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Strategies for gene cloning

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Abstract Cancer, including genitourinary malignancy, is a consequence of accumulated genetic aberrations in genes involved in crucial regulatory pathways. The result is a deregulation of cellular behaviour, leading to neoplastic transformation, uncontrolled cell proliferation and acquisition of metastatic ability. The development and perfection of techniques in the field of recombinant DNA technology, gene cloning, (differential) analysis of gene expression, and sequencing of genes and proteins have provided a wealth of information about the genetic aberrations associated with cancer development. This "recombinant DNA and gene cloning" technology *and* the recently developed DNA chip technology may provide new molecular diagnostic tools. Furthermore, the technology of gene cloning in combination with the progress in in vivo gene delivery techniques offers new treatment modalities, like gene therapy, additional to conventional therapies. This review is intended to provide a general introduction to the fundamentals or strategies of recombinant DNA and gene cloning techniques as a basis for understanding the rapidly expanding range of new diagnostic and therapeutic opportunities. Some illustrative examples are provided, addressing basic and biomedical research and possible clinical applications in genitourinary oncology.

Key words Genitourinary malignancies · Recombinant DNA · Gene cloning · DNA chip technology · Diagnostics · Gene therapy

Introduction

The introduction of new tools or techniques in genetic research, comprehending isolation (and manipulation) of DNA fragments or genes, inserting of DNA derived from one species of organism into the DNA of another (*recombinant DNA*), and introducing and propagating recombinant DNA in a suitable host (*gene cloning*), has contributed immensely to an improved understanding of the genetic basis of a large array of both inherited and acquired (human) diseases. In the field of medical care, this explosive advance in basic knowledge has already offered a new avenue for the development of diagnostic tools [71, 74], and therapies, such as gene therapy. Gene therapy consists of delivering genetic material into a diseased individual with the intention to cure or alleviate particular diseases [53]. On the eve of possible revolutionary changes in medicine a profound revision of thinking among biomedical investigators and medical practitioners seems to be required. A fruitful participation in the discussions about the value of these new technologies, encompassing clinical exploitation, and ethical and financial implications makes it necessary or even mandatory for both (basic) life scientists *and* medical practitioners to gain some, at least general insight into the field of recombinant DNA technology [59].

Contrary to widespread belief, that recombinant DNA, gene cloning, construction of genomic libraries, etc. are complex and difficult subjects, the general principles are not so extremely difficult to comprehend, in spite of the large range of sometimes difficult, but technical procedures. In this article, focusing on readers who are relatively unfamiliar with the subject, we will discuss initially the molecular structure of genetic information, the control of this information and its expression into protein, being the fundamentals of gene technology. Subsequently, a general outline of the principles of recombinant DNA technology and gene cloning will be presented. Finally, the potential power of these techniques will be illustrated with a few, representative examples, focusing on the use of these new technologies in the development of new "tools" in the diagnosis of cancer and their (potential) therapeutic value for human malignancies, especially emphasizing gene therapy for (due to the authors' bias) genitourinary

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cancers. Considering the extent of the subject, space and citation constraints, readers interested in (more) extensive discussions on gene technology are referred to a number of excellent reviews and books, cited within the (sub)headings of the various sections. Citations referring to more specific research topics are mentioned where appropriate.

Recombinant DNA technology and gene cloning: the goals

The ultimate goal of recombinant DNA technology and gene cloning as ''tool-orientated'' technologies can be considered from a basic or a medical point of view, although these views are not mutually exclusive. The aim in basic research is to (1) unravel the specific molecular structure or *nucleotide sequence* of the genes of a given organism (genotype), underscored in the Human Genome Project [48, 60], and (2) understand the regulation of this genetic information and the collective in vivo function of the gene products (proteins), which ultimately determine the functional and structural appearance (phenotype). Focusing on the clinical impact of these techniques, the central challenge is to (1) identify genetic aberrations causally related to inherited and acquired human diseases, (2) provide nucleotide sequences of (relevant) genes for diagnostic and prognostic purposes, (3) isolate and propagate relevant genes, and alter the genome by ablation, addition, substitution or augmentation of specific genes of the diseased host (gene therapy). Alternatively, the treatment targets may be indirect, occurring via an intermediate (protein) expressed by an inserted gene. A (essential) condition for the accessibility of such goals is the availability of genomic or cDNA libraries, containing the genes or DNA sequences of interest.

