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# Review Article

# Yeast and the AIDS Virus: The Odd Couple

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Despite being simple eukaryotic organisms, the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* have been widely used as a model to study human pathologies and the replication of human, animal, and plant viruses, as well as the function of individual viral proteins. The complete genome of *S. cerevisiae* was the first of eukaryotic origin to be sequenced and contains about 6,000 genes. More than 75% of the genes have an assigned function, while more than 40% share conserved sequences with known or predicted human genes. This strong homology has allowed the function of human orthologs to be unveiled starting from the data obtained in yeast. RNA plant viruses were the first to be studied in yeast. In this paper, we focus on the use of the yeast model to study the function of the proteins of human immunodeficiency virus type 1 (HIV-1) and the search for its cellular partners. This human retrovirus is the cause of AIDS. The WHO estimates that there are 33.4 million people worldwide living with HIV/AIDS, with 2.7 million new HIV infections per year and 2.0 million annual deaths due to AIDS. Current therapy is able to control the disease but there is no permanent cure or a vaccine. By using yeast, it is possible to dissect the function of some HIV-1 proteins and discover new cellular factors common to this simple cell and humans that may become potential therapeutic targets, leading to a long-lasting treatment for AIDS.

#### 1. Introduction

Since the genetics and cell biology of higher eukaryotes are extremely complex, scientists looking for a good model have turned to the use of yeast as a simpler system for the study of various pathologies including virus proliferation and assay new drugs against these pathogenic agents.

The yeast *Saccharomyces cerevisiae* is a simple eukaryotic organism with approximately 6,000 genes. It is inexpensive to cultivate, can exist in either haploid or diploid states, and is extremely simple for genetic manipulations. The complete sequence of this yeast genome, the fruit of a worldwide collaboration of more than 100 laboratories from 1989 to 1996, was the first determined for a eukaryote cell. More than 75% of the genes have an assigned function, while more than 40% share conserved sequences with at least one known or predicted human gene ([1]; http://www.yeastgenome.org/).

Owing to the high conservation of fundamental biochemical pathways, yeast has been used as a model to unravel biological processes in many higher eukaryotes (for a short review see [2]). A very important tool facilitating the use of these cells is the availability of yeast libraries in which each nonessential gene has been deleted. It has been used for multiple studies including genome wide screenings for human disease genes and host factors that support virus replication [3–5]. The above collection is commercially available and covers more than 90% of all yeast genes [6].

An exciting example of the beneficial using this model system is found in two very recent reports, although not related to virus research, on the use of the yeast model to study a severe human pathology. Amyotrophic lateral sclerosis (ALS), also known as Lou Gehrig's Disease, is a neurodegenerative disease in which the motor neurons of the central nervous system that control muscles die off leading to paralysis and death within 3–5 years of onset. The cause of the disease is unknown, and there is no treatment to stop or slow it. A gene related to ALS, called FUS, has been used to explore its biology in yeast and highlight the potential for modeling elements of complex diseases in this simple eukaryotic cell. Results from both studies suggest

that defects in RNA processing and transport may be a key element of ALS pathophysiology. In the cytoplasm of motor neurons of ALS patients, proteins aggregate to form insoluble aggregations, called inclusions, that may involve FUS and another ALS causing protein, called TDP-43 [7]. When the two research groups overexpressed human FUS in yeast, they observed cytoplasmic inclusions. In humans, the majority of FUS is found in the nucleus, and some ALSassociated mutations reduce the nuclear/cytoplasmic ratio of the protein, suggesting that transfer to the cytoplasm, rather than mutation, may be important in this pathology. This hypothesis was supported by the observation that decreasing the overexpression of wild-type FUS in the yeast nucleus diminished its toxic effect. Both FUS and TDP-43 are RNA binding proteins. Genome-wide screens to identify yeast genes that specifically decreased cell toxicity were performed and the identified genes included some coding for other DNA/RNA binding proteins. One of these yeast genes, called ECM32, has a human homolog, hUPF1, which was found to eliminate toxicity. Among other functions hUPF1 plays an important role in mRNA quality control, supporting the idea that pathways involving RNA are defective in FUSinduced ALS. Interestingly, expression of hUPF1 was able to rescue FUS toxicity in yeast without driving FUS out of the inclusions or sending it back to the nucleus, suggesting that it may be possible to overcome therapeutically the effects of abnormally localized FUS without addressing the difficult problem of restoring it to its original compartment. Two important consequences may result from this approach. By identifying new genes that can diminish ALS-linked toxicity, they point to RNA processing as a therapeutic target in this disease. It has also been shown that yeast has the potential to be a much simpler and exciting system for modeling aspects of ALS. Since testing ideas about pathogenesis and treatment is much faster and cheaper in yeast, these results may open the way for more rapid progress in understanding the disease, its treatment, and the role of this new gene in ALS development. Important details of other neurodegenerative diseases have been obtained using yeast. For example, protein misfolding associated with many human diseases, including Alzheimer's, Parkinson's, and Huntington's disease, has been studied in yeast. As in the case of ALS mentioned above, protein misfolding often results in the formation of intracellular or extracellular inclusions whose role in protein misfolding diseases is unclear. Studies on the implication of protein aggregation and toxicity in yeast provide an excellent experimental and conceptual paradigm that contributes to understanding the differences between the toxic and protective roles of protein conformation changes. Results from these studies using yeast have the potential to transform basic concepts of protein folding in human diseases and may help in identifying new therapeutic strategies for their treatment [8].

In virus research, *S. cerevisiae* is a very useful organism. In terms of public health, the use of yeast to produce vaccines was a remarkable breakthrough. For example, the first recombinant vaccine, the hepatitis B surface antigen expressed in yeast, has become a safe and efficient prophylactic vaccine worldwide [9]. In addition, yeast has proved

useful for drug discovery as illustrated by the power of applying genomic approaches using the yeast model to characterize the biological activity of small molecules and to identify their cellular targets, an important step towards understanding the mode of action of human therapeutic agents [10]. This study and other recent reports show that the path from sequence and functional analysis to direct therapeutic applications is not necessarily long, at least using the yeast model, a simple organism, which could well be considered among "man's best friends."

