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HISTONE HYPERACETYLATION DURING SV40 TRANSCRIPTION IS REGULATED BY p300 AND RNA POLYMERASE II TRANSLOCATION

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Abstract

The effects of the RNA Polymerase II (RNAPII) translocation inhibitors alpha amanitin and 5,6-dichloro-1-beta-D-riboenzimidazole (DRB) and an siRNA targeting p300 on the presence of RNAPII, p300, hyperacetylated H4 and H3 and unmodified H4 and H3 in transcribing SV40 minichromosomes were determined. Following treatment with alpha amanitin we observed a profound reduction in the occupancy of the promoter by RNAPII, the loss of p300 from chromatin fragments containing RNAPII, and an increase in the amount of unmodified H4 and H3 associated with the RNAPII. Treatment with DRB had little effect on the presence of RNAPII or p300 but also resulted in a significant increase in the amount of unmodified H4 and H3 present in chromatin fragments associated with RNAPII. Following treatment with a p300 siRNA, we observed a significant decrease in late transcription and a corresponding reduction in the amounts of p300 and hyperacetylated histones associated with the transcribing SV40 minichromosomes. We conclude that in transcribing SV40 minichromosomes histone hyperacetylation and deacetylation is dependent upon the presence of p300 and an as yet unknown histone deacetylase associated with the RNAPII complex and occurs coordinately as the RNAPII complex moves through a nucleosome.

Keywords

Chromatin; Transcription; SV40; RNA Polymerase II; Histone Hyperacetylation

INTRODUCTION

DNA within a eukaryotic cell is tightly compacted with the help of histone proteins and packaged into the nucleus as chromatin with the consequence that the DNA is less accessible to the various regulatory proteins and enzyme complexes that are involved in processing genetic information. However, dynamic post translational modifications to the histone tails is thought to play an important role in changing the chromatin structure of packaged DNA, thereby regulating gene expression by either causing gene activation or repression^{1; 2}. In this model, Histone Acetyltransferases (HATs) and Histone Deacetylases (HDACs) act as molecular switchboards in regulating gene expression by adding or removing acetyl groups from the ε-amino of lysine residues on the histone tails respectively. Though the exact mechanism of action of HATs and HDACs on transcriptional regulation has still not been

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elucidated there have been a number of studies that have shown a correlation between hyperacetylation and increased transcription of specific genes by the eukaryotic enzyme RNA Polymerase II (RNAPII)³.

Recently we have presented evidence that histones undergo sequential hyperacetylation and deacetylation during transcription⁴. Specifically we showed that in the early and late coding regions of transcribing SV40 minichromosomes immune selected with antibody to RNA polymerase II, hyperacetylated histone H4 and H3 were found associated with and independent of the polymerase while unacetylated H4 and H3 were preferentially found independent of the polymerase. Moreover, using the HDAC inhibitor sodium butyrate we showed that the unacetylated H4 and H3 present in the coding regions resulted from the action of an HDAC on previously hyperacetylated histones. Finally, we showed that the histone acetyltransferase (HAT) p300 was associated with the RNA polymerase II in the coding regions of the SV40 genome but was not present in the absence of RNA polymerase II in these regions.

Based upon these results we proposed a dynamic model for histone hyperacetylation during active transcription in the coding region of the SV40 genes in which hyperacetylated H4 and H3 were the result of the action of p300 bound to RNA polymerase II acting on nucleosomes ahead of the polymerase complex, while unmodified H4 and H3 were the result of the action of one or more HDACs acting on histones after passage of the RNA polymerase II complex through a nucleosome⁴.

Two aspects of this model which were not extensively explored in our publication⁴ were the relationship between histone deacetylation and translocation by the RNAPII complex and whether p300 was required for histone hyperacetylation and transcription. In order to address these issues we now report on the effects of RNAPII translocation inhibitors and a siRNA to p300 on the SV40 transcription process. Alpha amanitin and 5,6-dichloro-1-beta-Dribobenzimidazole (DRB)⁵ although functioning by different mechanisms are both known to inhibit translocation by RNA polymerase II. Alpha amanitin binds strongly to the large subunit of RNAPII complex, the Rpb1 subunit, which is the active site for addition of nucleotide triphosphates (NTPs) and causes irreversible inhibition of transcription^{6; 7}. Even though the binding of alpha amanitin does not block the addition of NTPs in the active site it significantly reduces the addition of NTPs from several thousand NTPs per minute to few nucleotides per minute^{8; 9}. For RNAPII to be functionally active, the C terminal domain (CTD) of the large subunit containing a heptad repeat, Tyr¹-Ser²-P-Pro³-Thr⁴-Ser⁵-P-Pro⁶-Ser⁷, needs to be phosphorylated on the serine residues^{10; 11; 12; 13}. Cyclin dependent kinase (Cdk) 7 has shown to phosphorylate the fifth serine residue in the heptad repeat, causing transcription initiation¹⁴ and Cdk 9 has been shown to phosphorylate the second serine residue, causing transcription elongation and promoter clearance^{15; 16}. DRB, a nucleoside analog reversibly inhibits the function of the cyclin dependent kinases thereby inhibiting the phosphorylation of the CTD and in turn inhibiting the translocation of RNAPII^{15; 17}.

Using similar techniques as described in our previous publication⁴ we studied the effects of inhibiting RNAPII dependent transcription by alpha amanitin and DRB on the presence of RNAPII and p300 and the levels of histone hyperacetylation. Using siRNA mediated knockdown of p300 followed by real time reverse transcription PCR we also determined the effect of inhibition of p300 on the transcription of the SV40 late genes and the chromatin structure of the SV40 minichromosome. Together, the results from these studies add further evidence supporting a dynamic model for histone hyperacetylation in the coding regions of SV40 genes undergoing transcription.

RESULTS

1. Inhibition of transcription by alpha amanitin but not DRB leads to reduced occupancy of transcribing SV40 minichromosomes by RNAPII

In preliminary studies we showed that Alpha amanitin and DRB both inhibited the uptake of tritiated uridine into RNA in a dose dependent manner and that there was a significant reduction in the incorporation of tritiated uridine following treatment with both inhibitors at a concentration of 250 μ M (supplemental data Figure SF1). Since the inhibitors were functioning as expected we next determined whether inhibition of transcription by either alpha amanitin or DRB affected the organization of RNAPII on the SV40 minichromosome during late transcription. We have previously used a technique which we called “immune selection and fragmentation” (*ISF*) to measure the occupancy of RNAPII on the SV40 minichromosome during the course of infection¹⁸. Using this technique we have previously shown that RNAPII was preferentially associated with the early coding region early in infection and the late coding region late in infection. Furthermore, using an SV40 mutant cs1085 virus, which had a clean deletion in the T Antigen Site I binding region and hence did not downregulate early transcription we confirmed that the shift in RNAPII occupancy from the early to the late coding regions was a consequence of a change in transcription¹⁸.

Depending upon the specific mechanism of inhibition of translocation by each inhibitor, one might expect to see no effect on occupancy or an increase/decrease in the occupancy at one or more sites in the genome. For example, if an inhibitor blocked translocation of all the RNAPII on the genome without affecting the binding of the RNAPII to the chromatin, we would not expect to see any change in occupancy. However, if the inhibitor specifically blocked the translocation of RNAPII in the promoter or coding region or affected the binding of the RNAPII to the chromatin, we might expect to observe an increase or decrease in the occupancy of RNAPII at one or more sites in the genome.

