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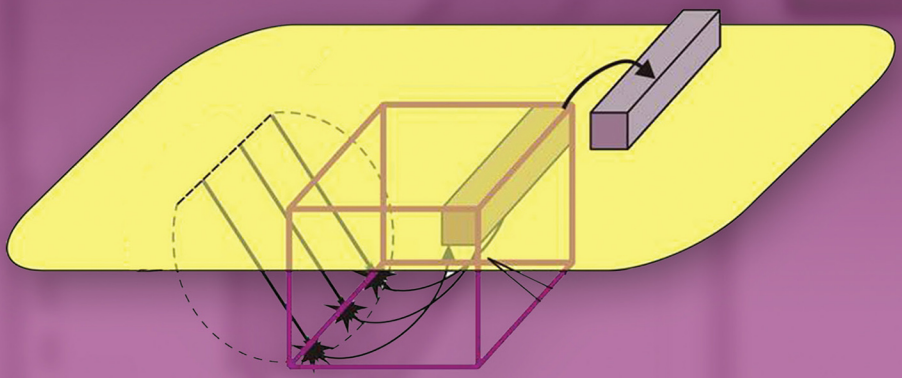
Nanobiotechnology in Energy, Environment, and Electronics

Methods and Applications



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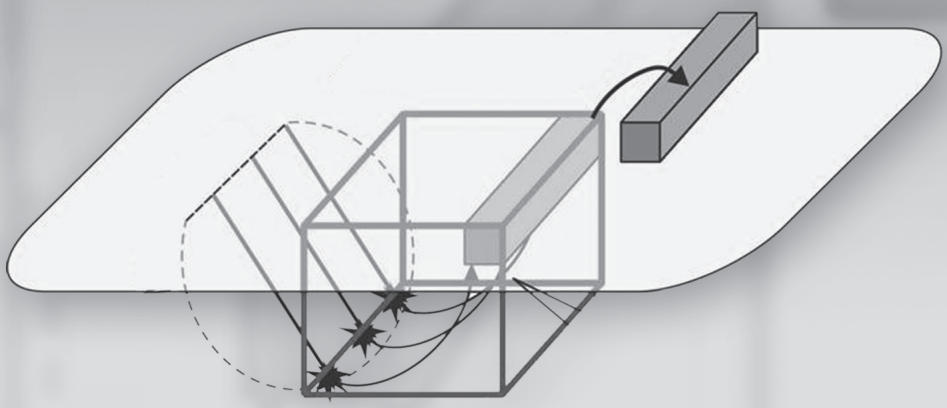

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To the memory of Dr. Alexey A. Peshkov, my very dear friend, outstanding Russian scientist, and correspondent member of the Russian Academy of Sciences, for his long scientific activity towards new megatechnologies—namely the development of mineral deposits by the technogenic initiation of directed geological processes—that a premature departure has suddenly terminated, leaving, however, to us his important scientific contribution witnessed also in this volume.

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Contents

Introduction: Present Challenges and Future Solutions via Nanotechnology for Electronics, Environment and Energy

Claudio Nicolini

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Oil fuels the modern world, but oil is a finite resource. It brought great changes to economies and lifestyles in less than 200 years and nothing else to date equals the enormous impact which the use of oil has had on so many people in so many ways around the world. The critical question is, however, "When is or was the maximum daily amount of world oil production at its peak?" After that, oil is going to become an irreversibly declining resource facing an increasing demand, which will not be met. The world passed its peak of rate of oil discoveries in the 1960s, and it seems that the peak of world oil production will then be reached by 2020, and possibly within the next decade (Campbell, 1997; Campbell and Laherrere, 1998; Ivanhoe, 1995).

What are then going to be realistic alternative energy sources for humanity among the existing renewable or non-renewable one (Table 1)?

Fusion involves the fusion of either of two hydrogen isotopes, deuterium or tritium. Deuterium exists in great quantities in ordinary water, and from that perspective fusion is theoretically an almost infinitely renewable energy resource. This is the holy grail of ultimate energy. Fusion is the energy that powers the Sun, and that is the problem. The temperature of the Sun ranges from about

10,000 degrees Celsius on its surface to an estimated 15–18 million degree Celsius in the interior where fusion takes place. Containing such a temperature on Earth in a sustainable way and harnessing the heat to somehow produce power has so far escaped our search. However, even if commercial fusion will be accomplished, the end product again is likely to be electricity, and not a replacement for fuel sources such as oil and gas.

