ugation, rinsed in buffer I, resuspended in 1.7 volumes of buffer II (10 m*M* Hepes, *p*H 7.9, 0.4*M* NaCl, 1.5 m*M* $MgCl_2$, 0.5 m*M* DTT, 0.5 m*M* PMSF, 5 percent glycerol), agitated for 30 minutes, and then centrifuged 50 minutes at 34,000 rpm in a Beckman Ti50 rotor (*8*). The supernatant was dialyzed overnight against buffer III (20 m*M* Hepes, *p*H 7.9, 50 m*M* NaCl, 0.5 m*M* DTT, 0.5 m*M* PMSF and 20 percent glycerol), recentrifuged, and stored in equal portions at −70°C. Chelated extracts were prepared with 0.1 m*M* EGTA in buffer II and 0.1 m*M* EDTA in buffer III, whereas $CdCl_2$-treated extracts were prepared with 5 μ*M* $CdCl_2$ in all buffers.

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Megabase-Scale Mapping of the HLA Gene Complex by Pulsed Field Gel Electrophoresis

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In the study of the genetic structure of mammalian chromosomes, there exists a "resolution gap" between molecular cloning experiments and meiotic linkage analyses. This gap has discouraged attempts to construct full-scale genetic maps of mammalian chromosomes. The organization of the human major histocompatibility complex was examined within this range by pulsed field gel electrophoresis. The data obtained indicate that the complex spans over 3000 kilobases and enable the construction of a megabase-scale molecular map. These results indicate that the techniques employed in DNA extraction, enzymatic digestion, electrophoresis, and hybridization are suitable for the efficient analysis of megabase regions of mammalian chromosomes and effectively bridge the resolution gap between molecular cloning and classical genetics.

THE MAJOR HISTOCOMPATIBILITY complex (MHC) is an extended multigene family composed of several classes of genes, many of which play central roles in the function of the immune system. The multiplicity of phenotypes controlled by the MHC has made the solution of its genetic structure a major goal of immunogeneticists. Considerable progress has been made in studies of both the human (HLA) and the murine (H-2) MHCs. Meiotic linkage analyses have defined three major regions and several subregions of the MHC (*1*). Molecular cloning and chromosome walking experiments have resolved the structure of individual genes and, in some cases, the organization of subregions (*2, 3*). Nevertheless, the limits of resolution of each technique have precluded the synthesis of a full-scale structural map of the HLA complex.

Recently, several methods for the molecular analysis of DNA sequences separated in the genome by distances of up to 1000 kb have been developed (*4, 5*). Pulsed field gel electrophoresis (PFG) has been used to separate and analyze DNA fragments as large as yeast chromosomes (*6*) and to generate a full-scale molecular map of the *Escherichia coli* genome (*7*). This report describes the extension of these methodologies to the human genome in an analysis of the organization of the human MHC.

The fragments of mammalian DNA generated by the majority of restriction enzymes are one to two orders of magnitude smaller than the size range necessary for effective mapping of large chromosomal regions such as HLA. The enzymes used in this study were initially selected as those which recognize eight–base pair cleavage sites and/or contain CpG in their recognition sequences. Because of the rarity of CpG in mammalian genomes, these enzymes were expected to generate fragments that are much larger than would be predicted statistically in random sequence DNA. Figure 1 shows PFG fractionation of human DNA digested with the restriction nucleases Not I, Sal I, and Nru I. Linear concatemers of λ *vir* DNA, coelectrophoresed with the digests of human DNA, enable precise determinations of the sizes of the human DNA fragments. In contrast to conventional gel electrophoresis, in which linear ladders of DNA become

