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Developmental Biology

Cloning and antisense oligodeoxynucleotide inhibition of a human homolog of *cdc2* required in hematopoiesis

(antisense phosphorothioate oligodeoxynucleotides/butyrylcholinesterase/cholinergic signaling/control of cell division/megakaryocytopoiesis)

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ABSTRACT **Mechanisms triggering the commitment of pluripotent bone marrow stem cells to differentiated lineages such as mononuclear macrophages or multinucleated megakaryocytes are still unknown, although several lines of evidence suggested correlation between cholinergic signaling and hematopoietic differentiation. We now present cloning of a cDNA coding for CHED (cholinesterase-related cell division controller), a human homolog of the *Schizosaccharomyces pombe* cell division cycle 2 (cdc2)-like kinases, universal controllers of the mitotic cell cycle. Library screening, RNA blot hybridization, and direct PCR amplification of cDNA reverse-transcribed from cellular mRNA revealed that CHED mRNA is expressed in multiple tissues, including bone marrow. The CHED protein includes the consensus ATP binding and phosphorylation domains characteristic of kinases, displays 34–42% identically aligned amino acid residues with other cdc2-related kinases, and is considerably longer at its amino and carboxyl termini. An antisense oligodeoxynucleotide designed to interrupt CHED's expression (AS-CHED) significantly reduced the ratio between CHED mRNA and actin mRNA within 1 hr of its addition to cultures, a reduction that persisted for 4 days. AS-CHED treatment selectively inhibited megakaryocyte development in murine bone marrow cultures but did not prevent other hematopoietic pathways, as evidenced by increasing numbers of mononuclear cells. An oligodeoxynucleotide blocking production of the acetylcholine-hydrolyzing enzyme, butyrylcholinesterase, displayed a similar inhibition of megakaryocytopoiesis. In contrast, an oligodeoxynucleotide blocking production of the human 2Hs cdc2 homolog interfered with cellular proliferation without altering the cell-type composition of these cultures. Therefore, these findings strengthen the link between cholinergic signaling and cell division control in hematopoiesis and implicate both CHED and cholinesterases in this differentiation process.**

Early proliferative and commitment steps in hematopoiesis appear to be modulated separately from one another (1) and from subsequent maturation events (2), suggesting distinct regulators for proliferation and differentiation of bone marrow cells (3). In developing megakaryocytes, the progenitors of blood platelets, both phases involve nuclear multiplications (4), implicating cell division control (CDC) genes in these processes. Involvement of cholinergic signaling in hematopoiesis was independently inferred from observations that the cholinesterase genes amplify in leukemias (5) and that antisense oligodeoxynucleotides complementary to butyrylcholinesterase (BCHE) mRNA (AS-BCHE) block megakaryocyte maturation (6). Moreover, acetylcholine stimulates inositol phospholipid hydrolysis in cholinoceptive cells (7, 8), which induces the phosphorylation of cdc2 kinases, altering their biochemical properties and enhancing cell proliferation (9–11). Therefore, the question arose whether cholinergic signaling is involved in the control of hematopoiesis through specific *CDC* genes. We now report molecular cloning, sequence determination,‖ and antisense oligodeoxynucleotide inhibition studies that strongly support this notion.

METHODS

Libraries. Several cDNA libraries, all prepared in the λgt10 phage vector (by Stratagene or Clontech), were screened in the course of this study, first with cholinesterase-specific antisense oligodeoxynucleotide probes (12, 13) and subsequently with ^{32}P-labeled cholinesterase-related cell division controller (CHED) cDNA. The screened libraries originated from poly(A)$^+$ RNA from primary glioblastoma tumors (14) as well as from brain, liver, and muscle of fetal origin (12) and from cultured IMR32 neuroblastoma cells (14). Polymerase chain reaction (PCR) amplification was performed with libraries from adult whole brain, hippocampus, and bone marrow. Screening procedures were as detailed (12–14). DNA sequencing was performed both on double-stranded plasmid DNA and on single-stranded phage DNA after subcloning into the pBS M13(+/−) vector (Stratagene)—all as detailed elsewhere (12). Enzymatic digestion, gel electrophoresis, blot hybridization, and autoradiography of genomic DNA were performed as described (5). DNA/protein data management and EMBL data search were performed with the GCG (Genetics Computer Group, University of Wisconsin, Madison) software.

