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Using Simian Virus 40 As A Model To Determine The Effects Of Replication And Transcription On Histone Methylation

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USING SIMIAN VIRUS 40 AS A MODEL TO DETERMINE THE EFFECTS OF
REPLICATION AND TRANSCRIPTION ON HISTONE METHYLATION

By

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Bachelor of Science, Bemidji State University 2008

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This dissertation submitted by Les Kallestad in partial fulfillment of the requirements for the Degree of Doctor of Philosophy from the University of North Dakota, has been read by the Faculty Advisory Committee under whom the work has been done and is hereby approved.

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ABSTRACT

Simian Virus 40 (SV40) is a well-characterized virus whose small circular DNA genome is organized into chromatin and, as a consequence, undergoes many of the same biological processes observed in cellular chromatin. SV40 early transcription is repressed when the product of early transcription, T-antigen, binds to its cognate regulatory sequence, Site I, in the promoter of the SV40 minichromosome. We have subsequently shown that T-antigen binding to Site I results in the replication-dependent introduction of H3K9me1 into SV40 chromatin late in infection. Since H3K9me2 and H3K9me3 are also present late in infection, we determined whether their presence was also related to the status of ongoing transcription and replication.

In order to determine the capacity of SV40 epigenetic regulation, we have analyzed SV40 chromatin from minichromosomes and virions for the presence of modified histones using various ChIP techniques and correlated these modifications with specific biological effects on the SV40 life cycle. Since repression is frequently epigenetically marked by the introduction of specific forms of methylated histone H3, we characterized the methylation of H3 tails during transcription and replication in wild-type SV40 minichromosomes and mutant minichromosomes which did not repress T-antigen expression. While repressed minichromosomes following replication were clearly marked with H3K9me1 and H3K4me1, minichromosomes repressed during early transcription were not similarly marked. Instead repression of early transcription was marked by a significant reduction in the level of H3K9me2. The replication dependent introduction of

H3K9me1 and H3K4me1 into wild-type SV40 minichromosomes was also observed when replication was inhibited with aphidicolin.

We observed that H3K9me2/me3 was specifically introduced when transcription was inhibited during active replication. The introduction of H3K9me2/me3 that occurred when transcription was inhibited was partially blocked when replication was also inhibited. The introduction of H3K9me2/me3 did not require the presence of H3K9me1 since similar results were obtained with the mutant cs1085 whose chromatin contains very little H3K9me1.

Our results demonstrate that, like its cellular counterpart, SV40 chromatin is capable of passing biologically relevant transgenerational epigenetic information between infections. Our data suggest that methylation of H3K9 can occur either as a consequence of a specific repressive event such as T-antigen binding to Site I or as a result of a general repression of transcription in the presence of active replication. The results suggest that the nonproductive generation of transcription complexes as occurs following DRB treatment may be recognized by a 'proof reading' mechanism, which leads to the specific introduction of H3K9me2 and H3K9me3.

CHAPTER I

INTRODUCTION

DNA

All biological systems have blueprints which are used to construct mature living organisms, whether these be single cell bacteria or complex sentient multi-organ beings such as ourselves. These blueprints are made up of a pair of 2 nm thick deoxyribonucleic acids strands (DNA), which are macromolecules built from nucleotides consisting of a base (guanine, adenine, thymine or cytosine), a sugar (deoxyribose), and a phosphate group. These macromolecules can vary in size, with SV40 DNA consisting of 5,243 base pairs (BP) and human chromosome number 1 being about 220 million BP in length.

By a process referred to as transcription this double stranded DNA can be read by RNA polymerase II and an RNA molecule called messenger RNA (mRNA) is created which is complementary to the DNA sequence read except thymines are replaced with uridines. This mRNA gets transported out of the nucleus, finds ribosomes, and forms proteins in a process referred to as translation. These steps happen in a highly controlled manner, starting with DNA and ending up with protein products that make up the majority of an organism.

While bacteria and higher level organisms such as humans both use DNA as the form of nucleic acid in their blueprints, higher order organisms (eukaryotic cells) have an added obstacle of having large amounts of DNA that needs to be efficiently stored. For example, each human cell contains about 2 meters of DNA that needs to be packaged into a cellular nucleus that is four to five microns in diameter. Eukaryotic cells not only overcame this obstacle through the

condensation of DNA, but in the process added a highly complex mechanism to transcriptional control that we are beginning to more fully understand.

Nucleosomes

The first unit of DNA condensation is the nucleosome. A single nucleosome comprises 8 proteins, two copies each of the histones H2A, H2B, H3, and H4. Histone proteins are small basic proteins and have a globular domain containing about 75% of the protein's mass and a flexible amino terminus (referred to as the “histone tail” containing the remaining 25%). These proteins aggregate together to form a thick disk-like structure with 146 BP of DNA wrapped around to make a structure that is 11nm thick known as a nucleosome. In between nucleosomes there is a variable amount of linker DNA and another protein named H1 which binds DNA at the entry and exit site on the nucleosome, helps stabilize nucleosomes, and can promote higher order structure for these nucleosomes.(1) This genetic information is no longer naked DNA and is now coiled around nucleosomes, which is referred to as chromatin. It is important to the cell no naked DNA be present in healthy conditions, because naked DNA serves as one of the signals to cells that there is an abnormal condition like DNA damage or viral DNA present. SV40, the model system used for the studies described in this dissertation has its genetic information in the basic nucleosome DNA form referred to as chromatin. Eukaryotic organisms can further condense this chromatin by packing the nucleosomes into a tight 30nm fiber. This 30 nm fiber can be connected to a scaffold and be further condensed and ultimately packaged into a tight structure called a chromosome. These higher order chromatin structures play a role in regulating gene expression in eukaryotic organisms. (1)

Chromatin Remodeling

Chromatin structure originally was thought to exist solely to ensure efficient packaging of cellular DNA. However, this idea has subsequently changed based on studies on nucleosome positioning. If packing was the sole function of chromatin nucleosomes would be expected to have a random position on chromatin. Early experiments using endonuclease digestion showed that random nucleosome location was primarily the case for cellular chromatin. However, there were also domains of chromatin which were free of nucleosomes known as nucleosome-free (NFR) regions. The NFRs are most often found in promoter regions or transcriptional start sites and can be formed in a variety of ways. (2) Alternatively an NFR can be formed by a more dynamic mechanism in which chromatin remodeling complexes, such as SWI/SNF (SWItch/Sucrose NonFermentable-B) and RSC (Remodels the Structure of Chromatin), use ATP to either remove or slide nucleosomes. (3) It is now clear that nucleosomes and higher order chromatin structure not only efficiently packages DNA but also plays important developmental and regulatory roles. (4)

Heterochromatin is the term for condensed tightly packed DNA that is typically located at the periphery of the nucleus and comes in at least two forms, constitutive, which is always repressed and facultative, which can respond to signals and become transcriptionally active. Lightly packed chromatin is called euchromatin. Euchromatin is the portion of the genome that is gene rich and is able to undergo active transcription. Euchromatin also lacks the higher order chromatin structure and resembles beads on a string, with the individual nucleosomes being the beads and the interconnecting DNA being the string. (4)

Histone Modifications

Histones can be post-translationally modified at over 60 amino acid residues. These known modifications include methylation, acetylation, propionylation, butyrylation, formylation, phosphorylation, ubiquitylation, sumoylation, citrullination, proline isomerization, ADP-ribosylation, and most recently discovered, crotonylation. (5) (Table 1) The studies presented here focus on methylation and acetylation, though other modifications also have biologically significant functions.

As previously mentioned, histones have amino terminal tails that protrude from the globular region of the nucleosome. These tails are lysine and arginine rich and both of these two unmodified amino acids bear a positive charge. (6) The phosphate backbone of DNA contains a negative charge resulting in a strong interaction between DNA and histones, causing DNA to be tightly wound around the nucleosomes, creating an obstacle for proteins, namely transcription factors, to recognize and bind to their target DNA sequence. Modification of the histone tails provide modulation of this interaction both by neutralization of these native charges and by acting as a signal that can be recognized by other proteins, resulting in a wide range of tightly regulated and specific outcomes. (7)

Histone Methylation

Histones can be methylated on their lysine and arginine amino acid residues by a variety of lysine methyltransferases and can conversely be demethylated by lysine demethylases allowing for dynamic regulation mechanism. Unmodified lysines have a side group bearing a positive charge. Methylation of individual lysines can occur in three different forms, monomethylation, dimethylation, and trimethylation, all of which leave the positive charge intact. (8) In contrast, arginine can undergo only monomethylation and dimethylation. These modifications instead act by recruiting proteins possessing specific domains on them with tudor,

Table #1

Writers and Erasers in Mammals

Modification	Writer	Eraser
DNA methylation	DNA methyltransferases: DNMT1, DNMT3A/3B	Enzymes of demethylation pathway: AID/APOBEC; DNA glycosylases: TDG, SMUF1, MBD4; TET family
Histone methylation	HMTs, lysine and arginine-specific	HDMs
Histone acetylation	HATs	HDACs
Histone phosphorylation	Kinases	Dephosphatases
Histone ubiquitination	Ubiquitin ligases	Isopeptidases
Histone sumoylation	Serin-specific peptidases (SENP)—remove C-terminal residue, ligases—catalyzed conjunction SUMO to lysine	Isopeptidases
Histone biotinylation	Biotinyl ligases: BTD, HCS	Debiotinidase: BTD?
Histone citrullination	Peptidylarginine deaminases: PADI2, PADI4	
Histone ADP-ribosylation	ADP-ribosyltransferases (ARTs)	ADP-ribosyl hydrolases (ARHs) poly-(ADP ribosylase) glycohydrolase (PARD)
Histone β -N-glycosylation	Transferase: <i>O</i> -linked β -N-acetylglucosamine transferase (OGT)	Hydrolase: <i>O</i> -linked β -N-acetylglucosaminidase (OGA)
Histone proline isomerization	Proline isomerase: FK506 binding proteins (FKBPs)	
Histone propionylation	Histone acetyltransferases: p300, CBP	HDACs
Histone butyrylation	Histone acetyltransferases: p300, CBP	HDACs

chromo, and PHD domains being the predominant ones. (9)Histone modifications can recruit proteins with a high degree of specificity, depending on which lysine is modified and what degree of methylation is present. (10)For example, H3K9 methylation can specifically recruit proteins with the chromo domain present on them, which generally result in heterochromatin formation and cause a more stable and permanent gene silencing. H3K4 methylation is generally thought to be associated with transcriptional start sites and active gene transcription. H3K27 methylation has been linked with transcriptional repression. Interestingly, H3K4 methylation and H3K27 methylation can be bivalently located on a nucleosome which can result in genes poised for transcription yet at the same time prevented from initiating transcription. It is also important to note that these above stated general associations between histone lysine methylation and biological functions do not take into account that lysines can be mono-, di-, or tri-methylated, leading to a more complex regulatory network. Some general types of methylation modification marks and their associated biological roles are outlined in atable 2. (11)

Histone Acetylation

Select lysines on histone's amino terminus tail can be acetylated. Similarly to lysine methylation, lysine acetylation is a dynamic process of regulation with acetyl groups being added by histone acetyltransferases (HATs) and removed by histone deacetylases (HDACs). The addition of an acetyl group to a lysine neutralizes the positive charge normally present on the amino acid, which in turn weakens the interaction between the nucleosome and DNA allowing the chromatin to be more loose in structure. This is thought to allow the DNA to be more accessible to transcription factors and chromatin remodeling complexes. (12)

Acetylation of histone H3 is associated with activation of transcription and acetylation of histone H4 is associated with activation and also chromatin assembly. (11)Some models suggest

that HATs acetylate histones before RNA polymerase II, with HDACs acting behind the RNA polymerase II, removing the acetylation after transcription, with the whole thing moving as a complex. (13) Histone acetylation is enriched in the promoter region of actively transcribing genes and can function by recruiting proteins which contain a bromo domain. Two examples of proteins recruited by their bromo domains to lysine acetylation are SWI/SNF (SWItch/Sucrose NonFermentable-B) and RSC (Remodels the Structure of Chromatin), which function to remove or slide histones away from the transcriptional start site prior to initiation of transcription. (14) There is also something referred to as global lysine acetylation which takes place at low levels throughout the genome. Not much is known about this and will not be the subject of this dissertation.

Histone Code

Individual histone modifications can at times be correlated to specific biological processes. The histone code hypothesis states that histone modifications form specific patterns and these combinations of histone modifications confer a specific biological outcome. In trying to understand the biological function of a potential histone code one major obstacle is the sheer number of potential modifications. Taking histone H3 as an example, there are 280 million different lysine methylation patterns on its nineteen potential lysine residues, 4^{19} , and this does not take into account all the other types of histone modifications which are also possible. With a nucleosome being made up of two copies each of H2A, H2B, H3, and H4 it is obvious that mapping all possible combinations would be very difficult if not impossible. Instead, various laboratories have focused on select methylation and acetylation modifications in order to test the histone code hypothesis and determine the relationship between combinatorial histone

modifications and biological function. The ones most studied were listed previously in the table of methylation and acetylation modifications.

Simian Virus 40

Simian Virus 40 (SV40) is a small DNA tumor virus that was initially discovered in 1960 as a contaminant in the polio vaccine as a result of using rhesus macaque cells which were contaminated with indigenous viruses. (15) The vaccine was licensed in 1955 in the United States. The vaccine lots produced in 1961 or later were required to be free of SV40 but lots previously produced could still be used. 98 million United States residents received at least one dose of this vaccine from 1955-1961. (15) Not all lots contained SV40, and the formalin process used to produce the virus should have inactivated most or all of SV40, but it is impossible to determine the number of people who were actually infected with SV40 from the administration of this vaccine. It is also known that people born after 1961 have become infected with SV40 so it appears that human to human transmission might be involved. (16)

In SV40's natural host, the rhesus macaque, the virus is typically a harmless passenger. After SV40's discovery it was injected into newborn rodents and discovered to be oncogenic. It was also discovered to be able to transform rodent and human cells in culture. This raised serious concerns over the possibility of increased cancer rates in humans who received the potentially contaminated vaccine produced prior to 1961. (15) The debate continues on today as to the role of SV40 in human cancer development although there is little evidence in support of a role. Nevertheless, as a result of this concern in the 1960s there was tremendous interest in investigating the biological and oncogenic properties of SV40 and other related polyomaviruses.

Simian Virus 40 Structure

SV40 has 5243 base pairs of DNA, making it one of the smaller known viruses. It is encapsulated in 3 viral capsid proteins called VP1, VP2, and VP3 and is icosahedral in shape. Inside the protein coat the genetic information is stored with histones present on the DNA. (17) These histones can possess post-translational modifications, such as methylation and acetylation, that result from conditions of the previous infection, allowing for the potential of variable epigenetic information to be passed on to subsequent infections based on the environmental conditions of the previous infection. (18)

Simian Virus 40 Chromosomes

SV40 chromosomes are small, containing only 5243 base pairs of DNA, are circular, and are negatively coiled. Two copies each of host cell produced histones H2A, H2B, H3, and H4 come together and form nucleosomes which SV40 DNA wraps around. (21) An SV40 chromosome contains 24-26 nucleosomes with H1 not being found in virion particles but is found in intracellular chromosomes. (19)

Simian Virus 40 Life-cycle

SV40 undergoes a number of distinct states during its life cycle in order to go from incoming infecting virions to newly created and released progeny virions. The first of which occurs when SV40's VP1 comes into contact with carbohydrate moiety of the glycosphingolipid GM1 in the extracellular plasma membrane. The interaction of VP1 and GM1, along with other cellular receptors, is quite complex, with at least 4 GM1 receptors required for stable binding to the membrane, along with substantial 3-dimensional rotation in the GM1 bound virion resulting

in 28 pentamers coming into contact with cellular receptors. (20)(21). Once inside the cell, the protein coat is partially removed and the chromosomes inside the virion are at least partially exposed. (22) SV40's PKKKRKV nuclear localization signal (NLS) facilitates the transportation to the nucleus where it enters through the nuclear pore complex. (23) Within an hour of entering the nucleus early transcription begins, coding for T-antigen and small T-antigen (t-antigen). Initiation of late transcription begins at about 20 hours post infection and encodes for the viral capsid proteins VP1, VP2, and VP3. Replication begins at 20-24 hours post-infection and newly replicated SV40 chromosomes can become encapsulated by the VP proteins and form virions. After about 72 hours post-infection these newly formed virions are transported to the cytoplasmic membrane and released from the cell.

If all of these steps happen as they should in a given cell type and the infection is productive and makes progeny virions the cell is said to be permissive. However, if the incoming virions are unable to establish an infection the cell type is said to be non-permissive. In non-permissive infections the viral chromosome can become integrated into the hosts genome at a random location. Gene products can then be produced, such as T-antigen, which in turn can result in a transformed cell and may result in cancer.

SV40 Genome Organization

The SV40 genome can be divided into two categories based on their function, the coding region for proteins and the regulatory region. The coding region can be further divided into two genetic units, the early coding region and the late coding region. The early coding region is the part of the genome that becomes expressed at the beginning of an infection when the SV40 chromosomes first get into the nucleus. The late coding region is expressed at very low levels early in infection and becomes expressed at greatly increased levels after replication begins at

around 20-24 hours post-infection. The regulatory region consists of the promoters for both the early and late coding regions, SV40 enhancer, and the origin of replication. (24)

Early Region

The early region of SV40 produces mRNA that codes for two proteins, T-antigen and t-antigen. This mRNA is transcribed from only one of the two strands of DNA, called the E (early) strand or – (minus) strand. This strand is transcribed in a counterclockwise direction starting from the regulatory region. Both T-antigen and t-antigen are transcribed from the same DNA sequence but due to differential splicing have different mRNAs that get translated, yielding two distinct gene products. (24)

Large T Antigen

Large T Antigen (T-antigen) was first detected in 1977 as a 90-100 kDA polypeptide. Shortly thereafter it was determined that T-antigen was encoded by one of the three variations of the early SV40 mRNA. T-antigen was found to bind DNA over a 120 bp region, centered around bp 67, which contains SV40's origin of replication, Site 1 which when bound by T-antigen down-regulates early transcription, and Site 2 which when bound by T-antigen is involved in initiation of DNA replication. T-antigen has been shown to be able to function as a DNA helicase and when bound to single stranded DNA can bind and hydrolyze ATP, providing energy for the unwinding of DNA during replication. (25)

Another function of T-antigen is to bind to retinoblastoma (Rb) and the tumor suppressor protein p53. By binding Rb T-antigen overrides the G1 checkpoint and entry into S phase is permitted, allowing SV40 to replicate its DNA. T-antigen also binds p53 and inactivates some of its functions as a tumor suppressor. (26) These interactions result in transformed cells with T-antigen being the sole protein involved in transforming many of the cell lines in use today.

Late Region

The late proteins are transcribed from the L (late) strand or + (plus) strand of DNA in a clockwise direction starting from the regulatory region. Two mRNAs are transcribed from the late region, each mRNA being translated into two protein products. One mRNA codes for VP2 and VP3, while the other mRNA strand encode for VP1 and VP4. VP1, VP2, and VP3 are capsid proteins, with VP4 being involved in disruption the cellular membrane. VP1 and VP2 are thought to be the major structural components of the capsid, with VP3 proposed to be an intermediary between VP1/2 and the chromatin which needs to be packed inside. (24) VP1, VP2, and VP3 also possess a nuclear localization sequence directing their biological function. (27) Binding of VP1 is thought to prevent the function of histone deacetylases (HDACs) on the attached chromosome while still allowing histone acetyltransferases (HATs) to acetylate the chromosome, resulting in increased levels of histone acetylation in virion particles compared to the pool of chromosomes at late times in infection when encapsulation is occurring.

Simian Virus 40 Regulatory Region

SV40's regulatory region spans about 420 bps (nt 5171-346) and contains the origin of replication, the enhancer, and both the early and late promoters. The regulatory region contains many of the elements required for the control and regulation of transcription and replication and is located between the early and late regions of the genome. (24)

Simian Virus 40 Replication

SV40 chromosomes contain a single origin of replication (ori) located within the regulatory region and is 64 bp (nt 5211-31) in length. Replication occurs in both directions from the ori and terminates at approximately 180 degrees around the circular genome from the ori where the two replication forks meet. (28) T-antigen, which was described earlier is involved in replication in two primary ways. Firstly, it binds to SV40 DNA near the origin of replication and acts to recruit other needed proteins for replication. Secondly, it has the ATPase and helicase activity required to unwind DNA during replication. Interestingly, other than T-antigen all of the proteins necessary for replication and chromatin assembly are host derived.

Simian Virus 40 Transcription

For what is known about transcriptional regulation in SV40 it closely resembles what has been seen in eukaryotic and viral systems, making it an ideal model system to study transcription. The regulatory region contains the DNA sequences required to control initiation of transcription. Specific DNA sequences in the regulatory region interact with host cell regulatory proteins tightly regulating this biological process, with many general transcription factors binding at SV40s 'TATA' box about 30 bp upstream from the transcription initiation site. Early transcription is regulated by the early promoter, which can bind T-antigen. The binding of T-antigen prevents

the binding of other required proteins required for early transcription, effectively downregulating early transcription. (29) Repression of early transcription allows the focus of transcription to fall up the late transcript products, resulting in the production of VP1-3 and angoprotein.

SV40 in relation to other polyoma viruses

Polyoma viruses are small and have closed circular double stranded DNA of around 5.3 Kbases. SV40 is the most extensively researched polyoma virus due to its initial discovery of SV40's ability to both transform cells and cause tumors in newborn mice. Along with SV40, polyoma viruses include mouse polyomavirus, human JC virus, human Merkel cell virus, and human BK virus, as well as others. (30) These viruses' possess a region which contains both the origin of replication and transcriptional regulatory sequences. These regulatory sequences exert control over both the early and late genes which are located on either side of this region. (15) These viruses posses small genomes with limited encoding regions which when paired with their ease which they can be cultured makes them a good candidate for use in model systems.

CHAPTER II

STATEMENT OF THE PROBLEM

Regulation of post-translational modifications, especially histone modifications, is critical for normal cellular function. Disregulation of these histone modifications has been known to be associated with many disease states and cancer types. The mechanism behind regulation of these modifications is not yet well understood. Understanding of these mechanisms will allow better insight into initiation and progression of specific disease states and cancer types.

Most research on histone modification has focused on histone methylation. Histone methylation can be an immediate response to a biological process such as transcription; it can also provide stable, long term heritable epigenetic information to subsequent generations. Methylation resulting from transcription is an example of intra-generational epigenetic regulation, whereas passing epigenetic directing progeny to specific fates is an example of trans-generational epigenetic regulation.

Within the broad category of epigenetic regulation different input events can result in different epigenetic outcomes. The problem is to determine which input events correlate with particular epigenetic outcomes, how these epigenetic outcomes are involved in the progression of the lifecycle of an infection, and how current environmental conditions can be transmitted to subsequent generations.

This study has three goals:

(1) Determining the effects of replication and transcription on histone methylation.

(2) Understanding the epigenetic progression in the lifecycle of an infection.

(3) Ascertaining the trans-generational ability to maintain current epigenetic information.

