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A NOVEL ADENYLYL CYCLASE SEQUENCE CLONED FROM THE HUMAN ERYTHROLEUKEMIA CELL LINE

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SUMMARY. The polymerase chain reaction (PCR) was used to detect several forms of adenylyl cyclase (AC's) expressed in human erythroleukemia (HEL) cells. Degenerate oligonucleotide primers were synthesized based on the conserved sequences in the C_{2a} area of the AC's. HEL cells were found to contain mRNA for type III and type VI AC. In addition, a novel AC message was identified. The cloned sequence, excluding primer areas, represented 69 amino acids with most similarity to rat AC's II and IV. Northern analysis of RNA from HEL cells demonstrated a 6.7 kilobase message. RNase protection assays revealed that in HEL cells the novel AC mRNA was dominant compared to types III and VI. Human embryonic kidney cells (HEK293) were also used as a source of mRNA to synthesize cDNA for PCR reactions. The HEK293 cells were found to contain message corresponding to type II, III, VI AC as well as the novel AC message. The novel AC message was also detected in human brain tissue and was most abundant in the caudate, cerebellum and hippocampus. The smallest amount of novel AC mRNA in the tested brain tissue was found in the cortex. The mRNA for the novel AC was relatively abundant in human liver.

Adenylyl cyclases (AC's) [ATP pyrophosphatelyase (cyclizing) EC 4.6.1.1.] are enzymes that catalyze the synthesis of adenosine 3',5'-cyclic monophosphate (cAMP), an important second messenger. Following the cloning of the first mammalian AC, type I (1), several studies have shown that AC's form a gene family with distinct subfamilies (2,3). Individual members in subfamilies are known to have characteristic functional properties and tissue distribution. Full length cDNAs encoding six distinct mammalian forms (types I-VI) have been isolated from bovine, rodent and dog tissue (1-9). Furthermore, a partial C-terminal sequence of an AC from human brain has been determined (10). Krupinski and coworkers (2) also reported two partial amino acid sequences for additional AC's from different rat tissues. Thus, there appear to be at present at least eight different forms of the enzyme.

Our main interest has been to characterize the AC system and to ascertain the enzyme forms present in blood platelets. Since platelets, as non-nucleated cells, contain only residual

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RNA, we used the human erythroleukemia (HEL) cell line (11) as a model for platelets. The HEL cells exhibit a "multilineage-affiliated phenotype" which includes "a strong expression of megakaryocyte-platelet determinants" (12). We also examined the forms of AC present in the human embryonic kidney cell line HEK293 and other human tissues.

MATERIALS AND METHODS

HEL and HEK293 cell cultures. Human HEK293 cells were cultured as described earlier (8). HEL cells (11,12) were grown in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) supplemented with 10 % heat inactivated fetal bovine serum (HyClone, Logan, UT). The cells were cultured in Corning disposable tissue culture flasks at 37°C in a humidified 5 %/95 % CO_2/air atmosphere and maintained at a concentration of 5 x 10^5 - 1 x 10^6 cells/ ml during the growth phase. For RNA extraction, HEK293 cells and HEL cells were harvested from the flasks and centrifuged at 700 x g for 10 min at 4°C. The cell pellet was washed by careful suspension in phosphate-buffered-saline (pH 7.4) and centrifugation. Human brain tissues were autopsy material, in which the post-mortem delay was 20 hours or less and the freezer storage time was less than five months. Human autopsy material stored in these conditions have been shown to be useful in studies which require RNA extraction (13).

RNA extraction, isolation of poly $(A)^+$ RNA and first strand cDNA synthesis. Total RNA was extracted from the cultured HEK293 cells, HEL cells, human brain (cortex, hippocampus, caudate, cerebellum) and human liver tissue using the guanidinium/CsCl centrifugation method (14). Oligo(dT)-cellulose (type 3, Collaborative Research Inc., Bedford, MA) was used when poly$(A)^+$ RNA was isolated (15). Poly$(A)^+$ RNA from HEL and HEK293 cells was used as a template for the synthesis of first strand cDNA (Riboclone cDNA Synthesis System, Promega, Madison, WI). The AC region amplified by PCR is located in the conserved C_{2a} area present in the putative C-terminal intracellular domain of AC's (6). The first strand cDNA synthesis was primed with the degenerate antisense oligonucleotide primer adc4 at a concentration of 5 μM (8). This primer was complementary to the conserved amino acid sequence YDIWGNTVNV: 5'-GGGATCC A(ACG)(AG) TT(ACGT) AC(ACGT) GT(ACGT) TT(ACGT) CCC CA(AGT) AT(AG) TC(AG) TA3'. The antisense primer adc4 contained a *Bam*HI site in the 5' end for cloning purposes.