Central concept of molecular biology [34, 36, 69, 79]

The ability to isolate and clone human gene fragments or even complete genes in ''technically'' suitable hosts of different origin is based ultimately on the similarity of molecular organization in *all* organisms, from bacteria through to mammals. The molecular structure of human DNA is compatible with that of any other organism and, as a consequence, human DNA segments, including complete genes, can be inserted in DNA of other organisms, such as bacteria. Understanding of such a ''cut-and-paste'' technology requires some insight into the molecular structure of DNA, its characteristics as a medium of ''informational storage'' and the processes involved to translate this (genetic) information into protein, together known as the ''central concept of molecular biology'' [75–77]. The genome of human (somatic) cells consists of 46 chromosomes, each composed of a single, linear double-stranded DNA molecule and associated proteins. In native DNA, two strands or chains of deoxyribonucleic acids are coiled round the same axis in a helical fashion, orientated antiparallel, which results in a polarity with an unpaired 5′ phosphate at one end and a 3′ hydroxyl group at the other (Fig. 1). Each chain is composed of four kinds of covalently linked (deoxyribose) nucleotides or *bases*, being adenine, cytosine, guanine and thymine (abbreviated A, C, G and T). In the double helix the chains are joined together through hydrogen bonds between these nucleotide bases (base pairs or bp). It is of importance to appreciate that the bases are complementary, that is adenine pairs with thymine (A–T) and guanine with cytosine (G–C). So, whereas the nucleotide sequence on a single strand does not seem to be restricted, the complementary, specific pairing of the nucleotides determines the base sequence of one chain automatically, given the sequence of the other chain (Fig. 1). This mechanism of comple-

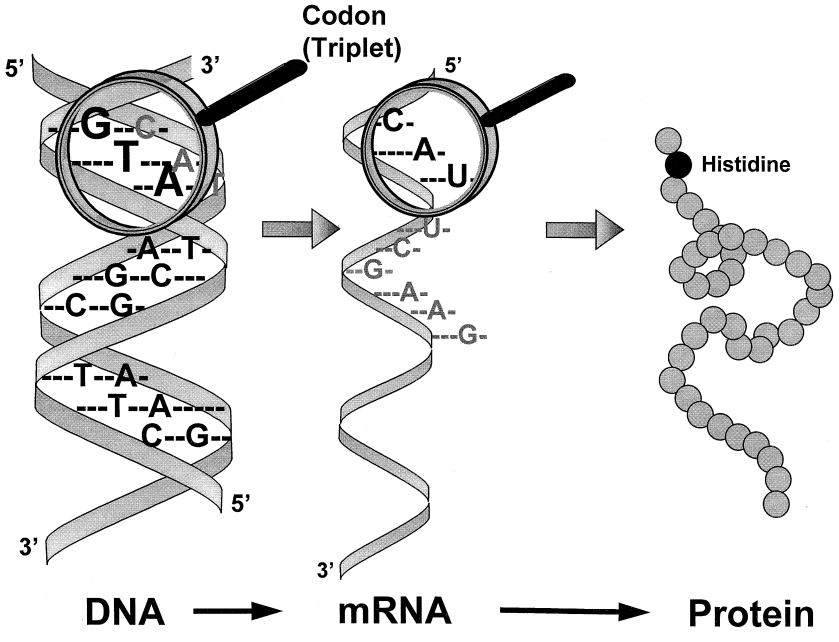

Fig. 1 Structure of the complementary base pairing of two DNA strands. Base pairing always occurs between the complementary nucleotides Adenine and Thymine (A–T; T–A), and Cytosine and Guanine (C–G; G–C) on opposite strands. A series of three successive bases (triplet or codon) on one strand specify each of the amino acids (*grey and black filled circles*) of proteins. For example, the codon CAU on mRNA specifies the amino acid histidine. Note that in mRNA the pyrimidine Uracil (U) replaces the structurally similar pyrimidine thymine found in DNA. Genetic information ''flows'' from DNA through mRNA to protein (see also Fig. 2)

mentary base pairing provides the basis for a proper replication of the genetic, DNA material and its stored information. Furthermore, within the context of the present review this complementary nucleotide pairing is crucial for the technology of recombinant DNA construction and gene cloning.

Within the chromosomes, the genes (or a specific sequence of nucleotides) are the carriers of genetic information, and each single gene codes, in the majority of cases, for a single polypeptide or protein. The basic units of genetic information are embodied in the particular sequences of three nucleotides or *triplets*, where a specific triplet (or *codon*) corresponds with a specific amino acid within a protein (Fig. 1). Taken together, a unique sequence of codons within a gene reflects the unique sequence of amino acids found in a particular protein. The details of the mechanism by which the genetic information, stored in a gene and written in the codon "language", is controlled and deciphered into the other molecular "language" composed of amino acids are enormously complex. However, in essence, this process of gene expression can be divided in three major steps (Fig. 2):

Control of the transcription of a gene, being either stimulatory or inhibitory

The control of messenger RNA (mRNA) synthesis from DNA is achieved by promoter and enhancer elements, which are usually located at the 5′ site of the protein-coding region of the gene (the transcription unit). These elements serve as starting and controlling points of mRNA synthesis by the enzyme *RNA polymerase*, using DNA as template and growing in the 5′ to 3′ direction.

