Gene Characterization, Promoter Analysis, and Chromosomal Localization of Human Bleomycin Hydrolase*

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The human gene encoding bleomycin hydrolase, a cysteine proteinase involved in chemotherapy resistance, has been cloned, and its overall organization has been established. The gene is composed of 12 coding exons and 11 introns and spans more than 30 kilobases. The number and distribution of exons and introns differ from those reported for other human cysteine proteinases, indicating that these genes are only distantly related. Nucleotide sequence analysis of about 1.2 kilobases of the 5′-flanking region of the human bleomycin hydrolase gene revealed a high GC content, a ratio of CpG/GpC close to unity, and the absence of consensus transcriptional sequences such as TATA box or CCAAT box. All these features are characteristic of housekeeping genes and provide an explanation for the widespread expression of bleomycin hydrolase in human tissues. The 5′-flanking region of the gene also contains a polymorphic CCG trinucleotide repeat that may be a target of genetic instability events and affect its transcriptional activity. Chromosomal localization of the human bleomycin hydrolase gene revealed that it maps to chromosome 17q11.2, very close to the locus of the neuromelanin polymerase. The 5′-flanking region of the gene also contains a polymorphic CCG trinucleotide repeat that may be a target of genetic instability events and affect its transcriptional activity. Chromosomal localization of the human bleomycin hydrolase gene revealed that it maps to chromosome 17q11.2, very close to the locus of the neuromelanin polymerase. The 5′-flanking region of the gene also contains a polymorphic CCG trinucleotide repeat that may be a target of genetic instability events and affect its transcriptional activity. Chromosomal localization of the human bleomycin hydrolase gene revealed that it maps to chromosome 17q11.2, very close to the locus of the neuromelanin polymerase.

Bleomycin is a glycopeptide produced by Streptomyces verticillus that is clinically used as a chemotherapeutic agent in the treatment of human malignancies because of its ability to cleave double-stranded DNA (1, 2). However, a general utilization of bleomycins in cancer therapy is limited by the finding of both pulmonary toxicity and resistance of certain tumor cells to the cytotoxic effects of these compounds (3–6). Different mechanisms including reduced bleomycin uptake, increased metabolic drug inactivation, or enhanced DNA repair activity have been proposed to explain why certain tumors are refractory to therapy by these antibiotics. However, the finding in animal tissues of an enzymatic activity capable of hydrolyzing the amide bond of bleomycin to render an inactive metabolite strongly suggested that drug inactivation could play a major role in the tumor resistance to these compounds (7). In recent years, studies from different groups have contributed to elucidate structural and functional characteristics of this bleomycin inactivating enzyme, which has been designated bleomycin hydrolase. Thus, biochemical characterization of the enzyme purified from rabbit tissues has revealed that this enzyme is a cysteine proteinase sensitive to cysteine proteinase-specific inhibitors like E-64 (8–10). More recently, we have reported the molecular cloning and nucleotide sequence of a cDNA coding for human bleomycin hydrolase (11). The isolated cDNA codes for a polypeptide of 455 amino acids that contains all of the structural features characteristic of cysteine proteinases and displays a significant sequence similarity to different yeast and bacterial proteins. These proteins, called yeast bleomycin hydrolase and bacterial aminopeptidases C, have about 35–40% identities with the human enzyme and also possess proteolytic activity (12–17). The conservation of this gene from bacteria to mammals points to an important although as yet undefined function for this protein. Consistent with this proposal, human bleomycin hydrolase is expressed in all examined tissues, suggesting that besides its highly specific role in the degradation of antitumor antibiotics, this enzyme may play a housekeeping role in human tissues as a protease involved in normal cellular protein turnover (11, 18). In fact, biochemical characterization of the recombinant human bleomycin hydrolase has confirmed that it acts as an aminopeptidase with a relatively broad substrate specificity (18). Finally, in addition to this role of bleomycin hydrolase as a peptidase, it has been recently reported that the yeast bleomycin hydrolase also has DNA binding activity and acts as a repressor in the GAL4 regulatory system (19, 20). These results provide additional interest to the functional analysis of this enzyme.

