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Viral Epigenetics

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Abstract

DNA tumor viruses including members of the polyomavirus, adenovirus, papillomavirus, and herpes virus families are presently the subject of intense interest with respect to the role that epigenetics plays in control of the virus life cycle and the transformation of a normal cell to a cancer cell. To date, these studies have primarily focused on the role of histone modification, nucleosome location, and DNA methylation in regulating the biological consequences of infection. Using a wide variety of strategies and techniques ranging from simple ChIP to ChIP-chip and ChIP-seq to identify histone modifications, nuclease digestion to genome wide next generation sequencing to identify nucleosome location, and bisulfite treatment to MeDIP to identify DNA methylation sites, the epigenetic regulation of these viruses is slowly becoming better understood. While the viruses may differ in significant ways from each other and cellular chromatin, the role of epigenetics appears to be relatively similar. Within the viral genome nucleosomes are organized for the expression of appropriate genes with relevant histone modifications particularly histone acetylation. DNA methylation occurs as part of the typical gene silencing during latent infection by herpesviruses. In the simple tumor viruses like the polyomaviruses, adenoviruses, and papillomaviruses, transformation of the cell occurs via integration of the virus genome such that the virus's normal regulation is disrupted. This results in the unregulated expression of critical viral genes capable of redirecting cellular gene expression. The redirected cellular expression is a consequence of either indirect epigenetic regulation where cellular signaling or transcriptional dysregulation occurs or direct epigenetic regulation where epigenetic cofactors such as histone deacetylases are targeted. In the more complex herpesviruses transformation is a consequence of the expression of the viral latency proteins and RNAs which again can have either a direct or indirect effect on epigenetic regulation of cellular expression. Nevertheless, many questions still remain with respect to the specific mechanisms underlying epigenetic regulation of the viruses and transformation.

Keywords

Cancer; Epigenetics; Methylation; Transformation; Virus

1 Epigenetics

Epigenetic regulation consists of those changes in gene regulation resulting from environmental cues which can be passed from mother to daughter cells during replication which are not a result of changes in DNA sequence. Because the biologically functional state of DNA in eukaryotes is in the form of chromatin in which the DNA is wrapped around a histone core, the major contributors to epigenetic regulation are DNA and the histones H2A, H2B, H3, and H4. The major forms of epigenetic regulation are indicated in Fig. 1.

DNA can be epigenetically regulated through the introduction of a methyl group onto the cytosine in the sequence CpG [1]. There are three known mammalian DNA methyltransferases. One of which, Dnmt1 prefers hemi-methylated substrates and may be responsible for maintenance of the methylation state of DNA during replication. The other two methyltransferases, Dnmt3a and Dnmt3b appear to be responsible for the de novo introduction of methylated cytosine into DNA [2]. Methylation of DNA is usually thought to be associated with repression of gene expression [1].

Histones are also known to contribute to epigenetic regulation. Histones can affect epigenetic regulation either through the presence of different variants or by posttranslational modification of amino acids in the histones. Histone H2A and H3 are known to exist in multiple forms which differ in their primary amino acid sequence at a limited number of sites in the histone [3, 4]. Generally the changes in amino acid sequence consist of replacement of serines by valine or alanine in regions other than the amino terminal tails.

There are four well known variants of H3 and three of H2A. The variants of H3 include H3.1, H3.2, H3.3, and CENP-A. H3.1 and H3.2 are primarily associated with newly replicated chromatin in higher eukaryotes in contrast to H3.3 which is associated with actively transcribing chromatin and CENH3 which is typically found in the centromeres of chromosomes [5, 6]. Interestingly, the specificity of association of the H3 variants in chromatin appears to be based primarily upon how the variant is introduced into the chromatin. Interactions between the histones and DNA in nucleosomes are typically disrupted during processes in which DNA sequence information is read such as replication and transcription and regenerated after the process is complete. The regeneration of nucleosomes typically involves the interaction between histones, specific chaperones, and proteins involved in replication or transcription. For example, H3.1 and H3.2 interact with CAF-1 and ASF-1 through interaction with PCNA at replication forks [7], while H3.3 interacts with HIRA during transcription and CENP-A interacts with HJURP which recognizes centromeric region [7].

The variants of H2A consist of H2A.Z, H2A.X, and macroH2A. H2A.X appears to be primarily associated with DNA repair, macroH2A appears to be associated with repressed regions, while H2A.Z appears to be located at the flanks of nucleosomefree regions [8–10]. This may be due to the fact that H2A appears to be dynamically regulated with the histone constantly being displaced from and reassociated with nucleosomes [5].

Posttranslational histone modifications are also thought to play a major role in epigenetic regulation. A number of modifications are known to occur in histones including acetylation, methylation, phosphorylation, ADP ribosylation, and ubiquitylation. However, of these, acetylation and methylation are arguably the most studied. Acetylation of H3 (lysines 9 and 14) and H4 (lysines 5, 8, 12, 16, and 20) is generally thought to be primarily associated with transcriptionally active chromatin. In its role associated with transcription, acetylation appears to be a very dynamic process in which H3 and H4 undergo continuous acetylation and deacetylation along with movement of RNA Polymerase II through the transcribed chromatin [11]. There are a number of well- characterized cellular acetyltransferases responsible for histone acetylation including p300 and others [12]. Similarly, there are at

least 18 known histone de-acetylases, divided into four classes, Class I (HDAC1, 2, 3, and 8), Class II (HDAC4, 5, 6, 7, 9, and 10), Class III (the Sirtuin family), and Class IV (HDAC 11) [13, 14].

The other major form of well-studied histone posttranslational modification is methylation of H3 and H4. This form of regulation appears to be more complex in part because a particular lysine can be either mono-, di-, or tri-methylated. As a consequence while there are general relationships between methylation of a particular lysine, there are also major exceptions to many of these general relationships depending upon cell type, differentiation status and location within the genome. Nevertheless, methylation, particularly trimethylation of H3K4, H3K36, and H3K79 are generally associated with transcriptional activation while trimethylation of H3K9, H3K27, and H4K20 are generally associated with transcriptional repression [15]. Our own studies, to be discussed below in the SV40 section, suggests that in particular the mono- methylated form of many of these modifications can have a significantly different regulatory effect than the tri-methylated form [16, 17]. At the present there are a number of histone methylases known and in general each form of histone methylation has its own enzyme [15]. Similarly, enzymes capable of demethylating each of the forms of methylated histones with the present exception of H4K20me3 and H3K79 have been identified [15]. The latter suggests that none of the modifications with the possible exception of methylation on H3K79 or H4K20me3 are stable. However, histone methylation is not thought to be as dynamic as histone acetylation.

