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EPIGENETIC PATTERNS: PROCESS-DRIVEN OR SEQUENCE-DRIVEN?

by

Amanda Marie Gefroh
Bachelor of Science, University of North Dakota, 2010

A Thesis

Submitted to the Graduate Faculty

of the

University of North Dakota

In partial fulfillment of the requirements

for the degree of

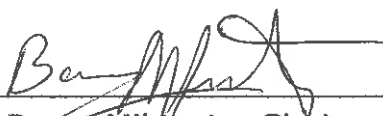
Master of Science

Grand Forks, North Dakota

August

2013

This thesis, submitted by Amanda Marie Gefroh in partial fulfillment of the requirements for the Degree of Master of Science 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.



Dr. Barry Milavetz, Chairperson of Committee




Dr. Katherine Sukalski, Committee Member



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This thesis is being submitted by the appointed advisory committee as having met all of the requirements of the Graduate School at the University of North Dakota and is hereby approved.



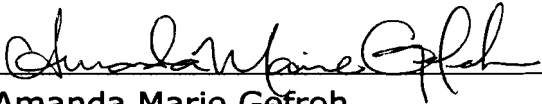
Dr. Wayne Swisher
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Title Epigenetic Patterns: process-driven or sequence-driven?
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Amanda Marie Gefroh

7.25.13
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It has been said that “knowing yourself is the beginning of all wisdom” (Aristotle). I feel as though I have been a testament to that in these last few years, and I never would have had the opportunity to explore exactly what that means in the way that I have without Dr. B’s patience and support.

Beginning as an undergraduate, curious about ‘real science’, I managed to be set up with an opening in the biochemistry lab of Dr. Barry Milavetz. Knowing the impact of genetic and epigenetic research, I felt privileged to be involved – even in a small way. After graduating, my ambitions of attending medical school were put on hold and Dr. B approached me to stay on in the lab and apply for the graduate program. Although the past three years have been some of the most difficult and exhausting, through them I have come to learn things I am convinced I would not have otherwise learned.

Not only did Dr. B and the Department of Biochemistry and Molecular Biology give me the chance to further my study of the small molecules of life and financially support me to conduct the experiments that were a part of my thesis project presented here, but

also give me the gift of the opportunity to pursue a Masters of Science. What an honor that has been to receive, and for it I give my utmost gratitude to Dr. Barry Milavetz and my department.

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I truly feel that I have grown to know myself better immensely through the challenges of graduate school. I am certain that what I have learned here – in the classroom, in the lab, and outside as well – will greatly contribute to the quality life and career decisions I make going forward.

Lastly, I would most humbly like to give a huge amount of gratitude to the close friends who have been so giving, so understanding, and so incredibly supportive. I would not have seen this through without them.

For those whom I consider a part of my Family

ABSTRACT

Some of the most rapidly-evolving fields of study in the medical and research communities presently are those which investigate our genetic code, the elements and factors that control the expression of our genetic code, as well as fields of study which analyze theories about an organisms' physiology and phenotype from the information that both of the previous topics concomitantly provide. The work of the Milavetz lab, among many others, is striving to fill the gaps in knowledge about epigenetic regulation of biological processes – here by using the well-suited model virus, Simian Virus 40.

It has for some time now been agreed upon that epigenetic modifications of histone proteins not only exists, but play a crucial role in biological processes related to the availability or condensation of genetic material. Certain modifications have continuously been shown to either silence or expose genes^{1,2}. It has also been shown that these modifications are not static but in a dynamic state³. However, the details of how, when, and why changes in the epigenetic modifications on the histone tails occurs is still being investigated.

This project is intended to explore the effect of novel sequence inserts into the SV40 genome related to the enhancer region, Sp1 binding site, and late promoter. Specific variants of SV40 were created to contain either one or two copies of the enhancer region (T-129 and T-143 respectively), two copies in succession of the Sp1 site (RH-43), or a full late promoter region (T-165). Chromatin immunoprecipitation (ChIP) and real-time quantitative polymerase chain reaction (q-PCR) of the histone modifications H3K4me1/2/3, H3K9me1/2/3, H4K20me1 and RNAPII were analyzed.

We found that certain modifications were affected by each sequence addition – some by a notable increase and some by a notable decrease. While more data is needed to more wholly characterize this phenomenon, these are some of the first results indicating an association between the DNA sequence present and the regulation of epigenetic histone modifications.

CHAPTER I

INTRODUCTION

Deoxyribonucleic acid, or DNA, has sometimes been referred to as the molecule of life. While many small molecules are essential in the biological interworkings which support life, DNA is considered especially important because it is the molecule that holds all of the information necessary for the making of the proteins that carry out the moment-to-moment interactions that define life, growth, development, and even death. Since the discovery of DNA in 1869 by Friedrich Miescher⁴, many great findings since then have led to a now widely accepted understanding of the structure of DNA itself and the process by which this genetic material is used to direct the formation of functional molecules. The Central Dogma of Molecular Biology^{1,5} shows that the information encoded into strands of DNA are read by a collection of cooperating proteins to produce ribonucleic acid (RNA) in a process known as transcription. This RNA is ultimately read by another conglomeration of molecules to produce proteins by the process of translation (Figure 1).

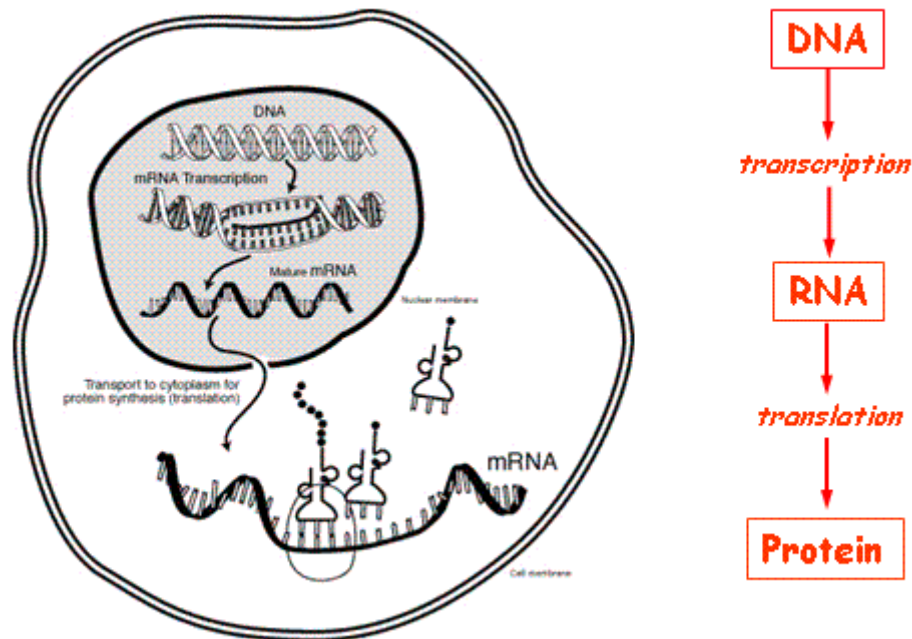


Figure 1. Central Dogma of Molecular Biology⁵.

Composition of DNA to form the Genome

DNA is composed of two long chains of nucleotides. The nucleotides of one chain, or strand, of DNA are held together by covalent bonds of the sugar-phosphate backbone to which the nucleotides are attached. There are four types of nucleotides which link, in a complementary and antiparallel fashion, to nucleotides of the second DNA strand by hydrogen bonds. Figure 2 portrays how the building blocks of DNA come together. The connections of the two strands of DNA form a double-helical structure. This particular