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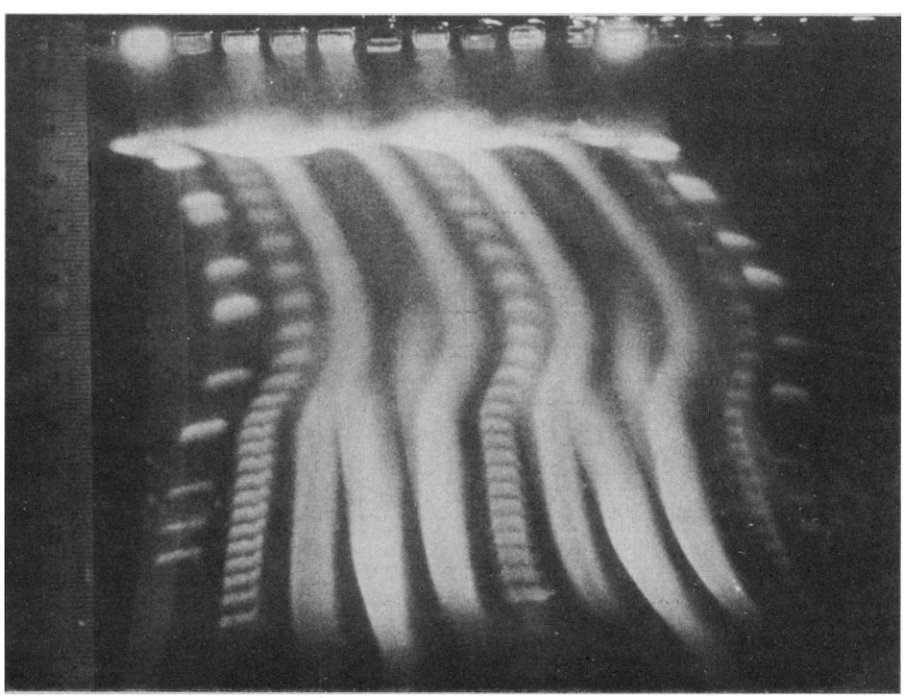

Fig. 1. Pulsed field gel electrophoresis of human DNA. The samples were separated in a 55-cm double, inhomogenous PFG apparatus for 72 hours at 500 volts with 2-minute pulses between 90° field reorientations. The lanes from left to right are: yeast chromosomal DNAs (*Saccharomyces cerevisiae*, strain DBY728); concatemers of λ *vir* (42.5-kb monomer); human (3.1.0) DNA digested with Not I, with Sal I, and with Nru I; λ *vir* concatemers; human (GM3104A) DNA digested with Not I, with Sal I, and with Nru I; λ *vir* concatemers; and yeast chromosomal DNAs. Further details of the DNA preparations, digestion, and electrophoresis are as described (*4*).

