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# cDNA cloning and functional activity of a glucocorticoid-regulated inflammatory cyclooxygenase

(prostaglandin G/H synthase/interleukin 1β/mRNA stability/fibroblasts/monocytes)

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ABSTRACT **The antiinflammatory glucocorticoids are potent inhibitors of cyclooxygenase, a key regulator of prostaglandin synthesis; yet, the mechanism(s) by which this occurs is not fully understood. We have cloned a 4.1-kilobase (kb) cDNA, distinct from the previously cloned cyclooxygenase (2.8 kb), that confers cyclooxygenase activity to transfected cells. The mRNA for this newly discovered cyclooxygenase is unique for its long 3′ untranslated region containing many AUUUA repeats. Levels of the 4.1-kb cyclooxygenase mRNA are rapidly increased by serum or interleukin 1β in mouse fibroblasts and human monocytes, respectively, and decreased by glucocorticoids, whereas levels of the 2.8-kb cyclooxygenase mRNA do not change. Similar effects are seen in the presence of cycloheximide where the 4.1-kb, but not the 2.8-kb, mRNA is greatly superinduced. Thus, there are both constitutive (2.8 kb) and regulated (4.1 kb) cyclooxygenase species, the latter most likely being a major mediator of inflammation.**

Prostaglandins represent a diverse group of autocrine and paracrine hormones that are important mediators of many cellular functions. Excessive local prostaglandin production is responsible for many of the clinical symptoms of inflammation as attested to by the use of aspirin and other nonsteroidal antiinflammatory drugs that inhibit the activity of cyclooxygenase, the first enzyme in the conversion of arachidonic acid to prostaglandins. Inflammatory mediators, such as interleukin 1β (IL-1β), IL-2, and lipopolysaccharide, increase cyclooxygenase activity (1–7). Glucocorticoids decrease cyclooxygenase activity, particularly activity induced by inflammatory mediators (2, 6, 7). Although the mechanism(s) by which this occurs is not fully understood, glucocorticoids inhibit the synthesis of cyclooxygenase in a number of systems (2, 6–10).

A cyclooxygenase [prostaglandin G/H synthase (PGHS; 8,11,14-icosatrienoate-hydrogen-donor:oxygen oxidoreductase, EC 1.14.99.1)] has been purified from sheep seminal vesicles (11) and cDNA clones were obtained from ovine (12, 13), murine (14), and human (15) sources. These cDNAs encode very similar proteins and hybridize to mRNAs of ≈2.8 kilobases (kb) on Northern blots. They have so far been the only reagents available for study of cyclooxygenase gene regulation. *In vitro*, the inhibition of cyclooxygenase activity by glucocorticoids occurs rapidly (within hours). Although some investigators have reported decreases in cyclooxygenase mRNA levels with glucocorticoid treatment (16), we and others have reported changes in the synthesis of cyclooxygenase and corresponding enzyme activity without concomitant changes in the level of the 2.8-kb mRNA (17, 18). We have recently identified a distinct 4-kb mRNA that varies with cyclooxygenase activity and encodes a protein specifically immunoprecipitated by antiserum against purified ovine cyclooxygenase (18). Here we report the full-length cloning of this cDNA, its functional expression as an active cyclooxygenase, and the initial characterization of its regulation in mouse fibroblasts and human monocytes.‡

## MATERIALS AND METHODS

**Cells, Cell Culture, and Two-Dimensional Gel Electrophoresis.** Cell cultures were maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% fetal calf serum (HyClone) without antibiotics. Cells were monitored for mycoplasma contamination by Hoechst 33258 staining (19). The C127 mouse fibroblast line (20) was obtained from Peter Howley. Adherent monocytes were isolated from the peripheral blood of healthy donors by overnight incubation of buffy coat preparations in tissue culture dishes (21). After repeated washing, adherent cells were scraped, suspended in M199 medium, counted in a Coulter counter, centrifuged, and resuspended in medium without serum at $1 \times 10^6$ cells per ml. Cells prepared in this manner were >95% monocytes based on morphologic appearance and staining for nonspecific esterase.

For two-dimensional gel analysis cells were metabolically labeled for 30 min with Tran$^{35}$S-label (200 μCi/ml; 1 Ci = 37 GBq; ICN) in DMEM without methionine. Cellular proteins were separated by giant two-dimensional gel electrophoresis as described (22) and visualized by autoradiography with Kodak XAR film.

**cDNA Cloning and Sequencing.** Fifty micrograms of poly(A)-enriched RNA (23, 24) from C127 cells treated for 2.5 h with serum and cycloheximide (25 μM) was fractionated on a 10–30% sucrose gradient in the presence of 10 mM $CH_3HgOH$ (25). Every other fraction was assayed for the presence of the 4-kb mRNA by Northern blot analysis (18) using the 1.6-kb 5′ end of the ovine PGHS cDNA (obtained from Oxford Biomedical Research) labeled by random priming. cDNA synthesis using an oligo(dT) primer/adaptor and library construction was performed according to the manufacturer's protocols (Stratagene). For screening of 250,000 plaques, the stringency of hybridization was lowered (30% formamide at 42°C, filters washed in 2× standard saline citrate/0.1% SDS at 55°C). Double-strand dideoxynucleotide termination sequencing of *Exo* III nested deletion subclones was carried out in both directions with T7 DNA polymerase (26, 27).

