DNA tumor viruses and human cancer

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There is a strong association between viruses and the development of human malignancies. A group of oncogenic DNA viruses exists in the human population today, members of which serve as infectious agents of cancer worldwide. The group includes the Epstein–Barr virus, Kaposi's sarcoma-associated herpesvirus, human papillomaviruses and human polyomaviruses. Globally, it is estimated that 20% of all cancers are linked to infectious agents. Studies of DNA viruses have contributed to our current understanding of the key molecular players in the transformation process. Research has also shed light on the molecular mechanisms of tumorigenesis that are employed by these viruses and there are indications that cofactors could be required for viral oncogenicity in some cases.

Linking DNA tumor viruses to human cancer

Cancer is a multistep process. Over the past three decades it has become increasingly evident that several viruses play a key part in the development of human malignancy. Studies on these oncogenic agents have been instrumental to our understanding of basic cell biology and how perturbations of cellular pathways contribute to the initiation and maintenance of cancer. Approximately 20–30% of all cancers can be linked to infectious agents worldwide [1]. Viruses might be involved in different steps of the oncogenic process and the association of a virus with a given malignancy can range anywhere from 15% to 100% (Table 1).

There are several DNA tumor viruses that are associated with cancer in the human population. These include the human papillomaviruses (HPVs), human polyomaviruses, Epstein–Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV, also known as HHV-8) [2]. We will discuss the association of HPV with cervical cancer, EBV with nasopharyngeal cancer and B-cell lymphomas, KSHV with Kaposi's sarcoma and B-cell lymphoproliferative disease, and human polyomaviruses with mesotheliomas and brain tumors (Table 1). In addition to a direct association of these viruses with cancer, other cofactors such as immune suppression and the environment have also been implicated in the development of viral-associated malignancies. Thus, infection with any or all of these viruses along with their cofactors has the potential to give rise to the development of cancer in the human population.

We will review the criteria that are used to establish a link between a virus and a specific cancer. In the context of the many associations between a virus and a given malignancy, the distinction between associated versus causative agent frequently arises and might not be easy to decide. zur Hausen [3] has proposed alternative criteria for defining a causal role for an infection in cancer: (i) epidemiological plausibility and evidence that a virus infection represents a risk factor for the development of a specific tumor; (ii) the consistent presence and persistence of the genome of the microbe in cells of the tumor; (iii) the stimulation of cell proliferation following transfection of the genome (or portions of it) in corresponding tissue culture cells; (iv) the demonstration that the genome of the agent induces proliferation and the malignant phenotype of the tumor. For each infectious agent, we will detail the disease association, oncogenic properties and required cofactors.

Human papillomavirus

Associated cancers

HPV is associated with cervical, anal and skin cancer [4]. There are more than 130 strains of HPV but certain strains are considered more oncogenic than others. The 'high-risk' strains include HPV-16 and HPV-18, on the basis of the consistency of their association with cervical and anal cancer [4]. HPV-31 and HPV-45 are also found in these cancers. In addition, vaginal and penile cancers are associated with HPV. 'Low-risk' viruses are associated with benign lesions such as condyloma accuminata. There have also been HPV associations with oropharyngeal cancer and with basal-cell and squamous-cell carcinomas of the skin [4].

It is well accepted that HPV is the causative agent of cervical and most anal cancers. However, linking HPV to skin cancer has been more difficult because the virus can also be found in normal skin. Most HPV-associated cervical cancers are monoclonal, although polyclonal lesions have also been observed to persist [5]. Unlike some of the other DNA tumor viruses, the association of HPV with cervical cancer is found throughout the world and there are no endemic pockets of HPV-associated viral cancer. A fairly recent advancement in the papillomavirus field has been the development of a promising papillomavirus vaccine against cervical cancer, using the HPV-16 L1 capsid protein [6].

