Nuclear receptors such as the glucocorticoid receptor (GR) are ligand-dependent transcription factors that mediate transcription of target genes by recruiting factors that modulate chromatin structure. In this study, curcumin, a compound known to inhibit GR-mediated transcription, was used to examine the different mechanisms by which GR regulates transcription. The mechanisms of transcription regulation of metallothioneine-2A (MT2A) and solute carrier family 19 member 2 (SLC19A2), two GR target genes where the hormone-dependent gene activation is inhibited or unaffected by curcumin treatment, respectively, were analyzed by chromatin immunoprecipitation and RT-PCR experiments. The data suggest that the loss of hormone-dependent MT2A gene expression is due to the inhibition of continued transcription activity after initial assembly of the transcription machinery. In contrast, the hormone-dependent SLC19A2 gene expression is maintained because the continued transcription output after assembly of transcription machinery is unaffected by curcumin. These results suggest that the two GR target genes use alternate mechanisms to regulate expression levels at the level of continued transcription output after transcription machinery assembly.

Signal-induced transcription regulation is an important part of the cellular response to physiological and environmental stimuli. Transcription response to these stimuli can involve the direct association of the signaling molecule to DNA binding transcription factors, which in turn recruit chromatin remodelers, modifiers, and the transcription machinery to target genes. In addition, physiological and environmental stimuli can activate or deactivate cellular signaling pathways that modulate activities of the transcription activators and repressors that lead to changes in expression of their target genes (1–3).

Steroid hormone receptors (SHRs) such as the glucocorticoid receptors (GR) are examples of sequence-specific DNA binding transcription factors that respond to hormonal signals to regulate various physiological processes such as cellular metabolism, homeostasis, development, and differentiation and have been the subject of decades of intense studies (4, 5). In addition to demonstrating their important roles in these physiological processes, studies of transcription regulation by SHRs have provided invaluable insight into the understanding of the mechanisms of transcription regulation by sequence-specific transcription factors in mammalian cells. Upon ligand binding, SHRs are directed to their hormone response elements located at target genes as dimers. The promoter-bound receptors recruit various coactivators, many of which are chromatin structure- and modification-altering enzymes such as the SWI/SNF chromatin-remodeling complex and histone acetyltransferases. The activities of these coactivators result in opening of the chromatin architecture at target genes to allow the transcription machinery access to GR target genes. In addition, sequential recruitment of GR and coactivator activities results in transcription activation and stabilization of the open chromatin architecture. GR also aids in the recruitment of the Mediator complex to bridge the communication between GR coactivator activity and the transcription machinery, thereby enhancing the efficiency by which transcription regulation of target genes takes place (6–9).

We have previously used a broad range of steroid hormone antagonists and inhibitors to enhance the understanding of how SHRs regulate promoter chromatin remodeling and recruit the transcription machinery to target genes (10–13). In our present study, we aimed to further characterize the different mechanisms by which GR targets the assembly of the transcription machinery. To this end, we utilized curcumin, one of several compounds that have been found to inhibit GR-mediated transcription by affecting the phosphorylation status of GR (14). We were interested in investigating the diversity in the manner by which GR-mediated transcription activation takes place by using curcumin as a chemical tool. We monitored transcription output of GR target genes after curcumin treatment in HeLa cells. Curcumin treatment led to the inhibition of most of the GR target genes such as the metallothioneine-2A (MT2A) gene. Chromatin immunoprecipitation (ChiP) experiments revealed that inhibition of GR-mediated transcription occurs without preventing the recruitment of GR to target promoters. Kinetic analysis of transcript accumulation and RNAPII recruitment to MT2A showed that curcumin does not affect the initial recruitment of the RNAPII machinery to MT2A but inhibits the continued transcript-
tional output of MT2A mRNA. In contrast, analysis of solute carrier family 19 member 2 (SLC19A2), a gene where the hormone-dependent activation of transcription was not affected by the curcumin treatment, demonstrated that curcumin affects the initial accumulation of nascent RNA but that the transcriptional activity above basal level is allowed to continue, and after 4 h of hormone treatment, leads to very little overall difference in the level of transcripts accumulated. These results suggest that the continual transcript output of the two GR target genes examined in this work is differentially regulated at a stage after initial assembly of the transcription machinery.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—HeLa cells (CCL-2; ATCC, Manassas, VA) were grown at 37 °C with 5% CO2 in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% fetal bovine serum (HyClone, Logan, UT) supplemented with 10 mM HEPES, 2 mM glutamine, and 100 μg/ml penicillin-streptomycin (Invitrogen).

