Down-regulation of cytokine-induced cyclo-oxygenase-2 transcript isoforms by dexamethasone: evidence for post-transcriptional regulation

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The immediate-early cyclo-oxygenase-2 (Cox-2) gene encodes an inducible prostaglandin synthase enzyme that has been implicated in inflammatory and proliferative diseases. We have shown that the inflammatory cytokine interleukin-1 (IL-1) induces the Cox-2 gene in a sustained manner and that post-transcriptional mRNA stabilization is an important event [Ristimäki, Garfinkel, Wessendorf, Maciag and Hla (1994) J. Biol. Chem. **269**, 11769–11775]. The anti-inflammatory glucocorticoid dexamethasone potently down-regulates IL-1-induced Cox-2 mRNA expression. Kinetic studies suggest that antagonism of IL-1-induced mRNA stabilization is, at least in part, responsible for the suppression of Cox-2 mRNA. The Cox-2 gene produces two major transcript isoforms, namely Cox-$2_{4.6}$ (4.6 kb) and Cox-$2_{2.8}$ (2.8 kb), which are derived by alternative polyadenylation in the 3′-untranslated region (UTR). In response to dexamethasone, the short Cox-$2_{2.8}$ transcript isoform, which lacks a highly conserved AU-rich region, decays with a longer half-life than the Cox-$2_{4.6}$ isoform. Furthermore, heterologous expression of the hybrid Cox-1 open reading frame and the Cox-2 3′-UTR results in the accumulation of high levels of the short isoform and lower levels of the long isoform. These data suggest that multiple elements in the 3′-UTR of the Cox-2 gene are involved in the determination of the differential mRNA stabilities of Cox-2 transcript isoforms. Because dexamethasone destabilizes the Cox-2 transcript, and because the decay of Cox-2 transcript isoforms induced by dexamethasone occurs with different half-lives, post-transcriptional mRNA destabilization may be an important mechanism in the action of anti-inflammatory glucocorticoids.

INTRODUCTION

Cyclo-oxygenase, also known as prostaglandin H synthase (EC 1.14.99.1), is the rate-limiting enzyme in the biosynthesis of prostanoids [1,2]. Two cyclo-oxygenase genes have been cloned (Cox-1 and Cox-2) that share approx. 60% identity at amino acid level [3–5]. The most striking difference between the Cox genes is in their regulation of expression. While Cox-1 is constitutively expressed, the expression of Cox-2 is low under basal conditions and is highly induced in response to cell activation by hormones, tumour promoters, growth factors and pro-inflammatory cytokines [3–5].

The genomic structures of both murine [6] and human [7,8] Cox-2 have been characterized. While the exon–intron format of the open reading frame (ORF) region is highly conserved between the Cox-1 and Cox-2 genes, the promoter and 3′-untranslated regions (UTRs) are highly divergent. Molecular analysis of the promoter region have shown the importance of C/EBP [9] and ATF/CRE enhancer elements [10] in the transcriptional induction by gonadotropic hormones in rat granulosa cells and by v-*src* in NIH 3T3 cells. In contrast, the functional role of the 3′-UTR of the Cox-2 gene is not known. The entire 3′-UTR (2.5 kb) of the human Cox-2 gene is encoded by exon 10 [7,8], which contains 22 copies of AUUUA mRNA instability motifs [11] and three canonical (AAUAAA) polyadenylation sequences [12]. As in other inducible genes [13], this region may be important in post-transcriptional regulation.

We have demonstrated that interleukin-1α (IL-1α) stimulates Cox-2 expression in a sustained manner in human endothelial cells, IMR-90 lung fibroblasts and rheumatoid synovial fibroblast cells [14,15]. However, IL-1α activation of Cox-2 transcription was only transient, and increased stability of the Cox-2 transcript appears to be an important component of the sustained induction [14]. Similar results demonstrating that IL-1 stabilizes Cox-2 mRNA were also reported in mesengial cells [16]. In contrast to IL-1, the anti-inflammatory steroid dexamethasone suppresses the induction of Cox-2 induced by growth factors, serum and cytokines (reviewed in [1–5]). However, the mechanism involved in the inhibition of Cox-2 expression by dexamethasone is poorly understood. Dexamethasone was shown to decrease the rate of transcription of the Cox-2 gene in serum-stimulated NIH 3T3 cells, as detected by nuclear run-on assays [17]. However, Evett et al. [18] did not detect a decrease in Cox-2 transcription in dexamethasone-treated 3T3 cells, but rather indicated that dexamethasone destabilizes the Cox-2 transcript. In addition, studies on the Cox-2 promoter have so far failed to uncover dexamethasone-responsive *cis*-acting elements in the 5′-flanking region of the Cox-2 gene ([5,19]; T. Hla, unpublished work). Dexamethasone is known to down-regulate several inducible genes by both transcriptional and post-transcriptional mechanisms [20,21]. It is not known whether the ability of dexamethasone to down-regulate IL-1-induced Cox-2 mRNA involves transcriptional and/or post-transcriptional components.

In this study, we have investigated the mechanisms involved in the suppression of IL-1-induced Cox-2 expression by dexamethasone. Our data suggest that destabilization of the Cox-2 mRNA is responsible, at least in part, for this effect.