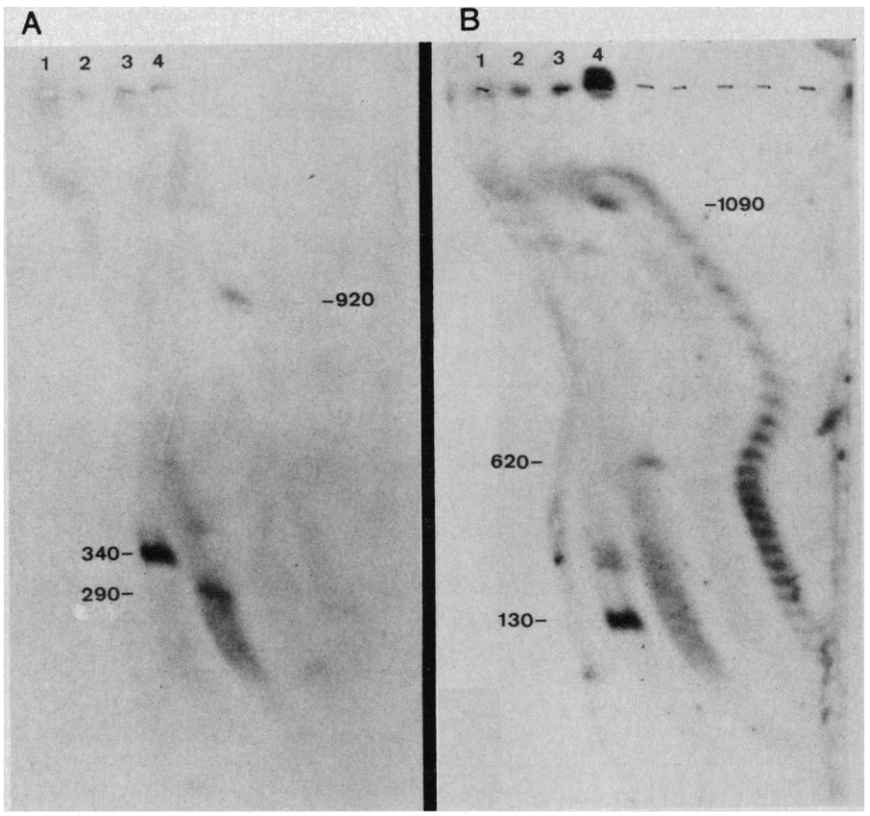

Fig. 2. Southern hybridization with PFG-separated DNA. The autoradiograms show the hybridization of the HLA-DR specific probe (**A**) and the HLA-B specific probe (**B**) with (lane 1) Mlu I, (lane 2) Sal I, (lane 3) Not I, and (lane 4) concatemers of λ *vir* (42.5-kb monomer). The λ ladder was illuminated by including a small quantity of ^{32}P-labeled λ DNA in the hybridization. Numbers indicate the sizes of the hybridizing bands in kilobases. The values are accurate ± 20 kb. Blotting and hybridization procedures followed the method of Southern (*13*). Further details are as described (*4*).

exponentially compressed toward the origin, the λ ladders are approximately linear. Consequently, the precision of fragment size estimations is uniform throughout the resolved range (<40 to 1100 kb in Fig. 1).

Individual restriction fragments were detected by hybridization with ^{32}P-labeled probes after Southern transfer to nitrocellulose. Figure 2 shows the results of a typical experiment. In this case, a filter containing PFG-resolved digests was hybridized with a probe specific for the class II region HLA-DR$_\alpha$ gene (Table 1). The filter was subsequently rehybridized with a probe specific for the class I region HLA-B gene. Specific fragments were detected without significant loss of signal intensity throughout the resolved size range. For example, the HLA-B probe shown in Fig. 2 detected a 130-kb Mlu I fragment, a 620-kb Sal I fragment, and a 1090-kb Not I fragment. Although migration of DNA in pulsed fields may be influenced by factors other than molecular weight, such as the amount of DNA in the gel lane, we found these measurements to be reproducible to within ± one half step of the λ ladder (±20 kb).

To detect large restriction fragments of the human MHC, we hybridized Southern blots of PFG-resolved human DNA with probes representing each of the three HLA regions. The genes of the class I region cross-hybridize by virtue of sequences encoding the β_2 microglobulin binding domain (exon 4) as well as other sequences that are highly conserved in class I genes (*2*). Similarly, each of the class II subregions includes β-chain genes that contain regions of conserved sequence, particularly in the second external domain (exon 3) (*3*). We used complementary DNA (cDNA) clones of the class I HLA-B gene (*8*) and the class II HLA-DR and DQ$_\beta$ genes (*9*) to investigate the extent of these HLA regions (Table 1). To ensure that the bands detected with these probes arose from the hybridization of syntenic fragments, HLA-hemizygous (3.1.0) (*10*) and HLA-homozygous (GM3104; NIGMS) DNAs were examined. To reduce the probability of missing internal non–cross-hybridizing regions of the complex, the most infrequently cutting enzymes, Not I and Mlu I, were employed.

The class I cross-hybridizing probe detected three fragments in Not I and five fragments in Mlu I–digested, HLA-hemizygous DNA (Table 2). The total sizes of these Not I and Mlu I fragments were 1820 kb and 2040 kb, respectively. The two cross-hybridizing probes for class II each detected the same two Not I fragments, totaling 1240 kb. The class III probe also reacted with the larger of these two Not I fragments. Results obtained with the HLA class II and III subregion probes indicate that these subregions span at least four distinct Mlu I fragments totaling 1080 kb in length. The combined data obtained with the probes suggest that the HLA complex spans at least five distinct Not I fragments totaling 3060 kb in length and at least nine distinct Mlu I fragments totaling 3120 kb in length.