Oligodeoxynucleotides. Phosphorothioate antisense oligodeoxynucleotides (15) were prepared and HPLC-purified as described (6). Phosphate oligodeoxynucleotides were synthesized in an Applied Biosystems 380B DNA synthesizer and were gel-purified. For PCR amplification of genomic DNA sequences, pairs of phosphorothioate and phosphate

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Abbreviations: CHED, cholinesterase-related cell division controller; AS-CHED, antisense oligonucleotide blocking CHED expression; BCHE, butyrylcholinesterase gene; cdc/CDC, cell division cycle; ORF, open reading frame; AS-BCHE and AS-2Hs, antisense oligonucleotide to BCHE and 2-Hs; PMN, polymorphonuclear leukocyte.

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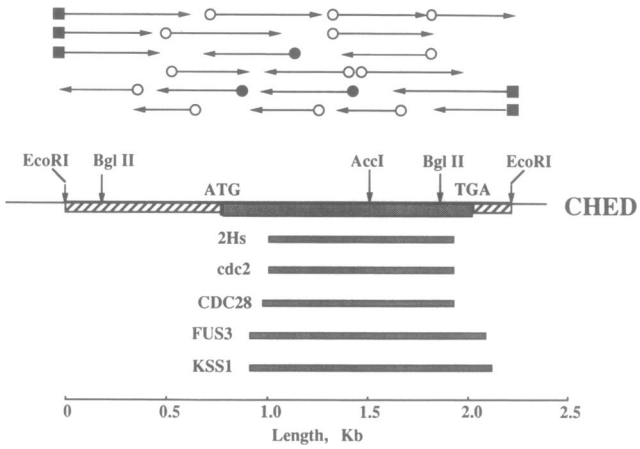
‖The sequence reported in this paper has been deposited in the GenBank data base (accession no. M80629).