Synthesis of single-stranded, mature mRNA or gene transcription with DNA as template

Besides the regulatory regions, each eukaryotic gene is arranged in *exons* or coding regions for amino acid sequences (protein), which are interspersed by non-coding segments or *introns*. Both the exons and introns are transcribed into a premature mRNA. Termination of the mRNA synthesis is accomplished by a "self-complementary" termination signal. Subsequently, primary mRNA is processed to mature RNA by removal or splicing of the introns from the primary RNA template. In addition, post-transcriptional modifications, like the addition of a sequence of 50–250 adenylic acid residues (A-A-….-A-A-; poly A tail) to the 3′ terminus of the mRNA molecule, are executed.

Synthesis of protein by the protein synthesis machinery with mRNA as template

The mature mRNA is transferred from the nucleus to the cytoplasm, where, dictated by the nucleotide sequence of the mRNA, amino acids are linked in a precise order by the ribosomes (and a variety of enzymes and RNAs), forming the polypeptide. After translation of mRNA information into a polypeptide, subsequent post-translational modifications (e.g. glycosylation) generally occur in eukaryotes, which finally results in mature, functional protein.

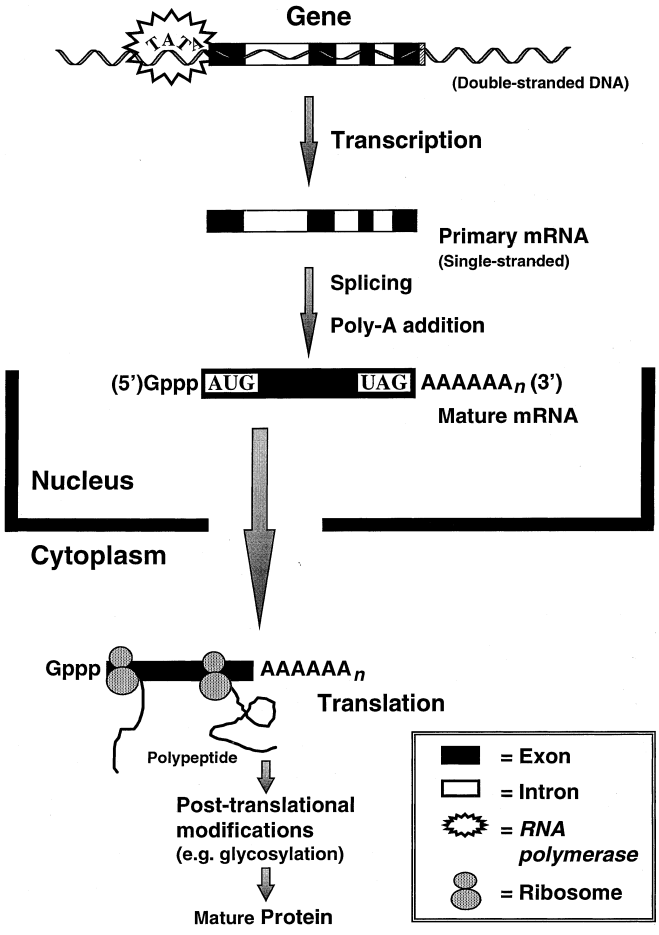

Fig. 2 Schematic representation of the structure of a typical eukaryotic gene transcription into mRNA, translation into a polypeptide and posttranslational modification into a mature protein. After synthesis of premature mRNA by RNA polymerase (initiated at the TATA box), splicing out of the introns and among others poly-A tail addition, mature mRNA is transported to the cytoplasm. Subsequently, mRNA is translated into a polypeptide using respectively the AUG and UAG codons as start and stop sites. Finally, posttranslational modifications lead to a mature protein. In this diagram, exons and introns are not drawn to scale; frequently most introns are longer than any of the exons.

Procedures of gene cloning [9, 33, 45, 64, 68, 78]

Complying with the outlined process of gene expression or "central concept of molecular biology" (DNA → RNA → protein) [77], a cloned gene of the target (e.g. human) genome should ideally, but not absolutely necessarily, encompass the *complete* nucleic acid sequence of a gene. The sequence of target DNA should embrace the

controlling or leader region, the protein coding regions (exons), the protein non-coding sequences (introns) and the sequence of the termination signal. Although the non-coding introns do not seem to be necessarily incorporated in the cloning sequence, it has been noted that artificially manufactured genes, which lack introns, sometimes fail to get transported from the cytoplasm to the nucleus.

