Repression of Cyclooxygenase-2 and Prostaglandin E\textsubscript{2} Release by Dexamethasone Occurs by Transcriptional and Post-transcriptional Mechanisms Involving Loss of Polyadenylated mRNA\textsuperscript{*}

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The two cyclooxygenase (COX) isoforms convert arachidonic acid to precursor prostaglandins (PGs). Up-regulation of COX-2 is responsible for increased PG production in inflammation and is antagonized by corticosteroids such as dexamethasone. In human pulmonary A549 cells, interleukin-1\textbeta (IL-1\textbeta) increases prostaglandin E\textsubscript{2} (PGE\textsubscript{2}) synthesis via dexamethasone-sensitive induction of COX-2. Nuclear run-off assays showed that COX-2 transcription rate was repressed 25–40% by dexamethasone, while PGE\textsubscript{2} release, COX activity, and COX-2 protein were totally repressed. At the mRNA level, complete repression of COX-2 was only observed at later (6 h) time points. Preinduced COX-2 mRNA was also potently repressed by dexamethasone, yet suppression of transcription by actinomycin D showed little effect. This dexamethasone-dependent repression involved a reduced COX-2 mRNA half-life, which was blocked by actinomycin D or cycloheximide, and was antagonized by the steroid antagonist RU38486. Repression of IL-1\textbeta-induced PGE\textsubscript{2} release, COX activity, and COX-2 protein by actinomycin D was only effective within the first hour following IL-1\textbeta treatment, while dexamethasone was effective when added up to 10 h later, suggesting a functional role for post-transcriptional mechanisms of repression. Following dexamethasone treatment, shortening of the average length of COX-2 mRNA poly(A) tails was observed. Finally, ligation of the COX-2 3′-UTR to a heterologous reporter failed to confer dexamethasone sensitivity. In conclusion, these data indicate a major role for post-transcriptional mechanisms in the dexamethasone-dependent repression of COX-2 that require de novo glucocorticoid receptor-dependent transcription and translation. This mechanism involves shortening of the COX-2 poly(A) tail and requires determinants other than just the 3′-UTR for specificity.

Prostaglandins (PGs)\textsuperscript{1} form a potent group of autocrine and paracrine lipid mediators (1, 2). These compounds are implicated in many normal cellular processes as well as pathophysiological processes such as inflammation, edema, bronchoconstriction, platelet aggregation, fever, and hyperalgesia (1–3).

PG synthesis involves phospholipase catalyzed release of arachidonic acid from membrane phospholipids and its conversion by the two cyclooxygenase (COX) enzymes to PGH\textsubscript{2}. Subsequently, cell-specific isomerases and reductases result in production of biologically relevant PGs. The two COX isoforms are encoded by distinct genes of which COX-1 is a constitutively expressed housekeeping gene and COX-2 shows low basal expression that is rapidly induced by inflammatory and mitogenic stimuli (1, 2). Pharmacologically, this pathway is important, since COX is the target for nonsteroidal anti-inflammatory drugs such as aspirin and indomethacin (1, 2). The anti-inflammatory benefits of nonsteroidal anti-inflammatory drugs are thought to derive from inhibition of COX-2, while many of the undesirable side effects are due to COX-1 inhibition (1, 2). This has now been confirmed by the use of selective COX-2 inhibitors (4, 5).

Despite the clinical usefulness of nonsteroidal anti-inflammatory drugs, the most effective drugs in the treatment of chronic inflammatory diseases, such as asthma, are synthetic glucocorticoids, which down-regulate inflammatory genes both in vitro and in vivo (6). Synthetic glucocorticoids, such as dexamethasone, act by mimicking the natural steroid, cortisol, in binding the glucocorticoid receptor (GR). GR then dissociates from its cytoplasmic protein complex and translocates to the nucleus, where it can activate transcription (transactivation) of anti-inflammatory genes via cis-acting promoter elements known as glucocorticoid response elements (GREs) (7). However, this fails to explain how glucocorticoids cause down-regulation of inflammatory genes such as COX-2. Other mechanisms for this effect may include transcriptional repression (transrepression) via negative GREs (8), repression of AP-1-dependent transactivation via direct AP-1/GR interactions (9, 10), and repression of NF-\kappaB-mediated transcription by up-regulation of the NF-\kappaB inhibitor 1\xBa and/or direct NF-\kappaB/GR interactions (11–14).