Preliminary analysis of bleomycin hydrolase expression in different human tumors has shown increased levels in some head and neck carcinomas when compared with paired adjacent normal mucosa (11). We have also observed a variable degree of bleomycin hydrolase expression in different types of lymphoma, with low or undetectable levels in Hodgkin’s disease samples and higher levels in Burkitt’s lymphomas that are refractory to bleomycin treatment (11). These results are consistent with a proposed role for human bleomycin hydrolase in the resistance of some tumors to bleomycin chemotherapy. In fact, overexpression of yeast bleomycin hydrolase induced resistance of NIH 3T3 cells to the antibiotic effect of bleomycin (21). According to these data, it seems that mechanisms controlling bleomycin hydrolase expression may be altered in human malignancies. As a previous step to elucidate these regulatory mechanisms and to further evaluate the relationship of...
this human gene to its bacterial and yeast homologues, we have undertaken studies directed to identify and characterize the human bleomycin hydrolase gene. In this work, we describe the isolation and characterization of genomic clones coding for this cysteine proteinase. We have also analyzed the promoter region of the bleomycin hydrolase gene with the finding of different motifs that could modulate its transcription. In addition, we have established that this gene maps at 17q11.2, close to the locus of the neurofibromatosis type 1 (NF1) gene (22, 23). Finally, we have examined the possibility that the human bleomycin hydrolase gene can be part of an amplicon recently described in this region of the human genome.

EXPERIMENTAL PROCEDURES

Materials—Human P1 artificial chromosome (PAC)1 and Centre d’Etude du Polyomorphisme Humain (CEPH) yeast artificial chromosome (YAC) genomic libraries were supplied by the Human Genome Mapping Resource Center (Cambridgeshire, UK). Restriction endonucleases and other reagents used for molecular cloning were from Boehringer Mannheim (Mannheim, Germany). Double-stranded DNA probes were radiolabeled with [32P]dCTP (3000 Ci/mmol) using a commercial random priming kit purchased from Amersham (Amersham, UK).

Screening of Human Genomic Libraries—Human PAC and YAC libraries were hybridized at 65 °C in 5× SSPE (1× SSPE = 150 mM NaCl, 10 mM NaH2PO4, 1 mM EDTA, pH 7.7), 1% SDS, 0.5% dried milk, and 6% polyethylene glycol 8000, with a full-length cDNA for human bleomycin hydrolase (11). Posthybridization washes were performed in 0.5× SSC, 0.1% SDS at 65 °C. Finally, the membranes were exposed to XAR-5 film (Kodak) at −70 °C with intensifying screens. Four PAC and three YAC positive clones were identified and characterized by endonuclease restriction and by PCR amplification and pulsed field gel electrophoresis analysis, respectively.

Nucleotide Sequencing and Computer Sequence Analysis—DNA fragments were inserted in the polylinker region of pBlueScript (Stratagene) and M13 and sequenced by the dideoxy chain termination method using the Sequenase version 2.0 kit (U. S. Biochemical Corp., Cleveland, OH). Computer analysis of DNA sequences was performed with the GCG software package of the University of Wisconsin Genetics Computer Group (24).

Identification of Intron-Exon Boundaries—The junctions between exons and introns and the size of the introns of the human bleomycin hydrolase gene were determined by restriction analysis of the PAC clones. Amplified PCR fragments and nucleotide sequences of two primer pairs used contained sequences from opposite strands of two putative neighboring exons of the cDNA. PCR conditions included 250 μM dNTP, 150 pmol of each primer, and 2.5 units of the expand long Taq DNA polymerase mixture (Boehringer Mannheim) in the XL PCR buffer. The PCR amplification was performed in a Perkin-Elmer 2400 thermocycler. Each cycle consisted of a 15-s denaturation at 94 °C, 15 s of annealing at 55 °C, and 30 s to 10 min of extension at 68 °C. Each PCR fragment was subcloned in the EcoRV site of the pBlueScript polylinker and sequenced. These sequences were compared with the human bleomycin hydrolase cDNA to identify the coding sequences and the intron-exon boundaries.

Chromosomal Localization—Fluorescent in situ hybridization (FISH) was carried out using as probes DNAs prepared from the isolated PAC and YAC DNA by standard procedures. Then, 2 μg of the PAC or of the yeast genomic DNA were nick translated with biotin-16-dUTP, whereas the centromeric probe DZ17 was labeled with digoxigenin-11-dUTP (Boehringer Mannheim). Both probes were hybridized to normal male metaphase chromosomes as described (25, 26). Chromosome slides were stained with diamidino-2-phenylindole dihydrochloride, and the images were captured in a Zeiss fluorescent microscope equipped with a CCD camera (Photometrics).