In basic research in Virology, yeast has assisted the elucidation of the function of individual proteins from important pathogenic viruses such as *Hepatitis C virus*, and *Epstein-Barr virus* ([11, 12]; for a review and further references see [13]). Furthermore, studies of viruses that infect yeast have provided many important contributions to the dissection of the life cycle of many other higher eukaryotes RNA viruses and the host factors involved [14].

The study of the replication of RNA plant viruses was the first to be attempted using yeast cells. The great majority of RNA+ viruses carry their own RNA polymerase and are replicated in the cytoplasm while entry to the nuclear compartment during the viral cycle is confined to retroviruses (see below). The first higher eukaryotic virus reported to replicate in yeast was *Brome mosaic virus*, a positive-strand RNA ((+)RNA) virus that infects plants ([15], for an excellent review see [16]).

Since the pioneering work of the Ahlquist laboratory, a growing list of viruses has been reported to undergo replication in yeast. These include RNA and DNA viruses that infect plants, insects, mammals, and humans (Table 1) [17–22].

This wide range of viruses emphasizes the general applicability of the yeast system. In this paper, we will focus on the contribution of the yeast model to the study of retroviruses with special emphasis on the human immunodeficiency virus type 1 (HIV-1).

### 2. The Search of Cellular and Viral Partners Using the Yeast Two-Hybrid System

The yeast two-hybrid system, also called double-hybrid, has been extensively used to search and characterize the potential partner(s) of a given protein ([23], for a review see [24]).

The basis of this method allows searching and characterizing proteins capable of interacting with a known protein leading to obtaining the cloned genes of these interacting proteins. Plasmids are constructed to encode two hybrid proteins (Figure 1). One hybrid consists of the DNA-binding domain of the yeast transcriptional activator protein GAL4 fused to the known protein; the other hybrid consists of the GAL4 activation domain fused to protein sequences encoded by a library of yeast (or other sources) genomic DNA fragments. Interaction between the known protein and a protein encoded by one of the library plasmids leads to transcriptional activation of a reporter gene easy to detect containing a binding site for GAL4. In addition of thousand of examples described in the literature using this system many cellular or viral partners of HIV-1 proteins have been

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| ARIE | ١. | Viruses | that | ren | licate | 111 | veast |
|      |    |         |      |     |        |     |       |

| (+)RNA viruses           | Virus   | Natural host |
|--------------------------|---|--------------|
| Bromoviridae             | Brome mosaic virus                            | Plants       |
| Tombusviridae            | Carnation Italian ringspot virus              | Plants       |
| Tombusviridae            | Tomato bushy stunt virus                      | Plants       |
| Nodaviridae              | Flock house virus                             | Animals      |
| Nodaviridae              | Nodamura virus                                | Animals      |
| DNA circular viruses     | Virus   | Natural host |
| Papillomaviridae (dsDNA) | Human papillomavirus                          | Animals      |
| rapinomaviridae (dsDNA)  | Bovine papillomavirus                         | Animals      |
| Geminiviridae (ssDNA)    | e (ssDNA) Mung bean yellow mosaic India virus |              |

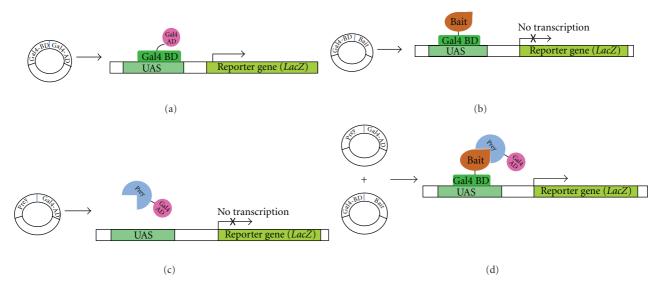


FIGURE 1: The two-hybrid assay, checking for interactions between two proteins, here called Bait and Prey. (a) Gal4 transcription factor gene produces two domain proteins (BD and AD), which are essential for transcription of the reporter gene (LacZ). (b) and (c), Two fusion proteins are prepared: Gal4BD + Bait and Gal4AD + Prey. None of them is usually sufficient to initiate the transcription (of the reporter gene) alone. (d) When both fusion proteins are produced and the Bait part of the first interacts with Prey part of the second, transcription of the reporter gene occurs (adapted from http://www.wikimediafoundation.org/).

detected using this strategy. In the case of the HIV-1 enzymes in addition of studies with RT and protease several cellular or retroviral partners of integrase have been described. Some examples are the interactions between the human and its ortholog yeast transcription factor SFN5/InI1 described below and that of the lens epithelium-derived growth factor (LEDGF/p75), the latter playing an important role by stimulating the integration step and facilitating the interaction with the host cell chromatin [25, 26]. The interaction of HIV-1 IN with microtubules proteins [27] using the two-hybrid approach was confirmed later by using other methods [28]. More recently were developed the three-hybrid and one-hybrid systems derived from the two-hybrid system.

## 3. Human Immunodeficiency Virus Type 1 (HIV-1)

Even though retroviruses carry an RNA+ genome, their replicative cycle involves the cell nucleus. These viruses

are characterized by the fact that they carry an RNA-dependent DNA polymerase (reverse transcriptase (RT)) that synthesizes a double-strand DNA called proviral DNA in the cytoplasm of the infected cell by using the viral RNA genome as template. Proviral DNA and other viral and cellular factors forming the preintegration complex (PIC) enter the nuclear compartment and integrate the proviral double-stranded DNA in the host genome via an encoded retroviral enzyme, integrase (IN). In this paper we will focus on the use of the yeast model system to study the following proteins encoded in the HIV-1: the enzymes reverse transcriptase, integrase and protease, Vpr, Rev and the translation machinery of HIV-1.