As illustrated in Fig. 3, the general scheme of the construction of recombinant DNA, gene cloning and the construction of a genomic or complementary DNA (cDNA, synthesized from mRNAs) library consists of a series of procedures or steps. Currently, an ever-increasing number of new techniques, comprising almost all of the various steps in isolation and propagation of DNA, is becoming rapidly available [9, 47]. It is, however, not the intention of this review to cover in complete detail all the currently available, highly sophisticated approaches and, consequently, these more recent developments will only be mentioned if appropriate. In the following sections the four basic steps in gene cloning are outlined.

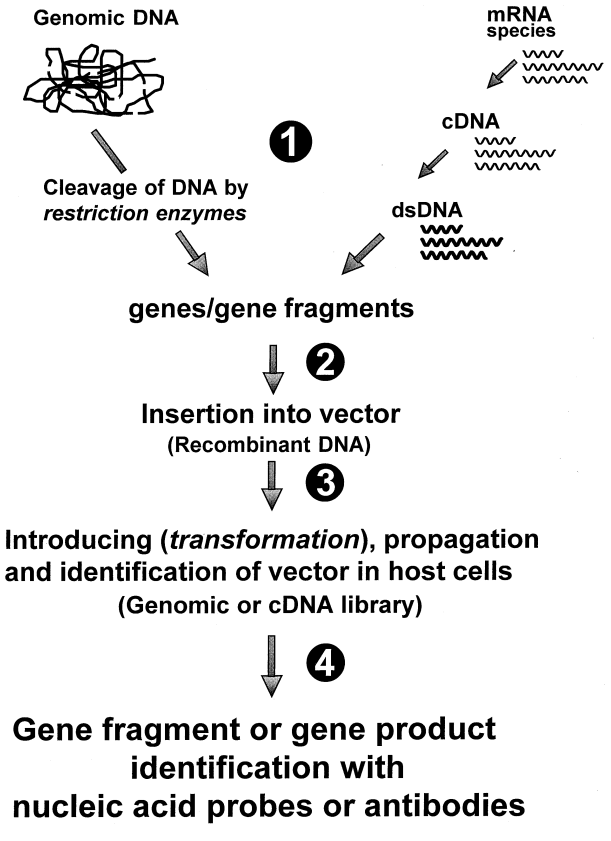

Fig. 3 Flow chart representing the basic plan for gene cloning. The four basic steps are indicated: (1) Preparation of target DNA (genomic DNA, cDNA), (2) recombination or insertion of target DNA into vector DNA (plasmids, bacteriophages, cosmids and yeast artificial chromosomes, etc.); (3) introducing and amplifying of recombinant vector in a suitable host (bacterium, eukaryotic cell) resulting in a "genomic DNA library" or "cDNA library"; and (4) identification of foreign DNA fragments of interest by various methods (DNA probes, immunochemically, functional analysis, etc.)

Step 1. isolation/preparation of target DNA (Fig. 3)

Genes or DNA fragments can be isolated and cloned from DNA, and are derived from two sources of nucleic acids, either genomic DNA or cDNA.

Genomic DNA

Omitting mitochondrial DNA, a preparation of human genomic DNA ($\approx 3 \times 10^9$ bp) contains all, approximately 90 000 *complete* genes, including the protein noncoding regions (Fig. 2). The identification of a battery (>150) of similarly acting enzymes, the *restriction endonucleases*, found in a wide range of bacteria, has appeared to be crucial in providing the basic tools to reduce the extremely long, double-stranded DNA (dsDNA) molecules of the chromosomes into a set of discrete DNA fragments (Fig. 4) [see ref. 1]. Moreover, these enzymes are of unequalled value to construct "recombinant DNA" molecules (see below). The various bacterial restriction enzymes cleave dsDNA at specific sites (restriction sites), determined by a well-defined nucleotide sequence of usually 4, 6 or 8 base pairs. Many of these enzymes make staggered, symmetrical cuts, yielding cohesive or "sticky" ends (Fig. 4) [5]. As a result of this enzymatic treatment, genomic DNA is digested into discrete reproducible fragments of different length, ranging from several hundred to several thousands of base pairs long, while each fragment contains sticky ends at each site of the gene or DNA fragment. The sizes of the DNA fragments depend on the frequency of occurrence of specific restriction sites, the restriction endonucleases used and time of enzymatic treatment. The final fragments are used to construct a "genomic library".