CHAPTER III

MATERIALS AND METHODS

Cells and Viruses

SV40 virus and chromatin were prepared in the BSC-1 cell line of African green monkey kidney cells (ATCC). The 776 SV40 wild-type and cs1085 mutant virus were a gift from Dr. Daniel Nathans and the SM virus from Dr. Chris Sullivan. PBM129-1 and pBM131-1 were created by Dr. Barry Milavetz, and the 39° epi-mutant created and grown in Dr. Milavetz's lab.

Cell Culture

African green monkey kidney BSC-1 cells were maintained in minimum essential medium (Gibco) and supplemented with 10% FBS (Gibco) and 1% gentamicin (Gibco)(10 mg/ml) in a 37°C NAPCO, Model 6200 CO₂ Incubator. Using sterile technique, all cell manipulations were done in a NUAIRE, Model NU 425-400 laminar air flow hood. Media from tissue culture flasks (Corning 75 cm²) containing confluent monolayers of cells was removed. The cells were washed with 3 ml of 0.05% trypsin-EDTA (Gibco) to remove protease inhibitors present in the growth media. The wash was also removed and 5 ml of free trypsin-EDTA was added to the flask and allowed to incubate at 37° for 1-2 minutes. Cells were then dislodged from the surface of the flask

by gentle tapping. Confirmation of cell detachment was checked using an Olympus CK 10 light microscope. Aliquots of the trypsin-EDTA solution containing the dislodged cells was removed then a 1 ml aliquot was introduced into each of the two new tissue culture flasks each containing 20 ml of fresh growth media for the expansion of the culture. 20 ml of fresh media was added to the original flask which still contained 1 ml of the trypsin-EDTA dislodged cells. The 3 flasks were then returned to the NUAIRE incubator and allowed to grow. The average time between subcultures was 2-3 days.

Infection of BSC-1 Cells

For the preparation of high titer working stocks of SV40 virus for subsequent studies, the following procedure was used. One ul of the virus to be cultured was added to the media in a large (75 cm²) T-flasks containing confluent monolayers of cells. The T-flasks were maintained at 37°C in a NAPCO Model 5100 incubator for approximately 2-3 weeks or until a vast majority of the cells were dead. The fluid was then placed in a 50 ml Corning centrifuge tube and frozen at -20°C. Verification of the presence of competent virus and to approximate the titer the HIRT method (described below) was performed prior to use. The 50 ml tubes were centrifuged at 3000 X G at 4°C for 10 minutes using a Beckman J6-MI centrifuge and the supernatant was placed into a new 50 ml centrifuge tube and the contents were divided into 1 ml aliquots for future use and stored at -20°C.

Isolation of Viral DNA

In order to determine if newly prepared virus was suitable for use in future experiments the amount of viral DNA produced in a typical infection was determined for each batch of virus. A batch is defined as coming from the same initial T75 flask as mentioned above. For these analyses, small T-flasks (25 cm²) containing confluent monolayers of cells were used. 333 ul of thawed virus was added to the medium and the T-flasks were incubated for 48 hours at 37°C in a NAPCO Model 5100 incubator. The viral DNA was harvested from the plate using 300 ul of lysing solution with gentle swirling to disrupt the infected cells. The disrupted cell solution was added to a 1.5 ml microfuge tube followed by 100 ul of 5 M NaCl and gently vortexed. The tube was refrigerated at 4°C overnight. The next morning the tube was centrifuged at 14,000 x G at 4°C for 30 minutes. The pellet was removed from the tube using a sterile wooden applicator stick and discarded. The supernatant was then purified and PCR'd as described below.

Infection of BSC-1 Cells for the Preparation of SV40 Chromosomes

Large T-flasks containing confluent monolayers of BSC-1 cells were infected with virus using sterile techniques in a Nuair Model NU 425-400 laminar flow hood that was located separately from the flow hood used for cell culture. First, nine ml of the medium from the T-flasks to be used were decanted into a 15 ml disposable centrifuge tube. Any remaining medium in the flask was discarded. The 9 ml of medium was then returned into the T-flask. Immediately prior to infection, one ml of the desired virus was thawed and transferred to the above T-flask containing 9 ml of medium and the flask was gently

swirled to mix virus throughout the media. To allow the virus to bind, the cells were incubated at 37°C for 30 minutes. Following virus adsorption to the cell, infected cells were either harvested immediately for 30 minutes chromosomes or washed twice with 5 ml of 37°C serum-free medium to remove any unbound virus from the media. The T-flasks were returned to the incubator after the addition of 10 ml serum-free medium. The infected cells were harvested after the desired period of incubation.

Preparation of SV40 Nuclei

After infected cells were incubated for the desired period cells were washed with around 4 ml of 4° PBS solution two times, both times pipetting the remaining PBS from the flask to insure complete removal. 14 ml of chromosome preparation nuclei buffer was placed in a 15 ml Corning centrifuge tube. One ml of the chromosome preparation nuclei buffer was placed in a sterile tube for later use. Another one ml of the nuclei buffer was removed from the centrifuge tube and added to the flask. The cells were gently scraped from the bottom of the flask using a Cell Scraper. 0.05% Triton X-100 was added to the flask and was used to wash the sides of the flask and to collect the nuclei in the corner of the flask. The contents were collected with a 1 ml pipette and layered gently on top of the remaining 12 ml of nuclei buffer in the 15 ml centrifuge tube. The one ml of chromosome preparation nuclei buffer previously set aside was added to the flask and used to rinse the sides to collect any remaining nuclei, which was then collected and added to the 15 ml centrifuge tube. The tube was centrifuged at 1,500 x G for 10 minutes at 4°C in a Beckman Model J6-MI centrifuge, the supernatant was

decanted and excess liquid was removed from the pellet with a 200 ul pipette. The nuclear pellet was frozen and stored at -20°C.

Nuclear Extraction

The nuclear pellet was thawed, resuspended in 100 ul of chromosome extraction buffer, transferred to a sterile microfuge tube, and centrifuged at 12,000 x G at 4°C for 30seconds. The supernatant was removed with a pipette and discarded. The pellet then had 200 ul of chromosome extraction buffer added to the tube and vortexed for 15 seconds to break up the pellet. The solution was then subjected to a 60 minute extraction at 4°C. After the incubation the contents were centrifuged at 12,000 x G at 4°C for 30 seconds then the supernatant was removed and placed in a sterile microfuge tube and stored at -20°C.

Chromosome Purification

100 ul of chromosome isolation buffer with 50% glycerol was placed in a microcentrifuge tube and stored at -20°C for at least 4 hours. 1 ml of chromosome isolation buffer with 10% glycerol was then gently layered on top of the 50% glycerol. The 200 ul nuclear extract was thawed and carefully layered on top of the 10% glycerol layer before being centrifuged using a TLA 100.3 rotor at 50,000 rpms for 35 minutes at 4°C in a Beckman TLA 100 ultracentrifuge. 400 ul was gently removed from the top of

the tube and discarded. 600 ul more was removed gently from the top and stored at -20°C.

Sodium Butyrate (NaBt) Treatment

Cells were infected as previously described. For the preparation of 12 hour NaBt treated chromosomes at 30 minutes post-infection following the addition of the fresh serum-free media the cells were treated with 250 uM of NaBt (25 ul of 1 M NaBt to a flask with 10 ml media) and incubated until 12 hours post-infection at 37°C. At 12 hours post-infection the cells were harvested as previously described. For the preparation of 48 hour NaBt treated chromosomes infected cells were treated at 24 hours post-infection cells with 250 uM of NaBt (25 ul of 1 M NaBt to a flask with 10 ml media) and incubated until 48 hours post-infection at 37°C. At 48 hours post-infection the cells were harvested as previously described.

5,6-Dichloro-1 beta-D-ribofuranosylbenzimidazole (DRB) Treatment

For the preparation of 2 hour DRB treated chromosomes cells were treated with 200 uM of DRB (20 ul of 1 M DRB to a plate containing 10 ml media) at 2 hours prior to infection and were incubated at 37°C. Cells were infected as previously described. After adding the fresh media at 30 minutes post-infection DRB was reapplied to the same concentration of 200 uM and the cells were incubated at 37°C until 2 hours post-infection. At 2 hours post-infection the cells were harvested as previously described.

For the preparation of 48 hour DRB treated chromosomes the cells were infected as previously described. At 24 hours post infection the cells were treated with 200 uM of DRB (20 ul of 1 M DRB added to a plate with 10 ml media) and were incubated at 37°C until 48 hours post-infection. At 48 hours post-infection the cells were harvested as previously described.

Aphidicolin Treatment

For the preparation of 48 hour aphidicolin treated chromosomes the cells were infected as previously described. Aphidicolin was applied at a concentration of 6 uM at 24 hours post-infection and incubated at 37°C until 48 hours post-infection. At 48 hours post-infection the cells were harvested as previously described.

Dual Treatment with DRB and Aphidicolin

For the preparation of 48 hour chromosomes dual treated with DRB and aphidicolin the cells were infected as previously described. At 24 hours post-infection DRB was applied at a 200 uM concentration and aphidicolin was applied at a 6 uM concentration and the cells were incubated at 37°C until 48 hours post-infection. At 48 hours post-infection the cells were harvested as previously described.

Chromatin Immunoprecipitation (ChIP)

SV40 chromosomes were immunoprecipitated with specific antibodies using the reagents and the protocol supplied by Millipore with minor modifications. Depending on the specific antibody used, either 7.5 ul or 10 ul of antibody was incubated with 125 ul of salmon sperm DNA/protein A agarose and 750 ul of ChIP dilution buffer for 5 hours at 4°C with constant rotation. After 5 hours of incubation the samples were centrifuged at 1000 x G for 1 minute to pellet the agarose, and the supernatant was discarded. 800 ul of ChIP dilution buffer was added to the agarose pellet along with 100 ul of chromatin. The mixture was then incubated from 7-12 hours at 4°C with constant rotation. Each tube containing immune complexes bound to protein A agarose was sequentially washed with low salt, high salt, lithium chloride, and two washes with Tris-EDTA (TE) buffer all supplied by Millipore according to their protocol. Following resuspension of the protein A agarose with TE buffer for the second wash with the buffer, the suspension was transferred to a clean microcentrifuge tube. Immune complexes were eluted from the protein A agarose by two sequential 15 minutes incubations in 110 ul of lysing buffer (0.6% SDS, 2 mM EDTA, pH 8.0) at room temperature. After each incubation the protein A agarose was pelleted by centrifugation for 1 minute at low speed with the supernatant removed and stored at -20°C.

Antibodies Used

The antibodies used included: H3K4me1 (07 = 436, Millipore (Temicula, California, USA), H3K4me2 (39141, Active Motif Carlsbad, California, USA), H3K4me3 (04 = 745, Millipore Temicula, California, USA), H3K9me1 (ab9045, Abcam,

Cambridge Massachusetts USA), H3K9me2 (ab1220, Abcam, Cambridge, Massachusetts, USA), H3K9me3 (ab8898, Abcam, Cambridge, Massachusetts, USA), and RNA PII 905 = 623, Millipore, Temecula, California, USA). All antibodies were ChIP validated by the respective vendors.

Immune-Selection Fragmentation Followed by a second Immunoprecipitation (ISFIP)

A standard ChIP was performed as described above with the following changes. 200 ul of input chromatin was used instead of the standard 100 ul. After the second TE wash the protein A agarose was transferred to a new tube using 200 ul of TE buffer. This resuspended agarose was sonicated with continuous pulse for 6 minutes in a cup horn at 50% amplitude of 115 V + 10% using a Branson Digital Sonifier followed by brief centrifugation at 3000 rpms for 30 seconds to separate the supernatant from the agarose. The supernatant was then centrifuged again at 3000 rpms for 30 seconds to pellet out any remaining agarose. This purified supernatant was used as the input for a second ChIP using a different antibody. This second ChIP was done by the standard protocol previously described.

Preparation of DNA for qPCR

Samples were prepared for PCR using an MP Bioscience GeneClean Spin Kit (#111101-200) with the following modifications. The glassmilk reagent (100 ul) was mixed with 100 ul of sample in a 1.5 ml centrifuge tube. The tube was mixed by repeated

inversion at 2 minutes and again at 4 minutes of incubation at room temperature. Following 5 minutes of incubation, the samples were centrifuged at 6000 rpm for 30 seconds in a Micro One (Tomy) to pellet the glass. The supernatant was discarded and 200 μ l of the wash buffer was added to the tube. While adding the wash the pipette tip was used to break up the pellet by both physically rubbing and vigorously pipetting up and down. The samples were inverted twice and centrifuged at 6000 rpm for 30 seconds again to pellet the glass. The supernatant was discarded and the pellets were dried in a vacuum for 5 min. The glass pellet with bound DNA was resuspended in 25 μ l of Tris EDTA (TE) buffer.

qPCR Amplification

DNA was amplified from the promoter region of the SV40 genome using the primers 5'-TTG CAA AAG CCT AGG CCT CCA AA-3' and 5'-TGA CCT ACG AAC CTT AAC GGA GGC-3' and from the early region using the primers 5'-TGCTCCCATTCATCAGTTCC -3' AND 5'-CTGACTTTGGAGGCTTCTGG-3' in a CFX Connect Real Time System thermal cycler (Bio-Rad) using "SSO Advanced DNA polymerase" (Bio-Rad). Immediately before use, the primers and DNase free water were added and 28 μ l of the mix was used per sample. Two microliters of the resuspended glass milk in TE buffer was added per sample. Samples were amplified by PCR in triplicate with a melt curve applied afterward to ensure specific amplification. All sample preparation for PCR was done in either a Nuair biological safety cabinet Model NU_425-400 or an AirClean 600 PCR Workstation (ISC BioExpress).

CHAPTER IV

SUMMARY OF RESULTS

NOTE: All figures and tables for this section are located in their respective publications presented as individual chapters V-VII in this dissertation.

Great advances have been made in the understanding of histone modifications over the years. Up until the early 1990s histones were thought to be only a highly efficient DNA storage protein. It is now clear that histones can have their individual amino acid side chains post-translationally modified. Not only can different amino acids on histones be modified, but at least eleven different types of modifications are now known to be present on histones, including methylation, acetylation, propionylation, butyrylation, formylation, phosphorylation, ubiquitylation, sumoylation, citrullination, proline isomerization, ADP ribosylation, and crotonylation. (5)

The above mentioned post-translational modifications and their specific location on histones are known to play critical roles in many diverse biological processes, such as regulation of gene expression, to direct the fate of the individual cell, and thus the organism. Many of the enzymes responsible for the addition and removal of these post-translational modifications are also known now, such as the related G9a and GLP

proteins which are thought to methylate H3K9me2 and H3K9me3 in euchromatin, and the protein Suv39H1 which causes H3K9me3 methylation in heterochromatin.(31)(32)(33)(32,33), While some of the proteins responsible to the addition and removal of post-translational modifications, such as lysine methylation, are now known a great deal remains to be understood about them in terms of their tightly controlled regulation and their diverse biological functions. (5)

The studies described in this dissertation used Simian Virus 40 (SV40) to examine post-translational modification introduction on histones under different conditions in order to further understand both the introduction of such modifications and also the biological outcomes of these modifications. Levels of H3K4 and H3K9 methylation and acetylation of histone 4 were measured and related to environmental conditions, active gene transcription, and productive genome replication in our model system.

Prior work on SV40 has shown that H3K4, H3K9, and histone H4 acetylation levels change over the course of the SV40 lytic infectious life cycle, starting with virions and transitioning from initial entry into the cell to late times in infection when future progeny are being replicated and encapsidated. (See Figure 1 in Chapter V) As hypothesized, these methylation modifications are not evenly distributed among the SV40 chromosomes but co-localize in distinct patterns, or epigenomes. It has been known that cells differentiate and have different physical properties based on their ultimate gene expression as a result of this differentiation, even though they have identical genetic information. This was the first evidence for viral chromosomes to also have different epigenomes, and at 48 hours post-infection at least 5 major epigenomes have been identified as shown in table 2 in Chapter V.

In order to test if the epigenomes present at late times were related to biological properties of an infection a mutant (CS1085) containing a 30 bp deletion and defective for repression of early transcription was compared to wild-type SV40. The deletion of Site 1 on CS1085 prevents the binding of T-antigen and results in the overproduction of early mRNAs that are normally repressed in a wild-type infection. (34)(35) Another mutant, SM (SV40 miRNA mutant), has a deleted regulatory miRNA which normally represses early gene expression. The SM mutant results in an over-production of T-antigen as a result of this mutation. (36)

These two mutants had noteworthy results. Levels of H3K9me1 were strongly correlated with binding of T-antigen. At 48 hours post-infection SM, which had increased T-antigen binding due to the increased presence of T-antigen, showed levels nearly double that of WT for H3K9me1, while CS1085 had H3K9me1 almost completely abolished (Table 3 in Chapter V). This provides strong evidence that T-antigen binding results in the introduction of H3K9me1 at late times in infection, whether through modulation of the repression of transcription or through T-antigen recruiting methyltransferases. While levels of H3K9me1 at 48 hours post-infection were increased in the SM mutant the levels of H3K9me1 in SM virions was reduced compared to WT virions, suggesting a different epigenome is responsible for encapsidation. (Table 4 in Chapter V)

Virions of the different mutants contained different epigenetic information stored on their encapsulated chromosomes. If these differences in epigenetic information within virion particles were at least in part due to the different cellular environmental conditions resulting from the mutants used, it was hypothesized that other environmental conditions

might also affect the epigenetic information stored in virion progeny. The environment was changed to 39°C in order to partially emulate a fever response to an infection. The resulting virions contained distinct epigenetic information and had a greatly reduced yield (8% compared to WT) of genetic material at 48 hours post-infection resulting from an infection. CS1085 virions contained different epigenetic modifications compared to WT virions, and CS1085 infections produced 9% of the genetic material compared to a WT infection at 48 hours post-infection. (Figure 2A in Chapter V)

While CS1085's Site 1 deletion would not have an effect on repression of early transcription as seen in WT infections it was unknown if the incoming chromosomes have any other differences that may result in altered properties that could play a role in effectively establishing a productive infection. As seen in Figure 2B in Chapter V, CS1085 had approximately a 50% reduction in the effective transportation of its chromosomes into the nucleus, which could in part explain CS1085's diminished effectiveness. Surprisingly, CS1085 was completely able to avoid degradation of its genetic material by cellular defenses, whereas WT infections resulted in 80% of the viral material in the nucleus being degraded from 30 minutes to 8 hours post-infection. (Figure 2C in Chapter V) The exact cause of CS1085's ability to evade degradation by cellular defenses is currently unknown.

When using CS1085 to determine the effect of early transcription on epigenetic marks it was discovered that at different times different epigenetic outcomes resulted from T-antigen binding. (37) At early times when only early transcription was occurring T-antigen's binding caused an inhibition of H3K9me2 introduction, (Figure 5 in Chapter VI) yet when replication was occurring at late times in infection when replication was

also occurring T-antigen caused the incorporation of H3K9me1 (Figure 4 in Chapter VI). To confirm this increase in H3K9me1 at late times was a result of T-antigen binding and not some other process a recombinant was created containing two copies of Site 1. In this construct, a second copy of Site was added and was located about 180° from the WT Site 1 location, and the construct had increased levels of H3K9me1 present on its chromosomes late times in infection, suggesting that Site 1 can function similar to an enhancer when directing the epigenetic affects on its chromosome.(38) (Figure 6 in Chapter VI)

We have presented strong evidence that T-antigen binding at late times results in the incorporation of H3K9me1, yet at early times inhibits the introduction of H3K9me2. This suggests that at late times in infection T-antigen's ability to incorporate H3K9me1 is related to DNA replication. Our lab has previously shown an association between actively replicating chromosomes and H3K9me1. (39) This research shows a second independent confirmation of this relationship by inhibiting replication with aphidicolin. The increase in H3K9me1 normally seen when replication was occurring was completely prevented when replication was inhibited. H3K9me3 incorporation still occurred even in the absence of replication, consistent with previous data showing that H3K9me3 uses H3K9me1 as a precursor independent of active replication.(40) (Figure 7 in Chapter VII) It is currently unclear how T-antigen binding blocks the incorporation of H3K9me2 at early times. T-antigen has different biologically epigenetic effects at different times and when active replication is inhibited, suggesting multiple distinct mechanisms related to the biological processes of early transcription and active replication in which histone modifications can be incorporated.

Expanding upon the inhibition of replication, transcription was repressed at both early and late times to determine its epigenetic effects. DRB, an inhibitor of RNAPII elongation, was used at two hours pre-infection with chromosomes being harvested at 2 hours post-infection, and also at 24 hours post-infections with chromosomes being harvested at 48 hours post-infection, in order to capture transcriptional repression in both the absence and presence of active replication. The results obtained as a result of transcriptional repression through the use of DRB both in the presence and absence of active replication tie the increase of H3K9me2 and H3K9me3 to chromosomes containing RNAPII but only when DNA replication is active. (Figure 8 in Chapter VII)

CHAPTER V

VIRION-MEDIATED TRANSFER OF SV40 EPIGENETIC INFORMATION

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Abstract

In eukaryotes, epigenetic information can be encoded in parental cells through modification of histones and subsequently passed on to daughter cells in a process known as transgenerational epigenetic regulation. Simian Virus 40 (SV40) is a well-characterized virus whose small circular DNA genome is organized into chromatin and, as a consequence, undergoes many of the same biological processes observed in cellular

chromatin. In order to determine whether SV40 is capable of transgenerational epigenetic regulation, we have analyzed SV40 chromatin from minichromosomes and virions for the presence of modified histones using various ChIP techniques and correlated these modifications with specific biological effects on the SV40 life cycle. Our results demonstrate that, like its cellular counterpart, SV40 chromatin is capable of passing biologically relevant transgenerational epigenetic information between infections.

Keywords: SV40, epigenetics, histone methylation, histone modification, transgeneration inheritance

Results

Epigenetic regulation through covalent modification of histones has been shown to play a critical role in a number of important biological processes including cell fate determination during differentiation,(41)(42)(43)(44)(45) regulation of gene expression(46,47)(47) and the development of cancer. (48)(49)(50)(51) Importantly, all of these examples of epigenetic regulation are characterized by the transfer of epigenetic information from parental cells to daughter cells during cellular division.

Simian Virus 40 (SV40) is a DNA tumor virus that is organized into chromatin in the intracellular minichromosome and virion, and undergoes hyperacetylation of H3 and H4 during transcription (52)(13)(53) and methylation of H4K20 during an infection. (54) Because of its similarity to cellular chromatin, we hypothesized that SV40 should be able to transfer epigenetic information recapitulating the conditions of an infection to a subsequent infection through the infecting virions. We tested this hypothesis by

characterizing the histone tail modifications in SV40 chromatin derived from minichromosomes and virions using ChIP analyses and correlating the observed variations in histone modification in the SV40 chromatin with subsequent effects on an infection.