Table 1 Alternative energy sources

Non-renewable	Renewable
Oil sands, heavy oil	Wood/other biomass
Coal	Hydropower
Shale oil	Solar energy
Gas hydrates	Wind energy
Nuclear fission	Wave energy
Geothermal	Tidal power
	Fusion
	Ocean thermal energy conversion

Questions are sometimes raised as to using **hydrogen and fuel cells** for fuel sources. Neither is a primary energy source. Hydrogen must be obtained by using some other energy source. Usually it is obtained by the electrolysis of water, or by breaking down natural gas (methane CH_4). Hydrogen is highly explosive, and to be contained and carried in significantly usable amounts, it has to be compressed to hundreds of pounds per square inch. Hydrogen is not easy to handle, and it is not a replacement for pouring 10 gallons of gasoline into an automobile tank. Fuel cells have to be fuelled; most use hydrogen or some derivative of oil. Fuel cells are not a source of energy in themselves.

Oil appears to be a unique energy source that up to now has no complete replacement in all its varied end uses. British scientist Sir Crispin Tickell concludes, "…we have done remarkably little to reduce our dependence on a fuel [oil] which is a limited resource, and for which there is *no comprehensive substitute in prospect*." Coming to realize that oil is finite, any and all suggestions of means to replace oil are obviously welcomed, but so far only cheerful myths are enthusiastically embraced. These include: that there are two trillion

barrels of economically recoverable oil in the Colorado Plateau oil shales; that dams and their reservoirs are a source of indefinitely renewable energy and that they are environmentally benign; that solar, wind, geothermal and hydro-electric power can supply the electrical needs, from the Arctic to the tropics, of the Earth's **over six billion people** (likely to further grow in the near future); that coal, oil from oil sands, and biofuels can replace the 72 million barrels of oil the world now uses daily; and that somehow electricity produced from various alternative energy sources can readily provide the great mobility which oil now gives to the more than 600 million vehicles worldwide. Regrettably, **none** of these cheerful myths appear up to now to be valid, including the mega-myth which represents the most popular public placebo that "The scientists will think of something" as I did witnessed myself few months ago at Orlando during a short visit to Disneyworld. The energy spectrum from burning wood to fusion that fuels the Sun (Table 1) is now well known. If there is some major exotic energy source beyond what is here listed, we have no evidence of it and the reality appears to be that the world is rapidly running out of a resource (oil) that in many ways appears irreplaceable. We have been living on a great fossil fuel inheritance accumulated during more than 500 million years that humanity has incredibly exhausted in the last period in less than 200 years. We will soon exhaust this capital, and we will have to go to work to try to live on current energy income. It will not be a simple easy transition as pointed out in a remarkably perceptive book written by Darwin in 1952, where were described historic changes in the human condition, calling them "revolutions." Darwin wrote that there is one more revolution clearly in sight: "The fifth revolution will come when we have spent the stores of coal and oil that have been accumulating in the earth during hundreds of millions of years…it is obvious that there will be a very great difference in ways of life… ."

The present energy situation is exemplified in Figs. 1 and 2 from what has been occurring in the United States in the recent time.

Similarly, the **natural disasters** have been increasing in number and frequency in the last few years permitted by the wrong myth that humanity could do without the Kyoto agreement attempting to control the devastating **carbon dioxide** effects. As in the preservation of oil reserve, the humanity and its governments have

failed also in the protection of natural environment. In summary, the result could become very shortly a great change in economies, social structures and lifestyles, and the opportunities for science and technology to make a difference are becoming constantly narrower. And this despite the striking development in **electronics at the nanoscale**, through the construction of new effective nanosensors and nanoactuators.

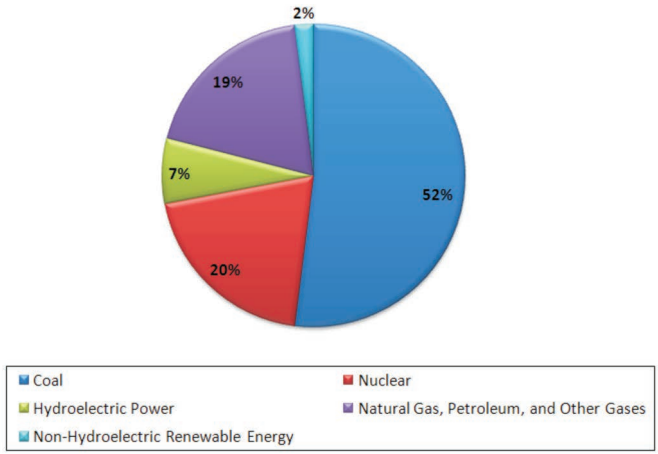

Figure 1 US electricity net generation by source for 2000 (EIA Annual Energy Review) *Source*: International Energy Agency.

