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Avian retroviral replication

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Avian retroviruses were originally identified as cancer-inducting filterable agents in chicken neoplasms at the beginning of the 20th century. Since their discovery, the study of these simple retroviruses has contributed greatly to our understanding of viral replication and cancer. Avian retroviruses continue to evolve and have great economic importance in the poultry industry worldwide. The aim of this review is to provide a broad overview of the genome, pathology, and replication of avian retroviruses. Notable gaps in our current knowledge are highlighted, and areas where avian retroviruses differ from other retroviruses are emphasized.

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Introduction

The study of avian retroviruses began with the work of two Danish researchers, Vilhelm Ellerman and Oluf Bang, more than a century ago. In a seminal 1908 publication, they showed that a form of leukemia and lymphoma that afflicts chickens, called avian leukosis, could be transferred by cell free-filtrates [1]. This was the first evidence that viruses could cause cancer, but because leukemia was not recognized as cancer, the significance of their work was not appreciated at the time. Three years later however, the American scientist Peyton Rous discovered a second virus that induced sarcomas in chickens [2]. Rous sarcoma virus (RSV) has been studied extensively and has greatly aided our current understanding of both retroviral replication and cancer. We now know that RSV is derived from the more common avian leukosis virus (ALV). These and related viruses are collectively referred to as the avian sarcoma/leukosis viruses (ASLV) and will be the focus of this review because they are the most common and well studied of the avian retroviruses. Other types of retroviruses discovered in birds include the gammaretrovirus, reticuloendotheliosis virus (REV) [3], and lymphoproliferative disease virus of turkeys (LPDV) [4,5].

ASLV genome/subgroups

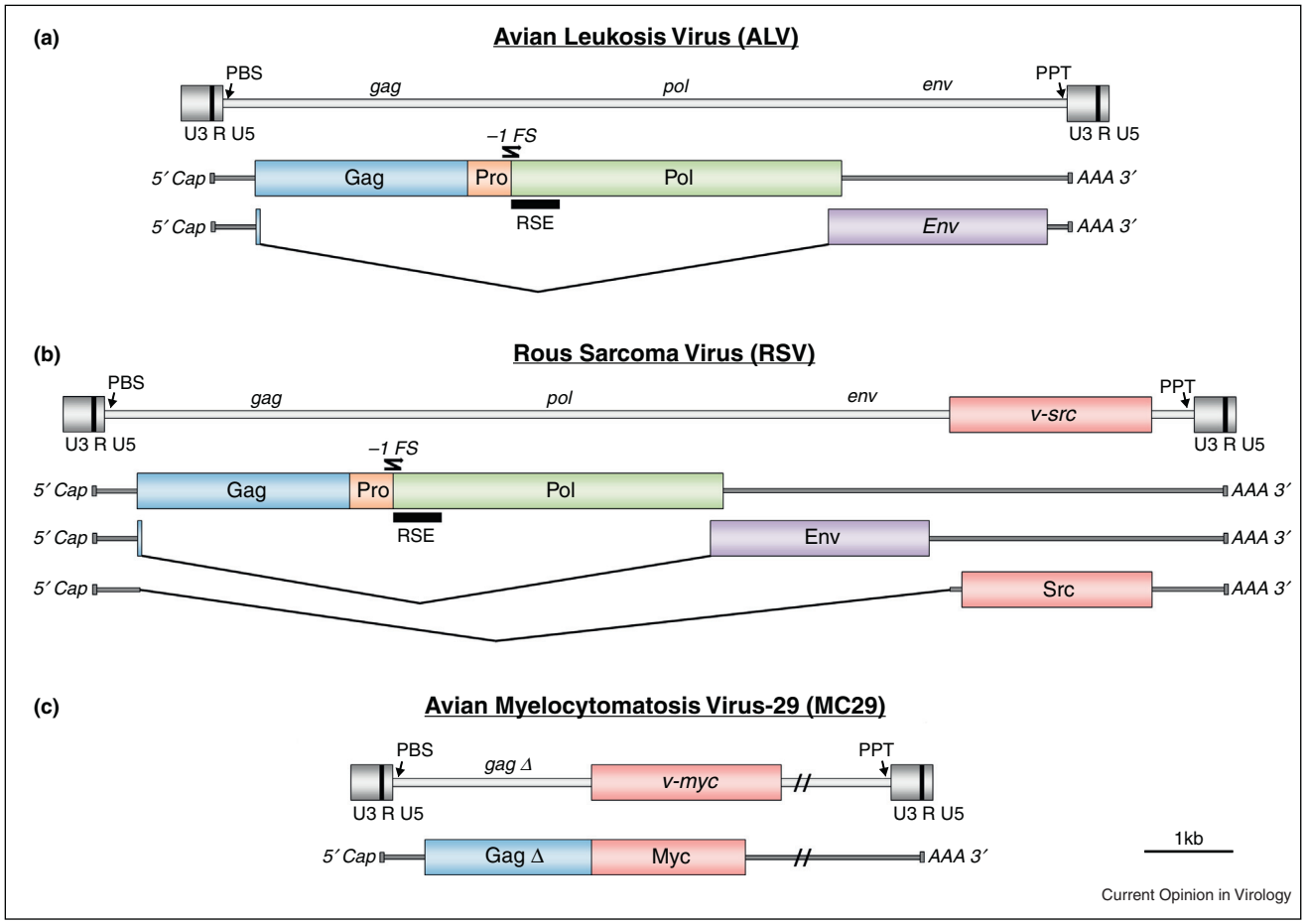
ASLVs are members of the Alpharetrovirus genus of the family Retroviridae [6]. The *gag-pol-env* gene order first determined for these simple retroviruses has proven to be common to all retroviruses. Unlike complex retroviruses, ASLVs do not encode accessory proteins in overlapping reading frames. The ASLV *gag* gene encodes the structural proteins capsid (CA), matrix (MA), and nucleocapsid (NC), as well as the viral protease (PR), which in most retroviruses is part of *pol*. Thus, ASLVs synthesize more PR than other retroviruses. The ASLV *pol* gene encodes the reverse transcriptase (RT) and integrase (IN) enzymes, and the *env* gene encodes the transmembrane (TM) and surface (SU) envelope glycoproteins. The viral genome is flanked on either side by long terminal repeats (LTRs). The most commonly studied ASLVs are classified into 6 distinct subgroups (A, B, C, D, E, and J) based on differences in their envelope glycoproteins, interference patterns, and host range in chicken cells of varying phenotypes. The chicken genome also encodes many endogenous retroviruses (ERVs), a minority of which are related to exogenous ASLVs [7••]. Surprisingly, the majority of the ERVs are related to betaretroviruses and gammaretroviruses, suggesting that they were once the dominant type of exogenous avian retrovirus [7••].