Extending our previous results,(54) we analyzed SV40 wild-type chromatin for the presence of methylated H3K4 and H3K9 during an infection, with the results shown in Figure 1. When SV40 chromatin was analyzed for the presence of methylated H3K4 (Fig. 5A), we observed mono, di and trimethylated H3K4 present at 48 h post-infection (PI), but little, if any, methylated H3K4 at other times. When SV40 was analyzed for the presence of methylated H3K9 (Fig. 5B), all three of the methylated forms of H3K9 were present in SV40 chromatin throughout the course of the infection, including disrupted virions, with the exception of H3K9me2 at 24 h PI, which was only present at low levels.

If the identified histone modifications actually represented epigenetic information, we expected that the modifications would be organized in the same way as in cellular chromatin, i.e., into various specific combinations (55) that would reflect the biological properties of the chromatin.

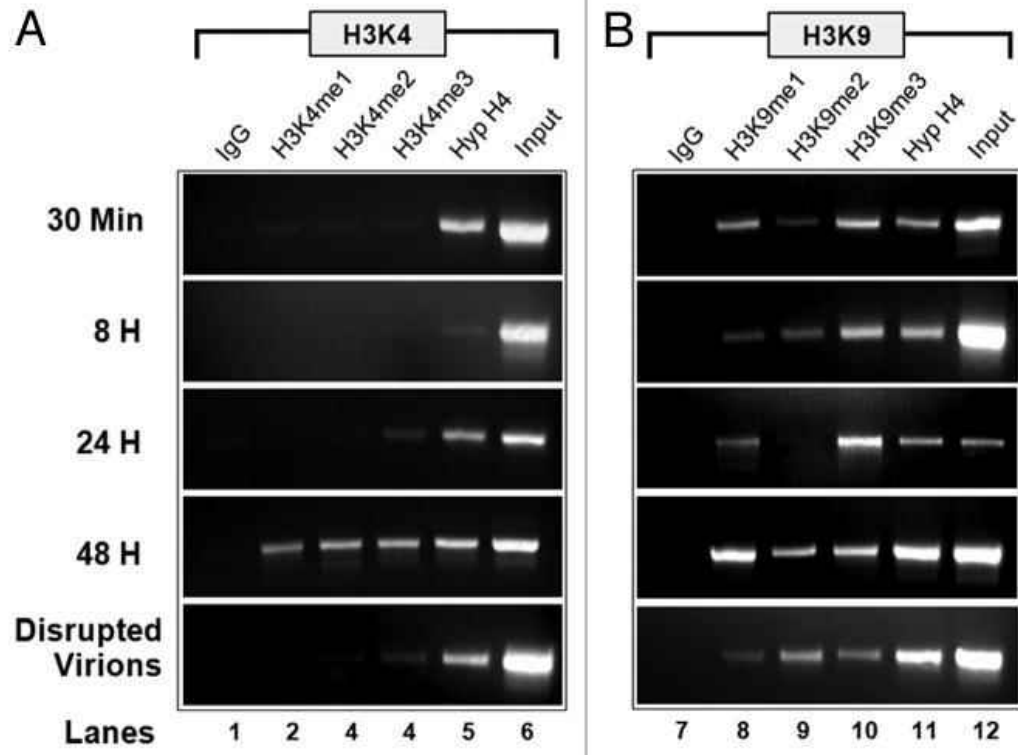


Figure 1. Methylated H3K4 (A) and H3K9 (B) are present in SV40 chromatin during a lytic infection. Unfixed SV40 minichromosomes were isolated from disrupted virions and infected cells at 30 min, 8 h, 24 h, and 48 h post-infection (PI), subjected to chromatin immunoprecipitation with 7.5 μ l of antibody to each target modified histone, and the resulting immunoprecipitates amplified by PCR with primers recognizing the SV40 early region as previously described. (A) Lane 1, IgG; Lane 2, H3K4me1; Lane 3, H3K4me2; Lane 4, H3K4me3; Lane 5 Hyperacetylated H4; Lane 6, 5% of input SV40 chromatin. (B) Lane 1, IgG; Lane 2, H3K9me1; Lane 3, H3K9me2; Lane 4, H3K9me3; Lane 5 Hyperacetylated H4; Lane 6, 5% of input SV40 chromatin. DNA present in immunoprecipitates from virions and minichromosomes isolated 48 h PI was amplified for 32 cycles. DNA from the other immunoprecipitates was amplified for 35 cycles.

SV40 minichromosomes were analyzed by a two-step ChIP process in which the minichromosomes were first immune selected with an antibody to a specific form of modified histone, the chromatin was fragmented, and the resulting fragments subjected to a second ChIP with an antibody to a different modified histone. If two modified forms of histones were found on the same minichromosomes, we would expect to see that a significant fraction of the input chromatin from the first ChIP would be bound in the second ChIP with antibody to the second form of modified histone. Table 2 shows the result of this analysis.

Based upon these results, we believe that there are at least five major SV40 epigenomes present in cells infected with wild-type virus at 48 h post-infection. One consisted primarily of H3K4me2 in conjunction with H3K9me1, H3K9me2, H3K9me3, and hyperacetylated H4 (HH4). A second form also consisted primarily of H3K4me2 but in conjunction with HH3 and HH4. This conclusion was based upon the fact that H3K9 cannot be methylated and acetylated at the same time, which we experimentally confirmed. A third epigenome consisted almost solely of H3K4me3 with some HH4 also present. The fourth epigenome consisted of H3K9me1 along with H3K9me2, H4K20me1 and HH4. The fifth epigenome consisted primarily of H3K9me3 with trace amounts of other modifications.

While this is the first demonstration that multiple distinct viral epigenomes defined by specific combinations of histone modifications are present in infected cells,

Table #2

Co-localization of modified histones on SV40 minichromosomes

Modification	H3K4me2	H3K4me3	H3K9me1	H3K9me3	H4K20me1
H4K20me1	Trace	None	+		Trace
H3K4me1	None	None	Trace	None	None
H3K4me2		None	Trace	None	Trace
H3K4me3	None		None	Trace	None
H3K9me1	+	None		None	++
H3K9me2	+	None	++	Trace	++
H3K9me3	+	None	None		None
HH3	+	None	None	None	+

None, less than 10% of input; Trace, 10–20% of input; +, 20–30% of input; ++, over 30% of input. SV40 chromatin is organized into distinct epigenomes late in infection. SV40 minichromosomes isolated 48 h PI were immune-selected with antibody to a modification of interest, the bound SV40 chromatin fragmented, and the fragments subjected to a second ChIP with antibody to a second modification of interest. The DNA present in the immunoprecipitates from the second ChIP was amplified for 32 to 40 cycles depending upon the amount of chromatin present in the immuno-selection step with primers recognizing the early region of the SV40 genome. The amount of co-localized modified histones were approximated from the semi-quantitative PCR using densitometry and confirmed by real time PCR. Each analysis was performed at least twice using different preparations of SV40 minichromosomes. The table represents the relative amounts of co-localized modified histones.

the co-localized modified histones are in agreement with previously reported results for cellular chromatin. For example, H3K9me1 has been previously shown to be co-localized within nucleosomes of transcribing regions with H3K4me1/2/3, H3K79me1/2/3 and H4K20me1 using a genome-wide strategy. H3K9me3 was found associated with H3K9me2 and H3K27me2/3 in silenced regions. (55)(56)

In order to test whether the modified histones in epigenomes present at late times reflected biological properties of the infection, we analyzed wild-type and mutant SV40 minichromosomes defective for the repression of early transcription for the presence of modified histones. The SV40 mutant cs1085 contains a 30 bp deletion of T-antigen binding Site I within the regulatory/promoter region and, as a consequence, does not repress early transcription as seen in a wild-type infection. (34)(35) This deletion results in an over-production of early mRNA and protein. We have previously exploited this aspect of cs1085 to investigate nucleosome phasing (57) and RNA polymerase II occupancy in transcribing SV40 minichromosomes. (52) SV40 early expression is also regulated by a miRNA found in the late region of the genome.(36) A mutant, SM (SV40 miRNA mutant), deleted for the regulatory miRNA without affecting any other aspects of the virus, was shown to result in the over-production of T-antigen during infection.(36)

We hypothesized that if one or more of the histone modifications observed in the epigenomes at 48 h PI in a wild-type infection was the result of T-antigen binding to Site I and subsequent repression of early transcription, these modified histones would likely be absent or significantly reduced in cs1085 minichromosomes late in infection.

Alternatively, if any of the histone modifications were a result of overexpression of T-antigen one might expect similar effects by both mutants.

SV40 minichromosomes were prepared at 48 h PI from cells infected with wild-type, cs1085, or SM virus and analyzed for the presence of methylated H3K4 and H3K9. The levels of the various modified histones in each of the minichromosomes were then quantitated by real time PCR and compared as shown in Table 3. In the absence of T-antigen mediated repression in cs1085 infections, we observed the greatest reduction in the amount of H3K9me1, and significant reductions in the amount of H3K4me1 and H3K4me2. In contrast, in the minichromosomes from SM virus infections with overproduction of T-antigen, we observed increases in the amounts of H3K9me1, H3K9me2 and H3K9me3. These results indicated that T-antigen mediated repression of early transcription had its primary effect on the introduction of H3K9me1, a modification thought to be associated with repression in other eukaryotes.⁽⁵⁸⁾ Unexpectedly, T-antigen mediated repression also appeared to have an effect on the introduction of H3K4me1 and H3K4me2 into SV40 minichromosomes, modifications generally thought to be associated with transcriptional activation.⁽⁵⁸⁾ Based upon these results, it would appear that the epigenome containing large amounts of H3K9me1 along with H3K4me1/2 was mostly likely associated with repression of early transcription.

We then directly tested the hypothesis that SV40 virions could carry epigenetic information by analyzing the histone modifications present in chromatin from virions. If the virions carried epigenetic information one would expect the patterns of histone modifications in the virions to relate to the patterns present in the minichromosomes. In

Table #3

Presence of modified histones in SV40 minichromosomes

Chromosome	H3K4me1	H3K4me2	H3K4me3	H3K9me1	H3K9me2	H3K9me3	HH4
Wt 776	0.1 ± 0.07%	0.4 ± 0.3%	0.08 ± 0.06%	22 ± 10%	0.04 ± 0.03%	12 ± 6%	3.9 ± 3%
cs1085	0.005 ± 0.007%	0.02 ± 0.02%	0.02 ± 0.02%	0.66 ± 0.6%	0.17 ± 0.2%	8.2 ± 5%	2.7 ± 2%
SM	0.02 ± 0.01%	0.5 ± 0.4%	0.13 ± 0.13%	39 ± 9%	0.93 ± 0.7%	18 ± 9%	13 ± 1%

Histone methylation is partially controlled by SV40 T-antigen late in infection and epigenetically propagated in virions. SV40 chromatin was isolated 48 h PI from cells infected with wild-type, cs1085, or SM mutant viruses. SV40 chromatin was also prepared from the corresponding disrupted virions and virions prepared at elevated temperature. The SV40 chromatin was subjected to ChIP analyses with antibodies to each of the methylated forms of H3K4 and K3K9, and the DNA present in the resulting immunoprecipitates quantitated by real time PCR. Analysis of wild-type, cs1085, and SM virus at 48 h PI.

contrast, if the pattern present in the virions was a result of other biological processes, one might expect the pattern in the virions to be independent of the patterns present in the minichromosomes. Virions were purified, disrupted with DTT, the chromatin present in the disrupted virions purified from intact virions,(54) and analyzed for the presence of modified histones with the results shown in Table 4. Compared with the pattern of H3K9 methylation and HH4 acetylation in wild-type chromatin from virions, we observed a significant reduction in the percentage of chromatin containing the three methylated forms of H3K9 and HH4 in cs1085 and a significant reduction in the percentage of H3K9me2, H3K9me3 and HH4 in the SM mutant. While the pattern of changes in the virions closely mirrored the pattern of histone modifications in the cs1085 minichromosomes, the pattern for the chromatin in SM virions did. Nevertheless, both mutants displayed patterns of histone modifications in the virions that were very different from the wild-type virion chromatin and each other, indicating that in each case the virion chromatin carried distinct epigenetic information.

Next, we confirmed that the pattern of histone modifications in virion chromatin reflected aspects of the infection by comparing virions produced at 37°C and 39°C. Since the SV40 DNA sequence is identical at both temperatures, any changes in the pattern of histone modifications in the virions must be a result of epigenetic changes. As shown in Table 4, the pattern of histone modifications for virions produced at 39°C was distinct and differed from the results obtained from the wild-type virions and both mutants particularly with respect to the amount of H3K9me3. These results were consistent with the hypothesis that the conditions of an infection contribute to the epigenetic modifications appearing in the virions produced during that infection.

Table #4

Presence of modified histones in SV40 virions

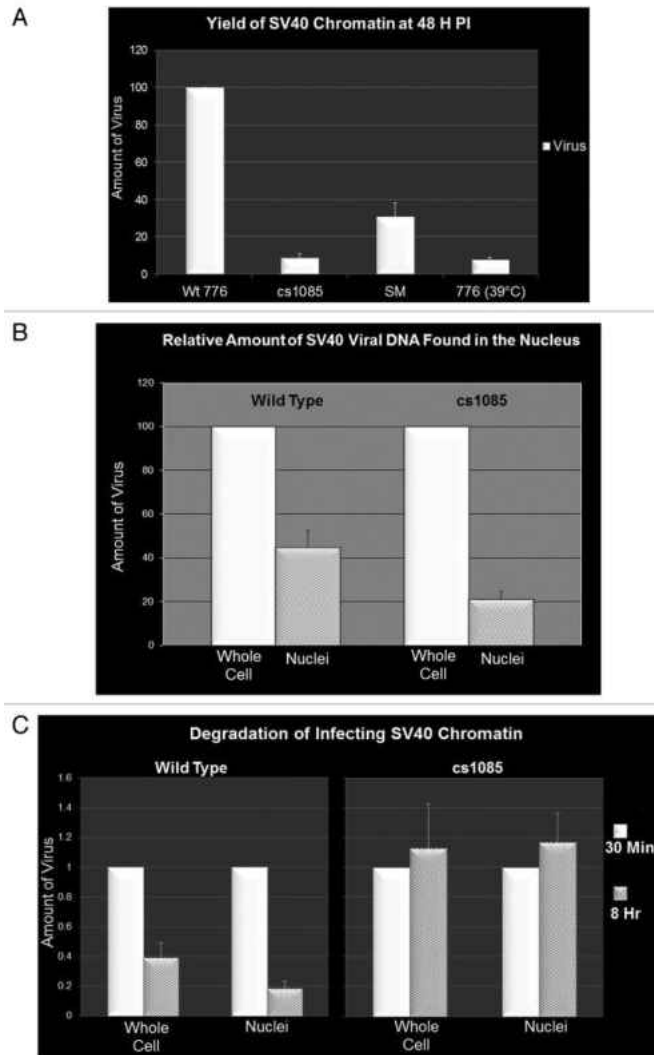
Chromosome	H3K9me1	H3K9me2	H3K9me3	HH4
Wt 776	2.9 ± 1%	1.9 ± 0.8%	0.9 ± 0.4%	22 ± 6%
cs1085	0.20 ± 0.1%	0.14 ± 0.1%	0.13 ± 0.09%	0.72 ± 0.3%
SM	1.8 ± 0.8%	0.08 ± 0.03%	0.29 ± 0.15%	0.43 ± 0.16%
776 (39°C)	3.1 ± 1.8%	0.81 ± 0.3%	3.4 ± 2%	0.93 ± 0.4%

Histone methylation is partially controlled by SV40 T-antigen late in infection and epigenetically propagated in virions. SV40 chromatin was isolated 48 h PI from cells infected with wild-type, cs1085, or SM mutant viruses. SV40 chromatin was also prepared from the corresponding disrupted virions and virions prepared at elevated temperature. The SV40 chromatin was subjected to ChIP analyses with antibodies to each of the methylated forms of H3K4 and K3K9, and the DNA present in the resulting immunoprecipitates quantitated by real time PCR. Analysis of chromatin obtained by disruption of virions from wild-type, cs1085, SM, and wild-type virus prepared at 39°C.

If the patterns of modified histones present in chromatin from virions represented inheritable epigenetic information, we expected that this epigenetic information would affect aspects of a subsequent infection. In order to test this hypothesis, we analyzed infections by the various viruses for the yield of SV40 DNA at 48 h PI as a measure of infection efficiency. SV40 DNA in virions and infected cells was isolated by the Hirt procedure (59) and measured by real time PCR. All comparative infections contained the same amount of input SV40 DNA in the pool of infecting virus. Consistent with our hypothesis that the viruses contained inheritable epigenetic information, in comparison to the wild-type virus prepared at 37°C, whose amount was set at 100%, we observed only $9 \pm 2\%$ for cs1085, $31 \pm 7\%$ for SM and $8 \pm 1\%$ for the virus prepared at 39°C (Fig. 6A).

Since the epigenetic information encoded in the histone modifications present in virion chromatin is first made available for reading following uncoating of the virus particle, we hypothesized that this epigenetic information might have an effect on the early stages of the establishment of an infection. In order for an SV40 infection to be established, virions must be transported through the cytoplasm, presented to the nuclear pore complex, and the protein coat of the virion removed or substantially modified so that cellular factors can access the chromatin to initiate early transcription. (60)(61) In a competing process, we have previously reported that a significant fraction of incoming viral chromatin is targeted for degradation by the cell. (54)

In order to test whether the observed differences in wild-type and cs1085 epigenetic information affected transport to the nucleus or cellular degradation of viral chromatin, we measured by real time PCR the amount of SV40 DNA present as chromatin in whole cells and nuclei at 30 min and 8 h following infection with the two



Methylation of histone H3 affects infectivity and stability of infecting SV40 chromatin. Cells were infected with equal amounts (based upon real time quantitation of the SV40 DNA present in virions) of wild-type 776, cs1085, SM or 776 grown at 39°C virus. SV40 DNA or minichromosomes were isolated at the times indicated and quantitated by real time PCR. (A) Yield of SV40 DNA at 48 h PI following infection with wild-type 776, cs1085, SM virus or 776 grown at 39°C. (B) SV40 DNA present in cells or nuclei infected with equal amounts of 776 or cs1085 virus at 30 min PI. C. SV40 DNA present in cells infected with equal amounts of 776 or cs1085 virus at 30 min and 8 h PI.

SV40 viruses. For these studies, nuclei were prepared according to our standard protocol, which was originally optimized for the yield of biologically active SV40 minichromosomes.(62) As shown in Figure 2B, a significantly smaller percentage of infecting SV40 chromatin was found in the nucleus following infection with cs1085 compared with wild-type virus at 30 min PI indicating that the epigenetic information carried by the two viruses had an effect on import into the nucleus. The epigenetic information carried by the infecting virions also had an effect on cellular degradation of the SV40 chromatin. As shown in Figure 2C, wild-type chromatin was degraded approximately 75% between 30 min and 8 h when measured in either whole cells or nuclei, consistent with our previous results,(54) while cs1085 chromatin appeared to be resistant to degradation (Fig. 6C). These results indicate that both the passage through the nuclear pore complex and the degradation of the incoming viral chromatin are sensitive to the specific epigenetic information carried by the viral chromatin.

While this first demonstration of virion-dependent transfer of biologically relevant epigenetic information between infections utilized SV40, which is generally thought to be nonpathogenic in humans, it is very likely that other DNA viruses that are organized into chromatin and are pathogenic (e.g., papillomaviruses) will also show this behavior. For those viruses that utilize epigenetics in the establishment of an infection, these results suggest that this process is much more complex and more highly regulated than previously thought. Finally, the results with SV40 virions prepared at elevated temperature indicate that fevers during viral infection may affect the disease process through the disruption of the normal epigenetic regulation pathways of the viral life cycle.

Materials and Methods

Cells, viruses and infections

SV40 DNA and minichromosomes were prepared in the BSC-1 cell line (ATCC) using wild-type 776 (from Dr. Daniel Nathans), cs1085 (from Dr. Daniel Nathans) or SM virus (from Dr. Chris Sullivan). Uninfected cells were maintained at 37°C in minimal essential medium containing 10% (v/v) fetal calf serum, gentamycin, glutamine, and sodium bicarbonate (GIBCO) as previously described. (52)(13)(53) Sub-confluent cell mono-layers were exposed to virus for 30 min, the virus containing medium removed, the plates washed twice with fresh warm medium, 10 ml of fresh (52)(13)(53) SV40 minichromosomes were prepared in 75 cm² T- flasks containing a total of 10 ml of medium, while experiments in which SV40 DNA was prepared were performed in 25 cm² T-flasks containing 3 ml of medium. Comparative infections with either different viruses or for different periods of infection were performed in parallel at the same time.

Preparation of SV40 DNA, Nuclei and Minichromosomes

Total SV40 DNA present in infected cells was prepared according to the Hirt procedure.(59) Briefly, the medium was removed from the infected cells, the cells washed twice with cold PBS, and 0.3 ml of lysing solution (0.6% SDS, 10 mM EDTA) added. The flask was rocked for a few minutes until all the cells in the flask appear to be solubilized and the solution transferred to a 1.4 ml Eppendorf tube. Following transfer the flask was washed with 0.2 ml of PBS and the wash added to the 0.3 ml transferred to the tube. 160 µl of 5M NaCl was added to the tube and the tube inverted 20 times to mix the contents. After storage overnight at 4°C, the tube was spun at 16,000 x G at 4°C for 30

min, and the soluble contents removed from the cellular DNA pellet. The soluble fraction was referred to as the Hirt supernatant and was used in subsequent analyses.

The SV40 DNA present in the nuclei of infected cells was prepared from infected cells (25 cm² T-flasks) by a modification of our previously described procedure for preparing SV40 minichromosomes. Nuclei were released from infected cells by a combination of scraping the cells in low-ionic strength buffer (0.5 ml) and TritonX-100 (0.5 ml) as previously described(52)(13)(53) and the liquid transferred to a 1.4 ml Eppendorf tube. Any remaining cells in the flask were obtained by a second scraping treatment in low-ionic strength buffer (0.5 ml) and TritonX-100 (0.5 ml) and transfer of the liquid to a second Eppendorf tube. The tubes were spun at 16,000 x G for 30 sec and the supernatant removed. The pellet in each tube was suspended in 0.2 ml of low-ionic-strength buffer, the supernatants combined and gently layered onto a new tube containing 0.6 ml of low-ionic-strength buffer. Following centrifugation at 5000 x G for 10 min, then supernatant was removed and the pellet resuspended in 0.2 ml of PBS. Lysing solution (0.3 ml) was added followed by 0.16 ml of 5M NaCl. The tube was inverted about 20 times and placed overnight at 4°C. The next day the tube was spun at 16,000 x G for 30 min at 4°C in an Eppendorf Model 5415 C centrifuge. The supernatant was transferred to a new tube labeled nuclear portion and the pellet was discarded. SV40 minichromosomes were prepared from infected cells and purified on glycerol step gradients as previously described. (52)(13)(53) Gradient fractions 3 through 5 were pooled for subsequent analyses.