**RNA Isolation and RT-PCR**—HeLa cells grown in 6-well plates were treated with 50 μM curcumin or vehicle control (DMSO) for 30 min or 50 mM nicotinamide (NAM) or vehicle control (H2O) followed by treatment with 100 nM dexamethasone (Dex) or vehicle control (ethanol) for the amount of times indicated in the figure legends. Total RNA was isolated using TRIzol reagent (Invitrogen) following manufacturer protocol. 1.5 μg of total RNA was subjected to DNase I treatment to digest contaminating genomic DNA, and the reverse-transcription reaction was performed according to the First strand synthesis protocols (Invitrogen). PCR analysis was performed by real-time PCR (see supplemental Tables 1 and 3 for primer sequences). Real-time PCR reactions were performed using the Stratagene SYBR Green quantitative PCR master mix and Stratagene Mx3000p instrument (La Jolla, CA). After normalizing the gene transcript levels to that of GAPDH, the level of transcription in the absence of any treatment was set to 1 as described in the legends for Figs. 1 and 5–8. The data presented are an average of three independent experiments with the S.E. indicated.

**Western Blot Analysis**—Nuclei from HeLa cells were isolated as described previously (15) and lysed in buffer X (100 mM Tris-Cl (pH 8.5), 250 mM NaCl, 1%(v/v) Nonidet P-40, and 1 mM EDTA) with protease inhibitor mixture (Sigma). Proteins were electrophoresed on 7.5 or 4–20% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad). Membranes were probed with antibodies against RNAPII (H-224), TBP (SI-1), MED1/TRAP220 (M-255), TFIIB (C-18), and TFIH p89 (S-19) (Santa Cruz Biotechnology). After immunoprecipitation, 60 μl of salmon sperm DNA-protein A-agarose was added for 1 h at 4 °C to capture the immune complexes. The agarose beads were washed, chromatin was extracted, protein-DNA cross-links were reversed, and the proteins were digested by proteinase K. DNA was purified by the QIAquick PCR purification kit (Qiagen, Valencia, CA) and analyzed by real-time PCR analysis using Stratagene SYBR Green quantitative PCR master mix and Stratagene Mx3000p instrument with primers specific to the genomic loci indicated in Figs. 2, 3, 4, 5, 7, and 8 (see supplemental Table 2 for primer sequences). The data presented are an average of three independent experiments with S.E. as indicated.

**RESULTS**

**Curcumin Inhibits Glucocorticoid Receptor-mediated Transcription**—We wished to use curcumin as an agent to identify distinct pathways by which GR is able to activate transcription from target genes by examining genes that respond differentially to curcumin treatment. HeLa cells expressing endogenous GR were treated with 50 μM curcumin or vehicle (DMSO) for 30 min followed by 100 nM synthetic glucocorticoid Dex or vehicle (EtOH) for 4 h. After the Dex treatment, cells were harvested, total RNA was collected, and expression levels of known GR target genes were determined by RT-PCR. The analysis revealed that curcumin inhibits Dex-induced transcription of many of the genes tested with ChIP assay as described previously (16). Immunoprecipitation was performed overnight with antibodies against GR (H-300), RNAPII (H-224), TBP (SI-1), MED1/TRAP220 (M-255), TFIIB (C-18), and TFIH p89 (S-19) (Santa Cruz Biotechnology). After immunoprecipitation, 60 μl of salmon sperm DNA-protein A-agarose was added for 1 h at 4 °C to capture the immune complexes. The agarose beads were washed, chromatin was extracted, protein-DNA cross-links were reversed, and the proteins were digested by proteinase K. DNA was purified by the QIAquick PCR purification kit (Qiagen, Valencia, CA) and analyzed by real-time PCR analysis using Stratagene SYBR Green quantitative PCR master mix and Stratagene Mx3000p instrument with primers specific to the genomic loci indicated in Figs. 2, 3, 4, 5, 7, and 8 (see supplemental Table 2 for primer sequences). The data presented are an average of three independent experiments with S.E. as indicated.
the exception of the SLC19A2, ABLIM3, and GADD45B genes (Fig. 1).

Curcumin Does Not Prevent GR and Mediator Complex Recruitment at GR Target Genes—To identify the mechanism by which curcumin is able to inhibit GR-mediated transcription, we decided to compare and contrast the mechanism of hormone-dependent transcription initiation of the two genes that responded differently to curcumin; the SLC19A2 gene where the hormone-dependent activation of transcription was not affected by curcumin treatment versus MT2A, a gene whose hormone-dependent activation of transcription was inhibited by curcumin (Fig. 1).

A series of ChIP analyses was performed to monitor recruitment of factors that are necessary for GR-mediated transcription. First, the recruitment of GR and the Mediator complex (MED1 subunit) was determined. HeLa cells were treated for 30 min with or without 50 μM curcumin followed by 100 nM Dex for 1 h, and the recruitment of GR and MED1 to the glucocorticoid response element (GRE) was monitored by real-time PCR (Fig. 2). Both GR and the Mediator complex are recruited to both the SLC19A2 and the MT2A glucocorticoid response element region in a hormone-dependent manner, and the levels of their association were not affected by curcumin treatment versus MT2A, a gene whose hormone-dependent activation of transcription was inhibited by curcumin (Fig. 1).