#### 3.1. Enzymes Encoded in the HIV-1 Genome

HIV-1 Encoded Enzymes. All retroviruses carry three enzymes, an RNA- and DNA-dependent DNA polymerase or reverse transcriptase (RT), integrase (IN), and protease

(PR) (see [29]). The organization of these proteins in several retroviruses is shown in Figure 2 while their 3D structure is shown in Figure 3. RT also contains an additional enzymatic activity, RNase H, which has been mapped to a separate, contiguous portion of the polypeptide. First identified in the avian viruses, the retroviral enzymes are organized in domains on the Gag-Pro or Gag-Pro-Pol precursor polypeptide. These domains are not always cleaved into separate mature proteins. In most genera, all enzymes are translated together as a Gag-Pro-Pol precursor, which are processed to yield the mature forms of the enzymes. Whether expression of pro and pol is due to frameshifting or termination suppression, they account for approximately 5% of RT and IN on a molecular basis but are synthesized and packaged in the virion at the same level as the Gag protein. For most retroviruses, the same holds for PR. A scheme describing the retroviral frameshifting process is shown in Figure 7. Since RT, IN, and PR are essential for viral replication and have characteristics that distinguish them from related cellular enzymes, they have all become privileged targets for drugs against the acquired immunodeficiency syndrome (AIDS).

3.2. Reverse Transcriptase (RT). At a time when the only assay to measure RT activity was in vitro assay, it seemed attractive to use the yeast S. cerevisiae as a model to test the in vivo activity of this enzyme. For this purpose constructions of hybrid retrotransposons composed of the endogenous yeast Ty1 element and the reverse transcriptase (RT) of HIV-1 were shown to be active in the yeast Saccharomyces cerevisiae. The RT activity of these hybrid Ty1/HIV-1 elements can be monitored by using a simple genetic assay. The reverse transcription of yeast carrying this hybrid RT depends on both the DNA polymerase and RNase H domains of HIV-1 RT.

Most HIV-1 RT inhibitors can be divided into two classes, nucleoside analog RT inhibitors (NRTIs) and nonnucleoside RT inhibitors (NNRTIs). NRTIs such as AZT (3-azido-3deoxythymidine) and ddI (dideoxyinosine) inhibit reverse transcription by a chain-termination mechanism; that is, when they are added to a growing DNA chain they block further synthesis while the NNRTIs act by blocking the synthesis by binding specifically an hydrophobic pocket of the HIV-1 RT. The reverse transcriptase activity measured in yeast cells was shown to be sensitive to inhibitors of HIV-1 RT like 8Cl-TIBO, a well-characterized nonnucleoside RT inhibitor of HIV-1 RT while the hybrid constructions that express NNRTI-resistant RT variants of HIV-1 are insensitive to8Cl-TIBO demonstrating in yeast the specificity of inhibition in this assay. These hybrid Ty1/HIV-1 (his3AI/AIDS RT), called HART, elements carrying NNRTI-resistant variants of HIV-1, RT were used to identify compounds that are active against drug-resistant viruses [30].

The reverse transcriptase enzyme purified from the virus is a heterodimer with subunits of 66 and 51 kDa called p66 and p51 (Figure 3(a)). p51 subunit is a shortened version of p66 after cleavage by the HIV-1 encoded protease. As with many other recombinant proteins the yeast *S. cerevisiae* has been used to express HIV-1 RT. Only the HIV-1 gene

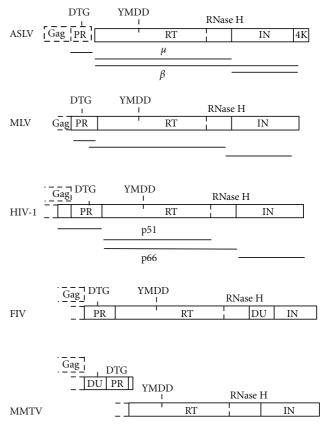


FIGURE 2: Organization of Pro and Pol proteins. Schematic representations of the mature Pro and Pol proteins and their precursors are drawn for examples from several retroviruses. The sequences representing the mature proteins PR, RT, and IN are indicated. Rectangles, precursor proteins, with solid vertical lines marking major cleavage sites and thick horizontal bars indicating mature proteins. DTG or DSG and YMDD indicate the conserved active site residues in PR and RT. The RNase H domain of RT is also indicated (ASLV: avian sarcoma leucosis virus, MMTV: murine mammary tumor virus, MLV: murine leukemia virus, and FIV: feline immunodeficiency virus).

for the p66 subunit was carried by the expression vector used and surprisingly the recombinant enzyme purified after expression in yeast was the heterodimeric p66/p51 form. Protein sequencing showed that the cleavage to produce the p51 subunit gave a product identical to the native viral enzyme showing that yeast possesses a protease with the same exquisite specificity as the HIV-1 protease [31].

3.3. Integrase. HIV-1 IN catalyzes the insertion of proviral DNA into the host-cell genome (for general reviews on HIV-1 integrase and further references see [32–36]). In the first step of the integration reaction, termed 3'-end processing, two nucleotides are removed from each 3'-end of the double strand viral DNA to produce new "hydroxyl ends" (CA-3'OH). This reaction occurs in the cytoplasm within a large viral nucleoprotein complex (PIC). After entering the nucleus via the PIC multimeric complex, the 5'-ends of processed viral DNA are joined covalently to the host

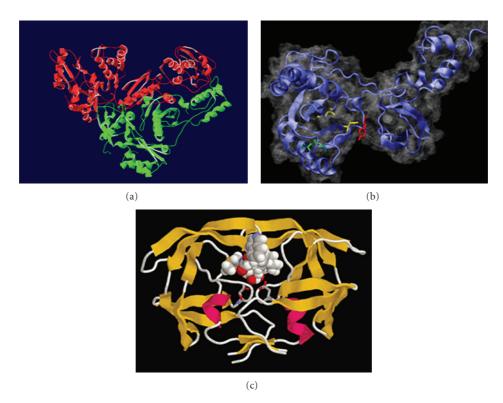


FIGURE 3: Tertiary structure of RT, IN, and PR: (a) RT: p66 in red and p51 in green; (b) catalytic core of IN in blue. Aminoacid 143 in red, 97 and 120 in green, and 64 and 116 in yellow. (c) Dimeric PR in complex with a protease inhibitor.