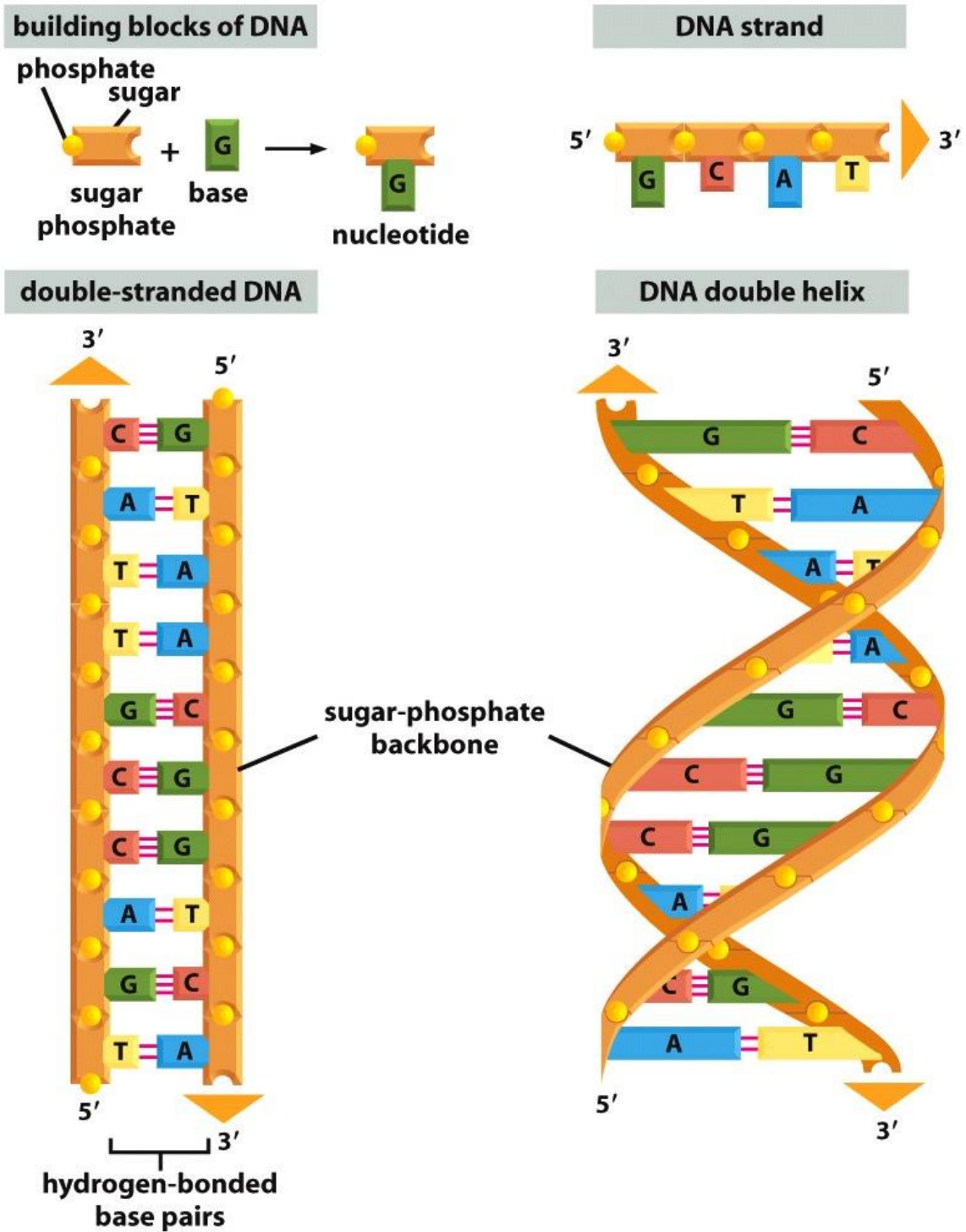


Figure 2. The building blocks and structure of DNA¹. Copyright 2008 from *Molecular Biology of the Cell*, Fifth Edition by Alberts et al. Reproduced by permission of Garland Science/Taylor & Francis LLC.

structure has characteristics that make it naturally advantageous for both protecting the integrity of the nucleotide sequence from damage while also presenting available binding sites for molecules essential to biological processes such as transcription or replication⁶.

The entirety of the DNA sequence and thus all of the genetic material is collectively referred to as an organisms' genome. This includes both protein-coding and non-coding sequences of DNA. The segments of the sequence of DNA which code for functional proteins are called genes. Although the human genome is approximated to contain between 20,000 and 25,000 genes, they make up a surprisingly small percentage of the total sequence – about 20% - while the actual sequences that code directly for proteins is only around 2-3%^{1,6}.

The enormity of the amount of genetic material present in a cell's nucleus drives its need for orderly compaction. In humans, our genetic material is divided into a set of 23 chromosomes. Figure 3 illustrates how genes are arranged within a particular chromosome. The figure also demonstrates how small a percentage of the genetic sequence is genes, and even smaller percentage is sequences that directly code for proteins. How then does a cell organize 3200 km of double-stranded DNA¹ into a chromosome that is less than 10 μm ? The answer is the nucleosome.

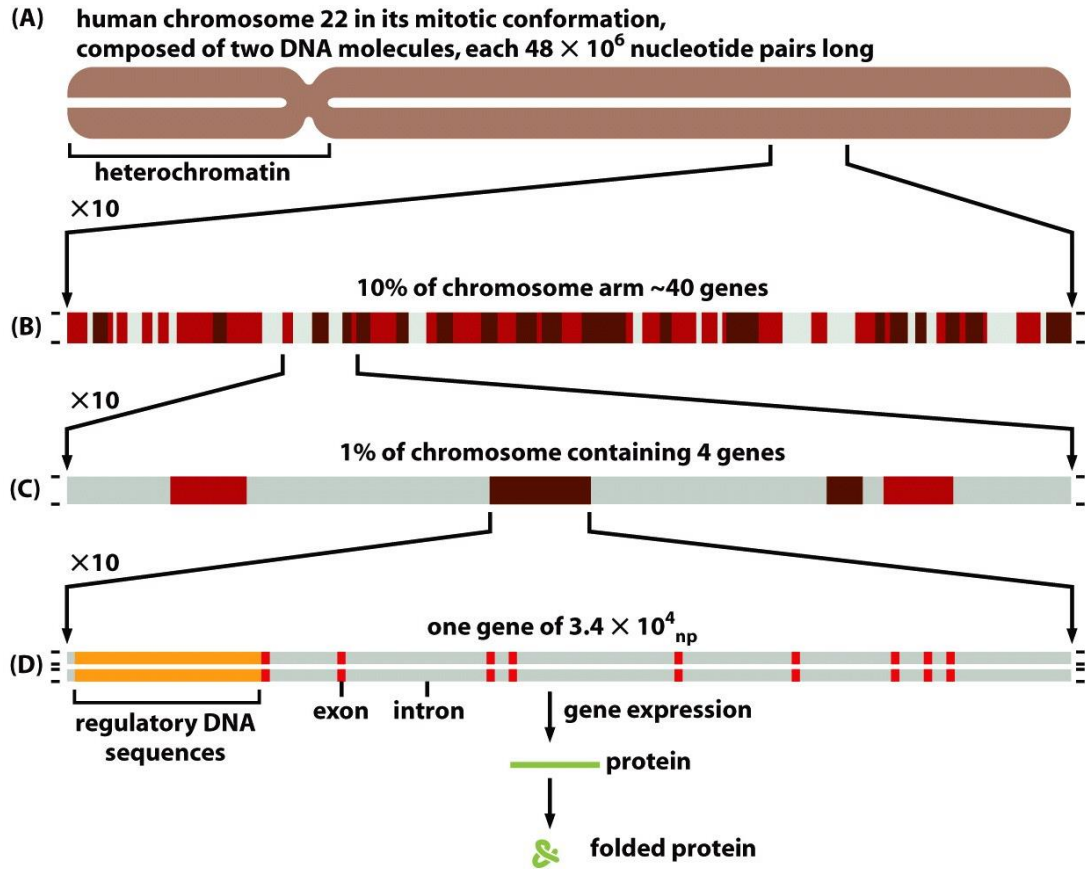


Figure 3. The arrangement of genes within a human chromosome¹.

Structure and Function of Nucleosomes

There are a variety of proteins that assist in the compaction of DNA into its higher order structures which are either histones or nonhistone chromosomal proteins. The term chromatin is used to describe the entire assembly of both the DNA and these two classes of proteins. DNA interacts with an arrangement of histone proteins to form the first level of compaction, the nucleosome (Figure 4).

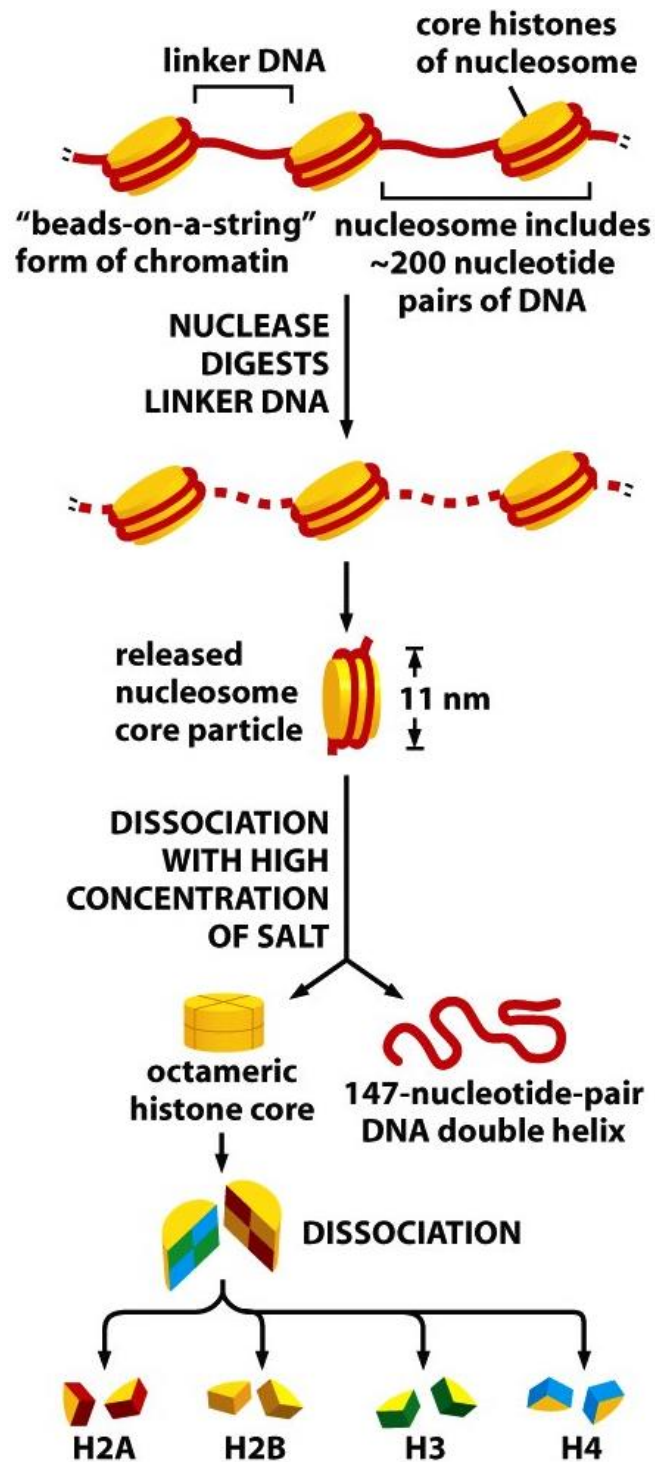


Figure 4. The structure of histones in the nucleosome¹. Copyright 2008 from *Molecular Biology of the Cell*, Fifth Edition by Alberts et al. Reproduced by permission of Garland Science/Taylor & Francis LLC.