**cDNA Expression and Prostaglandin $E_2$ ($PGE_2$) Determination.** Subconfluent COS A.2 cells in duplicate 60-mm plates

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Abbreviations: IL, interleukin; PGHS, prostaglandin G/H synthase; $PGE_2$, prostaglandin $E_2$; griPGHS, glucocorticoid-regulated inflammatory PGHS; PDGF, platelet-derived growth factor.

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‡The sequence reported in this paper has been deposited in the GenBank data base (accession no. M88242).

were transfected with different amounts of expression vector alone (SVL) or the expression vector containing the 4.1-kb cDNA (SVL-4.1) by a modified calcium phosphate precipitation procedure (28). Four plates were also treated with transfection reagents without added DNA. Plates were incubated for 24 h at 35°C in 3% $CO_2$/97% air, rinsed twice with DMEM without serum, and incubated for an additional 24 h in DMEM containing 10% fetal calf serum. For $PGE_2$ production, cells were rinsed once with prewarmed DMEM and then 1 ml of DMEM containing 30 $\mu$M arachidonic acid was added. After 10 min, the supernatants were collected, clarified by brief centrifugation, and assayed for $PGE_2$ by radioimmunoassay after conversion to the methyl oximated form (kit from Amersham). Monolayers were solubilized in 0.1 M NaOH, neutralized with 1 M HCl, and clarified by centrifugation before protein concentration determination.

**Northern Blot Analysis.** Poly(A)-enriched RNAs (2.5 $\mu$g) from C127 cells (23, 24) were fractionated by formaldehyde/agarose gel electrophoresis and transferred to a membrane (Duralon, Stratagene). Hybridization was carried out as described with the 5′ 1.2-kb *Eco*RI fragment of the 4.1-kb cDNA labeled with $^{32}P$ by random priming (29, 30). The membrane was later rehybridized with a similarly labeled portion (1.6-kb 5′ end) of the 2.8-kb ovine PGHS cDNA (Oxford Biomedical Research) and an end-labeled 40-mer complementary to $\beta$-tubulin (Oncor, Gaithersburg, MD). RNA molecular weight markers (BRL) were visualized by ethidium bromide staining. A similar analysis was performed on total RNA (5 $\mu$g per lane) isolated from human monocytes by a guanidinium/acid/phenol extraction method (31).

## RESULTS AND DISCUSSION

A directionally cloned cDNA library was constructed in $\lambda$ ZAP II from sucrose gradient fractions enriched in the 4-kb mRNA and screened with a radiolabeled portion of the 2.8-kb PGHS cDNA under conditions of lowered stringency. Several positive plaques were isolated and analyzed. One clone, ≈4.1 kb long, was fully sequenced. This clone encodes a 70-kDa protein specifically precipitated by anti-cyclooxygenase antiserum, which migrates identically with the immunoprecipitated protein product from *in vitro* translated poly(A) mRNA (data not shown). Comparison of the 4.1-kb sequence (Fig. 1) with that of the previously cloned 2.8-kb PGHS cDNA from mice [which is very similar to that cloned from sheep and human tissues (33)] revealed a single open reading frame with 64% amino acid identity to the protein encoded by the 2.8-kb PGHS cDNA. The deduced protein sequences are colinear except that the 4.1-kb cDNA has a shorter N terminus and longer C terminus (Fig. 2). Three of four potential N-glycosylation sites are conserved between the two molecules and there is particularly high similarity in the regions surrounding a putative axial heme-binding domain (amino acids 273–342) and the region around the presumed aspirin-modified serine[516] (amino acids 504–550) (8) (Fig. 2). By far the largest difference in the two cDNAs is the presence of a 2.1-kb 3′ untranslated region in the 4.1-kb cDNA. This region is rich in 5′-AUUU$_n$A-3′ repeats that are associated with the decreased stability of many cytokine and protooncogene mRNAs (34, 35). The presence of these motifs is consistent with the profound superinducibility of the 4.1-kb mRNA by cycloheximide (ref. 18; see Fig. 4), which is significantly greater than that for the 2.8-kb mRNA (see below). Xie *et al.* (36) have cloned a src-induced 4.1-kb cDNA from chicken embryo fibroblasts that shows similarities to the ovine PGHS. Comparison of our clone with theirs clearly shows that our 4.1-kb cDNA is the murine homolog of the chicken gene with 95% of all amino acid residues being identical. During preparation of this paper, Kujubu *et al.* (37) reported the cloning of a 4.1-kb cyclooxygenase-related