FIGURE 2. Curcumin does not affect GR and Mediator levels at GR target promoters. HeLa cells were treated with 50 μM curcumin or vehicle (DMSO) for 30 min followed by treatment with 100 nM Dex or with vehicle (EtOH) for 1 h. The ChIP assay was performed using antibodies against the indicated proteins. Nonspecific IgG (N.S. IgG) was used as background control. The graphs represent ChIP data quantified by real-time PCR using primers specific to the promoter region of the SLC19A2 gene or the MT2A gene as indicated. The error bars represent the S.E.

Curcumin Inhibits the Recruitment of RNAPII and General Transcription Factors—We next sought to determine the effect of curcumin on the recruitment of total RNAPII. The recruitment of total RNAPII to the transcription start site (TSS) region of SLC19A2 and MT2A was assessed after treatment of HeLa cells with curcumin and Dex as described above and in the figure legend (Fig. 3A). The ChIP results show that there is a basal level of RNAPII association near the TSS on both genes in the absence of Dex. These levels were determined to be significantly higher than levels seen at internal locations within the gene (supplemental Fig. 1). However, a Dex-dependent increase in RNAPII recruitment can be observed on the MT2A gene, which is inhibited in the presence of curcumin. Dex-dependent change in RNAPII association near the TSS of SLC19A2 was not observed, and curcumin did not have any consequence on RNAPII levels at this location (Fig. 3A).

To ensure that the differences seen in RNAPII association at the TSS are not due to changes in levels of RNAPII in the cell, Western blot analysis of HeLa nuclear extracts treated with curcumin and Dex was performed. The Western blot shows that the levels of RNAPII remain unchanged within the experimental conditions tested and are thus unlikely to influence the level of RNAPII recruited to the TSS of MT2A (Fig. 3B). The results of the RNAPII ChIP experiment support the RT-PCR results that indicate that curcumin inhibited Dex-induced MT2A gene activation while having little impact on the SLC19A2 gene activation (Fig. 1).

To further analyze the results of curcumin exposure on the RNAPII machinery assembly, ChIP experiments were conducted using antibodies against the general transcription factors TFIIIB, TBP, and TFIIH (p89) after treatment of HeLa cells with curcumin and Dex as described in the figure legend (Fig. 4A). Basal levels of TFIIIB and TBP could be found near the TSS of both genes prior to hormone treatment. These basal levels were found to be significantly higher than levels found at nonspecific loci away from the TSS (supplemental Fig. 2). Association of TFIIIB, TBP, and TFIIH all increased in a hormone-dependent manner near the TSS of the MT2A gene, which was inhibited in the presence of curcumin, simi-
lar to what we have observed for RNAPII association at this locus. Neither curcumin nor Dex treatment leads to changes in levels of association of these three factors at the SLC19A2 gene, indicating that curcumin specifically inhibits the hormone-dependent recruitment of the members of the transcriptional machinery. Interestingly, in our ChIP assays, we could not detect the association of TFIIH(p89) at the SLC19A2 TSS under any of the conditions tested. Western blot analysis of the cellular levels of TFIIB, TBP, and TFIIH(p89) shows that they remain unchanged under the experimental condition tested and cannot be attributed to the differences in recruitment of these factors to the MT2A gene (Fig. 4B). These ChIP experiments showcase the disparity in the mechanism of transcription machinery assembly upon hormone treatment on MT2A and SLC19A2 and may explain their differential response to curcumin treatment.

Curcumin Inhibits Zinc-induced MT2A Transcription without Significantly Affecting RNAPII Recruitment—To further understand how curcumin may inhibit the recruitment of RNAPII, we wished to determine whether the impact of curcumin on transcription is specific to GR-mediated recruitment of RNAPII machinery. If curcumin affects RNAPII recruitment by other signaling pathways, it would suggest that curcumin has a general mechanism by which it inhibits activator-mediated recruitment of transcription.

To test the generality of the effects of curcumin on transcription activator-mediated transcription, we took advantage of the MT2A gene whose transcription can be activated by both glucocorticoids and metals such as zinc (17, 18). MT2A is a metallothionein protein that plays an important role in metal homeostasis in cells upon exposure to metals such as zinc, cadmium, and copper (19, 20). The transcription factor, metal-regulatory transcription factor (MTF1), accumulates in the nucleus upon metal exposure and is targeted to gene promoters containing the metal regulatory elements to activate metallothionein and other genes involved in metal homeostasis and detoxification (20).