To orient these restriction fragments with respect to one another, we took advantage of available genetic data. By recombination, the HLA genes reside in the order centro-

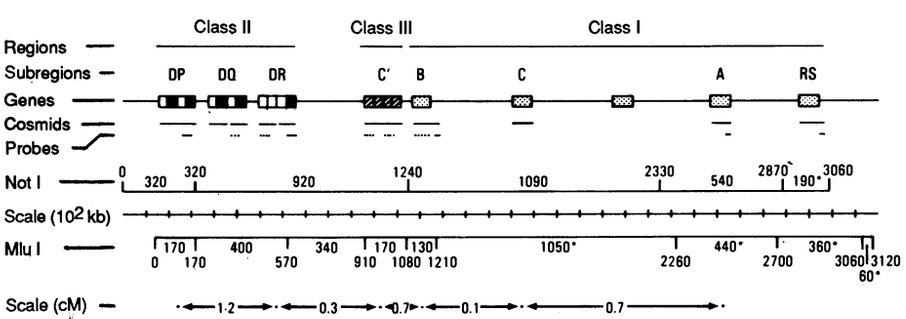

Fig. 3. Molecular and genetic map of the HLA gene complex. The HLA genes are represented by boxes: class II α (closed), class II β (open), class III (striped), and class I (stippled). Regions of the complex isolated in overlapping cosmid clones are indicated by the solid lines beneath the genes. The locations of the genomic and complementary DNA fragments used as probes are indicated, respectively, by the solid and broken lines beneath the cosmid clusters. The genes, cosmids, and probes are not to scale. Numbers indicate the individual and cumulative sizes in kilobases of the restriction fragments. These values are accurate ± 20 kb. Recombination frequencies are as described (*1*). Divisions on the kilobase scale represent hundreds of kilobases. The positions of the starred class I fragments are uncertain. The exact alignment of the Not I and Mlu I maps is not known, although preliminary double digest data are consistent with that shown. Assuming no additional internal fragments, the Mlu I fragments harboring C' and B align the maps to within less than 100 kb; cM, centiMorgans.

mere–DP–DR–C′–B–C–A–telomere (*1*). Specific probes for each of these genes were prepared (Table 1) and used to probe PFG blots. The DP-specific probe hybridized exclusively with the 320-kb Not I fragment and the 170-kb Mlu I fragment previously detected with the cross-hybridizing class II probes. Consequently, these fragments map as the most centromeric of the HLA complex. In contrast, the DR-specific probe hybridized exclusively with the 920-kb Not I fragment. Therefore, in accord with the genetic data, the 320- and 920-kb fragments are oriented as shown in Fig. 3. The probe specific for class III hybridized with the same 920-kb Not I fragment as was detected with the DR-specific probe, indicating that the class III region also resides within this DNA fragment. To orient the Not I bands representing class I, an HLA-B–specific probe was prepared. This probe reacted exclusively with the 1090-kb Not I fragment and the 130-kb Mlu I fragment detected previously with the cross-hybridizing probe, locating these fragments as the most centromeric of the class I region. The most telomeric of the mapped MHC genes, HLA-A, was located within the 540-kb Not I fragment. Thus, as shown in Fig. 3, with the exception of the 190-kb Not fragment from class I, these data orient all of the HLA-related Not I restriction fragments. By analogy with the murine MHC, the 190-kb fragment may contain Qa/Tla-like genes and reside telomeric to the classical class I genes. Indeed, our results indicate that at least one nonclassical class I gene, RS5 (*11*), is located in this fragment (Table 2).

This analysis of the structure of the HLA complex relies on the detection of HLA genes by region-specific, cross-reactive probes. It is possible that weakly cross-hybridizing fragments, nonreactive fragments internal to the complex, or fragments out of range of PFG electrophoresis were missed in this analysis. However, the similarity of the values obtained with different enzymes as well as the confirmation of the cross-hybridizing patterns with the subregion-specific probes, suggest that the values provide an accurate estimation of the extent of the HLA complex. It is also uncertain whether the detected HLA restriction fragments are directly contiguous or whether additional smaller Not I fragments reside between them. In view of the clustering of CpG residues in the genome, it seems possible that Not I and Mlu I sites might also be clustered. Polymorphisms might also be expected to affect PFG hybridization patterns. Comparison of the 3.1.0 (HLA A-2, B-27, C-1, and D-1) and GM3104 (HLA-A-3, B-35, C-4, and D-1) cell lines, however, indicates a strong conservation of the restriction fragments shown in Fig. 3. An exception is the 1090-kb Not I fragment, which appears to be divided into three smaller class I–hybridizing Not I fragments in the GM3104 cell line.