EXPERIMENTAL

Cell culture

Diploid human lung cells (IMR-90; passages 10–13; American Type Tissue Collection CCL-186) and transformed human embryonic kidney cells (293; American Type Tissue Collection CRL-1573) were grown in Dulbecco's modified Eagle's medium

Abbreviations used: Cox, cyclo-oxygenase; DRB, 5,6-dichlorobenzimidazole; DMEM, Dulbecco's modified Eagle's medium; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL-1, interleukin-1; ORF, open reading frame; RT-PCR, reverse transcriptase-PCR; UTR, untranslated region.

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(DMEM; Mediatech) containing 10% (v/v) fetal bovine serum (HyClone) and antibiotics (JRH Biosciences). Human rheumatoid synoviocytes were cultured as previously described [15]. Prior to the initiation of the experiments, the cells were maintained in 0.5% fetal bovine serum for 48 h, and the experiments were conducted in the presence of 5% fetal bovine serum with 10 ng/ml human recombinant IL-1β (Biological Response Modifiers Program, NCI-FCRDC) or 1 μM dexamethasone (Sigma) for the time periods indicated.

Total RNA isolation and Northern assays

Total RNA was isolated by the method of Chomczynski and Sacchi [22]. The RNA concentration was determined by absorbance at 260 nm. Total RNA (10–20 μg) was electrophoresed through a 1.2% agarose gel containing 2.2 M formaldehyde and the integrity of the RNA was analysed by ethidium bromide staining. The RNA was then electroblotted on to a Zeta-probe membrane (Bio-Rad) in 10 mM Tris/acetate, pH 8.0, and 1 mM EDTA at 25 V for 6 h at 4 °C. The membrane was then UV-irradiated (Stratalinker, Stratagene). The human cDNAs {Cox-1 ORF (1.8 kb), Cox-2 ORF (1.8 kb) [23] and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 0.8 kb)}, restriction enzyme fragments of the genomic DNA of human Cox-2 [probe A: *Bst*EII–*Bgl*II, bases 7941–8597; probe B: *Sac*I–*Bst*EII, bases 5976–7940], and a purified PCR fragment of genomic Cox-2 DNA (probe C: bases 7406–8209) [7] were labelled to high specific radioactivity using [α-^{32}P]dCTP and a random primer labelling system (Amersham). Filters were hybridized and washed by the protocol of Church and Gilbert [24]. The bands were visualized by autoradiography and quantified using a BioImage analytical imaging system (Millipore). RNA molecular size standards were a 0.24–9.5 kb RNA Ladder and a 0.16–1.77 kb RNA Ladder (Gibco-BRL).

Poly(A)$^+$ RNA isolation

Poly(A)$^+$ RNA was isolated from the total RNA (500 μg) of IL-1-treated IMR-90 cells using an oligo(dT)–cellulose column [Poly(A) Quik mRNA Isolation Kit; Stratagene]. Poly(A)$^+$ (1 μg) and poly(A)$^-$ (20 μg) RNA were analysed by Northern blot hybridization.

RNase H treatment

RNase H digestion was modified from the protocol of Brewer and Ross [25]. Total RNA (10–40 μg) from IL-1-treated IMR-90 cells and oligonucleotides (0.5–2.0 μg) were incubated in 10 μl of water in 65 °C for 5 min and placed on ice for 5 min. The samples were then incubated with 40 μl of buffer [50 mM Tris/HCl, pH 8.0, 100 mM KCl, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol and 40 units of rRNasin (Promega)] at 37 °C for 30 min, after which RNase H (1–3 units; Pharmacia) was added and incubation was continued at 37 °C for 30 min. The samples were then extracted with phenol/chloroform (1:1, v/v), precipitated with ethanol and analysed by Northern analysis. To remove the poly(A) tails, oligo$(dT)_{12-18}$ (Pharmacia) was used. The following human Cox-2 antisense primers were used: 5′-TTACTTCTAATGCATCATGGAAG-3′ (oligo A; bases 6445–6467), and 5′-AGTCCTGAGCTGAGGTTTACCTGA-3′ (oligo B; bases 7466–7489).

Expression vectors

The human Cox-1 ORF (1.8 kb) [23] was fused to the Cox-2 3′-UTR (2.0 kb) [7)] and subcloned into pcDNAI/Neo (Invitrogen Corp.), which contains a Cytomegalovirus promoter and a Simian Virus 40 intron and polyadenylation signal. This expression vector adds approx. 0.6 kb of Simian Virus 40 3′-UTR sequences to the transcripts. The Cox-2 3′-UTR was a *Sac*I–*Bst*EII fragment of exon 10, which contains 97 bases of the 3′-end of the Cox-2 ORF and the first 1868 bases of the 3′-UTR [7]. This part of the Cox-2 gene is intronless and ends before the first of the three canonical polyadenylation signals. This was done so as to avoid the use of insert-derived canonical polyadenylation signals, which would eliminate the use of the vector intron that is necessary for the optimal expression of the transfected cDNAs [26].