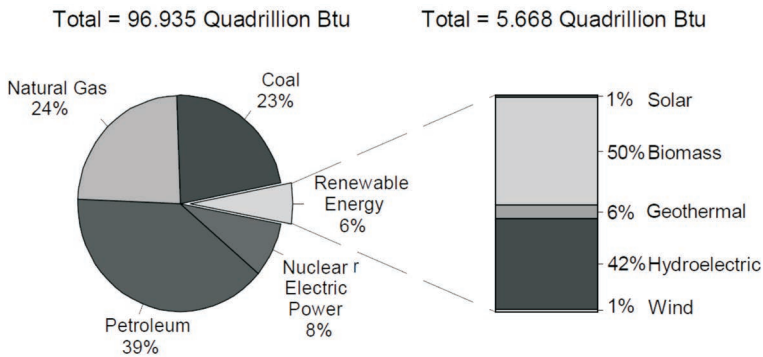

Figure 2 Renewable energy in proportion to total US energy supply. *Source*: Energy Information Administration (EIA).

It is, therefore, essential to find concrete solutions to the growing problems in all sectors of the economy, such as energy,

environment and electronics, and worldwide the situation could be overcome only by a wide internationalisation involving the leading countries in economic, military and financial terms, namely the **Russian Federation**, the **United States of America and Europe**. Furthermore, only at the nanoscale, we can hope to embark on such undertaking with some degree of success. It is the right moment for the United States and Russia to unite with Europe their forces and to concentrate all the possible resources to solve the dramatic problems affecting the entire world as those illustrated above, involving resources and facilities from leading multinational companies, as well manpower coming from citizens of the above three large countries which should change their priorities and return to science and technology as for the past full of long stories of successes. I know this will work for the entire world's benefit and for my personal direct experience having passed my entire life in these three large communities extremely productive in R&D. Attempt to do this at the national scale with large recruitment from underdeveloped countries is bound to failure because the magnitude of the crisis induced by the derivatives has far lasting devastating effects (continuously coming to light) and because the magnitude of the technological problems long time underestimated is unmatchable at any single isolated nation scale. The scheme based on multinational companies, that in the past was able to work for Italy in Bioelectronics with Polo Nazionale Bioelettronica and CIREF (both centred around Italian-based multinational companies as ABB, Montedison, FIAT, ST Microelectronics, Olivetti, Farmitalia, Elsag-Bailey), may still be valid but this time only if the above named three large countries, frequently in the past on opposite sites, find means to cooperate in order to achieve the required critical mass at the world scale. An institution (Fondazione EL.B.A.) indeed in the past was born and did grow with participation of organizations at the crossing of Europe, Russia and the United States and constitute the proof of principle that something similar (Nanoworld Institute) could become again the triggering factor between Europe, Russia and the United States. In the past, the Biochip Project initiated by President Gorbachev through Academician Velikov (USSR) and President Craxi through myself (Italy) did work and I do not see why should not work now, despite the larger scale and the more ambitious objectives. I hope that the time has passed for science to be at the service of arms race

among between the United States and Russia, and the reduction of nuclear arsenals and waste being pursued in START (STrategic Arms Reduction Treaty) should further aim not only to avoid their falling in wrong hands and to increase global security (as suggested by President Obama in the 2013 State of the Union), but also to transfer all these military resources in **joint civilian project**. Recently in an open debate with President Putin organized by Russian television I raised via the Internet a question (subsequently acknowledged) about the opportunity of final disarmament within the "Measures to Further Reductions and Limitation of Strategic Offensive Arms" treaty between Russia and the United States such that the enormous resources spent could be used for the development of joint projects in nanoscience and nanotechnology for energy, health, electronics and environment.

Cancer at the molecular scale is strongly interlinked to differentiation, ageing and proliferation, but also to ecology, and solving it we will solve major correlated problems in life sciences. Energy is strongly interlinked with power generation, automation and environment, while similarly is happening (at the nanoscale) for really intelligent hardware, being strongly interlinked to communication, defence and environment. Indeed the risk of upcoming ecological disasters, including global warming, can be reduced or avoided with the development of new energy sources nanotechnology-based from sun, wind and hydrogen. The far-reaching effects will be beneficial for the entire humanity and for the survival and growth of earth. Last but not least is the objective to bring back the prestige of science among young people to correct the economical disasters caused by bankers and financial institutions.