1.1 Life Cycle of Tumor Causing Viruses

Because epigenetic regulation occurs in the context of a virus infection, it is necessary to first consider the typical biological events which occur during an infection. Infection by DNA tumor viruses can either result in productive infections in which progeny virus is obtained, transformation of the infected cells in which case tumor cells arise, or latent infections in which the virus exists as a semifunctional episome. The initial steps of a typical virus infection includes binding to an external cellular receptor, internalization into the infected cell, and transport to the nucleus of the cell usually with concomitant uncoating of the genetic information. Once delivered to the nucleus the viral genome then undergoes initiation of certain genes required for beginning the infection usually described as early genes or immediate early genes. As discussed in detail in subsequent chapters the initiation of transcription is dependent upon the virus. Some viruses like SV40 are present in virions as chromatin and capable of carrying transgenerational epigenetic information [18]. Schematic genomic structures of different viruses are shown in Figs. 2, 3, 4, and 5. Other DNA viruses are like herpes virus and enter the cell as DNA and then become organized into chromatin upon transport to the nucleus [19] (Fig. 5). These differences are significant with respect to epigenetic regulation.

Following the initiation of early transcription there is generally a series of regulatory events leading to the repression or activation of both cellular and viral genes frequently in a cascade which ends with two events: the initiation of late transcription which generally includes the viral proteins required for generation of the virus coat and the initiation of replication. The order of these events and whether they actually occur during infection can vary by particular

virus. For example, certain viruses including members of the Herpes virus family may go into a latent state in which there is minimal transcription along with long term stability of the chromatin containing the genome as an episome. At other times the viruses integrate into the host genome generally resulting in transformation of the infected cell and subsequent generation of a tumor.

In a productive infection when an appropriate level of replication is reached along with the necessary amounts of coat proteins from late transcription, the viral chromosome begins the encapsidation process. Ultimately, this leads to the formation of new viable virus ready for a subsequent round of infection. Most if not all of these biological processes are likely to be regulated through epigenetic mechanisms. The role that epigenetics plays in regulation of each viral life cycle will be discussed in detail in subsequent sections.

1.2 Special Epigenetic Problems with Viruses

While the epigenetic regulation of the chromatin from cells and tumor-causing viruses appears to be very similar with respect to the mechanisms involved, there is a major difference between the epigenetics of cellular and viral chromatin. Typically it is thought that cells whether undergoing differentiation or terminally differentiated contain only a single epigenetic state or epigenome defined by the particular set of modifications present in the chromatin. Viral chromatin, on the other hand, must exist as multiple epigenomes at the same time in the same cell in order to complete its life cycle. For example, following infection with SV40 and similar viruses, minichromosomes capable of early transcription, repressed early transcription, late transcription, replication, and encapsidation all must coexist within a single infected cell at late times in infection. Moreover, the relative proportion of each of the species must also be tightly controlled. One would expect that if one of the above processes was deregulated there would be a profound effect on the amount of virus ultimately generated by the infection. For example, in the SV40 system if late proteins were over-produced and no mechanism was present to ensure that a pool of chromosomes capable of replication was maintained, one might expect the infection to end with only a small amount of actual virus being produced. However, what is clear is that the levels of transcribing, replicating, and encapsidating SV40 chromosomes are optimized for viral yield by the infection. This control is most likely mediated through epigenetic regulation since all the minichromosomes still retain the same genomic information. In the SV40 system we have shown that at these late times a minimum of five distinct SV40 epigenomes are present in infected cells [18] based upon the combinations of histone modifications present in H3 and H4. As discussed in more detail below we believe that at least some of these epigenomes represent the biologically functional minichromosomes which are expected to be present. While the presence of multiple tightly regulated epigenomes within an infected cell appears to be only associated with these viral infections at this time, it would not be surprising to see similar mechanisms involved in regulation of cellular genes present in multiple copies on one chromosome or on diploid chromosomes.

1.3 Overview of Epigenetic Consequences of Viral Infection on Cellular Genes

The small DNA tumor viruses including polyomaviruses, adenoviruses, and papillomaviruses promote their productive infections and cellular transformation events by a

common strategy in which one or more virus-specific early proteins disrupt normal cellular control processes by binding to critical cellular regulatory factors. For example, p53 which is a checkpoint regulator and transcription factor is a common target of a number of these early gene products [20–22]. The binding of the viral protein can then lead to either specific activation or repression of critical cellular genes or global effects on cellular expression patterns. Not surprisingly, factors involved in epigenetic regulation can be targets of the viral early proteins. For example, e1a of adenovirus binds to p130 and the related lysine acetyltransferases, EP300 and CREBBP [23–26]. The role of the viral early proteins in global modification of gene expression will be discussed in more detail with respect to each relevant individual virus.

1.4 Overview of Transformation by Relevant Viruses with Emphasis on Latency Regulation

Under certain circumstances related to regulation of viral gene expression and cellular context, the small DNA tumor viruses can transform infected cells. When this occurs, all or critical portions of the viral genome become integrated into the host cell genome. The integration event then results in both dysregulation of the viral gene expression and certain cellular genes. Typically the viral genes involved in reordering cellular gene expression to allow for replication of the virus such as p53 are over-expressed. This leads ultimately to the increased growth rate and dysregulation of the cellular control genes. For the larger herpesviruses transformation is a consequence of gene expression associated with latency. Presumably epigenetics could play a critical role in this process in a number of ways. Chromatin structure could affect the sites of DNA integration and subsequent expression of viral genes. In addition, viral proteins such as latency factors could directly modulate the activities of global epigenetic regulators. The influence of the different viruses on epigenetic regulation during integration or latency will be discussed below. An overview of the viruses to be discussed and the forms of epigenetic regulation and methods of detection are shown in Table 1.