Transfections

Batches of 293 cells were seeded on 35-mm tissue culture wells (4×10^5 cells/well) and grown for 24 h. Lipofectamine (6 μl; Gibco-BRL) and 1 μg of vector DNA were diluted with 100 μl of DMEM, mixed and incubated at room temperature for 30 min, after which 800 μl of DMEM was added. The cells were washed twice with DMEM and incubated for 6 h with the lipid/DNA mixture (1 ml), after which 1 ml of complete growth medium was added. This medium was replaced by 2 ml of complete growth medium after incubation for 24 h. Total RNA was extracted 48 h after transfection, as described above.

Reverse transcriptase-PCR (RT-PCR), subcloning, sequencing and Southern blotting

Total RNA (1–2 μg) from untreated and IL-1-treated IMR-90 cells and from 293 cells transfected with an expression vector containing the Cox-1 ORF plus the Cox-2 3′-UTR was converted to cDNA using the $(dT)_{17}$-adaptor primer (5′-GACTCGAGTCGACATCGAT$_{17}$-3′), amplified with PCR using the adaptor primer [7] and a human Cox-2 sense primer (primer A: 5′-CAGGTATCAGTGCATTATTAAATG-3′; bases 6523–6546), and electrophoresed through a 1% agarose gel [7]. A 200 bp fragment was amplified from the cDNA of IL-1-treated IMR-90 cells and from the transfected 293 cells, and subcloned into the vector pCRII (Invitrogen Corp.); three clones from each cell type were sequenced. To study the use of the three canonical polyadenylation sites, the cDNA from IL-1-treated IMR-90 cells was amplified by PCR using the adaptor primer and a human Cox-2 sense primer (primer B: 5′-AGCTATCTGTAACCAAGATGGATG-3′; bases 7600–7623), electrophoresed through a 1% agarose gel, Southern-blotted [24] and probed with probe C.

RESULTS

Dexamethasone down-regulates IL-1-induced Cox-2 mRNA

Glucocorticoids such as dexamethasone potently inhibit inducible prostanoid synthesis by down-regulation of Cox-2 expression [1–5,17,18]. However, the molecular mechanisms involved in the inhibition of Cox-2 expression by dexamethasone remain to be elucidated. Because our recent data implicated the importance of post-transcriptional mRNA stabilization in the sustained induction of Cox-2 by IL-1 [14], we investigated whether similar mechanisms are involved in the down-regulation of Cox-2 by dexamethasone.

As previously shown, treatment of IMR-90 diploid lung fibroblasts and human synovial fibroblasts with IL-1 resulted in the sustained induction of Cox-2 mRNA levels [14,15]. Because Cox-2 mRNA turns over with a half-life of 1 h [14], and because the transcriptional activation of the Cox-2 gene by IL-1 is transient (from 1 to 3 h) [14], post-transcriptional mRNA

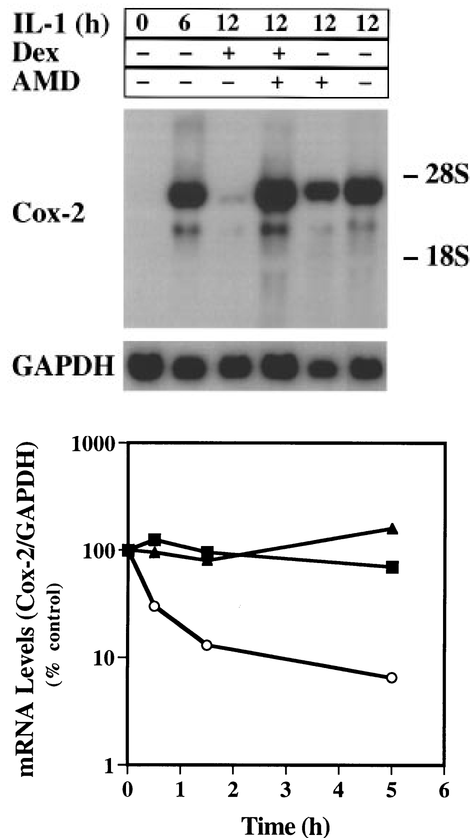

Figure 1 Effect of transcription inhibitors on dexamethasone suppression of IL-1-induced Cox-2 mRNA expression

Upper panel: IMR-90 cells were treated with IL-1β (10 ng/ml) for 6 h or 12 h as indicated. Dexamethasone (Dex; 1 μM) was present between 6 and 12 h in the presence or absence of the transcription inhibitor actinomycin D (AMD; 2 μM). RNA was analysed by Northern blot analysis. Lower panel: human synovial fibroblasts were pretreated wih IL-1β for 24 h (time 0). Cells were then treated with 1 μM dexamethasone or the transcription inhibitor DRB (20 μg/ml) either alone or in combination for indicated times: ■, DRB; ○, dexamethasone; ▲, DRB plus dexamethasone. RNA was isolated and Northern blot analysis was conducted as described. Densitometric quantification of the blot is shown.