To determine the outcome of curcumin exposure on zinc-mediated transcription, HeLa cells were treated with 50 μM curcumin or vehicle (DMSO) for 30 min followed by 100 μM zinc sulfate (Zn2+) or vehicle (water) for 4 h. After the Zn2+ treatment, cells were harvested, total RNA was collected, and the expression level of MT2A was determined by RT-PCR. The resulting analysis revealed that curcumin inhibits Zn2+-induced transcription activation of MT2A (Fig. 5A). The effect of curcumin on the recruitment of MTF1 to metal regulatory element (MRE) sequences located approximately −50 to −300 bp from the TSS of MT2A (17) was monitored by a ChIP experiment. Real-time PCR analysis of the MTF1 ChIP experiments shows that curcumin does not inhibit MTF1 recruitment to the MT2A promoter, consistent with the lack of impact of curcumin on GR recruitment upon Dex activation of MT2A (Fig. 5B). However, although curcumin inhibits Zn2+-induced transcription, the ChIP experiment probing for the effect of curcumin treatment on RNAPII recruitment demonstrated that curcumin has very little effect on the RNAPII recruitment to the MT2A TSS after 1 h of Zn2+ treatment (Fig. 5C). This is in contrast to the GR-mediated transcription inhibition of MT2A transcription where curcumin prevented the recruitment of RNAPII upon Dex treatment (Fig. 3A).

Curcumin Allows the Initial Zn2+-induced Transcription Activation of MT2A—In contrast to the Dex-induced transcription activation of MT2A, the above ChIP experiment has demonstrated that curcumin does not affect RNAPII recruitment detected after 1 h of Zn2+ treatment. This suggested that curcumin inhibition of Zn2+-induced MT2A transcription occurs at a step downstream of initial RNAPII recruit-
ment. To test this idea, we monitored the expression level of not only the total MT2A mRNA expressed (RT-PCR primers spanning an exon) but also the presence of pre-spliced, nascent MT2A mRNA in HeLa cells at different time points of Zn\textsuperscript{2+}/H11001 treatment in the presence or absence of curcumin. The nascent pre-spliced mRNA was detected by using RT-PCR primers that amplify an exon-intron junction of the MT2A gene. To ensure that we are detecting little to no genomic DNA amplification during the RT-PCR analysis, the isolated RNA samples were treated with DNase I, and minus RT controls, where every component of the RT reaction except for the reverse transcriptase was added, were conducted (data not shown).

The time course RT-PCR experiments were consistent with the initial RT-PCR analysis (Fig. 5A); curcumin treatment led to very little MT2A overall transcript accumulating over the 4-h time course (Fig. 6, MT2A). In contrast, when the accumulation of pre-spliced MT2A over the Zn\textsuperscript{2+} treatment time course was assessed, the results showed an initial burst of accumulation of pre-spliced RNA that occurs within 30 min after Zn\textsuperscript{2+} treatment. This initial burst of pre-spliced RNA accumulation was not greatly affected by curcumin pretreatment (Fig. 6, MT2A pre-spliced), which supports the hypothesis that curcumin does not affect the initial recruitment of RNAPII upon Zn\textsuperscript{2+} treatment. However, cells treated with curcumin show a rapid decline in the levels of pre-spliced...
RNA when compared with control cells, which may suggest that curcumin inhibits the continued initiation of transcription, leading to the rapid drop in pre-spliced RNA as they are processed into mature RNA.

The Initial Transcription Activation of the MT2A Gene by Dexamethasone Is Not Inhibited by Curcumin—The ChIP experiments examining the recruitment of RNAPII have revealed that although curcumin treatment leads to decreased RNAPII occupancy at TSS at 1 h of Dex treatment, RNAPII occupancy is not significantly affected by curcumin at 1 h of Zn$^{2+}$ exposure. This suggested that curcumin does not inhibit the initial recruitment of RNAPII when MT2A gene expression is induced by Zn$^{2+}$. The time course RT-PCR experiment confirmed this idea, showing that the initial accumulation of pre-spliced RNA upon Zn$^{2+}$ was not affected by curcumin (Fig. 6, MT2A pre-spliced). Rather, curcumin may inhibit the continued production of MT2A mRNA, possibly through inhibition of RNAPII reinitiation. We thought that perhaps a similar phenomenon was occurring during curcumin inhibition of Dex-mediated MT2A transcription and that the 1-h time point did not capture this initial recruitment of RNAPII due to kinetic differences in the way the MT2A gene is activated by Dex versus Zn$^{2+}$ treatment. To test this idea, we conducted a time course RT-PCR experiment to determine whether curcumin affects the initial Dex-induced transcription activation of MT2A. HeLa cells were treated with or without curcumin for 30 min followed by Dex over a 4-h time course. Both the pre-spliced nascent MT2A mRNA as well as total MT2A mRNA levels were monitored as described in the legend for Fig. 6. Interestingly, RT-PCR analysis of pre-spliced MT2A mRNA level over the Dex treatment time course showed that curcumin did not have a significant effect on the initial burst of transcription of MT2A that occurs within 30 min after Dex treatment (Fig. 7, MT2A pre-spliced). To ensure that the burst of accumulation of nascent pre-spliced RNA analyzed in the time course RT-PCR analysis truly reflects the initial recruitment of the RNAPII machinery, HeLa cells were treated with NAM instead of curcumin. In our previous work, we have demonstrated that NAM inhibits progesterone receptor- and GR-mediated transcription by preventing the recruitment of the Mediator complex, thereby inhibiting the formation of the transcription machinery (13). We would expect that under NAM treatment conditions, the time course RT-PCR analysis of pre-spliced RNA will not show any burst of transcripts being generated within 30 min of Dex treatment. When HeLa cells were treated with NAM for 30 min followed by Dex treatment for the indicated amounts of time, we were not able to observe the initial burst of pre-spliced MT2A mRNA accumulation (Fig. 7B, MT2A pre-spliced), indicating that this burst of pre-spliced mRNA generated within 30 min of Dex treatment is most likely due to the initial rounds for transcription. Consistent with our
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FIGURE 7. Curcumin does not inhibit the Dex-induced initial burst of transcription of MT2A mRNA. HeLa cells were treated with 50 μM curcumin or vehicle (DMSO) for 30 min (A) or 50 μM NAM or vehicle (H2O) followed by treatment with 100 nM Dex or with vehicle (ETOH) (B) for the indicated amounts of time on the x axis. Total RNA was harvested and analyzed by RT real-time PCR with primers specific for the pre-spliced and total MT2A mRNA as indicated or GAPDH as control. The levels of transcripts for each gene as determined by real-time PCR were normalized to those of GAPDH, and the value for the untreated control (DMSO, vehicle) was set to 1. The error bars represent the S.E. Relative Transcript, relative transcript. C, HeLa cells were treated with 50 μM curcumin or vehicle (DMSO) for 30 min followed by treatment with 100 nM Dex or with vehicle (ETOH) for the indicated amounts of time on the x axis. The ChIP assay was performed using antibodies against total RNAPII. Nonspecific IgG (N.S. IgG) was used as background control. The graphs represent ChIP data quantified by real-time PCR using primers specific to the transcription start site region of the MT2A gene as indicated. The relative immunoprecipitation (Relative IP) value of the indicated antibody for the untreated control (−curcumin, −Dex) was set to 1. The error bars represent the S.E. Pol II, RNA polymerase II.