The minimum estimate for the size of the complex is in excess of three million base pairs, a figure not incompatible with that obtained from the linkage data, assuming that 1 centiMorgan is roughly equal to 1000 kb. Approximately two-thirds of this distance contains the more than 25 genes of the class I region of HLA. The remaining one-third contains the genes of the class II and

Table 1. HLA probes and their hybridization patterns in conventional Southern blots. Further detail of each probe can be found in the references. Abbreviations: HS, high stringency wash [0.1× SSC (standard saline citrate) at 65°C]; LS, low stringency wash (3× SSC at 50°C); UTS, untranslated sequence; cross-hyb, cross-hybridizing.

Probe	Description	Hybridization in Eco RI blot	Reference
	Class II		
DP specific (p8MI)	4.1-kb Msp I genomic fragment: DP_α exon 1 to DP_β exon 1	At HS, 10- and 7-kb fragments linked within DP subregion	(*14*)
DQ cross-hyb (2918.8)	0.7-kb Eco RI cDNA fragment: exons 1–5 of DQ_β	At LS, nine fragments between 3 and 12 kb	(*9*)
DR specific (pp34R13)	3.1-kb Eco RI genomic fragment: intron 2–3′ UTS of HLA-DR_α	At HS, a single 3.1-kb fragment	(*15*)
DR cross-hyb (2918.4)	1.1-kb Eco RI cDNA fragment: exons 1–5 of DR_β	At LS, nine fragments between 3 and 12 kb	(*9*)
	Class III		
C2 specific (pC2-2a)	1.6-kb Pst I cDNA fragment: not well characterized	At HS, a single 7-kb fragment	(*16*)
C4 specific (pC4AL1)	1-kb Pst I cDNA fragment: $C4_\gamma$ chain exons	At HS, a single 12-kb fragment	(*16*)
	Class I		
B specific (RS30.4)	3.4-kb Eco RI genomic fragment: intergenic DNA flanking HLA-B	At HS, a single 3.8-kb fragment	*
B cross-hyb (pDP001)	1.4-kb Pst I cDNA fragment: exon 2–3′ UTS of HLA-B	At LS, ten fragments between 5 and 20 kb	(*8*)
A specific (pHLA2a.1)	0.5-kb Pvu II–Msp II fragment: 3′ UTS of HLA-A2	At HS, a single 5.9-kb fragment	(*17*)
RS specific (RS5.10)	0.7-kb Eco RI genomic fragment: intergenic DNA flanking RS5	At HS, a single 4-kb fragment	(*19*)

*Described in this study.

Table 2. Results of PFG hybridization experiments with HLA hemizygous (3.1.0) DNA. Values are averages of at least two independent determinations and are accurate ± 20 kb. Bands hybridizing with more than one probe are indicated by the roman numerals. These identities were determined by successive hybridizations of each filter with the relevant probes. Although the co-migration of nonidentical fragments remains a possibility, preliminary partial and double digest data are also consistent with the identities indicated. ND, not determined.

Subregion	Probe specificity*	Hybridizing bands (kb)		
		Not I	Mlu I	Sal I
B	Specific	1090[i]	130[ii]†	620[iii]
B	Cross-hyb	1090[i] 540[iv] 190[v]	1050 440 360 130[ii] 60	620[iii]§
A	Specific	540[iv]	ND	ND
RS	Specific	190[v]	ND	ND
DP	Specific	320[vi]	170[vii]	250
DR	Specific	920[viii]	340	250[ix]†
DR	Cross-hyb	920[viii] 320[vi]	400‡ 170[vii]	250[ix]‡
DQ	Cross-hyb	920[viii] 320[vi]	ND	270† 250[ix]
C′	Specific	920[viii]	170	ND

*See Table 1 for further detail of probe specificities and stringency of washing conditions. †Cross-hybridization also observed with higher molecular weight bands probably due to partial digestion. ‡Hybridization also observed with higher molecular weight DNA not resolved in this experiment. §An undetermined number of hybridizing bands between 0 and 350 kb.