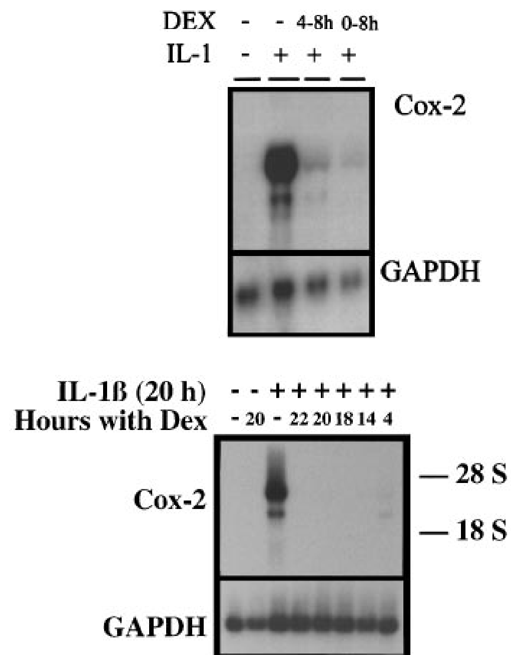

Figure 2 Kinetic analysis of dexamethasone suppression of IL-1-induced Cox-2 expression

Top panel: IMR-90 cells were treated for 8 h with IL-1β (10 ng/ml). Dexamethasone (Dex; 1 μM) was added from 4 to 8 h or from 0 to 8 h as indicated, RNA was purified and Northern blot analysis was conducted as described. A representative autoradiograph is shown. Lower panel: human synovial fibroblasts were treated for 20 h with 10 ng/ml IL-1β. Dexamethasone (Dex; 1 μM) was added at −2, 0, 2, 6 or 16 h relative to the time of IL-1 addition, as indicated. RNA was purified and Northern blot analysis was conducted as described. A representative autoradiograph is shown.

stabilization must occur to achieve sustained kinetics of Cox-2 induction in IMR-90 cells. The IL-1-induced expression of Cox-2 in IMR-90 lung fibroblasts and synovial fibroblasts is potently down-regulated by dexamethasone. As shown in Figure 1 (upper panel), treatment of IMR-90 cells with 1 μM dexamethasone for 6–12 h resulted in near-total suppression of IL-1-induced Cox-2 mRNA levels. In contrast, inhibition of transcription with 10 μg/ml actinomycin D for 6–12 h did not suppress Cox-2 mRNA levels, suggesting that transcription is not required to sustain the IL-1 effect. However, the ability of dexamethasone to down-regulate Cox-2 mRNA requires transcriptional mechanisms, because dexamethasone was unable to suppress Cox-2 mRNA levels when transcription was inhibited with actinomycin D. Similar data were also observed in human synovial fibroblasts. As shown in Figure 1 (lower panel), 5,6-dichlorobenzimidazole (DRB), an inhibitor of transcription, did not block the ability of IL-1 to induce Cox-2 mRNA in a sustained manner. However, even in the presence of IL-1, dexamethasone potently down-regulated Cox-2 mRNA with a half-life of approx. 0.4 h. The ability of dexamethasone to down-regulate Cox-2 mRNA was, however, blocked by concomitant incubation with DRB. These data suggest that: (i) continuous transcription is not required to sustain the IL-1-induced Cox-2 mRNA levels, (ii) dexamethasone potently destabilizes Cox-2 mRNA, and (iii) dexamethasone-induced transcription is required to down-regulate Cox-2 mRNA.

Dexamethasone potently inhibited IL-1-induced Cox-2 mRNA levels whether it was added concurrently with IL-1 (present between 0 and 8 h) or whether it was added 4 h after IL-1 addition (4–8 h) to IMR-90 cells (Figure 2, upper panel). Similarly, in human synovial fibroblasts, dexamethasone potently inhibited IL-1-induced Cox-2 mRNA levels even if dexamethasone was added 16 h after IL-1 treatment (Figure 2, lower panel). These data strongly suggest that: (i) IL-1-induced mRNA stabilization is critical to maintain high levels of Cox-2 mRNA in IMR-90 and synovial fibroblast cells, and (ii) dexamethasone down-regulates the Cox-2 mRNA in the post-transcriptional phase of Cox-2 induction.

Characterization of the Cox-2 transcript isoforms

Differential decay of mRNA is a major mechanism for the regulation of gene expression [13]. Some short-lived mRNAs such as that for c-fos are degraded by a mechanism involving poly(A) shortening, followed by rapid exonucleolytic decay [13]. In contrast, the mRNA for the cytokine gro/KC is first cleaved endonucleolytically, followed by rapid exonucleolytic decay [27]. One way to distinguish between these two distinct pathways is to characterize the degradation intermediates. The Cox-2 transcripts

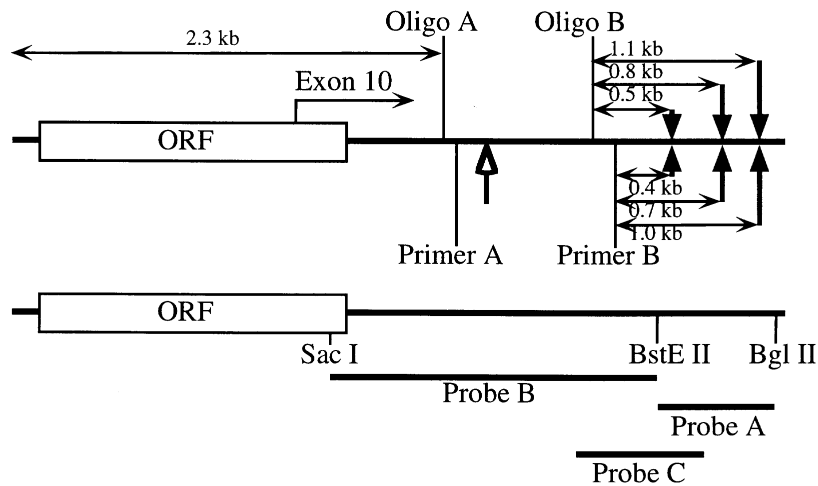

Figure 3 Schematic representation of the Cox-2 transcript

Oligo A and oligo B were used for RNase H mapping, primers A and B for 3′-mapping RT-PCR, probes A and B in Northern blot assays and probe C in the Southern blot assay. Closed arrows indicate the three canonical polyadenylation signals, and the open arrow indicates the non-canonical polyadenylation signal.