Epithelial cells are known to play an active role in inflammation by producing multiple mediators and therefore represent an important site for glucocorticoid action (15). Airway epithelial cells respond to proinflammatory cytokines, such as IL-1\beta, by induction of COX-2 and PGE\textsubscript{2} release (16). This response is also observed in human A549 cells, and in both cases the response is

\textsuperscript{1} The abbreviations used are: PG, prostaglandin; PGE\textsubscript{2}, prostaglandin E\textsubscript{2}; COX, cyclooxygenase; GR, glucocorticoid receptor; GRE, GR

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suppressed by dexamethasone (16, 17). We have therefore used these cells to examine the mechanisms of dexamethasone repression of IL-1β-dependent induction of COX-2 and relate these effects to functional changes in released PGE₂.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—A549 cells were grown to confluency as described (17). Cells were incubated overnight in serum-free medium before changing to fresh medium containing 1 ng/ml IL-1β (2 × 10⁵ units/μg) (Genzyme Corp., Cambridge, MA), 1 × 10⁻⁶ M phorbol 12-myristate 13-acetate (Sigma, Poole, UK), drugs, or vehicle. Dexamethasone, cycloheximide, and actinomycin D (all from Sigma) were used at 1 × 10⁻⁶ M (unless otherwise indicated), 10 μg/ml, and 10 μg/ml, respectively. Mifepristone, RU38486 (Roussel UCLAF, Romainville, France) was dissolved in ethanol at 1 × 10⁻⁶ M and diluted at least 1:1000 in culture medium prior to use.

**PGE₂ Release and COX Activity Determination**—Culture medium was removed and released PGE₂ measured by radioimmunoassay using anti-PGE₂ antibody (Sigma) essentially according to the manufacturer’s instructions (16). After rinsing cells twice, COX activity assays were performed as described (16). Fresh medium containing 30 μM arachidonic acid (Sigma) was added, and the plates incubated for 10 min at 37 °C. PGE₂ produced was measured by RIA and taken as an index of COX activity.

**Northern Blotting and Hybridization**—Total cellular RNA was extracted using acid guanidium thiocyanate/phenol/chloroform extraction prior to use. Northern blotting and hybridizations were performed according to standard procedures as described previously (19). Hybridization probes for COX-2 and GAPDH were as described (17, 20).

**Oligonucleotide-dependent RNase H Cleavage of RNA**—Total RNA, 15 μg, was incubated for 5 min at 65 °C with 500 ng of the COX-2-specific oligonucleotide H, 5'- AGA AGA TTC TTC GTA GAT ATC -3' (bases 9158–9178, Genbank™ accession no. HSU04636) and/or the GAPDH-specific oligonucleotide R, 5'- TGT AGC CAA ATT CTT GGT CAT ACC-3' (bases 4724–4747, accession no. J04393), either with or without 500 ng of oligo(dT)₁₅ (Promega) in 1× RNase H buffer (Promega). After cooling to 37 °C, 40 units of RNasin (Promega) and 1.5 units of RNase H was added, and the reaction was incubated for 30 min. The reaction was stopped by the addition of formamide/formaldehyde/MOPS gel loading buffer, and the RNA was run on 2.2% agarose/formaldehyde gels in 1× MOPS, and capillary blotting was performed. Hybridization of films for COX-2 were carried out using a 515-bp cDNA probe (BS) directed to the 3'-end of COX-2 3'-UTR as described previously (20). A 271-bp GAPDH 3'-end probe was generated by RT-PCR using the following primers: GAPDH-F2, 5'-GGT ATG ATC ACA AGT AAT TTG GCT ACA-3', and GAPDH-R2, 5'-GGT TGA GCA CAG GGT ATT TTA TT-3'. After cloning into pGEM T-vector (Promega) and double-stranded sequencing to confirm identity, this cDNA fragment was used for hybridization analysis.

**Nuclear Run-off Transcription Assay**—Nuclei, prepared as described by Greenberg and Ziff (21), were resuspended at 5 × 10⁹/100 μl in 10 mM Tris-HCl (pH7.5), 5 mM MgCl₂, 0.5 mM sorbitol, 2.5% Ficoll, 0.008% spermidine, 1 mM diithiothreitol, 50% glycero and stored at −70 °C. Run-off transcription reactions were performed using 5 × 10⁵ nuclei, and radiolabeled RNA was extracted as described (22). Hybridizations, using 2 × 10⁶ cpm of radiolabeled RNA in 2 ml of 50% formamide, 5× SSC, 0.1% SDS, 1 mM EDTA, 10 mM Tris-HCl (pH 7.5), 5× Denhardt's solution, 50 μg/ml yeast tRNA, 100 μg/ml salmon sperm DNA, 0.02 μg/ml poly(A) and poly(G) RNA, were carried out at 42 °C for 72 h against 10 μg of denatured plasmid DNA immobilized to Hybond-N (Amersham Pharmacia Biotech, Buckinghamshire, UK). Plasmids used were pGEM5a (Promega), cloned GAPDH cDNA (17), and 2029 bp of COX-2 cDNA (bases 2–2030) (23) cloned into pGEM5a (Promega). Following hybridization, filters were washed in Buffer A (300 mM NaCl, 10 mM Tris-HCl, pH 7.4, 2 mM EDTA, 0.1% SDS), at room temperature for 15 min; Buffer B (10 mM NaCl, 10 mM Tris-HCl, pH 7.4, 2 mM EDTA, 0.4% SDS) at 50 °C for 30 min; Buffer A plus 1 μg/ml RNase A and 10 units/ml RNase T₁ at 37 °C for 30 min; Buffer A at 55°C for 30 min; and finally Buffer B at 55°C for 30 min before autoradiography.