The characteristics of the different types of histone proteins in the nucleosome show how they interact with one another, how they condense DNA, and as we will see later the role they play in determining gene expression.

Eight subunits of 4 types of histones – two from each category of histone H2A, H2B, H3, and H4 – converge to form the disc-shaped protein core of the nucleosome. One-hundred and forty-seven base pairs of nucleotides wrap around this histone core almost twice and is held there by numerous, reversible interactions between the phosphodiester backbone of the DNA and the amino acid backbone of the histones. Also, the high number of basic side chains like lysine and arginine within the histones readily interact with the negatively charged DNA¹. A fifth type of histone, H1, is a linker histone which binds to both the DNA as it comes off the nucleosome and the histone core itself, assisting in the directionality of the DNA to bind with the next core of histone proteins. Nucleosomes interdigitate further to compact DNA into the 30 nm chromatin fibers of chromosomes.

As mentioned previously, the interactions between DNA and histone proteins is reversible and in fact highly dynamic^{1,7}. The cell has just as much need for the DNA to be uncondensed and exposed when replicating the genetic material or transcribing genes for protein production as it does for compaction to protect the integrity of the

genetic sequence and repress gene expression. Maintenance and control of the balance of appropriate response to the needs of the cell occurs mostly by means of epigenetic regulation^{1,6,7}.

Epigenetic Modifications Regulate Processing of Genetic Material

Epigenetics is a relatively new perception in the history of biological concepts, however the inquiries it lends an understanding to have long perplexed scientists. With the extraordinary amount of genetic information and the remarkable expanses of similarity between and even among species, how is it that life takes on such varied form and function?

The notion of epigenetics began forming over a century ago⁸ with the idea that factors outside the genetic sequence alone could have an impact on gene expression. The word epigenetics itself relates to its mechanism in that the Greek prefix 'epi' means 'upon' or 'over'⁹, here relating to its function upon or over the genetic sequence versus within^{1,8}. It is now the understanding of the scientific community that epigenetic modifications play a crucial role in most biological processes by regulating gene expression^{8,10}.

The two known types of epigenetic modifications occur on the DNA itself – namely DNA methylation of cytosine residues – and on the N-terminal tails of the histone proteins of the nucleosome where a

multitude of modifications can occur at a variety of sites (Figure 5). A 2007 review in *Cell* classified eight types of histone modifications and characterized their biological effect¹¹.

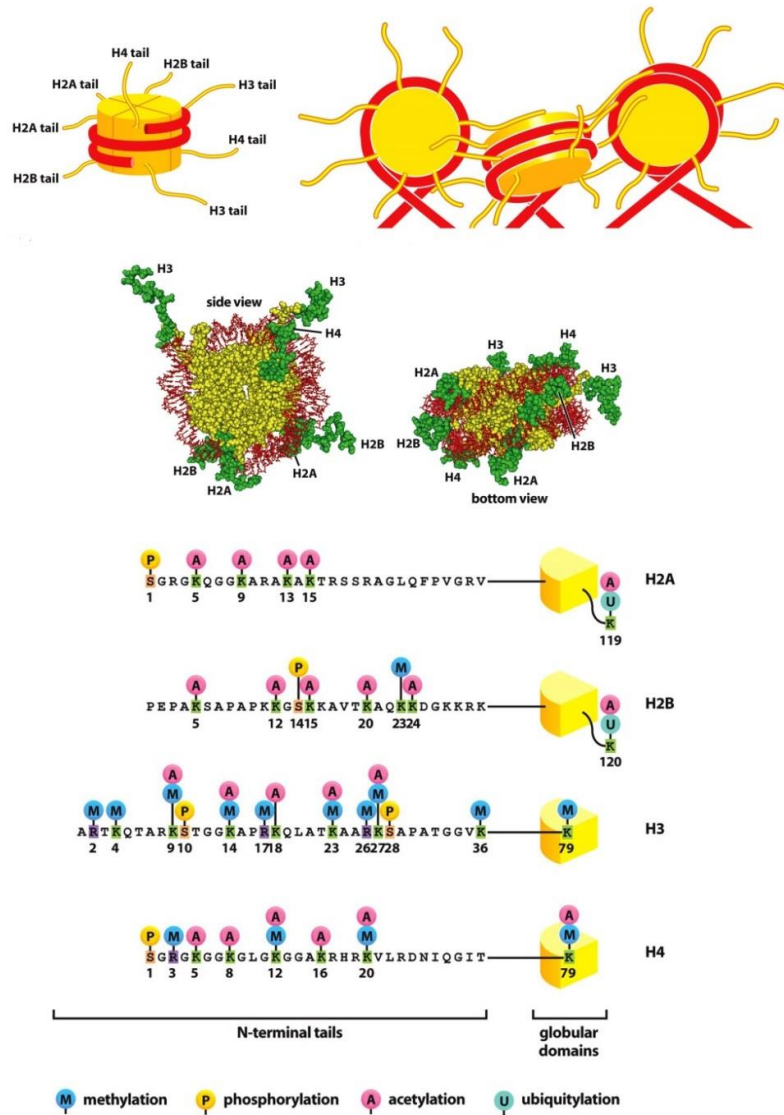


Figure 5. Histone tails and their epigenetic modifications¹. Copyright 2008 from *Molecular Biology of the Cell*, Fifth Edition by Alberts et al. Reproduced by permission of Garland Science/Taylor & Francis LLC.

Acetylation, methylation, phosphorylation, ubiquitylation, sumoylation, ADP ribosylation, deamination, and proline isomerization have been identified on specific residues. Table 1 list these modifications along with the particular residue it modifies and the current understanding of the biological process to which the modification relates.

Simian Virus 40

Simian virus 40, short-handed to SV40, was discovered in 1960. Its native environment is in certain primate species where it typically does not cause physiological issues except in animals with immune system deficiencies¹³. It became a subject of interest in the following years as it was found in the injected form of the polio vaccine which the Centers for Disease Control estimates that as many as 98 million Americans received between the years of 1955 and 1963¹⁴. While SV40 has not been proven to cause the formation of lesions or tumors, SV40 has been identified in certain types of human cancers in recent years^{15,16}. Because of this association, an increase in research about the characteristics and life cycle of SV40 has ensued, resulting in a plethora of novel, useful information regarding not only tumorigenesis but biological phenomena like DNA replication and transcription as well^{13,15,17,18}.

Table 1. Epigenetic modifications of histones and their biological effect¹².

Histone	Amino acid	Modification	Biological role
H3	R2	me2a	gene activation
	T3	ph	mitosis
	K4	ac	gene activation
	K4	me1, me2, me3	gene activation
	R8	me2s	gene repression
	K9	ac	gene activation
	K9	me1, me2, me3	gene repression
	S10	ph	mitosis
	S11	ph	mitosis
	K14	ac	gene activation
	R17	me2a	gene activation
	K18	ac	gene activation
	K23	ac	gene activation
	R26	me2a	gene activation
	K27	ac	gene activation
	K27	me1, me2, me3	gene repression
	S28	ph	mitosis
H4	K36	me1, me2, me3	gene activation
	K56	ac	gene activation
	K79	me1, me2, me3	gene activation
	S1	ph	mitosis
	R3	me2a	gene activation
	R3	me2s	gene repression
	K5	ac	gene activation
	K8	ac	gene activation
H2A	K12	ac	gene activation
	K16	ac	gene activation
	K20	me1, me2, me3	gene repression
	K91	ac	gene activation
	S1	ph	mitosis
	K4 [‡]	ac	gene activation
	K5	ac	gene activation
	K7 [‡]	ac	gene activation
H2B	T119 [*]	ph	mitosis
	K126 [‡]	su	gene repression
	K119	ub	gene activation
	S129 [‡]	ph	DNA repair
	K5	ac	gene activation
	S10 [‡]	ph	apoptosis
	K12	ac	gene activation
	S14	ph	apoptosis
	K15	ac	gene activation
	K16 [‡]	ac	gene activation
	K20	ac	gene activation
	S33 [*]	ph	gene activation
	K120	ub	meiosis
	K123 [‡]	ub	gene activation

[‡]S. cerevisiae, ^{*}D. melanogaster

SV40 is categorized as a polyomavirus – small, contains DNA, and has the potential to cause tumors. Figure 6 diagrams the SV40 genome along with the coded proteins that will be subsequently discussed. With no viral envelope, the capsid containing the 5243-base pair circular minichromosome enters the host cell via caveolin vesicle formation after the virion glycoprotein VP1 binds to MHC class 1 receptors on the surface of the cell. Once inside the nucleus, RNA polymerase II (RNAPII) initiates early gene expression and the production of three tumor antigens: the important and multifunctional large T antigen (LT), small T antigen (ST), and the less understood 17K T antigen.