Amplification of cDNA by PCR: cloning and sequencing of the products. One tenth of the first strand cDNA synthesis product was used as a template in PCR. In combination with adc4 a degenerate sense primer adc5 (8) was used for PCR amplification. Adc5 corresponded to the conserved amino acid sequence KIKTIGSTYMA: 5'-GAAGCTT AA(AG) AT(ACT) AA(AG) AC(ACGT) AT(ACT) GG(ACGT) (AT)(CG)(ACGT) AC(ACGT) TA(CT) ATG GC3'. The 5' end of the primer had a *Hind*III site to facilitate cloning. The final concentrations of the primers were 5 μM. GeneAmp PCR kit reagents with AmpliTaq polymerase (Perkin Elmer Cetus, Norwalk, CT) were used. Thermal cycler model 480 (Perkin Elmer Cetus, Norwalk, CT) was programmed according to the "touch-down" protocol (16) in which the annealing temperature was decreased by 1°C after every two cycles from 65°C to 55°C. Ten cycles were carried out at the final annealing temperature, followed by an extension of 15 minutes at 72°C. Aliquots of the 100 μl PCR reaction mixture were electrophoresed on a 2 % agarose gel and a 5 % polyacrylamide gel to verify products of expected size. The remainder of the reaction product was cut with *Hind*III and *Bam*HI, and separated on a 2% agarose gel. The DNA bands were excised from the gel and cloned into the pBluescript II SK(-) vector (Stratagene, La Jolla, CA). The cloned inserts were sequenced by the dideoxynucleotide method with forward/reverse primers and modified T7 DNA polymerase (Sequenase kit, Version 2.0, United States Biochemicals, Cleveland, OH) (17).

Northern analysis. Ten μg of poly(A)$^+$ RNA from HEL cells was electrophoresed on a formaldehyde-agarose (1.5%) gel. RNA molecular size standard (0.24-9.5 kilobase) was from Gibco-BRL (Grand Island, NY). RNA was transferred to a 0.45 μm Nytran membrane (Schleicher & Schuell, Keene, NH), and immobilized by UV crosslinking (Stratalinker; Stratagene, La Jolla, CA). For the synthesis of riboprobes corresponding to the antisense strand, the cloned AC sequences obtained by PCR were linearized at the 5' terminus of the insert in pBluescript II SK(-) vector. The linearized plasmid was used as a template in an [α-^{32}P]-labeled riboprobe synthesis reaction (Riboprobe Gemini System, Promega, Madison, WI; [^{32}P]-UTP, 800 Ci/mmol, DuPont-NEN, Boston, MA). Prehybridization of the filters and hybridization with the radioactive probe were performed at 65°C in a buffer consisting of 0.5 M NaCl, 0.1 M Na_2HPO_4, 5 mM EDTA, 1% N-lauroyl sarcosine, 0.1 mg/ml herring sperm DNA (pH 7). Filters were washed at 65°C with 0.1X SSC/ 0.1 % SDS. The Kodak XAR-2 film was exposed at -70°C.

RNase protection assays. All solution hybridization/RNase protection assays were performed using a RPA II kit from Ambion (Austin, TX). Total RNA from HEL cells, human brain and liver tissues was extracted as described and quantified spectrophotometrically at 260 nm/280 nm. [α-^{32}P]-labeled riboprobe was synthesized as described above. For the RNase protection assays the synthesized riboprobe was gel purified according to Ambion's protocol to remove transcripts shorter than full length. The solution hybridization/ RNase protection assay was performed according to the Ambion RPA II kit instructions using quantities of RNA listed in the legends of Figures 3 and 5. The protected fragments were separated in a 5% denaturing polyacrylamide gel. The Kodak XAR-2 film was exposed at -70°C. Quantitation of protected fragments was performed using a high resolution CCD video camera (Sierra Scientific, Sunnyvale, CA), a Macintosh IIx computer and Image 1.28 software for digital image processing.