**Reporter Plasmids**—The COX-2 3'-UTR reporter, pGL3.C2.3UT, was as described previously (20). This construct contains the entire COX-2 3'-UTR and part of the last exon cloned into an XhoI site downstream of the pGL3control (Promega) luciferase gene, which is driven by a SV40 promoter and enhancer.

**Transfections and Luciferase Assays**—For transient transfection, DNA, 1 μg, was incubated with 2.5 μl of FuX50 reagent (Promega)/ml of serum-free medium for 15 min. A549 cells at 60% confluency in six-well plates were washed and transfected by the addition of 1 ml of medium containing DNA/FuX50 for 2 h before changing to 1 ml of serum-free medium. Each transfection (well) contained 1 μg of DNA, 0.5 μg of reporter plasmid, and 0.5 μg of pSVβ-galactosidase. Cells were harvested for luciferase determination by resuspension in reporter lysis buffer (Promega). β-Galactosidase activity was measured using a kit (Promega).

**Immunoblot Analysis**—Cells were scraped into 100 μl of Hanks’ balanced salt solution (Life Technologies, Inc.). An equal volume of 100 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 0.05% bromphenol blue, 200 μM diethiothreitol, 2 mM phenylmethylsulfonyl fluoride was added, and the sample was boiled for 5 min. Total protein (70 μg) was separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to Hybond-ECL membranes (Amersham Pharmacia Biotech). Membranes were probed with a 1:2000 dilution of rabbit anti-human COX-2 antibody (Oxford Biomedical Research Inc., Oxford, MI). After washing, membranes were incubated with horseradish peroxidase-linked anti-rabbit immunoglobulin (Amersham Pharmacia Biotech) to allow detection by enhanced chemiluminescence (ECL) (Amersham Pharmacia Biotech).

**Statistical Analysis**—Statistical analysis was performed using analysis of variance, and data are presented as means ± S.E. Significance was taken as follows: *p < 0.05, **p < 0.01, and ***p < 0.001.

**RESULTS**

**Repression of COX Activity, PGE₂ Release and COX-2 mRNA by Dexamethasone**—In type II A549 cells, IL-1β causes a transcription- and translation-dependent induction of COX-2 protein, COX activity, and PGE₂ release (16, 22). Since COX-1 message is 100-fold less abundant and COX-1 protein is undetectable in both cytokine-stimulated and untreated cells, these effects are attributed almost exclusively to de novo COX-2 synthesis (16, 17). Furthermore, dexamethasone totally prevents these responses in both A549 and human airway epithelial cells (16, 17). To examine this effect, dexamethasone dose-response analyses were performed on IL-1β-induced COX activity and PGE₂ release in A549 cells (Fig. 1). In both cases, the IC₅₀ value lay between 10⁻⁸ and 10⁻⁹ M dexamethasone and is therefore consistent with a single predominant mecha-
nism of action at the level of COX-2 inhibition.

Previously, semiquantitative reverse transcriptase-polymerase chain reaction was used to demonstrate that dexamethasone (Dex) pretreatment totally suppressed IL-1β-induction of COX-2 mRNA at 6 h (17). We have used Northern analysis to confirm this result and now show similar inhibition characteristics to that observed for COX activity and PGE2 release (Fig. 2, right panels). In marked contrast, dexamethasone caused a maximum of only 50% repression of COX-2 mRNA levels after 2 h of IL-1β treatment, suggesting that effects on transcription may not fully explain the degree of repression observed by 6 h (Fig. 2, left panels).

**Effect of Dexamethasone on COX-2 Transcription**—As IL-1β increases COX-2 transcription by up to 8-fold (22), nuclear run-on transcription assays were performed to examine the effect of dexamethasone on COX-2 transcription rate (Fig. 3). Consistent with previous studies, 2-h IL-1β treatment produced a marked increase in COX-2 transcription, while by 6 h the transcription rate was falling. Dexamethasone pretreatment resulted in 29.8 ± 13.8% repression at 2 h. By 6 h, a 43.7 ± 18.9% repression in transcription rate relative to IL-1β at 6 h was observed. Consequently, while some degree of dexamethasone-dependent repression is exerted at the transcriptional level, we conclude that these changes alone cannot account for the almost complete repression observed at the mRNA level by 6 h poststimulation.

**Evidence for Post-transcriptional Repression**—In A549 cells, classical transcriptional arrest half-life experiments using actinomycin D showed that COX-2 mRNA was extremely stable (20). A similar approach was initiated to evaluate the effect of dexamethasone on COX-2 mRNA half-life. In these studies, the simultaneous addition of dexamethasone and actinomycin D resulted in little or no effect on COX-2 mRNA decay (data not shown and Fig. 4). We therefore examined the effect of dexamethasone on steady state levels of preinduced COX-2 mRNA. Cells were treated for 2 h with IL-1β to induce COX-2 mRNA. At this time point (t = 0), either dexamethasone or the RNA polymerase II inhibitor, actinomycin D, was added, and cells were harvested over the following 6 h as indicated (Fig. 4A). In IL-1β-treated cells, COX-2 mRNA levels continued to rise for 3 h before falling over the remaining 3 h (Fig. 4B). Following the addition of dexamethasone, COX-2 mRNA levels remained essentially unchanged for 2 h and then declined to almost undetectable levels. Actinomycin D resulted in almost no change in COX-2 mRNA levels during this time period. These data show that total RNA polymerase II-dependent transcriptional arrest failed to repress COX-2 levels, while the addition of dexamethasone, which suppressed COX-2 transcription rate by only 25–40% (Fig. 3), resulted in total loss of COX-2 message.