2 Polyomaviruses

2.1 Overview

The polyomavirus family consists of a number of related small DNA viruses including mouse polyomavirus, Simian Virus 40 (SV40), human Merkel cell virus, human JC virus, and human BK virus as well as others. The viruses are characterized by circular double strand genomes of approximately 5,000 basepairs organized with a regulatory region containing the origin of DNA replication and transcriptional regulatory sequences controlling the early and late genes located on either side of the regulatory region (Fig. 2). SV40 is not considered a human pathogen although SV40 DNA sequences are occasionally found in human tumors. Merkel cell virus, JC virus, and BK virus were all isolated from clinical samples. The latter viruses appear to be present in human hosts without causing disease. The viral DNA present in virions and infected cells is organized with cellular histones in typical chromatin structures and is known as a minichromosome [27]. With respect to epigenetic regulation, SV40 is the most extensively characterized polyomavirus and will be discussed first in detail. Because of the overall similarity of the viruses in this family, it is likely that the other polyomaviruses will show similar patterns of regulation.

2.2 Epigenetic Regulation of an SV40 Lytic Infection

The relationship between histone modification and the SV40 lytic infection has been studied using a number of variations of chromatin immunoprecipitation (ChIP) followed by PCR amplification. The histone modifications most extensively studied have been acetylation of H3 and H4 and methylation of H3 on lysine 4 and 9, and H4 on lysine 20. Because of the small size of the SV40 minichromosome and the ability to obtain fairly large quantities of material, one technique which has been particularly useful for these studies was immune selection and fragmentation followed by immunoprecipitation (ISFIP). In this modified ChIP procedure SV40 minichromosomes containing a particular modified histone or bound protein are immune selected with an antibody to the target protein and bound through the antibody to agarose. Following removal of all the nonspecifically bound minichromosomes the immune selected minichromosomes are fragmented by sonication and the minichromosome fragments remaining bound to the agarose separated from the unbound fragments. The chromatin bound to agarose is gently eluted and both forms of fragmented chromatin then subjected to a second ChIP using a second antibody targeting a protein or modification of interest. For example, to study the histone modifications associated with transcribing SV40 minichromosomes antibody to RNAPII can be used to select the transcribing minichromosomes. Importantly, because the immune selected minichromosomes were fragmented, it is possible to distinguish changes in histone modification occurring in regions where the RNAPII or protein of interest is bound compared to regions which do not contain RNAPII or the other protein. Again because of the small size of the minichromosome and quantities available, ChIP-SEQ and ChIP on chip would be very useful to identify the location of histone modifications related to the phasing of nucleosomes in immune selected minichromosomes.

In virus particles (virions) the SV40 DNA is organized into chromatin [27]. For many years it has been known that the histones H3 and H4 in present in these virions can be found acetylated [27]. More recently, it has been shown that the SV40 chromatin in virions can also contain H3K9me1, H3K9me2, H3K9me3, and H4K20me1 along with the hyperacetylated H3 and H4 based upon analyses of minichromosomes from disrupted purified virions using standard ChIP techniques [18]. Moreover, we have found that the presence of histone modifications on the SV40 minichromosomes present in virions is variable. We have identified distinct classes of minichromosomes in infected cells and virions which we have referred to as epigenomes which contain specific combinations of histone modifications [18]. In virions we have identified at least three classes including one that consists primarily of H3K9me3 with a little H4K20me1, H3K9me2 and hyperacetylated H4, a second class containing H3K9me1 along with H3K9me2, H4K20me1, and hyperacetylated H4, and a third class containing hyperacetylated H3 and H4 along with H4K20me1 (Milavetz, unpublished observations). Importantly, the pattern of histone modifications present in the epigenomes of virions reflects the circumstances of the infection in which the virions were produced and thus carry trans-generational epigenetic information which can profoundly impact the initiation of a subsequent infection [18]. The epigenetic heterogeneity of the minichromosomes present in SV40 virions raises a number of interesting questions. Is there a specific epigenome responsible for the initiation of early transcription, or can any epigenome serve as a substrate for early transcription? The answer

to this question will help to clarify the mechanism of early transcription. If one or more of the epigenomes is preferentially involved in the initiation of early transcription what is the function of the other epigenomes? How do they contribute to the infection?

All of the histone modifications observed in the minichromosomes in virions are also present in the minichromosomes that are transported to the nucleus during the early stages of an infection although the relative proportion of each modification may change [17, 18]. In particular from 30 min post-infection until approximately 10 h post-infection there is an increase in the relative level of H4K20me3 and a decrease in the levels of H3K9me1 and hyperacetylated H4 along with an overall decrease in the amount of the SV40 chromosomes present in the cell [17, 18, 28]. It was proposed that the reduction in SV40 chromatin along with changes in histone modification represented an inherent ability of the infected cell to recognize foreign chromatin and degrade that chromatin [17].

Transcription of the incoming SV40 minichromosomes from virions occurs very quickly during an infection. Within an hour of exposure to the virus early messenger RNA can be detected by RT PCR and RNAPII can be found bound to SV40 minichromosomes using ChIP analyses [29]. However, it has not yet been determined whether any of the incoming SV40 epigenomes can serve as the substrate for the initiation of transcription or if only a subset can bind RNAPII. The RNAPII can be found in the promoter, early region and late region at this time with highest occupancy on the early region, less on the promoter, and least on the late region [28]. These results are consistent with the physical organization of the SV40 genome and an absence of an insulator sequence between the early and late transcription units.

Hyperacetylated H3 and H4 are also found preferentially associated with the early region during the initial phases of an infection [28]. However, the presence of hyperacetylated H3 and H4 was found to be a dynamic process. Using the ISFIP/ReChIP procedure hyperacetylated H3 and H4 were shown to be preferentially associated with regions of the SV40 minichromosome which also contained RNAPII. In fact no unacetylated H3 or H4 were found in chromatin along with RNAPII. In regions where RNAPII was not present the levels of hyperacetylated H3 and H4 were much lower [11]. Moreover, the histone acetyltransferase p300 was also found closely associated with RNAPII [11]. This led to the suggestion that p300 was an integral component of the RNAPII transcription complex responsible for hyperacetylating H3 and H4 in front of the transcription complex.