RESULTS

The amplification of HEL and HEK293 cell poly(A)$^+$RNA-cDNA using degenerate primers in the PCR yielded products of 250 to 300 basepairs when analyzed in an agarose gel. Four different products were detected in HEK293 cells and three products in HEL cells. Fourteen HEK293 clones were sequenced and human homologs of AC II (one clone), AC III (seven clones), and AC VI (two clones) were identified (Fig. 1). Two of seven HEL clones

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II   rat     KIKTIGSTYMAATGLSAIPSQEHAQEPERQYMHIGTMVEFAYALVGKLDAINKHSFNDFKLRVGINHGPVIAGVIGAQKPQYDIWGNTVNV
II   human             ATGLSAVPSQEHSQEPERQYMHIGTMVEFAFALVGKLDAINKHSFNDFKLRVGINHGPVIAGVIGAQKPQ
                              +     +                 +

III  rat     KIKTIGSTYMAASGVTPDVNTNGFTSSSKEEKSDKERWQHLADLADFALAMKDTLTNINNQSFNNFMLRIGMNKGGVLAGVIGARKPHYDIWGNTVNV
III  human           ASGVTPDVNTNGFASSNKEDKSERERWQHLADLADFALAMKDTLTNINNQSFNNFMLRIGMNKGGVLAGVIGARKPH
                                    +     +  ++

VI   mouse   KIKTIGSTYMAASGLNASTYDQVGRSHITALADYAMRLMEQMKHINEHSFNNFQMKIGLNMGPVVAGFIGARKPQYDIWGNTVNV
VI   human           ASGLNASTYDQVGRSHITALADYAMRLMEQMKHINEHSFNNFQMKIGLNMGPVVAGVIGARKPQ
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FIG. 1.
Alignment of the partial human sequences for AC types II, III and VI, obtained by PCR, with the corresponding rat and mouse sequences. HEK293 cells have message corresponding to all of these human AC forms, whereas HEL cells have mRNA for types III and VI. Underlined area denotes a change of an amino acid in the human sequence. A conservative substitution is marked with plus (+). The reference sequences are rat type II (4), rat type III (5) and mouse type VI (8).

were recognized as the human homologs of AC VI. When the primer areas adc5 and adc4 were excluded, the identity of the AC II amplified sequence across species was 96% and similarity (conservative substitutions included) was 100%. The identity was 94% and similarity 99% between human and rat AC III in the amplified area. Human and mouse AC VI had identity and similarity of 98% (Fig. 1). In five of seven HEL clones and in four of fourteen HEK293 clones a novel human sequence was detected which represented 69 amino acids excluding primer areas (Fig. 2). It contained 31 of 34 consensus amino acids in this conserved area of AC's (Fig 2). A database search at the National Center for Biotechnology Information using the BLAST network showed that the novel sequence was most similar to rat AC types II (score 242, 45 identities/65) and IV (score 216, 40 identities/57). In this area the novel form was one amino acid shorter than rat AC's II and IV. The lowest score in the AC group was for rat type III (score 144, 25 identities/49). All other sequences including guanylyl cyclases had lower similarity scores than any of the AC's.

RNase protection assays also revealed that the novel human AC sequence was the major form of AC in HEL cells (Fig. 3). Northern blot analysis of HEL poly(A)$^+$ RNA using a riboprobe for the novel AC sequence detected a message of 6.7 kilobase, (average of two separate experiments [range 6.6-6.8 kilobase]) (Fig. 4). Of the human brain areas studied, the mRNA for the novel AC form was found to be most abundant in the caudate and cerebellum. A slightly lower signal was detected in the hippocampus. The smallest amount of the novel AC message was encountered in the cortex. The mRNA of the novel AC form was abundant in human liver (Fig. 5).