Transformation and oncogenesis

HPV is a small double-stranded DNA virus, ~8 kb in size [7]. It is a member of the papovavirus family. Primary infection occurs in the basal stem cells of the epithelium. The virus then traverses upwards and replicates in the terminally differentiated keratinocytes and is shed from the stratum corneum [8]. The virus lacks a polymerase

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Table 1. Human DNA tumor viruses and associated malignancies[a]

Virus	Human disease	Presence in tumor tissue	Causal role	Cofactors	Refs
HPV	Cervical cancer	100%	Yes	Genetic	[4]
EBV	Lymphoproliferative disease	100%	Yes	Immunodeficiency	[19]
EBV	Burkitt's lymphoma	98% in endemic areas	No (EBV is a cofactor)	Malaria	[19]
EBV	Hodgkin's lymphoma	40–50%	No	?	[19]
EBV	Nasopharyngeal cancer	100%	Yes	Genetic; environmental	[19]
KSHV	Kaposi's sarcoma	>95%	Yes	Immunodeficiency	[39]
KSHV	Primary effusion lymphoma	100%	Yes	Immunodeficiency	[39]
KSHV	Multicentric Castleman's disease	100% of AIDS lymphomas 50% of non-AIDS lymphomas	Yes	Immunodeficiency	[39]
JCV	Gliomas, medulloblastomas	50–80%	?	?	[56,60]
BKV	Brain tumors	20%	?	?	[60]

[a]Abbreviations: BKV, BK virus; EBV, Epstein–Barr virus; HPV, human papillomavirus; JCV, JC virus; KSHV, Kaposi's sarcoma-associated herpesvirus.

gene so the replication of the viral genome depends on the stimulation of cellular DNA synthesis in these cells. In most cervical carcinomas, HPV is integrated into the host genome resulting in a loss of expression of the E2 viral gene, which is a transcriptional repressor of E6 and E7 gene expression. This leads to enhanced expression of the E6 and E7 viral oncoproteins [8].

The E6 and E7 proteins of high-risk HPV strains have strong transforming abilities. E6 and E7 have been shown to immortalize cells *in vitro* and induce skin tumors in transgenic animals [9,10]. The HPV viral proteins target tumor suppressors resulting in the dysregulation of cell growth. E6 binds p53 and induces its degradation, whereas E7 binds retinoblastoma (Rb) family members [11,12]. The overall effect is the dysregulation of the cell cycle and the inhibition of apoptosis. E6 binds to a ubiquitin ligase, E6-AP, forming a complex that binds p53 and results in ubiquitination and proteosomal destruction of the protein [13]. In addition, E6 can induce telomerase activity and immortalize cells [14]. The E7 protein hinders Rb function and enables cells to progress into S phase. E7 binds the hypophosphorylated form of Rb and prevents it from binding to the transcription factor E2F. Free E2F transcription factors promote the expression of genes required for cell DNA synthesis, thereby pushing the cell into the cell cycle [15]. In addition, E7 stimulates cyclin-A- and cyclin-E-dependent kinase activity and inactivates the cyclin-dependent kinase inhibitors p21/WAF1 and p27/KIP1. E7 can also promote abnormal centriole synthesis and aneuploidy early in the oncogenic process. The ability of E6 and E7 to immortalize human cells is synergistic [10].

Cofactors
Sunlight and genetic backgrounds are cofactors for skin carcinomas caused by HPV strains 5 and 8. For example, epidermodysplasia verruciformis is a hereditary disorder affecting the skin and has been linked to chronic HPV infection [16]. However, there is also evidence for a genetic predisposition to cervical cancer [17] and HPV might play a part in this. Anal cancer associated with HPV is much more frequent in HIV-infected persons with immune deficiency, especially males [18]. Additionally, acute immunosuppression increases the risk of high-grade cervical dysplasia and progression to cancer [4].

It is important to recognize the differences between the oncogenic properties of various HPV strains. Without detailed strain analysis, the association of HPV with cervical cancer would have been obscured because non-oncogenic strains of HPV are widely prevalent.

Epstein–Barr virus
Associated cancers
EBV is associated with nasopharyngeal carcinoma (NPC), Burkitt's lymphoma (BL), subsets of Hodgkin's lymphomas and T-cell lymphomas, post-transplant lymphomas and gastric carcinomas [19].

NPC is an epithelial tumor that is found in the highest incidence among the Cantonese of Southern China, Hong Kong, Singapore and Taiwan. There is medium level of incidence in the Inuit populations in North America and in some populations in North Africa. The occurrence of NPC in the rest of the world is low, indicating that genetic or environmental factors are involved in the development of NPC. Nearly all undifferentiated NPC tumors contain EBV [20].