To further examine this effect, dexamethasone, actinomycin D, or cycloheximide (a translational blocker) or combinations of these were added to IL-1β-stimulated cells and harvested 6 h...
later (Fig. 4, C and D). Again, dexamethasone caused complete repression of COX-2 mRNA, while actinomycin D resulted in little change. The addition of actinomycin D with dexamethasone blocked the repression of COX-2 mRNA, suggesting that ongoing transcription was required for the dexamethasone effect (Fig. 4C). Alone, cycloheximide caused superinduction of COX-2, possibly via mRNA stabilization as well as increased transcription due to elevated NF-κB and c-Jun N-terminal kinase activation (24, 25). The addition of cycloheximide and dexamethasone again resulted in superinduction of mRNA levels over that observed for IL-1β alone; however, the induction was reduced by 36.8% compared with IL-1β plus cycloheximide (Fig. 4D). This correlates well with the degree of repression previously observed at the transcriptional level (Fig. 3), but the almost total dexamethasone-dependent post-transcriptional repression was not observed.

Since actinomycin D blocked dexamethasone-dependent inhibition of COX-2, the protocol was modified to allow dexamethasone-dependent transcription to occur prior to the addition of actinomycin D. Cells were stimulated with IL-1β for 2 h, to allow induction of COX-2 mRNA as in Fig. 4B, before the addition of dexamethasone. After incubation for a further 1.5 h, actinomycin D was added (t = 0), and samples were harvested at the times indicated for Northern analysis. In this time, dexamethasone-dependent transcription can occur, but this is prior to significant loss of COX-2 mRNA (Fig. 4B). During the 1.5-h dexamethasone treatment period, before to the addition of actinomycin D, steady state COX-2 mRNA levels are increasing in IL-1β-treated samples, while in the IL-1β plus dexamethasone samples COX-2 mRNA levels remain static or possibly start to decline (see Fig. 4B). This effect is reflected in the COX-2 mRNA levels at t = 0 as depicted in Fig. 5A. Using this protocol, we again found that IL-1β-induced COX-2 mRNA was very stable, showing less than 25% loss over 4 h (Fig. 5). By contrast, IL-1β plus dexamethasone-treated COX-2 was degraded with a half-life of around 3.5 h, clearly showing significantly increased turnover as a result of dexamethasone treatment. However, it is noteworthy that the degree of repression in the presence of actinomycin D was substantially less than without actinomycin D (Fig. 5A, last two lanes). This suggests a requirement for ongoing (dexamethasone-dependent) transcription for full repression.

The effect of the anti-steroid RU38486 was also tested. Alone, RU38486 had no effect on uninduced COX-2 mRNA levels (data not shown). Following IL-1β treatment, RU38486 resulted in some inhibition of COX-2 mRNA indicative of a partial agonist effect (Fig. 6). However, this effect failed to reach statistical significance. In contrast, the inhibitory effect of dexamethasone (10⁻⁷ M) was significantly antagonized by RU38486, indicating that the response was steroid receptor-mediated. The fact that complete antagonism was not observed is explained by partial agonist activity of RU38486 (14, 26–28). Taken together, these results indicate that both de novo GR-
and actinomycin D, we tested the requirement for de novo transcription and translation is required for full repression of COX-2 mRNA.

**Actinomycin D and Dexamethasone Inhibit COX Activity and PGE₂ Release**—The addition of actinomycin D (10 μg/ml) or dexamethasone (10⁻⁶ M) either just before or co-incident with IL-1β, totally abrogates the IL-1β-induced rise in COX-2 protein, COX activity, and PGE₂ release at 24 h (Figs. 1 and 7) (17, 22). However, the addition of actinomycin D 1 h after the IL-1β treatment followed by incubation for a further 23 h resulted in 50% of the full COX-2 protein and activity response, while the addition of actinomycin D 2 or more h after the IL-1β stimulation failed to cause appreciable inhibition of the COX-2 response (Fig. 7). These data show that after 2 h of IL-1β treatment, transcriptional inhibition is no longer effective in suppressing induction of COX-2. By contrast, dexamethasone was effective in repressing the IL-1β-induced COX-2 response even when added up to 10–14 h after the IL-1β. Thus, dexamethasone pretreatment or co-incubation is not necessary for inhibition, and simple repression of transcription cannot alone account for this effect. However, post-transcriptional mechanisms, for instance increased mRNA turnover, may partially explain these data. Since the dexamethasone-dependent repression of IL-1β-induced mRNA was blocked by cycloheximide and actinomycin D, we tested the requirement for de novo transcription in the effect described here. Cells were treated with IL-1β for 6 h before the addition of dexamethasome and/or actinomycin D to the medium and incubated for a further 18 h (total of 24 h) (Fig. 8). At this time, PGE₂ release and COX activity were measured. Cells were also harvested at 6 h to assess the contribution of COX activity and PGE₂ release prior to the addition of dexamethasome or actinomycin D. By 6 h, PGE₂ release and COX activity were 10.4 and 29.9%, respectively, of the levels at 24 h (Fig. 8). Alternatively, synthesis may be ongoing, and dexamethasone may cause repression via increased turnover. Actinomycin D had little effect on COX activity and partially inhibited PGE₂ release. However, the addition of actinomycin D with dexamethasone resulted in no inhibition of COX activity and only partial effect on PGE₂ release, again indicating a requirement for dexamethasone-dependent transcription.