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  25 CTTCAGGAGTCAGTCAGGACTCTGCTCACGAAGGAACTCAGCACTGCATCCTGCCAGCTC
  85 CACCGCCACCACTACTGCCACCTCCGCTGCCACCTCTGCGATGCTCTTCCGAGCTGTGCT
                                                 M  L  F  R  A  V  L
 145 GCTCTGCGCTGCCCTGGGGCTCAGCCAGGCAGCAAATCCTTGCTGTTCCAATCCATGTCA
   8  L  C  A  A  L  G  L  S  Q  A  A  N  P  C  C  S  N  P  C  Q
 205 AAACCGTGGGGAATGTATGAGCACAGGATTTGACCAGTATAAGTGTGACTGTACCCGGAC
  28  N  R  G  E  C  M  S  T  G  F  D  Q  Y  K  C  D  C  T  R  T
 265 TGGATTCTATGGTGAAAACTGTACTACACCTGAATTTCTGACAAGAATCAAATTACTGCT
  48  G  F  Y  G  E  N  C  T  T  P  E  F  L  T  R  I  K  L  L  L
 325 GAAGCCCACCCCAAACACAGTGCACTACATCCTGACCCACTTCAAGGGAGTCTGGAACAT
  68  K  P  T  P  N  T  V  H  Y  I  L  T  H  F  K  G  V  W  N  I
 385 TGTGAACAACATCCCCTTCCTGCGAAGTTTAATCATGAAATATGTGCTGACATCCAGATC
  88  V  N  N  I  P  F  L  R  S  L  I  M  K  Y  V  L  T  S  R  S
 445 ATATTTGATTGACAGTCCACCTACTTACAATGGTTACAAAAGCTGGGAAGC
 108  Y  L  I  D  S  P  P  T  Y  N  V  H  Y  G  Y  K  S  W  E  A
 505 CTTCTCCAACCTCTCCTACTACACCAGGGCCCTTCCTCCCGTAGCAGATGACTGCCCAAC
 128  F  S  N  L  S  Y  Y  T  R  A  L  P  P  V  A  D  D  C  P  T
 565 TCCCATGCGTGTGAAGGGAAATAAGGAGCTTCCTGATTCAAAAGAAGTGCTGGAAAAGGT
 148  P  M  G  V  K  G  N  K  E  L  P  D  S  K  E  V  L  E  K  V
 625 TCTTCTACGGAGAGAGTTCATCCCTGACCCCCAAGGCTCAAATATGATGTTTGCATTCTT
 168  L  L  R  R  E  F  I  P  D  P  Q  G  S  N  M  M  F  A  F  F
 685 TGCCCAGCACTTCACCCATCAGTTTTTCAAGACAGATCATAAGCGAGGACCTGGGTTCAC
 188  A  Q  H  F  T  H  Q  F  F  K  T  D  H  K  R  G  P  G  F  T
 745 CCGAGGACTGGGCCATGGAGTGGACTTAAATCACATTTATGGTGAAACTCTGGACAGACA
 208  R  G  L  G  H  G  V  D  L  N  H  I  Y  G  E  T  L  D  R  Q
 805 ACATAAACTGCGCCTTTTCAAGGATGGAAAATTGAAATATCAGGTCATTGGTGGAGAGGT
 228  H  K  L  R  L  F  K  D  G  K  L  K  Y  Q  V  I  G  G  E  V
 865 GTATCCCCCACAGTCAAAGACACTCAGGTAGAGATGATCTACCCTCCTCACATCCCTGA
 248  Y  P  P  T  V  K  D  T  Q  V  E  M  I  Y  P  P  H  I  P  E
 925 GAACCTGCAGTTTGCTGTGGGGCAGGAAGTCTTTGGTCTGGTGCCTGGTCTGATGATGTA
 268  N  L  Q  F  A  V  G  Q  E  V  F  G  L  V  P  G  L  M  M  Y
 985 TGCCACCATCTGGCTTCGGGAGCACAACAGAGTGTGCGACATACTCAAGCAGGAGCATCC
 288  A  T  I  W  L  R  E  H  N  R  V  C  D  I  L  K  Q  E  H  P
1045 TGAGTGGGGTGATGAGCAACTATTCCAAACCAGCAGACTCATACTCATAGGAGAGACTAT
 308  E  W  G  D  E  Q  L  F  Q  T  S  R  L  I  L  I  G  E  T  I