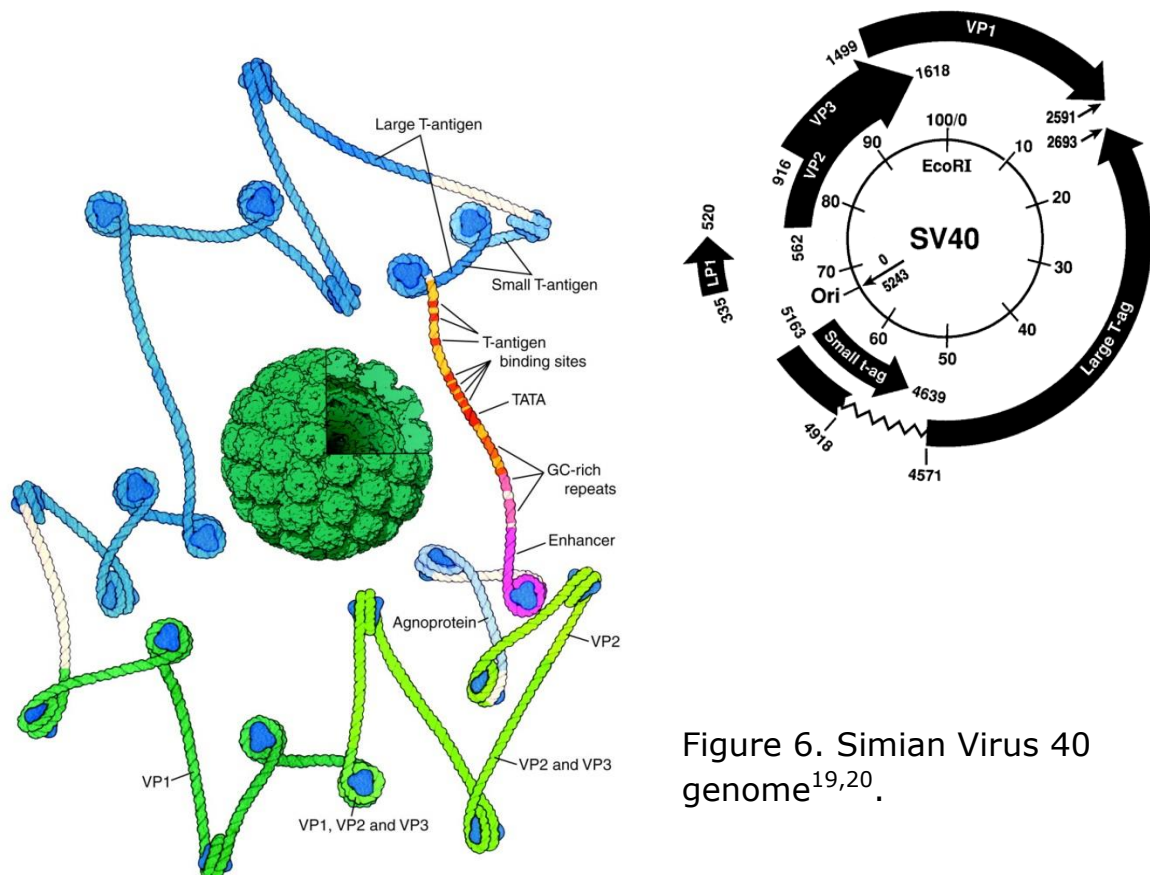


Figure 6. Simian Virus 40 genome^{19,20}.

Large T antigen has three distinctive regions to carry out its numerous functions. LT has been shown to inactivate cellular proteins, like p53, thereby preventing apoptosis or cell cycle arrest; bind to and recruit RNAPII and certain transcription factors essential for perpetuating transcription of the viral DNA to the origin of replication; as well as suppressing further early transcription and acting as a helicase in assisting directionality of RNAPII in the transcription of late genes^{21,22}. The structure of LT is shown in Figure 7.

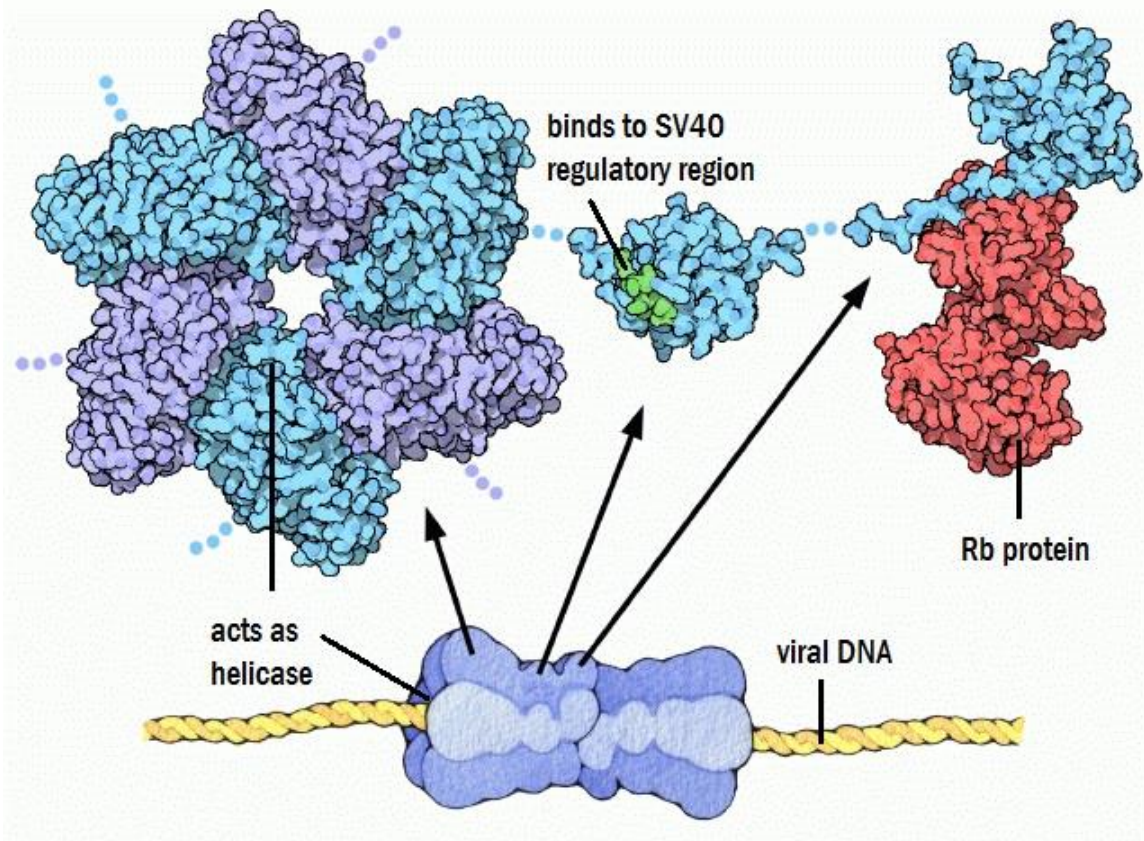


Figure 7. Large T antigen of Simian Virus 40¹⁹.

Late transcription gives rise to the structural viral capsid proteins VP1, VP2, and VP3. All protein products of the SV40 genome return to the nucleus and come together to form new virions. VP1 represents a majority of the proteins making up the icosahedral-shaped SV40 capsid and, as mentioned previously, is considered the dominant link between the virion and the new host cell membrane.

Because of its similarity to eukaryotic regulation of gene expression and the fact that it relies completely on the host cell's machinery, SV40 is a well-suited model for studying DNA replication, gene expression, and oncogenic transformation^{13,15,17,18,20} – and in the case of our lab, the epigenetic characteristics of these biological phenomena as well. Recent findings of this lab are continuing to elucidate connections between epigenetic markers, like acetylation and methylation, on specific lysine residues present within the tails of histones like H3 and H4 in the viral nucleosome, and their subsequent correlation with certain biological processes. For example, hyperacetylated histones H3 and H4 have been shown to be associated with p300 – a histone acetyl transferase – in the transcriptional complex²³. It has long been accepted that p300 works directly with RNA polymerase II (RNAPII) in transcribing genetic material^{1,23}, which therefore links the hyperacetylation of H3 and H4 with the biological process of transcription. The mono- and tri-methylation states of

histone H4 lysine 20 (H4K20me1 and H4K20me3, respectively) have been shown to be present at separate time points during the SV40 infection^{18,24}. Observed early in infection, H4K20me3 is thought to be associated with the uncoating of the virus. H4K20me1 is conversely found later on in the infection, both in replicating SV40 chromosomes (also referred to as minichromosomes) as well as the virions themselves suggesting a connection to encapsidation¹⁸. Most recently, we have published data that supports the existence of trans-generational epigenetic regulation by SV40²⁵. Along with this idea, we detected multiple distinct sets of histone modifications that occur together – which we termed an epigenome. Our lab has identified five epigenomes in SV40 minichromosomes²⁵ that will be explored as to their relation to certain biological processes as well.

The focus of the research project for my thesis is centered on the question of whether the epigenetic patterns we have noted to be present within the SV40 virus are a sequence-dependent phenomenon or a result of the initiation of a certain biological process like active transcription.