A non-denaturing polyacrylamide gel revealed three different sizes of PCR products in HEL cells (data not shown). However, we were unable to find a third type of AC among the clones derived from HEL cells. The size analysis suggested that the third AC form in HEL cells

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I        bovine    KIKTIGSTYMAAVGLAPT----------AGTKAKKCISSHLSTLADFAIEMFDVLDEINYQSYNDFVLRVGINVGPVVAGVIGARRPQYDIWGNTVNV
II       rat       KIKTIGSTYMAATGLSAI-------PSQEHAQEPERQYMHIGTMVEFAYALVGKLDAINKHSFNDFKLRVGINHGPVIAGVIGAQKPQYDIWGNTVNV
III      rat       KIKTIGSTYMAASGVTPDVNTNGFTSSSKEEKSDKERWQHLADLADFALAMKDTLTNINNQSFNNFMLRIGMNKGGVLAGVIGARKPHYDIWGNTVNV
IV       rat       KIKTIGSTYMAATGLNAT-------PGQDTQQDAERSCSHLGTMVEFAVALGSKLGVINKHSFNNFRLRVGLNHGPVVAGVIGAQKPQYDIWGNTVNV
V        dog       KIKTIGSTYMAASGLNDS------------TYDKVGKTHIKALADFAMKLMDQMKYINEHSFNNFQMKIGLNIGPVVAGVIGARKPQYDIWGNTVNV
VI       mouse     KIKTIGSTYMAASGLNAS------------TYDQVGRSHITALADYAMRLMEQMKHINEHSFNNFQMKIGLNMGPVVAGFIGARKPQYDIWGNTVNV
partial  human     KIKTIGSTYMAVSGLSPE-----------KQQCEDKWGHLCALADFSLALTESIQEINKHSFNNFELRIGISHGSVVAGVIGAKKPQYDIWGKTVNL
                          * **                    *   * ***  *      ** **** * ** * * **************

NEW      human               AAGLSVA--------SGHENQELERQHAHIGVMVEFSIALMSKLDGINRHSFNSFRLRVGINHGPVIAGVIGARKPQ
                             . ..                    .     .  .     .. .... . .. . . ..............
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FIG.2.
Comparison of the novel human AC sequence (NEW) obtained from HEL and HEK293 cells to the C-terminal conserved area of the currently known AC types I-VI and a partial human sequence. An asterisk denotes conservation of an amino acid residue in at least five of the seven previously published sequences. A single dot (NEW) indicates identity with the consensus amino acid. The reference sequences are: bovine I (1), rat II (4), rat III (5), rat IV (6), dog V (7), mouse VI (8), partial human sequence (10).

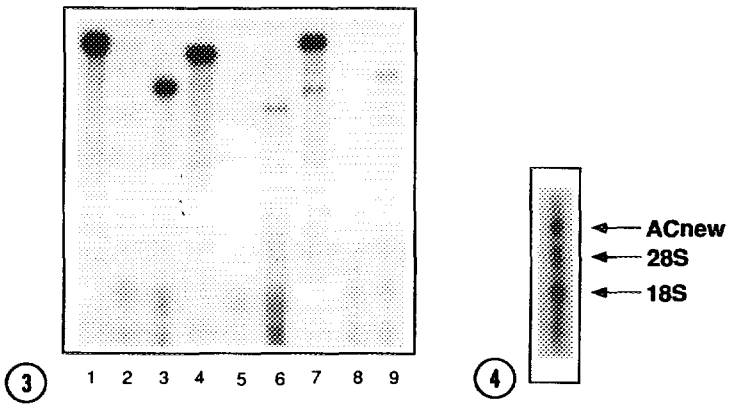

FIG. 3.
RNase protection assay of HEL poly (A)$^+$ RNA with novel human sequence (NEW) (lanes 1-3), type VI (lanes 4-6) and type III (lanes 7-9) AC riboprobes. Lanes 1, 4 and 7: riboprobes NEW, VI and III, respectively, hybridized with yeast RNA (no RNase treatment). Lanes 2, 5, and 8: riboprobes NEW, VI, and III, respectively, hybridized with yeast RNA and digested subsequently with RNase A/RNase T1. Lanes 3, 6, and 9: riboprobes NEW, VI, and III, respectively, hybridized with HEL poly (A)$^+$ RNA (three μg/lane) and digested with RNase A/RNase T1. The protected fragments were electrophoresed in a 5 % denaturing polyacrylamide gel. The film was exposed for 24 hours at -70°C.

FIG. 4.
Northern analysis of poly (A)$^+$ RNA from HEL cell line with the novel human AC sequence riboprobe (NEW). RNA size markers (not shown) and ten μg of HEL cell poly(A)$^+$ RNA were electrophoresed in a formaldehyde-containing gel (for details see Materials and Methods). The film was exposed for five hours at -70°C. A band corresponding to 6.7 kilobase was detected (average of two separate experiments, range 6.6-6.8 kilobase).