**Represion of COX-2 by Dexamethasone Involves Preferential Loss of Polyadenylated mRNA**—During the course of these investigations, it was noted that the degree of dexamethasone-dependent repression of COX-2 mRNA appeared greater on Northern blots using poly(A)-selected RNA as opposed to total RNA (data not shown). This result suggests that poly(A) selection may result in selective loss of dexamethasone-treated COX-2 mRNA. Since mRNA degradation often involves prior cleavage, we examined the effect of dexamethasone treatment on COX-2 poly(A) tail size distribution (29). To analyze COX-2 poly(A) tail size, total RNA from IL-1β-treated cells was subjected to RNase H-dependent cleavage. This was achieved by incubating RNA with oligonucleotide H, which hybridizes to a region 261–242 bases upstream of the polyadenylation site in full-length COX-2 mRNA. Subsequent hybridization with RNase H results in sequence-specific cleavage at the RNA/DNA heteroduplex. The presence of the 3’ fragment can be revealed by Northern hybridization with the COX-2 3' UTR end probe, BS (20). This probe detects the full-length, 4.5-kb, COX-2 mRNA and not the minor 3.0-kb transcript that arises by alternate polyadenylation and accounts for less than 10% of COX-2 mRNA in these cells (see Fig. 4C and Ref. 20). Hybridization of probe BS to cleaved RNA produces a heterogeneous hybridization signal ranging from about 250 to 450 bp,
actinomycin D (Act D) (10^{-6} M), Dexamethasone (Dex) (10^{-8} M) or actinomycin D (Act D) (10 μg/ml) was added either with IL-1β or at various times after IL-1β (without further changes of medium) as indicated. In all cases, cells were incubated for a total of 24 h prior to harvesting for COX activity determination, and PGE_2 release measurement. COX activity and PGE_2 release determinations from 4–8 independent experiments are expressed as a percentage of IL-1β-treated cells (after 24 h) as means ± S.E., and representative immunoblots are shown.

To test the effect of dexamethasone on IL-1β-induced COX-2 mRNA, cells were stimulated with IL-1β for 2 h (t = 0) before the addition of dexamethasone, and cells were harvested hourly as indicated. Cells treated with IL-1β for 2 h predominantly had long poly(A) tails (Fig. 9B, lanes 1, 4, and 10). Initially, dexamethasone had little effect on this size distribution. But after 2 h incubation with dexamethasone, loss of mRNA with the longest poly(A) tails was observed (compare Fig. 9B, lanes 1 and 6). This continued over the following hours until virtually all of the hybridization signal was lost (lane 9). This profile could arise by selective and rapid degradation of mRNA with long poly(A) tails, i.e., involving a processive mechanism whereby the poly(A) tail of individual RNA molecules is rapidly removed and the mRNA is degraded or by a distributive mechanism whereby uniform shortening of all poly(A) tails occurs with degradation and loss of mRNA with the shortest poly(A) tails (30). However, the presence of unadenylated intermediates (Fig. 9B, compare lane 2 with lanes 4–9) is consistent with a fast processive removal of the poly(A) tail and accumulation of unadenylated intermediates prior to degradation of the main mRNA body (30, 31). Furthermore, if a distributive (or synchronous) mechanism was occurring, one would expect the peak of mRNA with long poly(A) tails to retain its intensity but shift to shorter poly(A) tail lengths prior to disappearing with the degradation of the mRNA body. In this experiment, loss of mRNA with the longest poly(A) tails was observed with no shift to shorter tail lengths, suggesting a processive mechanism of degradation (30, 31). While loss of poly(A) tails is generally thought to precede degradation of the mRNA body (29), there remains the possibility that dexamethasone-dependent effects may simply cause increased mRNA degradation, for instance by increasing the frequency of cleavage within the main mRNA body. However, such a mechanism would result in a blank loss of COX-2 mRNA, and a consequent reduction in signal at all sizes of poly(A) tail length would be observed. The fact that selective loss of COX-2 mRNA with the longest poly(A) tails was observed indicates that deg-
radiation of the mRNA body only occurs after loss of the poly(A) tail.