CHAPTER II

METHODS

Cell Culture

The cells used for infections to produce the viral chromatin used in the experiments were African green monkey kidney BSC-1 cells obtained from American Type Culture Collection, Rockville MD, and held at 37° C in a Napco Model 6200 CO₂ incubator. The media used to support the growth of the cells was minimum essential media (MEM) containing Earle's salts and L-glutamine in a 500 ml bottle purchased from GIBCO, Invitrogen, supplemented with 5 mg/ml gentamicin reagent solution antibiotics and 50 ml fetal calf serum (FCS) containing growth factors, both also from GIBCO. The BSC-1 cells were maintained in Corning tissue culture flasks, Fisher Scientific Company, either 25 cm³ or 75 cm³ in size with approximately 10 ml or 20 ml FCS and antibody-supplemented MEM, respectively. Twenty-five cm³ flasks were generally used for HIRT preparations to check for efficiency of infections whereas 75 cm³ flasks were typically used for chromatin preparations – methodology presented in the following sections. Growing cells were subcultured in the sterile environment of a Nuaire

laminar air flow hood Model NU 425-400. The refrigerated, supplemented MEM and frozen bottle of trypsin-EDTA .05% dissociation reagent, also from GIBCO, were warmed to room temperature in a Stovall gyrating hybridization water bath. Then, the flask of cells was checked by an Olympus light microscope to ensure a confluent monolayer of cells present. The flasks were placed in the hood where all remaining manipulations were conducted to ensure sterility. The caps of the flasks were wiped with an Fisherbrand sterile alcohol prep pad. The bottles and caps of the supplemented MEM and trypsin-EDTA were also wiped with an alcohol prep pad. New 25 cm³ or 75 cm³ flasks for cell expansion were prepared with the addition of approximately 10 ml or 20 ml of fresh media, respectively. The media of the flask containing the confluent monolayer of cells was poured out into a waste flask. Using Fisherbrand 5 ml glass pipettes and rubber manual pipetter, 3 ml of trypsin-EDTA was added to the flask containing the cells to be removed. The trypsin wash was rotated over the cell layer manually for about 10 seconds to deactivate protease inhibitors within the MEM and then discarded into the waste flask. Using a new 5 ml pipette, 5 ml trypsin was added to the flask containing the cells to be removed and this time laid flat within the hood for approximately 1-2 minutes, allowing the trypsin to cover the cells for that time. The flask was then checked in the microscope to

approximate the level of detachment of the cells before a few gentle, manual taps were applied to the side of the flask to dislodge the cells completely. A new 5 ml pipette was used to remove 3 ml of the trypsin containing the now-detached cells and .5 ml was dispersed into each of six 25 cm³ flasks or 1 ml into each of three 75 cm³ flasks, all of which contain the appropriate amount of fresh media. Directly after the trypsin containing detached cells was dispersed to the new flasks, fresh MEM was added to the parent flask which still contains 2 ml trypsin and detached cells. Caps on all flasks were secured and then laid flat before returning to the CO₂ incubator at 37° C. BS-C-1 cells expanded to confluent monolayers in 2-3 days on average, at which time they became ready for further subculturing or infection.

Preparation of Stock Viral Material

The wild-type 776 SV40 virus was a gift from Dr. Daniel Nathans. The parent variant in the experiment (T-129) was created by Dr. Barry Milavetz from a mutant of the 776 virus²⁶, which was gift from Dr. Thomas Shenk. T-129 had a deletion of the enhancer region from nucleotide (nt) 128-200 and a deletion of the large T antigen region from nt 4739-4882. It also contained an inserted reporter segment with a polylinker at nt 2666 which contained sites recognized by restriction endonucleases *Mlu*I, *Apa*LI, *Pm*I, *Nci*I, and *Bgl*I. The experimental constructs RH-43, T-143, and T-165 were prepared

previously in the Milavetz lab from the T-129 parent^{26,27}. The variant RH-43 contained an addition of multiple Sp1 sites, the variant T-143 included a second enhancer region, and the variant T-165 contained a full late promoter region (figures in results section). Stock of the different strains of virus were accumulated after 1 μ l of the selected variant was placed into a flask containing a mono layer of the BSC-1 cells and the supplemented media, and incubated until the virus had infected most cells and released additional virions into the media (approximately 10-14 days). The entire contents of the infected flask were collected in a Corning 50 ml flat cap tube until being aliquoted for subsequent infections.

Infection of BSC-1 Cells

Flasks containing confluent monolayers of cells ready for infection were moved from the sterile CO₂ incubator to the virus incubator, a Napco Model 5100 CO₂ incubator. A 500 ml bottle of GIBCO minimum essential media, unsupplemented and dedicated for use with viral infections, was also placed in the virus incubator to warm. Stock virus that has been consolidated and aliquoted into 1.5 ml Eppendorf tubes via the protocol in the section above was thawed per amount needed. Typically .5 ml virus was used with 4.5 ml of media from the 25 cm³ flasks and 1 ml virus with 9 ml of media from the 75 cm³ flasks. Once ready for infection, the flasks were moved to the virus-dedicated

NuAire NU-425-400 isolation hood. The proper amount of media was poured off into a Corning 15 ml disposable flat-cap centrifuge tube (4.5 ml or 9 ml). The remaining media in the flask was poured into waste jars within the virus hood and the proper amount of media for the infection returned to the flask from the 15 ml centrifuge tube. The corresponding amount of virus was then placed into the proper flask with a virus-dedicated Gilson pipette man and TipOne pipette tips. Used tips were discarded in waste receptacle within virus hood. Immediately following the addition of the virus, the flasks were capped, laid flat, and rotated slightly to allow the virus to disperse evenly throughout the media and onto the cell layer. The flasks were incubated at 37° C for 30 minutes with the virus-media solution. At the end of 30 minutes, the virus-media solution was discarded from the flasks into the waste jars. The flasks were then washed twice with approximately 5 ml or 10 ml (for 25 cm³ flasks or 75 cm³ flasks, respectively) of the warmed, unsupplemented, virus-dedicated media by rotating the flasks in all directions to remove any remaining unbound virus from the inner surface of the flasks. The washes were discarded into the waste jars and any excess was pipetted out and also discarded. Next, 5 ml or 10 ml (for 25 cm³ flasks or 75 cm³ flasks, respectively) of the warmed, unsupplemented, virus-dedicated media was added to each flask. The flasks were then capped and placed back

into the 37° C virus incubator for 30 minutes or 48 hours, depending on the time-point of viral material needed.

HIRT

The HIRT method²⁸ was used to determine whole-cell chromatin levels. First, the desired 25 cm³ flasks that had been infected with virus 30 minutes or 48 hours previously were removed from the virus incubator and placed in the virus hood. The media was discarded into the waste jars. Dubecco's Phosphate Buffered Saine (PBS) was used for two washes of the flasks using approximately 5 ml of PBS each time and rotating the flasks all directions to wash the entire inner surface of the flasks. The washes were discarded into the waste jars and any excess was pipetted out and also discarded. Once the washes have been completed and all PBS removed, 300 µl lysing solution was added to the flask (description of lysing solution can be found in Appendix B). The flask was then held flat and rotated so that the lysing solution covered the cell layer. The flask was continually rotated as the lysing solution thickened, at which point it was pipetted into a 1.5 ml Eppendorf tube with a 101-1000 µl TipOne pipette tip and 10-1000 µl Gilson pipette man. Two-hundred microliters of PBS was used to rinse the sides of the flask, then pipetted off and added to the 1.5 ml Eppendorf tube. Next, 160 µl Sodium chloride was squirted into the 1.5 ml Eppendorf tube being careful not to touch the pipette tip to the

sample. The sample was incubated overnight at 4°C, then spun at 12000 x g for 30 minutes at 4°C in a Eppendorf Centrifuge, model 5415C. The spin down formed a pellet of material at the bottom of the 1.5 ml Eppendorf tube which was either pulled out of the tube with a sterile wooden stick or the supernatant was carefully pipetted without disturbing the pellet and placed into another 1.5 ml Eppendorf tube. The pellet was discarded. Samples were frozen at -20° C until ready for glassmilk preparation and quantitative real-time polymerase chain reaction (q-PCR), the protocols of which are in sections below.

Nuclei Isolation

The following procedure was used to isolate cellular nuclei for later extraction of viral chromatin material. In preparation, a set of one 15 ml Corning flat-cap centrifuge tube and one 1.5 ml Eppendorf tube per flask being harvested was filled as follows. Fourteen milliliters of nuclei preparation buffer (see Appendix B) was added to the 15 ml tube as well as 15 µl PMSF (Sigma), mixed gently, then 1 ml of the buffer plus PMSF was removed from the 15 ml tube and placed in the 1.5 Eppendorf tube. Both tubes were held at 4° C until ready for use in harvesting the cells. First, the desired 75 cm³ flasks that had been infected with virus 30 minutes or 48 hours previously were removed from the virus incubator and placed in the virus hood. The media was discarded into the waste jars. Dubecco's Phosphate Buffered Saine

(PBS) was used for two washes of the flasks using approximately 10 ml of PBS each time and rotating the flasks all directions to wash the entire inner surface of the flasks. The washes were discarded into the waste jars and any excess was pipetted out and also discarded. Once the washes were completed and all PBS has been removed, 1 ml of the buffer plus PMSF was taken from the 15 ml tube and added to the flask. The solution was rotated over the cell surface of the flask until completely covered with the liquid. A Fisherbrand disposable cell scraper was then used to gently remove the cells from the surface of the flask. Next, 1 ml 0.05% Triton X-100 (see Appendix B) was pipetted to wash the dislodged cells to the bottom of the flask where they were pipetted out and gently placed on top of the remaining 13 ml of buffer plus PMSF in the 15 ml tube. Once all material from the flask was added to the 15 ml tube, it was centrifuged at 2500 rpm for 10 minutes at 4° C in a Beckman J6-M1 centrifuge. The spin down formed a pellet of material at the bottom of the 15 ml tube. The supernatant was discarded and 100 µl of buffer plus PMSF from the 1.5 ml Eppendorf tube was used to resuspend the pellet and transfer it gently to the top of the remaining buffer plus PMSF in the 1.5 ml Eppendorf tube. This tube was centrifuged at 3000 x g for 10 minutes at 4° C. The spin down again formed a pellet at the bottom of the 1.5 ml Eppendorf tube. The supernatant was removed without disturbing the

pellet. Finally, the pellet was frozen at -20° C overnight before performing extraction and gradient steps.