Complementary DNA (cDNA)

The large size of the majority of human genes (partly determined by the presence of the non-coding introns) and the specific characteristics of the most commonly used cloning vectors and their hosts (*E. coli* plasmids and λ-phage; see below) may limit effective cloning. So, for example, the length of an average eukaryotic gene is approximately 2000 protein-coding nucleotides (exons) and one or more introns. So, as an alternative to genomic DNA cloning, cloning can start from smaller cDNAs, synthesized from mRNAs, devoid of introns. An additional advantage of this strategy is that only genes, really *expressed* in a particular tissue, are cloned, which allows the study of differential gene expression between various tissues, e.g. between malignant tissues and their normal counterpart(s).

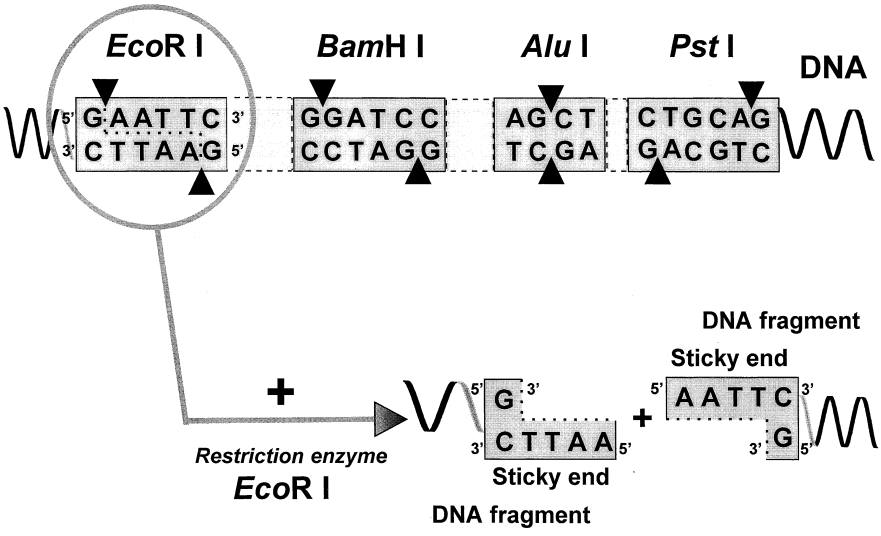

Fig. 4 Examples of restriction endonucleases, nomenclature, nucleotide sequence specificity and specific cleavage. The first three letters of the nomenclature (in italic) refer to the organism (*Eco Escherichia coli*, *Bam Bacillus amyloliquefaciens*, *Alu Arthrobacter luteus*, *Pst Providencia stuartii*), while, if relevant, the preceeding letter (e.g. R and d) indicates a particular strain. The roman numerals distinguish between multiple restriction enzymes from the same organism. As illustrated in detail for *Eco*R I, the enzymatic activity cuts (*filled triangles*) each strand of DNA between the G and A nucleotide within the base sequence 5′GAATTC 3′ resulting in single-stranded (5′AATT 3′) "sticky ends"

Protocols for cDNA synthesis with mRNA as template, and subsequent dsDNA synthesis vary greatly, but all follow the same scheme (Fig. 5). After purification of the mRNA population exploiting their poly-A tails (Fig. 2), each mRNA molecule is copied into a strand of cDNA, complementary to the nucleic acid sequence of a mRNA by the enzyme *reverse transcriptase* and oligo-dTs (short strings of thymine) as initiating *primers*, and the four nucleotides (dNTPs). After digestion of mRNA, single-stranded cDNAs are converted to dsDNAs through a series of steps using a variety of enzymes (DNA polymerase I, T7 DNA polymerase, terminal transferase, T4 DNA ligase), primers and dNTPs (Fig. 5). As a final step, so-called *Eco*R I linkers (or other restriction linkers) are added at each site of the dsDNAs, followed by cutting with (*Eco*R I) endonuclease. As a result of all these reactions, complete dsDNA molecules are generated which represent each molecule of the mRNA population and contain sticky ends at each site (Fig. 5). These dsDNA molecules are to used to construct a "cDNA library".

Steps 2 and 3: Cloning of target DNA in a vector, introduction and propagation in a host and selection of recombinant DNA containing hosts (Fig. 3)

Independent of the source of genetic material, either genomic DNA or cDNA, the final result is a population of double-stranded genes or DNA fragments with single-stranded, sticky ends. This material, also known as "target DNA" or "foreign DNA" has to be cloned in a suitable *cloning vector* and subsequently the "recombinant" vector has to be introduced and propagated in a suitable host. A cloning vector is a DNA molecule with three major properties: (1) a vector should be able to incorporate foreign DNA, (2) a vector should confer selectable properties to the host in order to identify single organisms carrying a "recombinant, target DNA" vector, and (3) a vector should be capable of replicating in a host, independently of the host chromosome(s). Traditional cloning vectors are the bacterial *plasmids* and *bacteriophages*, but gradually additional vector systems have emerged, each with particular advantages and disadvantages.

