Cloning the cystic fibrosis gene: implications for diagnosis and treatment

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In this article we trace the steps that led to the molecular cloning of the cystic fibrosis gene and identification of the major mutation, discuss detection assays that have been designed and applied to prenatal diagnosis and the problems associated with extending gene mutation assays to population carrier detection, and finally speculate on the prospects for improved treatment in the light of the likely biochemical function of the normal gene product.

The genetics of cystic fibrosis
Cystic fibrosis is the most common autosomal recessive genetic disease in white populations; with a carrier frequency of about 1 in 25; it affects around 1 in 2500 neonates. The most characteristic physiological defect is in chloride ion transport, which causes a raised chloride concentration in sweat, a long recognised diagnostic marker of the disease. Exocrine pancreatic insufficiency is a frequent complication, leading to meconium ileus and malabsorption. Several fetal intestinal and microvillar enzymes are characteristically present at reduced concentrations in affected prenancies as a consequence of the disease. These enzymes provided a basis for rapid prenatal diagnosis in the second trimester in “at risk” families, without the possible problems of “crossovers” and “non-informativeness” associated with the early DNA probes.

Candidate genes
Two complementary genetic approaches have been applied to cystic fibrosis. The “classical” approach is to study candidate genes for the proteins concerned either in terms of their inferred or proved biochemical function or through consistent association of their mutations (alteration or deletion) with the disease. A serum protein, CF antigen, consistently occurred in affected individuals, was absent from normal serum, and was found at intermediate concentrations in heterozygotes. Monoclonal antibodies to CF antigen made possible its purification and tissue localisation. Peptide sequencing facilitated cloning via complementary DNA (cDNA) libraries derived from tissues that expressed the peptide. These studies showed that CF antigen comprises two peptides, both of which have been mapped on the same region of chromosome 1 and show homology to the S-100 family of calcium binding proteins. Although this is not the primary defect, the intermediate concentrations of CF antigen seen in heterozygotes, and the expression profile in normal individuals (high concentrations in granulocytes and moderate concentrations in epithelial cells of the tongue, oesophagus, and buccal cavities and in cultured nasal epithelium in these carriers) may indicate a role in the functional expression of the cystic fibrosis gene.

Electrophysiological studies on intact secretory epithelial cells from patients with cystic fibrosis show defective chloride ion transport (see next article in this series). An obvious, if difficult, route to the basic defect would be the isolation of chloride ion channel proteins. The “reverse” genetic approach, however, succeeded in identifying the cystic fibrosis gene, which is most likely a mutant of the gene regulating chloride ion transport rather than that coding for the ion channel itself.

From genetic linkage to the disease gene
“Reverse” genetics does not presuppose the nature of the abnormal gene but depends essentially on establishing where the gene resides on the chromosome. Once a gene has been linked to a particular chromosomal region then somatic cell and molecular genetic methods can be applied to determine precisely where the gene lies.

Solomon and Bodmer first suggested that, just as protein variants can be used in pedigree analysis to track disease susceptibility, so the inherent variability in DNA sequence between different individuals could be exploited. DNA segments not genetically linked to the disease segregate randomly through the pedigree whereas linked fragments associate positively with the disease. The frequency with which the association breaks down, or a “crossover” is observed, provides an estimate of the genetic distance, in terms of recombination events, between the DNA marker and the disease gene. This method of genotyping relies on the specificity of restriction endonucleases as molecular scissors for revealing DNA sequence variation between individuals through the...
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Figure 1 Homing in on the cystic fibrosis gene. The figure shows the status of the map at the end of 1986 and indicates the physical location of the cystic fibrosis locus on the long arm of chromosome (Ch 7), flanked by the closely linked markers MET and D7S8. The estimated genetic distance separating MET and D7S8 was 1.5 cM, the physical distance 1.5 Mbp.

presence or absence of particular restriction, or cutting, sites. The resultant variations in the length of restriction fragments (hence polymorphisms of restriction fragment length) can be detected by using specific cloned DNA fragments as hybridisation probes to restricted and electrophoretically resolved DNA from different individuals.