Nuclear Extraction

Nuclear extraction was performed to open up the nuclear envelope and separate chromatin from other nuclear material. The desired nuclear pellet was thawed then neutralized by resuspending with 75 µl chromosome extraction buffer (see Appendix B). The solution was vortexed (Lab-line Instruments, Inc.) for about 5 seconds and spun down at 12000 x g for 30 seconds at room temperature. The supernatant was pipetted off and discarded. Four-hundred microliters of chromosome extraction buffer was used to again resuspend the pellet. The Eppendorf tube was vortexed before holding at 4° C for 1 hour. After this extraction period, the tube was centrifuged at 12000 x g for 10 minutes at 4° C. This spin down formed a pellet of material that was discarded once the supernatant was taken off and either transferred to a new, sterile 1.5 ml Eppendorf tube and frozen at -20° C until further use or immediately run on a gradient (see next section).

Chromatin Gradient

Glycerol gradients were used to purify DNA for use in chromatin immunoprecipitation (ChIP) protocols. The glycerol gradient was set up in a 1.5 ml Eppendorf microcentrifuge tube. Two-hundred microliters

of 50% glycerol (see Appendix B) were placed at the bottom of the tube. Next, 1 ml of 10% glycerol (see Appendix B) was gently added on top of the 50% glycerol base. The 400 μ l of nuclear extraction product from the previous step was added gently on top of the 10% glycerol layer then centrifuged at 50,000 rpm for 35 minutes at 4° C in a TLA 100.3 rotor within a Beckman TLA 100 ultracentrifuge. Upon completion of the spin, 400 μ l was carefully removed by pipette from the top-down and discarded. The next 600 μ l from the top of the current level down was carefully removed and saved in a new, sterile and appropriately labeled 1.5 ml Eppendorf tube. The content of this sample was concentrated by glassmilk protocol and PCR-checked (see steps below) before use in ChIP assays.

Chromatin Immunoprecipitation (ChIP)

Chromatin immunoprecipitation assays use antibodies to pull down selected targets. The antibodies used in the experiments for this particular study were from Millipore, Active Motif, abcam, Upstate, and Santa Cruz Biotechnology. For our experiments, 7.8 μ l of the selected antibody were added to an Eppendorf Protein LoBind tube along with 170 μ l protein agarose A and 750 μ l ChIP dilution buffer from the Millipore ChIP kit. This solution was rotated continuously at 4° C for approximately 4 hours in a Labnet mini labroller. The tube was then centrifuged at 2000 rpm in the Eppendorf Centrifuge model 5415C at

room temperature for 1 minute to encourage the agarose to collect at the base of the tube. The supernatant was removed and discarded. Nine-hundred microliters of fresh ChIP dilution buffer from the exact same ChIP kit was again added to the tube containing the agarose along with 125 μ l of the desired chromosomes that have previously been run on a gradient. This solution was rotated continuously at 4° C for approximately 12 hours. The tube was then centrifuged again at 2000 rpm at room temperature for 1 minute to encourage the agarose to collect at the base of the tube. The supernatant was removed and discarded. A series of 5 different washes of the agarose from materials within the Millipore ChIP kit was performed in the following order: Low Salt, High Salt, Lithium chloride (LiCl), Tris-EDTA (TE) buffer, and Tris-EDTA (TE) buffer again. For each wash, 750 μ l of the specified solution was added to the tube containing the agarose, rotated continuously at 4° C for 10 minutes, centrifuged at 2000 rpm at room temperature for 1 minute to collect the agarose to the bottom of the tube, then the supernatant carefully removed and discarded. After the fifth and final wash and centrifugation, the supernatant was carefully taken off the agarose and discarded as usual. One-hundred and twenty-five microliters of fresh TE buffer was used to resuspend the agarose and transfer it to a clean, sterile 1.5 ml Eppendorf tube. Two sequential 15 minute incubations at room temperature were conducted with 110 μ l

SDS lysing buffer each time. At the end of 15 minutes, the agarose had again settled and the supernatant was carefully collected and saved in a new, sterile and appropriately labeled 1.5 ml Eppendorf tube. The collection of supernatant from the second SDS lysis buffer incubation was added directly to the first in the new tube.

Concentrating with Glassmilk

Before quantitating with real-time PCR, all samples were concentrated using GeneClean Spin Kit glassmilk kits from MP Biomedicals, LLC. All glassmilk steps were conducted within the sterile environment of an Air Clean 600 PCR workstation (ISC Bioexpress) using clean technique. Fifty microliters of glassmilk was placed in a sterile 1.5 ml Eppendorf tube, then 25 μ l of the intended sample was gently mixed with the glassmilk using the suction of the pipette man. The glassmilk plus sample combination was allowed to sit for 10 minutes before being spun down for 90 seconds at room temperature in a Southwest Science microcentrifuge. The supernatant was carefully removed and discarded. An ethanol wash of 125 μ l was added to the tube where pellet present was resuspended gently with the pipette man. The solution was allowed to sit briefly before being spun down again at the same conditions as previously done. The supernatant was again carefully removed and discarded. The pellet was dried for 15 to 20 minutes, or until dry, in a Thermo Scientific Savant SPD Seppd Vac

SPD 131DDA with RVT 4104 refrigerated vapor trap. Once completely dried, 25 µl TE buffer was added to the pellet. Samples were stored frozen at -20° C or PCR-ed immediately.

Quantitative real-time Polymerase Chain Reaction (qPCR)

All PCR preparation steps were conducted within the sterile environment of an Air Clean 600 PCR workstation using clean technique. The hood was first cleaned of possible contaminants using DNA Zap two-part solution from Ambion. A 'master mix' solution was prepared of 600 µl Bio-Rad SsoAdvanced SYBR green SuperMix; 585 µl clean, nuclease-free water from Ambion; and 6 µl of both the forward and reverse parts of a primer set. The primer set was designed by Les Kallestad in the Milavetz lab using Primer-BLAST within the National Center for Biotechnology Information's website. The primers were of the promoter region with the sequences 5` TTG CAA AAG CCT AGG CCT CCA AA 3` and 5` TGA CCT ACG AAC CTT AAC GGA GGC 3`. The 'master mix' was thoroughly vortexed before using and prepared immediately before being aliquoted and combined with a sample to be PCR-ed. Twenty-nine microliters of 'master mix' was placed into a VWR 0.2 µl flat cap PCR tube. The glassmilk sample to be added was appropriately, manually agitated to form an evenly resuspended solution of the glassmilk within the TE buffer. Two microliters of this glassmilk-TE buffer solution was then immediately pipetted out with a

PCR-dedicated, sterile Gilson 0.1 – 2 µl pipette man and TipOne pipette tips, and added to the 'master mix' in the PCR tube. Samples were normally prepared in triplicate and quantitative real-time PCR was carried out using a Bio-Rad CFX Connect Real-Time System and the accompanying Bio-Rad CFX Manager 2.1 software. The cycle pattern was set up to begin with a 15 minute "hot start" of 95° C, then 55 cycles of one minute at 95° C to one minute at 60° C followed by one minute at 72° C. The program also performed a melt curve analysis – heating from 65° C to 95° C in five second intervals – at the end of the 55 cycles of amplification which we referred to for quality of amplified sample.

CHAPTER III

RESULTS

Construction of SV40 Variants

The parent construct in the experiment, T-129, is a variant of the SV40 wild type virus which has a deletion of an intron from the large T antigen coding region (nt 4739-4882) and a deletion of one of the enhancers from the nucleosome free region near the origin of replication (nt 128-200). The T-129 construct also contains an input reporter region with a polylinker (nt 2666) that contains sites sensitive to restriction endonucleases *Mlu* I, *Apa* LI, *Pml* I, *Nci* I, *Bgl* II^{26,27}. These sites allowed the insertion of the various sequences of interest producing the variants used in the experiment. Figure 8 diagrams the setup of the T-129 parent construct as well as three experimental variants. RH-43 contains an additional two copies of the Sp1 binding site (nt 40-59) between the *Mlu* I and *Apa* LI restriction sites. Variant T-143 contains the second enhancer region (nt 103-178), again between the *Mlu* I and *Apa* LI sites. The T-165 variant included the whole late promoter (nt 255-424) and was also positioned between the

same two restriction endonuclease sites as with the previously described variants.

Quality Control of Virus, Infection, and Experimental Conditions

The parameters of the experiments conducted were controlled for as much as possible by conducting quality control checks for the infectivity of the virus variants, optimal infection conditions, as well as ideal ChIP conditions. Newly produced virus was checked by HIRT procedure along with q-PCR to ensure new batches of virus were comparably infective of cells. The amount of virus used in infections,

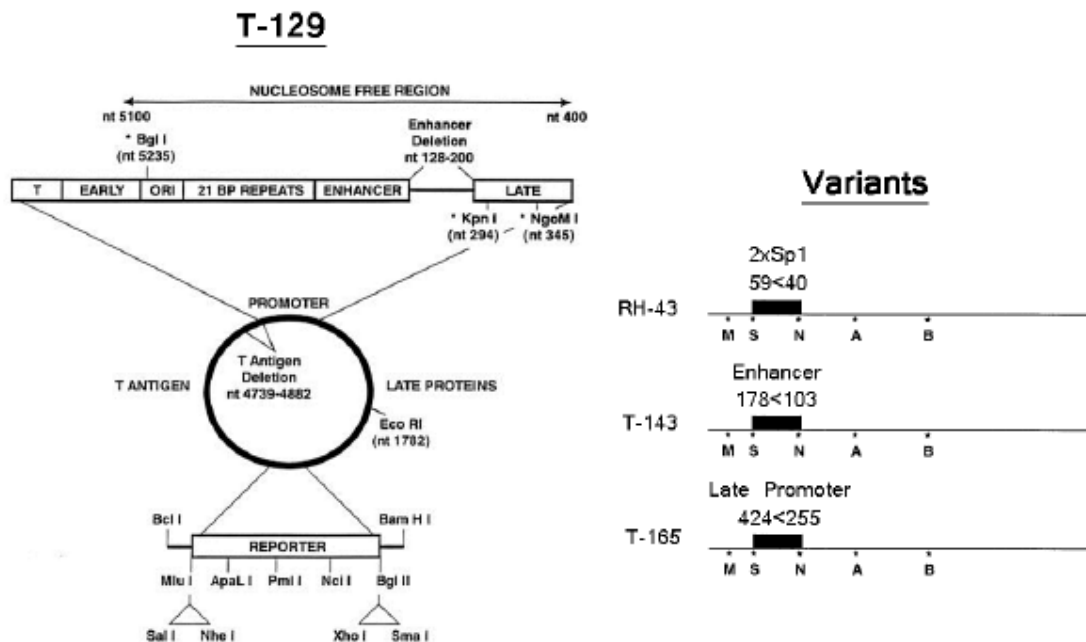


Figure 8. SV40 parent variant, T-129, and experimental variants RH-43, T-143, and T-165^{26,27}.

