA-DR α and β chains and the use of the α-chain probe for the isolation and partial characterization of an α-chain-specific genomic clone are described.

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MATERIALS AND METHODS

Materials. Avian myeloblastosis virus reverse transcriptase, polynucleotide kinase, deoxynucleotide triphosphates, and oligo(dT)cellulose were obtained as described (7). The Klenow fragment of DNA polymerase I was purchased from P-L Biochemicals; restriction enzymes were from New England BioLabs and Bethesda Research Laboratories. [γ-^{32}P]ATP (3,000 Ci/mmol; 1 Ci = 3.7×10^{10} Bq) and [α-^{32}P]dCTP (2,000–3,000 Ci/mmol) were purchased from Amersham.

Poly(A)$^+$mRNA and DNA Extraction. The human lymphoblastoid cell lines JY (DRw 4,6) (from C. Terhorst), LG2 (DR1, DR1) (from R. Reissfeld), and CA-SC (DR1, DR2) (from H. McDevitt) and the T-cell line Molt-4 were cultured as described (7) to a density of 10^6 cells per ml. Total poly(A)$^+$mRNA was extracted as described (7) and membrane-bound poly(A)$^+$mRNA was obtained by the method of Stetler *et al.* (8). Chromosomal DNA was extracted according to Gross-Bellard *et al.* (9).

Synthesis, Labeling, and Characterization of Oligonucleotide Primers. The oligonucleotide primers were synthesized by the triester method (10) and then characterized as described (8). The primers were labeled at the 5′ end (7) to a specific activity of 1,000–1,500 Ci/mmol, purified by electrophoresis in a 15% polyacrylamide gel in 40 mM Tris, pH 7.8/2 mM EDTA/20 mM Na acetate (TEA buffer), and eluted by electrophoresis in 0.1× standard saline citrate (NaCl/Cit; 1× is 0.15 M NaCl/0.015 M Na citrate, pH 7.0).

Primer-Directed cDNA Synthesis. The ratios of primer to RNA template are specified in figure legends. The ^{32}P-labeled primer in 0.1× NaCl/Cit and poly (A)$^+$mRNA were precipitated at −20°C overnight in 60% ethanol/100 mM NaCl and then centrifuged at 34,000 rpm for 1 hr in an SW 41 rotor. The desiccated pellet was dissolved in 130 μl of 80 mM KCl and centrifuged at 10,000 rpm for 10 min. The supernatant (120 μl) was heated to 90°C for 10 min, mixed with 10 μl of 1 M Tris·HCl at pH 8.3, and incubated for 2 hr at 42°C. For full-length cDNA synthesis, the above reaction mixture was made 5 mM in $MgCl_2$, 10 mM in dithiothreitol, and 0.5 mM in each deoxynucleoside triphosphate and then 300 units of reverse transcriptase was added. This mixture was incubated, treated, and electrophoresed as described (7) except that a 5% gel was used. The di-

Abbreviations: kb, kilobase(s); bp, base pair(s); NaCl/Cit, standard saline citrate, pH 7.0.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Mouse IE/C (2)	Ile	—	—	—	—	—	Ile	Ile	—	Ala	—	Phe	Tyr	Leu
Human HLA-DR α (2)	Ile	Lys	Glu	Glu	Arg	Val	Ile	Ile / Leu	Glu	Ala	Glu	Phe	Tyr	Leu
Codon choices for mRNA								AU$^{U}_{C}$	GA$^{A}_{G}$	GCN	GA$^{A}_{G}$	UU$^{U}_{C}$	UA$^{U}_{C}$	$^{U}_{C}$UN
Synthetic 11-mers								A			CT$^{T}_{C}$	AA$^{A}_{G}$	ATG*	GA
11-mer-primed cDNA								A	GTC	CGG	CTC	AAG	ATG*	GA
Synthetic 20-mer								TAA	GTC	CGG	CTC	AAG	ATG*	GA

FIG. 1. NH_2-terminal peptide sequence of mouse I-E/Cα and human HLA-DR α chain (from ref. 2) and the predicted mRNA sequence. N, choice of A, C, G, or U; *, mismatch with the analyzed genomic clone. Also shown are the nucleotide sequence of the four potentially complementary synthetic 11-mers, the 18-mer cDNA copy synthesized from the 5′ AGG-TAG-AA-CTC 3′ 11-mer, and the 20-mer synthesized on the basis of this cDNA sequence.

deoxyadenosine-terminated reactions were as described (7). The individual cDNA products were eluted by electrophoresis in 0.1× NaCl/Cit and subjected to sequence analysis (11).