Preparation of SV40 Chromatin from Virions

SV40 virus was prepared by infecting cells with 0.005 plaque-forming units of seed virus per cell in order to limit the amount of defective virus formed. Infected cells were incubated until approximately 99% of the cells were dead. Crude virus (1 ml) was transferred to a 1.4 ml Eppendorf tube and centrifuged in a Beckman TLA 100 ultracentrifuge at 50,000 x G for 35 min at 4°C on 10% glycerol buffer (10 mM HEPES, 5 mM KCl, 1mM EDTA, 0.2 mM MgCl₂, 0.5 mM DTT, 10% glycerol). The pellet of virus and cellular debris was resuspended in 175 µl of TE buffer, and 20 µl of a 10X digestion buffer supplied by NEB added and 5 µl of DNaseI (NEB) added. Following incubation at 37°C for 15 min to allow the DNase I to degrade any non-virion forms of SV40 chromatin, the mixture was centrifuged as above in the TLA 100 ultracentrifuge on 10% glycerol buffer. The pellet was resuspended in 194 µl of TE buffer, 6 µl of 5 M DTT was added, and the suspension incubated at room temperature for 15 min to disrupt the virions as previously described. (54) The mixture was again sedimented on 10% glycerol as described above. Fractions 2 through 5 which have previously been shown to contain SV40 chromosomes were pooled and used in subsequent analyses.

Standard Chromatin Immunoprecipitation Analyses

All of the antibodies used in these studies were ChIP validated by the supplier and included H3K4me1 (07–436, Millipore), H3K4me2 (39141, Active Motif), H3K4me3 (04–745, Millipore), H3K9me1 (ab9045, Abcam), H3K9me2 (ab1220, Abcam), H3K9me3 (ab8898, Abcam), H4K20me1 (39175, Active Motif), hyperacetylated H3 (06–599, Millipore), and hyperacetylated H4 (06–866 Millipore). We also used an antibody recognizing H4K20me1 which was a gift from Dr. Thomas Jenuwein, which we previously showed functioned similarly to the commercial antibody. (54) Unfixed SV40

chromatin (100 μ l) from infections and disrupted virions was immunoprecipitated with 10 μ l of antibody using the reagents and protocol supplied by Millipore in their kit for the analysis of hyperacetylated histone H4 with minor modifications. (52)(13)(53)

Preparation of DNA for PCR Analyses

DNA samples for standard PCR were prepared by phenol/chloroform extraction followed by ethanol precipitation in the presence of paint pellet (Novagen) as previously described. (52)(13)(53) DNA samples for real time PCR were prepared by phenol/chloroform extraction and used directly in amplifications. DNA samples from ChIP elutions (10 μ l) were mixed with 50 μ l of phenol/chloroform (AM9730, Ambion) and TE buffer (70 μ l). The mixture was vortexed for 10 sec and centrifuged for 10 min at 8,000 x G in an IEC Micromax centrifuge. The aqueous phase (40 μ l) was removed for subsequent PCR amplification.

PCR Amplifications

Purified DNA samples were amplified by standard PCR using primers which recognize the early region of the SV40 genome (5'GCTCCCATTCATCAGTTCCS3' and 5'CTGACTTTGGAGGCTTCTGG3') as previously described. (52)(13)(53) Amplification was initiated with a hot start at 95°C for 2 min followed by 35 to 45 cycles of amplification. Each cycle consisted of an annealing step at 60°C for 1 min, DNA syntheses at 72°C for 1 min, and denaturation at 95°C for 1 min.

Real Time PCR

SV40 DNA was amplified from the early region of the SV40 genome using the primers described above (0.3 μ M) in a Bio-Rad MyIQ2 real time thermal cycler using the Power SYBr Green PCR master mix (Applied Biosystems). The amplifications were initiated by a hot start at 94°C for 15 min. The DNA was amplified for between 40 and 50 cycles with each cycle consisting of annealing of primers at 60°C for 1 min, DNA synthesis at 72°C for 1 min, and denaturation of the DNA at 94°C for 1 min. All samples were analyzed in duplicate sets.

Analysis of PCR Amplification Products

Following standard PCR amplifications, amplification products were separated on 2.4% submerged agarose gels (Sigma) by electrophoresis. (52)(13)(53) Amplification products were visualized by staining with ethidium bromide and digitally recorded using a UVP GDS8000 gel documentation system (Ultra Violet Products).

CHAPTER VI

TRANSCRIPTION AND REPLICATION RESULT IN DISTINCT EPIGENETIC MARKS FOLLOWING REPRESSION OF EARLY GENE EXPRESSION

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Abstract

Simian virus 40 (SV40) early transcription is repressed when the product of early transcription, T-antigen, binds to its cognate regulatory sequence, Site I, in the promoter of the SV40 minichromosome. Because SV40 minichromosomes undergo replication and transcription potentially repression could occur during active transcription or during DNA replication. Since repression is frequently epigenetically marked by the introduction of specific forms of methylated histone H3, we characterized the methylation of H3 tails during transcription and replication in wild-type SV40

minichromosomes and mutant minichromosomes which did not repress T-antigen expression. While repressed minichromosomes following replication were clearly marked with H3K9me1 and H3K4me1, minichromosomes repressed during early transcription were not similarly marked. Instead repression of early transcription was marked by a significant reduction in the level of H3K9me2. The replication dependent introduction of H3K9me1 and H3K4me1 into wild-type SV40 minichromosomes was also observed when replication was inhibited with aphidicolin. The results indicate that the histone modifications associated with repression can differ significantly depending upon whether the chromatin being repressed is undergoing transcription or replication.

Keywords: simian virus 40, viral epigenetics, H3K9, H3K4, transcription, replication

Introduction

The selective methylation of the amino terminal tails of histone H3 and H4, a well-known form of epigenetic regulation, has been associated with a number of important biological regulatory processes including the control of transcription and cellular differentiation (41)(42)(46)(47)(43)(44)(45). Functionally, epigenetic regulation of transcription can occur either to control a particular gene's expression during a cell's life, or to pass along transcriptional information following cell division. The former would be an example of intra-generational epigenetic regulation while the latter would be an example of trans-generational regulation. While both forms of regulation might occur in association with a particular gene, it has not yet been established whether the same forms of histone methylation invariably mark the chromatin of the regulated gene during

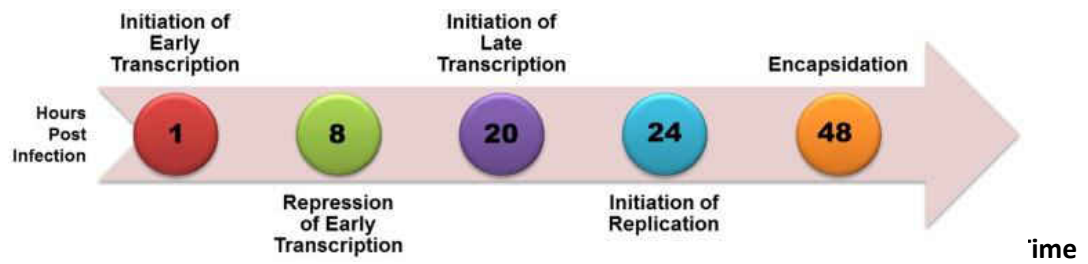
intra-generational and trans-generational regulation, nor how these two forms of epigenetic regulation might be related.

Since the passing of epigenetic information from a parental cell to daughter cells during cell division is critical to transgenerational epigenetic regulation, the mechanism of this inheritance has been the subject of much interest (63). A model for the inheritance of cellular transgenerational epigenetic information has emerged in which nucleosomes containing parental epigenetic information are randomly passed to daughter DNA during replication. These nucleosomes then act to direct the modification of histones present in the newly replicated nucleosomes added to the DNA in order to conserve the parental epigenetic modifications in the daughter chromatin (64)(65).

Simian virus 40 (SV40), a member of the polyomavirus family, has been extensively studied as a model for eukaryotic molecular biology since its initial identification in 1960 because of its small size, organization into typical chromatin structure, and almost complete use of cellular enzymes and factors to complete its life cycle. A time course of SV40 transcription, replication, and encapsidation is shown in Figure 3. Upon infection the SV40 is rapidly transported to the nucleus with removal of the virus coat proteins and within 2 h early transcription begins. As the level of the major product of early transcription, T-antigen, increases it serves to repress its own expression through a feedback mechanism in which it binds to a site in the transcriptional regulatory region known as Site I. By 8 h post-infection repression of early transcription is extensive. Between 12 and 24 h post-infection late transcription and DNA replication begin with late transcription slightly preceding replication. At approximately 48 h post-infection replication is maximal. Beginning at approximately 48 h, newly replicated

SV40 is bound by the products of late transcription, VP1, VP2, and VP3, to encapsidate new virus particles in a process which continues until the infected cell lyses and the newly synthesized virus is released (66).

We have recently shown using a SV40 mutant which does not repress early SV40 transcription, that repression is strongly associated at late times in infection with mono-methylation of H3K9 and weakly associated at this time with mono-methylation of H3K4 (37). Specifically, we compared the levels of methylated H3K4 and H3K9 at 48 h post-infection in wild-type SV40 which represses early transcription and the mutant cs1085 which contains a 30-bp deletion in the regulatory region encompassing T-antigen binding Site I and does not repress early transcription (35). We found that the percentage of SV40 minichromosomes containing H3K9me1 was reduced from $22 \pm 10\%$ in the wild-type minichromosomes to $0.66 \pm 0.06\%$ in the mutant which fails to repress. Similarly, we observed a reduction in H3K4me1 from $0.1 \pm 0.07\%$ in wild-type minichromosomes to $0.005 \pm 0.007\%$ in the mutant. In contrast, H3K4me2 went from 0.4 ± 0.3 to $0.02 \pm 0.02\%$, H3K4me3 went from 0.08 ± 0.06 to $0.02 \pm 0.02\%$, H3K9me2 went from 0.04 ± 0.03 to $0.17 \pm 0.2\%$, and H3K9me3 went from 12 ± 6 to $8.2 \pm 5\%$ comparing the wild-type to the mutant. Moreover, we also showed that the changes in methylation patterns which occurred in SV40 minichromosomes during infection in mutants or following other changes in environment could also be represented in the SV40 chromatin present in virions and transferred to a subsequent infection in the viral equivalent of trans-generational epigenetic regulation (37). However, we do not know whether transcriptional repression occurring prior to DNA replication also results in the same



Time course of biological processes during SV40 lytic cycle

effects on histone methylation. For this reason, we have extended our studies on early repression to early times in infection and characterized the changes which occur to the methylation patterns of SV40 minichromosomes. In addition, we have also investigated the role of DNA replication in introducing each of the methylated forms of H3K4 and H3K9.

Materials and Methods

Cells and Viruses

Wild-type and mutant SV40 minichromosomes were prepared in the monkey kidney BSC-1 cell line (ATCC) using either wild-type 776 virus, cs1085 virus (from Dr. Daniel Nathans) or SM virus (from Dr. Chris Sullivan). The recombinants pBM129-1 and pBM131-1 were prepared in our laboratory and previously described (67).

Cell Culture and Infection

BSC-1 cells were maintained and infected as previously described with the exception of incubating cs1085 virus with the cells for 1 h, in order to increase the minichromosome yield, instead of the typical 30 min (52)(37). SV40 minichromosomes were isolated at the indicated times post-infection as described for each of the analyses. DNA replication was inhibited with aphidicolin (final concentration 6 μ M). Aphidicolin in ethanol (4 μ l) was added at 24 h post-infection and minichromosomes were prepared from treated cells at 48 h post-infection.

Preparation of SV40 Minichromosomes

SV40 minichromosomes were harvested at the desired time as previously described (52)(37) with one minor modification. After transferring the lysed cells to the 15 ml centrifuge tube, an additional 1 ml of nuclei preparation buffer was used to rinse the flask and was subsequently added to the centrifuge tube in order to maximize the yield of minichromosomes from each infection.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) kits were obtained from Millipore and the protocol was followed as previously described (37). The antibodies used included: H3K4me1 (07-436, Millipore), H3K4me2 (39141, Active Motif), H3K4me3 (04-745, Millipore), H3K9me1 (ab9045, Abcam), H3K9me2 (ab1220, Abcam), H3K9me3 (ab8898, Abcam), and RNA polymerase II (RNAPII; 05-623, Millipore). All antibodies were ChIP validated by the respective vendors. Hundred microliters of protein A agarose, 800 μ l of ChIP dilution buffer, and 7.5 μ l of each antibody was used in a protein low-bind tube. The mixture was rotated for 5 h at 4°C on an end to end rotator in a refrigerator to bind the antibody to protein A agarose. Following binding of the antibody, the protein A agarose was spun down at 2,000 \times g for 2 min and the supernatant discarded. Eight hundred microliters of fresh ChIP dilution buffer was added and either 100 or 200 μ l of the chromatin to be analyzed was added. The samples containing antibody bound to protein A agarose and chromatin were incubated with end to end rotation for a further 7 h at 4°C. The chromatin bound to protein A agarose was washed according to the manufacturer's protocol and eluted as previously described (37).

Preparation of DNA

Samples were prepared for PCR using an MP Bioscience GeneClean Spin Kit (#111101-200) with the following modifications. The glassmilk reagent (100 μ l) was mixed with 100 of sample in a 1.5-ml centrifuge tube. The tube was mixed by repeated inversion at 2 min and again at 4 min of incubation. Following 5 min of room temperature incubation, the samples were centrifuged at 6,000 rpm for 30 s in a Micro One (Tomy) to pellet the glass. The supernatant was discarded and 200 μ l of the wash buffer was added to the tube. While adding the wash the pipette tip was used to break up the pellet by both physically rubbing and vigorously pipetting up and down. The samples were inverted twice and centrifuged at 6,000 \times g for 30 s to again pellet the glass. The supernatant was discarded and the pellets were dried in a vacuum for 5 min. The glass pellet with bound DNA was resuspended in 25 μ l of Tris EDTA (TE) buffer.

PCR Amplification

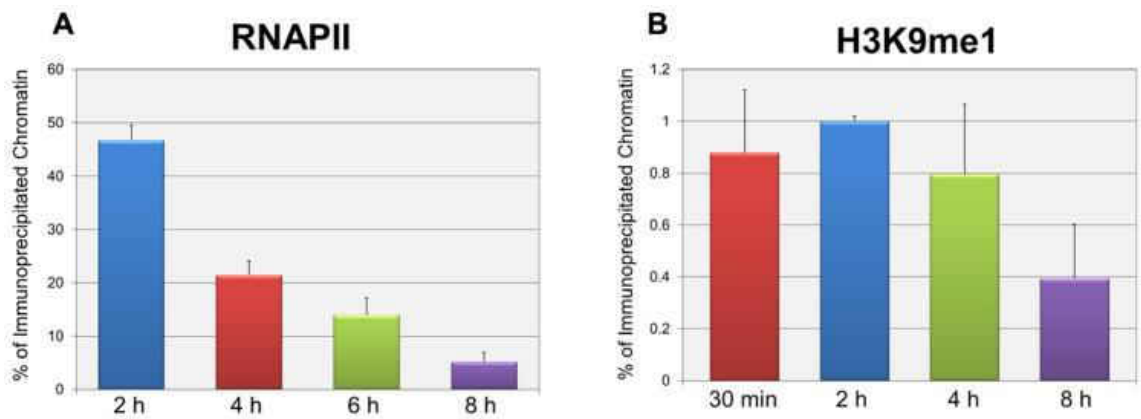
DNA was amplified from the promoter region of the SV40 genome using the primers 5'-TTG CAA AAG CCT AGG CCT CCA AA-3' and 5'-TGA CCT ACG AAC CTT AAC GGA GGC-3' in a CFX Connect Real Time System thermal cycler (Bio-Rad) using "SSO Advanced DNA polymerase" (Bio-Rad). Immediately before use, the primers and DNase free water were added and 28 μ l of the mix was used per sample. Two microliters of the resuspended glass milk in TE buffer was added per sample. Samples were amplified by PCR in triplicate with a melt curve applied afterward to ensure specific amplification. All sample preparation for PCR was done in either a Nuair biological safety cabinet Model NU_425-400 or an AirClean 600 PCR Workstation (ISC BioExpress).

Results

In order to test whether the repression of early transcription which occurs prior to replication was also associated with the same forms of histone methylation observed when replication was occurring, we used two distinct strategies. First, we determined whether there were changes in histone methylation during the first 8 h post-infection in a wild-type infection consistent with what we previously reported for repression of early gene expression late in infection during DNA replication (37). We hypothesized that if transcriptional repression occurring at early times was associated with mono-methylation (me1) of H3K9 as observed during DNA replication, we would observe an increase in H3K9me1 over the first hours of an infection perhaps approaching the 20% value seen at late times when transcriptional repression was occurring. In contrast if early transcriptional repression was not associated with mono-methylation of H3K9 we would expect no effect on the levels of H3K9me1. Since we previously reported that the fraction of SV40 minichromosomes containing RNAPII decreased during the first hours of infection consistent with the repression of early transcription (52), we first confirmed that this was the case. SV40 wild-type minichromosomes were isolated 2, 4, 6, and 8 h post-infection and analyzed by ChIP for the presence of RNAPII. As shown in Figure 4A, we observed a slow and continual decrease in the percentage of RNAPII bound to SV40 minichromosomes between 2 and 8 h post-infection. We next determined the percentage of minichromosomes isolated at 30 min, 2, 4, and 8 h which contained H3K9me1. We did not analyze for the presence of methylated H3K4 at these times because we have previously shown that minichromosomes contain very low levels of methylated H3K4 (37). As shown in Figure 4B, we did not observe an increase in the level of H3K9me1 as

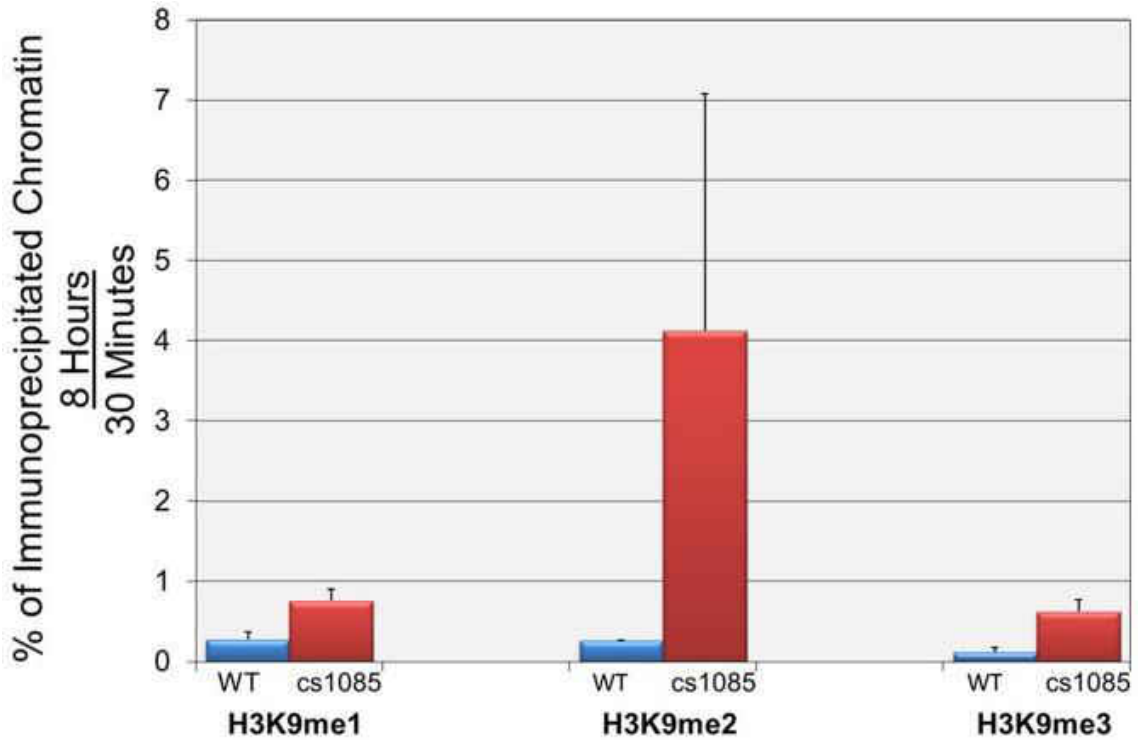
expected if it was associated with transcriptional repression. H3K9me1 remained present in approximately 1% or less of the minichromosomes at this time which was similar to the level that we previously reported present in the SV40 virus particles, $2.9 \pm 1\%$ (37), which was used for the infection.

Secondly, we determined whether infection by the mutant cs1085 which lacks Site I and fails to repress early transcription resulted in a changed pattern of histone methylation compared to wild-type virus during the same time. Again, we focused only on the methylated forms of H3K9 at this time because we have previously shown that there is very little if any methylated H3K4 at the very early times in question (37). SV40 minichromosomes were prepared at the indicated times, subjected to ChIP analyses and the percentage of minichromosomes containing each methylated form of H3K9 determined by real-time PCR. The data is represented as the percentage of minichromosomes containing the modification present at 8 h of infection divided by the percentage present at 30 min of infection. A ratio less than 1 indicates that the percentage of minichromosomes carrying a particular methylated H3 is reduced over this period. As shown in Figure 5, we observed that for both the wild-type and cs1085 mutant we observed a reduction in the relative amount of H3K9me1 and H3K9 tri-methylation (H3K9me3) present in minichromosomes between 30 min and 8 h post-infection. However, while the amount of H3K9 di-methylation (H3K9me2) was reduced during this period in the wild-type virus, the amount was significantly increased in the cs1085 mutant. These results suggest that repression of early gene expression during active transcription occurs by a process in which the levels of H3K9me2 are kept low.



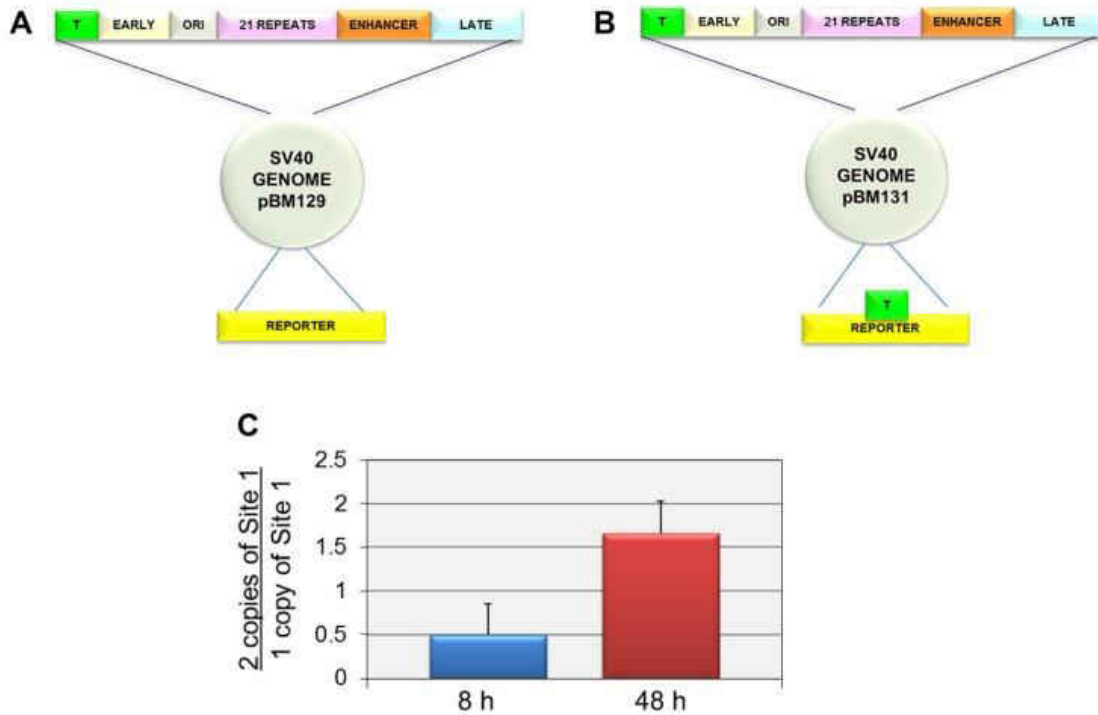
Repression of active early transcription does not result in an increase in H3K9me1. SV40 wild-type minichromosomes were isolated between 30 min and 8 h post-infection, and subsequently subjected to ChIP analyses with antibodies to either RNA polymerase II (RNAPII) or H3K9me1. The percentage of the input minichromosomes containing either RNAPII (A) or H3K9me1 (B) was determined by real-time PCR for each time point analyzed. All analyses were performed a minimum of three times using different preparations of SV40 minichromosomes.