Bacterial plasmid vector system

In addition to a single large, circular chromosome, bacteria may contain independently replicating, smaller loops of DNA, the plasmids. For recombinant DNA technology the most commonly used plasmids, either naturally occurring or redesigned, replicate in *E. coli*. A typical plasmid has a small (circular) length and possesses an origin of replication (ORI) and restriction sites (polylinkers) for insertion of foreign DNA (Fig. 6). In addition, a crucial property of these cloning vehicles is the presence of selectable markers, generally genes encoding for antibiotic-inactivating enzymes. The activity of these enzymes confers resistance to antibiotics on the host bacterium. This property allows selection of host bacteria that have incorporated a plasmid-containing target DNA (see below). All enzymes required for the replication of a plasmid are provided by the host, although the ORI site helps to regulate the initiation of replication. As illustrated in Fig. 6, the first procedure in the construction of a *recombinant* plasmid consists of in vitro annealing between target DNA ("genomic DNA fragments" or "cDNA") with plasmids, cleaved with the same (or functionally related) restriction endonuclease. Definite joining or ligation is established with the enzyme *DNA ligase*.

A variety of methods of introducing a single plasmid recombinant DNA molecule into a competent *E. coli*

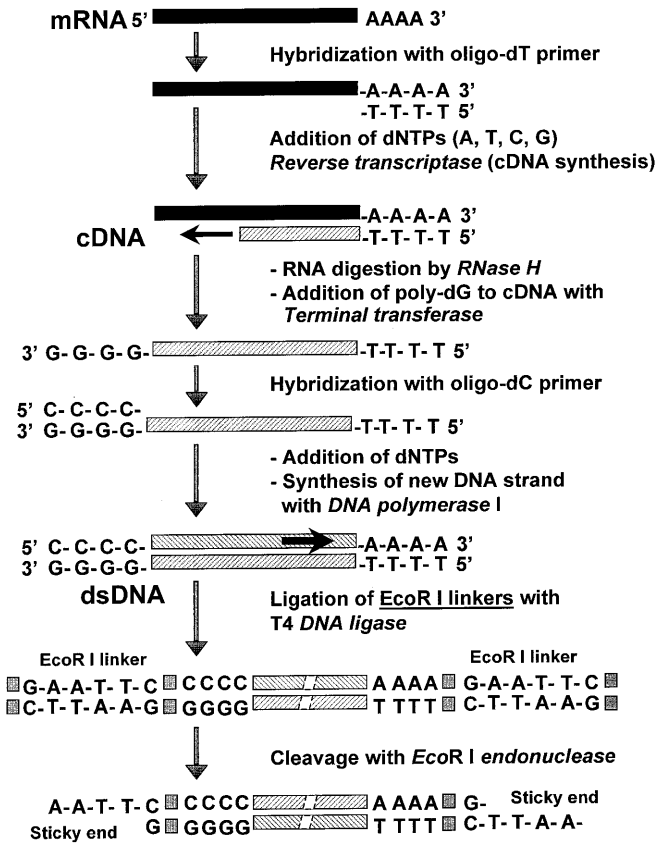

Fig. 5 Flow chart of in vitro dsDNA synthesis from purified mRNA. Following purification of the mRNA population (from other RNAs) by a column of matrix-linked oligo-dTs, the retroviral reverse transcriptase synthesizes a strand of cDNA by polymerizing deoxynucleoside triphosphates (dNTPs) using a mRNA molecule as template and a oligo-dT primer (T-T-T-T) base-paired to the 3′ poly-A tail. The subsequent step consists of enzymatic removal of RNA molecules (RNase H) and conversion of cDNA in double-stranded DNA (dsDNA). The latter is initiated by enzymatic (terminal transferase) addition of guanidine triphosphates, resulting in a series of guanines at the 3′ end of the cDNA. This oligo-dG (G-G-G-G) tail is used to synthesize enzymatically (DNA polymerase I) a DNA strand, complementary to the original cDNA, with oligo-dC (C-C-C-C) as primer. The result of these reactions is the production of dsDNA molecules corresponding to each of the mRNA molecules. Next, *Eco*R I linkers are added (with the enzyme T4 DNA ligase) to double-stranded oligo-dC-oligo-dG and oligo-dT-oligo-dA of the dsDNA molecules. Finally, treatment with *EcoR* I endonuclease provides the dsDNA molecules with "sticky-ends"

cell, a process known as *transformation*, is currently available. However, since transformation of *E. coli* is a highly inefficient process and cleaved plasmids may self-anneal, transformed *E. coli* cells with plasmids containing human DNA inserts have to be distinguished from *E. coli* cells without plasmids or plasmids without foreign DNA inserts. The outlined selection method, illustrated in Fig. 7, is an example of so-called negative (here, loss of antibiotic resistance) selection. More recently, positive selection methods have been developed using specially engineered plasmids (such as the pUC series), allowing for selection of transformed *E. coli* cells (colonies) by the colour of the colonies [72]. After selection of the transformed *E. coli* cells (colonies) a genomic DNA or cDNA library has been established. Since the transformation rate and stability of an *E. coli* plasmid decrease with the length of the foreign DNA insert, *E. coli* plasmids are mostly used for the construction of a cDNA library.