During the course of an SV40 infection early transcription is repressed by binding of the product of transcription, T-antigen, to a regulatory sequence found in the early promoter [27]. Thus, the SV40 system can serve as a useful model to study the epigenetic regulation associated with transcriptional repression. The epigenetics of early repression has been extensively studied using a mutant of SV40 which contains a deletion of the T-antigen binding site known as cs1085.

Repression of early transcription is associated with a significant reduction in the fraction of RNAPII bound to the promoter which is reversed in the mutant unable to repress early transcription [29]. Correspondingly repression also results in a significant reduction in the

presence of hyperacetylated H3 and H4 located in the early region. Not surprisingly this reduction is reversed in the mutant confirming the direct relationship between the presence of RNAPII and hyperacetylated histones [30].

T-antigen mediated repression of transcription at early times also affects histone methylation. Based upon a comparison between the wild-type and mutant viruses, the only effect of T-antigen mediated repression during active transcription was to inhibit the introduction of H3K9me2. No effects were observed on H3K9me1 or H3K9me3. Methylation of H3K4 was not studied since the levels of H3K4 methylation at this time were very low [16].

Between 12 and 24 h post-infection late transcription and DNA replication both begin with late transcription preceding replication by a few hours [27]. Like early transcription, late transcription is also closely linked to histone hyperacetylation with the hyperacetylated histones preferentially associated with regions of chromatin which contain RNAPII [11, 30]. Also like early transcription, late transcription is dynamic with p300 bound to RNAPII and responsible for histone hyperacetylation [30]. P300 was shown to be required to direct histone hyperacetylation for active late transcription to occur using siRNA knockdown of p300 [30].

Following the initiation of late transcription and DNA replication there is an increase in all forms of methylated H3K4 and H3K9 [18] and an increase in H4K20me1 [17]. Using the ISF/ReChIP technique the relationship between the different forms of modified histones was investigated at the late times [18]. The modified histones were shown to be nonrandomly associated with certain modified histones typically found together. Based upon the association between histone modifications, it was suggested that the SV40 minichromosomes present at this time were heterogeneous and consisted of at least five different epigenomes defined by the presence of specific combinations of modified histones. One epigenome consisted of H3K4me2 with H3K9me1, H3K9me2, H3K9me3, and HH4. A second also contained H3K4me2 but with HH4 and HH3. A third contained H3K4me3 with HH4, while a fourth contained H3K9me1 with some H3K9me2, H4K20me1, and HH4. Finally a fifth epigenome appeared to consist essentially of only H3K9me3. ISF/ReChIP was also used to show that H4K20me1 was present at low levels on transcribing minichromosomes in regions devoid of RNAPII at late times but not early times [17]. None of the methylated forms of H3 were found in association with transcribing minichromosomes at late times (Milavetz, unpublished observations).

The relationship between DNA replication and the introduction of methylated H3 has been directly investigated. Using a novel two step strategy with one antibody bound to agarose beads and a second antibody bound to magnetic beads called DAM chips, H3K9me1 was found to be present in actively replicating mini-chromosomes that contain the DNA replication protein RPA70 [31]. The role of DNA replication in the introduction of methylation was also investigated using the DNA replication specific inhibitor aphidicolin. Inhibition of replication by aphidicolin completely blocked the introduction of H3K4me1 and H3K9me1 into SV40 minichromosomes, but had little or no effect on the introduction of the di-methylated or tri-methylated forms of H3K4 and H3K9 [16]. This indicated that only

H3K4me1 and H3K9me1 were introduced along with active replication, while the di- and tri-methylated forms of H3K4 and H3K9 were being introduced without the necessity of active replication. Presumably other mini-chromosomes besides the actively replicating minichromosomes were acting as substrates for these modifications.

Repression of early transcription by T-antigen was also investigated at late times in infection using the mutant cs1085 [18]. Surprisingly repression at late times during active replication did not yield the same result as repression at early times when only transcription was occurring described above. During active replication repression resulted in the introduction of H3K9me1 and to a lesser extent H3K4me1 [18]. Instead of replication resulting in the conservation of preexisting chromatin structure, in the SV40 system replication appeared to serve as a switch in which new epigenetic information could be introduced [16, 18].

In the final stage of a lytic infection SV40 minichromosomes are bound by the coat proteins VP1, VP2, and VP3 and a new virion is produced. Comparing the histones modifications present at late times to those present in virions, it is clear that encapsidation is a selective process. There is enrichment for hyperacetylated histones [27] and very little if any methylated H3K4 [18]. The reasons for the selective encapsidation of minichromosomes containing either hyperacetylated H3 and H4 or methylated H3 and hyperacetylated H4 are not known at this time but seem likely to be related to the epigenetic consequences of these modifications.

DNA methylation does not appear to play a role in the epigenetic regulation of SV40 or the other polyoma viruses. The simplest explanation for the lack of DNA methylation regulation comes from an analysis of the DNA sequence of polyomaviruses. CpG which is the typical substrate for methylation in eukaryotic cells is very significantly under-represented in the genomes of the polyomaviruses which have been analyzed including SV40, JC virus, and BK virus with only 27 CpGs present in SV40, 16 present in JC virus, and 12 present in BK virus [32, 33]. The absence of DNA methylation during a lytic infection with BK virus has been confirmed [34].

2.3 Epigenetic Effects on Cellular Expression and Cellular Transformation

There have not been any specific studies investigating the genome-wide epigenetic consequences of an SV40 infection. However, T-antigen is known to interact with modulators known to be involved in cellular epigenetic regulation including p300 [20, 35], CBP [20, 36, 37], HDAC1 [36], HDAC3 [37], and lysine methyltransferase [38] and it is reasonable to expect that dysregulation of these modulators would have genome-wide consequences. Recently it has been shown that T-antigen's interaction with CBP/p300 results in an increase in the activity of the acetylase and a corresponding increase in the levels of acetylation of H3K56 and H4K12 [35]. This result confirms that T-antigen's interaction with histone acetylases can result in a cellular genome-wide effect on histone acetylation.