Construction and Screening of the Genomic Library. Chromosomal DNA from cell line JY was partially restricted with *Eco*RI and sized by sucrose density gradient, and 14-kilobase (kb) average-sized fragments were ligated into the *Eco*RI site of λ charon 4A (12). Plaque lifts at 10^4 recombinant phage per 15-cm plate were made (13), incubated in 2× NaCl/Cit containing 2× Denhardt's solution (14) at 65°C for 3 hr, and then hybridized under the same conditions with 175-mer (2×10^4 cpm/ml) for 24 hr. Filters were washed in 2× NaCl/Cit at 65°C and autoradiographed.

Subcloning the 4.4- and 3.1-kb *Eco*RI Fragments. Phage DNA was isolated (15), digested completely with *Eco*RI, and ligated into *Eco*RI-cut pBR328 (16). Plasmid-containing clones were screened with the 175-mer probe to identify the correct clones.

Restriction Map and Partial Sequence Analysis of λ HLA-DR α. The map was constructed by the method of Smith and Birnsteil (17) with the pBR328 subclones. Subfragments of these clones containing the coding sequences were labeled at the 5′ end with [α-^{32}P]ATP and T4 polynucleotide kinase or at the 3′ end with either the appropriate [α-^{32}P]dNTP and the Klenow fragment of DNA polymerase I or [^{32}P]cordycepin (18), and then subjected to sequence analysis by the procedure of Maxam and Gilbert (11).

RESULTS AND DISCUSSION

Construction of Short Oligonucleotide Probes and cDNA Extension. It is difficult to find unique oligonucleotides suitable for use as direct hybridization probes in eukaryotic genomic libraries for a number of reasons: the existence of multiple codon choices for most amino acids, incomplete amino acid sequence data for most polypeptides, amino acid polymorphism, and the complexity of the genome. Additionally, optimizing the hybridization conditions for a specific small probe can be difficult. The approach we have used circumvents some of these problems and generates a probe suitable for isolation of genomic clones containing the 5′ end of the gene.

A set of 11-base-long deoxyribonucleotide polymers was synthesized based on the NH_2-terminal amino acid sequence of HLA-DR α from a human B-cell line and the equivalent peptide from several mouse lines (2) (Fig. 1). To minimize potential mismatches, the region of the amino acid sequence chosen for the synthetic probes was one that corresponded to a region conserved between these two species (positions 11–14). Because codon assignment for this region was ambiguous, 4 of the 16 potentially complementary 11-mers were made. To ensure a complete match at the 3′ end of the primer, all four possibilities at this end (positions 6 and 9) of the probe were made and dG was utilized at positions 2 and 3 to minimize the effect of potential mismatches (19).

Because a full-length cDNA synthesis using the 11-mers was anticipated to give a complex extension product pattern, an intermediate cDNA synthesis step was used to learn more about the nucleotide coding sequence of the α peptide and to create a probe of greater specificity. Individual 11-mers were used to prime cDNA synthesis from B-cell poly(A)$^+$mRNA in the presence of dideoxyadenosine triphosphate. With the 5′ A-G-G-T-A-G-A-A-C-T-C 3′ primer an 18-base-long minor cDNA band was found (data not shown) whose sequence corresponded to amino acids identical to the published α peptide sequence (Fig. 1). On the basis of this sequence and two additional nucleotides inferred from the 3′-terminal isoleucine codon, a new 20-mer was synthesized. This 20-mer was then used as a primer to ex-

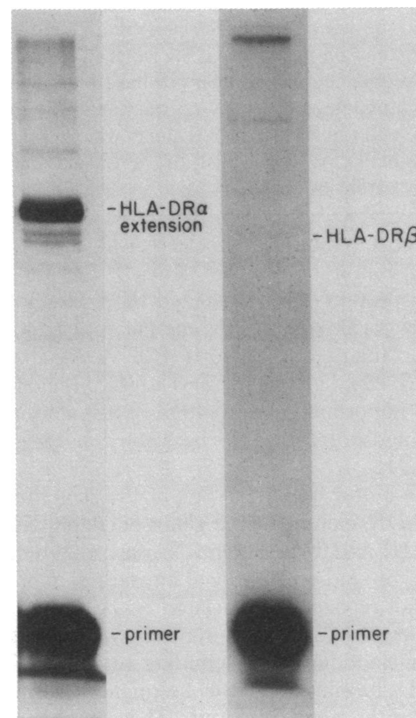

FIG. 2. Autoradiogram showing cDNA extension products. (*Left*) Extension primed by the HLA-DR α-chain complementary 20-mer (100 pmol) on human poly(A)$^+$mRNA from B-cell line LG-2 (160 μg). The major band (marked HLA-DR α-chain extension) is approximately 175 nucleotides long. (*Right*) Extension primed by the HLA-DR β-chain complementary 18-mer (60 pmol) on human poly(A)$^+$mRNA from B-cell line JY (60 μg). The extension product which is homologous to the β-chain of HLA-DR (marked HLA-DRβ) is approximately 175 nucleotides long.