PCR Amplification of Genomic DNA—Two oligonucleotides (5′-GACGCGCGTACTCGACCAAG-3′) and (5′-GGTAAACGGTTAGCTTCAAGGTT-3′) flanking a trinucleotide repeat present in the human bleomycin hydrolase gene were employed to PCR amplify this region in 10 unrelated genomic DNAs from healthy donors. PCR was performed in a Perkin-Elmer 2400 thermal cycler using 200 ng of genomic DNA.

RESULTS AND DISCUSSION

Isolation and Genomic Structure of the Gene Coding for Human Bleomycin Hydrolase—To identify the gene coding for human bleomycin hydrolase, a PAC genomic library was screened with the full-length cDNA for this enzyme (11). Four positive PAC clones were identified on the basis of their positive hybridization with the probe. After a preliminary restriction pattern analysis of the PAC DNA clones, followed by Southern blot analysis using two probes from the 5′- and 3′-ends of the bleomycin hydrolase cDNA, one of the isolated clones hybridized with both probes, suggesting that it was the only one that could encompass the entire human gene. This PAC clone, called 88O23 and spanning more than 100 kilobases of human genomic sequence, was used for all subsequent analysis. To determine the exon-intron organization of the human bleomycin hydrolase gene, we designed a series of primers derived from separated regions of the cDNA sequence. These primers were used for PCR amplification of total DNA from the isolated PAC clone or of selected fragments subcloned from this genomic clone.

After nucleotide sequencing of the restriction fragments and of the amplified products and comparison with the previously reported cDNA sequence (11, 18), we concluded that the human bleomycin hydrolase gene is composed of 12 exons and 11 introns (Fig. 1). The position and size of exons and introns, the sequences of the exon-intron junctions, and the intron phases are indicated in Table I. The size of the exons ranges from 54 (exon 9) to 938 bp (exon 12, which also contains the 3′-untranslated region). The size of the introns was determined either by agarose gel electrophoresis analysis of PCR amplified products using specific primers contained in consecutive exons or by Southern blot analysis using specific probes for the two exons flanking the analyzed introns. According to the results obtained by following both approaches, the intron sizes in the bleomycin hydrolase gene range from 125 (intron 8) to >8,500 bp (intron 10). It is noteworthy that phase 0, 1, and 2 introns were found, with a clear predominance (6 of 11) of phase 0 introns. In addition, it should be noted that all these intervening sequences conform perfectly to the canonical acceptor and donor splice sites described by Mount (27) (Table I).

The number and distribution of exons and introns in the bleomycin hydrolase gene differ from those reported for other human cysteine proteinases belonging to the papain superfamily, such as cathepsins B, L, S, C, and K. Thus, the number of
coding exons identified in the bleomycin hydrolase gene is higher than the number present in the coding sequences of the different human cathepsins so far analyzed: 9 in cathepsin B, 7 in cathepsins L, K, and S, and 2 in cathepsin C (28–33). The position of introns also displays marked differences between all of these cysteine proteinases. Unlike all these lysosomal cathepsins, which contain clearly defined prepeptide, propeptide, and mature enzyme domains, bleomycin hydrolase is a cytosolic protein that does not have a modular structure, thus hampering the assignation of the different exons to hypothetical functional units. Nevertheless, the occurrence in bleomycin hydrolase of conserved regions around the cysteine, histidine, and asparagine residues that are essential for the catalytic properties of these enzymes allowed us a comparative study of the exon-intron junctions at these regions. In this regard, it is of interest that a common feature in the gene structures of human cathepsins is that the conserved region (SGRCW) around the cysteinyl active site is located adjacent to an exon-intron junction. In cathepsin B this junction is located immediately after the conserved sequence, whereas in cathepsins L, K, and S, it is located immediately before the conserved sequence. In contrast, in the bleomycin hydrolase gene, this active site sequence is interrupted by an intron. On the other hand, the regions containing the histidine and asparagine residues proposed to be essential in the catalytic processes are encoded within the same exon in all cathepsins. In contrast, these sequences are codified by separate exons in the bleomycin hydrolase gene (Fig. 1). In summary, these marked differences