In this section and all subsequent sections in which we describe our investigations of SV40 chromatin structure, we have used unfixed SV40 minichromosomes for the figures and graphs. For quantitations, we have used at least eight separate analyses for each region of the SV40 genome studied. We used unfixed SV40 minichromosomes in these studies because in our experience unfixed SV40 chromatin yields more robust results and we have previously shown that there were no significant differences between the organization of histones, RNAPII or p300 in formalin fixed or unfixed SV40 minichromosomes^{4; 18; 19; 20}. Nevertheless, all of the chromatin analyses have been reproduced using formalin fixed SV40 minichromosomes with similar results (data not shown).

Typical examples of an *ISF* analysis of SV40 minichromosomes to determine occupancy by RNAPII following treatment with either alpha amanitin or DRB are shown in Figure 1. The amount of PCR amplification product obtained from the DNA present in the bound fraction from the early and late coding regions and promoter are shown in the odd numbered lanes, while the corresponding amount of amplification products from the DNA present in the released fraction are shown in the even numbered lanes. Compared to the untreated control we observed what appeared to be a small relative reduction in the amount of amplification products from the bound fractions and corresponding increase in the amount of amplification products from the released fractions obtained from the early (lanes 1 and 2) and late (lanes 3 and 4) coding regions following treatment with either alpha amanitin or DRB. In contrast, the effects of the inhibitors on RNAPII occupancy within the promoter (lanes 5 and 6) appeared to be significantly greater. We observed very little if any amplification product from the bound fraction following alpha amanitin treatment and a relatively large reduction in the relative amount of product in the bound fraction following DRB treatment.

In order to confirm that the occupancy of RNAPII was changing significantly in the promoter region but not the early or late regions, we semi-quantitated the amounts of products in the bound and released fractions obtained from a number of separate *ISF* analyses as previously described¹⁸. The data was then expressed as the percentage of the amplification products from the bound fraction over the total of the bound and released fractions. For this comparison we used a minimum of eight separate analyses for each of the regions of unfixed SV40 minichromosomes treated with inhibitors. Similar results were obtained from formalin fixed minichromosomes (data not shown). We have previously reported the occupancy of RNAPII at 48 hours post-infection and used these results for comparison. In the early coding region we have previously reported occupancy of $42 \pm 0.5\%$ bound, in the late coding region $53 \pm 1.0\%$ bound, and in the promoter $61 \pm 1.0\%$ bound¹⁸. Following alpha amanitin treatment RNAPII occupancy in the early region was $41 \pm 1.5\%$, in the late region $38 \pm 2.0\%$, and in the promoter 0%. Following DRB treatment we observed RNAPII occupancy in the early region to be $40 \pm 1.5\%$, in the late region to be $50 \pm 1.0\%$, and in the promoter $44 \pm 4.0\%$. It is clear from these data that there was a significant reduction in occupancy within the promoter following treatment with alpha amanitin (occupancy decreased from $61 \pm 1.0\%$ to 0%) and a lesser but still significant reduction following DRB treatment (occupancy decreased from $61 \pm 1.0\%$ to $44 \pm 4.0\%$). In the late coding region occupancy appeared to decrease significantly following alpha amanitin treatment ($53 \pm 1.0\%$ to $38 \pm 2.0\%$) but not following DRB treatment ($53 \pm 1.0\%$ to $50 \pm 1.0\%$). There did not appear to be a significant reduction in occupancy within the early coding region following treatment with either alpha amanitin ($42 \pm 0.5\%$ control and $41 \pm 1.5\%$ treated) or DRB ($42 \pm 0.5\%$ control and $40 \pm 1.5\%$ treated).

2. Inhibition of transcription by alpha amanitin and DRB results in an increase in the amount of unmodified histone H4 and H3 associated with RNAPII

In our recent publication⁴ we showed that histone deacetylation was occurring in the coding regions of SV40 minichromosomes undergoing extensive transcription. We hypothesized that the histone deacetylation was occurring after RNAPII had moved through a region of chromatin following its transcription since in regions of active transcription we did not observe unmodified histones associated with the RNAPII. There are two likely ways that an HDAC could deacetylate histones in a region of active transcription. Either the HDAC was associated with the RNAPII in a complex, as we observed for the HAT p300⁴, or the HDAC was functioning independent of this RNAPII/p300 complex. By blocking translocation by the RNAPII and analyzing the chromatin these two possible mechanisms can be differentiated.

SV40 minichromosomes treated with 250 μ M alpha amanitin, 250 μ M DRB, or an untreated control were prepared at 48 hours post-infection and analyzed by *ISFIP/ReChIP* using antibody to RNAPII in the first stage to immune select transcribing SV40 minichromosomes and antibody to hyperacetylated H4, hyperacetylated H3, unmodified H4, or unmodified H3 in the second stage to characterize the hyperacetylation status of the histones as previously described⁴. In this analysis the *ISFIP* portion allows for the determination of the hyperacetylation status of histones which are located independently of the RNAPII, while the *ReChIP* allows for the determination of the hyperacetylation status of histones colocalized with the RNAPII.

Typical examples of an *ISFIP/ReChIP* analysis of treated and untreated SV40 minichromosomes obtained at 48 hours post-infection are shown in Figure 2. In the untreated control we observed amplification products following immunoprecipitation with antibody to hyperacetylated H4 and H3 and unmodified H4 and H3 from the *ISFIP* portion of the analysis in the promoter, late, and early coding regions indicating that chromatin fragments containing these forms of histones were initially present in the immune selected chromatin. In contrast, we only observed amplification products following immunoprecipitation with antibody to

hyperacetylated H4 and H3 in the same regions following the *ReChIP* analysis indicating that only hyperacetylated H4 and H3 were present in the chromatin fragments which also contained RNAPII. These results were in agreement with our previous publication⁴.

When SV40 minichromosomes obtained from cells treated with 250 μ M alpha amanitin or 250 μ M DRB at 48 hours post-infection were subjected to an *ISFIP/ReChIP* analysis and the results compared to those obtained from untreated SV40 minichromosomes, we observed a number of differences. In the late coding region (Figure 2A) where active transcription was occurring we did not observe any changes in the pattern of amplification products in the *ISFIP* portion of the analysis from either inhibitor compared to the untreated sample, but we did observe the presence of amplification products following immunoprecipitation with antibody to unmodified H4 and H3 following inhibition with either compound in the *ReChIP* portion of the analysis. The presence of unmodified H4 and H3 associated with RNAPII in the *ReChIP* analysis was not previously observed in untreated SV40 minichromosomes obtained at 48 hours post-infection. This result indicated that blocking active translocation with alpha amanitin in this relatively actively transcribed region of chromatin resulted in the deacetylation of some of the histones associated with the RNAPII without greatly affecting the histones present in the rest of the chromatin lacking RNAPII.

A similar result was obtained when chromatin from the early region (Figure 2B) was analyzed. Neither inhibitor significantly affected the yield of amplification products in the *ISFIP* analysis of the early region compared to the untreated control. However, in the *ReChIP* analysis compared to the control both of the inhibitors caused a significant increase in the amount of amplification products obtained from immunoprecipitation with antibodies to unmodified H4 and H3. In addition to causing an increase in amplification products from unmodified histones, alpha amanitin but not DRB appeared to significantly reduce the amount of the amplification products obtained from immunoprecipitation with antibodies to hyperacetylated H4 and H3. Taken together these results suggest that at late times in infection at least some of the RNAPII transcription complexes present in the coding regions contain a HDAC which causes deacetylation upon inhibition of translocation with either alpha amanitin or DRB.