Pathology

Chickens are thought to be the natural hosts of ASLVs [8], although some wild bird species can also be infected [9]. The virus predominantly spreads horizontally by either direct or indirect contact, but can also be transmitted vertically from hen to egg.

ASLVs are divided broadly into two classes: slowly transforming and acutely transforming. ALVs (Figure 1a) make up the slow transforming class, causing tumors within a relatively long time frame of several weeks to months. ALV induces tumors by integrating within or near cancer-related genes, perturbing their expression or function. This process, first discovered with ALV, is called viral insertional mutagenesis and is common to other oncogenic simple retroviruses. Initial experiments detected ALV integration in the *myc* gene locus in tumors, causing deregulated expression of normal Myc protein from the ALV promoter [10•,11]. Similar integrations in the *myc* locus were seen with REV-induced lymphomas [12]. Subsequent work also detected common integrations into a locus called *bic* [13], which we now know is the precursor for oncogenic microRNA-155. Recently,

Figure 1



The ASLV proviruses and the transcripts of ALV **(a)**, RSV **(b)**, and MC29 **(c)** are shown. The location of viral oncogenes within the proviral genome are indicated when they exist. Protein coding portions of the transcripts are represented by colored rectangles. Locations of primer binding sites (PBS), RNA stability elements (RSE), polypurine tracts (PPT), and −1 frameshift sites (−1 FS) are indicated. Polyprotein cleavage sites are not shown. This figure was adapted from [62].

clonal ALV integrations into the telomerase reverse transcriptase (TERT) promoter were seen in B-cell lymphomas [14•]. In addition, ALV induces erythroblastosis by insertion into the EGF receptor (c-erbB) locus in certain chickens lines [15].

ALV-A is the most common ASLV found in commercial flocks. It most often induces lymphoid leukosis, a B-cell lymphoma that begins in the bursa of Fabricius and metastasizes to the spleen, liver, and occasionally other organs. Although the virus is common, only a small fraction of infected birds in commercial flocks develop neoplasms. In addition, selective breeding has had some success limiting viral spread, reducing the economic impact of the virus [16].

In contrast, a new virus with a new subgroup, ALV-J was discovered in the late 1980s. It has since spread rapidly resulting in great economic loss, especially in China. ALV-J is believed to have originated from a recombination event between ALV and an endogenous retroviral element [17–19]. The virus induces a different spectrum of tumors than ALV-A, primarily myeloid leukosis and hemangiomas [20,21]. The molecular basis of oncogenesis in these tumors is not well understood.