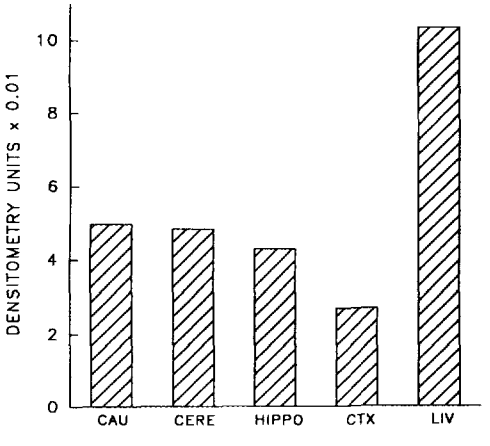

FIG. 5.
Relative abundance of the novel human AC sequence (NEW) in different human tissues. Sixty μg of total RNA from human caudate (CAU), cerebellum (CERE), hippocampus (HIPPO), cortex (CTX) and from human liver (LIV) were used in the RNase protection assay. Densitometry units were obtained by using Image 1.28 software (See Materials and Methods).

might be type III. This was verified by RNase protection assay using a human type III probe transcribed from the HEK293 kidney cell clone (Fig. 3). In further RNAse protection experiments type II AC was not detected in HEL cells after a film exposure time in which human cortex and hippocampus displayed a strong signal (data not shown).

DISCUSSION

A novel cDNA and corresponding amino acid sequence was obtained from human HEL cells using PCR with degenerate primers based on the conserved sequence in the C_{2a} area present in mammalian AC's. When compared in a database search to known amino acid sequences, the highest similarity scores were with various mammalian AC's. Thus, it can be postulated that this novel sequence represents a partial sequence for mRNA of an AC. The newly identified sequence most likely does not represent a human sequence of an already known AC type since there is one amino acid length difference when aligned with rat AC types II (4) and IV (6) which show the highest similarity scores to the novel human sequence. The identity of the novel human sequence with rat AC types II (4) and IV (6) is at most 69-70 % when primer areas are excluded. The differences across species have been smaller in the case of other AC types. For example the conservation of amino acids is 98 % in this particular area between dog (7) and rat type V AC (3). Similarly, if the same region from type VI AC is compared between mouse (8), rat (3) and dog (9) the identity of amino acids is 97-98 %. Additionally, there are no known length differences in the area in question when a given AC, e.g. type V (3,7) or type VI (2,3,8,9), is compared across species.

The 6.7 kilobase message of the novel human AC form in HEL cells differs from the message sizes observed for other AC's, especially from 4.1 kilobase of AC type II (4) and from 3.5 kilobase of AC type IV (6). Solution hybridization studies revealed that the mRNA of the novel AC form was the major type in the HEL cells. Different human brain areas and human liver also express this type of AC. The novel human AC was also obtained by PCR in human embryonic kidney cell line HEK293. These findings suggest that the novel AC form has a relatively wide tissue distribution.

Both HEL and HEK293 cell lines express AC types, which were identified as human homologs of AC forms III and VI. Originally it was suggested that the expression of type III was limited to olfactory neurons (5). The present study shows, however, that AC III may also be expressed in other cell types. This finding is consistent with the recent reports of Krupinski et al. (2) and Xia et al. (18) on the wider distribution of type III AC than originally reported. HEL and HEK293 cell lines also express type VI AC, which is found especially in heart, but also in brain and several peripheral tissues (8,9). AC type II was found to be present in

HEK293 cells, although earlier type II probe has failed to produce a signal in Northern blot analysis of kidney tissue (4).

The HEL cells were used in our current studies as a model of human platelets to study the expression AC's. Although the isolation of RNA from blood platelets has been reported (19,20) there is a possibility that even a small contamination of platelets with nucleated blood cells may lead to ambiguous results when using PCR. The proper characterization of human platelet AC systems will require use of type-specific antibodies and analysis of AC's at the protein level in addition to the studies we have performed with the HEL cell model. Such studies will also reveal whether the HEL cells can be further used as a system for studying the functional characteristics of the different AC forms in the platelets.

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