DNA. The joining reaction includes a coupled 4–6-base-pair staggered cleavage of the target host DNA and the ligation of the CA-3'OH viral DNA ends to the 5'-phosphate ends of the target DNA. Although the mechanism is not fully understood, host cell DNA repair enzymes probably accomplish the repair of the remaining gaps. HIV-1 IN is composed of three functional domains. The N-terminal region (residues 1–50) is characterized by an HHCC zinc finger-like sequence able to bind zinc. This binding induces proper folding of the N terminus and promotes tetramerization of IN. The central region (residues 50–160, Figure 3(b)) is characterized by three highly conserved amino acid residues, D64, D116, and E152, forming the catalytic triad DD(35)E. Each one of these residues is essential for enzyme activity. The Cterminal region is the least conserved domain and is involved in nonspecific DNA binding. The 3D structure of the three domains of HIV-1 IN has been determined by RMN or X-ray crystallography. Despite considerable efforts, the low solubility of the native HIV-1 integrase has hampered the determination of the crystallographic structure of the entire protein. The three HIV-1 IN domains are required for in vitro 3'-end processing and DNA strand transfer. Mutation analyses of the viral integrase gene showed that this enzyme is required for retroviral replication and that it is a legitimate target for the design of antiretroviral drugs. Recently, the 3D structure of the human foamy retrovirus integrase was determined, taking profit of the increased solubility and yield of this recombinant retroviral enzyme, and confirming a tetramer quaternary structure [37].

3.4. Expression of HIV-1 IN in the Yeast Saccharomyces cerevisiae. Based on results dating from 1985 where the expression of the bacterial EcoR1 restriction endonuclease expressed in yeast cells led to the appearance of a lethal phenotype [38] the idea emerged that the expression of retroviral IN also carrying an endonuclease activity may produce a similar phenotype. The idea behind this approach was that the feasibility of such a system may allow the setting up of an easy and rapid procedure for screening antiretroviral drugs. However, the weak entrance of molecules through the yeast wall and cell membrane hampered this project. The yeast model has been extremely useful to study many aspects of the integration step in retroviral replication. An expression plasmid containing the retroviral integrase gene under the control of the inducible promoter ADH2/GAPDH regulated by the glucose concentration of the medium was constructed. Haploid yeast strain W303-1A did not appear to be clearly sensitive to HIV-1 integrase expression (Figure 4). However, disruption of the RAD 52 gene, which is involved in the repair of double-strand DNA breaks, strongly increased the deleterious effects of the retroviral enzyme in this yeast strain. The diploid strain constructed with W303-1A and an isogenic strain of the opposite mating type also showed strong sensitivity to the HIV-1 IN. The lethal phenotype was suppressed by missense integrase mutations in the catalytic domain that are known to abolish HIV-1 IN activities in vitro (Figure 4) [39]. Subsequent studies were performed in order to determine the critical amino acid(s) and/or motif(s) required for the induction of the lethal phenotype in the

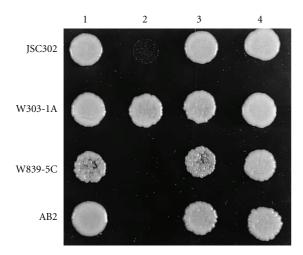


FIGURE 4: Drop test experiment:  $3\,\mu\text{L}$  droplets of plasmid-containing standard yeast suspensions containing about 20 000 ura+ colony-forming units were deposited on the appropriate selective media to allow expression of the HIV-1 integrase in yeast. When the drops had dried, plates were incubated for 3 days, and the effect of integrase expression on yeast was determined by visual observation of the Petri dishes. Lethality is observed when IN is expressed in yeast (2) but not in the presence of empty plasmid (1) or when expressing inactive D116A or E152A IN (3 and 4). JSC302 and W303-1A: RAD52+, W839-5C: identical to W303-1A except for *RAD52* gene disruption. AB2: diploid obtained from W303-1A (adapted from [39]).

yeast and to elucidate the molecular mechanism that causes this phenotype. The HIV-1 IN mutants, V165A, A179P, and KR186, 7AA, located in the C-terminal region of the catalytic core domain of IN were shown to be unable to induce the lethal phenotype in yeast. Further mutagenic and virologic experiments led to the conclusion that these mutations inhibit the IN-induced lethal phenotype in yeast by inhibiting the binding of IN to the host chromatin. These results demonstrate that the C-terminal region of the catalytic core domain of HIV-1 IN is important for binding to host chromatin and is crucial for both viral replication and the promotion of the IN-induced lethal phenotype in yeast [40]. The question remained whether the lethal effect was related to the nonspecific endonuclease activity of the viral IN or whether the mechanism involved was due to a pleiotropic effect of this protein. Lethality in yeast seems to be related to the mutagenic effect of the recombinant HIV-1 IN, most probably via the non-sequence-specific endonuclease activity carriedout by this enzyme. This nonsequence-specific endonuclease activity was further characterized. Although the enzyme was active on DNA substrates devoid of viral long terminal repeat (LTR) sequences characteristic of the retroviral proviral genome, the presence of LTR regions significantly stimulated this activity. Genetic experiments showed that both the mutagenic effect and the level of recombination events were affected in cells expressing the active retroviral enzyme, while expression of the mutated inactive IN D116A had no significant effect [41].

As mentioned above a nucleoprotein complex (PIC) comprising the proviral DNA, the IN, and other viral and cellular proteins is formed in the cytoplasm and enters the nucleus by an unknown mechanism where the retroviral DNA is inserted into the host nuclear genome. The exact composition of the PIC is a controversial matter since different laboratories have described various viral and cellular proteins. The yeast two-hybrid system was used to identify a human gene product that binds tightly to the human immunodeficiency virus type 1 (HIV-1) integrase in vitro and stimulates its DNA-joining activity. This protein has been suggested as being part of the PIC. The sequence of the gene suggests that the protein is a human homolog of yeast SNF5, a transcriptional activator required for highlevel expression of many genes. The gene, termed INI1 (for integrase interactor 1), may encode a nuclear factor that promotes integration and targets incoming viral DNA to active genes [25]. The analogy of the yeast and human orthologs prompted study of whether yeast SNF5 is involved in the lethal effect of HIV-1 IN expression in yeast. The effect of the inactivation of the yeast gene encoding for SNF5 on the lethality induced by the yeast expression of HIV-1 IN has been described. Results showed that the retroviral IN is unable to perform its lethal activity in cells where the SNF5 gene has been disrupted, suggesting that SNF5 may play a role in the lethal effect induced by IN in yeast [42]. SNF5 inactivation does not affect neither yeast viability nor expression of HIV-1 IN. Given the homology between SNF5 and its human counterpart INI1, these results suggest that this factor may be important for IN activity in infected cells. Moreover, given the important role proposed for this transcription factor in the integration step and the fact that it is not essential for cell viability, the interaction between INI1/ySNF5 and HIV-1 IN should become a potential target in the search for new antiretroviral agents. Given the proximity of many human and yeast proteins the expression of the human INI1 rescued the lethal phenotype in yeast cells where SNF5 was inactivated, again revealing the functional analogy of many human and yeast genes [42].