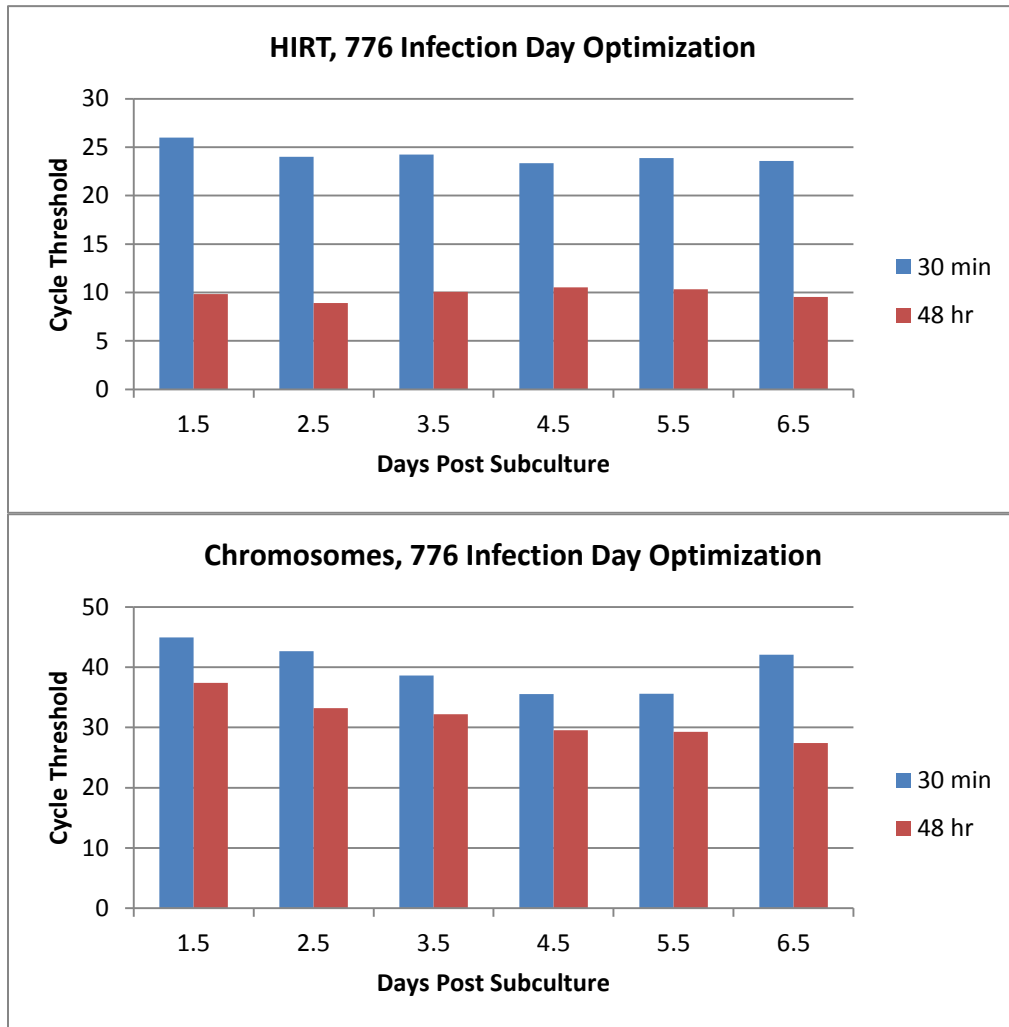


Figure 9. SV40 infection optimization.

the length of time the cells are first exposed to the virus, and the age of the cells at the time of infection were compared. The data collected used wild-type SV40 virus (776) and shows that the highest amount of whole cell viral material for a 48 hour infection is initiated when the cells are approximately 2.5 days post subculture (Figure 9, top). The highest amount of viral chromatin obtained from a 48 hour infection occurs when the infection is initiated 4.5 days post subculture or later

(Figure 9, bottom). This information was considered for the infections used to produce the selected viral chromatin for the ChIP experiments.

ChIP/q-PCR Reveal Changes in Ratios of Modifications Present

The ChIP experiments were carried out via protocols described in the methods section previously. After the ChIP products were subjected to q-PCR, statistics were used to calculate both the percentage of modified chromatin bound by the ChIP and the ratio of those percentages between the SV40 variants RH-43, T-143, and T-165 and the parent virus T-129. Cycle thresholds produced by the software of the q-PCR system indicate the number of cycles completed when fluorescence of the incorporated SYBR green are first able to be detected. These cycle thresholds (C_t) were used to calculate the comparisons (Table 2).

To compute the percentage of the input chromatin bound by the selected antibody, the C_t of the un-ChIPed input chromatin was subtracted from the C_t of the related ChIP product to give a C_t difference. Given that amplification by q-PCR reaction should yield twice the amount of product from each cycle, one divided by 2 to the power of the C_t difference revealed the percent of the whole input chromatin pulled down by the antibody (%) and inferring the

Table 2. Percent and Ratios of Histone Modifications in SV40 variants.

	H3K4me1		H3K4me2		H3K4me3		H3K9me1		H3K9me2		H3K9me3		H4K20me1		RNAPII		
	%	Ratio	%	Ratio	%	Ratio	%	Ratio	%	Ratio	%	Ratio	%	Ratio	%	Ratio	
T-129	Avg	0.017	0.358	0.363	6.423	0.850	0.435	0.074	0.008								
	Stdev	0.017	0.339	0.692	5.035	0.690	0.488	0.122	0.012								
	n																
RH-43	Avg	0.031	0.318	0.802	2.461	1.250	4.943	0.813	0.455	0.535	1.375	1.518	0.030	0.380	0.002	1.000	
	Stdev	0.040	0.382	0.654	1.063	0.157	3.553	0.249	0.601	0.149	1.874	1.3818	0.051	0.044	0.003	0	
	n	3	4	4	3	3	4	4	2	2	4	4	3	3	2		
T-143	Avg	0.017	0.720	0.106	0.510	1.867	10.58	1.125	0.573	0.375	0.995	1.440	0.026	0.155	0.004	1.500	
	Stdev	0.022	0.452	0.098	0.469	0.266	6.452	0.278	0.789	0.276	1.365	1.513	0.036	0.120	0.005	0.354	
	n	3	3	3	3	3	2	2	2	2	2	2	2	2	2		
T-165	Avg	0.001	1.020	0.263	0.460	0.700	6.337	1.606	0.393	0.373	0.263				0.010	0.890	
	Stdev	0.001	1.389	0.004	0.170	0	9.609	1.211	0.351	0.252	0.556	0.225	n/a		0.009	0.156	
	n	2	2	2	1	1	5	3	3	3	3	3	2	2	2		

percentage of the input chromatin containing the particular histone modification.

The ratio of the amount of a certain modification seen in the ChIP products of the variants to that of the ChIP product of the T-129 parent was calculated by dividing the percent (%) of a variant by the percent (%) of the T-129 parent within the same ChIP experiment. Average values of these computations are shown in Table 2. Ratios of close to 2 or higher indicate a prominent increase in the amount of the modification present as a result of the sequence addition of the variant. Ratios of down to 0.5 or less indicate a notable decrease in the amount of modification present as a result of the input sequence of the variant.