exhibit heterogeneity; in many cell types a predominant 4.2–4.6 kb isoform and a less-abundant 2.8 kb isoform are observed [3–5,28]. We set out to molecularly characterize these two isoforms in order to better understand the metabolism of Cox-2 mRNA in IMR-90 cells.

Poly(A)$^+$ and poly(A)$^-$ RNA from IL-1-treated IMR-90 cells were purified by affinity chromatography on oligo(dT)–cellulose. Both the 4.6 and 2.8 kb isoforms were found in the poly(A)$^+$ fraction, suggesting that they are polyadenylated (results not shown). This indicates that the Cox-$2_{2.8}$ isoform is not a stable endonucleolytic degradation intermediate. In addition, only the 4.6 kb isoform was detected when the Northern blot was probed with a 3′-UTR-specific probe (probe A, Figure 3), suggesting that the 2.8 kb transcript isoform lacks the distal part of the 3′-UTR. To further characterize the nature of the Cox-2 transcript isoforms, RNase H mapping experiments were conducted. The oligonucleotides to direct the RNase H cleavage of the Cox-2 transcript and the respective probes used in the subsequent Northern analyses are shown schematically in Figure 3. RNase H cleavage with oligo(dT) followed by Northern blot analysis with the Cox-2 ORF probe enhanced the mobility of both the 4.6 and 2.8 kb isoforms, confirming the presence of the poly(A) tail (Figure 4). However, RNase H cleavage with oligo A and similar Northern analysis detected only a 2.3 kb band (Figure 4). This indicates that sequences upstream from the oligo A site of both transcript isoforms are identical, and suggests that there is no alternative splicing within the existing intron–exon boundaries.

To precisely define the sequence of the 2.8 kb transcript isoform, we used the 3′-mapping RT-PCR protocol and cloned the 3′-end. The PCR products were subcloned and sequenced, which showed that the sequence of the 2.8 kb transcript aligned perfectly with the known human Cox-2 sequence [7], followed by a poly(A) tail (results not shown). A non-canonical poly-adenylation signal, AUUAAA [12], is located 15 bases upstream of the poly(A) tail. Together, these data demonstrate that the 2.8 kb Cox-2 transcript isoform is derived by alternative poly-adenylation in exon 10.

In the distal part of the 3′-UTR of Cox-2, there are three canonical (AAUAAA) polyadenylation sites [7]. We next determined the usage of these sites, which could potentially give rise

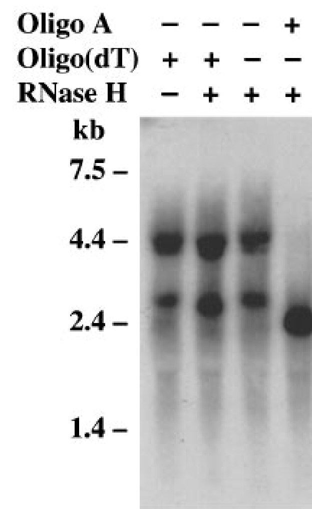

Figure 4 RNase H mapping of the Cox-$2_{2.8}$ and Cox-$2_{4.6}$ transcript isoforms

Total RNA (20 μg) isolated from IL-1-treated IMR-90 cells was hybridized with oligo(dT)$_{12-18}$ and/or oligo A and digested with RNase H as described. The resulting RNA was analysed by Northern blot analysis with the Cox-2 ORF probe.

to mRNA species of approx. 4.1, 4.4 and 4.6 kb. An RNase H mapping protocol using oligo B and probe B in the Northern assay gave rise to several bands (Figure 5). Similar analysis, together with use of oligo(dT) to remove the poly(A) tail, resulted in distinct bands of 3.3, 1.5, 1.1 and 0.5 kb. The mobility of the 3.3 kb band was not altered by poly(A) tail removal, suggesting that it is the 5′-fragment. Only the 1.1 kb and 0.5 kb bands possess the poly(A) tail. The identity of the 1.5 kb band is unknown; however, it may represent a de-adenylated stable degradation intermediate or a partial digestion product of RNase H. The remaining bands (III and I, Figure 5) correspond to the 4.6 kb and 4.1 kb Cox-2 mRNA isoforms, suggesting that the

