

Expression of a mitogen-responsive gene encoding prostaglandin synthase is regulated by mRNA splicing

(cyclooxygenase/Rous sarcoma virus/immediate-early gene/pp60^{v-src})

WEILIN XIE*, JEFFREY G. CHIPMAN*, DONALD L. ROBERTSON*, R. L. ERIKSON†, AND DANIEL L. SIMMONS*‡

*Department of Chemistry, 226 Eyring Science Center, Brigham Young University, Provo, UT 84602; and †Department of Cellular and Developmental Biology, Harvard University, Cambridge, MA 02138

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ABSTRACT Rous sarcoma virus was shown to induce in chicken embryo fibroblasts (CEF) a 4.1-kilobase mRNA (designated CEF-147) encoding a 603-amino acid protein. Analysis of the protein sequence showed that it shared 59% amino acid identity with sheep prostaglandin G/H synthase, the enzyme that catalyzes the rate-limiting steps in the production of prostaglandins. Significant differences, at both the protein and mRNA levels, existed between the *src* oncogene product-inducible prostaglandin synthase and the protein isolated and cloned from sheep seminal vesicle, suggesting that the *src*-inducible prostaglandin synthase may be a new form of the enzyme. A distinguishing feature of *src*-inducible prostaglandin synthase mRNA is its low abundance in nonproliferating chicken embryo fibroblasts and its relatively high abundance in *src*-transformed cells. Additionally, the majority of the *src*-inducible prostaglandin synthase RNA present in nonproliferating cells was found to be nonfunctional because of the presence of an unspliced intron that separated the signal peptide from the remainder of the protein. Upon mitogenic stimulation, this intron was removed, resulting in the induction of fully-spliced CEF-147 mRNA.

Neoplastic transformation of chicken embryo fibroblasts (CEF) by Rous sarcoma virus (RSV) results in the immediate-early activation of a set of cellular genes encoding secretory growth factors and transcription factors involved in the regulation of cell division (1–3). Induction of these genes is dependent on the activity of the *v-src* oncogene product pp60^{v-src}, a tyrosine kinase. The genes pp60^{v-src} induces are also activated by other cell-division stimulators such as the tumor promoter phorbol 12-myristate 13-acetate (PMA), as well as serum and epidermal growth factor (1, 2); however, transformation by a temperature-sensitive mutant of RSV produces different induction kinetics when compared with induction by serum or PMA. The most important difference is that pp60^{v-src} persistently elevates expression of many of its immediate-early genes, whereas serum and in most cases PMA cause only transient increases in mRNA levels that return to baseline levels within hours after mitogenic stimulation. Furthermore, pp60^{v-src} increases some mRNAs in biphasic or oscillating patterns, whereas serum treatment increases these same mRNAs in a monophasic fashion (1).

We have reported (1) the isolation of a set of cDNAs corresponding to pp60^{v-src}-inducible immediate-early genes in chicken embryo fibroblasts (CEF). In the present communication, we report that one of these genes, called CEF-147, encodes a protein related to prostaglandin G/H synthase (cyclooxygenase, EC 1.14.99.1; PGHS), the enzyme that catalyzes the rate-limiting steps in prostaglandin synthesis, and the site at which non-steroidal antiinflammatory drugs exert many of their pharmacological effects. Numerous stud-

ies have been done on the relationship of prostaglandin synthesis and cell division, and it is now well established that many mitogens induce PGHS activity (4–11). Significantly, it has also been shown that some nonsteroidal antiinflammatory drugs exert antiproliferative and antitumor activities *in vitro* and *in vivo*, suggesting that PGHS plays an important role in regulating or promoting cell proliferation in some normal and neoplastically transformed cells (12–15).

RSV-inducible prostaglandin synthase encoded by the CEF-147 cDNA showed several important features that distinguished it from the only form of the enzyme thus far cloned, which was first isolated from sheep seminal vesicles (this sheep form is hereafter termed PGHS_{ov}) (16–18) and later cloned by cross-hybridization techniques in mouse (19) and human (20). The pp60^{v-src}-inducible form we term “miPGHS_{ch}” for mitogen-inducible PGHS_{chicken}.