previous work, NAM treatment also inhibited the generation of total MT2A mRNA (Fig. 7B, MT2A). Much like the observed consequences of curcumin on Zn2+ induction of MT2A (Fig. 6A), the initial burst of transcription output was followed by a rapid decline in the levels of pre-spliced MT2A mRNA levels when compared with control, suggesting that curcumin does not affect the initial transcription initiation but does perhaps affect the continued initiation of transcription of MT2A. As expected, the RT-PCR analysis of total MT2A mRNA levels shows an overall inhibition of transcription over the 4-h time course (Fig. 7A, MT2A).

Although the RNAPII ChIP experiment suggested that curcumin treatment leads to loss of RNAPII occupancy at the TSS after 1 h of Dex treatment, the time course RT-PCR analysis indicated that the initial transcription of MT2A upon Dex treatment is not affected by curcumin. This indicates that RNAPII must be recruited to the MT2A gene earlier than 1 h of Dex treatment and that this initial recruitment of RNAPII is not inhibited by curcumin. To test this idea, HeLa cells were treated with or without curcumin for 30 min followed by a time course treatment of Dex to monitor RNAPII at the MT2A TSS at time points earlier than 1 h. The time course ChIP experiment showed that in the absence of curcumin, there is a rapid increase in RNAPII occupancy within 15 min of Dex treatment (Fig. 7C). This is consistent with the initial rapid increase in pre-spliced RNA generated within 30 min of Dex treatment as determined by the time course RT-PCR experiment (Fig. 7A, MT2A pre-spliced). The level of increase in RNAPII occupancy seen at 15 min after Dex treatment was significantly lower in cells treated with curcumin (Fig. 7C). This is despite the fact that curcumin does not seem to have an effect on the initial accumulation of pre-spliced MT2A mRNA (Fig. 7A, MT2A pre-spliced). It is possible that the RNAPII occupancy observed at 15 min after Dex treatment reflects a combination of initial recruitment of RNAPII as well as reinitiating RNAPII. Because the rapid decline of pre-spliced nascent MT2A mRNA in curcumin-treated cells when compared with control suggests that curcumin inhibits reinitiation of RNAPII (Fig. 7A, MT2A pre-spliced), the lower level of RNAPII in curcumin-treated cells at the 15-min time point may be due to the lack of signal from these reinitiating RNAPII.

The Initial Transcription Activation of the SLC19A2 Gene by Dexamethasone Is Inhibited by Curcumin—We have performed a similar time course RT-PCR experiment monitoring the nascent pre-spliced SLC19A2 mRNA and mature SLC19A2 mRNA levels as described previously for the MT2A gene (Fig. 7). The time course RT-PCR analysis showed that unlike the MT2A gene, curcumin inhibits the initial burst of pre-spliced SLC19A2 mRNA accumulation (Fig. 8A, SLC19A2 pre-spliced). The treatment of HeLa cells with the known inhibitor of RNAPII machinery recruitment, NAM, leads to the loss of this initial accumulation of pre-spliced SLC19A2, demonstrating that this is a good indicator of the initial RNAPII recruitment upon hormone treatment (Fig. 8B). Although curcumin inhibits the initial accumulation of pre-spliced SLC19A2 mRNA, there appears to be a steady increase in the levels of mature SLC19A2 mRNA species even in the presence of curcumin, and by 4 h of Dex treatment, both control and curcumin-treated cells have accumulated equivalent levels of mature SLC19A2 mRNA (Fig. 8, SLC19A2). These RT-PCR data suggest that the SLC19A2 gene is able to sustain a certain level of transcription initiation and output of SLC19A2 mRNA after curcumin and Dex treatment that is rapidly being converted to mature RNA.