The "reverse" genetic approach to identifying the defect was possible only because of the high prevalence of the disease and the availability of extended pedigrees of affected families covering several generations. The first real advance came from sophisticated DNA methods but through conventional study of protein variants. Linkage of cystic fibrosis to the enzyme paraoxonase was reported in 1985. This finding was followed rapidly by the discovery of the linkage of cystic fibrosis with DOCR1-917, a highly polymorphic DNA marker. But on which chromosome did these two markers and therefore the cystic fibrosis gene reside? Already nearly 40% of the genome had been excluded by negative linkage results. By the end of 1985 two markers, the met protooncogene (MET) and the arbitrary DNA marker pJ3.11 (locus designation D7S8), were shown to be very closely linked to the cystic fibrosis locus and were mapped on to the same region on the long arm of human chromosome 7.

As a direct consequence, early prenatal diagnosis for cystic fibrosis was dramatically improved, but the probes were informative only in those families with a previous affected child. The next step was to establish the relative order of MET and D7S8 with respect to the cystic fibrosis gene. This was a collaborative effort between seven research groups that had collected and typed over 200 families with cystic fibrosis. The answer was the best that could have been hoped for—MET and D7S8 flanked the disease gene. This increased the confidence with which these markers could be used for prenatal diagnosis and delineated the position of the gene (fig 1).

Now the solution was to search exhaustively between these two DNA markers. Put so simply, the task sounds trivial but an estimated one and a half million base pairs of DNA lay between these markers, of which only a few thousand were likely to code for the gene, with perhaps only one or a few base pairs differentiating the mutant from the normal. Through the application of powerful new molecular and somatic cell genetic methods for moving from linked markers to disease genes it took less than three years to accomplish this immense task. Two different, but complementary, approaches were taken. Lap-Chee Tsui and colleagues in Toronto used the chromosome "jumping" method, which was how the gene was eventually cloned, but not before a major step forward came from Williamson's team at St Mary's Hospital, London.

There was an element of luck in finding the markers MET and D7S8, which flanked the disease gene. It seemed possible that the cystic fibrosis region of chromosome 7 was potentially coselectable through the met oncogene by oncogenic transformation of cellular growth (fig 2). Human chromosome fragments can be transferred to suitable cultured rodent cell lines by chromosome mediated gene transfer (fig 2). These hybrid cell lines become greatly enriched for the region of interest and are ideal substrates for detailed physical mapping of DNA. More importantly, new and closely linked human DNA markers can be recovered with relative ease from any library constructed. The only known oncogenic activation of MET, however, required a complex chromosomal rearrangement between human chromosomes 7 and 1 in chemically mutagenised human osteosarcoma cells. There was a chance that the cystic fibrosis gene would not be cotransferred on MET selection, though D7S8 was consistently cotransferred with MET by chromosome mediated gene transfer. Furthermore, the hybrid cell lines had segregated most of the remaining chromosome 7 sequences with no obvious molecular rearrangement of the retained sequences.

The next step was to bypass the bulk of "junk" or "spacer" DNA and seek sequences likely to encode genes. This relied on the fact that the 5' ends of most genes were marked by islands of undermethylated DNA, relatively rich in the methylation sensitive dinucleotide pair CpG. Consequently, methylation sensitive restriction enzyme sites containing CpG dinucleotides are also relatively enriched in these regions. Using such an enzyme, XmaIII, as a cloning site and coselecting by hybridisation with total human DNA for clones of chromosome 7 origin, the St Mary's group identified a gene (originally designated IRP, for Int-I related protein, and later renamed INT1 L1) that was mapped on to almost the
(a) MET SELECTED CHROMOSOME MEDIATED GENE TRANSFER

MNNG-HOS cell line carrying activated MET oncogene on Ch 7

Mitotic arrest

Chromosome isolation

human MET selected CMGT mouse hybrid retaining ~3Mbp of Ch 7 DNA including MET and D7S8