Of the cDNAs isolated that encode miPGHS_{ch}, several contained a 553-base-pair (bp) unspliced intron located 17 amino acids from the amino terminus, which prohibited translation of the sequence. Northern blots showed that the majority of miPGHS_{ch} mRNA in contact-inhibited, nontransformed CEF grown in low serum contained this intron and, hence, was nonfunctional. Upon transformation and mitogenic stimulation by *v-src*, fully spliced miPGHS_{ch} mRNA increased rapidly in the cell.

MATERIALS AND METHODS

Cloning and Sequencing of CEF-147 cDNAs. To isolate a full-length CEF-147 cDNA, two λ ZAP (Stratagene) cDNA libraries were screened with a 2.7-kilobase (kb) CEF-147 cDNA insert. This clone had previously identified an \approx 5.0-kb immediate-early mRNA induced by pp60^{v-src} (1). One library screened was constructed from CEF infected with temperature-sensitive tsNY72-4RSV cultured at high density at the nonpermissive temperature (41.5°), and the other library was from virus-infected cells cultured at the permissive temperature (35°) for 4–6 hr in the presence of 75 μ M cycloheximide. From the former library a 4.4-kb cDNA was isolated. The 5'-terminal 100 bp of this cDNA contained a series of 16 nucleotide repeats. An antisense oligonucleotide, no. 773 (5'-AGCACCGGGACGACAGCCGGAGCCCCGCA-3'), complementary to a sequence in this region (nucleotides 388–417, Fig. 1) was used to rescreen both libraries, yielding several independent isolates \approx 4.7 kb in size. Both strands of one of these isolates were sequenced in their entirety by the dideoxy method with Sequenase from United States Biochemical (Fig. 1).

RNA Isolation and Blot-Hybridization (Northern) Analysis. RNA for gel blot analysis was obtained from CEF as de-

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Abbreviations: CEF, chicken embryo fibroblasts; PGHS, prostaglandin G/H synthase; PMA phorbol 12-myristate 13-acetate; RSV, Rous sarcoma virus; PCR, polymerase chain reaction.
‡To whom reprint requests should be addressed.

In Vitro Transcription and Translation of CEF-147. DNA clones isolated from the λ ZAP libraries were converted to Bluescript SK(-) phagemids by helper phage infection as described by the manufacturer (Stratagene). Sense and antisense cRNAs were transcribed from a spliced 4.1-kb CEF-147 cDNA by using T3 and T7 RNA polymerases. Transcription was done as described (25) in the presence of 0.3 mM $m^7G(5')ppp(5')G$ purchased from Pharmacia LKB to produce capped transcripts, which were translated in rabbit reticulocyte lysate containing [35 S]methionine as described (25). Cotranslational glycosylation and peptide cleavage were investigated by performing *in vitro* translation in the presence of canine pancreatic microsomes purchased from Promega.

In vitro translation products were visualized by sodium dodecyl sulfate/polyacrylamide gel electrophoresis followed by autoradiography (26).

RESULTS

Sequence and Expression of CEF-147 in Mitogen-Stimulated Cells. The cloning of a partial CEF-147 cDNA has been described (1). Briefly, the cDNA was isolated from CEF infected with tsNY72-4RSV, a temperature-sensitive mutant of Rous sarcoma virus. The CEF-147 cDNA detected rare 3.0-kb and \approx 5.0-kb mRNAs in nontransformed CEF. When pp60^{v-src} in tsNY72-4RSV-infected cells was activated by temperature shift, cellular levels of the \approx 5.0-kb mRNA were observed to increase in the presence or absence of cycloheximide, an inhibitor of protein synthesis (1, 2). Induction of the \approx 5.0-kb mRNA occurred biphasically, with an early increase being reached in 1 hr and a second 10-fold higher increase occurring at 4 hr and remaining elevated as long as pp60^{v-src} activity was present (1). Serum and PMA also were found to induce the \approx 5.0-kb CEF-147 mRNA, which increased in concentration briefly at 1–2 hr posttreatment, after which it quickly returned to basal levels (1). Similar to the effect of pp60^{v-src}, serum and PMA not only induced CEF-147 mRNA in the presence of cycloheximide but also induced CEF-147 mRNA to a higher level than that produced in the absence of translation inhibitor (1). In contrast, the 3.0-kb RNA was not induced by pp60^{v-src}, serum, or PMA.