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Part A

Methods

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Chapter 1

Influence of Chromosome Translocation on Yeast Life Span: Implications for Long-Term Industrial Biofermentation

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Ageing as always been part of the biological life and a topic that we understand very little of. In particular, ageing in microbial cultures undergoing long-term industrial fermentation is a very important factor determining the overall efficiency and in final analysis the economic outcome of the bio-production process. On the other hand, gross chromosomal rearrangements (GCR), specifically translocations, are genomic alteration well known to occur in fungal cells undergoing long-term growth. This work focuses primarily on the effects of chromosome translocation on the chronological life span (CLS) of *Saccharomyces cerevisiae* (SC), a model organism used both, for understanding the ageing process as well as for industrial large-scale production of re-cyclable

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biomass. A secondary goal of the research was to verify the feasibility of using telomeric DNA sequence length as a molecular marker to monitor population ageing during long-term fermentation. Therefore, a comparison between telomere length and CLS was performed. The data that have been gathered show that chromosome translocation has a different and sometimes opposite effect on the CLS of the budding yeast; furthermore, it was demonstrated that in some cases telomere length does not correlate with the life span of SC. This finding refutes the postulate that age and life expectancy can be deduced from the length of the telomeric DNA and thus precludes the possibility to use this parameter to monitor the health and viability of a long-term industrial fermentation process.

1.1 Introduction

1.1.1 The Yeast *Saccharomyces cerevisiae*

Saccharomyces cerevisiae, also known as the budding yeast, has been used since the dawn of the great civilizations for its fermentation properties in the production of bread, beer, and wine (Fig. 1.1).

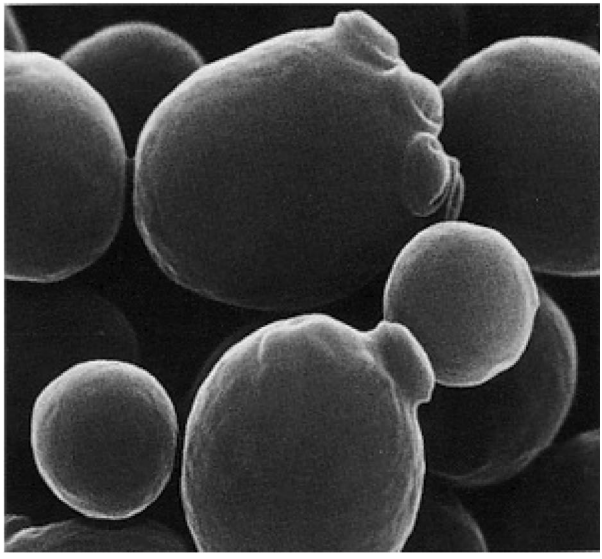

Figure 1.1 Electron microscope picture of *Saccharomyces cerevisiae*.

It is one the eukaryotic models of choice for the easiness with which it can be handled; in fact, it is one of the most studied eukaryotic microorganisms in the fields of molecular and cell biology.

Major discoveries have been made working on SC such as cell-cycle and genome instability, which have greatly contributed to the development of the recent techniques used in laboratories; furthermore, it presents the same structural complexity of plant and mammal cells.

Going deeper into the structural and molecular content of SC, it is important to understand some major characteristics that define the budding yeast.

SC cells differ from ovoid to round and have a diameter that varies from 5–10 depending if it is haploid or diploid.

The haploid form is due to the fact that it has gone into starvation, meaning that it has fewer nutrients on which it can survive and therefore it must conserve itself by using less energy as possible. This is achieved thanks to the production of four haploid spores, which remain held in a capsule called ascus. Since the different sexes are expressed by a couple of heterozygous alleles *MAT*a/*MAT*α, every ascus will hold two haploid cells for every sex type; this means two a and two α. If the amount of nutrients is re-established to normal, it returns to the diploid form by mating one α with one a.

1.1.2 The Yeast Genome

The yeast genome is organized in 16 chromosomes that vary in length between 200 and 1800 kb. The total amount of the genome is 12 Mb with approximately 6400 ORFs. Furthermore, there is the presence of extra-nuclear DNA such as 2 μ, which is an endogenous plasmid, and mitochondrial DNA. However, the yeast genome is very different from any other organism because it is very condensed; as a result, 72% of it is encoded.