A question that has not yet been fully answered concerning the HIV-1 cycle is the mechanism of entry of the PIC into the nucleus previous to integration in the host DNA. This question about intracellular HIV-1 trafficking has been tackled using the yeast system.Based on previous results using the two-hybrid strategy and showing that HIV-1 IN interacts with two yeast microtubule-associated proteins, Dyn2p (dynein light chain protein) and Stu2p, a centrosomal protein [43], the role of these proteins in intracellular trafficking and nuclear import in yeast was studied since it is thought that these biological mechanisms are evolutionarily conserved in eukaryotic cells. IN was found to accumulate at the perinuclear microtubule organizing center (MTOC) called the yeast spindle pole body (SPB) via Stu2p colocalization (Figure 5). Disruption of the microtubule network by nocodazole or IN expression in a dynein2-deficient yeast strain prevented IN accumulation in the nuclear periphery and additionally inhibited IN transport into the nucleus. By mutagenesis, the results indicated that trafficking of IN towards the SPB requires the C-terminus of the molecule.

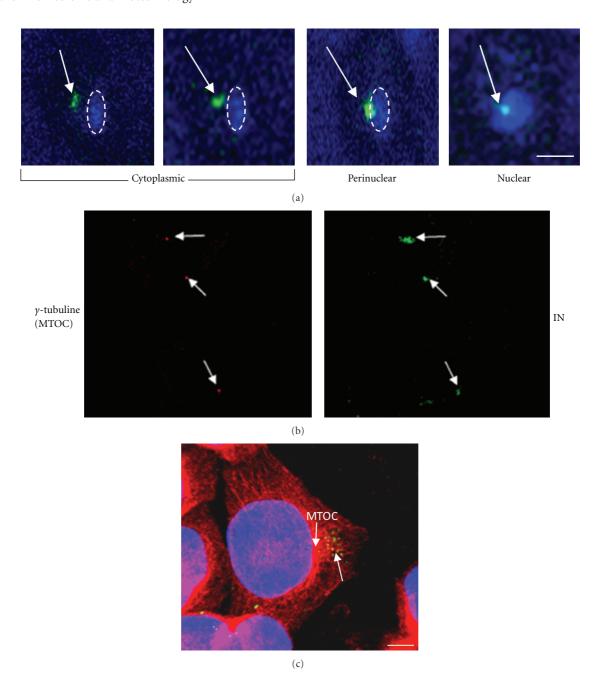


FIGURE 5: IN expressed in yeast and in human cell. (a) Expression of IN in yeast several times after induction. Cytoplasmic localisation: 6-7 h; perinuclear localisation: 8–10 h; nuclear localisation: 30 h. IN-GFP in green, nucleus in blue surrounded by dashed lanes. Barr = 1  $\mu$ m. (b) Expression of IN in H9 cells 20 h after transfection. Left, MTOC in red after immunodetection of  $\gamma$ -tubulin (arrows). Right, IN in green immunodetected with anti-IN antibody (arrows). (c) Expression of IN in H9 cells at higher magnitude. IN in green,  $\alpha$ -tubulin in red. Barr = 8  $\mu$ m (Desfarges, unpublished results).

These findings strongly suggest that IN nuclear import seems to depend on an essential intermediate step involving the SPB and that Dyn2p and Stu2p play an important role in driving IN toward MTOC and optimizing nuclear entry of the retroviral enzyme [44]. Intracellular transport of HIV-1 IN in yeast cells is in good agreement with later results using immunofluorescence and electron microscopy of the trafficking in human cells of IN tagged with GFP

and other labeling methods. These authors also observed an accumulation of labeled HIV-1 IN in the nuclear periphery and the involvement of the MTOC in the IN cellular *voyage* to the nucleus [28, 45].

The strong similarities between human infected cells and budding yeast demonstrate the relevance of the yeast model to obtain a detailed picture of the molecular mechanisms involved in the HIV-1 cycle (Figure 5).

As described above HIV-1 IN expressed in yeast in the absence of other viral proteins is able to enter the nucleus and cleave the host genome. An exciting question remained whether it is able to integrate a proviral-like DNA in the nuclear yeast genome when expressed alone. This was solved by setting up a system expressing only the IN and a short DNA mimicking the retroviral DNA by carrying the two LTRs flanking an antibiotic (zeocin) resistance gene (Figure 6). Multiple resistant yeast clones were observed under these conditions, while very few clones were obtained when using constructions carrying no IN or the inactive IN mutant D116A. Zeocin-resistant clones were confirmed to represent real integration events by detecting the 800 bp proviral-like DNA in the yeast genomic DNA by PCR or Southern experiments. Moreover, the sequence of the integration junctions showed that each integration event was disrupting a different open reading frame. Since the sequence of these links between integrated LTRs and the target DNA constitutes a specific signature of the IN involved in this process, they were carefully analyzed. The 5 bp repeats characterizing HIV integration were recovered for most clones, confirming that HIV-1 IN was responsible for the process. Therefore, in this simple eukaryotic model, the retroviral integrase is the only viral protein necessary for the insertion of a DNA containing viral LTRs into the genome, thereby allowing the study of the isolated integration step independently of other viral mechanisms. The identification of the yeast factors involved in the HIV-1 IN trafficking remains an interesting perspective. Another issue is whether these cellular factors, which are analogs to those forming the PIC in human infected cells, may be used to reconstitute a PIC-like complex [46]. Moreover these results may indicate that at least a fraction of the pool of integrase alone may enter the nucleus independent of the PIC.