The DNA sequence at the ends of the 2.7-kb partial CEF-147 cDNA showed that one end contained a consensus polyadenylation signal and poly(A) tract corresponding to the 3' end of the mRNA. The other end contained an open reading frame that was used in a global search of the GenBank and National Biomedical Research Foundation data bases. Significant sequence identity (62%) was detected between the translation product of this open reading frame and the carboxyl-terminal 119 amino acids of PGHS_{ov}. This 2.7-kb cDNA was used eventually to isolate a 4.7-kb cDNA as explained in *Materials and Methods*.

The cDNA sequence obtained from the 4.7-kb cDNA contained a long open reading frame extending from nucleotide 697 to 2409 (Fig. 1) that predicted a translation product with 57% sequence identity to PGHS_{ov}.

Comparison of the 4.7-kb CEF-147-predicted protein with PGHS_{ov} revealed that the former molecule lacked a proper hydrophobic amino-terminal signal peptide to target it to the endoplasmic reticulum; instead, the initiating methionine for the open reading frame (Met-33 in Fig. 1) appeared to be located in a hydrophilic stretch of amino acids and was encoded by an AUG that was unfavorable for initiation of translation by Kozak's criteria (27). Both of these facts made it unlikely that this methionine was the correct initiating site for translation.

Further analysis of the 4.7-kb CEF-147 sequence showed that a suitable initiating methionine codon (Met-1 in Fig. 1) with a hydrophobic signal peptide was located 648 nucleotides upstream of Met-33, but out of frame with the long open

reading frame encoding the PGHS-like protein. The sequence separating Met-1 from the long open reading frame contained 18 tandem copies of a 16-nucleotide repeat. This sequence also contained a near-consensus splice donor site at nucleotides 97–105 (which also encodes the cleavage site of the signal peptide). A splice acceptor site was found at nucleotides 642–653 (Fig. 1). These data suggested that the intervening sequence separating Met-1 and the body of the PGHS protein was caused by the nonremoval of an intron.

Identification of Spliced CEF-147. Polymerase chain reaction (PCR) was performed to identify the spliced form of CEF-147 by using a primer pair located at nucleotides 46–66 and 1383–1399 of the CEF-147 sequence shown in Fig. 1. The template for the PCR reaction was cDNA made from RSV-transformed CEF mRNA. A prominent product of the PCR reaction was a 0.8-kb cDNA, the size expected from mRNA that had experienced splicing of the putative intron at the splice junctions identified. This PCR product was cloned and sequenced, showing that the intron (nucleotides 100–652) had been removed, which resulted in the signal peptide being brought into frame with the CEF-147 long open reading frame (data not shown).

A 30-residue oligonucleotide (5'-AGCAGCAAGGGTTG-GCTGCGTGGCCGCAG-3'), oligo 790, specific for the spliced form was synthesized as the antisense sequence of the 15 exonic nucleotides flanking the splice junctions of the 5' intron and was used to isolate a full-length spliced cDNA from the cDNA libraries. The sequence of the spliced cDNA was 4.1 kb long and was identical to the sequence of the 4.7-kb CEF-147 clone except for the removal of the 5' intron. The predicted translation product of the long open reading frame of the spliced CEF-147 cDNA contained 603 amino acids and constituted the primary sequence of miPGHS_{ch} (Fig. 1).