The ChIP experiments performed in HeLa cells showed that at the SLC19A2 gene, there is no significant increase in RNAPII levels after 1 h of Dex treatment, and the level of RNAPII remains unchanged even in the presence of curcumin (Fig. 3A). It remains possible, however, that much like what was observed for the MT2A gene, the initial recruitment of RNAPII occurs earlier than the 1-h Dex treatment time point, and the levels of RNAPII observed at SLC19A2 TSS at this point reflect equilibrium of RNAPII recruitment and reinitiation. A Dex treatment time course ChIP experiment was conducted to monitor the association of RNAPII at the SLC19A2...
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FIGURE 8. Curcumin inhibits the Dex-induced initial burst of transcription of SLC19A2 mRNA. HeLa cells were treated with 50 μM curcumin or vehicle (DMSO) for 30 min (A) or 50 μM NAM or vehicle (H₂O) followed by treatment with 100 nM Dex or with vehicle (EtOH) (B) for the indicated amounts of time on the x axis. Total RNA was harvested and analyzed by RT real-time PCR with primers specific for the pre-spliced and mature SLC19A2 mRNA as indicated or GAPDH as control. The relative transcript levels of transcripts for each gene as determined by real-time PCR were normalized to those of GAPDH, and the value for the untreated control (DMSO, vehicle) was set to 1. The error bars represent the S.E. Rel.Transcript, relative transcript. C. HeLa cells were treated with 50 μM curcumin or vehicle (DMSO) for 30 min followed by treatment with 100 nM Dex or with vehicle (EtOH) for the indicated amounts of time on the x axis. The ChIP assay was performed using antibodies against total RNAPII. Nonspecific IgG (N.S. IgG) was used as background control. The graphs represent ChIP data quantified by real-time PCR using primers specific to the transcription start site region of the SLC19A2 gene. The relative immunoprecipitation (Relative IP) value of the indicated antibody for the untreated control (−curcumin, −Dex) was set to 1. The error bars represent the S.E. Relative IP, RNA polymerase II.

with or without curcumin treatment. As with the MT2A gene, we observed a rapid increase in the association of RNAPII at the SLC19A2 gene around 15 min after Dex treatment. Interestingly, curcumin treatment led to a slight decrease in the levels of RNAPII association at 15 min after Dex treatment in comparison with control (Fig. 8C).

DISCUSSION

Transcription regulation by nuclear receptors such as GR has been a target of extensive study due to their role in various physiological processes such as development, immune response, and metabolism. In addition, nuclear receptor-mediated transcription has played a vital role as a model system to study ligand- and signal-induced transcription response by transcription activators in mammalian cells (1, 6, 7, 21). A generalized view of transcription activation by nuclear receptors such as GR has emerged where, upon hormone binding, GR dimerizes and is targeted to its cognate hormone response elements within chromatin. Ligand binding induces a conformational change in the GR that allows it to recruit various coactivators to remodel chromatin and increase accessibility of general transcription factors to GR target genes to initiate transcription (8, 21, 22).

One of the important questions in the understanding of transcription regulation by DNA binding transcription factors such as GR is the mechanisms behind their ability to uniquely regulate each target gene with respect to their magnitude and timing of expression. Depending on the cell type, target genes may be encompassed in very different epigenetic environments where a specific group of promoters of target genes may be more accessible to binding and recruitment of transcription factors, thereby resulting in these specific sets of genes to be regulated by SHRs (21, 23, 24). The specificity by which transcription factors such as GR activate each gene may also be regulated by the expression levels of co-activators and co-repressors in a given cell type that are required for the regulation of a particular set of genes. For example, the underlying chromatin architecture may influence and be influenced by the binding of factors such as FOXA1, which has been suggested to facilitate the binding of estrogen receptor in MCF7 breast cancer cells (25, 26). Interestingly, in a cell line that does not express FOXA1, such as the osteosarcoma cell line U2OS, the overlap of estrogen receptor-regulated genes identified is minimal, and FOXA1 has also been shown to have cell type-specific binding regions when compared between MCF7 and LNCaP cells, resulting in a different set of genes to be regulated by estrogen receptor (27). In addition, in U2OS cells, it has been found that there is a distinct requirement for specific Mediator complex subunits to activate individual GR-regulated genes that allows for differential regulation of these genes upon a glucocorticoid signal (28), and our previous work has found that the differences in the requirement for the Mediator complex for GR- and progesterone receptor-mediated transcription can be distinguished based on the variability in their ability to elicit hormone-dependent transcription in the presence of chemical agents such as NAM (13).