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5' pppNNN~29GACTCCCAACAGAGCGCCCAAGAAGAAAATGGCCATAAGTGGAGTC
                                         Met Ala Ile Ser Gly Val

CCTGTGCTAGGATTTTTCATCATAGCTGTGCTGATGAGCGCTCAGGAATCA
Pro Val Leu Gly Phe Phe Ile Ile Ala Val Leu Met Ser Ala Gln Glu Ser
—————————————— Leader Peptide ——————————————

TGGGCTATCAAAGAAGAACATGTGATCATTCAGGCC[GAGTTCTACCT] 3'
Trp Ala Ile Lys Glu Glu His Val Ile Ile Gln Ala Glu Phe Tyr Leu

        Ile Lys Glu Glu Arg Val Ile Ile Gln Ala Glu Phe Tyr Leu
         1   2   3   4   5   6   7   8   9   10  11  12  13  14
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FIG. 3. Nucleotide sequence of the 175-nucleotide-long cDNA product which was primed by the 20-mer and which encodes part of the HLA-DR α chain. The nucleotides of the original 11-mer are enclosed in a box; the position of the 20-mer is indicated by dotted underlining. The proposed leader peptide is designated by solid underlining. The bottom line, under the sequence predicted by the 175-mer, is the published amino acid sequence of B-cell line RPMI-4265.

tend on poly(A)$^+$ B-cell mRNA, and a major 175-nucleotide-long cDNA band was synthesized (Fig. 2). The sequence of this product predicted an amino acid sequence consistent with the published sequence for the α chain (Fig. 3).

From concurrent work by Stetler *et al.* (8) it is known that mRNA for HLA-DR α chain is present in unfractionated poly(A)$^+$mRNA from the B-cell line CA-SC at a level of about 0.02%. If the level is similar in B-cell line LG2, then it can be concluded that specific probes 20 nucleotides long can be used to synthesize specific cDNA probes on low-copy-number mRNA.

The NH_2-terminal amino acid sequence for the β chain is known in several different human cell lines (20–22), and in this highly polymorphic peptide the only region that is highly conserved is at positions 20–25 (Fig. 4). Because, during the course of this work, a cDNA clone encoding the HLA-DR β chain was isolated from Raji cells (22), an 18-mer was synthesized to match the Raji sequence in this conserved region. This 18-mer was used as a primer to extend poly(A)$^+$mRNA from B-cell line JY and two major cDNA products were obtained (Fig. 2). Preliminary analysis of both bands indicates that the sequence of the lower 175-nucleotide band matches the Raji sequence. The reason for the lower yields of cDNA products in this extension compared to the α-chain extension is not known.

In the process of carrying out the above sets of experiments some data were generated which provide additional information concerning the use of probes in extension experiments. The original four 11-mers complementary to HLA-DR α chain were used individually in full-length extension experiments on total poly(A)$^+$mRNA from B cells, on membrane-bound poly(A)$^+$-mRNA from B cells in which HLA-DR α-chain mRNA was enriched 10-fold over unfractionated (8) and on total poly(A)$^+$mRNA from a human T-cell line (Fig. 5). Only the primer shown to be homologous to α-chain mRNA, designated GC, gave rise to a band at 175 nucleotides. When a mismatch occurred in either