In order to independently confirm that Site I was responsible for the introduction of H3K9me1 at late times but not early times, we compared the level of H3K9me1 in an SV40 recombinant containing two copies of Site I (pBM131-1) to a parental recombinant containing only a single copy of Site I (pBM129-1). We hypothesized that if Site I was responsible for the introduction of H3K9me1 in a replication dependent manner, we would observe an increase in the percentage of H3K9me1 in the recombinant compared to the parental virus at late times but not at early times when replication was not occurring. For these studies we used recombinant viruses originally prepared to study the ability of SV40 regulatory sequences to phase nucleosomes and generate nucleosome free regions in SV40 chromatin. The parental recombinant and its construction as well as the recombinant containing two copies of Site I have been previously described (67). The structures of both of these constructs are shown in Figure 5. The parental construct pBM129-1 has a single copy of Site I in the regulatory region as in the wild-type virus (Figure 6A). pBM131-1 has two copies of Site I, one located as in pBM129-1 and a second copy present in the reporter region as shown in Figure 6B. The results of this analysis are graphically represented in Figure 6C. As shown at 8 h post-infection when Site I should be active down-regulating early transcription we observed a ratio of 0.50 ± 0.35 indicating that there was less methylation of H3K9me1 at this time in the recombinant carrying two copies of Site I than in the parental recombinant with only one copy. In contrast at 48 h post-infection when replication is occurring we observed a ratio of 1.66 ± 0.37 confirming that Site I is capable of directing the introduction of H3K9me1 when SV40 is replicated. Interestingly it is also apparent that the second copy of Site I can function during replication outside of its normal location within the virus genome.



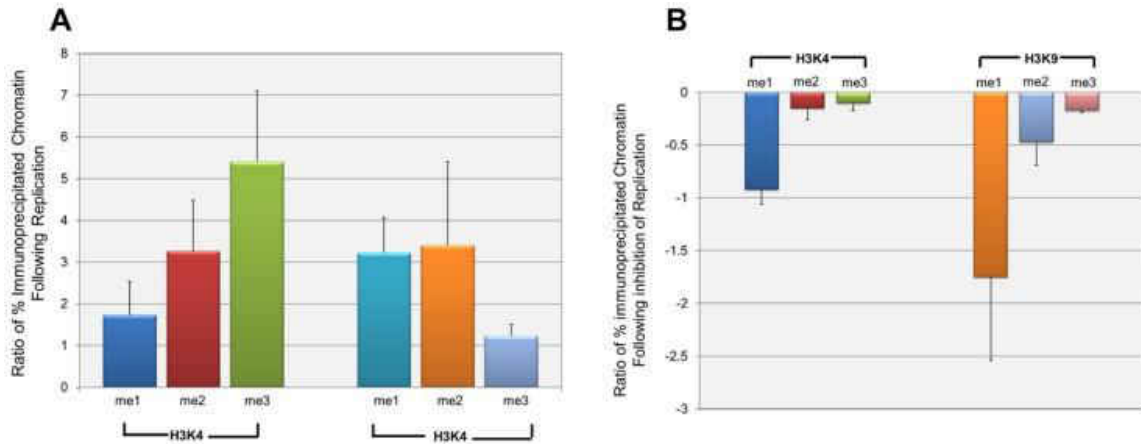
H3K9me2 is significantly increased during active early transcription in the site I deletion mutant cs1085. Wild-type and cs1085 SV40 minichromosomes were isolated from appropriately infected cells at 30 min and 8 h post-infection. Isolated minichromosomes were subjected to ChIP analyses with antibodies against H3K9me1, H3K9me2, and H3K9me3, and the percentage of input minichromosomes containing each form of methylated H3 determined by real-time PCR. The results are displayed as the ratio of the percentage of minichromosomes isolated at 8 h which contain a particular modification divided by the percentage of minichromosomes isolated at 30 min which contain the same modification. Ratios greater than 1 indicate that a modification is increasing during the period from 30 min to 8 h, while a ratio less than 1 indicates that the modification is decreasing during this period of infection. All analyses were performed a minimum of three times using different preparations of SV40 minichromosomes.

Since the effect of repression on H3K9me1 was only seen at late times in infection, it seemed likely that it was either directly or indirectly related to the replication of SV40 DNA which was occurring at this time. In order to test his hypothesis we determined the effect of the inhibition of replication on the introduction of methylated H3K4 and H3K9. SV40 minichromosomes were prepared at 24 h post-infection when replication was beginning and at 48 h post-infection in the presence or absence of aphidicolin, a specific inhibitor of eukaryotic DNA replication (68). SV40 minichromosomes were then subjected to ChIP analysis with antibodies to methylated H3K4 and H3K9. We first investigated the introduction of methylated H3 during the increase in SV40 chromatin resulting from replication between 24 and 48 h post-infection. Since we generally observe a 50- to 200-fold increase in the pool size of SV40 minichromosomes between 24 and 48 h post-infection, we compared the increase in a particular form of modification to the increase in the amount of SV40 minichromosomes. We expected that this ratio would be 1 if both the SV40 minichromosomes and form of modification were increasing at the same rate, greater than 1 if the newly replicated minichromosomes were more likely to contain the form of modification, or less than 1 if the minichromosomes were increasing faster than the introduction of the modified histone H3. The results of this analysis are graphically represented in Figure 7A. Based upon the observed ratios, all methylated forms of H3K4 and H3K9 were being introduced into the newly replicated minichromosomes at a rate faster than the increase in SV40 chromatin. However, H3K4me2 and H3K9me3 appeared to be introduced at rates close to the rate of increase of chromatin (1.74 and 1.23, respectively), while the other methylated forms of H3 were introduced at rates much greater than 1.



Two copies of Site I directs the incorporation of more H3K9me1 compared to one copy of Site I in SV40 minichromosomes isolated at 48 h post-infection but not at 8 h post-infection. SV40 minichromosomes were prepared from cells infected with pBM129-1 (one copy of Site I) or pBM131-1 (two copies of Site I) at 8 and 48 h post-infection. The percentage of SV40 minichromosomes containing H3K9me1 was determined by ChIP analyses for each preparation of minichromosomes at each time point followed by real-time PCR. The results are displayed as the ratio of the percentage of minichromosomes containing two copies of Site I immunoprecipitated by antibody to H3K9me1 over the corresponding percentage for minichromosomes containing one copy of Site I. A schematic of the structure of the SV40 recombinants pBM129-1 is shown in (A) and pBM131-1 in (B). pBM131-1 contains a second copy of Site I introduced into the reporter region of the basic recombinant, pBM 129-1. The results of this analysis are shown in (C). All analyses were performed a minimum of three times using different preparations of SV40 minichromosomes.

Next, we determined whether the introduction of a particular form of methylated H3 was actually dependent upon ongoing DNA replication. If ongoing DNA replication was necessary for the introduction of a particular methylated form of H3, inhibition of replication with aphidicolin should also block the introduction of the methylated form of H3. In contrast if the introduction of a methylated form of H3 was due to some other biological process, one would expect little if any effect on the introduction of the methylated form of H3 following inhibition of replication. SV40 minichromosomes were isolated from cells treated with aphidicolin from 24 to 48 h post-infection or from untreated cells at 48 h post-infection and subjected to ChIP analysis and real-time PCR. For each methylated form of H3, we then calculated the ratio of the decrease in methylated H3 to the decrease in the amount of SV40 minichromosomes following inhibition of replication. A ratio of 1 or greater would indicate that the introduction of methylated H3 was equal to or even greater than the reduction in the amount of SV40 chromatin, while a ratio near 0 would indicate that the introduction of methylated H3 was independent of DNA replication. The results of this analysis are graphically represented in Figure 7B. As shown in the figure the ratios for H3K9me1 (1.75) and H3K4me1 (0.92) were similar to or greater than 1 indicating that the introduction of these two methylated forms of H3 into SV40 chromatin were directly dependent upon DNA replication. The ratios for three of the methylated forms of H3 were very low including H3K4me2 (0.15), H3K4me3 (0.10), and H3K9me3 (0.17) indicating that these methylated forms of H3 were being introduced in the absence of direct DNA replication. The ratio for H3K9me2 (0.47) was intermediate between the other forms of methylated H3 suggesting that it was at least in part dependent upon replication



H3K4me1 and H3K9me1 are introduced into wild-type SV40 minichromosomes primarily during active replication. Wild-type SV40 minichromosomes were isolated at 24, 48, and 48 h post-infection following treatment with the DNA replication inhibitor aphidicolin from 24 to 48 h post-infection. The percentages of SV40 minichromosomes containing methylated H3K4 and H3K9 were determined by ChIP analyses followed by real-time PCR. The relative increase of each methylated form of H3K4 and H3K9 following DNA replication from 24 h to 48 h post-infection is shown in (A). The relative increase is shown as the ratio of the fold increase of a particular form of methylated H3 between 24 and 48 h post-infection divided by the corresponding fold increase in the amount of SV40 minichromosomes between these times. Ratios greater than 1 indicate that a particular methylated form of H3 is preferentially being introduced into newly replicated minichromosomes at a rate faster than the increase in the pool size of SV40 minichromosomes. The effects of the inhibition of DNA replication from 24 to 48 h post-infection on the introduction of methylated H3K4 and H3K9 are shown in (B). The results are shown as the ratio of the fold decrease in the amount of a particular form of methylated H3 in minichromosomes following inhibition of DNA replication divided by the fold decrease in minichromosomes resulting from inhibition of replication. Ratios less than or equal to 1 indicate that a particular methylated form of H3 is inhibited to a greater or the same extent as the inhibition of replication of the total SV40 minichromosomes. All analyses were performed a minimum of three times using different preparations of SV40 minichromosomes.

While we believe that the changes observed following aphidicolin treatment are primarily a result of the extensive inhibition of replication, we cannot exclude the possibility that indirect effects on transcription or induction of the DNA damage response following aphidicolin might also be contributing to changes in histone modifications.

Discussion

In SV40 minichromosomes, repression of early gene expression by T-antigen binding to Site I in the viral regulatory region was shown to result in distinct epigenetic marks at early and late times post-infection. At early times when only early transcription was occurring T-antigen binding resulted in the inhibition of the introduction of H3K9me2, while at late times when replication was occurring T-antigen binding resulted in the introduction of H3K9me1. The latter was first shown in a previous publication (37).

These results raise interesting questions concerning the mechanisms responsible for the introduction of epigenetic marks at the two time points in infection. Clearly, T-antigen binding is required for the introduction of the majority of H3K9me1. However, T-antigen binding does not appear to be the only signal for the introduction of H3K9me1 since a low level of H3K9me1 is still present in SV40 minichromosomes in a mutant in which T-antigen binding cannot occur. While Site I is necessary for the late introduction of H3K9me1, the Site I does not have to be located in the regulatory region since a recombinant containing an extra copy of Site I near the terminus of transcription showed an increase in H3K9me1 at late times but not early times. The location independent

increase in H3K9me1 in this recombinant suggests that Site I may be functioning like an enhancer to direct epigenetic changes (38).

It seems likely that the T-antigen directed introduction of H3K9me1 is mechanistically related to DNA replication. First, we have previously shown that at late times in infection H3K9me1 was specifically associated with SV40 minichromosomes actively undergoing replication using a two-step ChIP protocol (39) in which actively replicating minichromosomes were immunologically selected for subsequent analysis using an antibody to RPA70 a replication protein (39). Second, this association was confirmed by characterizing SV40 chromatin following inhibition of replication by aphidicolin. H3K9me1 appeared to be directly related to replication since it increased when replication occurred and was completely blocked when replication was blocked. Although H3K4me1 also appeared to be a direct result of replication the other methylated forms of H3K4 and H3K9 appeared to result from post-replication maturation. The introduction of H3K9me3 following replication has been shown in HeLa cells to occur via a maturation process in which the H3K9me3 is introduced into previously replicated chromatin containing H3K9me1 (40). It is not clear how the binding of T-antigen to Site I at early times results in the inhibition of the incorporation of H3K9me2. Potentially T-antigen might be disrupting the normal biological pathways linking H3K9me1 to H3K9me2 and H3K9me3.

These results are not consistent with a model of chromatin replication in which the pre-existing histone modifications present in the parental chromatin are duplicated in the daughter chromatin during replication (64)(65). Instead these results suggest that in SV40 minichromosomes DNA replication can serve as an epigenetic switch in which

newly replicated chromatin can be epigenetically modified in response to specific signals such as T-antigen binding to Site I. It seems unlikely that the H3K9me1 present during replication is simply a consequence of H3K9me1 being present in parental chromatin. If this were the case one would expect similar levels of H3K9me1 in both the cs1085 mutant and the wild-type virus since both contain H3K9me1 at early times. Secondly, a model in which pre-existing H3K9me1 drives the introduction of H3K9me1 following replication does not fit with the data obtained with the recombinant containing an extra copy of Site I. At early times the recombinant and its parental strain both contain similar levels of H3K9me1 yet at late times there is a significant increase in the amount of H3K9me1 present in replicated minichromosomes. This epigenetic switching hypothesis is consistent with a recent publication showing that replication of *Drosophila* chromatin occurs through a process in which pre-existing histone modifications are lost at the replication fork and histone modifications are re-introduced following replication by modifying complexes which remain closely associated with the replicating chromatin (69). The results differ in that in the publication pre-existing modifying complexes are thought to drive the introduction of post-replicative histone modifications while in SV40 the post-replicative changes are driven by the binding of the repressive factor T-antigen.

The most likely reason for the epigenetic switch is to ensure that newly replicated minichromosomes are not capable of activation for early transcription at late times in infection. Allowing activation of early transcription as in the case of the mutant cs1085 has been shown to result in a significant reduction in the pool size of SV40 minichromosomes and yield of virus late in infection (37). This epigenetic switch may

also play a critical role in controlling the relative pool sizes of transcribing, replicating, and encapsidating SV40 minichromosomes.

While an epigenetic switch associated with replication appears to have a biological relevance for SV40 it is not yet clear whether a similar process functions in cellular chromatin. However, it is interesting to speculate that a similar process could act during cellular differentiation to prepare newly replicated chromatin for subsequent activation or repression of transcription in response to specific signals introduced during replication as part of the differentiation pathway.

CHAPTER VII

TRANSCRIPTIONAL REPRESSION IS EPIGENETICALLY MARKED BY H3K9 METHYLATION DURING SV40 REPLICATION

Peer Reviewed and Published

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Abstract

Background

We have recently shown that T-antigen binding to Site I results in the replication-dependent introduction of H3K9me1 into SV40 chromatin late in infection. Since H3K9me2 and H3K9me3 are also present late in infection, we determined whether their presence was also related to the status of ongoing transcription and replication. Transcription was either inhibited with 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole (DRB) or stimulated with sodium butyrate and the effects on histone modifications early and late in infection determined. The role of DNA replication was determined by concomitant inhibition of replication with aphidicolin.

Results

We observed that H3K9me2/me3 was specifically introduced when transcription was inhibited during active replication. The introduction of H3K9me2/me3 that occurred when transcription was inhibited was partially blocked when replication was also inhibited. The introduction of H3K9me2/me3 did not require the presence of H3K9me1 since similar results were obtained with the mutant cs1085 whose chromatin contains very little H3K9me1.

Conclusions

Our data suggest that methylation of H3K9 can occur either as a consequence of a specific repressive event such as T-antigen binding to Site I or as a result of a general repression of transcription in the presence of active replication. The results suggest that the nonproductive generation of transcription complexes as occurs following DRB treatment may be recognized by a ‘proof reading’ mechanism, which leads to the specific introduction of H3K9me2 and H3K9me3.

Background

Five distinct but potentially related elements are thought to contribute to the epigenetic regulation of eukaryotic gene expression: DNA methylation, nucleosome location, histone variation, covalent histone modifications, and interactions by regulatory RNA. Of these elements, the post-translational modification of histones has been of particular interest because of the diversity of the available forms of modification and the

number of target amino acids and their physical location within histones. Importantly, there is an abundance of published reports demonstrating the association between certain forms of histone modification and activation or repression of transcription in one or more well characterized biological systems.

In general, it is thought that acetylation of certain lysines in histones is associated with active biological processes like transcription while methylation of lysines or arginines may be associated with either activation or repression of gene transcription. For example, in the nucleosome core, histone H3 acetylation on lysine 9 and 14 (H3K9 and H3K14) have been shown to be associated with transcription along with methylation of lysine 4 (H3K4), while methylation of lysine 9 (H3K9) has been shown to be associated with repression (70)(71).

While the association between the methylation of H3K9 and gene repression has been well established, much less is known about the circumstances that lead to the introduction of methylated H3K9 into chromatin. What has added to this uncertainty is the complexity of methylation, including mono-, di-, and tri-methylation, and the involvement of multiple methylating enzymes (72).

In order to better understand the factors that contribute to epigenetic regulation, we have been investigating the role of histone modifications in the regulation of gene expression during the Simian Virus 40 (SV40) life cycle (52)(13)(53)(37)(73)(74). In a recent publication we confirmed that the mono-methylation of H3K9 in SV40 chromatin was associated with repression of transcription (74). Specifically, we showed that the introduction of H3K9me1 was a consequence of the repression of early transcription by

the product of transcription, T-antigen, binding to a critical regulatory sequence known as Site I (74). Moreover, importantly, we observed that the introduction of H3K9me1 during repression required DNA replication. Repression occurring prior to the initiation of replication was not associated with the introduction of H3K9me1. Our results demonstrated that replication could serve as an epigenetic switch and that the same biological event could have different epigenetic readouts depending upon whether replication was occurring.

During the course of these studies, we investigated the introduction of all three methylated forms of H3K9 during replication. We noted that unlike the introduction of H3K9me1 which absolutely required replication, the introduction of H3K9me2 and H3K9me3 appeared to occur even when replication was substantially blocked by inhibitor (74). These results raised two important questions. First, was the introduction of H3K9me2 and H3K9me3 also associated with the repression of transcription in SV40 chromatin, and second, what biological factors contributed to the introduction of these epigenetic marks?

In order to address these questions in SV40 chromatin, we have investigated the effects of a general inhibition or stimulation of transcription on the introduction of H3K9 methylation in the presence or absence of active replication. Transcription was inhibited with 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole (DRB) (75), a well-characterized inhibitor of RNA Polymerase II (RNAPII) elongation, and stimulated with sodium butyrate, an inhibitor of histone deacetylase (76)(77) known to stimulate gene expression. DNA replication was inhibited with aphidicolin (68). We show that the introduction of H3K9me2 and H3K9me3 are the result of a general inhibition of transcription at late

times in infection but not early times, and that the introduction is partially dependent upon replication but not the prior introduction of H3K9me1.

Results

Inhibition of Transcription by DRB Stimulates the Introduction of H3K9me2 and H3K9me3 into SV40 Chromatin Late in Infection but not Early in Infection

Since we previously observed that the introduction of H3K9me2 and H3K9me3 was not directly related to repression by T-antigen binding to Site I (74) we hypothesized that their incorporation into SV40 chromatin might be the consequence of a more general inhibition of transcription. In order to test this hypothesis we determined the epigenetic consequences of inhibiting transcription with the RNAPII elongation inhibitor 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole (DRB). We have previously used DRB to investigate the relationship between RNA polymerase II translocation and the acetylation and deacetylation of histones during transcription (13). For these studies, we chose to investigate SV40 chromatin isolated at 2 hours post-infection when early transcription was occurring but prior to DNA replication, and at 48 hours post-infection when early and late transcription were occurring along with active replication.

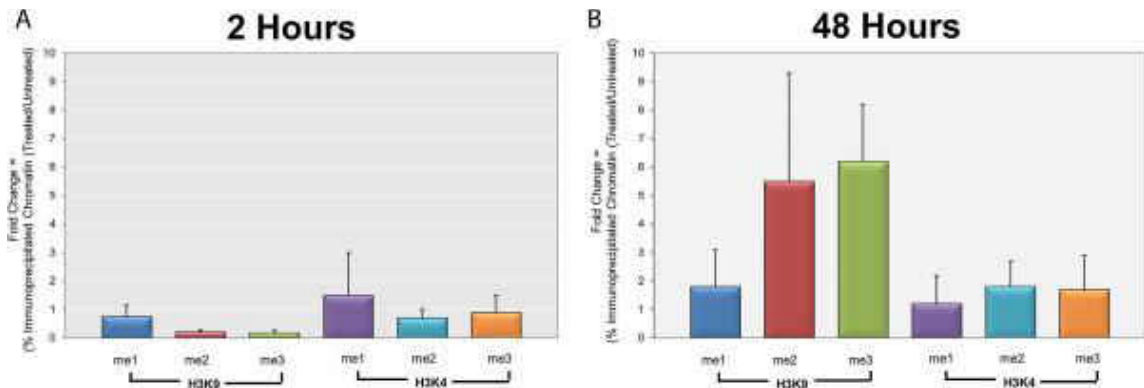
SV40 minichromosomes were isolated and purified from cells infected with wild-type 776 virus following treatment with DRB or no treatment. When minichromosomes were isolated at 2 hours, cells were pretreated with DRB for 2 hours prior to infection, while for minichromosomes isolated at 48 hours post-infection infected cells were treated from 24 to 48 hours. In these experiments, we observed a 112 ± 35 fold increase in the

size of the pool of SV40 chromosomes present in glycerol gradient fractions following purification from cells infected for 48 hours compared to 24 hours post-infection. While the increase was somewhat variable we always observed at least a tenfold increase. This increase was reduced 10 ± 6 fold following treatment of the SV40 infected cells with DRB from 24 to 48 hours post-infection. The latter analysis was determined by comparing the increase in the presence and absence of inhibitor and indicates that on average there was about a tenfold reduction in the pool of SV40 minichromosomes following inhibition. As expected, treatment with DRB substantially inhibited the generation of mRNA (data not shown). The effect of DRB on the introduction of methylated H3K4 and H3K9 was determined by subjecting treated and untreated samples of intact SV40 minichromosomes obtained at 2 hours and 48 hours post-infection to ChIP analyses with antibodies that recognize mono-, di-, or tri-methylated H3K4 or H3K9. Intact SV40 minichromosomes were used because they are easily obtained in relatively large amounts and yield the maximum PCR signal compared to fragmented chromatin. Because the SV40 genome was intact, this analysis only yielded information relative to changes in the numbers of minichromosomes carrying a particular modification following treatment. No information was obtained concerning the location of any specific histone modifications. The results of these analyses are graphically shown in Figure 8. The data is displayed as the ratio of the percentage of minichromosomes that contain a modification following treatment divided by the percentage of untreated minichromosomes containing the same modification. If treatment had no effect on the introduction of a particular modification the ratio will be one. Ratios greater than one indicate that treatment results in an increase in the minichromosomes containing the

modification while ratios less than one indicate that treatment resulted in a decrease in the presence of a modification, As shown in Figure 8A and 8B, DRB treatment had no significant effect on the methylation of H3K4 at either 2 hours or 48 hours post-infection. For each form of methylation, the ratio of treated sample to untreated sample was approximately one. In contrast, DRB treatment had a significant effect on the presence of H3K9me2 and H3K9me3 at 48 hours post-infection and little effect at 2 hours post-infection (Figure 5A and B). We observed a 6 ± 4 fold increase for H3K9me2 with a range from 3 to 12 for four independent samples and a 6 ± 2 fold increase for H3K9me3 with a range of 3 to 8 for four independent samples. As expected from our previous published work, DRB treatment had no significant effect on the presence of H3K9me1 at either time point.