### Table I

| Number | Exon-intron junction | Exon size | Intron size |
|--------|----------------------|-----------|-------------|
| 2      | ctcctaacagGA         | AGC       | AGC         | TCG Gttgaggtgc Gttgaggtgc ~1000 |
| 3      | cttgttcagGG          | L         | N           | S S 198 G ttgaggtgc ~2500 |
| 4      | taacttttagGG         | GTT       | GAA         | CGC 110 G ttgaggtgc ~1700 |
| 5      | ttcttttttagAA        | V         | E           | R 142 G tcaataata ~1000 |
| 6      | tctgctataagGG        | M         | R           | E 93 G ttaataaca >8000 |
| 7      | gcttcctcagGG         | I         | F           | R 156 G ttggtgaat ~1500 |
| 8      | atctttttagGG         | ATT       | TAT         | TTA 159 G ttggttttgg ~125 |
| 9      | ctgtgctttagGG        | GCT       | GTG         | TGG 68 G Ttggagttc ~1200 |
| 10     | ctttttacagGG         | TAT       | GAC         | CAT 118 G tggatgc ~7000 |
| 11     | ttttttccagGG         | GAT       | GAT         | CAG 70 G tggatgc ~8500 |
| 12     | ttttttccagGG         | TAC       | CTG         | TGC 638 G tggatgc ~7000 |
in both number and distribution of introns between the gene for bleomycin hydrolase and those encoding other cysteine proteinases, support the proposal that these molecules are only distantly related from an evolutionary point of view (34).

**Structural Analysis of the Promoter Region of the Human Bleomycin Hydrolase Gene**—The human bleomycin hydrolase gene is widely expressed by normal tissues, which is consistent with a proposed role for this proteolytic enzyme in the normal protein catabolism occurring in all cell types (11, 18). However, the expression of this gene is increased in a series of tumor conditions including some head and neck carcinomas and lymphomas, thus contributing to explain why some of these tumors may become refractory to chemotherapeutic treatment with bleomycins (11). Furthermore, several groups have reported a diminished expression of this gene in lung tissue, which could be the cause of the high pulmonary toxicity of this antineoplastic drug (1–4). As an initial attempt to investigate the molecular mechanisms regulating the expression of this gene, we performed a structural analysis of its promoter region, searching for sequence motifs that could explain its wide expression pattern in human tissues. Simultaneously, we also looked for putative regulatory motifs that could affect the bleomycin hydrolase gene transcription in pathological conditions. For these purposes, we cloned and determined the nucleotide sequence of a genomic fragment comprising about 1.2 kilobases of the 5'-flanking region of the human bleomycin hydrolase gene, and the results obtained are shown in Fig. 2.

**Fig. 2.** Nucleotide sequence of the 5'-flanking region of the human bleomycin hydrolase gene. Nucleotides are numbered relative to the translation initiation site, which is designated as nucleotide +1. The ATG and the trinucleotide repeat are underlined. AP-1, SP-1, AP-2, PEA-3, NFE-1, and H4TF-1 consensus motifs are boxed.

Further support for this proposal was provided by analysis of the GC content and the ratio of CpG/GpC in the 650-bp sequence upstream of the start codon. As can be deduced from Fig. 2, the percentage of GC in this 5'-flanking region is 75%, whereas the ratio of CpG/GpC dinucleotides in the same region is close to unity. Because both high GC content and lack of CpG suppression are common features in promoter regions of constitutively expressed genes (35), their occurrence in the bleomycin hydrolase gene is again compatible with its ubiquitous expression pattern.