BL contains a chromosomal translocation that places the *c-myc* oncogene under the control of the immunoglobulin heavy or light chain promoters, resulting in the deregulation of *c-myc* in these cells [21]. Overall, 20% of BL tumors are associated with EBV infection but this varies widely by region. For instance, only 5% of BLs in USA is associated with EBV, whereas in endemic regions such as eastern Brazil or Africa nearly 90% of pediatric BLs carry EBV. Interestingly, BL has a high incidence in African regions that are also endemic for malaria.

Hodgkin's lymphoma is a B lymphoproliferative disease in which 1% of the tumor population is comprised of Hodgkin/Reed-Sternberg (HRS) cells, which are derived from germinal center B cells. The HRS cells are multinucleated giant cells that have distinct nucleoli and marginated heterochromatin. There are three types of Hodgkin's lymphoma: lymphocyte-depleted, nodular sclerosis and mixed-cellularity. Each of these differs in their association with EBV infection; 20% of nodular sclerosis Hodgkin's lymphoma is associated with EBV, whereas 100% and 70% of lymphocyte-depleted Hodgkin's lymphoma and mixed-cellularity Hodgkin's lymphoma are associated with EBV infection, respectively [22].

Transformation and oncogenesis
EBV is a double-stranded DNA virus belonging to the γ subfamily of herpesviruses. Herpesviruses have a latent phase and a lytic phase to their lifecycle. Similar to other γ-herpesviruses, EBV establishes a lifelong latent infection

in the B lymphocytes of its host. The infection of naïve B cells with EBV in culture results in the establishment of immortalized B cell lines [23].

EBV encodes several viral proteins that have transforming potential. For example, EBV latent membrane protein 1 (LMP1) has been shown to transform a variety of cell types including rodent fibroblasts. LMP1 is essential for the ability of EBV to immortalize B cells because deletion of LMP1 from EBV renders the virus non-transforming [19]. LMP1 has multiple transmembrane-spanning domains and the carboxyl terminus can interact with several tumor necrosis factor receptor associated factors (TRAFs) [24,25]. The interaction of LMP1 with these TRAFs results in the high expression of nuclear factor κB (NF-κB) in LMP1-expressing epithelial and B cells [26]. LMP1 also upregulates the expression of several anti-apoptotic and adhesion genes, including *A20*, *bcl2* and *ICAM-1*. Additionally, it activates the expression of interferon regulatory factor 7 (IRF-7) [27], matrix metalloproteinase-9 (MMP-9) and fibroblast growth factor-2 (FGF-2) [28]. Another viral gene, LMP2, located on the opposite end of the linear genome, has been shown to inhibit B-cell-receptor (BCR) signaling [29]. LMP2 sequesters the Src family members Fyn and Lyn and prevents them from translocating into lipid rafts with BCR, thereby inhibiting BCR activation [30]. In epithelial cells, LMP1 has been shown to inhibit differentiation and induce cell proliferation through the activation of the phosphoinositol-3-kinase (PI3K)/Akt pathway [31,32].

Other viral genes that encode transforming potential include EBV nuclear antigen 2 and 3 (EBNA2 and EBNA3). EBNA2 is a promiscuous transcriptional activator, activating the promoters of both viral and cellular genes [33,34]. Like LMP1, EBNA2 is essential for B-cell transformation because deleting the EBNA2 gene from wild-type EBV renders the mutant virus incapable of immortalizing B lymphocytes [35].

The genes encoding EBNA3A, EBNA3B and EBNA3C lie in a tandem array in the viral genome. EBNA3A and EBNA3C are essential for B-cell transformation, whereas EBNA3B is dispensable [36]. The three EBNA3 nuclear proteins are hydrophilic and share a common amino-terminal domain but have different carboxyl termini. All three EBNA3 proteins can interfere with EBNA-2 activation by disrupting its interaction with the DNA-binding protein RBP-Jk, thereby suppressing EBNA2-mediated transactivation [37]. EBNA3C has been shown to cooperate with the proto-oncogene Ras to immortalize and transform rodent fibroblasts. It can directly interact with the Rb tumor suppressor protein, rendering it inactive and promoting tumor progression [38]. In summary, EBNA3C can promote cellular proliferation and over-ride the G1–S phase checkpoint, and it can co-operate with EBNA2 and EBNA3A to modulate cellular gene expression in EBV-infected lymphocytes.