Introduction of H3K9me2 and H3K9me3 Following Inhibition of Transcription is Partially Dependent Upon Replication

Since we have previously shown that the introduction of H3K9me2 and H3K9me3 into SV40 chromatin late in infection does not require DNA replication (88), we next tested whether replication played a role in the enhanced introduction of these modifications following DRB inhibition of transcription. SV40 minichromosomes were obtained from infected cells that were untreated or treated from 24 to 48 hours post-infection with a combination of DRB and aphidicolin to inhibit both transcription and replication. The minichromosomes were then subjected to ChIP analyses with antibody to H3K9me2 and H3K9me3 with the results shown in Figure 9. The data is again shown as



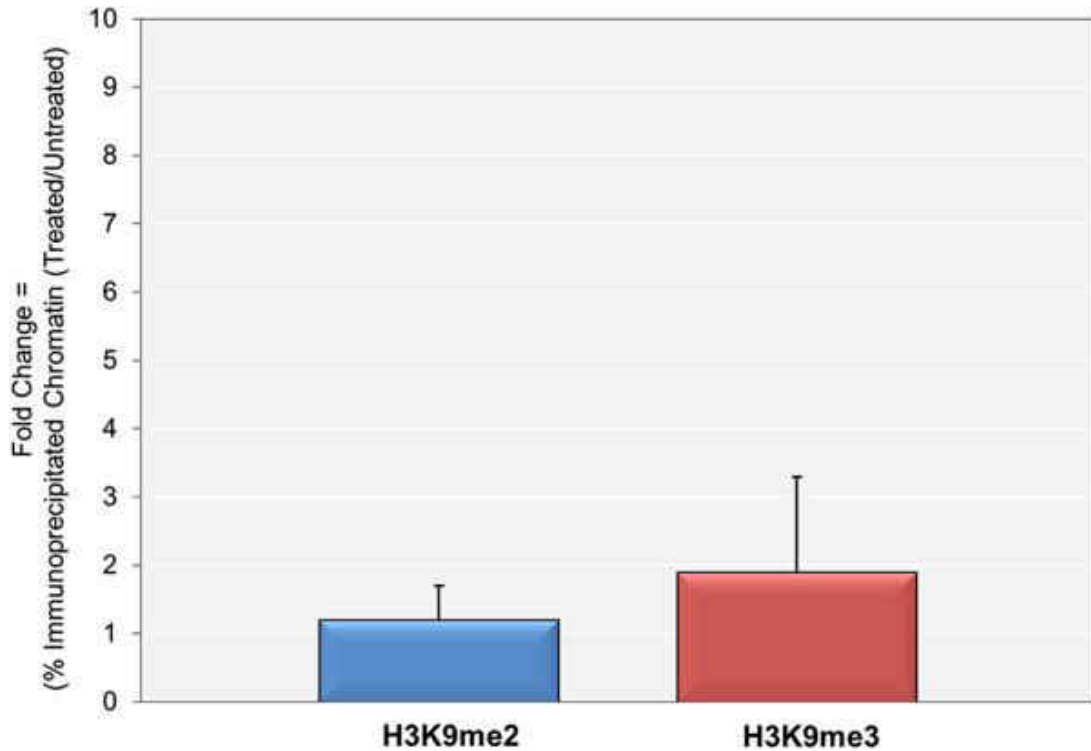
Inhibition of transcription with 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole (DRB) stimulates the incorporation of H3K9me2 and H3K9me3 into SV40 chromatin late in infection. Wild-type SV40 minichromosomes were isolated at 2 hr post-infection with or without incubation with 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole (DRB) from minus 2 hr until isolation and at 48 hr post-infection with or without incubation with DRB from 24 to 48 hr post-infection. The percentages of intact minichromosomes containing methylated H3K4 and H3K9 were determined by ChIP analyses followed by purification of the intact genomic DNA and PCR amplification with primers recognizing the promoter region. For each form of histone modification, the ratio of the percentage present in the treated minichromosomes compared to the untreated minichromosomes was calculated. The effects of DRB treatment on the introduction of methylated H3K4 and H3K9 from minus 2 hr to isolation at 2 hr post-infection is shown in (A). The corresponding effects of DRB treatment on the introduction of methylated H3K4 and H3K9 from 24 to 28 hr post-infection are shown in (B).

the ratio between the percentages of input minichromosomes containing the modification of interest in the treated sample compared to the untreated sample.

Treatment with aphidicolin and DRB resulted in approximately a 99% reduction in the amount of SV40 minichromosomes obtained at 48 hours post-infection compared to the amount obtained from untreated cells. Following inhibition we observed no increases in the ratio for H3K9me2 (1 ± 0.5) and a small increase in the ratio for H3K9me3 (2 ± 1). These increases were significantly lower than the values obtained when transcription was inhibited while DNA replication was occurring as shown in Figure 8. These results suggest that ongoing replication plays at least a small role in the introduction of H3K9me2 and H3K9me3 at late times in infection.

Introduction of H3K9me2 and H3K9me3 Following Inhibition of Transcription does not Require the Presence of H3K9me1

Since H3K9me1 can be present in as much as 22% of the SV40 minichromosomes present at late times (37), it seemed reasonable that the H3K9me1 containing minichromosomes might serve as substrates for the introduction of H3K9me2 and H3K9me3. To test this possibility we characterized the effect of DRB treatment on the mutant cs1085 SV40 virus. This mutant lacks T-antigen binding Site I and as a consequence fails to down-regulate early transcription. We have previously shown that minichromosomes from this mutant contain very low levels of H3K9me1 in contrast to parental wild-type viral chromatin (37). If the minichromosomes containing H3K9me1 served as a substrate for the introduction of H3K9me2 and H3K9me3,

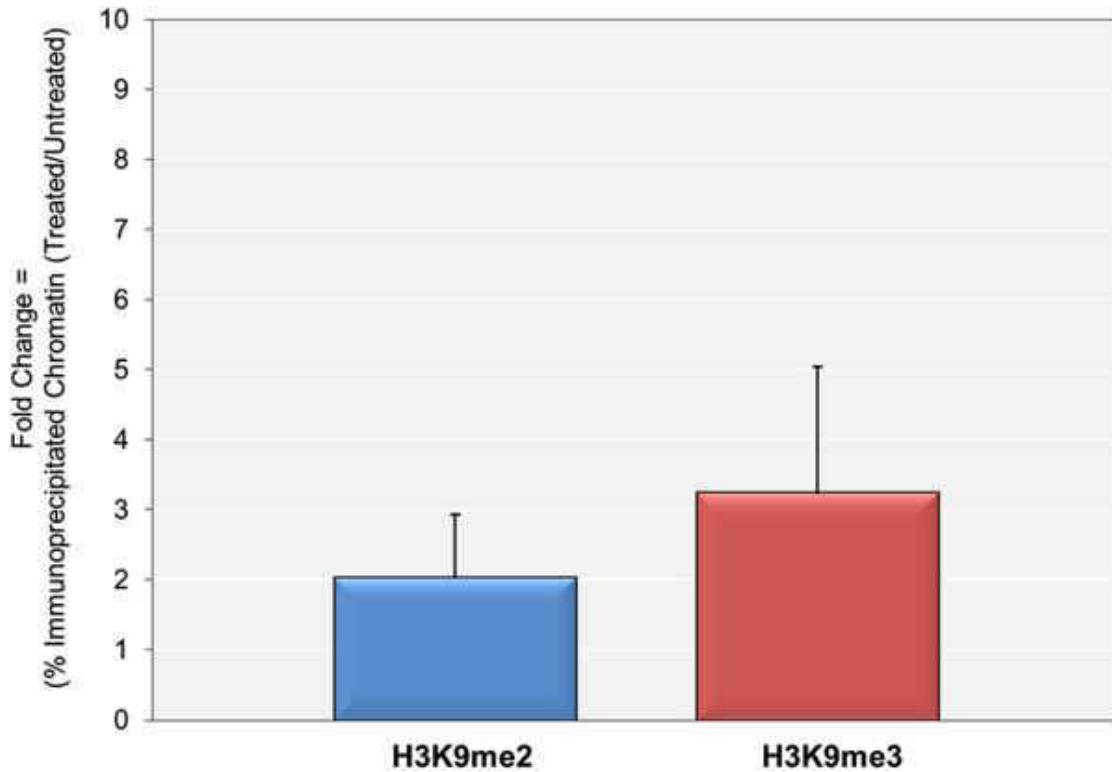


The 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole (DRB)- stimulated introduction of H3K9me2 and H3K9me3 is partially dependent upon ongoing DNA Replication. Wild-type SV40 minichromosomes were isolated at 48 hr post-infection with or without treatment with 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole (DRB) and aphidicolin from 24 to 48 hr post-infection. The treated and untreated intact minichromosomes were subjected to ChIP analyses with antibodies to H3K9me2 and H3K9me3 and the percentages of the minichromosomes containing the modified H3K9s determined by real-time PCR amplification of the bound intact SV40 genomic DNA with primers recognizing the promoter region. The fold increase in the percentages of minichromosomes containing H3K9me2 and H3K9me3 was then calculated.

we would expect that inhibition of transcription of cs1085 with DRB would not result in a large increase in the amounts of H3K9me2 and H3K9me3 in the treated minichromosomes compared to the untreated minichromosomes. Cells infected with SV40 cs1085 virus were treated with DRB from 24 to 48 hours post-infection or left untreated and the minichromosomes present in the cells isolated and purified. The minichromosomes were subjected to ChIP analyses with antibodies to H3K9me2 and H3K9me3 with the results shown in Figure 10. Treatment with DRB resulted in significant increases in the amounts of H3K9me2 (3 ± 1 fold) and H3K9me3 (5 ± 3 fold) present in the cs1085 minichromosomes indicating that the presence of H3K9me1 in the minichromosomes was not necessary for the introduction of the higher levels of methylated H3K9.

Introduction of H3K9me2 and H3K9me3 are Associated with SV40 Minichromosomes that Contain RNAPII

In order to determine whether H3K9me2 and H3K9me3 were being added to minichromosomes that contain RNAPII following treatment with DRB, we analyzed SV40 minichromosomes that contain RNAPII for the presence of the two methylated forms of H3K9 following treatment with DRB using our ISFIP procedure (52)(73). In this procedure, SV40 minichromosomes containing RNAPII were immune-selected with antibody to RNAPII bound to protein A agarose in a standard ChIP assay. Following purification of the bound chromatin and prior to elution, the minichromosomes bound to agarose were sonicated to fragment the chromatin into nucleosome-sized pieces and the



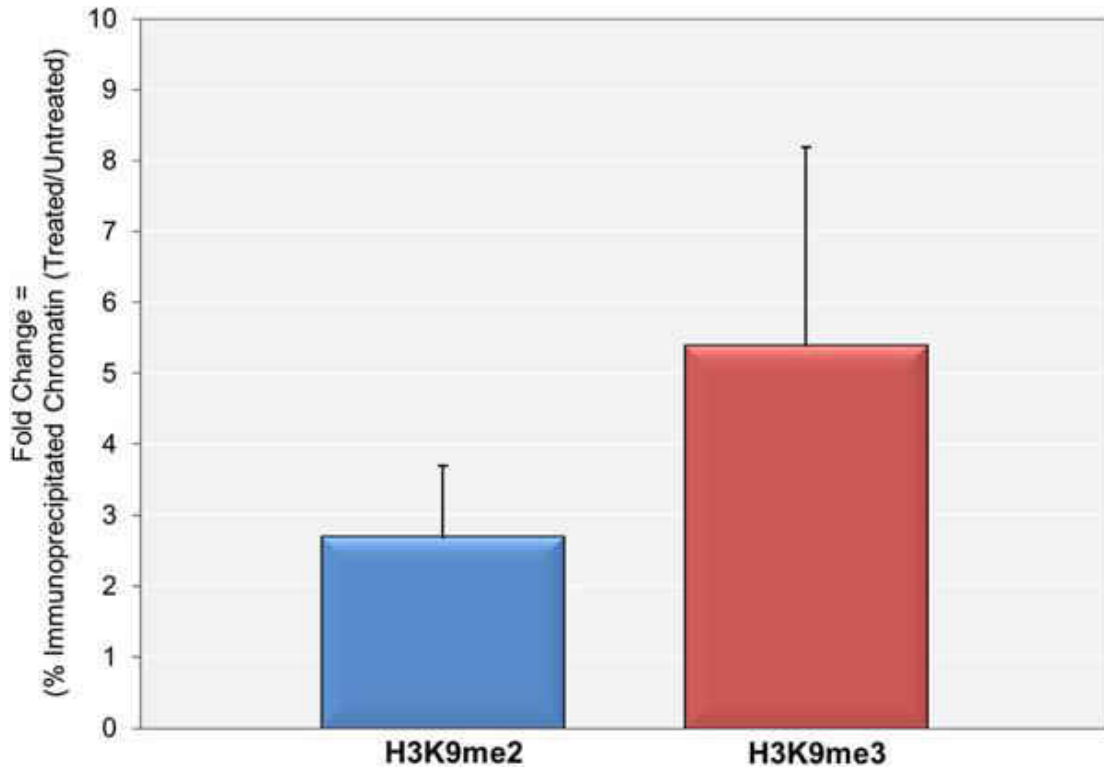
The 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole (DRB)- stimulated introduction of H3K9me2 and H3K9me3 does not require the presence of H3K9me1. SV40 minichromosomes from the mutant virus cs1085 were isolated at 48 hr post-infection with or without treatment with 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole (DRB) from 24 to 48 hr post-infection. Intact minichromosomes were subjected to ChIP analyses with antibody recognizing H3K9me2 and H3K9me3 and the intact SV40 genomic DNA present in the bound fraction quantitated by real-time PCR with primers recognizing the promoter region. The percentages of minichromosomes in the treated and untreated samples were determined and the fold increase in the percentage in the treated samples compared to the untreated calculated.

bound fragments separated from the released fragments. The released fragments were then subjected to a second ChIP with antibodies to H3K9me2 and H3K9me3 to determine whether H3K9me2 and H3K9me3 were present in the minichromosomes containing RNAPII and if so whether the amount changed upon treatment with DRB. As shown in Figure 11 we observed increases in the percentage of the RNAPII containing minichromosomes which also contained H3K9me (2 ± 1 fold) and H3K9me3 (3 ± 2 fold) although not as significant as those observed in Figure 8. These results suggest that minichromosomes containing RNAPII may serve as the substrate for H3K9 methylation.

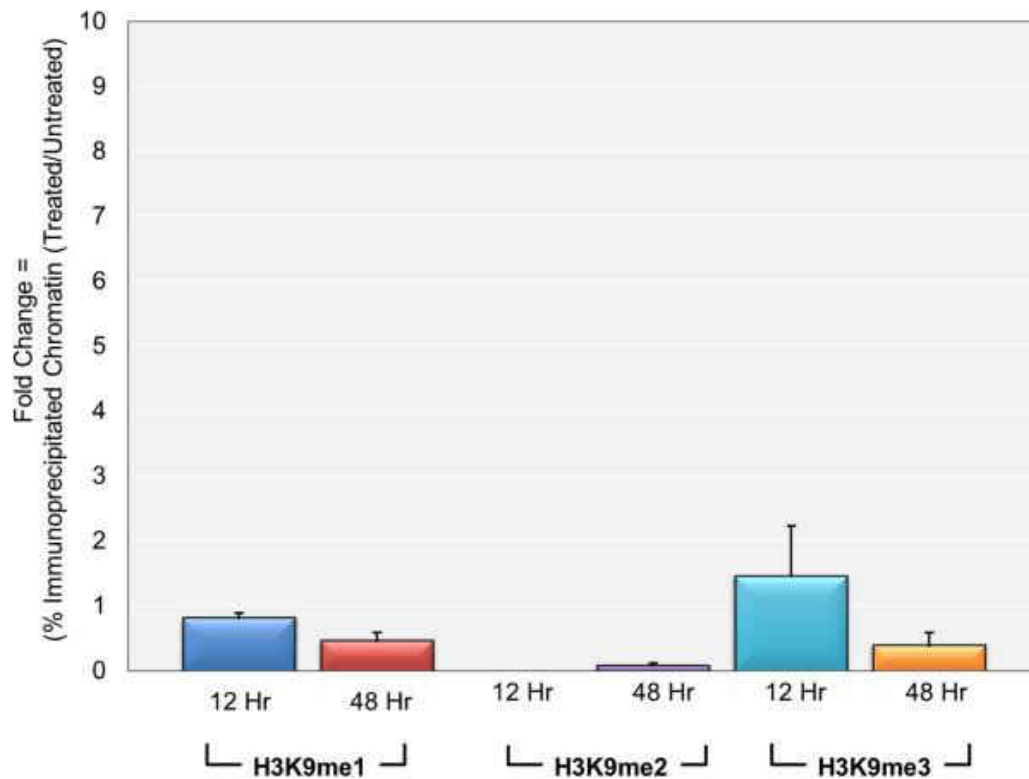
Stimulation of Transcription with Sodium Butyrate Inhibits the Incorporation of H3K9me2 and H3K9me3 into SV40 Chromatin Late in Infection

Since the introduction of H3K9me2 and H3K9me3 appeared to be associated with repression of transcription, we then determined whether stimulation of transcription would have the opposite effect. Sodium butyrate is a well-known inhibitor of many histone deacetylases and for this reason has been used extensively to investigate the relationship between histone acetylation and various biological processes including transcription (76)(77). We have previously shown that inhibition of histone deacetylases by sodium butyrate results in an increase in transcription of actively transcribed genes as well as an increase in histone acetylation (53)(78). If increased transcription and/or histone acetylation prevented the introduction of H3K9me2 or H3K9me3, we would expect to see a reduction in the ratio of these two modified forms of H3 in the treated minichromosomes compared to the untreated minichromosomes.

SV40 wild-type infected cells were harvested at 24 hours post-infection, 48 hours post-infection, or 48 hours post-infection following treatment with 50 μ M sodium butyrate from 24 to 48 hours post-infection to determine the effects of sodium butyrate during the period of active replication. In a parallel analysis, infected cells were harvested at 12 hours post-infection and 12 hours following a 12-hour treatment with sodium butyrate to determine the effects of sodium butyrate in the absence of replication. The minichromosomes were purified and subjected to ChIP analyses with antibodies to methylated H3K4 and H3K9 with minichromosomes isolated late in infection but only methylated H3K9 when isolated at very early times. The reason for this was our previous observation that relatively little of the SV40 chromatin contained methylated H3K4 at the very early times (37) and because there was no change in the levels of methylated H3K4 following DRB treatment. As shown in Figure 12, we observed ratios close to 1 for H3K9me1 (0.9 ± 0.1) and H3K9me3 (1.5 ± 0.8) in minichromosomes isolated at 12 hours post-infection. We saw very low levels of H3K9me2 in both treated and untreated minichromosomes, which were too variable to quantitate at this time. In contrast, for minichromosomes isolated at 48 hours post-infection, we observed significant inhibition of H3K9 methylation. The ratios were 0.46 ± 0.13 for H3K9me1, 0.08 ± 0.04 for H3K9me2, and 0.39 ± 0.2 for H3K9me3. While H3K4me2 (0.75 ± 0.13) and H3K4me3 (1.26 ± 0.38) did not seem to be affected by sodium butyrate treatment at late times, H3K4me1 (0.47 ± 0.27) appeared to be moderately inhibited (data not show). The decrease in the levels of H3K9me2 and H3K9me3 when transcription was stimulated were consistent with the idea that these modifications were being introduced as a consequence of a general repression of transcription at late times.



The 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole (DRB)- stimulated introduction of H3K9me2 and H3K9me3 is partially associated with minichromosomes containing RNAPII. Wild-type SV40 minichromosomes were isolated from cells at 48 hr post-infection with or without treatment with 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole (DRB) from 24 to 48 hr post-infection. Intact Minichromosomes were subjected to an ISFIP ChIP analysis in which minichromosomes were first immune selected with antibody to RNAPII. The minichromosomes bound by antibody to RNAPII were sonicated and the soluble chromatin fraction subjected to a second ChIP with antibody to either H3K9me2 or H3K9me3. The percentage of the treated and untreated chromatin containing H3K9me2 and H3K9me3 was determined by real-time PCR using primers that recognize the early region of the genome and the fold increase resulting from treatment calculated.