Acutely transforming viruses are distinct from ALV in that they contain one or two viral oncogenes in their genome, and induce neoplastic transformation more rapidly, within days or weeks. These viral oncogenes are derived from host cellular genes that have been incorporated into the virus by gene capture and are over-expressed by the strong viral promoters. For example, RSV picked up the v-*src* oncogene, a mutated version of the c-*src* tyrosine kinase gene found in host cells (Figure 1b) [22,23]. Many acutely transforming ASLV strains have been isolated, carrying a wide variety of viral oncogenes, including *myc*, *myb*, *fps*, *yes*, *jun*, *ets* and

erbB. With the exception of some strains of RSV, acutely transforming avian retroviruses are replication deficient (Figure 1c), requiring a helper virus to replicate [24].

Viral entry

The surface of all retroviral virions is studded with Env proteins, which bind to specific receptors on the target cell surface, beginning the infection cycle. Four distinct cellular receptors have been identified that mediate ASLV entry. The *tva*, *tvc*, and *chNHE1* [25] genes confer susceptibility to ASLV of subgroup A, C, and J respectively, while *tvb* encodes the host cell receptor for subgroups B, D, and E. Two different alleles of *tvb* have been identified, tvb^{s1} and tvb^{s3}, which confer susceptibility to ASLV subgroups B and D, and B, D, and E respectively [26–28]. ALV has a broad cellular tropism, able to replicate in a range of tissues and organs in the chicken [29,30].

Once bound to its receptor on the host cell, virion/cell fusion is activated through a two-step mechanism. First, the envelope glycoprotein undergoes a conformational change at the cell surface that mediates viral uptake and endosomal trafficking. Then, the acidic environment of the endosome activates hemifusion and release of the capsid into the cytoplasm [28]. Once in the cytoplasm, reverse transcription of the viral RNA can begin. Reverse transcription is an intricate multi-step process in which cellular dNTPs and packaged tryptophan tRNAs are utilized by the RT to convert viral RNA into a double stranded DNA provirus. Detailed reviews of the reverse transcription process are available elsewhere [31].

Nuclear entry/integration

Before nuclear entry, the DNA copy of the viral genome associates with integrase and other viral and cellular proteins to form the preintegration complex (PIC). The ability of the PIC to gain access to nuclear DNA varies among retroviruses. Gammaretroviruses can only integrate after nuclear envelope disassembly in mitosis, while lentiviruses are able to infect non-dividing cells via active transport into the nucleus [32]. For decades it was thought that alpharetroviruses, like gammaretroviruses, were only able to infect dividing cells [33]. Contrary to this idea, it was observed that cells infected with avian sarcoma virus (ASV) upon the release of serum starvation, could be stably integrated into host genomic DNA before mitosis [34], suggesting that nuclear envelope breakdown is not necessary for ASV integration. It is now thought that ASV can infect a variety of non-cycling cells [35–37,38•], and the ASV integrase contains a nuclear localization signal (NLS) that can mediate active transport of the ASV integrase through the nuclear pore [39]. This work, together with earlier observations [40], suggests a block at reverse transcription rather than nuclear import may be responsible for the low infectivity of ASLV in non-dividing cells [38•].

Once in the nucleus, the PIC mediates integration of the provirus into the host genomic DNA. Integration site selection varies among retroviruses, with some showing strong selection for certain areas of the genome. ASLV integration is more indiscriminant that most retroviruses, only slightly preferring areas of active transcription. This difference in target site selection is true regardless of whether the viruses are infecting chicken or human cells [41•]. The reason for this pattern of integration targeting has not been completely elucidated [42].

Transcription/splicing/nuclear export

Transcription of the ASLV provirus is directed by the viral LTRs, which contain strong promoter and enhancer sequences. Because ASLVs do not encode transcriptional transactivators, they rely entirely on host transcription factors, which bind to the U3 region of the LTR and drive RNA Polymerase II transcription of the provirus. All viral transcripts have an m^7G cap at the 5′ end and undergo 3′ end cleavage and polyadenylation by cellular machinery before export from the nucleus.

All replication-competent ASLVs produce a single primary RNA transcript. This full-length, unspliced viral RNA serves as mRNA for translation of *gag* and *pol* genes, as well as the genomic material that is packaged into new virions. In addition, a fraction of these primary transcripts are spliced to generate the subgenomic *env* mRNA. In the case of RSV, a second spliced transcript is generated which encodes the v-*src* oncogene (Figure 1b). Replication-deficient ASVs such as myelocytomatosis virus (MC29) (Figure 1c) typically produce only a single unspliced *gag-onc* fusion transcript.

The full-length viral RNA transcript (~7–9 kb) is an aberration in the cellular context, in which intron-containing transcripts are not usually exported from the nucleus for translation. To ensure successful replication, ASLV has evolved a variety of RNA elements that aid in the efficient transcription, export, and translation of these long unspliced viral RNAs.