The last step of the integration process involves the repair of the DNA junctions. It has been widely reported that the cellular DNA repair system plays a crucial role in the retroviral integration step. DNA repair factors like RAD18, RAD51, and RAD52 modulate this process [47]. The effect of RAD51 and other DNA repair factors can be easily studied in yeast since, contrary to what has been observed in human cells, no deleterious effects are observed when RAD51 or RAD18 are deleted in yeast. When RAD51 was deleted, a striking increase in integration events was observed after expression of IN and the viral-like DNA in yeast [46]. Very recently, the close interaction between IN and RAD51 and the molecular mechanism played by this protein in HIV-1 integration was described in yeast and human cells. In vitro integration assays performed under various conditions promoting or inhibiting hRAD51 activity demonstrated that the formation of an active hRAD51 nucleofilament is required for optimal inhibition involving the dissociation of the IN-DNA complex. Furthermore, the availability of the RAD51 stimulatory agent, RAS-1, made it possible to show that this inhibition mechanism could be promoted in HIV-1-infected cells by chemical stimulation of the endogenous hRAD51 protein. Stimulation of RAD51 induced both an enhancement of the endogenous DNA repair process and the inhibition of the integration step. These results showing

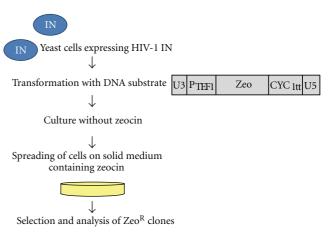


FIGURE 6: Integration assay in yeast. Adapted from [46].

the restriction of viral proliferation by RAD51 pave the way for a new concept of antiretroviral therapy based on the enhancement of endogenous hRAD51 recombination activity [48].

3.5. Protease. A lethal effect similar to that obtained by the expression of HIV-1 IN in yeast was observed more recently when the retrovirus-encoded protease (HIV-1 PR, Figure 3(c)) was expressed both in Saccharomyces cerevisiae and in mammalian cells. These findings contribute to a deeper understanding of HIV-1-induced cytopathogenesis. Expression of HIV-1 PR stopped yeast growth followed by cell lysis. The lytic phenotype included loss of plasma membrane integrity and cell wall breakage leading to the release of cell content to the medium. Interestingly, this effect seems to be specific for HIV-1 PR since neither poliovirus 2A protease nor 2BC protein, which are both highly toxic for S. cerevisiae, was able to produce similar effects. Drastic alterations in membrane permeability preceded the lysis in yeast expressing the HIV-1 PR. The morphological changes after expression of HIV-1 PR in yeast and mammalian cells were similar in many aspects [49].

# 4. Expression of Retroviral Enzymes Is Controlled by Frameshifting

During the replication of retroviruses, large numbers of Gag molecules must be generated to serve as precursors to the structural proteins of the virions. However, the enzymes encoded by the *pro* and *pol* genes (PR, RT, and IN) are generally needed in lower amounts to carry out their catalytic functions. Retroviruses have developed a mechanism leading to the synthesis of the Gag protein at higher levels relative to the *pro* and *pol* gene products, while retaining coregulated expression (Figure 7). This is due to the use of the same initiation codon in the same mRNA to express the *gag*, *pro*, and *pol* genes. Translation of this RNA leads occasionally to synthesis of a fusion protein that is usually called the Gag-Pro-Pol precursor. Typically, 10–20 structural Gag molecules are made for every molecule of Gag-Pro-Pol. This device

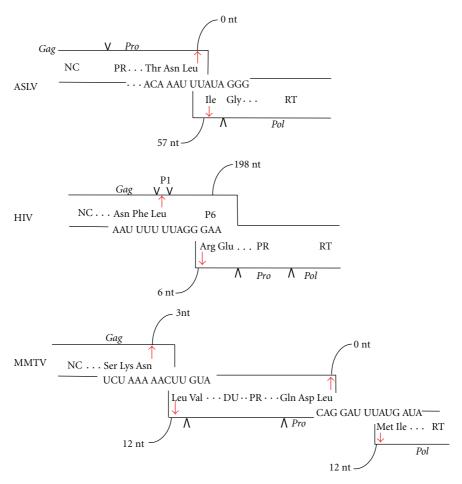


FIGURE 7: Frameshift suppression in the synthesis of Gag-Pro-Pol. Shown are the nucleotide sequences at the frameshift site and the amino acids encoded in the Gag-Pro-Pol precursors of the indicated viruses. The upper amino acid sequence is read from either the *gag* or *pro* reading frame, and the lower sequence is read from either the *pro* or *pol* reading frame, as shown. The boxes represent the indicated reading frames. The colored arrows indicate the position of the nucleotide (shown in color) that is read in both reading frames, the Vs represent the positions in the sequence that encode PR processing sites, and the numbers represent the number of nucleotides between the frameshift site and the end of the reading frame (irrespective of the nucleotide that is read in both frames). The nucleotides in the boxes include the beginning and ending codons in the reading frames shown (ASLV: avian sarcoma leucosis virus and MMTV: murine mammary tumor virus).

allows the same mechanism that targets the Gag precursor to the site of virion assembly also to direct the Gag-Pro-Pol precursor. In all retroviruses, the gag gene is positioned at the 5' end of the viral genome, upstream of the pro and pol genes. The Gag-Pro-Pol precursor is generated using a strategy in which the termination codon that defines the 3' terminus of the gag reading frame is bypassed, allowing translation to continue into the adjacent pro and pol reading frames. Bypass of the termination codon occurs by one of two mechanisms. The first mechanism (used by the mammalian type-C retroviruses) is read through (termination) suppression, in which the gag termination codon is occasionally misread as a sense codon. Translation then continues past the termination codon and into the pro-pol reading frame. The second mechanism, which is used by most retroviruses, is ribosomal frameshifting. Here, occasional ribosomes slip backward one nucleotide (-1 frameshift, i.e., in the 5' direction) during translation of gag. Thus, the ribosome leaves the gag reading

frame (with its downstream termination codon) and shifts into an overlapping portion of the *pro-pol* reading frame.