Cofactors

Both EBV-associated BL and NPC have endemic patterns of incidence. This strongly suggests that both environmental and genetic factors contribute to the neoplastic process. Tumor-promoting chemicals have been found in salted fish

and other food products in Southern China. In the case of BL, it is thought that the high incidence of malarial infection in certain regions of Africa results in an expansion of the germinal centers. This combined with increased viral reactivation raises the probability for EBV to infect additional B lymphocytes in the germinal center.

Kaposi's sarcoma-associated herpesvirus
Associated cancers

KSHV/HHV-8 has been linked to several malignancies in the human population. These include Kaposi's sarcoma (KS), primary effusion lymphomas (PELs) and multicentric Castleman's disease (MCD).

KS is a multifocal vascular tumor of mixed cellular composition that is most often seen as a cutaneous lesion. KSHV is always found in the spindle cells of the lesion, which are thought to be endothelial in origin. There are four epidemiological forms of KS. (i) Classic KS is seen in men of Mediterranean and Eastern European descent. (ii) AIDS-associated KS is a highly aggressive tumor and is primarily detected in HIV-infected individuals. In these individuals, the KS lesion is not restricted to the skin and often disseminates to the liver, spleen, gastrointestinal tract and lung. Today KS is the most frequently detected tumor in AIDS patients. (iii) Endemic KS is a third type of KS, which is seen in Africa and affects HIV-positive and HIV-negative individuals, and even children. It seems to be more aggressive than classic KS. (iv) The fourth type of KS lesion is an iatrogenic form of KS that besets post-transplant patients receiving immunosuppressive therapy [39]. Greater than 95% of all KS lesions, regardless of type, have been shown to contain KSHV viral DNA, thereby indicating a strong epidemiological link between KSHV infection and KS [40].

PELs are malignant B-cell lymphomas representing a specific subset of body cavity based lymphomas (BCBLs) that arise as body cavity effusions. The term PEL was designated to represent a distinct clinicopathologic group of lymphomatous effusions [41]. All PELs are KSHV positive, indicating a strong epidemiological link between the presence of KSHV and the induction of PEL. These lymphomas often contain EBV as well. PELs are observed in both HIV-positive and HIV-negative individuals, with both types of PELs invariably containing KSHV viral DNA.

MCD is a B-cell lymphoproliferative disorder. There are two types of MCD: a hyaline vascular form, which presents as a solid mass, and a plasmablastic variant, which is associated with lymphadenopathy. MCD is sometimes referred to as multicentric angiofollicular hyperplasia and is characterized by vascular proliferation of the germinal centers of the lymph node. Nearly 100% of AIDS-associated MCD is positive for KSHV, whereas ∼50% of non-AIDS-associated MCD contains KSHV viral DNA. AIDS-associated MCD is usually accompanied by the development of KS in the affected individuals. MCD is a polyclonal tumor and is dependent on cytokines such as interleukin-6 (IL-6) [42].

The epidemiological evidence linking KSHV to KS, PELs and MCD is solid and has been confirmed by several laboratories, establishing that KSHV is necessary for the development of these malignancies. Epidemiological

studies linking KSHV to disease, the ability of KSHV to transform endothelial cells, and the identification of transforming and mitogenic genes encoded by KSHV all support the notion that KSHV is a tumor virus with oncogenic properties.

Transformation and oncogenesis

KSHV is also a member of the γ-herpesvirus family and also establishes lifelong latency in B cells. The viral genome is ~160 kb in size and codes for > 80 open reading frames (ORFs). KSHV encodes a diverse set of genes involved in transformation, signaling, prevention of apoptosis and immune evasion.

It is believed that KSHV transforms cells through a paracrine mechanism because several studies have shown a high level of cytokines and growth factors in lesions of KS and MCD. KSHV can immortalize primary bone marrow derived endothelial cells and induce cell proliferation, anchorage independence and survival of these cells [43]. Interestingly, only a subset of the transformed endothelial cells contained viral DNA, suggesting that the neighboring uninfected cells survived through a mechanism involving cytokines secreted by the infected cells [43]. These observations suggest that KSHV transformation is dependent on paracrine factors in addition to the cellular microenvironment, a concept also gaining favor in other tumor models.