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121  CCCTACAGCCCTGTGCTCAGACGGTCTGGAAAATCCCGAAGCAGAAGCCCGTATTCATCT
181  AGGCATTCAAGATCTCGTAGCAGGCACAATTGTCTAGAATCCAGAAGTCGTCATTCTAGT
241  ATTTCTCCTAGCACACTAACTCTGAAGAGTAGCCTGGCAGCTGAATTGAACAAGAATAAA
301  AAAGCACGAGCAGCAGAGGCAGCAAGAGCCGCAGAAGCAGCGAAAGCTGCAGAAGCAACT
361  AAGGCTGCTGAGGCTGCTGCCAAGGCTGCAAAAGCTTCAAACACTTCTACACCTACCAAG
421  GGGAACACGGAAACTAGTGCCAGTGCATCACAAACAAACCATGTGAAGGATGTGAAGAAA
481  ATTAAAATTGAACATGCACCTTCTCCCTCAAGTGGTGGAACTTTAAAAAATGACAAAGCA
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601  ACCAAGAAAGCAGTCATAGTTGGAAAGGAGAGTAAATCTGCTGCTACAAAGGAGGAATCA
661  GTATCTCTTAAAGAGAAAACCAAACCACTTACACCAAGCATAGGAGCCAAGGAGAAGGAG
721  CAACATGTAGCTTTAGTCACCTCTACATTACCACCGTTACCTTTGCCTCCCATGCTGCCT
                                                        M  L  P   3
781  GAAGATAAAGAAGCTGATAGCTTACGAGGAAATATTTCAGTAAAAGCAGTTAAAAAAGAA
     E  D  K  E  A  D  S  L  R  G  N  I  S  V  K  A  V  K  K  E  23
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901  GGAGATGATCTTTCAAAGAGTCCAGAGGAAAAGAAAACAACAACACAGTTACATAGTAAA
     G  D  D  L  S  K  S  P  E  E  K  K  T  T  T  Q  L  H  S  K  63
961  AGGAGGCCTAAAATATGTGGGCCTCGCTATGGTGAAACCAAAGAAAAAGATATTGACTGG
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1021 GGAAAACTCTGCGTGGATAAATTTGATATCATCGGAATTATTGGAGAAGGTACTTACGGA
     G  K  L  C  V  D  K  F  D  I  I  G  I  I  G  E  G  T  Y  G  103
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     Q  V  Y  K  A  R  D  K  D  T  G  E  M  V  A  L  K  K  V  R  123
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     L  D  F  K  K  D  K  G  A  F  Y  V  A  F  E  Y  M  D  H  D  183
1321 CTGATGGGACTACTGGAATCAGGCTTGGTTCATTTTTATGAAAATCACATAAAGTCATTT
     L  M  G  L  L  E  S  G  L  V  H  F  Y  E  N  H  I  K  S  F  203
1381 ATGAGACAGCTCATGGAGGGTCTGGATTATTGTCATAAGAAGAACTTTTTGCATAGAGAT
     M  R  Q  L  M  E  G  L  D  Y  C  H  K  K  N  F  L  H  R  D  223
1441 ATTAAATGTTCCAATATCCTTCTAAATAATAGAGGGCAGATAAAACTTGCAGACTTTGGA
     I  K  C  S  N  I  L  L  N  N  R  G  Q  I  K  L  A  D  F  G  243
1501 CTTGCTCGATTGTATAGCTCAGAAGAAAGTCGGCCGTATACTAACAAGGTAATTACTTTA
     L  A  R  L  Y  S  S  E  E  S  R  P  Y  T  N  K  V  I  T  L  263
1561 TGGTACCGTCCACCTGAACTGCTACTGGGAGAAGAACGATACACACCAGCCATTGATGTA
     W  Y  R  P  P  E  L  L  L  G  E  E  R  Y  T  P  A  I  D  V  283
1621 TGGAGCTGTGGCTGTATCCTTGGCGAACTCTTCACTAAAAAACCTATATTTCAAGCAAAT
     W  S  C  G  C  I  L  G  E  L  F  T  K  K  P  I  F  Q  A  N  303
1681 CAGGAACTTGCACAACTAGAATTAATAAGCCGAATATGTGGGAGTCCATGTCCTGCAGTG
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1921 CTCCGAGATGTGGAACCCTCAAAATGCCTCCACCAGATCTCCCTTTATGGCAAGATTGTC
     L  R  D  V  E  P  S  K  C  L  H  Q  I  S  L  Y  G  K  I  V  403
1981 ATGAGTTATGGAGTAAAAAGCGAAGAAGACAGAAGCAGATGGGCATGACTGATGTTTCCA
     M  S  Y  G  V  K  S  E  E  D  R  S  R  W  A  *              418
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2101 CCCAGGGTGTGCTGCCATCTTCACAGCTGAAATCTCAGGGCAGCTCAAATGTGGCACCTG
2161 GTGAAAAACAGACAGATCCATCAACACCACAACAGGAGTCTTCGAAACCGTTGGGAGGA 2219
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FIG. 1. CHED cDNA sequence and its characterization. (*Upper*) A representative CHED cDNA insert, isolated by screening a phage λgt10 cDNA library of human glioblastoma (14) is shown. DNA sequencing was performed by the dideoxy chain-termination technique using the universal 17-mer primer (Amersham; no. 4511, indicated by a filled symbol at the beginning of arrows), or unique 17-mer primers synthesized from confirmed cDNA sequences (indicated by empty circles at the beginning of arrows). Sites for restriction endonucleases (vertical arrows) were located by sequence data analysis and confirmed experimentally. ATG, first methionine in the open reading frame; TGA, putative termination codon. Computer-aligned ORFs from human 2Hs cDNA (17), *S. pombe* cdc2 cDNA (18), and *S. cerevisiae CDC28* (19), *FUS3* (20), and *KSS1* (21) genes are presented below. (*Lower*) Nucleotide sequence of the 2.2-kb CHED cDNA and amino acid sequence of its encoded translation product. Nucleotides are numbered 5′ to 3′ (left). Amino acid numbers (right) start with the initiator methionine (nucleotide 772). Five upstream ATG codons (boldface type) in a single reading frame are all followed by termination codons (underlined). Cysteine residues, the putative ATP binding (residues 98–120) and phosphorylation (residues 222–269) domains, one potential site for N-linked glycosylation (amino acids 14–16), and a presumed polyadenylylation site (nucleotide 2043) are in boldface type. Four potential splice sites are marked at positions 1069, 1273, 1280, and 1548, close to sites where the *cdc2* gene includes introns bordered by the same sequence (18). PCR amplifications of genomic DNA templates using primers from both ends of these introns have failed, reflecting the existence of large introns.

oligodeoxynucleotides were used as follows: for CHED sequences, p1026(−) (antisense oligonucleotide blocking CHED expression, AS-CHED) (5′-TTTTCCCCAGTCAAT-3′) and p861(+) (5′-TCTTCTTGCTGATTTACCGCTGC-CCCCTGAGCTACCAGGA-3′) (see Fig. 1 *Lower*); for BCHE sequences, p76(+) (sense BCHE oligonucleotide, S-BCHE) (5′-ATGCATAGCAAAGTC-3′) and p1589(−) (5′-ATTTTGCAAAATTTGCCCACCGTTTCACTATGGA-3′) (13); and for 2Hs sequences, p155(−) (antisense oligonucleotide blocking 2Hs expression, AS-2Hs) (5′-GG-TATAATCTTCCAT-3′) and p71(+) (5′-CGGCTTGTTG-TAGC-3′) (16). PCR amplification of CHED cDNA was performed by using the primers p1565(+) (5′-ACCGTC-CACCTGAACTGCTACTGGGAGAAG-3′) and p1887(−) (5′-CGCTTACTAGGATCCAAGGCAAGCATGTAA-3′). Muscle β-actin cDNA was amplified by using the primers ACT 822(+) (5′-TGAAACAACATACAATTCCATCAT-GAAGTGTGAC-3′) and 996(−) (5′-AGGAGCGATAATCT-TGATCTCATGGTGCT-3′) (17). For all of these primers, numbers indicate the 5′-end position in each primer within the relevant cDNAs and (+) or (−) signs depict upstream or downstream orientations, respectively.