1105 CAAGATAGTGATCGAAGACTACGTGCAACACCTGAGCGGTTACCACTTCAAACTCAAGTT
 328  K  I  V  I  E  D  Y  V  Q  H  L  S  G  Y  H  F  K  L  K  F
1165 TGACCCAGAGCTCCTTTTCAACCAGCAGTTCCAGTATCAGAACCGCATTGCCTCTGAATT
 348  D  P  E  L  L  F  N  Q  Q  F  Q  Y  Q  N  R  I  A  S  E  F
1225 CAACACACTCTATCACTGGCACCCCCTGCTGCCCGACACCTTCAACATTGAAGACCAGGA
 368  N  T  L  Y  H  W  H  P  L  L  P  D  T  F  N  I  E  D  Q  E
1285 GTACAGCTTTAAACAGTTTCTCTACAACAACTCCATCCTCCTGGAACATGGACTCACTCA
 388  Y  S  F  K  Q  F  L  Y  N  N  S  I  L  L  E  H  G  L  T  Q
1345 GTTTGTTGAGTCATTCACCAGACAGTTGCTGGCCGGGTTGCTGGGGGAAGAAATGTGCC
 408  F  V  E  S  F  T  R  Q  I  A  G  R  V  A  G  G  R  N  V  P
1405 AATTGCTGTACAAGCAGTGGCAAAGGCCTCCATTGACCAGAGCAGAGAGATGAAATACCA
 428  I  A  V  Q  A  V  A  K  A  S  I  D  Q  S  R  E  M  K  Y  Q
1465 GTCTCTCAATGAGTACCGGAAACGCTTCTCCCTGAAGCCGTACACATCATTTGAAGAACT
 448  S  L  N  E  Y  R  K  R  F  S  L  K  P  Y  T  S  F  E  E  L
1525 TACAGGAGAGAAGGAAATGGCTGCAGAATTGAAAGCCCTCTACAGTGACATCGATGTCAT
 468  T  G  E  K  E  M  A  A  E  L  K  A  L  Y  S  D  I  D  V  M
1585 GGAACTGTACCCTGCCCTGCTGGTGGAAAAACCTCGTCCAGATGCTATCTTTGGGGAGAC
 488  E  L  Y  P  A  L  L  V  E  K  P  R  P  D  A  I  F  G  E  T.
1645 CATGGTAGAGCTTGGAGCACCATTCTCCTTGAAAGGACTTATGGGAAATCCCATCTGTTC
 508  M  V  E  L  G  A  P  F  (S)  L  K  G  L  M  G  N  P  I  C  S
1705 TCCTCAATACTGGAAGCCGAGCACCTTTGGAGGCGAAGTGGGTTTTAAGATCATCAATAC
 528  P  Q  Y  W  K  P  S  T  F  G  G  E  V  G  F  K  I  I  N  T
1765 TGCCTCAATTCAGTCTCTCATCTGCAATAATGTGAAGGGGTGTCCCTTCACTTCTTTCAA
 548  A  S  I  Q  S  L  I  C  N  N  V  K  G  C  P  F  T  S  F  N
1825 TGTGCAAGATCCACAGCCTACCAAAACAGCCACCATCAATGCAAGTGCCTCCCACTCCAG
 568  V  Q  D  P  Q  P  T  K  T  A  T  I  N  A  S  A  S  H  S  R
1885 ACTAGATGACATTAACCCTACAGTACTAATCAAAAGGCGTTCAACTGAGCTGTAAAAGTC
 588  L  D  D  I  N  P  T  V  L  I  K  R  R  S  T  E  L
1945 TACTGACCATATTTATTTATTTATGTGAAGAATTTAATTTAATTATTTAATATTTATACT
2005 GAATTTTTTTTCATGTAACATCTTCCATAACAGAAGGCAATGTTCTTGAACAATGTTACA
2065 TTTGTGAAGATTCCCTCCGGTGTTTGTCCTTTAAATATGTGTTACCTGAAACTGAAAGGA
2125 AATCAGCATTCATTCCTCTACATAAGCCAGTGAGAAGGGAAATGAATTTTGATATCTTTA
2185 TACTTGAATTTCAGATCATGATTAGCTTAACAAGAACCAAGGAAAAATGTATGAATATGT
2245 GAGTGTTGTTACAAGATGAAAAATGCTGCAGGTATCAACActgttGGTTACAACTGTGTC
2305 TTCTTTACTATGATAGGAGCATGTAATGTGGAATTCTTCTTAAACTTGCATATCTTTAT
2365 CTCATCAAACAAAGGGGTCCAAGTTCAGTTTTAAATAAGCATTTAAGGCAGATACTGACA