SV40 transforms cells through the interaction of its large T-antigen with p53 and Rb and the interaction of its small t antigen with PP2A [20]. As noted above large T-antigen binds to a number of potential epigenetic regulators and would be expected to dysregulate a number

of cellular genes through modulation of the levels of the epigenetic regulators. The increase in histone acetylase activity by T-antigen could account at least in part for the increase in gene transcription for many genes typically seen in transformed cells.

2.4 Epigenetics of Other Polyomaviruses

The other polyoma viruses have been much less studied with respect to epigenetic regulation. Nevertheless, one epigenetic process which appears to be consistent across the family of polyomaviruses is histone acetylation. The virions of mouse polyoma virus like SV40 contain hyperacetylated forms of H3 and H4 [39]. In addition, mouse polyoma virus large T antigen interacts with the histone acetyltransferases PCAF and GCN5 suggesting one possible way that the polyoma virus histones become methylated [40]. Polyoma virus small T also has been shown to play a critical role in regulating histone hyperacetylation presumably through control of the lytic infection and dysregulation of the cell cycle [41].

JC virus also appears to be regulated by acetylation. Both early transcription and late transcription appear to be stimulated by inhibitors of histone deacetylase and co-transfection with a vector expressing p300 results in a stimulation in the level of transcription [42]. The other human polyoma viruses Merkel and BK have not been investigated yet with respect to their epigenetic regulation.

2.5 DNA Methylation Effects of Polyomavirus Integration

In non-permissive cells, the polyomaviruses frequently integrate into the host cell genome with resulting dysregulation of both viral and cellular genes and transformation of the cells. Surprisingly there have been only a few reports on the role that DNA methylation plays in this transformation process. SV40 integration in fibroblasts has been shown to result in a marked increase in the level of DNA methyltransferase activity and corresponding increase in the level of methylation of the promoter of alpha2 collagen VI [43]. In human mesothelial cells the integration of SV40 was shown to have minimal effects on methylation in early passage cells but showed a significant increase in methylation on late passage when morphological changes in the cell were also observed. The morphological changes were correlated with an increased methylation of the RASSF-1 gene which is frequently found repressed in mesotheliomas [44]. The RASSF-1 has also been shown to be hypermethylated in Merkel cell carcinoma in conjunction with integration of the Merkel cell virus [45].

3 Adenoviruses

3.1 Overview

The adenoviruses (Ad) are a family of nonenveloped viruses whose genome consists of a small linear double strand of DNA of approximately 30–40 kb in size. Like the polyomaviruses the Ads have a similar lytic life cycle consisting of binding to a cell, transport of the genome to the nucleus, early transcription, replication, late transcription, and generation of new packaged virus. Adenoviruses are associated with human respiratory infections [46]. Within the virion Ad DNA is complexed with three virus coded polypeptides, V, VII and Mu [47–51]. Protein VII a highly basic protein similar to protamine is thought to condense the Ad DNA [52]. The interaction between Ad DNA and

these proteins has been reported to result in a chromatin-like repeating structure [53]. However, since histones are not involved it is unlikely that the complexes in virions will be regulated through the same epigenetic pathways associated with chromatin containing histones.

Because Ad DNA was associated with viral proteins in the virion, there was significant interest in determining the corresponding organization of the DNA in the nucleus of infected cells. The initial reports using available technology of the 1970s and 1980s were conflicting, with some laboratories reporting that intracellular DNA remained bound to the viral protein VII and others reporting that the Ad DNA was associated with histones into nucleosomes [54–58]. More recently the question has been reexamined using ChIP technology with the result that there is general consensus that at least some of the Ad DNA is organized as chromatin during the course of the infection [59–63].

During the initial stages of an infection the Ad genome is thought to be transported to the nucleus in association with VII [64–66]. Within approximately 1 h of infection the Ad DNA becomes associated with cellular histones although there is also evidence that the VII remains associated as well [60]. As noted above there are two major variants of histone H3, a replication dependent variant, H3.1, and a replication independent variant, H3.3. Based upon ChIP analysis it is the replication independent variant H3.3 that is deposited onto the incoming Ad genome during the initiation of infection [62]. Interestingly, when Ad DNA is transfected into cells as an expression vector it also becomes associated with histones very rapidly [61]. The organization of the infecting Ad genome with histones appears to be biologically significant, since siRNA knockdown of the H3.3 chaperone HIRA leads to both a reduction in the introduction of H3.3 and a reduction in expression of early genes [61]. Moreover, acetylation of H3 on early genes increases for the first hours of infection consistent with the general observation that histone acetylation is associated with genes undergoing active transcription at early times [61, 63]. Recently, CTCF has been shown to play a critical role in the replication dependent shift to late transcription [67]. However, the status of the histone modifications on the Ad DNA was not addressed.

At late times in infection there appears to be a shift in the protein complement associated with the newly replicated Ad DNA from histones back to protein VII in order to compact the DNA for virion formation. This has been hypothesized to be due in part to the ability of the virus at late times to inhibit cellular histone synthesis [57, 68, 69].

With the exception of the ChIP studies, demonstrating that intracellular Ad DNA is organized as chromatin and can be acetylated described above, there has not been an extensive characterization of the role of histone modifications in the regulation of the genes of Ad. However, it seems that this is an area that is likely to see much growth since like the polyomaviruses the Ad viral life cycle is very likely to be regulated through epigenetic mechanisms.

3.2 Epigenetic Effects of Adenoviruses on Cellular Expression and Cellular Transformation

One hallmark of infection by Ad is the relatively rapid dysregulation of cellular gene expression. Within 24 h of infection the expression of most cellular genes is repressed while

a few genes required for cellular replication are induced [46]. Since such an extensive change in expression was likely to be associated with corresponding changes in epigenetic regulation, the organization of certain histone modifications and transcription cofactors within infected cells has been analyzed by ChIP-chip and ChIP-seq strategies [25, 63]. In these genome-wide studies acetylation of H3K9 and H3K18 was followed along with certain transcription factors known to be affected by the e1a protein of Ad during the course of infection including retinoblastoma (Rb) family proteins and EP300 histone acetyltransferase. Consistent with the dysregulation of expression, many of the genes observed to be repressed during infection demonstrated a significant reduction in the levels of acetylated H3K18 with less effect on acetylation of H3K9. Moreover, the replication associated genes which were induced became associated with acetylated H3K18 following infection. Interestingly, the acetylated H3K18 was found more or less throughout the genes induced for expression [63].