The KSHV K1 and viral G-protein-coupled receptor (vGPCR) genes have oncogenic potential. The K1 protein can transform rodent fibroblasts *in vitro*, and, when injected into nude mice, these cells induce multiple and disseminated tumors. Furthermore, K1 can functionally substitute for the saimiri transformation protein (STP) of herpesvirus saimiri (HVS) *in vitro* and *in vivo* to induce lymphomas in common marmoset monkeys. Transgenic animals expressing K1 develop sarcomas and lymphomas [44]. K1 can elicit B-cell signaling and proliferation through its immunoreceptor tyrosine-based activation motif (ITAM) and by blocking Fas-induced apoptosis of these cells [45,46]. Furthermore, K1 can activate the NF-κB and PI3K pathways. In endothelial cells, K1 has been shown to upregulate the expression and secretion of vascular endothelial growth factor (VEGF) and MMP-9 [47,48].

Similar to K1, the KSHV vGPCR protein can transform NIH 3T3 cells *in vitro*. vGPCR immortalizes primary endothelial cells and transgenic mice expressing vGPCR develop angioproliferative KS-like lesions [49,50]. vGPCR can activate the phospholipase C (PLC) and PI3K pathways [49]. Additionally, vGPCR expression in many cell types results in the upregulation of many cytokines and paracrine factors. Thus, this viral protein might contribute to KSHV-associated neoplasia by inducing and sustaining cell proliferation. Aside from K1 and vGPCR, KSHV also encodes a viral interferon regulatory factor 1 (vIRF-1) and the Kaposin/K12 gene, both of which have transforming potential *in vitro* [51]. In addition, the latency associated nuclear antigen (LANA), which has been shown to be crucial for the establishment and maintenance of the viral episome [52,53], has also been shown to immortalize endothelial cells and induce B-cell hyperplasia and

lymphoma in mice [54,55]. This list is by no means complete, but identifies a minimal set of viral genes that are likely to contribute to KSHV-associated cancers.

Cofactors

As with other tumor viruses, HIV is a cofactor for the development of malignancy because the prevalence of KS in AIDS patients is unusually high. Immunosuppresive therapy is also a cofactor for the iatrogenic form of KS. However, KSHV-associated KS can occur in healthy, HIV-negative individuals, as has been observed in Africa and the Mediterranean. Finally, because the classic form of KS is predominantly observed in elderly men of Mediterranean and East European descent, age, sex, genetic and environmental cofactors might also play a part in the development of the disease.

Human polyomaviruses

Associated cancers

The human polyomaviruses JC virus (JCV) and BK virus (BKV) have been linked to several different human cancers. However, this is currently a subject of much controversy. JCV is associated with brain tumors found in patients with or without progressive multifocal leukoencephalopathy (PML). JCV has also been shown to be associated with glial tumors and pediatric medulloblastomas [56] and other associations include colon cancer and central nervous system (CNS) lymphoma [57,58]. Although a role for BKV in brain tumors is less well established than for JCV, BKV DNA has also been found in pancreatic islet tumors and brain tumors [59,60]. The ability to find JCV and BKV virus sequences in brain tissues might be a result of one of two possibilities. First, it might actually reflect the involvement of these viruses in the development of brain tumors. Alternatively, it might reflect the increased propensity for these viruses to enter or replicate in cells of glial origin. Currently, it is not possible to differentiate between these two scenarios. Establishing a tight epidemiological association of polyomaviruses with specific human tumors has been difficult because of the wide prevalence of polyomaviral infection and the resulting lack of virus-negative controls. Whether the role of the virus is causal or incidental has been the subject of much debate. However, because these viruses are highly tumorigenic in cell culture and animal model systems, it is likely that they are indeed true oncogenic agents. In conclusion, although the role of the human polyomaviruses in malignancy is still under scrutiny, the presence of viral DNA and viral gene expression in a subset of human tumors has been firmly established.