PCR Amplification. Amplification was performed with *Taq* DNA polymerase, 20-ng samples of human or mouse genomic DNA, the cycling conditions recommended by Perkin–Elmer/Cetus unless otherwise stated, and the programmable Automated Thermal controller (MJ Research, Boston). Annealing and elongation for cDNA amplification was at 65°C. Agarose gel analysis of the polymerization products was as detailed elsewhere (12). The specificity of the PCR reaction was confirmed by blot hybridization of PCR-amplified DNAs with ^{32}P-labeled probes from the relevant genes.

Cell Cultures. Bone marrow cells from C3H/HeJ mice were grown in liquid cultures as described (6). Briefly, culture medium contained 10% of serum-free medium conditioned in WEHI-3B cells as a source for interleukin 3, 1% bovine serum albumin, and 100 μM thioglycerol (Sigma) in either Iscove's modified Dulbecco's medium (IMDM; GIBCO) or LPM medium (Beit Ha'emek, Beit Ha'emek, Israel). Cells were analyzed after Cytospin centrifugation (Shandon model II; London) and staining with May–Grunwald–Giemsa (Sigma). Photography was in a Zeiss Axioplan microscope equipped with a HC100 camera and a X100 Neofluar lense. [^{35}S]Methionine incorporation, in cpm/mg of protein, was determined by 24-hr pulse-labeling on day 3 of cell cultures with 50 μCi (1 μCi = 37 kBq) of [^{35}S]methionine (1000 Ci/mmol) per 300 μl of liquid culture, protein determination by the Bradford technique (6), and precipitation with trichloroacetic acid (6).

RESULTS AND DISCUSSION

The CHED human cdc2 homolog was discovered in our laboratory by serendipity while screening a phage λgt10 cDNA library of human glioblastoma origin (14) with cholinesterase-specific oligodeoxynucleotide probes (12). One of the isolated clones included a 5′ fragment from BCHE cDNA (nucleotides 1–248; ref. 13) followed by a 2.1-kilobase (kb)-long sequence with no homology to BCHE cDNA. This sequence, designated CHED cDNA, was subcloned and used as a probe in subsequent screenings. CHED cDNA clones were found in libraries from brain, liver, and muscle of fetal

origins and in libraries from neuroblastoma and glioblastoma tumors but lacked the BCHE cDNA fragment to which the original clone was ligated. RNA blot hybridization revealed 2.2- to 2.3-kb transcripts hybridizing with CHED cDNA probes in all of these tissues (not shown). Moreover, CHED cDNA-specific PCR primers supported the amplification of CHED cDNA fragments from cDNA libraries of adult human whole brain, hippocampus, and bone marrow (Clontech). Thus, the *CHED* gene, like *BCHE* (16), appeared to be expressed in hemopoietic cells, various brain regions, and several fetal and tumor tissues.

CHED cDNA was found to be an unusual sequence, with a single 1253-base-pair (bp)-long open reading frame (ORF, Fig. 1). Its long 5′ nontranslated region includes five ATG codons, all in a frame shift with the apparently functional ATG codon at the 5′ end of the ORF (Fig. 1 *Lower*); this conforms with the Kozak consensus sequence (22), suggesting translational control. Potential splice sites were detected in CHED cDNA (Fig. 1 *Lower*) at positions where alignment with the *Schizosaccharomyces pombe cdc2* gene (18) predicts the presence of three introns, and several restriction sites for *Eco*RI and *Bgl* II were observed in the CHED gene but not in CHED cDNA. Thus, the CHED gene apparently includes introns, unlike the intronless *Saccharomyces cerevisiae CDC28* gene (19), and covers an overall length of at least 20 kb (Fig. 2 *Upper*).