3.3 Epigenetic Consequences of Integration of Ad

Ad causes tumors in appropriate hosts in part by integrating its genome into the genome of the host cell [46]. Insertion of a foreign DNA into the cell genome might be expected to have significant epigenetic consequences and for this reason the effects of integration on the extent of DNA methylation in the Ad and the cellular DNA has been investigated. Ad DNA in virions and in the nucleus of a lytically infected cell is not methylated [70, 71].

The effects of Ad integration on DNA methylation of the integrated Ad DNA, proximal cellular DNA, and distal cellular DNA have all been analyzed using bisulfate sequencing. Not unexpectedly, the Ad DNA is organized like the rest of the cellular DNA in typical chromatin structure [72]. Within the integrated Ad DNA there tends to be significant methylation of most of the Ad DNA with the exception of parts of the early region [72, 73]. The regions of Ad DNA which are methylated are also characterized by the presence of hypoacetylated H3 and H4 in the chromatin associated with the DNA.

Integration of Ad DNA also has effects on the methylation of cellular DNA located adjacent to the integration site. In comparison to the methylation patterns present in the DNA of cells prior to integration of Ad, there was a significant reduction in the levels of methylated DNA adjacent to the Ad DNA at the site of integration [74, 75].

Finally, integration of Ad DNA has effects on cellular DNA at sites distal to the site of integration [76]. For example, within a region of DNA known as the intracisternal A particle retrotransposon there was a significant increase in DNA methylation following integration of Ad DNA. However, it should be noted that changes in DNA methylation were also observed when non-transforming DNAs were integrated into cellular DNA so that the latter effect may be more a result of a general phenomenon associated with integration of foreign DNA than one specific to Ad DNA [77]. What is most interesting from the epigenetic perspective is the observation that the changes in DNA methylation in the cellular chromatin persist even after the integrated Ad is lost. Revertants which had lost the integrated Ad DNA continued to maintain the DNA methylation patterns which resulted from the original introduction of the Ad DNA [76].

4 Human Papillomavirus

4.1 Overview

The human papillomaviruses (HPVs) are a family of nonenveloped viruses whose genome consists of approximately 8 kb of double strand closed circular DNA [46]. The family is quite large with a number of members capable of causing human disease from warts to cancer. The high-risk papillomaviruses are now thought to be the causative agent for most of the cervical cancers in the world, many anogenital cancers, and head and neck cancers. The life cycle of papillomaviruses is very similar to the other small tumor viruses with one major exception. The lytic cycle is tied very closely to the differentiation status of the infected cell. The virus infects the basal layer of epithelium where it expresses its early genes and undergoes replication. As the cell differentiates the virus switches to late genes and new virions are generated in the terminally differentiated cells. This complex lytic cycle has made the epigenetic characterization of the virus challenging.

4.2 Epigenetic Regulation of a Papillomavirus Lytic Infection

There have been two reports concerning the role of DNA methylation in the epigenetic regulation of a lytic HPV16 infection [78, 79]. Both of these reports have focused on methylation within the long control region (LCR) which regulates transcription of early genes in non-integrated episomal HPV. In the former study using bisulfite treatment to distinguish methylated from unmethylated DNA, the LCR was found to go from a methylated to an unmethylated state during the course of cell differentiation and corresponding virus production. The latter study used laser microdissection to isolate cells at various stages of differentiation including transformed cells, and tested for the presence of methylated HPV16 in the various cells using bisulfite treatment and sequencing. This study also demonstrated the shift from methylated to unmethylated in the LCR. But this study also showed that one specific region of the LCR, the E2 viral protein binding site became hypermethylated during this transition, despite the overall reduction in methylation. The status of histone modifications in HPV 31 during a lytic infection has also been addressed. Hyperacetylated H3 and H4 have been found in both the early and late regions prior to and following differentiation of the host cells [80]. Because significant differences were observed in this study for transcription factor binding, it was concluded that the factor binding was likely playing a larger role in control of gene expression than the acetylation of H3 and H4.

4.3 Epigenetic Consequences of HPV Integration

HPV causes cancer usually through the integration of one or more copies of the virus into the host cell genome and subsequent dysregulation of viral and cellular gene expression. Because the change from a lytic infection to cancer goes through a series of pathologically defined stages it is possible to analyze the different stages of the disease progression for specific forms of DNA methylation in the virus genome. A number of laboratories have studied the role of DNA methylation within the viral genome and the cellular genome following the establishment of a neoplastic transformation (reviewed in ref. 81). Almost all of these studies have relied on bisulfite treatment followed by PCR, cloning, and sequencing. There appears to be consensus that disease progression and deteriorating

prognosis is associated with increased methylation of regions of the late structural proteins [81]. For example, in a set of samples taken from women with increasing severity of disease there was increased methylation of L1, L2, and the regulatory protein E2 [82]. Analysis of the LCR for methylation has led to less consistent results. In some cases the LCR appeared to be hypermethylated while in other studies the LCR was hypomethylated, for example [83, 84].

The effect of HPV integration on methylation of host genome genes has also been analyzed [85, 86]. Using either laser capture dissection of HPV positive and negative head and neck cancer samples in conjunction with either arrays or MeDip-seq technology, 453 differentially methylated regions were identified many of which were located in gene regulatory regions. Of potentially greatest significance was the dysregulation by methylation seen in the cell adhesion genes for cadherin.

While no studies to date have specifically focused on the effect of HPV integration on changes in histone modifications during disease progression, ChIP-seq data generated in other studies using HeLa cells which are transformed with HPV18 have been reanalyzed to identify the histone modifications associated with the viral genome [81]. Histone modifications generally thought to be associated with gene activation including H3K4me1, H3K4me2, H3K4me3, H3K9ac, H3K27ac, H3K36me3, and H3K79me2 were all associated with the HPV18 LCR. In addition, a number of transcription binding factors appeared to be present within the LCR. Taken together these results suggest that the integrated copies of HPV18 in HeLa cells are transcriptionally active [81].