Foci

weeks

NIH3T3 monolayer

(b) CpG ISLAND CLONING OF INT1L1

~3Mbp human Ch 7. MET and D7S8 +ve

prepare DNA

cleave with XmaIII + HindIII, clone into Lorist 6

Hybridisation select for ~0.1% human recombinants

Probable location of the CF gene

1. map to MET-D7S8 interval
2. isolate overlapping clones from standard DNA library
3. identify RFLPs
4. map closer to CF
5. sequence to identify INT1L1

met D met H

XV2c CS.7 KM19

pJ3.11 RFLPs

CpG island Loci

Transcription

Linkage Disequilibrium Gradient

100 kbp

Scale

Figure 2  Met selected chromosome mediated gene transfer and CpG island cloning of INT1L1. (a) Method of chromosome mediated gene transfer was used by the St Mary’s team to derive a somatic cell hybrid whose human component was reduced to about 3 Mbp of DNA subtending the cystic fibrosis (CF) locus from the MNNG-HOS chromosome. (b) Restricting the DNA with XmaIII and screening for human sequences enriched for CpG island associated fragments of chromosome (Ch) 7. One such fragment identified a gene, INT1L1, that was mapped between MET and D7S8 and was in strong linkage disequilibrium with cystic fibrosis.

The finding of informative polymorphisms and strong linkage disequilibrium with the XV2C, KM19, and CS7 probes derived from around this CpG island improved prenatal diagnosis still further, but neither the nature of the gene as an inferred secreted growth factor nor its pattern of expression fitted with that predicted for the cystic fibrosis gene and no differences were detected by sequencing between normal and affected individuals. Finally and conclusively, crossovers between the CpG island associated with INT1L1 and cystic fibrosis were discovered. The hunt for the real CF gene was not over.
Saturation cloning and chromosome "jumping" and "walking" identify the cystic fibrosis gene

The St Mary's team continued to "walk" from the INT L1 gene towards the cystic fibrosis gene while the teams in Toronto and Michigan collaborated to saturation clone the region between MET and D7S8. As a first step, Tsui and colleagues searched through 258 different human chromosome 7 probes from a FACS sorted library to find two that were mapped between MET and D7S8. These became the starting points for "jumps" along the chromosome in either direction (fig 3).

Chromosome jumping libraries are constructed by cleaving genomic DNA to produce a representative set of long, linear molecules—upwards of 100,000 base pairs (100 Kbp). These are ligated with a "tag" molecule under conditions favouring the formation of circular monomers rather than linear concatamers. The DNA is then cleaved to produce many small fragments and the selectable tag molecule is used to isolate only those derived from either end of the original molecule, originally separated on the chromosome by 100 Kbp or more (fig 3). Two consecutive jumps in the same direction took them to the CpG island associated with INT L1. This indicated that they were moving towards D7S8 and the region of maximum linkage disequilibrium. Further jumps, complemented by extensive isolation of overlapping, intervening clones ("walking"), enabled them to bypass the unclonable region that had stalled the progress of the St Mary's team. In the region with maximum linkage disequilibrium with cystic fibrosis they searched for sequences conserved across species boundaries (that is, homologous DNA segments present in mouse, hamster, bovine, or chicken DNA, and sometimes all of these—referred to as zoo blots) as an indication of...
coding rather than junk or spacer DNA.\textsuperscript{32}

Unfortunately, the new candidate gene segments failed to detect RNA transcripts in tissues predicted to express the cystic fibrosis gene. DNA sequence analysis did show, however, a short length of open reading frame distal to a CpG rich, undermethylated stretch of DNA. Thus the region had some of the hallmarks of a bona fide gene. For verification, they searched for homologous sequences in complementary DNA (cDNA) libraries constructed from cultured epithelial sweat glands, a tissue predicted to express the gene.\textsuperscript{40} Eventually, a single cDNA clone was isolated. It recognised a 6-5 Kbp transcript, the expression of which was essentially restricted to exocrine tissues. Sequentially isolated cDNAs were clearly related one to another, but contained a high level of sequence rearrangement, plus extraneous sequences of uncertain origin. No full length cDNA clones could be isolated, but eventually the entire coding sequence was abstracted from overlapping clones. Much of the difficulty of first identifying genetic sequences in the cystic fibrosis region and then isolating large cDNAs is explained by the fact that the mRNA is transcribed from 27 short exons stretched over about 250 Kbp of genomic DNA. Nevertheless, DNA sequence homology searches and translation of the cDNA sequence into a predicted protein structure have already told us much about the likely function of the cystic fibrosis gene product (to be discussed in detail in the next article). Furthermore, identification of disease associated mutations in the cystic fibrosis transmembrane regulator have not only aided prenatal diagnosis but have also contributed to a growing understanding of demographic and clinical anomalies.