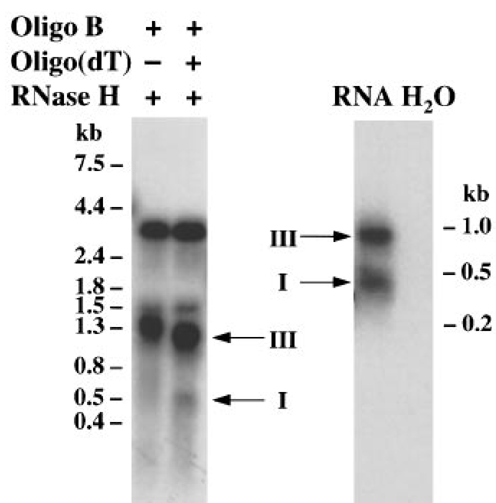

Figure 5 RNase H mapping analysis of the full-length Cox-2 mRNA isoform

Total RNA (10 μg) purified from IL-1-treated IMR-90 cells was hybridized with oligo B with or without oligo(dT)$_{12-18}$ and then digested with RNase H. The resulting RNA was analysed by Northern blot hybridization with probe B (left panel). Note the 3.3 kb 5′ fragment of Cox-2 transcripts and two polyadenylated bands of 1.1 kb and 0.5 kb that represent the use of the last (III) and the first (I) canonical polyadenylation signals respectively. To confirm this result, we carried out a RT-PCR/Southern blot protocol (right panel) using primer B and the adaptor primer [7] to amplify cDNA derived from IL-1-treated IMR-90 cells. The PCR products were run through an agarose gel, blotted and hybridized with probe C.

first and the last canonical polyadenylation signals are utilized in IL-1-treated IMR-90 cells. To further confirm the existence of the Cox-$2_{4.6}$ and Cox-$2_{4.1}$ transcript isoforms, we used 3′-mapping RT-PCR with primer B and an oligo(dT) adaptor. As shown in Figure 5 (right panel), two mRNA isoforms corresponding to the 4.1 and 4.6 kb isoforms were amplified. These isoforms correspond to the use of the first and the last polyadenylation sites. Thus the ‘full-length’ Cox-2 transcript is composed of two alternatively polyadenylated mRNA species of 4.1 and 4.6 kb. Northern blot analysis using the ORF probe and 4.6 kb specific probe (probe A, Figure 3), as well as RNase H analysis (Figure 5, left panel), indicated that Cox-$2_{4.6}$ is the major isoform, constituting $> 90\,\%$ of the larger-transcript band. A schematic representation of the three Cox-2 transcript isoforms is shown in Figure 6. These data indicate that alternative polyadenylation is used to generate heterogeneity in Cox-2 transcript isoforms. Polyadenylation at two distal canonical sites (AAUAAA) gives rise to a major 4.6 kb isoform and a minor 4.1 kb isoform, both of which constitute the ‘large’ 4.6 kb band on Northern blots. However, polyadenylation at a non-canonical site (AUUAAA) results in the production of significant levels of the ‘small’ 2.8 kb isoform.

Differential kinetics of turnover of Cox-2 transcript isoforms

The Cox-2 3′-UTR is distinct from its Cox-1 counterpart in that it contains multiple copies of the AUUUA motif, which is implicated in the rapid post-transcriptional turnover of mRNAs [7,8,28]. In the human Cox-2 gene, exon 10, which encodes the entire 3′-UTR, contains 22 copies of the AUUUA sequence [7]. Interestingly, two AU-rich regions found in the proximal and distal parts of the 3′-UTR are highly conserved between the Cox-2 genes of human, rat and mouse (Figure 6) [7,28,29]. Both of these potential regulatory motifs are present in the major 4.6 kb and minor 4.1 kb isoforms, whereas only the proximal motif is present in the 2.8 kb isoform (Figure 6). Since AUUUA motifs have been implicated in determining mRNA stability, and because Cox-2 mRNA is destabilized by dexamethasone, we examined the kinetics of turnover of the Cox-2 transcript isoforms in IMR-90 cells.

IMR-90 cells were pretreated with IL-1 to maximally stimulate Cox-2 mRNA levels and subsequently challenged with 1 μM dexamethasone. Detailed kinetics of the down-regulation of Cox-2 transcript isoforms were determined by Northern blot analysis using the Cox-2 ORF probe (which detects all three mRNA species) and probe A (which is specific for the 4.6 kb isoform). As shown in Figure 7, the large Cox-2 band decayed with a half-life of 0.94 h. The half-life of this band was identical when the Northern blots were probed with either the ORF probe or probe A. This is in agreement with our previous data (Figures 3 and 6) indicating that the 4.6 kb transcript isoform is the predominant species. In contrast, the Cox-$2_{2.8}$ isoform decayed with a significantly longer half-life after dexamethasone treatment (Figure 7). These data indicate that the alternatively polyadenylated Cox-$2_{4.6}$ and Cox-$2_{2.8}$ mRNA isoforms show