5 Herpesviruses

5.1 Overview

The herpesviruses are a family of enveloped viruses characterized by a double strand of linear DNA between approximately 150 and 250 kb in size. The viral genome is organized as a series of genes with terminal repeats at each of the ends of the linear DNA. Following infection the terminal repeats serve to circularize the genome and allow the virus to exist in the nucleus of an infected cell as a circular episome. Interestingly, the viral DNA in the infecting virion is not chromatinized but upon episome formation becomes chromatinized like cellular chromatin. The episome can either exist as a fully functional virus capable of replication and the generation of daughter virus, or the episome can exist in a latent form in which only a limited number of viral control elements coming from the early regions of the virus are produced with no replication or subsequent daughter virus production. Unlike the other viruses discussed which integrate as part of the transformation of normal cells into cancer cells, the cancer causing herpesviruses transform as a consequence of the latent infection. Because cells can be either lytically infected or latently infected with subsequent reactivation of the virus, there has been a great deal of interest in investigating the role that epigenetics plays in control of these biological processes.

Importantly, two members of the herpesvirus family, Epstein-Barr virus (EBV) and Kaposi's sarcoma herpes virus (KSHV) have been shown to be at least partially responsible for human cancers. EBV appears to be the causative agent for Burkitt's lymphoma and is

associated with other lymphoproliferative diseases such as Hodgkin's lymphoma and nasopharyngeal carcinoma (reviewed in ref. 87, 88). KSHV appears to be the causative agent for Kaposi's sarcoma and is linked to other B cell lymphomas [89, 90].

5.2 Epigenetic Regulation of an EBV Infection

The role of epigenetic modification in the regulation of EBV latency has recently been analyzed using a newly developed software program and existing high-throughput ChIP-derived sequencing data from many laboratories [91]. This extensive analysis has focused on histone modifications, location of transcription factors and cofactors, nucleosome locations, and DNA methylation yielding a fairly complete picture of the organization of epigenetic regulators during EBV latency. During EBV latency 9 proteins, 21 microRNAs, and several noncoding RNAs are expressed which are associated with B-cell proliferation and survival. The proteins involved in lytic replication and subsequent virion formation are repressed.

Not surprisingly, RNAP II and RNAPIII, responsible for transcription of many of the latency protein and RNA genes, are located at the appropriate sites in the EBV genome along with H3K4me1, me2 and me3. Similarly, acetylated H3K9 and H3K27 are also located at these genes consistent with transcription. EBV does not appear to contain any regions with bivalent histone modifications as observed in KHSV (*see below*) [91] or specific regions enriched with the potential repressive mark H3K9me3. Significantly, certain latency genes containing RNA polymerase also contain a number of transcription cofactors including TAFs, GCN5, and P300 with potential epigenetic consequences [91].

DNA methylation is not found in the EBV genome derived from virions but is present in EBV episomes during latent infection. DNA methylation is found throughout the genome with the exception of certain of the latency genes [92, 93].

5.3 Epigenetic Consequences of EBV on the Cell

Because of the well-known ability of EBV to transform cells during a latent infection, there has been a great deal of interest in determining how the virus redirects the normal cellular expression patterns to that of a transformed cell. Increasingly epigenetics has been implicated in this process. A number of genome-wide and gene specific studies have investigated the binding partners of the latency proteins [94, 95], changes in gene expression [96, 97], changes in histone modifications [96, 98, 99], and changes in DNA methylation [97, 100, 101]. Latent infection by EBV appears to cause a significant increase in transcription of a number of cellular genes. This increase is associated with an overall reduction in the levels of putative repressive histone modifications including H3K9me3 and H3K27me3 as well as H3K4me3 [98]. Transformation is also associated with hypomethylation of the activated cellular genes [96, 97, 100] and hypermethylation of certain tumor suppressor genes [101]. These changes in gene methylation are consistent with the changes in the levels of gene expression. While a number of questions remain unanswered concerning the specific mechanisms by which EBV directs these epigenetic modifications the picture is becoming clearer how the changes resulting from infection can be maintained epigenetically even when the virus itself is lost from the infected cell.

5.4 Epigenetic Regulation of a KHSV Infection

The role that epigenetics plays in a KHSV infection has been recently reviewed [102]. Since this review is quite extensive, in this section we will focus only on some of the major aspects of epigenetic regulation. The role of histone modification in KHSV infection has been assessed in two cell lines using a ChIP-on chip strategy [102, 103] focusing on acetylated H3K9/14, H3K4me3, H3K9me3, and H3K27me3. In general the early genes responsible for establishing latency have H3K4me3 and acetylated H3K9/14 suggesting transcription while the silent genes have H3K9me3 and H3K27me3 suggesting repression. The genes associated with reactivation tend to contain bivalent epigenetic marks including H3K4me3 and H3K27me3.

Not surprisingly, acetylated H3K9/14 was found to be enriched on certain genes during latency and to be increased at these genes upon reactivation based upon ChIP-chip analyses [102, 103]. These results are consistent with a low level of transcription during latency and increased transcription during reactivation. When latently infected cells are treated with HDAC inhibitors reactivation of the virus is typically observed presumably as a consequence of the increased transcription at these genes. Two specific latency associated genes have been studied with respect to the role of histone acetylation during reactivation. The RTA promoter was shown to be hypoacetylated and associated with HDAC1, 5, and 7 during latency, while upon reactivation the HDACs are lost and acetylation occurs [104]. Along with the change in acetylation, a disruption of a nucleosome within the promoter was also observed. Histone acetylation was also observed in the origin of latent replication region of the genome where recruitment of DNA replication factors during latency occurs [105].