We also determined the effects of the inhibitors on histone hyperacetylation in the promoter region (Figure 2C). Unexpectedly we observed that the two inhibitors had very different effects in the promoter. Following treatment with alpha amanitin we observed that there were no amplification products derived from chromatin containing hyperacetylated histones either in the *ISFIP* or *ReChIP* analyses. This result suggested that compared to the untreated control alpha amanitin treatment was associated with the extensive deacetylation of the histones present in the promoter. In contrast the pattern of amplification products from SV40 minichromosomes treated with DRB appeared to be indistinguishable from the untreated controls suggesting that DRB had no effect on histone hyperacetylation in the promoter.

3. Inhibition of transcription in the SV40 mutant cs1085 by alpha amanitin and DRB also results in deacetylation of H4 and H3

Since inhibition of RNAPII translocation by alpha amanitin and DRB at late times in infection appeared to have its effects primarily on histones associated with RNAPII, we then determined whether the inhibitors acted similarly during early transcription. In order to address this we utilized a mutant of SV40 (cs1085) which contained a deletion of T antigen binding site I and as a consequence over expressed the early genes. We have previously used this mutant to investigate the organization of RNAPII on the SV40 chromatin¹⁸ and the organization of hyperacetylated and unmodified histones in transcribing SV40 minichromosomes⁴.

Because alpha amanitin appeared to have a profound effect upon the occupancy of RNAPII in the promoter at late times, we first determined the occupancy of RNAPII at early times as well

using an *ISF* analysis. As shown in Figure 3A and the accompanying graph, in comparison to an untreated control there was little effect of alpha amanitin or DRB on RNAPII occupancy in the early region ($60\pm2\%$ in alpha amanitin and $59\pm3.0\%$ in DRB treated compared to $57\pm2\%$ in the untreated control) or the late region ($41\pm5.0\%$ in alpha amanitin treated and $34\pm1.0\%$ in DRB treated compared to $31\pm2.0\%$ in the untreated). However, in agreement with the results observed at late times there was a profound effect on RNAPII occupancy in the promoter at early times ($48\pm7.0\%$ in alpha amanitin treated and $73\pm2.0\%$ in DRB treated compared to $97\pm1.0\%$ in the untreated control). The 49% reduction in occupancy within the promoter at early times in the cs1085 mutant following treatment with alpha amanitin is similar although somewhat less than the 61% reduction seen at late times. The 24% reduction in occupancy within the promoter following DRB treatment is also similar although somewhat greater than the 17% reduction observed at late times in infection.

Next, we analyzed the transcribing cs1085 minichromosomes obtained from cells treated with alpha amanitin or DRB or untreated for the presence of hyperacetylated and unmodified H4 and H3 using an *ISFIP/ReChIP*. Typical results from these analyses are shown in Figure 3B. Each *ISFIP/ReChIP* analysis was performed at least three times with three different preparations of treated or untreated cs1085 minichromosomes with similar results. In agreement with our previously published results we observed that untreated cs1085 minichromosomes contained only hyperacetylated H4 and H3 in both the *ISFIP* and the *ReChIP* fractions⁴. In contrast, cs1085 minichromosomes from cells treated with alpha amanitin or DRB contained both hyperacetylated H4 and H3 and unmodified H4 and H3 in both the *ISFIP* and *ReChIP* fractions. The presence of unmodified H4 and H3 in the *ReChIP* fractions from treated cells infected with cs1085 was consistent with the results described above when cells infected with wild-type virus were similarly treated at late times in infection. However, unlike the results obtained with wild-type virus at late times in infection, unmodified H4 and H3 were also observed in the early region chromatin which was not associated with RNAPII in the *ISFIP* analysis.

4. Alpha amanitin but not DRB causes dissociation of p300 from RNAPII in the coding region

As noted above, treatment of cells with alpha amanitin appeared to cause a greater increase in the amount of unmodified H4 and H3 present in SV40 transcribing minichromosomes than treatment with DRB. Because the relative proportions of hyperacetylated and unmodified histones is thought to depend upon the activity of the HATs and HDACs responsible for hyperacetylation and deacetylation respectively, one possible explanation for this result was that alpha amanitin had an effect on both the HAT activity and HDAC activity while DRB only had an effect on the HDAC. Having previously shown that the HAT p300 was colocalized with RNAPII in the coding regions of transcribing SV40 minichromosome⁴, we hypothesized that alpha amanitin may be having an effect on the presence of p300 in the regions also containing RNAPII. In order to test this hypothesis SV40 infected cells were treated with inhibitors, the SV40 minichromosomes isolated, and the transcribing SV40 minichromosomes subjected to an *ISFIP/ReChIP* analysis using in the first stage antibody to RNAPII to immune select chromosomes undergoing transcription and in the second stage antibody to p300 to determine the location of the p300 relative to RNAPII in the chromatin.

A typical example of the results obtained from this type of analysis is shown in Figure 4. Similar results to those shown in Figure 4 were obtained from at least three separate preparations of SV40 minichromosomes. Following treatment of SV40 infected cells with alpha amanitin (Figure 4A) no amplification products were obtained from the *ISFIP* or *ReChIP* fractions immunoprecipitated with antibody to p300 (lanes 3 and 6) although amplification products were obtained from the chromatin present in each input fraction (lanes 1 and 4) and following immunoprecipitation with antibody to unmodified H4 (lanes 2 and 5). In contrast, when SV40

minichromosomes from cells treated with DRB (Figure 4B) were analyzed by immunoprecipitation with antibody to p300 we observed an amplification product from the *ReChIP* fraction (lane 6) but not the *ISFIP* fraction (lane 3). The results obtained from amplification of the DNA present in the starting materials (lanes 1 and 4) and following immunoprecipitation with antibody to unmodified H4 (lanes 2 and 5) were similar to those obtained from the alpha amanitin treated minichromosomes. The results obtained from the DRB treated SV40 minichromosomes were similar to those which we have previously reported for wild-type SV40 minichromosomes obtained at late times in infection where we observed that p300 was found in the *ReChIP* fraction following immune selection with RNAPII antibody but was not found in the *ISFIP* fraction in both the early and late regions⁴. The absence of an amplification product following immunoprecipitation of the chromatin present in the *ReChIP* fraction with antibody to p300 from minichromosomes treated with alpha amanitin in contrast to the results obtained from untreated and DRB treated SV40 minichromosomes indicates that p300 was lost from chromatin fragments which contain the RNAPII complex in the coding regions of chromatin undergoing active transcription following treatment with alpha amanitin.

5. Downregulation of p300 expression with siRNA causes a reduction in SV40 late gene expression

The loss of p300 from RNAPII in the coding regions of SV40 transcribing minichromosomes following treatment with alpha amanitin coupled with the greater inhibition of transcription with this inhibitor raised the possibility that p300 activity might be necessary for optimal SV40 transcription. If p300 were necessary for SV40 late transcription, we reasoned that a reduction in the amount of p300 in a cell would result in a significant reduction in late transcription. In order to address this possibility, we used siRNA targeting p300 to knock down expression of p300.

In preliminary studies we determined that 5 nM of siRNA targeting p300 was optimal for the inhibition of p300 expression at 24 hours after transfection of the siRNA as judged by Western blotting (*data not shown*). Using this concentration of siRNA to p300 and an identical concentration of a random sequence siRNA as a control, we then determined the effects of the siRNAs on the protein levels of p300, the major SV40 late protein, VP1, and a loading control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Figure 5A). As shown in Figure 5A the levels of p300 and VP1 were unaffected by treatment with the random sequence siRNA (lane 2) compared to a control (lane 1) but were significantly reduced following treatment with siRNA targeting p300 (lane 3). In contrast no effect was observed on the level of GAPDH protein following either control siRNA (lane 2) or siRNA to p300 (lane 3) compared to the control (lane 1).