2425 ACAATCTCATTTTTTAAAATGTTGTCTTGAGACAAATAATTTGAAATTTCTAAATTGGGA
2485 GTTTGAATCACTTTTGAAAGCTCTTACTTTCTTAAGCTGTCAGGTTTGTACCGACATGGA
2545 GTAAACAGCTATCATAAACGTAAATCTCCAAAACTAGTAGAAATTATGTCATGATTGATG
2605 GTTAAGATACCATGTCAGGGATTGTCTTTTCTTAGAAGTAGTGAAAGCTACTTACTATGA
2665 CAATCAGACCTTCCTTGTATGTCAAAATGCTGGTGTGGAAGGTGGTGGAGCCCGTGCTGC
2725 TCTGTCTTAACTATGAGTGTGAGCTTTAAAGCTCGTTGATGAGTGGTAGCCAGCAAAGCC
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2965 TTTTTCTGTTGCACAGTATGATGTAACAGTCCATCTCTCAATGCAATAGGTATCAGTGGC
3025 CTCGTGAGCTTCTTCACAATATTGATATGTCTTCCAGCCCATTGAACCTGGACTGCAGAA
3085 GGCCCCATGTCATGTGTGAGCTCAGCCTGGATGCCAGCATTGCTGCTCCTCTTAGTTCCG
3145 TTTCTCGTGGTCACTTTACTACGAGAAACGCTGATTGGGTTTTCGTAGCTGTGTTCCAGG
3205 TTTTTAGTATCAGAACTATTCTTTCTTTAACCTCTATTCATATTTTCTCTACTTGAAGTT
3265 TTACATTCAGGAAAACCTCAGCTCAGGACTACTATGTACCTCCCCTTTGGAGGGAAAAAT
3325 TATTTTAGGTAAAAGGCAAAAATTTTTTAAAAATATTTTTATTTATAATTATATGGAAGG
3385 GCCCTACCAAGATGCTAGAAATATAGGGAGTTCCTGACAAGAAATTTCCATTCTTATTCT
3445 GAAGAATTGCTTTCTTACTTAAAAACAAAGACAGTTTGTGAGTAGTTCTGGGCAATAGGG
3505 ATAAATATAAAACAATAATGATGATCATTTTCTACATCTCATTATCAGCTGAGGTACTGT
3565 ATATTACTGAATTTATTGAAGATAGTTATGTCTTTTAGACATTGTTGTTATAAACTATGT
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3745 GATAAATTCTAAGAAAACTAGTGTATTTTTTTGAAAAGTTTGAAGTTAGAACTTAGGCTG
3805 TTGGAATTTACGCATAAAGCAGACTGCATAGATCCAATATTGACTGACCCAAGCATGTTA
3865 TAAAGACTGACATTTTAGACATTTTGAAGGCCCTGTAAGTGTTTATTAATTAGTTAGAAC
3925 TTAATTGATTAAAAAATATATCCAAAGCACTATAGGCATTAGAATTCGGGCATCAAGAAA
3985 TGATGACAAATAATACTGTTATTTATATAAATAACTAAAAAGGGTGTCTAATGAAGAAAT
4045 ATATTTTATTACAAAGAAATTATAAAACATTTTGAAGATTATATGCTTTAAAAGTTTAAG
4105 ATTGAAAAAATAATCAACCTTAGAAAAAATGTATAAAAATATATAAATTGTTAATGTCAT
4165 TGATTAAAAAAA  4176
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FIG. 1. cDNA and predicted amino acid sequence of glucocorticoid-regulated inflammatory PGHS (griPGHS). Based on a transcription start site determined by primer extension at −24 (M.K.O., unpublished data), the numbering of this sequence starts at 25. A predicted signal peptide cleavage site between amino acids 17 and 18 (32) is marked with an arrowhead. The position of the putative aspirin-modified serine is indicated by a circle and potential N-glycosylation sites are double underlined. A total of 18 5′-AUUU$_n$A-3′ motifs are single underlined.