Bacteriophage vector system

A disadvantage of plasmids as cloning vectors is their intracellular instability and low transformation rate if they contain large (>15 kb) DNA fragments. Bacteriophages or bacterial viruses provide the opportunity to clone larger DNA inserts more efficiently. The well-known and most often applied viral vector is the bacteriophage λ (phage lambda; phage λ), which contains a dsDNA genome of 49 kb length [57]. The life cycle of phage λ in *E. coli* has two modes of replication [45]. The first is the so-called *lysogenic pathway*, which involves attachment of the phage to the bacterial cell, injection of phage DNA, and integration of phage DNA into the host cell chromosome where it remains in a dormant state. The alternative mode, the *lytic pathway*, follows the same initial path of infection, but rather than integration, the phage DNA replicates in the cytosol of the host. Replication of phage λ DNA and DNA packaging in phage λ protein coats result in lysis of the host and the release of phages.

Genes involved in the lysogenic pathway, but not essential for the lytic pathway, are irrelevant for the use of phage λ as a cloning vector. These non-essential genes are located in the middle region of the phage λ DNA and can be (partially) replaced by a foreign DNA insert of approximately 20–25 kb (Fig. 8). These types of phage λ vectors are known as *replacement* vectors, unlike *insertion* phage λ vectors, in which target DNA is simply ligated in, without replacement of, phage λ DNA. As illustrated in Fig. 8 for a replacement vector, human genomic DNA fragments and phage "arms" (obtained by restriction endonucleases) are annealed and ligated, followed by packaging into an infectious *phage coat*. This latter process is accomplished in vitro by a phage-assembly package set, consisting of preassembled λ heads and tails. Finally, the recombinant (λ) phages are detected and multiplied on a lawn of *E. coli*. Each genomic recombinant λ phage is unique and collectively they constitute a λ genomic DNA library. In addition, a λ cDNA library can be established with the outlined procedure.

Other vector systems

As noted, both *E. coli* plasmids and λ phages have been recognized as vectors well suited for cloning of relatively small (<25 kb) DNA fragments. As was gradually realized, many human genes appear to be considerably larger, spanning hundreds of kb or even megabases. In recent years more efficient and versatile vectors have been developed which have made the previously discussed techniques less labour intensive. So, for example,

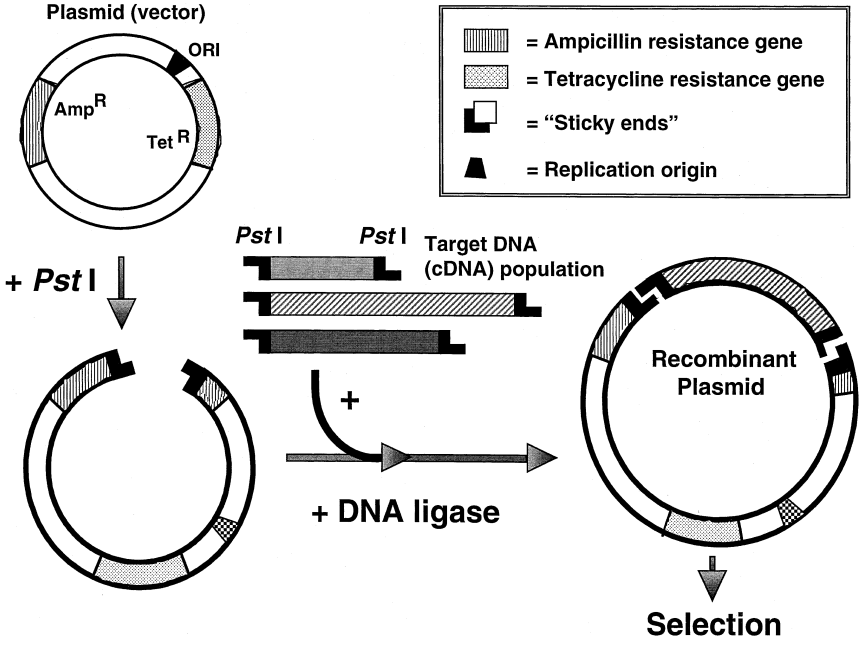

Fig. 6 Construction of a recombinant plasmid. The shown plasmid is similar to the most succesful, engineered plasmid pBR 322 with a length of 4.3 kb. Annealing and ligation of a foreign DNA insert into a *Pst* I restriction endonuclease site (see Fig. 4) are shown. Amp^R and Tet^R represent plasmid genes which render *E. coli* to ampicillin and tetracycline resistance, respectively. ORI is the DNA sequence necessary for attachment of DNA polymerase, the enzyme that duplicates the plasmid. For detailed explanation see text

these "new" vectors generally allow cloning of larger DNA fragments. These new vectors consist of cosmids [3, 78], phagemid vectors [73], yeast artificial chromosomes (YACs) [7, 78] and eukaryotic plasmid vectors [2, 43].