One of the reasons why SC has become so important is that it was the first eukaryotic genome to be sequenced. The completed sequence has been published in 1996 (Goffeau et al., 1996).

The availability of the genome has turned SC in an optimal model for the studies concerning homologous genes in humans, plants and other organisms. In addition, presumably 50% of the yeast genes have a homologous gene in humans.

1.1.3 DNA Double-Strand Break

Different DNA lesions can occur; one of them is the double-strand break (DSB). This break can be induced by different kinds of factors: endogenous such as accumulation of oxygen reactive species, replication stresses and replication errors, whereas the exogenous factors are chemical reagents, different kinds of drug species and IR radiation.

In human cells, these breaks normally occur during the entire life span of the cell due to their metabolism and environmental factors therefore by leading to a major structural damage of the DNA molecule, which can dramatically change how the cell reads the gene information. Consequently, the cell needs to frequently repair the damage using different repair mechanisms. However, when the cell ages these repair mechanism become slower and less efficient to a point that it cannot repair itself anymore, now the cell can choose different paths to follow, such as senescence, apoptosis or carcinogenesis.

The majority of the cells in our body are senescent. However, if they are hit by a non-repairable DNA damage, they will follow the path of apoptosis, this is the last resort to prevent the formation of tumorigenic cells.

In somatic tissues, since the recurrence of repair is high, the genome instability can lead to the loss of heterozygosity and carcinogenesis.

This means that you can have the activation of proto-oncogenes through an allelic mutation or the inactivation of oncosuppressor (i.e. the alteration of the gene that gives mammary gland cancer *BRCA1*).

When a DSB occurs in a eukaryotic cell a complex web of proteins activate, these are damage sensor enzymes, signal transductors and effectors. Some of the proteins that are activated are kinases (Mec1 and Tel1), which in terms activate by phosphorylation Rad9 and Rad53.

Rad53, moreover, acts on a series of substrates (Brca1, Nbs1, p53 and Cdc25C) activating the damage response mechanism. Therefore, Rad53 stops the cell-cycle and enhances the repair mechanism and if necessary triggers apoptosis (Zhou et al., 2000).

1.1.4 DBS Repair Mechanisms

There are different DBS repair mechanisms (Fig. 1.2):

- homologous recombination (HR)
- single-strand annealing (SSA)
- synthesis-dependent strand annealing (SDSA)
- break-induced replication (BIR)
- crossing over
- non-homologous end joining (NHEJ)

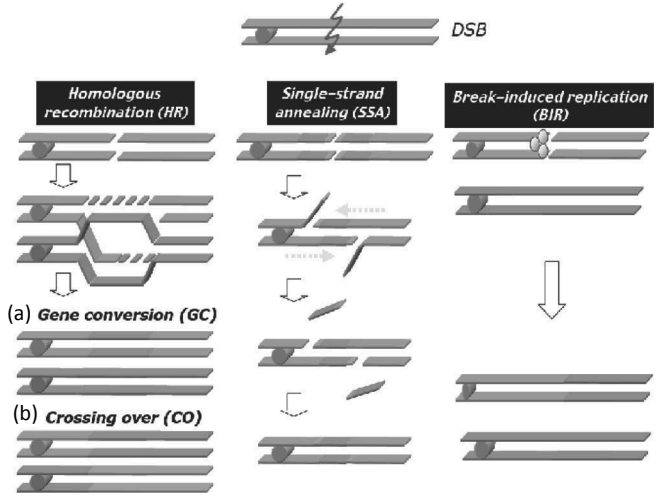

Figure 1.2 Schematic representations of some DNA repair mechanisms.

The different repair mechanisms that arise after the DSB are used differently in the different cells; NHEJ is used by the mammalian cells, whereas HR is used by the yeast (Kanaar et al., 1998; Kolodner, 2002). In addition, the HR is more efficient in the diploid yeast cell rather than the haploid yeast (Shrivastav et al., 2008).

Defects in these repair systems cause genome instability, which can lead to tumorigenesis.

DSB in SC activates different repair mechanisms, which are kinetically different but all activated by Mec1 and Tel1. Mutations to Mec1 and Tel1 cause different chromosome alterations such as the shortening of the telomeres, the increase in mitotic recombination,

chromosome loss, and the sticking of the telomere ends, which ultimately leads to translocations and circular chromosomes (Craven et al., 2002).