In order to confirm that the observed changes in protein levels following treatment with the siRNA targeting p300 were due to an effect on the levels of mRNA by the p300 siRNA, total RNA was also prepared from SV40 infected cells treated with p300 siRNA, a control siRNA, or left untreated and the RNA subjected to real-time RTPCR analysis with primer sets recognizing either p300, VP1, or GAPDH. All analyses were performed on at least six separate sets of treated or untreated cells. As shown in the table and product analyses in Figure 5B, the levels of mRNA for p300 and VP1 were both significantly reduced following treatment with the siRNA targeting p300 but were not affected by treatment with the control siRNA. Following treatment with the p300 siRNA we observed a shift of -9.6 ± 0.52 cycles in the cycle threshold for p300 mRNA which corresponded to approximately an 800 fold reduction in the amount of p300 mRNA. We also observed a shift of -16.4 ± 1.06 cycles in the cycle threshold for the SV40 late gene VP1 which corresponded to approximately an 85,000 fold reduction in late mRNA. Following treatment with a control siRNA we observed less than a single cycle change in the

cycle threshold for either of the genes. Similarly no effect was observed on the level of mRNA for GAPDH following either treatment with siRNA targeting p300 or a control siRNA. The cycle thresholds were changed by less than a single cycle following either treatment.

Since the levels of VP1 protein and mRNA were both significantly reduced along with the levels of p300 protein and mRNA following treatment with a siRNA targeting p300, the hypothesis that p300 was necessary for SV40 late transcription appeared to be correct. Based upon our previous studies showing that p300 could be found within the coding regions of SV40 associated with RNAPII but also independent of RNAPII in the promoter⁴, we hypothesized that the p300 siRNA might be having its effect on late transcription by acting on RNAPII. In order to test this hypothesis, we first determined whether the reduction in p300 caused by p300 siRNA affected the percentage of SV40 minichromosomes carrying RNAPII. We reasoned that if p300 was acting primarily during elongation at late times we would not expect to observe a significant reduction in the percentage of input SV40 minichromosomes which carried RNAPII. However, if p300 was necessary for the initiation of transcription and affected the binding of RNAPII to the minichromosome, we would expect to observe a significant reduction in the percentage of SV40 minichromosomes carrying RNAPII.

SV40 minichromosomes prepared from infected cells treated with p300 siRNA, a control siRNA or left untreated were subjected to a ChIP analysis with antibody to RNAPII or p300 and amplified by real-time PCR with a primer set that recognized the late region. The analysis of PCR amplification products and a table showing the change in the cycle threshold during real time PCR amplification of the DNA present in the immunoprecipitated chromatin compared to the input chromatin is shown in Figure 5C.

Following ChIP analysis of untreated SV40 minichromosomes we observed approximately a -3.3 ± 0.0 shift in cycle threshold with antibody to RNAPII and approximately a -4.0 ± 0.6 shift in cycle threshold with antibody to p300. Although the differences in cycle threshold between the amounts of chromatin immunoprecipitated with antibody to RNAPII compared to p300 did not appear to be significant, the shift in threshold indicated that approximately 10% and 6% of the input SV40 minichromosomes were carrying RNAPII and p300 respectively. The percentage of SV40 minichromosomes carrying RNAPII from untreated cells based upon the ChIP and real-time PCR analysis was in good agreement with a previous report for the percentage of SV40 minichromosomes at late times in infection which were actively undergoing transcription²¹. The slightly smaller percentage of SV40 minichromosomes carrying p300 compared to RNAPII was consistent with our previous studies⁴. Following treatment with the control siRNA we observed a -3.6 ± 0.3 shift in cycle threshold in the chromatin immunoprecipitated with antibody to RNAPII and a -4.6 ± 1.8 shift in cycle threshold in the chromatin immunoprecipitated with antibody to p300. These values while slightly smaller than those obtained from the untreated control were not statistically significant. However, in ChIP analyses of SV40 minichromosomes treated with p300 siRNA we observed a -4.1 ± 1.3 shift in cycle threshold using antibody to RNAPII and a -16.6 ± 1.5 following an analysis with antibody to p300. While the effect on immunoprecipitation with antibody to RNAPII appeared to be minimal following treatment with p300 siRNA, the effect on the presence of p300 was highly significant. There was approximately a 100,000 fold reduction in the amount of SV40 chromatin immunoprecipitated with antibody to p300.

The results obtained from an analysis of the PCR amplification products generated during the real time PCR amplification were in good agreement with the results from real-time PCR. We observed little effect on the amount of the amplification products from SV40 minichromosomes present in infected cells following treatment with either control siRNA (lane 4) or p300 siRNA (lane 7) compared to the untreated SV40 minichromosomes (lane 1). Similarly, we observed little difference in the amount of amplification products from the SV40 minichromosomes

immunoprecipitated with antibody to RNAPII in the control siRNA treated cells (lane 5) and the p300 siRNA treated cells (lane 8) compared to the untreated cells (lane 2). However, when SV40 minichromosomes from cells treated with p300 siRNA were immunoprecipitated with antibody to p300 we observed a large decrease in the amount of amplification product following PCR amplification (lane 9) compared to the amount obtained from the untreated (lane 3) and control siRNA treated (lane 6) cells.

These results indicated that treatment of SV40 infected cells with p300 siRNA had little if any effect on the binding of RNAPII to SV40 minichromosomes, but a large effect on the binding of p300 to SV40 minichromosomes consistent with the hypothesis that p300 was functioning during elongation and not initiation.

6. Effects of p300 knockdown on the organization of SV40 minichromosomes containing RNAPII

Since late transcription was significantly reduced following treatment with siRNA to p300 without a significant reduction in the amount of RNAPII bound to SV40 minichromosomes, we then determined whether the reduction in transcription was related to either the organization of RNAPII or a change in the extent of histone hyperacetylation in the SV40 minichromosomes. Since we have previously shown that p300 is normally found associated with an RNAPII complex in the coding regions of SV40 minichromosomes undergoing transcription⁴, we hypothesized that in the absence of p300 the organization of RNAPII and histone hyperacetylation might reflect the original function of the p300. For example, if p300 was loaded onto an RNAPII complex in the promoter and remained associated until transcription terminated, we would not expect to find many RNAPII complexes in the coding regions following siRNA knockdown because of the small amount of transcription occurring but instead find the majority of the RNAPII in the promoter. In contrast, if p300 was cycling on and off the RNAPII complex during the course of transcription we would not expect to see much effect on the organization of RNAPII on the genome following siRNA knockdown compared to a control. Similarly, we reasoned that if p300 was responsible for histone hyperacetylation in the SV40 coding regions we would expect to see a significant reduction in the level of histone hyperacetylation following treatment with p300 siRNA.