4.1. Vpr. The 14-Kd, 96-amino-acid HIV-1 encoded protein Vpr (viral protein R) [50, 51], plays several roles in the replication cycle of this retrovirus. Thus, it has been proposed that Vpr regulates the nuclear import of the preintegration complex (PIC) carrying the proviral DNA, the viral integrase, and other viral and cellular proteins. It is also required in the replication of HIV-1 in nondividing cells like the macrophages, and much evidence indicates that it is able to induce the arrest of cell cycle at the G2 step in proliferating cells, the latter effect likely playing an important role in the immunosuppression process in AIDS patients (for a recent review see [52]).

4.2. Arrest of G2/M by Vpr. Several lines of evidence in yeast and human cells show that Vpr has an important

effect on the G2 to M transition of the cell cycle [53]. It has been reported that the induction of Vpr expression in the fission yeast *Schizosaccharomyces pombe* leads to several defects in the assembly and function of the mitotic spindle. Some spindle pole body proteins were delocalized in Vprexpressing yeast cells perturbing its integrity. In addition, nuclear envelope structure, contractile actin ring formation, and cytokinesis were also disrupted. As similar problems in mitosis and cytokinesis were observed in human cells, it has been suggested that these defects account for some of the pathological effects associated with HIV-1 infection.

4.3. Apoptosis. When VPR was expressed in the fission yeast Schizosaccharomyces pombe the induction of cell death observed was reminiscent of the effect of HIV-1 infection in mammalian cells, suggesting that VPR may affect a conserved cellular process. In recent years proteins have been identified as Vpr suppressors that are able to overcome Vpr-induced cell death in fission yeast as well as arrest Vpr-induced apoptosis in mammalian cells. Although not conclusive these results may suggest that Vpr-induced cell death in yeast resembles some of the apoptotic processes in mammalian cells. Like Vpr-induced apoptosis in mammalian cells where mitochondria play an essential role, Vpr in yeast promotes phosphatidylserine externalization and induces hyperpolarization of mitochondria, leading to changes in mitochondrial membrane potential. Moreover, Vpr triggers production of reactive oxygen species (ROS), indicating that cell death via an apoptotic-like mechanism might be mediated by these reactive species. As mitochondria play a crucial role in apoptosis, it is intriguing that, in fission yeast, Vpr induces unique morphologic changes in these organelles. The Vpr suppressor factors EF2, Hsp16, and Skp1 that suppress Vpr-induced apoptosis in mammalian abolished cell death mediated by Vpr and restored normal mitochondrial morphology in yeast cells [54]. The similarity of Vpr-induced cell death in fission yeast with the mammalian apoptotic process reinforces the idea that fission yeast may be used as a simple model organism to study the apoptotic-like process induced by Vpr and other proapoptotic agents.

4.4. Rev. The 116-amino-acid HIV-1 Rev, an 18 kDa phosphoprotein discovered in 1986, is capable of being imported into the nucleus and binding specially to the Rev responsive element (RRE) a viral sequence-specific RNA. Rev is able to form multimers and direct the nuclear export of large RRE-containing RNP complexes. Rev activity is crucial in the nuclear export of intron-containing HIV-1 RNAs [55– 57]. Rev shuttles between the nucleus and the cytoplasm and has a nuclear localization signal (NLS) as well as a nuclear export signal (NES). These essential peptide motifs have now been shown to function by accessing cellular signal-mediated pathways for nuclear import and nuclear export. In addition to NLS and NES a nuclear import inhibitory signal (NIS) that inhibits the entry of low molecular weight proteins has been described in Rev [58]. HIV-1 Rev is therefore an excellent system to study aspects of transport across the nuclear

envelope [59]. Human immunodeficiency virus type 1 (HIV-1) replication requires the expression of two classes of viral mRNA. The early class of HIV-1 transcripts is fully spliced and encodes viral regulatory gene products. The functional expression of Rev induces the cytoplasmic expression of the unspliced or incompletely spliced mRNAs that encode the viral structural proteins, including Gag and Env. Based on experiments that indicate a similar function of Rev in the yeast S. cerevisiae, a yeast protein interacting with the effector domain of Rev was found [60]. This protein called Rip1p is a novel small nucleoporin-like protein, some of which is associated with nuclear pores. Its closest known yeast relative is a nuclear pore component also involved in mRNA transport from nucleus to cytoplasm. Analysis of yeast strains that overexpress Rip1p or which are deleted for the RIP1 gene show that Rip1p is important for the effect of Rev on gene expression, indicating that the physical interaction is of functional significance in vivo. These results suggest that Rev directly promotes the cytoplasmic transcripts transport by targeting them to the nuclear pore.

The NES domain of the Rev protein is required for Rev-mediated RNA export in mammals as well as in the yeast *Saccharomyces cerevisiae* [61]. As mentioned above Rev NES has been shown to specifically interact with a human (hRIP/RAB1) and a yeast (yRip1p) protein in the two-hybrid assay. Both of these interacting proteins are related to FG nucleoporins on the basis of the presence of typical repeat motifs. Rev is able to interact with multiple FG repeat-containing nucleoporins from both *S. cerevisiae* and mammals. Moreover, the ability of Rev NES mutants to interact with these FG nucleoporins parallels the ability of the mutants to promote RNA export in yeast, Xenopus oocytes, and mammalian cells [62].

HIV-1 Rev-mediated nuclear export of viral RNAs involves the interaction of its leucine-rich NES with nuclear cofactors. Using an extensive panel of nuclear export positive and negative mutants of the functionally homologous NES of the HIV-1 Rev, physiologically significant interactions of human Nup98 (hNup98) with NESs from various viral sources were demonstrated. Recently, a cellular factor called CRM-1 was shown to be an essential nuclear export factor interacting directly with nuclear export signals including the Rev NES in a RanGTP-dependent manner. It was shown that NLP-1, like the previously described Rev-interacting protein hRIP/Rab and several nucleoporins, also interacts with CRM-1 in both yeast and mammalian cells. Missense mutations in the yeast nuclear export factor Crm1p abrogated Rev NES interaction with the XXFG repeat-containing nucleoporin Rab/hRIP. These mutations had minimal effects on the interaction with GLFG repeat-containing hNup98. Functional analysis of the Nup98 domains required for nuclear localization demonstrated that the entire ORF was required for efficient incorporation into the nuclear envelope. A putative nuclear localization signal was identified downstream of the GLFG repeat region. Whereas overexpression of both full-length Nup98 and the amino-terminal GLFG repeat region, but not the unique carboxy-terminal region, induced significant suppression of HIV unspliced RNA export, lower levels of exogenous Nup98 expression resulted in a relatively modest increase in unspliced RNA export. These results suggest a physiological role for hNup98 in modulating Rev-dependent RNA export during HIV infection [63].