To further explore the relationship between COX-2 mRNA degradation and poly(A) tail shortening, dexamethasone treatment was carried out in the presence of actinomycin D. As already observed, actinomycin D blocked dexamethasone-dependent COX-2 mRNA degradation (Figs. 4C). Likewise, actinomycin D also prevented loss of COX-2 poly(A) tails, highlighting the possibility of a causal relationship between loss of poly(A) tails and dexamethasone-dependent repression of COX-2 mRNA (Fig. 9C). Furthermore, loss of COX-2 poly(A) tails was not simply a function of time, since RNA from cells solely treated with IL-1β for 7 h (2 plus 5 h) showed full-length poly(A) tails (Fig. 9C). To investigate the specificity of these effects, poly(A) tail variation was examined in GAPDH mRNA. RNase H oligonucleotide-dependent cleavage reactions were performed using GAPDH-specific primer R3. Heterogeneous cleavage products ranging from about 260 to 400 bp were observed, indicating poly(A) tail variation (Fig. 9A). This was confirmed by a single discrete band of about 260 bp (expected size 246–270 bp, depending on cleavage site) after the incorporation of oligo(dT) into the cleavage reaction. Failure of either RNase H or oligonucleotides alone to cause cleavage of the full-length GAPDH transcript indicates that the products are the result of oligonucleotide-dependent RNase H cleavage (Fig. 9A, lanes 3 and 4). Distribution of GAPDH poly(A) lengths was unaffected during the time course of this experiment by IL-1β, dexamethasone, or dexamethasone plus actinomycin D, indi-
Dexamethasone Repression of COX-2

Repression of gene expression by activated GR accounts for two major functions of glucocorticoids, namely classical negative feedback of the hypothalamo-pituitary-adrenal axis and immunosuppression. Clinically, the immunosuppressive nature of glucocorticoids is important in the treatment of inflammatory disease by down-regulating expression of many proinflammatory genes (6). One target of glucocorticoid repression is COX-2, whose expression is responsible for inflammatory prostaglandin synthesis and plays a major role in inflammation (1–5). Cytokine-induced COX-2 and PG synthesis is strongly repressed by dexamethasone in a number of experimental systems; however, the molecular basis for this effect is presently unclear (16, 17, 34–36).

Two mechanisms of glucocorticoid-mediated repression that have recently received much attention are those involving suppression of AP-1 and NF-κB-dependent transcription (9–14). Promoter analyses in nonhuman cells have previously identified the NF-κB, CCAAT/enhancer-binding protein, and cAMP response element sites as important in induction of COX-2 in response to phorbol esters, lipopolysaccharide, and tumor necrosis factor-α (37, 38). Since AP-1 components, for example c-Jun, can activate transcription via the COX-2 cAMP response element (39), this site may provide sensitivity to dexamethasone. Furthermore, involvement of NF-κB in the transcriptional control of COX-2 is also suggested in human cells, including A549 cells where overexpression of NF-κB subunits activates the COX-2 promoter (22, 40, 41). Analysis of COX-2 transcription rate indicated dexamethasone-dependent repression of between 30 and 40%, suggesting a partial role for GR-mediated transcription in this system. Interestingly, these data are consistent with the fact that NF-κB-dependent transcription from a β-dependent reporter stably transfected in A549 cells also showed 30–40% repression by dexamethasone (42).

The role of mRNA stability as a major determinant in the control of gene expression is now well established (29). However, the actual mechanisms for these processes are only now being elucidated. One prevailing theme is that shortening and/or loss of the poly(A) tail precedes degradation of the main body of many mRNA species. Consequently, we examined the effect of dexamethasone on COX-2 poly(A) length variation. Consistent with the above hypothesis, we observed a shortening of the average length of COX-2 poly(A) tails following dexamethasone treatment via what appeared to be a processive mechanism. This involves rapid shortening of the poly(A) tail.

**DISCUSSION**

Repression of gene expression by activated GR accounts for two major functions of glucocorticoids, namely classical negative feedback of the hypothalamo-pituitary-adrenal axis and immunosuppression. Clinically, the immunosuppressive nature of glucocorticoids is important in the treatment of inflammatory disease by down-regulating expression of many proinflammatory genes (6). One target of glucocorticoid repression is COX-2, whose expression is responsible for inflammatory prostaglandin synthesis and plays a major role in inflammation (1–5). Cytokine-induced COX-2 and PG synthesis is strongly repressed by dexamethasone in a number of experimental systems; however, the molecular basis for this effect is presently unclear (16, 17, 34–36).