In order to determine the effects of p300 siRNA on the organization of RNAPII, we subjected SV40 minichromosomes obtained from cells treated with p300 siRNA to an *ISF* analysis using antibody to RNAPII as previously described¹⁸. A typical example of this type of analysis is shown in Figure 6A. Comparing the relative amounts of PCR amplification products from the bound (odd numbered lanes) and the released fractions (even numbered lanes) in chromatin originally present in the early (lanes 1,2), late (lanes 3,4) and the promoter (lanes 5,6) regions we observed relatively similar amounts in each lane. Moreover, the relative amount of amplification product in each fraction was similar to what we showed previously for the control in Figure 1A. In order to confirm that the results of the *ISF* analysis of RNAPII from SV40 minichromosomes treated with siRNA to p300 were similar to a control, we semi-quantitatively analyzed treated minichromosomes using our *ISF* approach¹⁸. Using multiple preparations of treated SV40 minichromosomes we averaged the results from at least 8 separate analyses to obtain the results shown in the table in Figure 6B. Compared to a control we observed RNAPII occupancy to be only slightly reduced. In the early region occupancy was 39 ± 0.5 in the treated and 42 ± 0.5 in the control; in the late region occupancy was 48 ± 0.5 in the treated and 53 ± 1.0 in the control; and in the promoter occupancy was 55 ± 1.0 in the treated and 61 ± 1.0 in the control. We concluded that knockdown of p300 did not have a major effect on the occupancy of RNAPII in the SV40 minichromosomes. In addition, the occupancy results suggested that p300 was capable of cycling on and off the RNAPII complex during transcription and was not simply staying bound to the complex from initiation to termination.

In order to determine whether reduction of p300 affected histone hyperacetylation, we subjected siRNA treated SV40 minichromosomes to an *ISFIP/ReChIP* analysis as previously described⁴. SV40 minichromosomes treated with siRNA to p300 were immune selected with antibody to RNAPII to select only those SV40 minichromosomes carrying RNAPII and the supernatant and bound fractions obtained following sonication analyzed with antibodies to p300, hyperacetylated H4 and H3 or unmodified H4 and H3. As shown in Figure 6C, in the *ISFIP* portion of the analysis we obtained amplification products only from the input chromatin and unmodified H4 and H3 indicating that there was little if any p300 or hyperacetylated H4 or H3 present in chromatin fragments lacking RNAPII. In the *ReChIP* portion of the analysis all fractions gave some PCR product although immunoprecipitation with p300 antibody gave the least product indicating that some p300 and hyperacetylated and unacetylated H4 and H3 were present in chromatin fragments containing bound RNAPII. Compared to an untreated control shown in Figure 2 it was clear that there was a significant increase in the levels of unmodified H4 and H3 and corresponding decrease in the levels of hyperacetylated H4 and H3 consistent with a critical role for p300 in controlling the extent of histone hyperacetylation in transcribing SV40 minichromosomes. Moreover, the fact that p300 was present in the chromatin fragments which contained hyperacetylated H4 and H3 but not in chromatin fragments lacking hyperacetylated H4 and H3 suggested that p300 was likely to be responsible for the hyperacetylation of histones present in the coding region and not another HAT.

DISCUSSION

The results obtained in these studies were in good agreement with a model for histone hyperacetylation in the coding regions of genes during active transcription which we recently proposed⁴. In our model histones may undergo hyperacetylation by an RNAPII associated HAT as the RNAPII complex approaches or passes through a nucleosome and deacetylation as the RNAPII complex leaves the nucleosome. The increase in unmodified H4 and H3 in chromatin fragments associated with RNAPII from the coding regions of transcribing SV40 minichromosomes following inhibition of translocation by alpha amanitin and DRB was consistent with this model since the results suggested that HDACs function in regions of active transcription in association with the RNAPII transcription complex. A critical role for p300 in histone hyperacetylation during SV40 transcription was also confirmed by these studies, since alpha amanitin but not DRB appeared to cause the loss of p300 from the RNAPII complex and a corresponding reduction in the amount of hyperacetylated H4 and H3 associated with the RNAPII. Moreover, the reduction in transcription and histone hyperacetylation following downregulation of p300 with siRNA confirmed that p300 played a critical role in SV40 transcription.

Histone hyperacetylation and deacetylation in transcribing chromatin has been considered to be a dynamic process for some time^{22; 23; 24}. While this idea is primarily based upon studies characterizing events in the promoter during initiation and subsequent re-initiation of transcription, for example^{25; 26}, we believe that continuous cycling of histone hyperacetylation may also take place in the coding regions of a gene as the RNA polymerase II complex passes through a nucleosome.

We would like to suggest that dynamic histone hyperacetylation may play a critical role in determining the overall rate of RNAPII translocation and by extension the rate of transcription through the coding regions of SV40 minichromosomes and potentially cellular genes as well. In this model the rate of translocation would depend upon the status of the histones present in nucleosomes in front of the RNAPII complex and whether a HAT and/or an HDAC were present on the RNAPII complex. If the histones in front of the complex were hyperacetylated, the RNAPII could translocate relatively quickly through a nucleosome. In contrast, if the histones were not hyperacetylated, an associated HAT would need to function before

translocation could occur and the time required for the HAT to function would slow down the rate of translocation. Similarly, if an RNAPII complex contained an associated HDAC activity, the HDAC would act to deacetylate histones following translocation through a nucleosome. While this would probably not directly affect the translocation of the RNAPII complex carrying the HDAC as it passes through the nucleosome, it would tend to slow down the next RNAPII complex since that complex would have to use its associated HAT in order to be able to translocate through the nucleosome. If this next RNAPII complex did not have an associated HAT, the RNAPII complex would have to pause until a HAT could cycle onto the complex.

In this model we propose that there are two likely ways that regulation of histone hyperacetylation in the coding region might occur. First, during the generation of an initiation complex within the promoter we propose that the RNAPII complex can be loaded with either a HAT or an HDAC, or both a HAT and an HDAC. The greater the relative number of RNAPII complexes loaded with only a HAT, the greater the level of overall histone hyperacetylation in the coding region and the faster the rate of translocation. Second, we propose that within the coding region the HAT and possibly the HDAC can cycle off and on the RNAPII complex in response to external signals. This model is consistent with the observation that the rate of RNAPII elongation is variable and dependent upon the influence of external factors^{27; 28}. While the rate of elongation has been thought to be controlled by the phosphorylation status of the RNAPII^{27; 28}, based upon our results we believe that chromatin structure might also impact this rate either as a consequence of RNAPII phosphorylation status^{27; 28} or through other as yet undefined control mechanisms. This model is also consistent with recent evidence showing that Set2 associated with RNAPII can direct histone deacetylation following passage of an RNAPII transcription complex through chromatin thereby affecting the rate of transcription and blocking intragenic transcription initiation²⁹. Our model extends this idea of histone hyperacetylation/deacetylation controlling the rate of transcription by suggesting that the rate of transcription can be “fine tuned” by controlling the proportion of RNAPII complexes containing an associated HAT and/or HDAC.

HDACs and HATs are thought to be recruited to chromatin either through interactions with other proteins which bind sequences in the promoter or by binding directly to chromatin in which the histones contain specific modifications^{24; 30}. HDACs have been shown to interact with transcriptional factors in the promoters of certain genes^{25; 26; 30; 31; 32; 33; 34} and potentially could be loaded onto an RNAPII complex during the initiation process. This proposal is consistent with the view of RNAPII as an organizer for the elements present in the transcription complex. A number of proteins which may modulate chromatin structure such as Brm, Swi/Snf components, p300, Paf1, and PCAF have been shown to be bound to RNAPII in the transcription complex^{35; 36; 37; 38; 39}.

Alternatively, specific histone methylation may serve as a marker for recruitment of HDACs to coding regions^{29; 40; 41}. Our results would also fit with this mechanism as long as the marker for recruitment was generated by passage of the transcription complex. Regardless of the specific mechanism by which the RNAPII complex directs HDAC activity, it seems clear from these results that the HDAC does not act through a “scavenger” mechanism in which there is random deacetylation of all hyperacetylated histones. If the latter had been the case we would have expected to observe greater histone deacetylation throughout the coding region and in particular in the regions of chromatin lacking RNAPII.