III regions. The multiplicity of hybridizing fragments in the class I region suggests that the class I genes are dispersed throughout the 1800 to 2000 kb comprising the class I region. In contrast, since no class II beta genes were found in the 340-kb Mlu I fragment that hybridized with DR_α, the class II genes appear to be clustered in the centromeric half of the 920-kb Not I fragment. These data are in accord with those recently determined by Hardy *et al.* (*12*) who have shown that the DO and DX class II subregions also reside between DP and DR and that DZ is closely linked to DP. Thus, there may be as much as 300 kb between the class II and class III subregions in which classical HLA genes are absent. In addition to the class I, II, and III genes, other genes are interspersed in or located near the MHC (*5*). Since the PFG estimation of the extent of HLA exceeds the amount isolated in molecular clones by a factor of at least 2, these data indicate ample room for these and other nonclassical HLA-linked genes or gene families.

This study has examined the application of pulsed field gel electrophoresis and hybridization to the molecular organization of the HLA gene complex. The preparation of essentially intact human DNA suitable for restriction digestion, the identification of restriction enzymes that generate fragments suitable for megabase-scale restriction mapping, and the development of electrophoresis and hybridization procedures that detect fragments throughout a range of 10 to 1000 kilobases demonstrate that the techniques employed are suitable for the efficient analysis of megabase regions of mammalian DNA. The potential now exists for the use of these techniques in conjunction with classical genetics, standard cloning techniques, and chromosome linking and chromosome jumping libraries (*4, 5*), in the construction of an extensive molecular map of the human genome.

Note added in proof: Partial digestion data confirm the placement of the 190-kb Not I fragment adjacent to the 540-kb Not I fragment.

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β Amyloid Gene Duplication in Alzheimer's Disease and Karyotypically Normal Down Syndrome

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With the recently cloned complementary DNA probe, λAm4 for the chromosome 21 gene encoding brain amyloid polypeptide (β amyloid protein) of Alzheimer's disease, leukocyte DNA from three patients with sporadic Alzheimer's disease and two patients with karyotypically normal Down syndrome was found to contain three copies of this gene. Because a small region of chromosome 21 containing the *ets*-2 gene is duplicated in patients with Alzheimer's disease, as well as in karyotypically normal Down syndrome, duplication of a subsection of the critical segment of chromosome 21 that is duplicated in Down syndrome may be the genetic defect in Alzheimer's disease.

A COMPLEMENTARY DNA (cDNA) probe λAm4 for the chromosome 21 gene encoding the amyloid polypeptide (β amyloid protein) of Alzheimer's disease (AD-AP gene) was recently cloned and sequenced (*1*). The polypeptide encoded by this gene is found in the brains of patients with Alzheimer's disease, as well as in adult patients with Down syndrome (*2*). We present results of gene dosage measurements in leukocyte DNA, using this cDNA as a probe, that show that the AD-AP gene is duplicated in patients with Alzheimer's disease and in patients with karyotypically normal Down syndrome (*3, 4*).

DNA was obtained from leukocytes from seven healthy control subjects (four young adults and three aged adults), five patients with trisomy 21 Down syndrome, two patients with karyotypically normal Down syndrome (and their four normal parents), and three patients with sporadic Alzheimer's disease. The clinical diagnosis of Alzheimer's disease was based on criteria defined by the National Institute of Neurological and Communicative Disorders and Stroke (NINCDS) (*5*). Preliminary experiments established appropriate autoradiographic exposure times for calibrating dose-response curves for the quantity of DNA as a function of band intensity measured by transmission densitometry. The linear response range of the curves was used in all experiments. An 8.6-kb band, which is recognized by the human AD-AP cDNA probe (λ Am4) located on chromosome 21, and a 4.1-kb reference band, which is recognized by the human proα2 (I) collagen probe (COL1A2) located on chromosome 7 (*6*), are seen on Eco RI–digested DNA from the patients and control subjects (Fig. 1). Actual densitometric measurements were carried out by using autoradiographs. By assuming that the DNA from the control subjects had a gene dosage number of two for both the COL1A2 reference probe and the AD-AP gene, AD-AP gene dosage numbers in the patients were deduced from the ratio of the intensities of the 8.6-kb band to the 4.1-kb reference band. Four additional chromosome 21 probes were used or are referred to in this study: human superoxide dismutase-I (*SOD1*), anonymous DNA sequence D21S11 located proximal to *SOD*I, human proto-oncogene *ets*-2, and the estrogen-inducible sequence expressed in breast cancer BCEI located in 21q22.3 (*7*).

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