The predicted translation product of CHED cDNA contains putative ATP binding and phosphorylation domains, both homologous with parallel regions in CDC proteins, which are known to directly regulate the "start" point of the cell cycle and entry into mitosis in yeast (23) and in man (17) or which regulate conjugation in yeast (20, 21). The CHED protein is longer than other CDC-related proteins at its amino and carboxyl termini (Fig. 1 *Upper*). Since the three experimentally interchangeable CDC proteins (2Hs, cdc2, and CDC28) (17, 18) are quite uniform in length, the size divergence of CHED might reflect differences in its biological activities and/or cellular and subcellular distributions. The CHED protein displays 34–42% identically aligned amino acid residues with the above mentioned proteins in the common sequence domain (residues 88–388), similar to the cdc2Sp homolog reported in HeLa cells (17), and is richer in cysteine residues. Most conserved are the consensus ATP binding and phosphorylation sequences, implying a cdc2-like protein kinase activity (Fig. 2 *Lower*).

Cdc2-related protein kinases phosphorylate both nuclear and cytoplasmic proteins at the consensus motif (Ser or Thr)-Pro-Xaa-Zaa, where Xaa is a polar residue and Zaa is a generally basic residue (24) [for example, $pp60^{c\text{-}src}$ (25)]. Since BCHE includes this motif (Ser-Pro-Gly-Ser, residues 210–213 in the mature protein; ref. 13), and in view of the effects of BCHE gene expression on hematopoiesis (6), we examined the possibility that CHED or another cdc2-like gene may also be involved in blood cell development. For this purpose, nuclease-resistant phosphorothioate antisense oligodeoxynucleotides (15) complementary to CHED mRNA (AS-CHED, nucleotides 1026–1012; Fig. 1 *Lower*), human BCHE mRNA (AS-BCHE; nucleotides 90–76; ref. 13) and 2Hs mRNA, encoding the 2Hs human cdc2 homolog (AS-2Hs, nucleotides 155–141; ref. 16) were used to interfere selectively with the expression of CHED, BCHE, or 2Hs in differentiating bone marrow cells. To ascertain that these oligodeoxynucleotides are capable of hybridizing in culture with their corresponding mouse mRNAs, we used them as primers in polymerase chain reactions (PCR) with human and mouse genomic DNA at the annealing temperature of 38°C. The selective synthesis of discrete hybridizable PCR fragments of the exact same sizes in human and mouse (not shown) implied that the mouse homologs of CHED, BCHE, and 2Hs were sufficiently similar to these human genes to