Transformation and oncogenesis

Human polyomaviral genomes do not encode their own replication machinery. To replicate they express two proteins, large T antigen and small t antigen, which push the host cell into the cell cycle [61]. Large T antigen has both Rb- and p53-binding domains that interact with these two tumor suppressor proteins and inactivate their function. This releases the E2F transcription factor from Rb suppression, resulting in the activation of cyclin promoters. The interaction of large T antigen with p53 suppresses its

apoptotic function and prevents the activation of inhibitors of the cellular cyclins [59,62]. As a result, cell proliferation ensues and precedes oncogenic transformation. It is thought that small t antigen co-operates with large T antigen in the transformation of differentiated cells [61].

BKV is highly oncogenic in rodents and hamsters. The subcutaenous injection of BKV is weakly oncogenic but intracerebral intravenous injections result in a high incidence of tumors. The types of tumors induced include neuroblastoma, pineal gland tumors, pancreatic islet cell tumors, fibrosarcoma and osteosarcoma, which suggests a tropism of the virus for certain cell types [59]. Transgenic mice expressing the BKV T antigens develop kidney carcinomas and thymoproliferative disease [63].

Complete BKV genomic DNA or fragments that encode the large T and small t antigen can transform a multitude of cells including human embryonic fibroblasts and cells cultured from the kidney or brain of macaques, rodents and hamsters. The transforming ability of JCV occurs mainly in cells of neural origin [59]. JCV also has high oncogenicity in animals, including primates. Transgenic mice expressing JCV large T antigen develop demyelinating disease, adrenal neuroblastomas and neural tumors.

Cofactors
Although environmental and host cofactors for polyomaviral malignancies are not well defined or established, it is possible that one cofactor for JCV and BKV transformation might be immune suppression such as HIV co-infection. Additionally, environmental cofactors that might contribute to disease progression could include the inhalation or ingestion of carcinogens.

Concluding remarks and future perspectives
Historically, research on the biology of both RNA and DNA viruses has led to the development of key tenets in cell biology, including the discovery of cellular proto-oncogenes and tumor suppressor genes. These discoveries, in turn, led to an understanding of how both viral and non-viral cancers arise. Thus, the study of tumor viruses has been essential to our present knowledge of the neoplastic process.

Many tumor viruses stimulate the proliferation of the infected cell and the analysis of viral genes associated with transformation has revealed different strategies by which viruses can deregulate cell proliferative pathways. Additionally the small tumor viruses such as polyomaviruses and papillomaviruses, which do not encode their own replication machinery, induce the cell into the cell cycle to generate all the factors needed for viral replication and packaging. By contrast, the large DNA tumor viruses such as EBV and KSHV encode their own viral DNA polymerase. Another contrasting feature between the small and large DNA tumor viruses is that the small viruses generally integrate into the host chromosomal DNA during or before the neoplastic event, whereas the large herpes viral genomes are maintained episomally through the tethering of the viral genome to the host chromosome by viral latent proteins such as EBV EBNA1 and KSHV LANA. Among the smaller DNA tumor viruses such as human polyomaviruses and papillomaviruses, only one or two genes show transforming potential. The function of the large T antigen of polyomavirus has been divided between two viral proteins, E6 and E7, in papillomaviruses. However, the larger DNA tumor viruses such as EBV and KSHV encode several transforming genes and can cause cancers in many different cell types.

Tumorigenesis is considered to be a multistep process and infection with the DNA tumor viruses can substitute for one or many of the mutational events that result in complete transformation, neoplasia and metastasis. As described above, JCV/BKV, HPV, KSHV and EBV encode a diverse array of viral genes that help contribute to the neoplastic process. These viruses have evolved strategic means of deregulating and perturbing normal cellular pathways that would otherwise lead to apoptosis, activation of the host immune system and cell growth arrest. The rest of the genes encoded by these viruses have roles in viral replication, packaging, entry and immune evasion.

Although our understanding of the mechanisms by which DNA tumor viruses induce transformation has greatly advanced, we are still lacking specific drugs that inhibit these viral proteins. Today, most anti-tumor therapies against virus-induced cancers target cellular proteins that have a role in these processes rather than viral proteins [64]. Thus, it will be crucial for future studies to be aimed at designing therapeutics that specifically target viral proteins because these therapies will be more specific and reduce drug cytotoxicity. Another future goal is to develop vaccines against these DNA tumor viruses. We currently have a promising vaccine against human papillomavirus, but none against EBV, KSHV and the human polyomaviruses.

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