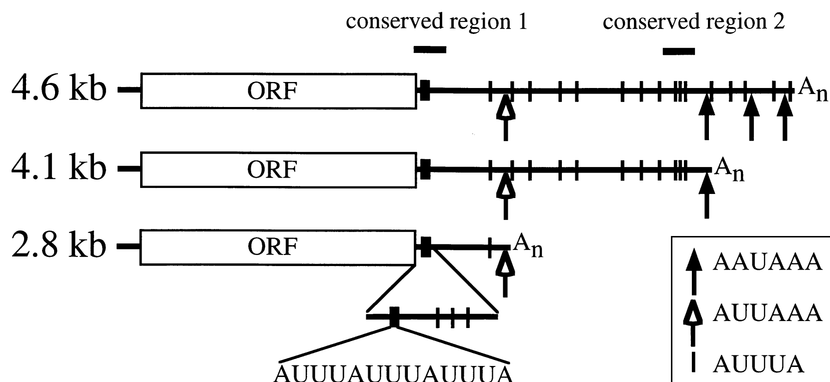

Figure 6 Schematic representation of the three alternatively polyadenylated Cox-2 transcript isoforms

The three canonical polyadenylation signals (AAUAAA) are indicated by closed arrows and the non-canonical polyadenylation signal (AUUAAA) by an open arrow. The AUUUA mRNA instability elements are indicated as black bars, and the conserved regions in the 3′-UTR are marked.

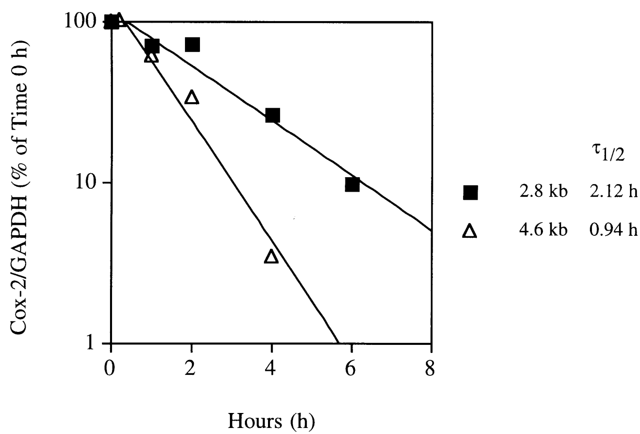

Figure 7 Kinetics of dexamethasone-induced degradation of Cox-2 mRNA isoforms

IMR-90 cells were preincubated with IL-1β (10 ng/ml) for 6 h (time 0 h). After the preincubation period dexamethasone (1 μM) was added for 1–6 h to the preincubation medium. RNA was analysed as described. The levels of the mRNA isoforms were quantified by densitometry, normalized to GAPDH levels and plotted. Values are derived from three independent experiments. The mean values are shown; S.D.s were less than 10% of the mean in all cases.

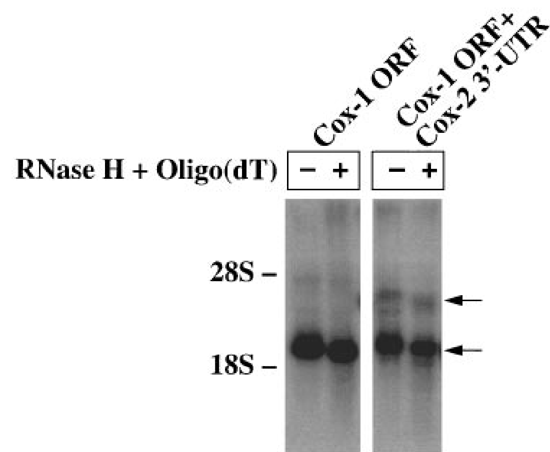

Figure 8 A chimaeric cDNA of the Cox-1 ORF and the Cox-2 3′-UTR is alternatively polyadenylated

293 cells were transiently transfected with a mammalian expression vector containing the Cox-1 ORF or the Cox-1 ORF plus the Cox-2 3′-UTR. Untreated total RNA (—) and RNA hybridized with oligo(dT)$_{12–18}$ and digested with RNase H (+) was analysed by Northern blot hybridization using the Cox-1 ORF probe. No endogenous Cox-1 mRNA was detected in non-transfected 293 cells (not shown).

differential rates of mRNA decay after dexamethasone treatment. Because both transcript isoforms are derived from a unique transcription start site, the differential decay of the transcript isoforms provides strong evidence for the existence of dexamethasone-induced post-transcriptional mRNA destabilization mechanisms.

To further demonstrate the functional role of the Cox-2 3′-UTR in the alternative polyadenylation and the differential stability, we prepared a chimaeric transcript containing the Cox-1 ORF fused to the entire 3′-UTR encoded by exon 10 of the human Cox-2 gene. The resulting chimaeric cDNA was placed under the transcriptional control of the cytomegalovirus immediate-early promoter in the vector pCDNA-Neo. The 1.8 kb Cox-1 ORF cloned in the same vector constituted a control. As shown in Figure 8, transfection of the Cox-1 ORF construct resulted in the accumulation of a dominant transcript of the expected size (approx. 2.4 kb). However, expression of the Cox-1 ORF/Cox-2 3′-UTR construct yielded low levels of the long transcript isoform (approx. 4.4 kb), and higher levels of the short isoform (approx. 2.5 kb). The mobility of both isoforms was increased after hybridization with oligo(dT) followed by RNase H digestion, which suggested appropriate polyadenylation. To exclude the possibility of vector-dependent aberrant splicing [26,27], we used RT-PCR to amplify cDNA from total RNA from cells transfected with the expression vector containing the Cox-1 ORF/Cox-2 3′-UTR construct. The PCR products were subcloned and sequenced, showing that the mRNA is an alternatively polyadenylated isoform and that the poly(A) tail is added to a identical position when compared with the wild-type 2.8 kb Cox-2 transcript isoform. Because both transcripts are derived from a single viral promoter, these data confirm the existence of post-transcriptional mechanisms. Moreover, the data suggest that: (i) the Cox-2 3′-UTR contains sufficient information to direct the alternative polyadenylation, and (ii) the Cox-$2_{2.8}$ transcript isoform is more stable than the Cox-$2_{4.6}$ isoform. It is highly likely that instability elements present in the distal part of the 3′-UTR contribute to the extreme lability of the Cox-$2_{4.6}$ transcript isoform.