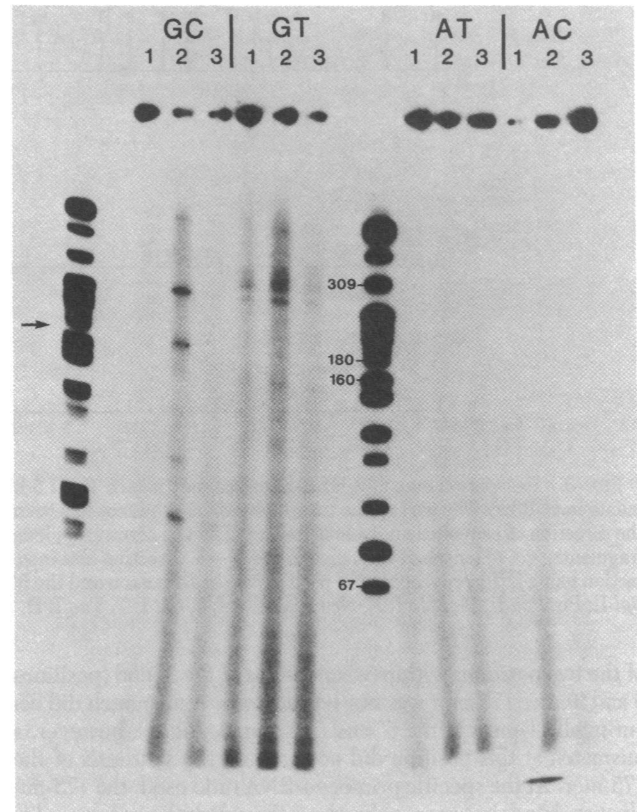

FIG. 5. Autoradiogram showing cDNA extension products primed by the 11-mers on different poly(A)$^+$mRNA fractions. The four 11-mers are: GC, 5′ A-G-G-T-A-G-A-A-C-T-C 3′; GT, A-G-G-T-A-G-A-A-T-T-C; AT, A-G-G-T-A-A-A-A-T-T-C; and AC, A-G-G-T-A-A-A-A-C-T-C. The cDNA extensions depicted for each 11-mer were prepared by using 18 pmol of specific primer: with 3 μg of total B-cell poly(A)$^+$mRNA from the CA-SC cell line (lanes 1), with 3 μg of membrane-bound B-cell poly(A)$^+$mRNA (lanes 2), and with 3 μg of total poly(A)$^+$mRNA from T-cells (column 3). Markers shown are pBR322 digested with *Hpa* II; arrow indicates the position of 175 nucleotides.

Strain	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
HLA-DW2,2/DR2,2 (20)	Gly	Asp	Thr	Arg	Pro	Arg	Phe	Leu	Trp	Gln	Pro	Lys	Arg	Glu	Cys	His	Phe	Phe	Asn	Gly	Thr	Glu	Arg	Val	Arg
LG2 HLA-DR1,1 (21)	Gly	Asp	Thr	Arg	Pro	Arg	Ser	Leu	Trp	Gln	Leu	Lys	Phe	Glu	Cys	His/Tyr	Phe	Phe	—	Gly	Thr	Glu	Arg	Val	Arg
Raji HLA-DW3,6 (22)	Arg	Asp	Ser	Pro	Glu	Asp	Phe	Val	Tyr	Gln	Phe	Lys	Gly	Met	Cys	Tyr	Phe	Thr	Asn	Gly	Thr	Glu	Arg	Val	Arg
Codon choices for mRNA																				GGN	ACN	GA$^{A}_{G}$	CGN	GTN	CGN
cDNA sequence from Raji (21)	AGA	GAC	TCT	CCC	GAG	GAT	TTC	GTG	TAC	CAG	TTT	AAG	GGC	ATG	TGC	TAC	TTC	ACC	AAC	GGG	ACA	GAG	CGC	GTG	CGT
Synthetic 18-mer																				CCC	TGT	CTC	GCG	CAC	GCA

FIG. 4. NH_2-terminal peptide sequence of human HLA-DR β chain from three sources. The amino acid sequence from Raji cells is inferred from the nucleotide sequence of the cloned gene (22). N, choice of A, C, G, or U. The mRNA sequence is predicted from the DNA sequence of the cloned gene. The 18-mer probe synthesized based on this sequence is shown at the bottom.