Mitogen-Stimulated Splicing of the 5' CEF-147 Intron. Northern blots of total cellular RNA appeared to detect only one inducible \approx 5.0-kb mRNA (1). Further characterization showed that the \approx 5.0-kb mRNA seen in previous Northern blot experiments (1) was a mixture of both spliced and unspliced forms that could be electrophoretically separated by extending the electrophoresis time (Fig. 2). (The term "unspliced" here refers only to the nonremoval of the 5' CEF-147 intron; the remainder of the CEF-147 transcript is fully processed.) Since the unspliced CEF-147 mRNA was relatively rare, it was also necessary that Northern blots be performed with poly(A)-selected mRNA.

Oligonucleotides 790 and 773 were used in Northern blot experiments to detect the spliced and unspliced forms of CEF-147 mRNA, respectively. These oligonucleotides, as well as a cDNA from the 3' end of CEF-147 that cross-reacted with both spliced and unspliced forms, were used to probe mRNA from CEF infected with tsNY72-4RSV. The results obtained with all three probes confirmed our previous observation that CEF-147 was a very rare mRNA in nonmitogen-stimulated cells, since none of the probes effectively detected a transcript in CEF held at 41.5°C (0-hr cells) except upon very long exposures of the autoradiogram (Fig. 2; ref. 1). Furthermore, the data in Fig. 2 also clearly indicated that the transcript induced by pp60^{v-src} was fully spliced and that no appreciable induction of the unspliced transcript occurred.

In the light of its low abundance, these data raised the question as to why the unspliced form, which was originally isolated as a 4.4-kb cDNA from CEF cultured at 41.5°C in medium with low-serum content, was ever isolated. Therefore, we analyzed CEF-147 expression by Northern blots in contact-inhibited cells grown in low-serum medium and found that unspliced CEF-147 represented \sim 70% of CEF-147 transcripts (Fig. 2). Shifting these cells to 35°C caused a rapid increase in the spliced form such that by 16 hr after temper-

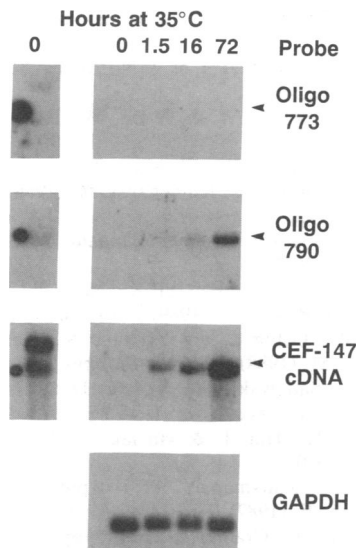


FIG. 2. Induction of CEF-147 by pp60^{v-src}. CEF infected with tsNY72-4RSV were seeded on 150-mm dishes at a density of 2×10^7 cells per plate and allowed to grow at 41.5°C in medium containing 5% (vol/vol) calf serum for 48 hr, after which they were placed in medium containing 0.5% serum and cultured for an additional 24 hr. (Right) At time 0, cells were shifted from the nonpermissive temperature (41.5°C) to the permissive temperature (35°C). In the time periods shown, RNAs [7 μ g of poly(A)⁺ mRNA per lane] were electrophoresed on a formaldehyde denaturing gel, blotted, and hybridized to ³²P-labeled probes. In the two uppermost panels, oligonucleotides specific for the spliced (oligonucleotide 790) and unspliced (oligonucleotide 773) CEF-147 transcripts were used as probes. The mRNAs in the third panel were hybridized to CEF-147 cDNA, which detected both the spliced and unspliced transcripts at a 10-fold higher sensitivity. (Left) RNAs were from noninfected CEF cells grown to high density, after which they were cultured for 36 hr in medium containing 0.5% serum. The Northern blots were performed with 12 μ g of poly(A)⁺ mRNA and were hybridized to the probes (at 8×10^6 cpm/ml) indicated. Autoradiograms were exposed to x-ray film for 3 days. Similar results have been obtained with tsNY72-4RSV-infected cells grown at the nonpermissive temperature in low serum. The position of 28S rRNA in Right is designated by an arrowhead and in Left by a dark spot on the left edge of the autoradiogram.

ature shift, >95% of steady-state CEF-147 transcripts were spliced.