Interestingly the presence of H3K9me3 was inversely correlated with the presence of JMJD2A and KDM3A/JMJD1A the demethylases responsible for removal of methyl groups from H3K9 [106]. Instead, the demethylases were co-located at sites containing H3K4me3 and acetylated H3. This observation suggested that the role of the demethylases might be to prevent the introduction of H3K9me3 at locations which would be subsequently involved in reactivation [106]. LANA which is involved in reactivation was shown to collocate with JMJD1A at sites depleted for H3K9me2 [107]. Together these results suggest that histone demethylases may be directed to critical gene locations to prevent the introduction of H3K9 potentially repressive histone modifications.

H3K4me3 also appears to play a critical role in the regulation of KHSV gene expression. H3K4me3 is located on critical genes during latency and the amount of H3K4me3 present increases during reactivation [102, 103].

DNA methylation has also been implicated in the regulation of latency. First, treatment of cells latently infected with methylation inhibitors results in reactivation of virus [108]. Second, MeDIP analysis indicates that DNA methylation occurs extensively throughout the KHSV genome during latency with the exception of the latency-associated locus [103]. However, the DNA methylation was found to occur slowly during the establishment of a latent infection unlike the changes described above for histone modifications which occurred rapidly [103]. The slower time course for the introduction of DNA methylation compared to

histone modifications suggests that methylation is unlikely to be involved in establishing the latent chromatin structures but instead serve to ensure that when established that they are maintained.

5.5 Epigenetic Consequences of KHSV on the Cell

KHSV is also capable of reprogramming the epigenetics of cellular genes. LANA an early KHSV gene product associated with latency of the viral episome [109] has been implicated in modulating the expression of certain master transcriptional factors [110]. LANA is also capable of interacting with a large number of cellular chromatin modifiers including the histone acetyltransferase CBP [111]. Consistent with this capability LANA has been found bound to a number of sites in cellular chromatin using ChIP-seq [112].

6 Conclusions

Since the development of simple and rapid procedures like ChIP, MeDIP, and deep sequencing to analyze chromatin for nucleosome and binding factor location, histone modifications, and DNA modifications, there has been a huge increase in our understanding of the relationship between chromatin structure and the life cycle of tumor causing DNA viruses. Nevertheless, the field remains at a very early stage of development. Most of the published studies to date are primarily associative. That is, epigenetic changes are correlated to biological events such as the regulation of specific gene expression. Because of the emphasis on correlations at this time, there have been conflicting results where a particular epigenetic mark results in different biological consequences depending upon the system being studied. These conflicts are likely to be resolved in the next few years as the focus of research shifts to the mechanisms which signal the introduction of epigenetic marks and the reading of existing marks.

In addition to the new emphasis on mechanisms defining epigenetic regulation, there are a number of specific questions that are likely to be answered in the coming years. For those viruses like adenovirus and herpesvirus which become chromatinized upon infection, it is still not known how chromatin is formed and how the newly formed chromatin influences the initiation of appropriate early transcription. Similarly, for the viruses that exist as chromatin throughout their life cycle like the polyomaviruses and HPVs it is not clear how selective activation for early transcription occurs in the context of infecting chromatin. Does activation occur on a particular epigenome or can any infecting epigenome regardless of the epigenetic marks which it carries serve as a substrate for the initiation of transcription? Later in infection, when multiple genes may be transcribed along with replication and encapsidation, how does epigenetics contribute to the control of the various pool sizes of the functional chromosomes? In SV40 we find that the pool of chromosomes containing RNAPII is fairly constant for a given set of conditions. How is this regulated? Finally, the role of DNA replication in epigenetic regulation needs to be further characterized. To what extent does replication play a role in conserving existing epigenetic marks compared to our demonstration that replication can serve to switch epigenetics marks? As these questions are answered it is likely that a much better understanding of the role that epigenetics plays in the regulation of biological function will become evident.

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Fig. 1. Major forms of epigenetic regulation

Fig. 2. Schematic representation of the polyomavirus genome. The organization of a typical polyomavirus (SV40) is shown. The early proteins T-antigen and t-antigen, the late proteins VP1-4 and the agnogene protein, and the origin of replication (ori) within the regulatory region are indicated

Fig. 3. Schematic representation of the papillomavirus genome. The organization of a typical oncogenic human papillomavirus (HPV16) is shown. The early proteins E1–7, the late proteins L1 and L2, and the origin of replication (ori) within the upstream regulatory region (URR) are indicated

Fig. 4. Schematic representation of the adenovirus genome. The organization of a typical adenovirus (adenovirus 5) genome including the early proteins E1–4, the late proteins L1–5, and the terminal repeats (ITR) are indicated

Fig. 5. Schematic representation of the herpesvirus genome. The organization of the episomal form of a typical oncogenic human herpesvirus (EBV) is shown. The location of the latency proteins EBNA1–3, EBNA-LP, LMP1, and LMP2 are indicated. The lytic origin of replication is also indicated (ori)

Table 1
Forms of viral epigenetic regulation and methods of detection

Virus	Form	Method of detection		
		DNA methylation	Histone variants	Histone modifications
Polyoma Virus—SV40	Virus	NA	ND	ChIP [16, 18] ISFIP/ReChIP [11, 30] DAM ChIP [31]
	Cell	Methylation-specific PCR [44]	ND	ND
Polyoma Virus—other	Virus	NA	ND	Gel electrophoresis [41] Gel electrophoresis and Western blotting [39]
	Cell	Methylation-specific PCR [45]	ND	ND
Adenoviruses	Virus	Radioactivity based [70] Restriction endonuclease [71] Bisulfite [72, 73]	ChIP [62]	ChIP [61, 63]
	Cell	Restriction endonuclease [74 – 76]	ND	ChIP-ChIP [25] ChIP-Seq [63]
Human papilloma virus	Virus	Bisulfite [78, 79]	ND	ChIP [80]
	Cell	Bead-arrays [86]	ND	ChIP-Seq [81]
		MeDIP-Seq [85]		
Herpesvirus—EBV	Virus	Restriction endonuclease [93]	ND	ChIP-Seq [91]
	Cell	Bisulfite [98, 99, 101]	ND	ChIP [96, 98, 100]
		Bead-ChIP arrays [97]		
Herpesvirus—KH	SV Virus	MeDIP [103]	ND	ChIP-ChIP [102, 103]
	Cell	ND	ND	ChIP-Seq [112]