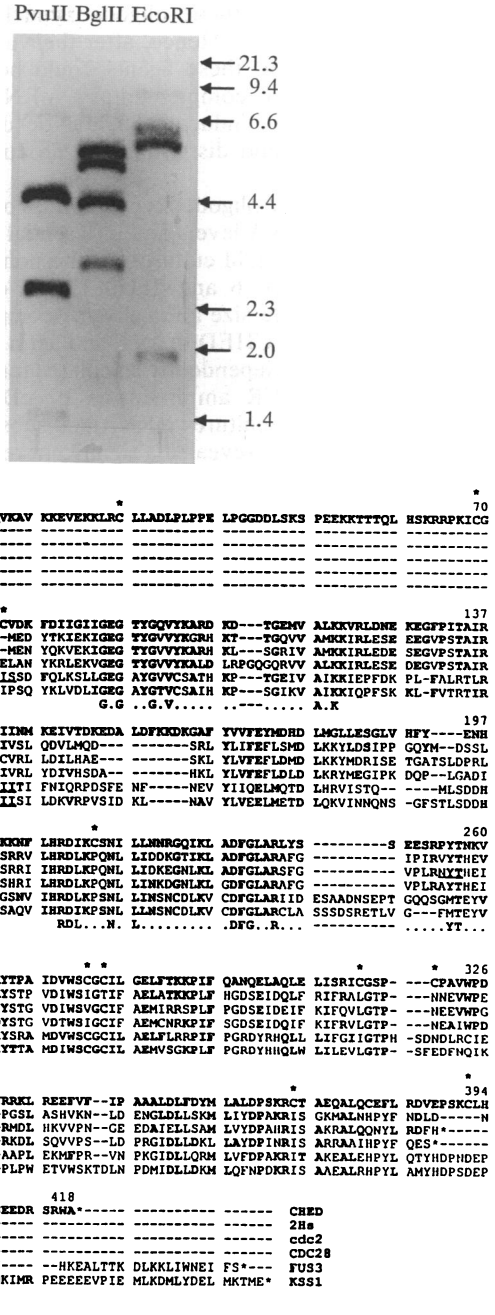

FIG. 2. The CHED gene and its protein product. (*Upper*) Human genomic DNA digested with *Eco*RI, *Bgl* II, or *Pvu* II was subjected to blot hybridization with a *Bgl* II-digested 1.8-kb ^{32}P-labeled fragment of CHED cDNA (nucleotides 190–1955; Fig. 1). *Hin*dIII-digested phage λ DNA provided known size markers. Parallel hybridization with ACHE and BCHE cDNA probes (not shown) verified the completeness of the enzymatic digestions. Note four distinct bands covering a total size of at least 20 kb following digestion with *Eco*RI, which presents no restriction sites in CHED cDNA. (*Lower*) Amino acid sequences inferred from the cDNAs coding for human CHED (Fig. 1 *Lower*) and 2Hs (17), yeast cdc2 cDNA (18) and *CDC28* (19) gene, and yeast *FUS3* (20) and *KSS1* (21) conjugation control genes. Amino acids identically aligned to CHED are in boldface type. Dashes note gaps in the sequences. Cysteine residues (C) are starred. N-linked glycosylation sites are underlined. Consensus amino acid residues involved in ATP binding (residues 98–120) and phosphorylation (residues 222–269) in all protein kinases appear below the sixth line (dots denoting any amino acid). Note identically aligned amino acids (42%, 37%, 40%, 33%, and 34% of residues 88–388 in CHED for 2Hs, *cdc2*, *CDC28*, *FUS3* and *KSS1*, respectively), and additional permissive substitutions (adding up to 62%, 58%, 61%, 56%, and 55%, respectively), including substitutions of consensus leucine for isoleucine-224 and alanine for proline-267.

hybridize with the synthetic oligodeoxynucleotides under physiological conditions. Hence, after their uptake into the cultured cells (6, 26), these agents could be expected to hybridize with their complementary mRNA sequences through base pairing, inducing nucleolytic degradation of these mRNAs (26) and disrupting the production of their protein products.

The effects of these oligodeoxynucleotides on mouse bone marrow CHED mRNA levels and differential cell composition were tested in liquid cultures, at the nontoxic concentration of 5 μM (ref. 6 and Table 1). To overcome the variabilities in sample size and cell-type composition, arbitrary ratios between CHED mRNA and actin mRNA levels for reference were independently calculated for each culture, based on direct PCR amplification of cDNA reverse-transcribed from cell cultures mRNA and subsequent densitometry. This analysis revealed a significant reduction in the CHED/actin transcript ratio, which was observed in AS-CHED-treated cultures as early as 1 hr after AS-CHED addition and persisted for 4 days (Fig. 3). This indicated that AS-CHED was actively incorporated into the cells, where it induced a stable, selective destruction of CHED mRNA. Interestingly, CHED/actin ratios increased with time in both control and AS-CHED-treated cultures. Since actin mRNA levels increase with megakaryocytopoietic differentiation (27), this implied a parallel absolute increase for CHED mRNA, which was not prevented by the presence of AS-CHED, demonstrating that the effect of AS-CHED was limited to the posttranscriptional level.

Reduced numbers of late, polynuclear, mature megakaryocytes and corresponding increases in early, mononuclear or binuclear megakaryocytes, macrophages, and/or polymorphonuclear leukocytes (PMNs) were observed in cultures challenged with either AS-CHED or AS-BCHE but not in those treated with the "sense" BCHE oligodeoxynucleotide. The common shift towards the myeloid, mononuclear lineages emphasized the specificity of the AS-CHED/AS-BCHE effects (Table 1 and Fig. 4). This can possibly imply that blocking the expression of these genes modified the M phase of the cell cycle (28), during which nuclear multiplication, dissociated from cytoplasmic division, accompanies megakaryocyte maturation (1–3). In view of the high concentration of actin in mature megakaryocytes (27), this experiment also implied that the AS-CHED-treated cultures