The conclusion that p300 plays an essential role in transcribing through SV40 coding regions is consistent with a number of studies showing that p300 has a major functional role during transcription^{42; 43; 44; 45}. In particular the inhibition of late transcription which we observed following siRNA knockdown of p300 was very similar to the results presented in a recent comparable study⁴⁶. In many of these studies p300 has been thought to play a role in events

occurring within the promoter,^{44; 47}. However, there have been reports that p300 plays a critical role in transcription during elongation and not necessarily during the generation of an initiation complex^{48; 49}. Our results with SV40 minichromosomes undergoing transcription which were obtained in vivo agree with this interpretation.

Because of the involvement of p300 in the transcription of a number of genes, it is possible that the effects of siRNA knockdown may be due to the inhibition of another critical transcriptional factor. However, we believe that this is unlikely given the strong correlation between the reduction in p300 occupancy on the SV40 minichromosomes, reduction in histone hyperacetylation, and reduction in late transcription following siRNA knockdown. Similarly, it is possible that another HAT besides p300 may be responsible for some of the histone hyperacetylation associated with the RNAPII complex in cells treated with p300 siRNA.

Although both alpha amanitin and DRB caused an increase in the levels of unmodified H4 and H3 in treated transcribing minichromosomes, it was clear from our results that each inhibitor appeared to cause its effects through different mechanisms. Alpha amanitin is known to bind RNAPII near the catalytic site and to lead to ubiquitination of the RNAPII^{50; 51}. However, it is not clear why the binding or ubiquitination should also affect p300 association or the ability of the RNAPII to bind to the promoter as was observed in our studies. Although there are two recent reports concerning the effects of alpha amanitin on chromatin structure^{52; 53}, it is difficult to compare our results to these publications because the analyses were performed very differently. For example, our studies focused only on transcribing chromatin based upon the presence of RNAPII while the two publications used unselected chromatin. We used alpha amanitin treatment for only one hour while the other studies used different concentrations for from two to five hours of treatment. Finally, we used antibodies to p300 and unmodified H4 and H3 which were not used in the previous studies. Nevertheless, there were certain similarities between our results and the previous publications. For example, hyperacetylated H4 and H3 were also observed previously in the coding regions of genes following treatment with alpha amanitin^{52; 53}. Additionally, some but not all HATS tested were found to dissociate from the coding regions depending upon the gene analyzed⁵². The effects of alpha amanitin on RNAPII in these two publications were conflicting. In one⁵³ the RNAPII was essentially unaffected by alpha amanitin treatment, while in the other publication treatment resulted in a significant loss of RNAPII from the whole gene⁵².

DRB, in contrast to alpha amanitin, is thought to act as an inhibitor by blocking the phosphorylation of the carboxy terminal domain (CTD) of RNAPII which is necessary for binding of cofactors and translocation^{54; 55; 56}. The lessened effects which we observe with DRB most likely reflects the fact that it is only the CTD that is affected by DRB and not the rest of the RNAPII molecule.

MATERIALS AND METHODS

Cells and Viruses

SV40 virus and chromatin were prepared in the BSC-1 cell line of monkey kidney cells (ATCC). The 776 SV40 wild type virus and cs1085 mutant virus were a gift from Dr Daniel Nathans.

Cell Culture and Infections

BSC-1 cells were maintained at 37° C in minimal essential medium containing 10% fetal calf serum, gentamycin, glutamine, and sodium bicarbonate (GIBCO). Sub-confluent monolayer of cells were infected with either the wild-type 776 virus or mutant cs1085 virus as previously described⁵⁷. The virus was allowed to adsorb onto the cells for 30 min. The virus was washed

off, fresh serum free medium was added and the plates were incubated further for 8 hours and 48 hours at 37° C in an incubator. SV40 minichromosomes were harvested at 8 hours and 48 hours post-infection respectively. SV40 chromatin was fixed with formalin by adding 0.275 ml of 37% formalin to the cells and incubating the cells with the formalin for 10 min at 37° C in an incubator. The formalin-fixed SV40 chromatin was isolated and prepared the same as the unfixed chromatin described below. Treated cells were grown in the presence of 250µM of alpha amanitin or 5,6-dichloro-1 beta-D-ribofuranosylbenzimidazole (DRB), [Sigma] for 1 hour prior to harvesting the minichromosomes. SV40 minichromosomes from treated cells were harvested in a similar manner as described above.

Preparation of SV40 minichromosomes

SV40 minichromosomes were harvested from infected cells and purified by glycerol gradient centrifugation as previously described^{58; 59}. Gradient fractions four and five, which contained SV40 minichromosomes, were combined for subsequent analysis.

Small Interfering RNA Transfection

Seventy percent confluent BSC-1 cells in a 75 cm² flask were infected with wild type 776 SV40 virus as previously described. Twenty four hours later the cells were transfected with 5 nM of either p300 siRNA (sc-29431) or control siRNA - A (sc-37007) oligonucleotides; Santa Cruz Biotechnology using Lipofectamine 2000 (Invitrogen). The SV40 minichromosomes from the siRNA transfected cells were harvested as previously described above. Total RNA was extracted from the transfected cells using the PARIS Kit (Ambion, Austin, TX) according to the manufacturer's protocol. Proteins for western blotting were extracted using 1 X RIPA buffer.

Chromatin Immunoprecipitations followed by Chromatin Fragmentation (ISF)

Alpha amanitin treated, DRB treated or untreated, unfixed and fixed SV40 minichromosomes were immunoprecipitated with either 10µl antibody to RNA Polymerase II (sc-900; Santa Cruz Biotechnology) using the reagents and protocol supplied by Upstate for the analysis of hyperacetylated H4 with minor modifications as previously described^{18; 19; 20} with the exception of the addition of 175µl of chromatin to the pelleted agarose after 4 hours of incubation with the RNAP II antibody. In the final step of chromatin immunoprecipitation the pelleted agarose was resuspended in 400 µl of TE buffer. 200µl of resuspended agarose was sonicated and prepared for subsequent analysis as previously described^{18; 19}

Immune Selection Fragmentation followed by a second Immunoprecipitation (ISFIP)

Alpha amanitin treated, DRB treated or untreated, unfixed and fixed SV40 minichromosomes were immunoprecipitated with antibody to RNAP II as described above for the *ISF* procedure. In the final step of *ISF*, the soluble fraction obtained after sonication was used as the secondary input sample (200 µl) and immunoprecipitated with antibody to unacetylated histone H4 (sc-8653), unacetylated histone H3 (sc-8657), p300, (Santa Cruz Biotechnology) hyperacetylated histone H4 (06-866), hyperacetylated histone H3 (06-599) (Upstate). The immunoprecipitation was carried out as described previously^{18; 19; 20}.

Re Chromatin Immunoprecipitation (Re-ChIP)

ReChIP was performed according to the procedure described by IJpenberg et al (2004) with minor modifications⁶⁰. Alpha amanitin treated, DRB treated or untreated, unfixed and fixed SV40 minichromosomes were immunoprecipitated with antibody to RNAPII as described above for the *ISF* procedure. In the final step of *ISF*, the bound fraction was eluted twice with 200 µl Immunopure Gentle Ag/Ab Elution Buffer (Pierce). The bound fraction was incubated for 15-minute with elution buffer at room temperature and the eluted chromatin recovered by

centrifugation. The eluates were pooled as the secondary input sample (200 μ l) and immunoprecipitated with antibody to p300, unacetylated histone H4 and unacetylated histone H3 (Santa Cruz Biotechnology) or hyperacetylated histone H4 or hyperacetylated histone H3 (Upstate). The immunoprecipitation was carried out as described previously^{18; 19; 20}. Specificity of the antibodies against hyperacetylated histone H3 and H4 and unmodified H3 and H4 is shown in the supplementary figure 2.