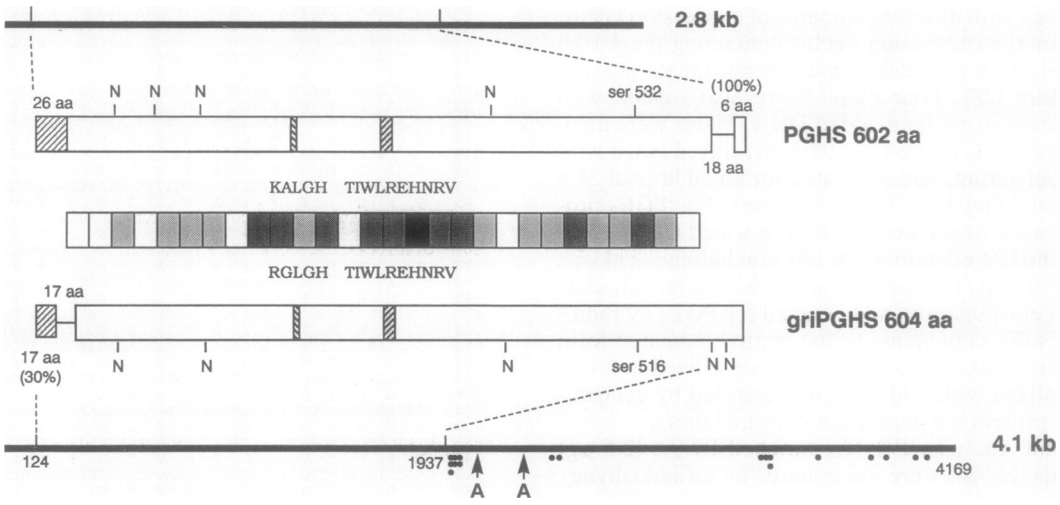


FIG. 2. Comparison of cDNA and protein sequences for the murine 2.8- and 4.1-kb mRNA-encoded cyclooxygenases. cDNA structures for the 4.1-kb cDNA cloned from C127 cells and the murine 2.8-kb cDNA (14) are drawn as thick lines at top and bottom. Alternative polyadenylylation sites established from other cDNA clones are indicated with an "A" and the 5′-AUUU$_n$A-3′ motifs are identified by dots below the sequence. These motifs are not found in the 2.8-kb cDNA. Deduced protein sequences are drawn colinearly with gaps [17 amino acids (aa) at the N-terminal end of the 4.1-kb mRNA product and 18 aa at the C-terminal end of the 2.8-kb mRNA product] indicated by connecting lines. The 26-aa leader sequence for the 2.8-kb PGHS is indicated. Although its extent has not been precisely defined, a shorter, nonhomologous leader appears to exist for griPGHS with a mature N-terminal end at amino acid 18 (32). The positions of potential N-glycosylation sites (NXS/T; "N") and the conserved aspirin-modified serines are noted on each molecule. Hatched areas near the center of each molecule denote presumed axial (TIWLREHNRV, identical between the two molecules) and distal (KALGH/RGLGH) heme-binding sites as suggested by DeWitt *et al.* (14). Bar with different levels of shading represents similarities between the two mouse PGHS proteins (omitting the nonconserved N and C termini) as the percentage of identical residues for groups of 20 amino acids with increasing shading indicating 40–55% (no shading), 60–75%, 80–95%, and 100% identity. The overall identity is 64% and with conservative changes the similarity index is 79%.

cDNA (TIS10) from phorbol ester-stimulated mouse fibroblasts that is nearly identical to the cDNA described here. Our sequence differs from TIS10 at one amino acid (an isoleucine instead of threonine at position 98). In addition, our sequence includes the polyadenylylated tail, making it 197 bases longer than the TIS10 clone.

To determine whether the 4.1-kb mRNA encodes a protein with cyclooxygenase activity, the cDNA was inserted into a simian virus 40 late promoter expression vector [SVL (38)]. COS cells, which have little or no endogenous cyclooxygenase activity (14), were transfected with 2.5 or 5 μg of either the vector alone or the vector containing the 4.1-kb cDNA. Two-dimensional gel electrophoresis of $^{35}$S-labeled proteins from transfected cells showed a protein doublet (72/74 kDa; pI 7.5) in the 4.1-kb cDNA-expressing cells that corresponds exactly to the immunoprecipitated cyclooxygenase protein doublet we observed in C127 mouse fibroblasts (18) whose synthesis is increased by growth factors and decreased by glucocorticoid hormones (Fig. 3). Transfected cells were also assayed for cyclooxygenase activity. COS cells expressing the 4.1-kb cDNA produced nearly 2 orders of magnitude more $PGE_2$ than control cells (Table 1). Furthermore, prostaglandin production increased with the amount of transfected DNA. These results unequivocally demonstrate that the 4.1-kb mRNA encodes an active cyclooxygenase, which we call griPGHS.

Based on our previous two-dimensional gel experiments, griPGHS protein synthesis increases by as much as 35-fold upon 3-h exposure of quiescent Swiss 3T3 mouse fibroblasts to serum, platelet-derived growth factor (PDGF), or the phorbol ester phorbol 12-myristate 13-acetate (ref. 39; H. B. Sadowski and M.K.O., unpublished data). These increases in synthesis were abrogated in the presence of dexamethasone. Similar results were obtained in mouse C127 fibroblasts treated with serum or PDGF (18) (Fig. 3). We have also shown that v-*src* activation induces griPGHS protein synthesis. This induction correlates with an increase in prostaglandin synthesis and is repressed by dexamethasone (8). These results are consistent with those of Xie *et al.* (36), who isolated the chicken homolog of the 4.1-kb PGHS on the basis of its induced expression in *src*-transformed cells.

We have compared expression of the 4.1- and 2.8-kb mRNAs in two systems. In murine fibroblasts, serum and dexamethasone have little or no effect on 2.8-kb mRNA levels (Fig. 4). In contrast, addition of fresh serum increases the basal level of the 4.1-kb mRNA 5-fold within 2 h. Dexamethasone abrogates this response and reduces basal levels 4-fold (Fig. 4). Moreover, there is a >10-fold accumu-