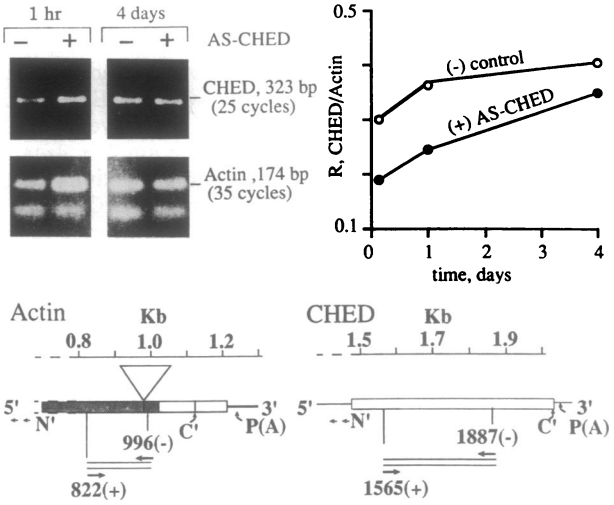

FIG. 3. Selective reduction in CHED mRNA/actin mRNA ratios in bone marrow cultures treated with the AS-CHED oligodeoxynucleotide. RNA was extracted from 1-ml samples of bone marrow liquid cultures at the indicated incubation times and treatment conditions (Table 1). Briefly, cells were centrifuged, washed with phosphate-buffered saline, lysed with RNasol (CINN; Bioteck, Houston), and subjected to chloroform extraction and isopropyl alcohol precipitation according to the manufacturer's instructions. Reverse transcription of the resultant RNA was performed by using random hexamers as primers and was followed by PCR amplification of the produced cDNA for the indicated number of cycles, all with the RNA-PCR kit (Perkin–Elmer/Cetus) with CHED and actin cDNA-specific primers. (*Lower*) The positions of these primers within the coding regions as related to the exon/intron structures of CHED and β-actin genes is shown. N' and C' denote the amino and carboxyl termini of the mature proteins; p(A), the polyadenylylation signal; ▽, the position of an intron. The length is shown in kb. Control PCR amplifications with non-reverse-transcribed RNA were performed for both genes to exclude the possibility that the amplified fragments were produced from contaminating genomic DNA (not shown). (*Upper Left*) Ethidium bromide staining of electrophoresed RNA PCR products. (*Upper Right*) Arbitrary ratios (R) between signal intensities of CHED and actin RNA PCR products as determined by densitometry for each culture. ○, Control cultures; ●, AS-CHED-treated cultures. Note the selective reduction in R values in treated cultures.

Table 1. Interference with hematopoiesis by antisense phosphorothioate oligodeoxynucleotides directed toward CHED and BCHE mRNAs

	Cell-type composition					Other parameters		
		Differential cell composition,† %					Liquid culture	
Oligonucleotide*	Cells counted	Mϕ	PMN	Megakaryocytes Early	Megakaryocytes Late	Colonies per semisolid culture dish,‡ ratio ± SEM	Total cells,§ no. × 10^{-5} per ml	[^{35}S]Met incorp.,¶ cpm × 10^{-6}/mg of protein per day
None	1239	14.5	19.9	21.4	44.2	1.00 ± 0.07 (119–296)	5.0	2.7
AS-CHED	1540	18.6	27.7	24.2	29.5	0.81 ± 0.05 (78–291)	14.4	1.3
AS-2Hs	999	10.1	21.9	14.9	53.0	0.59 ± 0.03 (46–210)	3.2	8.0
AS-BCHE	1324	27.7	22.4	23.9	25.8	0.58 ± 0.03 (66–134)	2.8	4.5
S-BCHE	1916	13.7	15.9	21.0	48.8	1.03 ± 0.04 (153–246)	4.5	2.0

Mϕ, macrophages; S-BCHE, sense BCHE oligodeoxynucleotide.

*Four 15-mer phosphorothioate oligodeoxynucleotides were prepared as recently detailed (6).

†Differential cell composition determined microscopically (6) for liquid cultures containing 5 μM of the specific oligodeoxynucleotide. Early megakaryocytes, defined as immature forms with one or two nuclei, were distinguished from late types characterized by their large size, multinucleation (≥4 nuclei), and tendency to shed cytoplasmic fragments. Cumulative fractions of cell types in percentage values (for five different cultures) and the total numbers of cells counted for each type of culture are indicated.

‡Ratio of colony counts per semisolid culture dish on day 4, where the control culture with no oligodeoxynucleotide (None) equals 1.0. Means ± SEM of six different experiments are shown. The range of colony numbers for each type of culture is in parentheses. Five μM of each oligodeoxynucleotide was added to the cultures. Megakaryocyte colonies contained at least four cells; myeloid colonies consisted of ≥50 cells.

§Total cell counts per ml on day 4 for liquid cultures (6) without or with the specific oligodeoxynucleotides. Values represent the average of three different cultures with deviations of up to 30%.

¶Note the general similarity in the rate of protein synthesis in cultures treated with oligodeoxynucleotides, demonstrating no toxicity (average of two experiments).