Sequence Analysis of miPGHS_{ch} Protein. *In vitro* transcription-translation of a full-length spliced CEF-147 cDNA yielded a 70-kDa protein (Fig. 3). Cotranslational glycosylation of miPGHS_{ch} in the presence of canine microsomal enzymes produced a protein of 79 kDa, comparable to the size of PGHS_{ov} *in vivo* (Fig. 3) (10).

The cDNA sequence of miPGHS_{ch} predicted a protein of 68 kDa, which, although almost identical to PGHS_{ov} in size, differed with this protein in subtle but potentially important ways. At its amino terminus, PGHS_{ov} and its homologs in mouse and human have a characteristic hydrophobic signal peptide that is 23–24 amino acids long; in contrast, miPGHS_{ch} had only 17 amino acids in its signal peptide. The cleavage site of the miPGHS_{ch} signal peptide was also the location of a 5' intron, the splicing of which appears to be regulated during cell transformation. The position of this intron was not conserved in the gene encoding the human homolog of PGHS_{ov} (20). In addition, although miPGHS_{ch} and PGHS_{ov} differed in size by only 3 amino acids, comparison of their sequences showed that miPGHS_{ch} lacked 15 amino acids near its amino-terminal end with respect to PGHS_{ov}, but contained additional amino acids at its carboxyl terminus that compensated for this deficiency. In this carboxyl-terminal sequence was an additional potential glycosylation site at amino acid

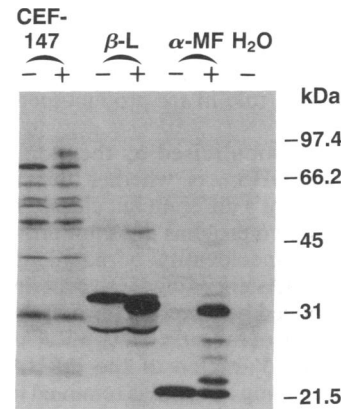


FIG. 3. *In vitro* translation of spliced CEF-147 mRNA. RNA transcribed from the spliced 4.1-kb CEF-147 insert was translated in rabbit reticulocyte lysate in the presence (lanes +) and absence (lanes -) of canine pancreatic microsomes. The RNAs translated in each lane were 4.1-kb CEF-147 sense cRNA (lanes CEF-147), β -lactamase mRNA (lanes β -L), yeast α -mating factor mRNA (lanes α -MF), and the H₂O control. α -Mating factor RNA was translated to show the ability of the canine microsomes to catalyze glycosylation of the 19-kDa α -mating factor precursor to produce the 32-kDa form. *E. coli* β -lactamase mRNA was translated to show the ability of the canine microsomes to cleave the signal peptide from the 32-kDa protein to form the 29-kDa protein. The many sub-full-length translation products seen in the translation of CEF-147 cRNA appear to occur because of internal initiation of translation caused by cRNA breakage in the lysate (data not shown).

579 (Fig. 1). PGHS_{ov} has been hypothesized to traverse the endoplasmic reticulum lipid bilayer by means of a transmembrane domain located in the center of the primary sequence of the protein, thus placing half of the PGHS_{ov} polypeptide in the lumen and the other half in the cytosol (16). Therefore, miPGHS_{ch} would be predicted to contain more sequence that projected into the cytosol and less sequence that projected into the lumen than PGHS_{ov}.