In this present study, we wished to further examine the different modes of transcription activation process employed by GR. We utilized the molecule curcumin as a tool to identify genes that respond differently to curcumin and compare and contrast their transcription regulation processes. We focused on two genes on which curcumin had very little impact (SLC19A2) or had an inhibitory effect (MT2A) on their hormone-induced gene expression levels (Fig. 1). Kinetic RT-PCR and ChIP experiments demonstrated that following hormone treatment, both MT2A and SLC19A2 genes are induced rapidly with a burst of pre-spliced transcript accumulating within 30 min. Curcumin treatment does not seem to affect the initial burst of mRNA transcription, but rather than affecting the initial recruitment of the RNAPII machinery, curcumin may also play a role in the initial burst of pre-spliced transcript accumulation.
mRNA transcription, but some level of continued transcription takes place, and over the course of 4 h of hormone treatment, leads to no discernable difference in the level of hormone-dependent transcripts accumulated in the presence or absence of hormone (Fig. 8). These time course experiments imply that there are distinct differences between MT2A and SLC19A2 in the manner by which transcriptional output is continued and perhaps the rate at which pre-spliced mRNA is processed into mature RNA. These differences are highlighted by the dissimilarity in their response to curcumin treatment.

Interestingly, both MT2A and SLC19A2 genes had substantial levels of RNAPII associated with the TSS prior to hormone treatment. For MT2A, this may be due to the relatively high level of basal expression of the gene (Ref. 29 and data not shown). In addition, studies have shown that RNAPII can be localized to promoters of low expressing genes prior to gene activation, and these genes are poised for activation by environmental cues (30–34). It is possible that both SLC19A2 and MT2A genes employ mechanisms involving release of the already present RNAPII from the TSS to increase transcriptional output.

Time course RNAPII ChIP analysis suggests a transient large increase in ChIP signal within 15 min of hormone treatment at both genes (Figs. 7C and 8C), which suggests an increase in residence time of RNAPII (35) at the TSS and/or recruitment of additional RNAPII that leads to overall increase in transcriptional output. It is also possible that upon hormone treatment, the fraction of the promoter alleles being occupied by the RNAPII machinery increases, leading to the overall increase in transcription output of MT2A mRNA. Curcumin may inhibit the functional hormone-induced assembly of the RNAPII machinery without affecting the activity of the preformed transcription complex, resulting in the transient increase in pre-spliced RNA output. Consistent with this idea, when the RNAPII machinery is allowed to assemble by treatment with Dex, curcumin treatment has no effect on the MT2A transcription output and level of RNAPII occupancy at the promoter (supplemental Fig. 3).

We have also tested the consequences of curcumin on gene expression induced by an alternate signaling pathway. We wished to examine whether curcumin also affects the transcription machinery assembly and continued transcriptional process when driven by a transcription factor other than GR to determine whether the effects observed were specific to GR-regulated transcriptional events. We took advantage of the fact that the MT2A, a metallothionein gene, can be regulated by MT1F in the presence of Zn$^{2+}$ (17). Much like MT2A expression in the presence of Dex, curcumin treatment led to the inhibition of transcription as observed after 4 h of Zn$^{2+}$ treatment, and the time course RT-PCR analysis of pre-spliced and total MT2A mRNA suggested that curcumin does not inhibit the initial burst of pre-splice mRNA transcription but inhibits the continued transcription output throughout the 6 h of Zn$^{2+}$ treatment (Fig. 6). This is consistent with the results obtained when MT2A is induced with Dex. These results suggest that curcumin does not specifically disrupt GR signaling but affects transcription output after initial transcription machinery recruitment.

Comparing and contrasting the mechanism of transcription activation and steps at which curcumin seems to have an effect on GR-mediated regulation of the SLC19A2 gene and MT2A genes revealed the differences in the mechanisms by which the two genes regulate gene output after transcription initiation. Such a difference may be attributed to alternate usage of transcription factors such as TFIIH. Our ChIP assay demonstrated that although the TFIIH (p89) signal was observed at the MT2A TSS, it was not observed on the SLC19A2 TSS. Much like transcription activators and coactivators, the members of the basal transcription machinery such as TFIIH have also been shown to have lineage- and promoter-specific residence times on target genes, leading to diversity in the manner by which genes are regulated by a signal, and this difference may explain their divergent regulation of transcription output throughout the hormone treatment (36). In addition, the requirement for mechanisms such as RNAPII phosphorylation, which is also influenced by the presence of TFIIH and the Mediator complex, and the use of factors such as negative elongation factor (NELF) and 5,6 dichloro-β-d-ribofuranosyl benzimidazole (DRB) sensitivity inducing factor (DSIF) to restrain and release RNAPII upon hormonal signal as shown for estrogen receptor signaling may be different between the two genes, resulting in the diversity in the manner by which these two genes are regulated after transcription initiation (37, 38).