Preparation of DNA for PCR

Samples were prepared for PCR by phenol/chloroform extraction followed by ethanol precipitation in the presence of paint pellet co-precipitant (Novagen) as previously described^{18; 19; 20}. Non-fluorescent paint pellet co-precipitant (Novagen) was used to precipitate DNA that was amplified by real time PCR. Approximately 100 μ l of protein A agarose eluates was purified using phenol/chloroform. The aqueous phase (125 μ l) was added to a PCR tube that contained 3 μ l of pellet paint co-precipitant or 3 μ l of non-fluorescent pellet paint co-precipitant and 12.5 μ l of 3 M sodium acetate, pH 5.2 (Novagen). The samples were mixed and 280 μ l of 100% ethanol added to each. Following 10-min incubation at room temperature, the samples were centrifuged at 8000 $\times$ g for 5 min and the supernatant discarded. The samples were washed with 70% ethanol, vortexed, incubated for 5 min, and then centrifuged at 8000 $\times$ g for 5 min. The supernatant was again discarded and the samples were dried in a vacuum.

PCR amplifications

DNA was amplified from three different regions of the SV40 genome (the early coding region, the late coding region and the promoter) in a Perkin-Elmer Model 480 thermal cycler using Ampli Taq Gold DNA Polymerase (Applied Biosystems) with primer sets 5'GCTCCCATTCATCAGTTCCA3' and 5' CTGACTTTGGAGGCTTCTGG3' for the amplification of the early region (nt 4540-4949), 5' CAGTGCAAGTGCCAAAGATC3' and 5'GCAGTTACCCCAATAACCTC3' for amplification of the late region (nt 1566-1878) and 5'GCAAAGCTTTTTTGCAAAAGCCTAGGCCT3'and 5'CGAACCTTAACGGAGGCCTGGCG3' for amplification of the promoter region (nt 5168-420). A master mix containing all the required constituents was prepared according to the instructions supplied with the DNA polymerase in advance and kept at -20°C until required. Immediately before use, the master mix was thawed and a volume corresponding to 30 μ l for each sample to be amplified was removed to prepare a working mix. The working mix was then prepared by adding the DNA polymerase to the master mix in the ratio of 0.5 μ l per 30 μ l of master mix. Following thorough mixing, the 30 μ l of working mix was added to each previously prepared PCR tube containing a sample of template DNA to be amplified. The tubes were gently vortexed to suspend the pelleted DNA present in the tubes. When suspension was complete, the samples were overlaid with two drops of molecular biology grade mineral oil (Sigma). All previous manipulations were performed in a Nuair biological safety cabinet Model NU_425-400. The samples were centrifuged for 1 min at 10,000 $\times$ g in an Eppendorf micro centrifuge, and the PCR amplifications were hot started by heating the tubes for 2 min and 30 sec at 95°C . The DNA was amplified for 45 cycles with each cycle consisting of annealing at 60°C for early region, 64°C for late region and 70°C for the promoter region for 1 min, DNA synthesis for 1 min at 72°C and denaturation at 95°C for 1 min.

Real Time PCR

DNA was amplified from the late region of the SV40 genome in a Cepheid Smart Cycler 2.0 System using the QuantiTect SYBR Green Real Time PCR Kit (QIAGEN, Valencia, CA) using the same primer set for the late region as described above. The working mix was prepared by adding the late primer set (0.3 μM each primer) to the master mix. The PCR amplifications were hot started by heating the tubes for 15 min at 95°C . The DNA was amplified for 40 cycles

with each cycle consisting of annealing at 64° 30 sec, DNA synthesis for 30 sec at 72° C and denaturation at 94° C for 15 sec. All samples were analyzed in duplicate sets.

Real Time Reverse Transcription PCR

Real Time RT-PCR analysis was performed using the QuantiTect SYBR Green RT-PCR kit (QIAGEN, Valencia, CA). A master mix containing 25 µl of 2X QuantiTect SYBR Green RTPCR Master Mix (1X), primer sets (0.5 µM of each primer), 0.5 µl of QuantiTect RT Mix (0.5 µl/reaction), template RNA (500 ng/reaction) and RNase free water to make the final volume of the mix 50 µl. The primer set used for the late region were the same as above, primers for p300 (sc-29431-PR) and GAPDH (sc-35448-PR) were purchased from Santa Cruz Biotechnology. The samples were centrifuged for 30 sec in a micro centrifuge before placing them in a Cepheid Smart cycler. First strand cDNA was synthesized at 50° C for 30 min, followed by a 15 min initial PCR activation step at 95° C to activate the HotStarTaq DNA Polymerase. The DNA was amplified for 40 cycles with each cycle consisting of annealing at 64° C for late region, 60° C for GAPDH and p300 for 1 min, DNA synthesis for 1 min at 72° C and denaturation at 94° C for 1 min and a final extension at 72° C for 10 min.

Western Blotting

Protein extracted from siRNA transfected, 48 hour wild type 776 SV40 virus infected cells were harvested and lysed using 1X RIPA buffer. Protein levels were determined with the Micro BCA™ Protein Assay kit (Pierce Biotechnology, Inc., Rockford, IL). The proteins were separated on a 4–20% PAGER® polyacrylamide gel (Cambrex BioScience Inc., Walkersville, MD) under denaturing conditions and electroblotted onto PVDF membrane (Millipore, Billerica, MA). Antibodies used for western blotting were rabbit polyclonal anti p300 (sc-584), goat polyclonal anti GAPDH (sc-20357); Santa Cruz Biotechnology and rabbit GST-fusion anti VP1⁶¹ (a gift from Dr Ariella Oppenheim).

Analysis of PCR Amplification Products

Following PCR amplification of the DNA samples, the products were separated on 2.4% submerged agarose gels (Sigma) by electrophoresis^{18; 19; 20; 58; 59; 62}. The separated products were visualized by staining with ethidium bromide and electronically photographed using UVP GDS8000 Gel Documentation System (Ultra Violet Products).

Scanning Densitometry

Quantitation of agarose gels was done with Molecular Analyst (Version 1.4) from BioRad. Using Molecular analyst images were obtained by importing those that were captured with UVP GDS8000 Gel Documentation System. On importing the image, quantitation was performed with the Volume Analysis function to determine the percent volume of DNA bands of interest. The Local Background subtraction function was utilized to normalize background noise.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Relative occupancy of RNA Polymerase II on the SV40 genome after treatment with transcription inhibitors

Unfixed SV40 minichromosomes either untreated or treated with 250 μ M of alpha amanitin or DRB were isolated from cells infected with 776 wild-type virus for 48 hours. Purified SV40 minichromosomes were immunoprecipitated with antibody to RNA polymerase II using the ISF technique. Data represented in the graph is Mean $\pm$ S.E.