**Disease associated mutations in cystic fibrosis transmembrane regulator**

Comparison of the cDNA sequences of the cystic fibrosis transmembrane regulator isolated from normal and affected individuals revealed a major mutation comprising a three base pair deletion, causing the loss of a single phenylalanine at residue 508 in the first ATP binding domain. This mutation accounts for about 70% of cystic fibrosis mutations in North American\textsuperscript{33} and slightly more in northern European populations,\textsuperscript{34} 46% in southern European populations,\textsuperscript{35} 30% in Ashkenazi families,\textsuperscript{36} and 35% in the community of Hutterites.\textsuperscript{37} Initially, there appeared to be a good correlation between homozygosity for the Δ508 mutation and pancreatic insufficiency,\textsuperscript{38} but this is not absolute.\textsuperscript{39} Similarly, although mild lung disease is often associated with pancreatic sufficiency, individuals with only moderate lung disease in a severely affected family, all carrying the Δ508 mutation, have been described.\textsuperscript{40}

The search for mutations accounting for the remaining 30%, or more, of affected chromosomes continues. This relies on the polymerase chain reaction to amplify regions of the gene for detailed analysis (fig 4). Analysis may be by various methods, including high resolution polyacrylamide gel electrophoresis, to look for length polymorphisms (micro deletions or insertions)\textsuperscript{41} chemical cleavage methods to detect and position single base mismatches (for example, the hydroxylamine osmium tetroxide method)\textsuperscript{42}, neutral gel analysis of single stranded DNA mobility to look for sequence composition or length variations that lead to conformational polymorphisms,\textsuperscript{43} or simply by direct DNA sequence analysis. The first few documented cases used the last two methods to detect mutations on non-Δ508 chromosomes.\textsuperscript{44,45} In one case neither parent of an affected child carried the Δ508 mutation, but a two nucleotide insertion in exon 13 was found in the mother that would result in a severely truncated and non-functional protein.\textsuperscript{46} The nature of the paternal mutation was not discovered. In a study searching for mutations in the transmembrane region three different base change mutations, each causing an amino acid change from positively charged to less polar, were detected in exons 4 and 7 of non-Δ508 chromosomes in 46 patients.\textsuperscript{47} One of these mutations was common to two of the Δ508 compound heterozygote families where the penetrance was quite variable. Most of the patients had a mild form of the disease, but one individual was severely affected. Additional environmental, epigenetic, or genetic factors clearly contribute to the clinical picture with these atypical mutations.

It has been assumed that chloride ion transport is an essential function and that individuals homozygous for null mutations of the cystic fibrosis transmembrane regulator, such as frameshift terminations, will not survive. Such mutations will be found only in compound heterozygotes with dysfunctional mutations, such as Δ508. It will be of considerable interest to identify further mutations of the dysfunctional class (amino acid substitutions or, possibly, aberrantly spliced products) as these will indicate functionally important regions of the gene. Possibly the common Δ508 mutation may represent a partial "gain of function" or "over expression" defect. An advantage for Δ508 heterozygotes—for example, increased resistance to chloride secreting diarrhoea, might explain the high prevalence of cystic fibrosis in white populations,\textsuperscript{48} although this is hard to reconcile with the data for the southern Mediterranean and Ashkenazi populations, where the Δ508 mutation accounts for less than half the mutations in the closed community of Hutterites.\textsuperscript{49} In turn accounts for over three quarters of the mutated cystic fibrosis transmembrane regulator chromosomes.\textsuperscript{50,51} Certainly, a second mutation (perhaps three or more) must have arisen on the major haplotype. The peak of linkage disequilibrium in these atypical populations points clearly to the region between INT1 L1 and the cystic fibrosis gene. Additional intervening sequences, coding or controlling, may play a part in the aetiology of cystic fibrosis and contribute to the heterozygous advantage of the B haplotype and the high prevalence of cystic fibrosis.
Prenatal diagnosis