However, despite the fact that all of these genomic features are characteristic of a weakly regulated gene, the expression of the bleomycin hydrolase gene appears to be regulated at least in some pathological circumstances. Consequently, we performed an additional analysis of its 5'-flanking region looking for putative elements that could be relevant in a regulated expression of the gene. Interestingly, a perfect match with the AP-1 consensus site (TGACTCA) is found 224 bp upstream from the translation initiation site. This motif is recognized by members of the c-Fos, c-Jun, and ATF-1 families of transcription factors and has been found to mediate induction of different genes by a variety of tumor promoters, cytokines, and growth factors (36). The promoter sequence also contains several consensus sequences for Sp1 binding, as well as AP-2, PEA-3, NFE-1, and H4TF-1 sites. Functional analysis of these sequences will be required to elucidate their potential role in the altered expression of the bleomycin hydrolase gene in some pathological conditions. Finally, it is worthy to note the presence of a CCG trinucleotide repeat in the 5'-flanking region of the human bleomycin hydrloase gene (Fig. 3A). These repeated sequences are characterized by their high genetic instability causing an increasing number of human diseases (37, 38). In fact, more than 10 neurological and neuromuscular disorders associated to expansion of trinucleotide repeats have been dis-
covered in the past years. In two of these diseases, Fragile X syndromes FRAXA and FRAXE, the CGG or CCG trinucleotide repeats are located in the 5'-untranslated region of two genes called FMR1 and FMR2, respectively (39–43). The underlying mechanism of these diseases seems to be an increased methylation of both the expanded repeats and adjacent CpG islands, leading to transcriptional silencing of the gene. The finding of a CCG repeat in the 5'-untranslated region of the human bleomycin hydrolase, which is also located immediately adjacent to a CpG island, makes it a candidate for undergoing genetic instability. To evaluate the possible occurrence of polymorphism in this sequence, we performed a PCR-based amplification of this region in genomic DNA obtained from 10 unrelated healthy donors. As can be seen in Fig. 3B, these studies revealed that the trinucleotide repeat identified in the 5'-flanking region of the bleomycin hydrolase gene is polymorphic, with at least two different alleles being present in our population. The high frequency of this polymorphism makes it useful as a genetic marker for linkage studies in this region of the human genome. In addition, it will be of interest to examine the possibility that this trinucleotide repeat could be target of some of the increasing number of microsatellite expansion events associated with human diseases.

Chromosomal Localization of the Human Bleomycin Hydrolase Gene and Analysis of Its Amplification in Breast Carcinomas—To determine the chromosomal location of the human bleomycin hydrolase gene, PAC DNAs were used as probes in FISH experiments using human male chromosome metaphases. After diamidine-2-phenylindole dihydrochloride banding of the metaphase cells with specific hybridization signals corresponding to a CpG island, makes it a candidate for undergoing genetic instability. To evaluate the possible occurrence of polymorphism in this sequence, we performed a PCR-based amplification of this region in genomic DNA obtained from 10 unrelated healthy donors. As can be seen in Fig. 3B, these studies revealed that the trinucleotide repeat identified in the 5'-flanking region of the bleomycin hydrolase gene is polymorphic, with at least two different alleles being present in our population. The high frequency of this polymorphism makes it useful as a genetic marker for linkage studies in this region of the human genome. In addition, it will be of interest to examine the possibility that this trinucleotide repeat could be target of some of the increasing number of microsatellite expansion events associated with human diseases.

Chromosomal Mapping of the Human Bleomycin Hydrolase Gene—Fluorescent in situ hybridization with a centromeric probe of chromosome 17 and with a probe specific for human bleomycin hydrolase. Metaphase cells were counterstained with diamidine-2-phenylindole dihydrochloride. The human bleomycin hydrolase gene maps at this unique position among all cysteine proteinases mapped to date is consistent with the above structural data suggesting that this gene is only distantly related from an evolutionary point of view to the other members of the papain superfamily.

In addition to its possible significance in the evolutionary history of the human cysteine proteinases, knowledge of the chromosome location of the bleomycin hydrolase gene may be useful in searching for putative diseases related to abnormalities in this protein. In this regard, it is noteworthy that the cytogenetic region containing this gene coincides with that of the NF1 gene, which is altered in neurofibromatosis type 1, a common autosomal dominant disorder characterized by abnormalities in multiple tissues derived from the neural crest and associated to an increased risk of malignancy, especially glioma and neurofibrosarcoma (22, 23). Because the NF1 gene consists of 59 exons and spans about 350 kilobases (45), we examined the possibility that the human bleomycin hydrolase gene could be located close to the NF1 gene. To this aim, the human CEPH YAC genomic library, which encloses genomic inserts up to 2 megabases, was screened with the bleomycin hydrolase cDNA. Three positive YAC clones (765D1, 929F8, and 947G11) were identified on the basis of its positive hybridization with the probe. FISH mapping confirmed that all these YAC clones corresponded to the expected 17q11.2 chromosomal region. In addition, the finding that no other sites were labeled demonstrated the absence of chimerism in these clones. Because previous studies have shown that one of these YAC clones (947G11) contained the entire NF1 gene (data not shown), subsequent studies were performed with this clone. To further refine the physical location of both genes, high molecular weight DNA from the YAC 947G11 was digested with several rare restriction enzymes, and their restriction fragments separated by pulsed field gel electrophoresis and analyzed by Southern blot. After reprobing the same filter with the bleomycin hydrolase cDNA, as well as with two different probes cor-