DISCUSSION

The Cox-2 gene is an immediate-early gene that is induced by a variety of stimuli involved in cell activation, proliferation and differentiation [1–5]. Cox-2 expression is exaggerated in proliferative and angiogenic diseases such as rheumatoid arthritis [15] and colon cancer [32–34]. Inhibitors of the Cox isoenzymes provide temporary relief from inflammatory symptoms [1], and long-term administration of the non-steroidal anti-inflammatory drug aspirin results in a significant decrease in mortality from cardiovascular disease [35] and colon cancer [30]. Thus dysregulated expression of the Cox-2 enzyme is thought to play an important role in the pathogenesis of a variety of pathological conditions. However, the precise biological role of the Cox-2 enzyme is not well understood.

Since dysregulated expression of the Cox-2 enzyme is associated with human proliferative diseases, the molecular mechanisms involved in the regulation of expression of this gene have been studied intensively [1–5]. Early studies by Raz et al. [31] demonstrated that inducible Cox synthesis can be divided into early transcriptional and late post-transcriptional phases. We noted that the kinetics of transcriptional activation (1–3 h) of the Cox-2 gene cannot account for the sustained kinetics of induction demonstrated by IL-1 [14]. Since Cox-2 mRNA is highly unstable (half-life of approx. 1 h), and since IL-1 stabilizes Cox-2 mRNA in the absence of transcription, we suggested that post-transcriptional mRNA stability is important in IL-1 action [14]. It is known that glucocorticoids inhibit the induction of Cox-2 by a variety of mediators including IL-1 [5,17,18]. However, it is not known whether dexamethasone inhibits IL-1-induced transcription or post-transcriptional mRNA stabilization.

The results of the present study indicate that transcription is not required to sustain the IL-1-induced increase in Cox-2 mRNA in IMR-90 and synovial fibroblasts. This is consistent with our previous data from endothelial cells [14]. However, dexamethasone down-regulates Cox-2 mRNA with a half-life of approx. 0.4 h. Transcription is required for the action of dexa-

methasone, suggesting that dexamethasone-induced cellular factors are involved in the rapid degradation of Cox-2 mRNA. In synovial fibroblasts, the addition of dexamethasone 16 h after the addition of IL-1 completely inhibited IL-1-induced Cox-2 expression. Thus dexamethasone-induced destabilization of Cox-2 mRNA occurs primarily during the post-transcriptional phase.

Since the molecular characterization of the different transcript isoforms of Cox-2 may shed some light on the degradative pathways and post-transcriptional regulatory mechanisms involved, we characterized the molecular basis for Cox-2 transcript heterogeneity in IMR-90 cells. These studies clearly demonstrate that alternative polyadenylation within exon 10 results in the production of the Cox-$2_{2.8}$ and Cox-$2_{4.6}$ transcript isoforms. Interestingly, the 4.6 kb Cox-2 isoform is less stable than the 2.8 kb isoform. Since both transcripts are derived from a unique transcription start site and thus are under the same transcriptional controls, post-transcriptional mechanisms appear to be important. The Cox-$2_{4.6}$ isoform contains additional AU-rich regions in the distal part of the 3′-UTR, including an AU-rich stretch of sequence that is conserved between the Cox-2 genes of human, rat and mouse. Such sequence elements or the secondary structure dictated by these elements may be involved in the rapid destabilization of Cox-2 mRNA [13]. However, the Cox-$2_{2.8}$ isoform is degraded as well, albeit with a longer half-life. Conserved elements in the 5′-proximal region of the 3′-UTR (conserved region 1, Figure 6) may be important in this respect. These data suggest that the sequence elements in the proximal and distal parts of the 3′-UTR co-operate to achieve rapid degradation of the 4.6 kb isoform. Much less is known about the mechanisms of regulation of mRNA stability compared with the transcriptional regulatory mechanisms. While AUUUA elements are known to be involved in determining basal degradation, elements involved in induced stabilization or destabilization are not understood [13]. Detailed understanding of IL-1- and dexamethasone-regulated Cox-2 mRNA stability mechanisms may yield novel approaches to therapeutic intervention.

In conclusion, the down-regulation of IL-1-induced Cox-2 transcript isoforms in IMR-90 and synovial fibroblasts has been characterized. The data indicate that: (i) dexamethasone induces the degradation of Cox-2 mRNA during the post-transcriptional phase of induction, and (ii) alternatively polyadenylated Cox-2 transcripts possess differential mRNA stabilities in response to dexamethasone. Thus post-transcriptional mechanisms may be important in the regulation of Cox-2 by glucocorticoids.

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