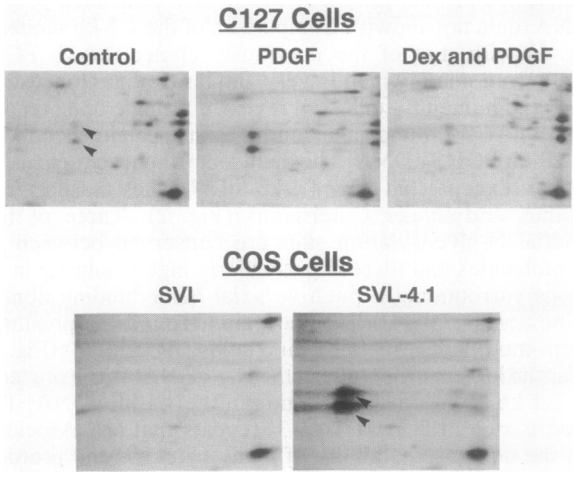


FIG. 3. The 72/74-kDa PGHS doublet is encoded by the 4.1-kb mRNA. Subconfluent C127 cells were metabolically labeled in the last 30 min of a 2-h treatment with PDGF (50 ng/ml; BB homodimer; Collaborative Research) or PDGF/dexamethasone (1 μM) in serum-free medium. Cells were lysed and subjected to giant two-dimensional gel electrophoresis. (*Upper*) Equivalent regions of autoradiographs from the basic end of the gels are shown. Arrowheads indicate griPGHS. (*Lower*) $^{35}$S-labeled proteins in the same region of two-dimensional gels from COS cells transfected with 5 μg of vector alone (SVL) or 5 μg of vector containing the 4.1-kb cDNA (SVL-4.1). COS cell proteins were metabolically labeled 48 h after transfection.

Table 1. Expression of 4.1-kb cDNA in COS cells leads to prostaglandin synthesis

| DNA | Amount, μg | $PGE_2$, pg per μg of protein |
|---|---|---|
| None | — | 0.56, 0.58, 0.51, 0.50 |
| SVL | 2.5 | 0.55, 0.68 |
| SVL | 5.0 | 0.63, 0.65 |
| SVL-4.1 | 2.5 | 14.8, 24.6 |
| SVL-4.1 | 5.0 | 63.8, 42.4 |

Subconfluent COS A.2 cells in duplicate 60 mm plates were transfected with the indicated amounts of expression vector alone (SVL) or expression vector containing the 4.1-kb cDNA (SVL-4.1) and assayed for $PGE_2$ production 2 days later.

lation of 4.1-kb mRNA in the presence of cycloheximide, consistent with the long 3′ untranslated region containing AUUUA repeats. Cytokine mRNAs containing AUUUA motifs have longer half-lives in cells treated with growth factors and are less stable in cells exposed to glucocorticoids (34, 40–42). Thus, griPGHS mRNA levels may in part be determined by effects on mRNA stability. In addition, shorter exposures of the filter in Fig. 4 clearly showed modulation of 4.1-kb mRNA levels by serum and dexamethasone in the presence of cycloheximide, suggesting transcriptional regulation. The relative contributions of each of these mechanisms (mRNA stability and transcription) to overall 4.1-kb mRNA levels have yet to be determined.

Interestingly, we detected a diffuse set of less-abundant transcripts in the 2.0- to 2.3-kb range by using the 4.1-kb cDNA probe that are distinct from the single band detected in all lanes when the same filter was probed with the 2.8-kb cDNA (Fig. 4; particularly evident in the cycloheximide-treated samples). We also observed a similar situation in human monocyte RNAs probed with the 4.1-kb cDNA (Fig. 5). The appearance of these bands on Northern blots is consistent with our analysis of the mouse cDNA library: two of four isolated clones were polyadenylylated at nucleotides

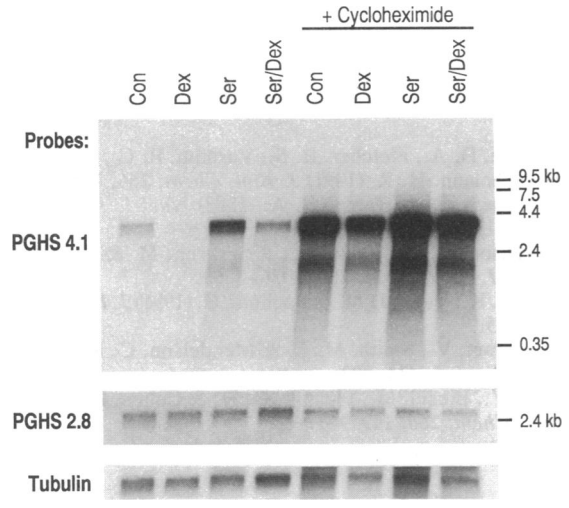


FIG. 4. Regulation of the 4.1-kb, but not the 2.8-kb, PGHS mRNAs in murine fibroblasts. Subconfluent C127 cells grown for 2 days in DMEM supplemented with 10% fetal bovine serum were treated with dexamethasone (Dex) (1 μM), fresh serum (10%), or both in the presence or absence of 25 μM cycloheximide (added 15 min earlier) for 2 h. Poly(A)-enriched RNAs (2.5 μg) were fractionated by formaldehyde/agarose gel electrophoresis, transferred to a membrane, and hybridized with the indicated probes. Tubulin signals in the cycloheximide-treated RNA lanes [particularly control (Con) and serum (Ser) lanes] are partially obscured in this reproduction by residual signal from the PGHS 4.1-kb probe. Densitometric scanning shows that the amount of tubulin in each of the cycloheximide-treated samples is essentially equivalent.