FIG. 3. Identification of a polymorphic trinucleotide repeat in the 5'-flanking region of the human bleomycin hydrolase gene. A, sequencing gel showing the region containing the trinucleotide repeat. B, polyacrylamide gel electrophoresis of a DNA fragment amplified from the genomic DNA of eight individuals with oligonucleotides flanking the trinucleotide repeat. The gel was stained with silver nitrate. PCR double bands in each allele are due to the slightly different molecular weights of the two DNA strands.

FIG. 4. Chromosomal mapping of the human bleomycin hydrolase gene. Fluorescent in situ hybridization with a centromeric probe of chromosome 17 and with a probe specific for human bleomycin hydrolase. Metaphase cells were counterstained with diamidine-2-phenylindole dihydrochloride.
responding to exons 1–4 and 45–49 of the NF1 gene, common NotI and SfiI restriction bands hybridizing with both the bleomycin hydrolase probe and the 3′-end of the NF1 gene were detected (Fig. 5). According to these data, together with those reported by Li et al. regarding NF1 gene orientation (45), we can conclude that the human bleomycin hydrolase gene is telomeric to the NF1 gene, with the maximum distance between them of about 90 kilobases, which is the size of the common SfiI restriction band. On this basis, NF1 and bleomycin hydrolase can be somewhat considered as neighbor genes sharing the same chromosomal environment, thereby being potentially subjected to similar alterations such as loss of heterozygosity or small genomic amplification.

In relation to this, it is of interest that this segment of chromosome 17 containing the human bleomycin hydrolase gene is one of the most commonly amplified DNA regions in human tumors and more specifically in breast carcinomas (46). Several lines of evidence have suggested that the EBB2 protooncogene is the gene responsible for the emergence of this amplicon (47, 48). However, recent studies have revealed that amplification of 17q11-q21 DNA sequences is a more complex event, involving additional genes (49). Two of these genes, called MLN 62 and MLN 64, have been identified as targets of two distinct amplified regions within 17q11-q21 in breast carcinomas, one located at 17q11–12 and containing the MLN 62 locus, and the other at 17q12-q21 and including EBB2 and MLN 64 loci (50, 51). To evaluate the possibility that the bleomycin hydrolase gene could also be located within one of these amplicons, normal DNA from peripheral blood leukocytes and autologous tumor DNA from 70 breast cancer patients were screened by Southern blot with a probe specific for human bleomycin hydrolase (Fig. 6). Densitometric analysis of filters revealed that a low grade of amplification (about 2-fold) was only detected in three primary tumors (T1290, T284, and T309). Nevertheless, the amplification rate of the bleomycin hydrolase gene in these three cases was lower than that of other genes present in the amplicon that were amplified more than 10-fold (51). Taken together, these data suggest that amplification events are not important in the acquisition of resistance of these tumors to bleomycin chemotherapy.

In summary and according to the results presented in this work, the human bleomycin gene shows clear differences with the genes coding for other members of the papain superfamily of cysteine proteinases. The number and distribution of exons and introns as well as the chromosomal location of this gene are unique among all the cysteine proteinase genes characterized to date. In addition, the analysis of its promoter region has revealed some features that could explain the ubiquitous expression of bleomycin hydrolase in human tissues. This fact distinguishes this enzyme from other cysteine proteinases such as cathepsins S, K or W, which are expressed in a more restricted fashion (Refs. 32, 51, and 52; reviewed in Ref. 53), and is consistent with a proposed role for bleomycin hydrolase in the normal protein degradation taking place in all cell types. The availability of the genomic structure of the bleomycin hydrolase gene reported in this work will be helpful for future studies directed to generate animal models lacking this gene, which could contribute to clarify the precise functional role of this widely distributed enzyme.

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