Each mutation discovered sheds light on the functional constraints of the cystic fibrosis transmembrane regulator protein, but complicates unequivocal prenatal diagnosis as a specific detection assay must be devised for each mutation. For this reason, an immunological assay to distinguish functional and dysfunctional protein in accessible tissue would be ideal. Efforts are focusing on the putative cell surface domain, with peptides as immunogens or fusion protein constructs. The polymerase chain reaction makes detection of the Δ508 mutation and screening for new mutations at the DNA level much easier (fig 4). The polymerase chain reaction allows any segment of DNA lying between two sequence specific sites defined by two chemically synthesised oligonucleotide primers to be repeatedly amplified, generating unlimited copies of the intervening DNA corresponding to the genotype of the original template. The source of DNA template can be a minute quantity derived from a blood spot, Guthrie card, mouth wash, microvillar sample, or even a single cell removed from the 8 cell stage of a human embryo fertilised in vitro.

The Δ508 mutation can be detected in several ways—for instance, on the basis of the intrinsic size difference between the amplification products derived from normal chromosomes and those with the deletion of three base pairs, and by using allele specific oligonucleotides that encompass the mutation. Unaffected, carrier, and affected individuals are typed according to the presence or absence of hybridisation with the polymerase chain reaction products (fig 4). Substituting fluores-
Figure 5  Gene targeting in mouse stem cells to create an animal model for cystic fibrosis (CF).

Outline of the experimental scheme for deriving chimeric mice from stem cells mutated at the mouse cystic fibrosis transmembrane regulator (CFTR) locus by gene targeting.

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cently labelled primers and probes offers exciting opportunities for simultaneous screening for several different mutations in a single test tube, the result being indicated simply by the absorption spectrum of the product.

Despite the power of the polymerase chain reaction and an increasingly elegant set of mutation screening and detection assays, scanning all 27 exons, totalling 6-5 Kbp of coding sequence, for new mutations is an enormous task. If, as seems likely, some defects are due to non-coding mutations affecting transcription the task will be greater as the cystic fibrosis locus encompasses about 250 Kbp of genomic sequence. It may be of practical value to subdivide cystic fibrosis into $\Delta 508$ and non-$\Delta 508$ disease for the purpose of prenatal diagnosis and population screening. Recognising that clinical variation among the haemoglobinopathies may be related to mutational differences within the globin locus has been a great help in population screening, prenatal diagnosis, genetic counselling, and clinical treatment. With cystic fibrosis, the frequency of $\Delta 508$ approaches 75%, in most populations where the mutation is prevalent, and prenatal diagnosis for this one mutation is available now for almost half the families with cystic fibrosis.35

Population screening

The arguments for and against population screening are complex and strongly felt.52

There is much to learn from the failure of the screening programme for sickle cell anaemia and the success of the Tay-Sachs and $\beta$ thalassemia screening programmes. The scientific knowledge and technology required for screening for the major cystic fibrosis mutation already exists. The major outstanding requirement is a comprehensive programme of information, education, counselling, and support. The American Society of Human Genetics considers it premature to offer carrier detection as part of antenatal care. Meanwhile, commercial companies are eager to tap the perceived public demand for testing, but are
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unable or unwilling to accept the responsibilities for genetic counselling that must accompany the test results. In Britain pilot schemes designed to ascertain the acceptability and effectiveness of screening through schools, general practitioners, or antenatal clinics are under way. Whatever programmes are favoured, carrier screening gives prospective parents informed choice and may reduce the incidence of cystic fibrosis, freeing valuable resources for the care and treatment of existing patients. Screening for Δ508 alone in the United Kingdom would identify over 100 fetuses with cystic fibrosis annually. In North America the figure would exceed 1000. Such a programme would reduce the a priori risk estimate to 1 in 10 000 if one parent is shown not to carry the Δ508 mutation, and 1 in 40 000 if both parents are found not to carry it. Of course, the opposite is also true, with the attendant risks proportionally increased if one or both parents are found to be carriers. The need for informed consent to testing and a comprehensive genetic counselling and follow up programme cannot be overemphasised.

Prospects for treatment

The prospects for survival with cystic fibrosis to early adulthood have increased over recent years. The most aggressive population screening programme imaginable would still take a generation to have full effect. Until then, most new cases of cystic fibrosis will continue to arise in families with no previous history of the disease. As a direct result of the molecular cloning of the cystic fibrosis gene, we can confidently anticipate progress by several complementary approaches to improved treatment.