Further studies combining the use of curcumin and a more genome-wide assay such as a microarray and ChIP-on-chip/sequencing will help identify more GR target genes that respond differentially to curcumin treatment. The data obtained from such an analysis can be used to further compare and contrast the differences in the mechanisms by which these genes are regulated, such as alternate requirements for histone modifications and CpG methylation status as well as the presence of TATA boxes and other transcription factor binding sites that add to the complexity of GR regulation of genes. The results presented in this work demonstrate that small molecules such as curcumin can be used to identify different modes of transcription mechanism driven by a transcription factor such as GR and provide a much broader view of the diversity in the ways in which each gene can be regulated uniquely in individual tissues at specific time points in organism development and function.

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REFERENCES
1. Rosenfeld, M. G., Lunyak, V. V., and Glass, C. K. (2006) Genes Dev. 20, 1405–1428
2. Weake, V. M., and Workman, J. L. (2010) Nat. Rev. Genet. 11, 426–437
3. White, R. J., and Sharrocks, A. D. (2010) Trends Genet. 26, 214–220
4. Mangelsdorf, D. J., Thummel, C., Beato, M., Herrlich, P., Schütz, G.,
Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., and Evans, R. M. (1995) Cell 83, 835–839
5. Evans, R. M. (2005) Mol. Endocrinol. 19, 1429–1438
6. Cheung, E., and Kraus, W. L. (2010) Annu. Rev. Physiol. 72, 191–218
7. Biddie, S. C., John, S., and Hager, G. L. (2010) Trends Endocrinol. Metab. 21, 3–9
8. Kinyamu, H. K., Jefferson, W. N., and Archer, T. K. (2008) Environ. Mol. Mutagen. 49, 83–95
9. O’Malley, B. W., and Kumar, R. (2009) Cancer Res. 69, 8217–8222
10. Mymryk, J. S., and Archer, T. K. (1995) Mol. Endocrinol. 9, 1825–1834
11. Lee, H. L., and Archer, T. K. (1994) Mol. Cell. Biol. 14, 32–41
12. Mymryk, J. S., and Archer, T. K. (1995) Genes Dev. 9, 1366–1376
13. Lee, H. L., and Archer, T. K. (1998) EMBO J. 17, 1454–1466
14. Aoyagi, S., and Archer, T. K. (2008) Mol. Cell. Biol. 28, 30–39
15. Koizumi, S., Suzuki, K., Ogra, Y., Yamada, H., and Otsuka, F. (1999) Eur. J. Biochem. 259, 635–642
16. Heuchel, R., Radtke, F., Georgiev, O., Stark, G., Aguet, M., and Schaffner, W. (1994) EMBO J. 13, 2870–2875
17. Alonso-Gonzalez, C., Mediavilla, D., Martinez-Campa, C., Gonzalez, A., Cos, S., and Sanchez-Barcelo, E. J. (2008) Toxicol. Lett. 181, 190–195
18. Laity, J. H., and Andrews, G. K. (2007) Arch. Biochem. Biophys 463, 201–210
19. John, S., Sabo, P. J., Johnson, T. A., Sung, M. H., Biddie, S. C., Lightman, S. L., Voss, T. C., Davis, S. R., Meltzer, P. S., Stamatoyannopoulos, J. A., and Hager, G. L. (2008) Mol. Cell 29, 611–624
20. Trotter, K. W., and Archer, T. K. (2007) Mol. Cell. Endocrinol. 265–266, 162–167
21. John, S., Johnson, T. A., Sung, M. H., Biddie, S. C., Trump, S., Koch-Paiz, C. A., Davis, S. R., Walker, R., Meltzer, P. S., and Hager, G. L. (2009) Endocrinology 150, 1766–1774
22. Eeckhoute, J., Lupien, M., Meyer, C. A., Verzi, M. P., Shivdasani, R. A., Liu, X. S., and Brown, M. (2009) Genome Res. 19, 372–380
23. Carrell, J. S., Meyer, C. A., Song, J., Li, W., Geistlinger, T. R., Eeckhoute, J., Brodsky, A. S., Keeton, E. K., Fertuck, K. C., Hall, G. F., Wang, Q., Bekiranov, S., Sementchenko, V., Fox, E. A., Silver, P. A., Gingeras, T. R., Liu, X. S., and Brown, M. (2006) Nat. Genet. 38, 1289–1297
24. Lee, H. L., and Archer, T. K. (1994) Mol. Cell. Biol. 14, 32–41
25. Kymber, J. S., and Archer, T. K. (1995) Mol. Endocrinol. 9, 1825–1834
26. Trotter, K. W., and Archer, T. K. (2007) Mol. Cell. Endocrinol. 265–266, 162–167
27. John, S., Sabo, P. J., Johnson, T. A., Sung, M. H., Biddie, S. C., Trump, S., Koch-Paiz, C. A., Davis, S. R., Walker, R., Meltzer, P. S., and Hager, G. L. (2009) Endocrinology 150, 1766–1774