Two mechanisms of glucocorticoid-mediated repression that have recently received much attention are those involving suppression of AP-1 and NF-κB-dependent transcription (9–14). Promoter analyses in nonhuman cells have previously identified the NF-κB, CCAAT/enhancer-binding protein, and cAMP response element sites as important in induction of COX-2 in response to phorbol esters, lipopolysaccharide, and tumor necrosis factor-α (37, 38). Since AP-1 components, for example c-Jun, can activate transcription via the COX-2 cAMP response element (39), this site may provide sensitivity to dexamethasone. Furthermore, involvement of NF-κB in the transcriptional control of COX-2 is also suggested in human cells, including A549 cells where overexpression of NF-κB subunits activates the COX-2 promoter (22, 40, 41). Analysis of COX-2 transcription rate indicated dexamethasone-dependent repression of between 30 and 40%, suggesting a partial role for GR-mediated transcription in this system. Interestingly, these data are consistent with the fact that NF-κB-dependent transcription from a β-dependent reporter stably transfected in A549 cells also showed 30–40% repression by dexamethasone (42). Since GR-dependent transrepression of AP-1 and NF-κB involves direct interactions between pre-existing proteins, there is no requirement for protein synthesis, so repression would be expected to occur even in the presence of cycloheximide (13, 43). Thus, the 35–40% repression of COX-2 mRNA observed in the presence of cycloheximide further substantiates these transcriptional effects.

The fact that COX-2 transcription rate was only reduced by a maximum of 40%, while repression of COX-2 protein and mRNA (at 6 h) was complete indicates that other, post-transcriptional, mechanisms of repression must also exist. This was confirmed by the addition of dexamethasone to cells that had been preinduced with IL-1β. In this case, dexamethasone resulted in total loss of preformed COX-2 mRNA within 6 h, presumably while COX-2 transcription was still occurring at up to 60% of the IL-1β-induced level. This contrasted with total inhibition of transcription by actinomycin D, which had little effect on preformed COX-2 mRNA levels. Furthermore, the dexamethasone-dependent repression took place after a lag of almost 2 h, suggesting the need for new transcription and translation. This was confirmed by the ability of actinomycin D and cycloheximide to block the dexamethasone effect.

In addition, the steroid antagonist, RU38486, also inhibited the dexamethasone-dependent repression, yet showed little repressive effect by itself. This is explained by RU38486 being an effective antagonist and only a weak agonist of GR-dependent transactivation (26, 28, 44). Yet in AP-1 transrepression assays, RU38486 showed much of the activity of dexamethasone (26, 44), while in NF-κB transrepression assays significant transrepression by RU38486 was also observed (14, 28). Taken together, this information, along with the actinomycin D, cycloheximide, and RU38486 data reported here, indicates a major role for post-transcriptional mechanisms in the dexamethasone-dependent repression of COX-2 mRNA. This mechanism (or mechanisms) requires GR-mediated transactivation, which leads to de novo transcription and translation of genes that either directly or indirectly suppress COX-2 mRNA levels.

To evaluate the functional significance of these effects, we examined the ability of dexamethasone and actinomycin D to inhibit COX-2 protein, COX activity, and PGE2 release when added with or after IL-1β. To our surprise, actinomycin D was only effective in suppressing these indicators of COX induction when added with IL-1β or within the first hour of IL-1β treatment, whereas dexamethasone effectively suppressed induction of COX-2 when added up to 10–14 h after the IL-1β. Since total suppression of transcription is not observed with dexamethasone, these data point to an important functional role for post-transcriptional, possibly including translational and post-translational, mechanisms in the dexamethasone inhibition of COX-2. Again, the dexamethasone effect was blocked by actinomycin D, indicating the requirement for de novo gene synthesis.

The role of mRNA stability as a major determinant in the control of gene expression is now well established (29). However, the actual mechanisms for these processes are only now being elucidated. One prevailing theme is that shortening and/or loss of the poly(A) tail precedes degradation of the main body of many mRNA species. Consequently, we examined the effect of dexamethasone on COX-2 poly(A) length variation. Consistent with the above hypothesis, we observed a shortening of the average length of COX-2 poly(A) tails following dexamethasone treatment via what appeared to be a processive mechanism. This involves rapid shortening of the poly(A) tail.

**FIG. 10.** The COX-2 3′-UTR is not sufficient for dexamethasone-dependent repression. Cells, in six-well plates, were transiently transfected with 1 μg of DNA made up of 0.5 μg of luciferase reporter pGL3control or pGL3.C2.3UT plus 0.5 μg of pSV-β-galactosidase. Following transfection, cells were changed to serum-free medium, and IL-1β (1 ng/ml) (solid bars) and IL-1β plus dexamethasone (10−7 M) (open bars) were added. After 24 h, cells were harvested for luciferase and β-galactosidase assay. Data, n = 3 (in duplicate for pGL3.C2.3UT), were expressed as the ratio of luciferase activity to β-galactosidase activity as a percentage of IL-1β-treated activity as means ± S.E. 

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and accumulation of deadenylated intermediates prior to degradation and loss of the main body of the RNA (31).