4.5. Antifungal-Effect of Rev NIS-Derived Peptide. The NIS signal encoded in Rev has been used as a therapeutic agent with anticandidal effects. In addition this property was used to study the mechanism of action of this factor by studying the intracellular localization of the peptide. The result showed that Rev rapidly accumulated on the fungal cell surface. The cell wall regeneration test also indicated that Rev exerts its anticandidal activity on the fungal plasma membrane rather than on the cell wall. By using fluorescent probes, the membrane-disruption mechanism of Rev was further confirmed, suggesting that it may be a potential therapeutic agent for treating fungal diseases caused by Candida species in humans. Further studies showed that the antifungal effects of the nuclear entry inhibitory signal peptide of HIV-1 Rev protein had no hemolytic effects [64, 65].

# 5. Yeast and HIV-1 Translation (for a Recent Review See [66])

Retroviral frameshifting (see above) is a change in reading frame during gene expression and is a mechanism that allows to keep at a low level the synthesis rate of the functional proteins relative to that of the structural proteins. Using S. cerevisiae to decipher viral frameshifting mechanisms Wilson et al. were the first to provide an in vivo demonstration of a frameshifting event, in S. cerevisiae [67]. They inserted the Gag-Pol fragment containing the potential frameshifting site of HIV-1 (without the stimulatory element) into a yeast expression plasmid, upstream from the interferon (IFN) cDNA. They monitored production of the frameshifted protein by Western blotting. It is now clear that a stimulatory secondary structure is required for maximal frameshifting efficiency although the precise nature of this structure remained unclear for a long time. Results using a dual reporter system in yeast showed that there is a direct correlation between HIV frameshifting efficiency and the stability of the stem loop [68]. The stem loop analyzed in these studies was the upper part of the complete stimulatory element observed by NMR. Under these conditions, the stability of this structure is clearly linked to frameshifting efficiency. A structure was recently identified on the basis of the complete yeast genome sequence, and it seems interesting to explore frameshifting efficiency with the complete sequence. The structure of the tetraloop is similar to the motif found in the RNase III recognition site from S. cerevisiae [69]. As the ACAA motif in the tetraloop is poorly recognized by RNase III, the possibility of engineering the S. cerevisiae RNase III for selective targeting of the HIV-1 tetraloop followed by the expression of this protein in HIV-1-infected cells has been suggested. This hypothetical approach remains to be confirmed but would provide an interesting therapeutic

strategy, derived from experiments in yeast, to limit HIV-1 proliferation. The similar slippage efficiencies of the HIV frameshifting site in vivo in yeast and in vitro in a mammalian system demonstrate the high level of conservation of frameshifting mechanisms. Bidou et al. [66] suggested that the demonstration that frameshifting is conserved from yeast to humans paves the way for the use of yeast mutants to analyze retroviral frameshifting as already reported by several groups for other viruses. For instance, SARS coronavirus (sometimes shortened to SARS-CoV) is the virus that causes severe acute respiratory syndrome. SARS-CoV carries a frameshifting signal [70]. The minimal frameshifting signal in this virus is a U UUA AAC slippery sequence and a stimulatory structure folding into a pseudoknot [71]. This pseudoknot has several unusual features, including the third stem in loop 2 and the presence of two unpaired adenosine residues within the structure ([70, 72]. Plant and Dinman [70] demonstrated the ability of this new site to frameshift in S. cerevisiae. The frequency of frameshifting in yeast was much lower (3%) than for other coronavirus sites tested in yeast (12% for infectious bronchitis virus (IBV)). This may indicate the existence of subtle differences in terms of frameshifting mechanisms. Indeed, the importance of the unpaired adenosine residues remains unclear as this part of the pseudoknot is thought to lie outside the ribosome. It would be interesting to investigate the possible binding of a transacting factor, although the binding of such a factor has never been detected with the well-studied IBV coronavirus pseudoknot. The yeast mak8-1 mutant is known to have a specific defect in frameshifting [73]. It carries an altered form of ribosomal protein L3 in the ribosomal peptidyl-transferase center. This strain was reported to have a slightly higher SARS-CoV frameshifting efficiency than wildtype strains [70], in the first demonstration that this newly discovered frameshifting site is used.

#### 6. Summary and Perspectives

In summary, although much of the potential promise of yeast is still to be revealed, it has proved extremely valuable in virus research. In addition to techniques like the double-hybrid system, this simple eukaryotic cell has been very useful for producing recombinant viral proteins whose purification from native virions is difficult or simply impossible. It also allows the mechanism of action of viral proteins to be studied thanks to the close analogy between human and yeast proteins, and this has led to the emergence of new therapeutic targets. By associating the toxic phenotype induced by some viral proteins in yeast cells with the genetic manipulation facilitating the entry of drugs with potential therapeutic properties, it may become possible to establish a simple cheap system allowing faster screening of the antiviral agents of the future. Moreover, future work should lead to the discovery of new cellular factors involved in virus proliferation, thus shortening the time necessary to develop new therapies against current and new viruses. The ultimate information on the behavior of viruses or virus proteins inside the cell should be attained with plant, animal, and human cells or in vivo in whole organisms, although it may be difficult and costly for most laboratories to develop these approaches. Since experiments with yeast will always be technically easier, more rapid, and cheaper than those with human and other complex eukaryote cells, yeast will remain a method of choice for studying virus infections mechanisms and the search for new drug targets.

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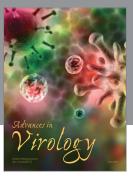
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