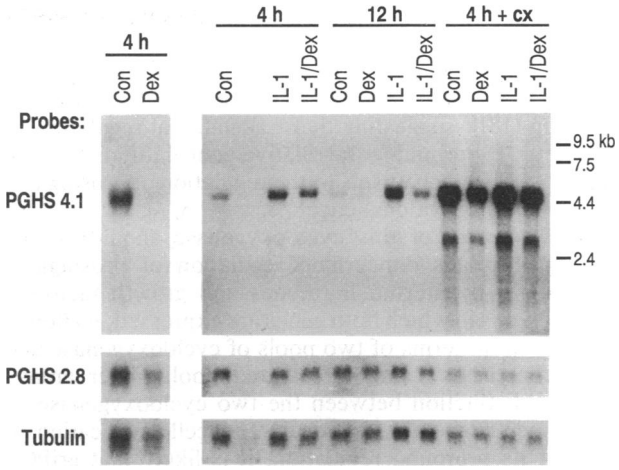


FIG. 5. griPGHS mRNA expression in human monocytes. Adherent human monocytes isolated from healthy donors were suspended in medium without serum at $1 \times 10^6$ cells per ml. One-milliliter aliquots in 5-ml polypropylene tubes were incubated with loosened caps in 5% $CO_2$/95% air at 37°C with occasional shaking. Monocytes were incubated with dexamethasone (Dex) (1 μM), IL-1β (10 half-maximal units; Collaborative Research), or both for the indicated times before total RNA isolation. Cycloheximide (cx) (25 μM) was added to one set of incubations 15 min before the addition of cytokine or hormone. Five micrograms of each RNA was subjected to Northern blot analysis with the indicated probes. Two separate experiments are represented. Con, control.

2052 and 2256 of the cDNA (Fig. 2). Therefore, in addition to regulation at the level of transcription and mRNA stability, griPGHS expression may also be regulated by alternative polyadenylylation.

We have also examined the expression of the two cyclooxygenase genes in freshly isolated human monocytes. In Northern blots of total monocyte RNA, a 4.8-kb mRNA species is detected with the mouse griPGHS 4.1-kb probe. When normalized to the hybridization signal for β-tubulin, griPGHS mRNA levels are down-regulated by dexamethasone at 4 h (5-fold in this example), while the level of the 2.8-kb PGHS mRNA is not affected (Fig. 5). In this same experiment, the level of accumulated $PGE_2$ in the supernatant after 4 h of incubation was reduced by dexamethasone from 122.5 to 52.5 pg per $10^4$ monocytes. In another experiment, monocytes treated with IL-1β showed increased levels of griPGHS mRNA at 4 h (2.5-fold relative to control) and 12 h (14-fold) (Fig. 5). These increases were significantly blunted when dexamethasone was present. Furthermore, the IL-1β induction and dexamethasone repression of griPGHS mRNA abundance occurred in the presence of cycloheximide, where superinduction of the 4.8-kb mRNA was clearly evident (Fig. 5). In contrast, levels of the 2.8-kb mRNA were not significantly altered relative to β-tubulin by IL-1β, dexamethasone, or cycloheximide treatment. Needleman and coworkers (6, 7) have shown that lipopolysaccharide induces cyclooxygenase in human monocytes and mouse macrophages and that this induction is abrogated by dexamethasone treatment, whereas control levels of prostaglandin production are only modestly affected. Their experiments suggest that two pools of cyclooxygenase exist, a model that our results now confirm.

Regulation of the 4.1-kb mRNA for griPGHS has the potential for occurring at several levels including transcription, stability, and alternative polyadenylylation. The rapid turnover of griPGHS mRNA allows tightly controlled responses to multiple stimuli, including inhibition by glucocorticoids and stimulation by PDGF, epidermal growth factor, IL-1β, fibroblast growth factor, phorbol 12-myristate 13-acetate, the calcium ionophore A23187, forskolin, and *src*

activation (refs. 8, 18, and 39; H. B. Sadowski and M.K.O., unpublished data). This dynamic modulation of message level may not fully account for the very rapid decline of griPGHS protein synthesis in cells exposed to dexamethasone [>75% in 45 min (18)], suggesting that translational regulation may also occur. Determining the relative contributions of each of these possible mechanisms to the regulation of prostaglandin synthesis remains a challenge.

The discovery of this cyclooxygenase should clarify a number of issues concerning regulation of prostaglandin biosynthesis by steroid hormones and growth factors. In those systems in which both genes are expressed, it allows an examination in terms of two pools of cyclooxygenase activity: a constitutive and a regulated pool. Other areas of potential distinction between the two cyclooxygenase species include tissue distribution and subcellular location. Because of its profound regulation, it is likely that griPGHS plays a major role in some if not all of these processes. Indeed, based on its regulation in human monocytes and similarities between the 3′ untranslated regions of griPGHS and many cytokines, we speculate that griPGHS is the predominant cyclooxygenase mediating the inflammatory response.

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