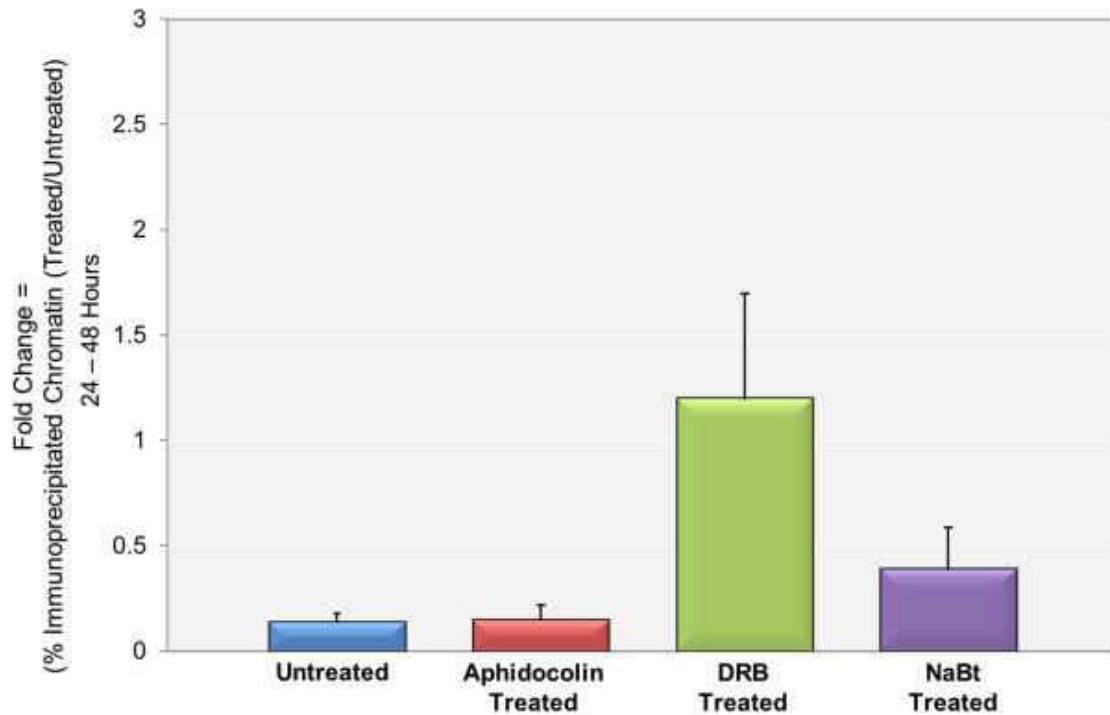
The introduction of H3K9me2 and H3K9me3 is inhibited by sodium butyrate stimulation of transcription during active replication. Wild-type SV40 minichromosomes were isolated at 12 hr post-infection with or without treatment from the initiation of infection with sodium butyrate and at 48 hr post-infection with or without treatment with sodium butyrate from 24 to 48 hr post-infection. Intact minichromosomes isolated at 12 hr post-infection were subjected to ChIP analyses with antibodies to methylated H3K9. Intact minichromosomes isolated at 48 h post-infection were subjected to ChIP analyses with antibodies to methylated H3K4 and methylated H3K9. The percentage of treated and untreated minichromosomes containing each form of methylated H3K4 or H3K9 was determined by real-time PCR amplification of the intact SV40 genomic DNA with primers recognizing the promoter region. The fold change resulting from treatment was then calculated from the percentages.

Pools of transcribing SV40 minichromosomes are Dynamic During Replication

Since DRB had a major effect on transcription and the introduction of H3K9me2 and H3K9me3, we also determined whether it affected the proportion of minichromosomes carrying RNAPII and was, therefore, potentially capable of transcription. We have previously shown that with short-term treatment with DRB there was little effect on the percentage of minichromosomes carrying RNAPII (13). However, long-term treatment when replication was occurring could be different. In order to better understand the factors contributing to determining the pool size of minichromosomes containing RNAPII during this time frame, we also investigated the effects of aphidicolin and sodium butyrate. If the pool of minichromosomes containing RNAPII was simply related to the overall size of the pool of minichromosomes, we would expect to observe that the percentage containing RNAPII would remain constant despite the overall changes in the size of the pool following the different treatments. For this analysis, we compared the percentage of RNAPII containing minichromosomes at 24 hours post-infection when replication was beginning to 48 hours post-infection when replication was active in the presence or absence of the replication inhibitor aphidicolin, transcription inhibitor DRB, or transcription stimulator sodium butyrate.

As indicated above, between 24 and 48 hours post-infection we observed a 112 ± 35 fold increase in the size of the minichromosome pool. Following treatment with aphidicolin the pool size at 48 hours post-infection was essentially the same as at 24 hours post-infection indicating substantial inhibition by the drug. Following treatment with DRB we again observed a substantial increase in the pool of minichromosomes although the increase was 10 ± 6 fold less than observed in the absence of the inhibitor.

Treatment with sodium butyrate was seen to increase the size of the pool of minichromosomes by 10 ± 4 fold above the level of increase without the added sodium butyrate. The latter two results indicate that the inhibitors had a modest effect on pool sizes when replication was occurring. SV40 minichromosomes from treated or untreated infections were subjected to ChIP analysis with antibody to RNAPII with the results shown in Figure 13. We observed that the ratio of the percentage of minichromosomes containing RNAPII was only 0.14 ± 0.04 comparing minichromosomes isolated at 48 hours post-infection to 24 hours post-infection indicating that only a small fraction of the newly replicated minichromosomes became associated with RNAPII. Following treatment with aphidicolin to block replication the ratio was reduced even further. Compared to untreated controls at 48 hours the ratio was 0.64 ± 0.16 . Since there was no increase in the overall pool size this indicated that the actual amount of SV40 chromatin containing RNAPII following inhibition of replication was lower in the treated samples than was present at 24 hours post-infection. Following treatment with DRB from 24 to 48 hours post-infection the ratio of minichromosomes containing RNAPII increased to 1.2 ± 0.5 , indicating that the pool size of transcribing minichromosomes was increasing along with replication in the presence of DRB. Finally, we observed that treatment with sodium butyrate during replication also had a profound effect. The ratio of RNAPII containing minichromosomes was 0.39 ± 0.20 following treatment with sodium butyrate from 24 to 48 hours post-infection. This indicated that compared to untreated controls a larger fraction of minichromosomes were capable of transcription.



The pool size of SV40 minichromosomes containing RNAPII is dynamic and dependent upon replication and rate of transcription. SV40 minichromosomes were isolated from cells infected with wild-type virus at 24 and 48 hr post-infection. Infected cells were either untreated or treated from 24 to 48 hr post-infection with DRB, sodium butyrate (NaBt), or aphidicolin. Intact minichromosomes were subjected to ChIP analyses with antibody to RNAPII and the percentage of untreated and treated minichromosomes containing RNAPII was determined by real-time PCR amplification of the intact SV40 genomic DNA with primers recognizing the promoter region. The fold increase or decrease from 24 to 48 hr post-infection with or without treatment was then calculated from the percentages.

Discussion

The introduction of H3K9me2 and H3K9me3 into SV40 chromatin following repression of transcription through the inhibition of RNAPII elongation by the inhibitor DRB is consistent with previous reports showing that the presence of H3K9me2 and H3K9me3 in chromatin is associated with transcriptional repression (reviewed in (79)(80)). H3K9me3, in particular, is now considered a characteristic mark of repression by many laboratories (71).

H3K9me2 and H3K9me3 are typically introduced into chromatin by a member of the SET domain family of histone methyltransferases (80). The related proteins G9a and GLP are thought to be primarily responsible for H3K9me2 methylation in euchromatin, whereas Suv39H1 is thought to be responsible for H3K9me3 methylation in heterochromatin (31)(33)(32). G9a/GLP also appears to be capable of introducing H3K9me3 into euchromatin (81). While these proteins contribute to the bulk of the H3K9me2 and H3K9me3 in a cell, there are other methyltransferases that may also play a role since deletion of these enzymes cause a significant but not complete reduction in H3K9 methylation (80).

There are two well-characterized mechanisms for the introduction of H3K9me2 and H3K9me3. H3K9me2 is associated with repression of nuclear hormone receptor regulated transcription (reviewed in (79)). In this model system, the introduction of H3K9me2 is the consequence of the binding of the receptor along with the histone methyltransferase to maintain a repressive environment. Introduction of the ligand results

in the selective loss of the methyltransferase and subsequent corresponding loss of H3K9me2 and activation of transcription (79).

The introduction of H3K9me3 into heterochromatin has also been extensively characterized (reviewed in (80)). The introduction of H3K9me3 in heterochromatin is thought to be closely linked with replication and to occur through the targeting of the relevant methyltransferase by associated proteins including CAF1, HP1, and MBD1 in higher eukaryotes (40)(82) and Clr4 and Swi6 in fission yeast (80). The presence of MBD1 in higher eukaryotes links H3K9me3 to DNA methylation and suggests a possible mechanism for targeting the complex to the appropriate sites (hemi-methylated DNA) following replication.

It seems unlikely that either of these mechanisms is responsible for the introduction of H3K9me2 and H3K9me3 reported here. With respect to the introduction of H3K9me2, SV40 transcription does not appear to be regulated by a nuclear hormone type mechanism as previously described for H3K9me2. Similarly the observed increase in H3K9me2 occurred as a consequence of the inhibition of transcription by DRB not the binding of normal transcription factors. The introduction of H3K9me3 also is unlikely to occur by the same mechanism as described for heterochromatin. Notably, SV40 DNA in chromatin is not known to be methylated because of an absence of DNA methylase substrates in the DNA. Also our observed increase in H3K9me2 and H3K9me3 appeared to be only secondarily a result of DNA replication since the increase was observed on minichromosomes containing RNAPII.

To explain our results we propose the following model. As DNA replication takes place, some of the newly replicated minichromosomes are committed to transcription through the binding of specific and general transcription factors. Concurrently, for reasons as yet unknown, some of the preexisting transcribing minichromosomes stop transcription. These nonfunctioning minichromosomes are recognized as being repressed, and H3K9me2 and H3K9me3 are introduced into their chromatin as a mark of repression. We propose that following replication and the activation for new transcription there is a 'proofreading mechanism,' which ensures that the newly initiated minichromosomes are capable of productive transcription. In the event that this is not the case, the minichromosomes are again recognized as repressed, as with the nonfunctioning minichromosomes, and marked by H3K9me2 and H3K9me3. Since treatment with the inhibitor DRB results in a large increase in the number of minichromosomes in which transcription is stopped, presumably at initiation, there is a corresponding increase in the incorporation of H3K9me2 and H3K9me3.

This proofreading mechanism could be specific to SV40 and other similar viruses in which case it could function to ensure that the size of the pool of transcribing minichromosomes is tied to the level of replication and encapsidation. Conversely, this mechanism could be true for higher eukaryotes in general and serve as a way to ensure that when genes are activated for transcription, only those genes correctly activated are allowed to persist in the activated state.

Assuming that the introduction of H3K9me2 and H3K9me3 following the inhibition of elongation by RNAPII reflects a normal biological process for SV40, we

believe it is likely to play a role in two aspects of normal SV40 molecular biology. First, the introduction of H3K9me2 and H3K9me3 could play a critical role in regulating the pool size of transcriptionally competent minichromosomes during an infection. We have presented evidence that the pool size is dynamic with new transcribing minichromosomes entering the pool as a result of replication and old minichromosomes leaving the pool. We believe that old minichromosomes leaving the pool of transcribing minichromosomes would be labeled with H3K9me2 and H3K9me3 in order to prevent their reactivation.

Second, H3K9me2 and H3K9me3 could also play a critical role during the initiation of a subsequent infection. We have shown that minichromosomes in virions contain significant amounts of H3K9me2/3 (37). If the minichromosomes, which contain these modifications, result primarily from the minichromosomes that originally transcribed the late genes following replication, it seems desirable that they be silenced during a new infection when only the transcription of the early genes is required. Allowing transcription of the late genes during the establishment of an infection would seem to be very undesirable for the virus.

Because DRB treatment blocks RNAPII elongation, we believe that it is a way to model the effects of repression of transcription in order to characterize the subsequent changes in epigenetic modifications. However, we recognize that the DRB treatment, as well as the treatment with the other inhibitors themselves, may be responsible for the observed effects on the levels of H3K9me2 and H3K9me3 and may not accurately reflect the epigenetic changes occurring *in vivo*.

Conclusions

Methylation of H3K9 can occur during active expression either as a consequence of a specific repressive event such as T-antigen binding to Site I or as a result of a general repression of transcription. Moreover, these results suggest that the nonproductive transcription complexes which are generated by DRB treatment may be recognized by a ‘proof reading’ mechanism, which leads to the specific introduction of H3K9me2 and H3K9me3.

Methods

Cells and Viruses

Wild-type and mutant SV40 minichromosomes were prepared in the monkey kidney BSC-1 cell line (ATCC) using either wild-type 776 virus (from Dr. Daniel Nathans) or cs1085 virus (from Dr. Daniel Nathans).

Cell Culture and Infections

BSC-1 cells were maintained and infected as previously described [5–52]. An RNA polymerase II inhibitor, 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole (DRB), was used at a concentration of 200 μ M. For 2-hour infections, DRB was added two hours prior to infection and re-applied at 30 minutes post-infection when the media was replaced by fresh media without virus. For 48-hour infections, DRB was added at 24 hours post-infection. Sodium butyrate was used at a concentration of 250 μ M. Sodium

butyrate was added at infection and along with fresh media following removal of unbound virus for 12-hour infections and at 24 hours post-infection for 48-hour infections. Replication was inhibited with aphidicolin added at 24 hours post-infection at a final concentration of 6 μ M as previously described (74).

Preparation of SV40 Minichromosomes

SV40 minichromosomes were isolated at the indicated times from 2 hours to 48 hours post-infection as described for each of the analyses using our standard purification protocols with one minor modification. After transferring the lysed cells to a 15-ml centrifuge tube, an additional one ml of nuclei preparation buffer was used to rinse the flask and was subsequently added to the centrifuge tube in order to maximize the yield of minichromosomes from each infection.

Chromatin Immunoprecipitation

ChIP kits were obtained from Millipore (Temicula, California, USA) and the protocol was followed as previously described (74). The antibodies used included: H3K4me1 (07 = 436, Millipore (Temicula, California, USA), H3K4me2 (39141, Active Motif Carlsbad, California, USA), H3K4me3 (04 = 745, Millipore Temicula, California, USA), H3K9me1 (ab9045, Abcam, Cambridge Massachusetts USA), H3K9me2 (ab1220, Abcam, Cambridge, Massachusetts, USA), H3K9me3 (ab8898, Abcam, Cambridge,

Massachusetts, USA), and RNA PII 905 = 623, Millipore, Temecula, California, USA). All antibodies were ChIP validated by the respective vendors. A total of 100 μ l of Protein A agarose, 800 μ l chip dilution buffer, and 7.5 μ l of each antibody were combined in a protein low-bind tube. The mixture was rotated for 5 hours at 4°C on an end to end rotator in a refrigerator to bind the antibody to Protein A agarose. Following binding of the antibody, the Protein A agarose was centrifuged at 2,000 rpms for 2 minutes and the supernatant discarded. Next, 800 μ l of fresh ChIP dilution buffer was added, and 100 μ l of the SV40 chromatin to be analyzed was added. The samples containing antibody bound to Protein A agarose and chromatin were incubated with end to end rotation for a further 7 hours at 4°C. The chromatin bound to Protein A agarose was washed according to the manufacturer's protocol and eluted as previously described (74).

Immune Selection Fragmentation Immunoprecipitation

ChIPs were performed as previously described with the following changes. The amount of input chromatin added was increased to 200 μ l and the amount of Protein A agarose was doubled to 200 μ l. Following the final wash of the protein A agarose containing antibody and bound chromatin, the agarose was resuspended in 200 μ l of buffer and transferred to a clean low-bind Eppendorf centrifuge tube. The suspension was sonicated using a Branson Digital Sonifier for 6 minutes with the amplitude set at 50%. The sonicated samples were centrifuged at 2,000 rpms for 2 minutes to separate the chromatin that remained bound to the agarose from the fragmented chromatin in the supernatant and the supernatant chromatin saved. The chromatin that remained bound to

agarose following sonication was removed with lysis buffer according to the ChIP kit's instructions. The supernatant was then used as the starting material for a subsequent ChIP.

Preparation of DNA: Samples were prepared for PCR using an MP Bioscience (Solon, Ohio, USA) GeneClean Spin Kit (#111101-200) with the following modifications. The glassmilk reagent (100 μ l) was mixed with 50 μ l of sample in a 1.5-ml centrifuge tube. The tube was mixed by repeated inversion at 2 minutes and again at 4 minutes of incubation. Following 5 minutes of room temperature incubation, the samples were centrifuged at 6,000 rpm for 30 seconds in a Micro One (Tomy) to pellet the glass. The supernatant was discarded and 200 μ l of the wash buffer was added to the tube. As the wash buffer was being added, the pipette tip was used to break up the pellet by physically rubbing the pellet and vigorously pipetting up and down. The samples were inverted twice and centrifuged at 6,000 rpm for 30 seconds to again pellet the glass. The supernatant was discarded and the pellets were dried in a vacuum for 5 minutes. The glass pellet with bound DNA was resuspended in 25 μ l Tris EDTA (TE) buffer.

PCR Amplification

For most of the amplifications, DNA was amplified from the promoter region of the SV40 genome using the primers 5' TTG CAA AAG CCT CCA AA 3' and 5' TGA CCT ACG AAC CTT AAC CGA GGG 3' in a CFX Connect Real Time System thermal cycler (Bio-Rad, Ipswich, Massachusetts, USA) using 'SSO Advanced DNA polymerase

(Bio-Rad, Ipswich, Massachusetts, USA). In the immune selection fragmentation experiment, DNA was amplified from the early region using the primers 5'TGCTCCCATTCATCAGTTCC3' and 5'CTGACTTTGGAGGCTTCTGG3' because the promoter region is extremely sensitive to sonication. Immediately before use, the primers and DNase free water were added, and 28 µl of the mix was used per sample. 2 µl of the resuspended glass milk in TE buffer was added per sample. Samples were amplified by PCR in triplicate with a melt curve applied afterwards to ensure specific amplification. All sample preparation for PCR was done in either a Nuair biological safety cabinet Model NU_425-400 or an AirClean 600 PCR Workstation (ISC BioExpress).

Statement on the Use of Human Subjects

This research did not involve the use of any human subjects, materials, or data.

CHAPTER VIII

DISCUSSION AND CONCLUSIONS

Epigenetics Relating to Cancer and Disease States

One critical function of an epigenome is its ability to vary itself depending on current cellular needs. These needs can be short term, for example in the ability to respond to stressor events such as an elevated environmental temperature, or they can be long term, such as the ability to differentiate into different cell types in an organism. (83)(84) Many disease states and cancer types result in part from improper epigenetic regulation of these natural variations in an organism's epigenome. In order to better understand how these illnesses result in part from dysregulated epigenetic control more research is needed to understand the factors that go into properly regulated epigenetic control.

Viral Epigenetics as a Eukaryotic Epigenetic Model

Individual cells are understood to have a single epigenetic state in terminally differentiated cells that is passed on during replication. Inheritance of these epigenetic patterns is critical to properly maintain cell lineages and preserve cellular function.

Histone modifications have been identified as one of the ways chromatin maintains its epigenetic identity through the semi-conservative process of DNA replication. (39) One model is that the histones from the replicated chromatin are randomly deposited onto the newly formed chromatin strands. These conserved histones then promote the existing modifications onto newly incorporated histones. (65)

While cellular chromatin in terminally differentiated cells is understood to have a single epigenetic state that is passed on during replication, this body of research shows that viral chromatin in the SV40 model system exists as multiple epigenetic states which vary throughout the lifecycle of the virus. These states are in a controlled and dynamic flux through the lifecycle of the virus, thus having replication function as an epigenetic switch is not supported by the theory of histones maintaining the epigenome of the parental strand of chromatin. The incorporation of new epigenetic patterns following replication as seen when using SV40 as a model system may parallel epigenetic mechanisms in differentiating cell lineages and more research should be done in this area.

Mechanisms of SV40 Epigenetic Regulation

Our initial studies mapping the patterns of H3K4 and H3K9 led us to question whether the binding of T-antigen to site 1 results in an increase in the amount of H3K9me1 present on SV40 minichromosomes. When comparing WT SV40 to the two mutants CS1085 and SM, H3K9me1 was notably reduced at 48 hours in the CS1085 ($0.66 \pm 0.6\%$) mutant compared to wild type virus ($22 \pm 10\%$), whereas the SM mutant had increased incorporation of H3K9me1 ($39 \pm 9\%$) relative to wild type. This supports

the hypothesis that T-antigen binding results in the incorporation of H3K9me1, as CS1085's inability to bind T-antigen resulted in a near absence of H3K9me1 contrasted by SM's overproduction of T-antigen resulting in an increase of H3K9me1 at 48 hours.

As T-antigen is the agent responsible for the repression of early transcription we wanted to determine if the H3K9me1 patterns we saw at late times as a result of T-antigen binding to Site 1 were similar at early times during repression of transcription. If H3K9me1 was related to the repression of early transcription we would see these levels become enriched during the 30 minute to 8 hour post infection time period. However, we instead saw a reduction of H3K9me1 during this time period which correlated to the reduction of RNAPII bound to minichromosomes.

Since this was an unexpected result we wanted to confirm that T-antigen binding to Site 1 was responsible for the H3K9me1 levels we documented, both the reduction at 8 hours post infection and the enrichment at 48 hours post infection. A mutant with two Site 1's was used to determine if T-Antigen binding to Site 1 had the effects on H3K9me1 incorporation. Confirming what we observed in earlier experiments, two copies of Site 1 resulted in a reduction of H3K9me1 at 8 hours post infection yet resulted in an enrichment of H3K9me1 at 48 hours post infection.

Replication was inhibited using aphidicolin in order to examine if the changes we were seeing between 8 hours and 48 hours post infection in H3K9me1 levels were due to T-antigen binding. Inhibition of replication resulted in the reduction of levels of H3K4me1 and H3K9me1, indicating a dependence of replication for the incorporation of these epigenetic marks.

While investigating H3K9me1's relationship to Site 1 and replication, incorporation of H3K9me2 and H3K9me3 were shown to be independent of replication. In order to determine if inhibition of transcription was related to the incorporation of H3K9me2 and H3K9me3, 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole (DRB) was used to inhibit transcription at both the 2 hour time point and at the 48 hour time point. Inhibition of transcription by DRB had little effect on H3K4 methylation at any time point measured. H3K9me1 levels also experienced little change at the 2 hour time point measured. However, at the 48 hour time point we observed a 6 ± 4 fold increase for H3K9me2 with a range from 3 to 12 for four independent samples and a 6 ± 2 fold increase for H3K9me3 with a range of 3 to 8 for four independent samples.

To further characterize the differences we were seeing at 2 hours compared to 48 hours in the presence and absence of transcriptional inhibition by DRB in H3K9me2 and H3K9me3, replication was inhibited in conjunction with transcription. These results were significantly lower than previously seen with DRB inhibition alone, suggesting that replication plays a role in the introduction of H3K9me2 and H3K9me3 at late times in infection.

Finally, to determine if repression of transcription was causing the changes in H3K9me2 and H3K9me3 that we documented, transcription was stimulated to determine if increased transcription had the opposite effect. We observed ratios close to 1 for H3K9me1 (0.9 ± 0.1) and H3K9me3 (1.5 ± 0.8) in minichromosomes isolated at 12 hours post-infection. We saw very low levels of H3K9me2 in both treated and untreated minichromosomes, which were too variable to quantitate at this time. In contrast, for minichromosomes isolated at 48 hours post-infection, we observed significant inhibition

of H3K9 methylation. The ratios were 0.46 ± 0.13 for H3K9me1, 0.08 ± 0.04 for H3K9me2, and 0.39 ± 0.2 for H3K9me3. This is consistent with the idea that these modifications were being introduced as a consequence of a general repression of transcription at late times.