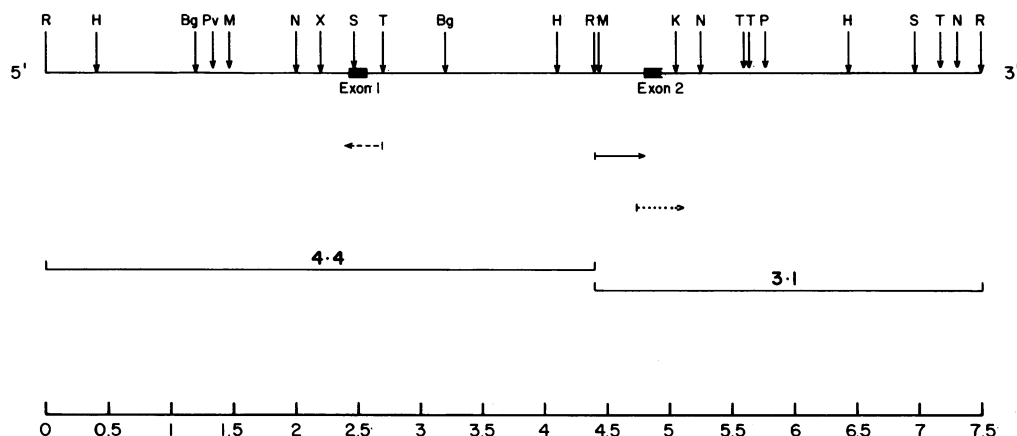

FIG. 6. Restriction map of λ HLA-DRα genomic insert. The 7.5-kb insert was mapped by using subclones of the 4.4-kb and 3.1-kb *Eco*RI fragments in pBR328. The part of the insert where the sequence has been determined is indicated by the bold black line below the restriction map and the direction of sequence analysis is indicated by the arrows: →, length of the sequence obtained from the 5'→3' direction by using α-^{32}P-labeled fragments; ←---ı, length of sequence in the 3'→5' direction obtained by 3'-labeling with [α-^{32}P]dATP; ı······•, length of sequence in the 3'→5' direction using [^{32}P]cordycepin. The position of the first exon and the 5' end of the second exon are indicated by the heavy black boxes. E, *Eco*RI; Bg, *Bgl* II; Pu, *Pvu* II; M, *Msp* I; N, *Nco* I; X, *Xba* I; S, *Sst* I; T, *Taq* I; H, *Hin*dIII; K, *Kpn* I; P, *Pst* I.

of the two positions within 6 nucleotides of the 3' end (positions 6 and 9), the 175-mer was not synthesized. A mismatch did occur in all 11-mers at the 5' end (position 3, Fig. 1); however, a mismatch at this position did not prevent the synthesis of the 175-mer. At the specific primer-to-RNA ratio used, the 175-nucleotide band was seen only when the nearly homologous 11-mer was used to prime membrane-bound B-cell poly(A)$^+$mRNA; with total B-cell poly(A)$^+$mRNA it did not appear. Therefore, in this system it is possible to extend and obtain a characteristic cDNA band when a nearly homologous 11-mer primes on a message representing 0.2% of the total poly(A)$^+$ RNA population (8).

Screening of a Chromosomal Library and Characterization of a Clone λ HLA-DRα. A B-cell-specific human genomic library cloned in λ Charon 4A was screened with the 175-mer encoding part of the HLA-DR α-chain and one λ clone was chosen for further study. An *Eco*RI digest of DNA from this clone, λ HLA-DRα, indicated two insert bands, 4.4 and 3.1 kb (data not shown). Southern blot analysis showed that both *Eco*RI insert fragments hybridized to the 175-mer whereas the 20-mer bound strongly only to the 3.1-kb fragment (data not shown). These data imply that an *Eco*RI site splits the sequence encoded by the 175-mer and, because the actual sequence of the 175-mer contains no such site, they also imply that the 5' end of the gene contains an intervening noncoding sequence. This implication was confirmed by the establishment of a restriction map of the entire insert (Fig. 6) and a nucleotide sequence analysis of NH_2-terminal amino acid coding regions (Fig. 7). This sequence from B-cell line JY is consistent with the known NH_2-terminal amino acid sequence published for HLA-DR α chain from the B-cell line RPMI-4265 (Fig. 1) and for the leader peptide and NH_2-terminal amino acid sequence predicted from a cDNA clone from B-cell line CA-SC (8). These similarities among different B-cell lines confirm the belief that the HLA-DR α chain is not highly polymorphic. Further sequence analysis indicates that the entire coding sequence for the HLA-DR α chain is contained within the 7.5-kb fragment of λ HLA-DRα (unpublished data).

H.K.D. was supported by a Postdoctoral Fellowship from the Cancer Research Institute Inc., New York.

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FIG. 7. Partial nucleotide sequence of the λ HLA-DRα genomic insert. The positions of exon 1 and the start of exon 2 are marked. The 20-mer is marked by a bracket, [ ]; the 175-mer is underlined. The NH_2-terminal Ile residue is boxed; the amino acids of the proposed leader peptide are underlined with a wavy line. The upward arrow delineates a discrepancy between the genomic clone and the sequence of the original 11-mers. The dotted line indicates a portion of the intervening sequence containing the *Eco*RI site; its sequence has not yet been determined.

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