Comparison of the coding sequences of miPGHS_{ch} with PGHS_{ov} from sheep, human, and mouse showed 60%, 60%, and 59% sequence identity, respectively, at the nucleotide level and 59%, 61%, and 62% at the amino acid level. Conservative substitutions raised the similarity observed at the amino acid level to \approx 71%. Regions of least conservation (<40% identity at the amino acid level) were found at amino acids 1–94, 242–272, 377–449, and 543–603 of miPGHS_{ch}. Highly conserved regions (>80% identity over 30 or more amino acids) were found at amino acids 168–241, 273–376, 450–497, and 503–543. The sequence Thr-Ile-Trp-Leu-Arg-Glu-His-Asn-Arg-Val (residues 289–298, Fig. 1), the histidine of which has been proposed to provide the axial heme ligand in PGHS_{ov}, was perfectly conserved in miPGHS_{ch}. Also conserved was Ser-516, which is the acetylation site of aspirin (19).

DISCUSSION

The relationship between prostaglandin synthesis and tumorigenesis has been of interest to many laboratories. Prostaglandins of the E series, most often PGE₂, have been found to be overexpressed in carcinomas (14) and sarcomas (9) as well as in transformed cell lines (6, 9, 10). Increased prostaglandin synthesis in tumor cells is probably caused by increased synthesis of PGHS, since many studies *in vitro* have shown that mitogenic stimulation and transformation induce this enzyme (4–11, 28).

Recently Han *et al.* (10) reported that PGHS is induced in murine fibroblasts transformed by Rous sarcoma virus, and Barker *et al.* (28) have reported that arachidonic acid metab-

olism and prostaglandin synthesis are increased in RSV-transformed cells. The cloning of miPGHS_{ch} is consistent with these observations and suggests that prostaglandin synthesis may play a role in *src* product-mediated transformation.

An important question raised by the cloning of a cDNA (CEF-147) for miPGHS_{ch} is whether this is the chicken homolog of PGHS_{ov} or a different but closely related protein. Comparison of the two proteins does not provide conclusive evidence to determine identity or nonidentity. However, analysis of the mRNAs encoding these proteins suggests that miPGHS_{ch} may indeed be a second form of the enzyme. One unique characteristic of the miPGHS_{ch} mRNA is the presence of an intron in the 5' region of the mRNA that remains unspliced in nondividing cells but is removed from miPGHS_{ch} mRNA in *src*-transformed cells. The presence of the intron acts as a translational block *in vitro* and possibly serves to prevent translation of basal levels of miPGHS_{ch} in nondividing cells (data not shown).

Another unique feature is the 2.3-kb-long, AU-rich 3' untranslated region of miPGHS_{ch} mRNA that contains 16 copies of the repeat 5'-ATTTA-3' shown by Shaw and Kamen (29) to confer instability to transcripts. This sequence is found in multiple copies in the 3' untranslated regions of many mRNAs that encode proteins involved in growth control, including cytokines and transcription factors (29, 30). The 3' untranslated region of the sheep 2.8-kb mRNA is only 0.8 kb long, is not AU-rich, and contains only one copy of the Shaw-Kamen sequence.

Recently, Rosen *et al.* (31) used sheep PGHS_{ov} cDNA to probe RNA from sheep tracheal mucosa cells and found in addition to the 2.8-kb PGHS_{ov} transcript, a 4.0-kb transcript. The latter mRNA was detectable only under low-stringency conditions with two nonoverlapping PGHS_{ov} cDNA fragments and was responsive to mitogen induction. Induction of this 4.0-kb mRNA, but not the 2.8-kb species, paralleled the increased prostaglandin synthase activity observed in mitogen-stimulated cells, leading the authors to conclude that it, rather than the 2.8-kb mRNA, encoded the mitogen-inducible PGHS activity in these cells (31). The identity of this 4.0-kb murine transcript is uncertain until it is cloned and sequenced, but our data imply possible homology between Rosen's 4.0-kb mRNA and the CEF-147-encoded 4.1-kb mRNA, suggesting the existence of two forms of PGHS.

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