For example, ASLV employs several mechanisms to protect these transcripts from splicing, such as suboptimal 3′ splice sites [43], and cis-acting RNA elements. One cis-acting element is the negative regulator of splicing (NRS). The NRS acts as a faux 5′ splice site by interacting with the 3′ splice site and recruiting components of the spliceosome. This sequesters the 3′ splice site away from the 5′ splice site and further reduces the efficiency of splicing [44•].

Unlike complex retroviruses, which encode accessory proteins that mediate nuclear export, ASLV relies on a 100 nt direct repeat (DR) RNA sequence. ALV employs a single DR in its 3′ untranslated region (UTR), while RSV has two sequences, located on either side of the *src* oncogene. The DRs must form a highly stable stem loop

structure to mediated nuclear export, which is dependent on the nuclear export factor Tap, although the DR does not appear to bind Tap directly [45,46].

The full length virus faces yet another obstacle during translation. Because the viral RNA has a stop codon at the end of the *gag* gene, the region downstream appears to be a long 3′UTR, which often targets transcripts for degradation by the nonsense mediated decay (NMD) machinery. In order to avoid degradation, ASLV has evolved a 400 nt element, named the RNA stability element (RSE) [47]. Positioned immediately downstream of the *gag* termination codon, the RSE protects the full length viral RNA from NMD mediated degradation. Work is currently underway to determine the mechanism by which the RSE functions.

Translation

The full length viral transcript acts as template for synthesis of two different polyproteins, Gag-Pro and Gag-Pro-Pol. Because the virion is composed of more Gag structural proteins than Pol proteins, all retroviruses have developed means to synthesize more Gag than Pol, despite being encoded on the same transcript. ASLV accomplishes this with a short A-U rich 'slippery sequence' just upstream of the *gag* termination codon, followed by an RNA pseudoknot [48]. This pauses the ribosome over the slippery sequence, occasionally (~5% of the time) causing it to slip backwards a single nucleotide before continuing forward. This '−1 frameshift' places the *gag* termination codon out of frame and allows the ribosome to read through to the *pol* termination codon, generating the Gag-Pro-Pol polyprotein.

During synthesis, ASLV Gag undergoes further modification. Most retroviral Gag proteins undergo low levels of phosphorylation and myristylation at their N-termini. ASLV Gag does undergo low level phosphorylation [49], but is not myristylated. Instead ASLV Gag is acetylated at its N-terminus [50]. The function of this acetylation is currently unknown.

The Env polyprotein is synthesized from a separate spliced transcript. Unique among retroviruses, the ASLV *env* splice donor resides within *gag*, just downstream of the *gag* start codon. This appends the first six amino acids of Gag onto the beginning of each Env polyprotein [49,51,52]. The Env polyprotein undergoes additional processing and modification in the endoplasmic reticulum. During this process, the Env polyprotein is proteolytically cleaved into three fragments, is glycosylated, undergoes folding, forms a trimer [53], and is then exported to the cell surface. Once the trimer has formed, Env is competent to bind its cognate receptor which is also processed through the secretory pathway. This forms the basis of superinfection resistance. By binding the receptor in the ER and remaining bound as it is presented at the cell surface, infection of the cell with any virus that uses that receptor is prevented.

In addition to the canonical translation products, three small peptides are synthesized from upstream open reading frames (uORFs) adjacent to the *gag* gene. These uORFs are conserved among all ASLVs and may play a role in translation and packaging [54,55].

Virion assembly/budding

The assembly and budding process is mediated in large part by Gag. Following synthesis, the RSV Gag protein is transiently imported to the nucleus where the Gag NC domain interacts with a packaging sequence (Ψ) on the viral genomic RNA [56,57••]. After Gag dimerization, a nuclear export signal within the p10 domain of Gag then mediates export of the ribonucleoprotein (RNP) complex by the CRM1 Pathway [57••,58].

The RNP complex then undergoes phosphoinositide-dependent [59] trafficking from the nucleus and stably associates with the plasma membrane via a membrane binding domain (MBD) at the N-terminus of Gag [60]. The degree to which assembly occurs before plasma membrane localization is not well understood. At the plasma membrane the viral polyproteins, processed Env proteins, two covalently linked viral genomic RNAs and tryptophan tRNAs coalesce and the virion buds from the cell surface. Various domains of Gag, and multiple host proteins have been shown to be important in this process [49,61]. The viral PR mediates cleavage of the polyproteins, and the virion obtains a mature morphology capable of infection shortly thereafter.

Conclusion

Since the discovery of avian retroviruses more than a century ago, much has been learned about their replication and life cycle. ASLV has proven to be very adaptable. Its ability to capture and use cellular genes, evolve to use a variety of cellular receptors, and recombine to form ALV-J attests to this. Though our understanding of the virus has increased, there are still many aspects of ASLV replication that warrant further study.

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