The average ratios of the variants for the selected histone modifications from Table 2 was graphed to more readily display any notable changes (Figure 10). RH-43, the variant containing an input sequence of two Sp1 binding sites, saw a noteworthy increase in the amount of H3K4me1 compared to the T-129 parent. Distinguishable decreases in H3K9me2 and H4K20me1 modification were also observed for the RH-43 variant (Figure 10, top panel). T-143, having a reintroduced second enhancer region, showed a prominent increase in H3K4me3 while also displaying notable decreases in H3K9me2 and H4K20me1 (Figure 10, middle panel). T-165, the variant containing an

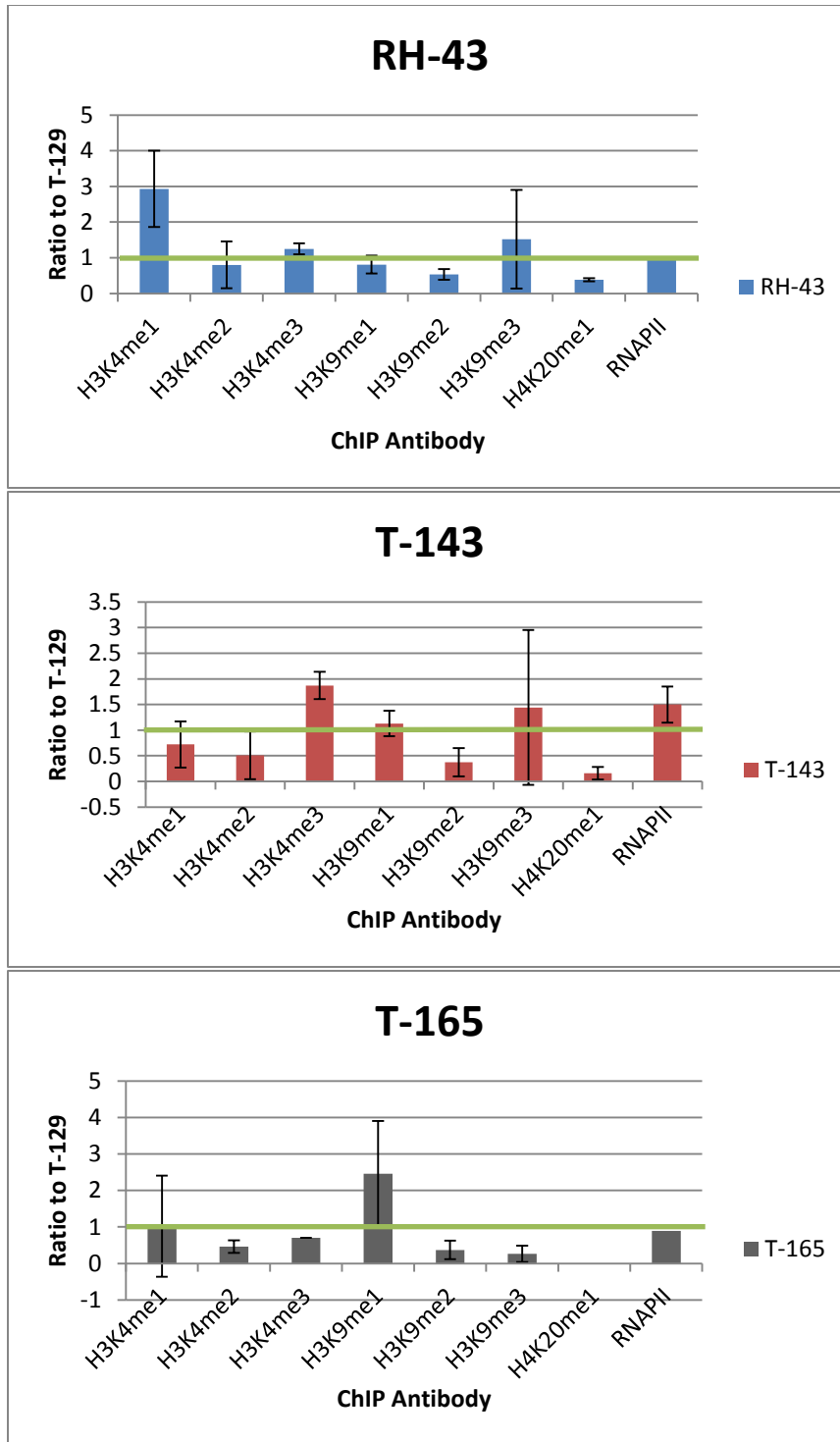


Figure 10. Ratios of the selected modifications present in SV40 variants to the T-129 parent.

addition of the late promoter sequence, resulted in decreases in H3K9me1 and H3K9me2 as well (Figure 10, bottom panel). The green line on the graphs is intended to assist in distinguishing notable increases or decreases in the ratio of modifications present in the variant versus the T-129 parent. The error bars represent one standard deviation of the average ratio of the variant to T-129. In this set of experiments, the antibody to RNAPII is intended as an internal control as RNAPII is traditionally a good marker for active transcription. As it relates to this experiment, the ratio of RNAPII does not increase or decrease significantly for the RH-43 or T-165 variants but perhaps a slight increase in T-143 (Figure 10, middle panel). This may suggest that the additional enhancer sequence in the T-143 variant could be associated with an increase in active transcription. However, more investigation is needed to confirm this observation.

CHAPTER IV

DISCUSSION

From the results of the initial experiments conducted and shown here, we can conclude that the presence of specific sequences is associated with changes in the amounts of certain histone modifications seen on SV40 minichromosomes. Additionally, it is important to note that the sequences inserted into the respective variants are known to be associated with gene activation (Table 1). The Sp1 binding sites added to RH-43 bind Specificity Protein 1 – a transcription factor known to be involved in gene expression in early development^{1,29}. The increase in the ratio of H3K4me1, a modification associated with gene activation (Table 1); as well as the decreases in H3K9me2 and H4K20me1, modifications associated with gene repression (Table 1), both suggest that the addition of sequences involved in transcription also affects the histone modifications related to that biological process. Similarly, enhancer regions – like the one added to variant T-143 – are segments of DNA that bind trans-acting factors and enhance gene transcription^{30,31}. Therefore the increase in the ratio of H3K4me3, again a modification associated with gene

activation, and the decreases in the ratios of H3K9me2 and H4K20me1 further support our hypothesis. The T-165 variant contained an additional late promoter, which is also a sequence involved in initiating the transcription of particular genes²⁶. The decreased ratios of H3K9me2 and H3K9me3 provide continued support for the notion that sequences can drive epigenetic modification of histones.

An additional piece of information that can be deduced from the data is one which is also recently being supported in other literature. Since the discovery of the different modifications that can be present on histones, it was assumed that methylation of any state would serve essentially the same purpose^{1,32,33,34}. Our data suggests this may not be the case and that different methylation states can be associated with the functioning of different sequences. The mono-methylated form of H3K4 was affected by the addition of Sp1 binding sites in RH-43, but the di- and tri-methylated states were unaffected (Figure 10, top panel). With the T-143 variant and its addition of a second enhancer region, the tri-methylated state of H3K4 saw an increase, however the mono- and di-methylated states did not show change (Figure 10, middle panel). Even though the Sp1 binding site and enhancer regions of a DNA sequence have both been associated with gene transcription and all three methylated states of H3K4 are

connected to gene activation (Table 1), the different methylated states appear to be regulated separately.

Our lab plans to continue investigating this phenomenon to better understand the maintenance and regulation of histone modifications and their biological relevance by experimenting with different sequence inputs to determine if other sequences have an effect on the amount of a particular modification present. We could also look into the outcome of the position of the input sequence within the SV40 genome to establish if there is a positional effect. There are also a multitude of antibodies to other histone modifications that can be tested. The overall aim of this and future research of epigenetic modifications is to better understand the mechanisms by which the cell controls, and the environment affects, gene expression. This information, we are already seeing, could ultimately have broad implications in the fields which study human diseases and development.

APPENDICIES

Appendix A
Commonly Used Abbreviations

DNA	deoxyribonucleic acid
RNA	ribonucleic acid
RNAPII	ribonucleic acid polymerase II
H3K4	histone 3 lysine 4
H3K9	histone 3 lysine 9
H4K20	histone 4 lysine 20
me1	monomethyl
me2	dimethyl
me3	trimethyl
km	kilometers
nm	nanometers
µm	micrometers
SV40	Simian virus 40
LT	large T antigen
°C	degrees Celsius
cm ³	cubic centimeters
L	liters
ml	milliliters

μl	microliters
nt	nucleotide
Sp1	Specificity protein 1
PBS	Phosphate Buffered Saline
PMSF	phenylmethylsulfonyl fluoride
x g	gravity constant
ChIP	Chromatin immunoprecipitation
q-PCR	quantitative real-time polymerase chain reaction
g	grams
M	molar
mM	millimolar

Appendix B
Solutions and Buffers

Lysing solution (HIRT)	6.0 ml 10% SDS, 2.0 ml 0.1 M EDTA, add Milli-Q water to 100 ml
10% SDS	100 g SDS, add Milli-Q water to 1.0 L
0.1 M EDTA (pH 8.0)	3.8 g EDTA, add water to 100 ml
Milli-Q water	Water purified by Milli-Q Water Purification System (Millipore)
Sodium chloride (HIRT)	292.2 g NaCl, add Milli-Q water to 1 L
Nuclei Preparation Buffer (pH 7.4)	50 ml glycerol (5%), 0.84 g HEPES (2 mM), 5 ml 0.1 M MgCl ₂ (0.2 mM), 5 ml 0.1 M PMSF* (0.5 mM), 5 ml 0.1 M DDT* (0.5 mM), add Milli-Q water to 1 L; autoclave sterilize *add after autoclaving, filter sterilize
0.1 M MgCl ₂	2.03 g MgCl ₂ : 6 H ₂ O, add water to 100 ml
0.1 M PMSF	1.74 g PMSF, add ethanol to 100 ml
0.1 M DDT	0.77 g DDT, add water to 50 ml
0.05% Triton	50 ml Triton X-100, add water to 100 ml
Chromosome Extraction Buffer (pH 7.5)	0.24 g HEPES (10 mM), 1 ml 0.1 M EDTA (1 mM), 0.5 ml 0.1 M DDT (0.5 mM), 0.1 ml 0.1 M PMSF (0.1 mM), add Milli-Q water to 100 ml, filter sterilize

Chromatin Gradient
Solutions (pH 7.5)

1.19 g HEPES (10 mM), 0.186 g KCl (5 mM), 5 ml 0.1 M EDTA (1 mM), 1 ml 0.1 M $MgCl_2$ (0.2 mM), 2.5 ml 0.1 M DDT (0.5 mM), add 50 ml glycerol for 10% glycerol solution or 250 ml glycerol for 50% glycerol solution, add water to 500 ml, autoclave sterilize

All glassware and solutions autoclaved in a Castle M/C 3500 Series Sterilizer (Gentige-Castle). All solutions adjusted to the indicated pH using either 6 M NaOH or 6 M HCl.

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