Figure 2. Level of histone hyperacetylation associated with transcribing SV40 chromatin after treatment with alpha amanitin and DRB

Inhibitor treated and untreated SV40 minichromosomes isolated from cells infected with 776 wild-type virus for 48 h were subjected to an ISFIP/ReChIP analysis. The samples were amplified by simplex PCR with primer sets to the late (2A), early (2B) and promoter (2C) regions. The position of the amplification product from the wild-type 776 DNA is indicated. Lane 1, ISFIP input fraction; lane 2, ChIP with 7.5 μ l of hyperacetylated histone H4 antibody (ISFIP); lane 3, ChIP with 10 μ l of unacetylated histone H4 antibody (ISFIP); lane 4, ChIP with 10 μ l of hyperacetylated histone H3 antibody (ISFIP); lane 5, ChIP with 10 μ l of unacetylated histone H3 antibody (ISFIP); lane 6, ReChIP input fraction; lane 7, ChIP with

7.5 μ l of hyperacetylated histone H4 antibody (ReChIP); lane 8, ChIP with 10 μ l of unacetylated histone H4 antibody (ReChIP); lane 9, ChIP with 10 μ l of hyperacetylated histone H3 antibody (ReChIP); lane 10, ChIP with 10 μ l of unacetylated histone H3 antibody (ReChIP). The PCR products in the ISFIP and ReChIP lanes were amplified from one-half of the total amount of DNA obtained from each of the samples. The PCR products in the input lanes were amplified from one-fourth of the total amount of DNA present in each of the input samples.

Figure 3. Effect of transcription inhibitors on mutant cs1085 minichromosomes

(3A): Relative occupancy of RNA Polymerase II on mutant cs1085 SV40 chromatin after treatment with transcription inhibitors. Inhibitor treated or untreated SV40 minichromosomes were isolated from cells infected with mutant cs1085 virus for 48 h. Purified SV40 minichromosomes were immunoprecipitated with antibody to RNA polymerase II using the ISF technique. Data represented in the graph is Mean $\pm$ S.E.

(3B): Level of histone hyperacetylation in transcribing mutant cs1085 minichromosomes treated with transcription inhibitors. Inhibitor treated and untreated SV40 minichromosomes isolated from cells infected with mutant cs1085 virus for 48 h were subjected to an ISFIP/ReChIP analysis. The samples were amplified by simplex PCR with primer set to the early region. The position of the amplification product is indicated. Lane 1, ISFIP input fraction; lane 2, ChIP with 7.5 μ l of hyperacetylated histone H4 antibody (ISFIP); lane 3, ChIP with 10 μ l of unacetylated histone H4 antibody (ISFIP); lane 4, ChIP with 10 μ l of hyperacetylated histone H3 antibody (ISFIP); lane 5, ChIP with 10 μ l of unacetylated histone H3 antibody (ISFIP); lane 6, ReChIP input fraction; lane 7, ChIP with 7.5 μ l of hyperacetylated histone H4 antibody (ReChIP); lane 8, ChIP with 10 μ l of unacetylated histone H4 antibody (ReChIP); lane 9, ChIP with 10 μ l of hyperacetylated histone H3 antibody (ReChIP); lane 10, ChIP with 10 μ l of unacetylated histone H3 antibody (ReChIP).

Figure 4. Association of p300 with transcribing SV40 minichromosomes treated with transcription inhibitors

Unfixed SV40 minichromosomes treated with **(4A)** alpha amanitin and **(4B)** DRB were isolated from cells infected with 776 wild-type virus for 48 h and subjected to an ISFIP/ReChIP analysis. The samples were amplified by simplex PCR with primer sets to the early, late and promoter regions. The position of the amplification product from the wild-type 776 DNA is indicated. Lane 1, ISFIP input fraction; lane 2, ChIP with 10 μ l of unmodified histone H4 antibody (ISFIP); lane 3, ChIP with 10 μ l of p300 antibody (ISFIP); lane 4, ReChIP input fraction; lane 5, ChIP with 10 μ l of unmodified histone H4 antibody (ISFIP); lane 6, ChIP with 10 μ l of p300 antibody (ReChIP).

A.

B.

Total RNA Analysis

C.

Chromatin Analysis

Figure 5. Effect of p300 siRNA transfection on SV40 infected BSC-1 cells

(5A): Effect of p300 siRNA transfection on SV40 protein encoded by the late region. Total protein extracted from 48 hour SV40 infected BSC-1 cells and either untreated or treated for 24 hours with control siRNA or p300 siRNA were separated using SDS PAGE followed by western blot analysis using antibody against p300, VP1 and GAPDH.

(5B): Effect of p300 siRNA transfection on SV40 late mRNA levels. Total RNA protein extracted from 48 hour SV40 infected BSC-1 cells and either untreated or treated for 24 hours with control siRNA or p300 siRNA were reverse transcribed and the amount was analyzed using real time PCR using primer sets against p300, late region and GAPDH. Similar results were obtained from at least six separate preparations of total SV40 RNA. C_T = Cycle Threshold. $\Delta C_T = C_T$ (Untreated Cells) - C_T (Treated Cells). The mean $\Delta C_T \pm$ S.E. obtained from the analyses is shown in the table.

(5C): Effect of p300 siRNA transfection on RNAPII binding to SV40 minichromosomes. 48 hour SV40 minichromosomes either untreated or treated for 24 hours with control siRNA or p300 siRNA were immune selected using antibody against RNAPII or p300. Similar results were obtained from at least three separate preparations of 48 hour unfixed SV40 minichromosome and two separate preparations of formalin fixed minichromosomes. $\Delta C_T = C_T$ (Input) - C_T (ChIP). The mean $\Delta C_T \pm$ S.E. obtained from the analyses is shown in the table.

Figure 6. Organization of RNAPII on SV40 minichromosomes treated with p300 siRNA
(6A): Relative occupancy of RNAPII on the SV40 genome after treatment with p300 siRNA. Unfixed SV40 minichromosomes treated with 5 nM of p300 siRNA were isolated from cells infected with 776 wild-type virus for 48 h. Purified SV40 minichromosomes were immunoprecipitated with antibody to RNA polymerase II using the ISF technique. The regions of the SV40 genome present in the bound and solubilized fractions were determined by simplex PCR amplification with primer sets to the early, late, and promoter regions.
(6B): Semi-quantitative analysis of RNAPII occupancy on the SV40 Genome. Data represented in the table is Mean ± S.E.
(6C): Level of Histone Hyperacetylation in p300 siRNA treated transcribing SV40 minichromosomes. Unfixed SV40 minichromosomes treated with 50 nM of p300 siRNA were isolated from cells infected with wild type 776 virus for 48 h, and subjected to an ISFIP/ReChIP analysis. The samples were amplified by simplex PCR with primer set to the late region. The position of the amplification product is indicated. Lane 1, ISFIP input fraction; lane 2, ChIP with 7.5 µl of hyperacetylated histone H4 antibody (ISFIP); lane 3, ChIP with 10 µl of unacetylated histone H4 antibody (ISFIP); lane 4, ChIP with 10 µl of hyperacetylated histone H3 antibody (ISFIP); lane 5, ChIP with 10 µl of unacetylated histone H3 antibody (ISFIP); lane 6, ChIP with 10 µl of p300 antibody (ISFIP); lane 7, ReChIP input fraction; lane 8, ChIP with 7.5 µl of hyperacetylated histone H4 antibody (ReChIP); lane 9, ChIP with 10 µl of unacetylated histone H4 antibody (ReChIP); lane 10, ChIP with 10 µl of hyperacetylated histone H3 antibody (ReChIP); lane 11, ReChIP with 10 µl of unacetylated histone H3 antibody (ReChIP); lane 12, ChIP with 10 µl of p300 antibody (ReChIP). Similar results were obtained from at least three separate preparations of SV40 minichromosomes for each region and also from two separate preparations of formalin fixed chromosomes.