Many cytokine genes and other acute phase genes are regulated by post-transcriptional mRNA stability as well as transcriptionally (45, 46). Indeed, COX-2 mRNA is stabilized by IL-1, and some evidence indicates a possible role for the 3′-UTR in this effect (20, 24). Furthermore, the human COX-2 3′-UTR contains 22 repeats of the sequence AUUUA, which is common in many cytokine genes and was shown to confer instability to granulocyte-macrophage colony-stimulating factor mRNA (33, 47). The addition of the COX-2 3′-UTR to a heterologous reporter failed to confer dexamethasone sensitivity, indicating that the region is not in itself sufficient to confer dexamethasone sensitivity. However, the actual mechanism of degradation appears to involve progressive loss of the poly(A) tail. This aspect of mRNA degradation can be conferred by AU elements from the granulocyte-macrophage colony-stimulating factor 3′-UTR, and similar elements in the COX-2 3′-UTR may be expected to show a similar function (31). Consequently, while the actual mechanism of degradation of COX-2 mRNA may be dictated by sequences in the 3′-UTR, this region is not sufficient to confer dexamethasone responsiveness.

Thus, we have established that repression of IL-1-induced COX-2 by dexamethasone involves some degree (<40%) of transcriptional repression in A549 cells. However, this is not sufficient to account for the observed degree of repression at the product level. In contrast, mouse 3T3 cells showed substantial transcriptional repression of COX-2 by dexamethasone (48, 49). However, in common with our data, the degree of transcriptional repression was again insufficient to account for the total dexamethasone-dependent inhibition of COX-2 protein, indicative of further repressive mechanisms (48). Our data point to the existence of significant post-transcriptional mechanisms that require the GR transactivation function for de novo transcription and translation of a gene or genes that mediate dexamethasone-dependent repression. Therefore, we demonstrate a functional relevance for this mechanism and indicate that loss of COX-2 mRNA as a result of dexamethasone treatment involves prior loss of the COX-2 poly(A) tail. However, the sequences involved in specificity and the events needed to initiate the response remain to be elucidated, and more detailed models are required to address these outstanding issues. Consequently, dexamethasone-dependent repression of COX-2 involves transcriptional as well as additional post-transcriptional or possibly translational mechanisms. It therefore seems that the exact contribution of each repressive mechanism to the overall repressive effect may depend on the cell type and the actual stimulation as well as the time of dexamethasone addition in relation to the stimulus.

Recently, the transactivation function of GR, in driving GRE or mouse mammary tumor virus (MMTV)-dependent transcription or expression of the GR-dependent gene tyrosine aminotransferase, was shown to be defective in transgenic mice carrying a point mutation in GR that prevents dimerization (50). In these mice, the repressive effect of dexamethasone on collagenase-3 and gelatinase B gene expression was essentially unaltered, whereas expression of the proopiomelanocortin and prolactin genes, which are thought to be negatively regulated via negative GRE sites, was markedly increased. These observations have been interpreted as showing that GR dimerization is required for GR-dependent transactivation via positive GREs and repression via negative GREs, while the transcriptional interference or transpression functions of GR are unaffected (51). While this is likely to be correct, one further activation function of GR, namely the positive synergistic activation of transcription via interactions with members, for example, of the STAT transcription factor family has been overlooked (52, 53). Cooperation with STAT proteins does not require GR dimerization and may therefore be unaffected in the GR dimerization-defective mice. We therefore speculate that positive cooperative functions of GR leading to post-transcriptional repressive mechanisms may, in addition to transpression with factors such as AP-1 and NF-κB, account for GR-dependent repression of inflammatory genes such as COX-2.
Herrlich, P. (1990) Cell 62, 1189–1204
44. Heck, S., Kullmann, M., Gast, A., Ponta, H., Rahmsdorf, H. J., Herrlich, P., and Cato, A. C. (1994) EMBO J. 13, 4087–4095
45. Yang, L., and Yang, Y. C. (1994) J. Biol. Chem. 269, 32732–32739
46. Razanajaona, D., Maroc, C., Lopez, M., Mannoni, P., and Gabert, J. (1992) Cell Growth Differ. 3, 299–305
47. Caput, D., Beutler, B., Hartog, K., Thayer, R., Brown-Shimer, S., and Cerami, A. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 1670–1674
48. DeWitt, D. L., and Meade, E. A. (1993) Arch. Biochem. Biophys. 306, 94–102
49. Herschman, H. R., Kujubu, D. A., Fletcher, B. S., Ma, Q., Varnum, B. C., Gilbert, R. S., and Reddy, S. T. (1994) Prog. Nucleic Acids Res. Mol. Biol. 47, 113–148
50. Reichardt, H. M., Kaestner, K. H., Tuckermann, J., Kretz, O., Wessely, O., Bock, R., Gass, P., Schmid, W., Herrlich, P., Angel, P., and Schutz, G. (1998) Cell 93, 531–541
51. Kari, M. (1998) Cell 93, 487–490
52. Zhang, Z., Jones, S., Haysod, J. S., Fuentes, N. L., and Fuller, G. M. (1997) J. Biol. Chem. 272, 30607–30610
53. Stocklin, E., Wissler, M., Gouilleux, F., and Groner, B. (1997) Nature 383, 726–728.