1.1.5 Homologous Recombination

The term "homologous recombination" describes a set of mechanisms, all of which use homologous sequences to repair DNA. Most current models of HR are initiated by a DSB; the most common models are double-strand break repair (DSBR), SDSA, SSA, and BIR models. These HR mechanisms have several common features: All HR reactions are catalyzed by a number of proteins that belong to the *RAD*52 epistasis group, although some enzymes are more important for specific pathways.

An important point in the HR is the formation of the Holliday junction, which is a mobile cross formation of DNA with four different strands paired in two duplex. This formation is achieved because when a DSB occurs, one strand of the duplex anneals with the strand of the other duplex (Agmon et al., 2009).

1.1.6 Synthesis-Dependent Strand Annealing

Homologous recombination via the SDSA pathway occurs in cells that mitotically divide and results in non-crossover products. The invading 3′ strand is extended along the recipient DNA duplex by a DNA polymerase and is released as the Holliday junction between the donor and recipient DNA molecules slides in a process called branch migration. The newly synthesized 3′ end of the invading strand is then able to anneal to the other 3′ overhang in the damaged chromosome through complementary base pairing. After the strands anneal, a small flap of DNA can sometimes remain. Any such flaps are removed, and the SDSA pathway finishes with the resealing, also known as ligation, of any remaining single-stranded gaps (Helleday et al., 2007).

1.1.7 Break-Induced Replication

During DNA replication, double-strand breaks can sometimes be encountered at replication forks as DNA helicase unzips the template strand. These defects are repaired in the BIR pathway of HR. The

precise molecular mechanisms of the BIR pathway remain unclear. Three proposed mechanisms have strand invasion as an initial step, but differ in how they model the migration of the D-loop and later phases of recombination (McEachern and Haber, 2006).

The BIR pathway can also help to maintain the length of telomeres, regions of DNA at the end of eukaryotic chromosomes, in the absence of (or in cooperation with) telomerase. Without working copies of the telomerase enzyme, telomeres typically shorten with each cycle of mitosis, which eventually blocks and leads to senescence. In budding yeast cells, where telomerase has been inactivated through mutations, two types of "survivor" cells have been observed to avoid senescence longer than expected by elongating their telomeres through BIR pathways (McEachern and Haber, 2006).

Maintaining telomere length is critical for cell immortalization, a key feature of cancer. Most cancers maintain telomeres by upregulating telomerase. However, in several types of human cancer, a BIR-like pathway helps to sustain some tumors by acting as an alternative mechanism of telomere maintenance (Morrish et al., 2009).

1.1.8 Single-Strand Annealing

The SSA pathway of HR repairs double-strand breaks between two repeat sequences. The SSA pathway is unique in that it does not require a separate similar or identical molecule of DNA, like the DSBR or SDSA pathways of HR. Instead, the SSA pathway only requires a single DNA duplex, and uses the repeat sequences as the identical sequences that HR needs for repair. The pathway is relatively simple in concept: After two strands of the same DNA duplex are cut back around the site of the double-strand break, the two resulting 3′ overhangs then align and anneal to each other, restoring the DNA as a continuous duplex (Haber et al., 2010; West, 2003).

1.1.9 Non-Homologous End Joining

NHEJ is referred to as "non-homologous" because the break ends are directly ligated without the need for a homologous template, in contrast to HR, which requires a homologous sequence to guide repair. The term "non-homologous end joining" was coined in 1996 by Moore and Haber.

NHEJ typically utilizes short homologous DNA sequences called micro-homologies to guide repair. These micro-homologies are often present in single-stranded overhangs on the ends of double-strand breaks. When the overhangs are perfectly compatible, NHEJ usually repairs the break accurately. Imprecise repair leading to loss of nucleotides can also occur but is much more common when the overhangs are not compatible. Inappropriate NHEJ can lead to translocations and telomere fusion, hallmarks of tumor cells (Boulton et al., 1996; Moore et al., 1996; Wilson et al., 1999; Budman et al., 2005).

1.1.10 Chromosome Translocation

Chromosome translocation is a rearrangement of parts between two non-homologous chromosomes (Fig. 1.3). When the two breaking points are within a gene coding sequence this could lead to the fusion of two genes, that is very common in cancer cells, and also is known to bring diseases such as the chronic myelogenous (or myeloid) leukemia (CML) which is the union between the *BCR* and the *ABL* protein genes (Klein et al., 1982).

There are two different main types of chromosome translocations in humans: the reciprocal translocation or non-Robertsonian translocation, which is usually an exchange of material between two non-homologous chromosomes, and the Robertsonian translocations, which are the fusion of the two acrocentric chromosomes near the centromere with loss of the short arm that therefore lead to an unbalanced karyotype of 45 chromosomes.