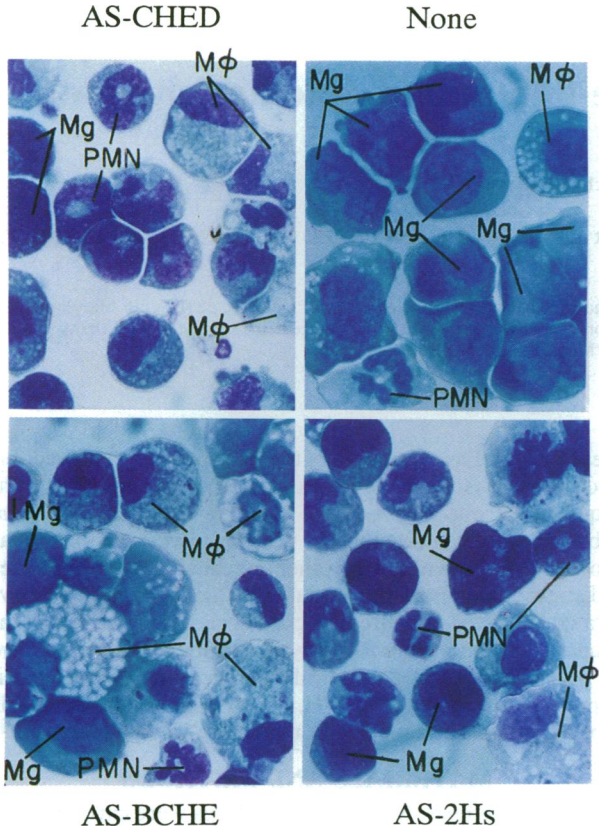

FIG. 4. Antisense oligodeoxynucleotides to the CHED and BCHE genes but not to the 2Hs cdc2 homolog divert hematopoiesis towards mononuclear lineages. Bone marrow cells were grown in semisolid or liquid cultures containing IMDM- and WEHI-3B-conditioned medium as in Table 1 and as recently detailed (6) with or without ("None") 5 μM specified phosphorothioate oligodeoxynucleotide. Cells were mechanically removed after 4 days in culture, cytocentrifuged, stained, and photographed. (*Upper Right*) Note heterogeneity of cellular morphologies, including mature megakaryocytes with multiple large nuclei (Mg), a polymorphonuclear neutrophil with its multilobed nucleus and lightly stained cytoplasm (PMN), and a macrophage with vacuole-rich cytoplasm (Mϕ). (*Upper Left*) Cells treated with AS-CHED. Note the abundance of macrophages and PMNs, presence of early Mg with dark-staining cytoplasm, and lack of mature Mg. (*Lower Left*) Cells treated with AS-BCHE. Note the absence of mature Mg and the abundance of Mϕ. (*Lower Right*) Cells treated with AS-2Hs. Note the heterogeneity of cells and the presence of multinuclear Mg.

contained far less actin-mRNA than did control cultures. This, in turn, further suggested that the absolute content of CHED mRNA transcripts in AS-CHED-treated cultures was far lower than that of controls.

In semi-solid cultures, AS-CHED, AS-BCHE, and AS-2Hs oligodeoxynucleotides inhibited colony formation to different extents, suggesting interference with the proliferation of certain precursor cells. However, in liquid cultures, AS-CHED induced increases in cell counts without greatly affecting the overall rate of protein synthesis as measured by pulse-labeling with [^{35}S]methionine (Table 1). This may parallel the shift from megakaryocyte colonies, composed of relatively few cells, to myeloid colonies, consisting of many cells. In contrast, liquid cultures incubated with AS-2Hs displayed reduced cell counts (Table 1), probably reflecting interrupted "start" activities. Yet, these cultures displayed no deviations from the differential cell composition of no-oligonucleotide control cultures (Table 1), indicating that the 2Hs protein does not affect bone marrow differentiation. In AS-BCHE-treated cultures, decreased colony numbers and cell counts were accompanied by an arrest of megakaryocytopoiesis (Table 1), which further indicated the qualitative similarity between the AS-CHED and AS-BCHE effects.

The existence in unicellular organisms of various cdc2-like proteins regulating detours from the mitotic pathway [i.e., FUS3 (20) and KSS1 (21)] predicts that in multicellular organisms certain cdc2-like proteins might control parallel processes in specific cell lineages [i.e., mak in germ cells (29)]. To the best of our knowledge, CHED is the first CDC-related gene whose product is required for and increases during the development of a particular somatic cell lineage(s). Further studies will be needed to reveal whether defects in CHED's production and/or function are involved in specific megakaryocytopoietic disorders. Although the initial connection between BCHE and CHED cDNAs remains unexplained, the similar modulation of hematopoietic differentiation by AS-CHED and AS-BCHE indicates that CHED and BCHE may be interrelated components responsive to cholinergic signals in the hematopoietic pathway. This, in turn, strengthens the evidence for an as yet undeciphered link between cholinergic signaling and cell division, and suggests that this link may be mediated through characteristic CDC proteins in a cell lineage-specific manner.

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