The major mutations, and perhaps most mutations, cause dysfunction rather than functional replacement and the prospects for drug or antibody treatment are good. Elucidation of the complete ion transport pathway in vivo and an understanding of the specific role of the cystic fibrosis transmembrane regulator protein in that complex process may provide a rational approach to compensatory intervention.

For this purpose we and others are establishing an animal model for the disease. This will complement studies with cultured human cystic fibrosis cells and provide a basis for physiological and pharmacological testing. The experimental approach is outlined in figure 5 and hinges on two recent developments. The first is the ability to derive pluripotent embryonal stem cells from mouse blastocysts, which can be maintained and manipulated in culture.51 On being reintroduced to mouse blastocysts, genetically manipulated stem cells can contribute to both the somatic and the germ line tissues of the resultant chimeric mouse.

The second requirement is to mutate, in the stem cells, the mouse equivalent of the cystic fibrosis transmembrane regulator gene by homologous recombination, or gene targeting.54 Cloned fragments of the cystic fibrosis gene are constructed in such a way that on transfection of cultured stem cells and homologous recombination with the corresponding chromosomal sequences they mutate the endogenous transmembrane regulator gene. This process is inherently inefficient, but various selection or screening strategies have been devised to identify the rare homologous recombination events. Such is the promise of this approach for the analysis of development, cancer, and genetic disease that despite its technical difficulty the approach has already been successful with genes as diverse as those coding for hypoxanthine phosphoribosyltransferase,55 the cellular homologue of the Abelson murine leukaemia virus (c-abl).56

In the simplest gene targeting experiment the homologous recombination event completely disrupts gene function, though mimicking the natural mutations will be important. We have designed vectors that may be used to introduce precise chain termination events and these will be valuable for recreating clinically interesting natural mutations.5657 Indeed, this strategy has been used successfully to create a truncation mutation of c-abl in mouse stem cells.56

Ideally, we wish to mimic the dysfunctional Δ508 mutation. This calls for a more precise approach to gene targeting, which we will test in various ways. One option is to microinject Δ508 specific oligonucleotides into stem cells and select for integration by a stringent polymerase chain reaction protocol for DNA strand displacement events. We will also isolate the entire mouse cystic fibrosis gene in the form of a yeast artificial chromosome recombinant58 and then be able to introduce any chosen mutation by standard yeast genetic procedures into the recombinant.

A final and direct approach to management of cystic fibrosis is somatic gene treatment. Developments in three directions encourage optimism and deserve consideration. Ethical dilemmas impinge on the use of fetal tissue transplants, safety considerations temper the use of differentiated epithelial cells from normal patients transformed in vitro. One solution to the consequences of pancreatic insufficiency might be to enclose secretory exocrine cells in Gor-Tex fibres to form a contained, implantable organoid.59

The second approach would be to use retroviral vectors to transfer a functional transmembrane regulator cystic fibrosis gene, or compensatory regulator gene, to secretory epithelial cells. Cell specificity of infection would be achieved by using ecotropic murine retroviruses, which can infect only the chosen target cell type through antibody mediated binding of a cell surface receptor.60 A third, related approach uses polycations such as poly-L-lysine to encapsulate and deliver the therapeutic DNA construct to the cell surface. Cell specific transfer is achieved by covalent linkage of an appropriate ligand to the polycation. This receptor mediated endocytosis gene transfer strategy has successfully directed intravenously injected DNA constructs to rat hepatocytes and achieved efficient transfection of cultured haemopoietic cells.61 Possibly a benign procedure, such as the regular inhala-
Conclusion

The cloning of the cystic fibrosis gene stands as a dual testament—to the power of molecular genetic approaches to human disease and to the dedication and ingenuity of the community of researchers whose immediate triumph this was. There has been talk of “winners” and “losers”, but without the spirit of competition and collaboration the race would still be in progress. The identification of the gene and the major mutation associated with the disease has had an immediate impact in improved prenatal and clinical diagnosis. This has spawned a wealth of studies to characterise new mutations, to deduce protein structure and function, and to construct valid disease models in culture and in whole animals from which to devise new immunological, drug, and gene replacement treatments. The challenge to the scientists, clinicians and counsellors are great, but the promised reward for the cystic fibrosis community is unquestionable and paramount.

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