The results presented here are inconsistent with the model of chromosomal replication where preexisting histone modifications contained on that parental chromosome are duplicated on the newly replicated daughter chromosome. (64)(65) These results point to a model in which replication acts as an epigenetic switch resulting in newly replicated chromatin responding to the binding of T-antigen to Site 1 and incorporating a specific epigenetic pattern. In SV40 evidence suggests newly replicated chromosomes are not directed to specific fates as a result of the parental chromosome due to the differences in H3K9me1 levels in CS1085 from 30 minutes to 8 hours compared to wild-type infections. Also, the recombinant with two copies of Site 1 present is inconsistent with the model of preexisting histone modifications driving duplication in daughter chromosomes following replication. The model supporting an epigenetic switch would occur through losing current histone modifications at the replication fork and new histones are modified following replication by histone modifying complexes associated with the replication fork. (69) This model allows for signals, such as T-antigen, to modulate post-replicative modifications on histones.

One biological benefit for possessing an epigenetic switch as our model suggests would be to control newly replicated chromosomes and direct their biological fate. For example, at late times in infection the virus would benefit from not producing chromosomes which would undergo early transcription, but rather would benefit from

chromosomes primed for encapsidation. An example of this can be seen in the CS1085 mutant which has over-expression of early transcription and thus a greatly reduced yield of genetic material at 48 hours post-infection. (37) It is likely that incorporation of H3K9me1 methylation, which is known to be repressive for transcription in SV40, helps prevent newly replicated chromosomes generated late in infection from undergoing active early transcription.

SV40 as a Model for a Proofreading Mechanism to Silence Unproductive Chromosomes

This is a proposed model to support this data. After DNA is replicated a portion of the replicated chromosomes initiate transcription. A portion of these newly replicated and transcribing chromosomes have their RNAPII halted. These halted chromosomes are detected and marked with H3K9me2 and H3K9me3 as part of a currently unidentified proof reading mechanism that can detect chromosomes which are unable to function in a productive manner. Treatment with DRB supports this model as it results in a large portion of chromosomes with halted RNAPII which become detected by this proof reading mechanism which silence the chromosomes preventing further transcription by the addition of H3K9me2 and H3K9me3.

Epigenetic Progression of SV40 Lifecycle

This research shows the existence of distinct epigenetic patterns on incoming virions, chromatin at early time points in infection, and chromatin at 48 hours post infection. One finding that makes the research presented here unique is evidence for the existence of at least 5 different SV40 epigenomes present at 48 hour point infection. One consisted primarily of H3K4me2 in conjunction with H3K9me1, H3K9me2, H3K9me3, and hyperacetylated H4 (HH4). A second form also consisted primarily of H3K4me2 but in conjunction with HH3 and HH4. This conclusion was based upon the fact that H3K9 cannot be methylated and acetylated at the same time, which we experimentally confirmed. A third epigenome consisted almost solely of H3K4me3 with some HH4 also present. The fourth epigenome consisted of H3K9me1 along with H3K9me2, H4K20me1 and HH4. The fifth epigenome consisted primarily of H3K9me3 with trace amounts of other modifications.

As discussed in chapter 5, while this is the first demonstration that multiple distinct viral epigenomes defined by specific combinations of histone modifications are present in infected cells, the co-localized modified histones are in agreement with previously reported results for cellular chromatin. For example, H3K9me1 has been previously shown to be co-localized within nucleosomes of transcribing regions with H3K4me1/2/3, H3K79me1/2/3 and H4K20me1 using a genome-wide strategy. H3K9me3 was found associated with H3K9me2 and H3K27me2/3 in silenced regions.

Mnichromosomes have different functions throughout the course of the viral lifecycle. Incoming virion particles need to be transported into the nucleus to initiate

early transcription. Once the early viral proteins have repurposed the cellular machinery, T-antigen binds to Site 1 and causes both repression of early transcription and progression in epigenetic patterns. At late times in infection the virus shifts to actively replicating chromosomes to be encapsulated and the transcribing late mRNA coding for viral proteins needed to encapsulate the chromosomes.

The existence and identification of 5 major epigenomes present at 48 hours post infection in SV40 correlates with the viral need of distinct simultaneous functions from the same genome. Presumably, at 48 hours these 5 epigenomes may each serve or have served distinct functions. For example, one could be actively replicating new chromosomes, another could be transcribing late mRNA to produce viral capsid proteins needed late in infection, while a third subset could be incorporated into newly created virions. The remaining epigenomes could have resulted from chromatin earlier in infection that are no longer needed and are now in a silenced state. Whether these epigenomes correspond to actual biological functions and to which function each epigenome would correspond to have not yet been researched.

SV40 as a Model to Transmit Epigenetic Information Trans-Generationally

In a typical SV40 infection the virions used to initiate an infection possess identical epigenetic patterns as virions that result from the infection, leading to the question of whether virions can contain and transmit epigenetic information. Two mutants, CS1085 and SM, were used to support the hypothesis that the virions could contain epigenetic information. If this was true, virions resulting from an infection would

relate to the patterns present in the minichromosomes. Both of these examined mutants contained distinct epigenetic patterns at both 48 hours post infection and in virion particles, showing that SV40 virions can contain epigenetic information and can preserve the epigenetic information in virion particles from infection to infection from a given set of environmental conditions.

SV40 as a Model for Host Fever Defense

The ability of SV40 to pass on epigenetic information in virion particles is further displayed when the cellular conditions in which the virus produces progeny virions have an elevated temperature. This is consistent with previous knowledge that fever can cause a heat response resulting in the change of expression and/or function of many proteins. (85) The virions resulting from these infections contain different epigenetic information and have reduced capacity to establish the next round of infection. This body of research displayed this by characterizing an epi-mutant which was created from culturing SV40 infected cells at 39°C instead of 37°C when creating virion particles. These epi-mutant virions with distinct epigenetic patterns could represent one of the host's innate immune defense mechanisms by epigenetically attenuating progeny virions through a fever response, though more research needs to be done in this area.

SV40 as a Model for the Human Papillomavirus Family

The human papillomaviruses (HPVs) are thought to be the agent responsible for many types of cervical, anogenital, head and neck cancers. This family of viruses has lifecycles very similar to SV40 with one important difference; the lifecycle of HPV is dependent on the differentiation status on the infected cells, making HPV a difficult candidate for use as a model system. Due to this limitation inadequate research into epigenetic factors relating to HPV's lytic lifecycle has been completed. (30) It is this author's opinion that research on SV40 may also correlate to HPV discoveries.

- (1) Igo-Kemenes T, Horz W, Zachau HG. Chromatin. *Annu Rev Biochem* 1982;51:89-121.
- (2) Kireeva ML, Walter W, Tchernajenko V, Bondarenko V, Kashlev M, Studitsky VM. Nucleosome remodeling induced by RNA polymerase II: loss of the H2A/H2B dimer during transcription. *Mol Cell* 2002 Mar;9(3):541-552.
- (3) Kadam S, Emerson BM. Mechanisms of chromatin assembly and transcription. *Curr Opin Cell Biol* 2002 Jun;14(3):262-268.
- (4) Kornberg RD, Lorch Y. Chromatin structure and transcription. *Annu Rev Cell Biol* 1992;8:563-587.
- (5) Tan M, Luo H, Lee S, Jin F, Yang JS, Montellier E, et al. Identification of 67 histone marks and histone lysine crotonylation as a new type of histone modification. *Cell* 2011 Sep 16;146(6):1016-1028.
- (6) Sadakierska-Chudy A, Filip M. A comprehensive view of the epigenetic landscape. Part II: Histone post-translational modification, nucleosome level, and chromatin regulation by ncRNAs. *Neurotox Res* 2015 Feb;27(2):172-197.
- (7) Cohen I, Poreba E, Kamieniarz K, Schneider R. Histone modifiers in cancer: friends or foes? *Genes Cancer* 2011 Jun;2(6):631-647.
- (8) Volkel P, Angrand PO. The control of histone lysine methylation in epigenetic regulation. *Biochimie* 2007 Jan;89(1):1-20.
- (9) Bannister AJ, Kouzarides T. Regulation of chromatin by histone modifications. *Cell Res* 2011 Mar;21(3):381-395.
- (10) Rothbart SB, Strahl BD. Interpreting the language of histone and DNA modifications. *Biochim Biophys Acta* 2014 Aug;1839(8):627-643.
- (11) Kustatscher G, Ladurner AG. Modular paths to 'decoding' and 'wiping' histone lysine methylation. *Curr Opin Chem Biol* 2007 Dec;11(6):628-635.
- (12) Peserico A, Simone C. Physical and functional HAT/HDAC interplay regulates protein acetylation balance. *J Biomed Biotechnol* 2011;2011:371832.
- (13) Balakrishnan L, Milavetz B. Histone hyperacetylation during SV40 transcription is regulated by p300 and RNA polymerase II translocation. *J Mol Biol* 2007 Aug 24;371(4):1022-1037.
- (14) Sarnowska E, Gratkowska DM, Sacharowski SP, Cwiek P, Tohge T, Fernie AR, et al. The Role of SWI/SNF Chromatin Remodeling Complexes in Hormone Crosstalk. *Trends Plant Sci* 2016 Feb 23.

- (15) Atkin SJ, Griffin BE, Dilworth SM. Polyoma virus and simian virus 40 as cancer models: history and perspectives. *Semin Cancer Biol* 2009 Aug;19(4):211-217.
- (16) Minor P, Pipkin P, Jarzebek Z, Knowles W. Studies of neutralising antibodies to SV40 in human sera. *J Med Virol* 2003 Jul;70(3):490-495.
- (17) Ben-nun-Shaul O, Bronfeld H, Reshef D, Schueler-Furman O, Oppenheim A. The SV40 capsid is stabilized by a conserved pentapeptide hinge of the major capsid protein VP1. *J Mol Biol* 2009 Mar 13;386(5):1382-1391.
- (18) Cremisi C, Pignatti PF, Croissant O, Yaniv M. Chromatin-like structures in polyoma virus and simian virus 10 lytic cycle. *J Virol* 1975 Jan;17(1):204-211.
- (19) La Bella F, Vesco C. Late modifications of simian virus 40 chromatin during the lytic cycle occur in an immature form of virion. *J Virol* 1980 Mar;33(3):1138-1150.
- (20) Luo Y, Motamedi N, Magaldi TG, Gee GV, Atwood WJ, DiMaio D. Interaction between Simian Virus 40 Major Capsid Protein VP1 and Cell Surface Ganglioside GM1 Triggers Vacuole Formation. *MBio* 2016 Mar 22;7(2):10.1128/mBio.00297-16.
- (21) Szklarczyk OM, Gonzalez-Segredo N, Kukura P, Oppenheim A, Choquet D, Sandoghdar V, et al. Receptor concentration and diffusivity control multivalent binding of Sv40 to membrane bilayers. *PLoS Comput Biol* 2013;9(11):e1003310.
- (22) Kuksin D, Norkin LC. Disassociation of the SV40 genome from capsid proteins prior to nuclear entry. *Virol J* 2012 Aug 10;9:158-422X-9-158.
- (23) Fontes MR, Teh T, Toth G, John A, Pavo I, Jans DA, et al. Role of flanking sequences and phosphorylation in the recognition of the simian-virus-40 large T-antigen nuclear localization sequences by importin-alpha. *Biochem J* 2003 Oct 15;375(Pt 2):339-349.
- (24) Knipe D. *Fields Virology* . ; 2013.
- (25) Manos MM, Gluzman Y. Simian virus 40 large T-antigen point mutants that are defective in viral DNA replication but competent in oncogenic transformation. *Mol Cell Biol* 1984 Jun;4(6):1125-1133.
- (26) Ali SH, DeCaprio JA. Cellular transformation by SV40 large T antigen: interaction with host proteins. *Semin Cancer Biol* 2001 Feb;11(1):15-23.
- (27) Wychowski C, Benichou D, Girard M. The intranuclear location of simian virus 40 polypeptides VP2 and VP3 depends on a specific amino acid sequence. *J Virol* 1987 Dec;61(12):3862-3869.

- (28) Lai CJ, Nathans D. Non-specific termination of simian virus 40 DNA replication. *J Mol Biol* 1975 Sep 5;97(1):113-118.
- (29) Myers RM, Rio DC, Robbins AK, Tjian R. SV40 gene expression is modulated by the cooperative binding of T antigen to DNA. *Cell* 1981 Aug;25(2):373-384.
- (30) Milavetz BI, Balakrishnan L. Viral epigenetics. *Methods Mol Biol* 2015;1238:569-596.
- (31) Rice JC, Briggs SD, Ueberheide B, Barber CM, Shabanowitz J, Hunt DF, et al. Histone methyltransferases direct different degrees of methylation to define distinct chromatin domains. *Mol Cell* 2003 Dec;12(6):1591-1598.
- (32) Ogawa H, Ishiguro K, Gaubatz S, Livingston DM, Nakatani Y. A complex with chromatin modifiers that occupies E2F- and Myc-responsive genes in G0 cells. *Science* 2002 May 10;296(5570):1132-1136.
- (33) Tachibana M, Sugimoto K, Nozaki M, Ueda J, Ohta T, Ohki M, et al. G9a histone methyltransferase plays a dominant role in euchromatic histone H3 lysine 9 methylation and is essential for early embryogenesis. *Genes Dev* 2002 Jul 15;16(14):1779-1791.
- (34) DiMaio D, Nathans D. Cold-sensitive regulatory mutants of simian virus 40. *J Mol Biol* 1980 Jun 15;140(1):129-142.
- (35) DiMaio D, Nathans D. Regulatory mutants of simian virus 40. Effect of mutations at a T antigen binding site on DNA replication and expression of viral genes. *J Mol Biol* 1982 Apr 15;156(3):531-548.
- (36) Sullivan CS, Grundhoff AT, Tevethia S, Pipas JM, Ganem D. SV40-encoded microRNAs regulate viral gene expression and reduce susceptibility to cytotoxic T cells. *Nature* 2005 Jun 2;435(7042):682-686.
- (37) Milavetz B, Kallestad L, Gefroh A, Adams N, Woods E, Balakrishnan L. Virion-mediated transfer of SV40 epigenetic information. *Epigenetics* 2012 Jun 1;7(6):528-534.
- (38) Calo E, Wysocka J. Modification of enhancer chromatin: what, how, and why? *Mol Cell* 2013 Mar 7;49(5):825-837.
- (39) Balakrishnan L, Milavetz B. Dual agarose magnetic (DAM) ChIP. *BMC Res Notes* 2009 Dec 14;2:250-0500-2-250.
- (40) Loyola A, Tagami H, Bonaldi T, Roche D, Quivy JP, Imhof A, et al. The HP1alpha-CAF1-SetDB1-containing complex provides H3K9me1 for Suv39-mediated K9me3 in pericentric heterochromatin. *EMBO Rep* 2009 Jul;10(7):769-775.

- (41) Atkinson SP, Koch CM, Clelland GK, Willcox S, Fowler JC, Stewart R, et al. Epigenetic marking prepares the human HOXA cluster for activation during differentiation of pluripotent cells. *Stem Cells* 2008 May;26(5):1174-1185.
- (42) Corry GN, Tanasijevic B, Barry ER, Krueger W, Rasmussen TP. Epigenetic regulatory mechanisms during preimplantation development. *Birth Defects Res C Embryo Today* 2009 Dec;87(4):297-313.
- (43) Lister R, Pelizzola M, Kida YS, Hawkins RD, Nery JR, Hon G, et al. Hotspots of aberrant epigenomic reprogramming in human induced pluripotent stem cells. *Nature* 2011 Mar 3;471(7336):68-73.
- (44) Shafa M, Krawetz R, Rancourt DE. Returning to the stem state: epigenetics of recapitulating pre-differentiation chromatin structure. *Bioessays* 2010 Sep;32(9):791-799.
- (45) Skinner MK. Environmental epigenetic transgenerational inheritance and somatic epigenetic mitotic stability. *Epigenetics* 2011 Jul;6(7):838-842.
- (46) Bonasio R, Tu S, Reinberg D. Molecular signals of epigenetic states. *Science* 2010 Oct 29;330(6004):612-616.
- (47) Gibney ER, Nolan CM. Epigenetics and gene expression. *Heredity (Edinb)* 2010 Jul;105(1):4-13.
- (48) Herceg Z. Epigenetic information in chromatin and cancer. *Eur J Cancer* 2009 Sep;45 Suppl 1:442-444.
- (49) Kubicek S, Schotta G, Lachner M, Sengupta R, Kohlmaier A, Perez-Burgos L, et al. The role of histone modifications in epigenetic transitions during normal and perturbed development. *Ernst Schering Res Found Workshop* 2006;(57)(57):1-27.
- (50) Sawan C, Herceg Z. Histone modifications and cancer. *Adv Genet* 2010;70:57-85.
- (51) Uribe-Lewis S, Woodfine K, Stojic L, Murrell A. Molecular mechanisms of genomic imprinting and clinical implications for cancer. *Expert Rev Mol Med* 2011 Jan 25;13:e2.
- (52) Balakrishnan L, Milavetz B. Reorganization of RNA polymerase II on the SV40 genome occurs coordinately with the early to late transcriptional switch. *Virology* 2006 Feb 5;345(1):31-43.
- (53) Balakrishnan L, Milavetz B. Histone hyperacetylation in the coding region of chromatin undergoing transcription in SV40 minichromosomes is a dynamic process regulated directly by the presence of RNA polymerase II. *J Mol Biol* 2007 Jan 5;365(1):18-30.

- (54) Balakrishnan L, Gefroh A, Milavetz B. Histone H4 lysine 20 mono- and trimethylation define distinct biological processes in SV40 minichromosomes. *Cell Cycle* 2010 Apr 1;9(7):1320-1332.
- (55) Jenuwein T, Allis CD. Translating the histone code. *Science* 2001 Aug 10;293(5532):1074-1080.
- (56) Wang Z, Zang C, Rosenfeld JA, Schones DE, Barski A, Cuddapah S, et al. Combinatorial patterns of histone acetylations and methylations in the human genome. *Nat Genet* 2008 Jul;40(7):897-903.
- (57) Kube D, Milavetz B. Generation of a nucleosome-free promoter region in SV40 does not require T-antigen binding to site I. *Virology* 1989 Sep;172(1):100-105.
- (58) Kouzarides T. Chromatin modifications and their function. *Cell* 2007 Feb 23;128(4):693-705.
- (59) Hirt B. Selective extraction of polyoma DNA from infected mouse cell cultures. *J Mol Biol* 1967 Jun 14;26(2):365-369.
- (60) Cohen S, Au S, Pante N. How viruses access the nucleus. *Biochim Biophys Acta* 2011 Sep;1813(9):1634-1645.
- (61) Greber UF, Kasamatsu H. Nuclear targeting of SV40 and adenovirus. *Trends Cell Biol* 1996 May;6(5):189-195.
- (62) Tsubota Y, Waqar MA, Burke JF, Milavetz BI, Evans MJ, Kowalski D, et al. Association of enzymes with replicating and nonreplicating simian virus 40 chromosomes. *Cold Spring Harb Symp Quant Biol* 1979;43 Pt 2:693-704.
- (63) Abmayr SM, Workman JL. Holding on through DNA replication: histone modification or modifier? *Cell* 2012 Aug 31;150(5):875-877.
- (64) Corpet A, Almouzni G. Making copies of chromatin: the challenge of nucleosomal organization and epigenetic information. *Trends Cell Biol* 2009 Jan;19(1):29-41.
- (65) Zhu B, Reinberg D. Epigenetic inheritance: uncontested? *Cell Res* 2011 Mar;21(3):435-441.
- (66) Acheson N. *Lytic Cycle of SV40 and Polyoma Virus*. Cold Spring Harbor NY: Cold Spring Harbor Laboratory 1981.
- (67) Hermansen R, Sierra MA, Johnson J, Friez M, Milavetz B. Identification of Simian virus 40 promoter DNA sequences capable of conferring restriction endonuclease hypersensitivity. *J Virol* 1996 Jun;70(6):3416-3422.

- (68) Ohashi M, Taguchi T, Ikegami S. Aphidicolin: a specific inhibitor of DNA polymerases in the cytosol of rat liver. *Biochem Biophys Res Commun* 1978 Jun 29;82(4):1084-1090.
- (69) Petruk S, Sedkov Y, Johnston DM, Hodgson JW, Black KL, Kovermann SK, et al. TrxG and PcG proteins but not methylated histones remain associated with DNA through replication. *Cell* 2012 Aug 31;150(5):922-933.
- (70) Shahbazian MD, Grunstein M. Functions of site-specific histone acetylation and deacetylation. *Annu Rev Biochem* 2007;76:75-100.
- (71) Kimura H. Histone modifications for human epigenome analysis. *J Hum Genet* 2013 Jul;58(7):439-445.
- (72) Krishnan S, Horowitz S, Trievel RC. Structure and function of histone H3 lysine 9 methyltransferases and demethylases. *Chembiochem* 2011 Jan 24;12(2):254-263.
- (73) Balakrishnan L, Milavetz B. Programmed remodeling of hyperacetylated histone H4 and H3 organization on the SV40 genome during lytic infection. *Virology* 2005 Mar 30;334(1):111-123.
- (74) Kallestad L, Woods E, Christensen K, Gefroh A, Balakrishnan L, Milavetz B. Transcription and replication result in distinct epigenetic marks following repression of early gene expression. *Front Genet* 2013 Jul 30;4:140.
- (75) Hensold JO, Barth D, Stratton CA. RNA polymerase II inhibitor, 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole (DRB) causes erythroleukemic differentiation and transcriptional activation of erythroid genes. *J Cell Physiol* 1996 Jul;168(1):105-113.
- (76) Boffa LC, Vidali G, Mann RS, Allfrey VG. Suppression of histone deacetylation in vivo and in vitro by sodium butyrate. *J Biol Chem* 1978 May 25;253(10):3364-3366.
- (77) Ayer DE. Histone deacetylases: transcriptional repression with SINers and NuRDs. *Trends Cell Biol* 1999 May;9(5):193-198.
- (78) Balakrishnan L, Milavetz B. HDAC inhibitors stimulate viral transcription by multiple mechanisms. *Virology* 2008 Mar 19;375(1):43-49.
- (79) Hublitz P, Albert M, Peters AH. Mechanisms of transcriptional repression by histone lysine methylation. *Int J Dev Biol* 2009;53(2-3):335-354.
- (80) Shinkai Y, Tachibana M. H3K9 methyltransferase G9a and the related molecule GLP. *Genes Dev* 2011 Apr 15;25(8):781-788.

(81) Yokochi T, Poduch K, Ryba T, Lu J, Hiratani I, Tachibana M, et al. G9a selectively represses a class of late-replicating genes at the nuclear periphery. *Proc Natl Acad Sci U S A* 2009 Nov 17;106(46):19363-19368.

(82) Sarraf SA, Stancheva I. Methyl-CpG binding protein MBD1 couples histone H3 methylation at lysine 9 by SETDB1 to DNA replication and chromatin assembly. *Mol Cell* 2004 Aug 27;15(4):595-605.

(83) Jaenisch R, Bird A. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nat Genet* 2003 Mar;33 Suppl:245-254.

(84) Herceg Z, Lambert MP, van Veldhoven K, Demetriou C, Vineis P, Smith MT, et al. Towards incorporating epigenetic mechanisms into carcinogen identification and evaluation. *Carcinogenesis* 2013 Sep;34(9):1955-1967.

(85) Hasday JD, Singh IS. Fever and the heat shock response: distinct, partially overlapping processes. *Cell Stress Chaperones* 2000 Nov;5(5):471-480.