Figure 1.3 Three-dimensional image of a chromosome.

Carriers of Robertsonian translocations are not associated with any phenotypic abnormalities, but there is a risk of unbalanced gametes, which lead to miscarriages or abnormal offspring. For example, carriers of Robertsonian translocations involving chromosome 21 have a higher chance of having a child with Down syndrome. These chromosome translocations can also be seen in yeast and can represent a way to evolve.

1.1.11 BIT Bridge-Induced Translocation

Bridge-Induced translocation method (Fig. 1.4), which was developed in this laboratory, induces chromosome translocation in unmodified yeast cells through targeted DNA cassette integration of the KANr selectable marker flanked by sequences homologous to two chromosomal loci randomly chosen on the genome (Tosato et al., 2005).

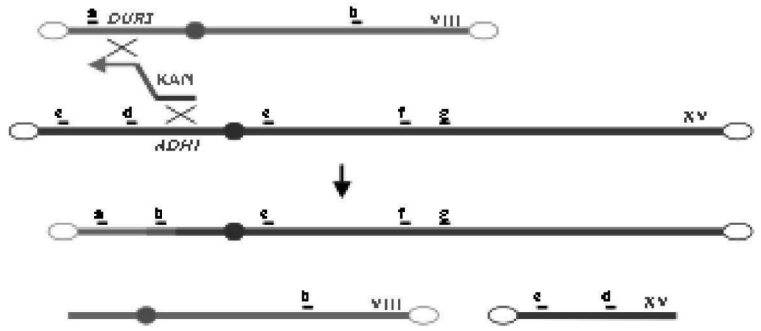

Figure 1.4 Scheme of a bridge-induced translocation between the gene ADH1 on chr. XV and the gene DUR3 on chr VIII.

The cassette carried at its ends two nucleotide sequences homologous to two distinct genomic loci each located on a different chromosome. Thus, once integrated, the selectable linear DNA fragment becomes a molecular bridge between two unique, naturally occurring pre-selected loci.

This method gives chromosome translocations without the need of previously engineering the cells. In literature, there are different techniques used to induce chromosome translocation, but in these cases, it is necessary to insert a site for an endonuclease such as HO or Sce1 by therefore promoting a DBS and a translocation, but they all occur between homologous chromosomes.

The length of the homology deeply influences the BIT system. In fact, a 40 nt homology has an efficiency of 1.6% integration into the desired locus, but by lengthening the homology to 65 nt the efficiency doubles. The low percentage in the desired translocation means that probably there is mechanism that suppresses the whole processes during mitosis.

Furthermore, the efficiency of the translocation is influenced by the region of homology. This means that the regions that regulate this process, also known as the promoter and the terminator regions, are more recombinogenic compared to the ORF.

The mechanism with which probably the translocation occurs follows two steps: First, one end pairs with its homologous sequence; second, the other end searches for its homology on the same chromosome, and if the homology is not met, the cell activates the mechanism to suppress the mitotic recombination. At this point, the free end is able to pair itself with the homology sequence found on the other chromosome and therefore induces a translocation. The pieces of DNA that are cut during the translocation can follow different paths such as degradation or chromosome rearrangements.

In most cases, the cassette can integrate ectopically in the genome, or with just one end of targeted locus. In this case, the free end can integrate in another chromosome using a micro-homology or it can integrate itself on the same chromosome without any homology at all. Finally, it can also integrate itself in the endogenous plasmid 2 μ.

It is also known that only 10–20% of the translocants show phenotype abnormalities such as polynucleated cells, longer buds, anucleated cells. In most cases the actin1 protein seems to be modified, this could explain in part the phenotype abnormalities. There is also a decrease of the Rad53p protein used in DNA repair mechanisms and also the missed phosphorylation of the same protein. These data suggest that the cells have undergone an adaptation after their arrest in the G2/M phase checkpoint (Nikitin et al., 2008).

It has been observed that from the same chromosome translocation you can obtain 10 translocants with different phenotype, morphology and different gene expression (Rossi et al., 2009).

1.1.12 Telomeres

Chromosomes to be determined as such need three essential structures: origin of replication, centromere, and a telomere.

The telomere (Fig. 1.5) has different functions in the chromosome, which differ from the maintenance of the chromosome structure to the protection of the ends. It has been known since the 1970s that the conventional DNA polymerase is unable to replicate the ends; therefore, the cell is unable to keep the telomere from shortening each round of cell division.

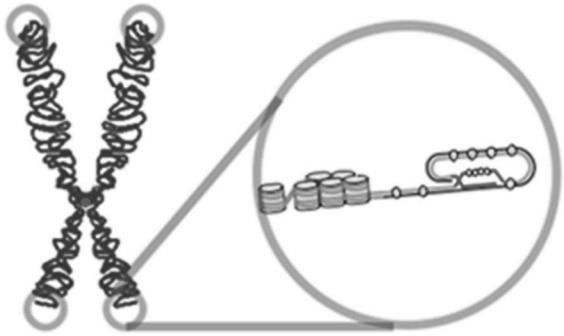

Figure 1.5 Representation of a chromosome and its telomere.

However, some cells such as stem and tumor cells have a very important protein that is expressed called the telomerase, which can increase the length of the telomeres every time they shorten since it carries a RNA template used for this task, this gives the cell a certain immortality, thereby the cell is able to divide limitlessly without entering senescence or a non-dividing stage.

In addition, there are some proteins that are associated to the telomere such as the TRF proteins that are considered the "ruler" of the telomere because they are used by the cell to understand how long is the telomere. Furthermore, another family of proteins known as sirtuins have shown to play an important role in the processes that bring the cell to senescence.

The Sir2 protein (silent information regulator) is known to suppress the rDNA recombination and extend the life span by 40% if an extra copy of the gene is added. However, if the telomeres are too long—and therefore there are a large number of sirtuin proteins attached, Sir2 could suppress essential genes of the cell

bringing it to a premature death (Kass-Eisler and Greider, 2000; Bitterman et al., 2003; Cheol Woong Ha and Won-Ki Huh, 2011).

1.2 Material and Methods

1.2.1 Materials

Table 1.1 Some of the translocants that were studied in this manuscript Yeast Strains

Yeast Strains Name	Chromosomes involved	Genotypic background	Produced by
D10	XV-VIII	SAN1	BIT with 40 nt of homology
D3	XV-VIII	SAN1	BIT with 40 nt of homology
D11	XV-VIII	SAN1	BIT with 65 nt of homology
T5	XV-VIII	Trisomic N2 (SAN1 + VIII)	BIT with 65 nt of homology
T12	XV-VIII	Trisomic N2 (SAN1 + VIII)	BIT with 40 nt of homology
AD5	V-VIII	SAN1	BIT with 65 nt of homology
N2	V-VIII	Trisomic N2	BIT with 40 nt of homology
POLY	XIII-XV	BY4743	Excision by Scel in vitro; transformation with 50 nt homology
Val	VII-II	SAN1	BIT with 40 nt of homology
Susu1	IX-XVI	SAN1	BIT with 40 nt of homology
Susu4	IX-XVI	SAN1	BIT with 40 nt of homology
Susu5	IX-XVI	SAN1	BIT with 40 nt of homology
77	VIII-VIII	SAN1	BIT with 40 nt of homology
San1	None	Wt	

1.2.1.1 Media

The media compositions follow the standards of "methods in yeast genetics" (Kaiser et al., 1994). Yeast used for genomic extraction was grown in YPD + G418 (final concentration 200 μg/mL) for selection, whereas for the CLS, all strains were grown in synthetic complete medium with G418 for those that needed selection.

Synthetic Complete Medium

Ingredients	% w/v
Bacto-yeast nitrogen base w/o amino acids	0.67
Ammonium sulfate	0.5
Glucose	2
Drop-out mix	0.2

Synthetic Complete Medium: Add 1000 mL of distilled water.
Dropout mix

Ingredients	mg. in 1000 mL
Adenine	18
Arginine	76
Histidine	76
Leucine	380
Lysine	76
Methionine	76
Proline	76
Threonine	76
Tryptophan	76
Tyrosine	76
Uracil	76

Adenine, threonine, and tryptophane were added after autoclaving the media, since they are thermolabile.

1.2.1.2 Solutions and enzymes

- Herring sperm (HS) (Roche), DNA MB grade, 10 mg/mL, for hybridization
- Marker 1 Kb plus 1 µg/mL
- Geneticine G418 50 mg/mL
- Stock solutions: ethanol, ethanol 70%, TBE 10×, ammonium acetate 4 M, maleic acid 0.5 M, Tween 20, NaOH 0.4 N, HCl 0.25 N, SSC 20×, SDS 10%
- Enzyme use for genomic digestion BanI