As an example of these new vectors, cloning into YACs is illustrated here in some detail. A YAC is a hybrid vector, combining *E. coli* plasmid segments and segments of yeast chromosomal DNA (Fig. 9). This hybrid can be transformed into and propagated in *E.coli* as well as yeast, generally *Saccharomyces cerevisae*. In the latter case, transformation occurs via yeast *spheroplasts*, i.e. wall-less yeast cells. A YAC vector includes, in addition to selectable markers, three types of chromosome-specific, essential elements: (1) a DNA segment, known as ARS, which allows autonomous replication in yeast cells; (2) a yeast-derived centromere (CEN), which ensures segregation of replicated YACs during mitosis; and (3) telomeres (TEL), which preserve the integrity of *linearized* YACs. Furthermore, *E. coli* plasmid sequences, such as ORI and drug resistance markers, are incorporated, which offers, to some extent, the opportunity to propagate and select YACs in *E. coli* cells as well. In essence, the procedures of recombinant DNA formation between foreign DNA and YACs and their cloning are highly comparable as described for "*E. coli*

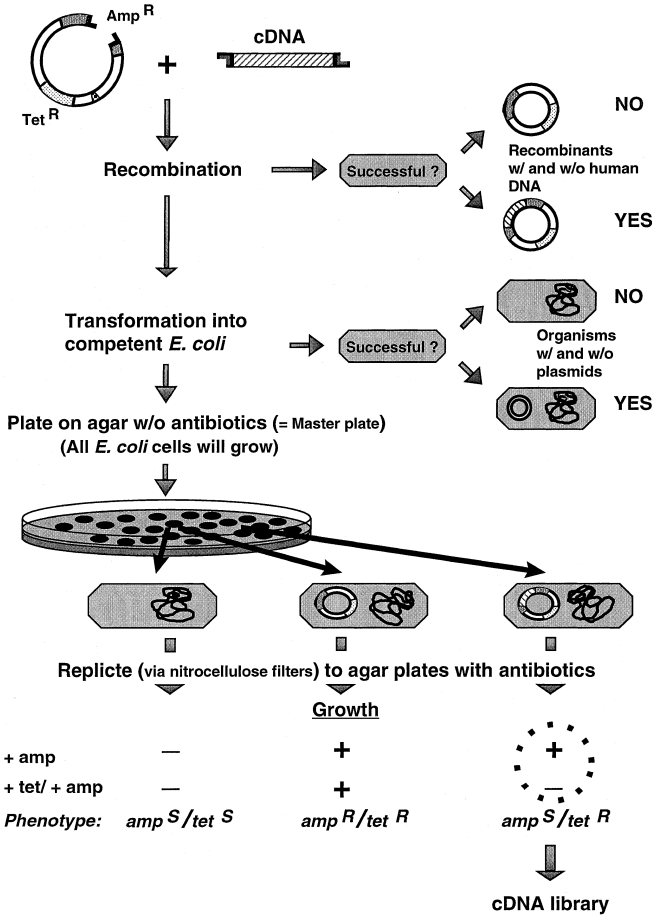

Fig. 7 Selection of *E. coli* bacteria containing recombinant DNA plasmids. Selection of bacteria transformed with plasmids containing foreign DNA, supposed to be inserted in the "ampicillin region" of the plasmids, can be achieved due to the presence of "drug-resistance genes" (Amp^R and Tet^R) (Fig. 6). *E. coli* colonies, originated from a single bacterial cell, are replicated from the master plate (w/o antibiotics) to plates with ampicillin (+amp) and ampicillin plus tetracycline (+tet/+amp). Antibiotic sensitive, amp^S/tet^S colonies consist of cells without plasmids, while antibiotic resistant, amp^R/tet^R colonies represent cells containing plasmids without inserts (self-annealing). The amp^S/tet^R (ampicillin sensitive/tetracycline resistant) colonies represent the colonies of interest, because they contain the *E. coli* cells with recombinant DNA plasmids. Matching with the master plate allows to identify and propagate these particular colonies

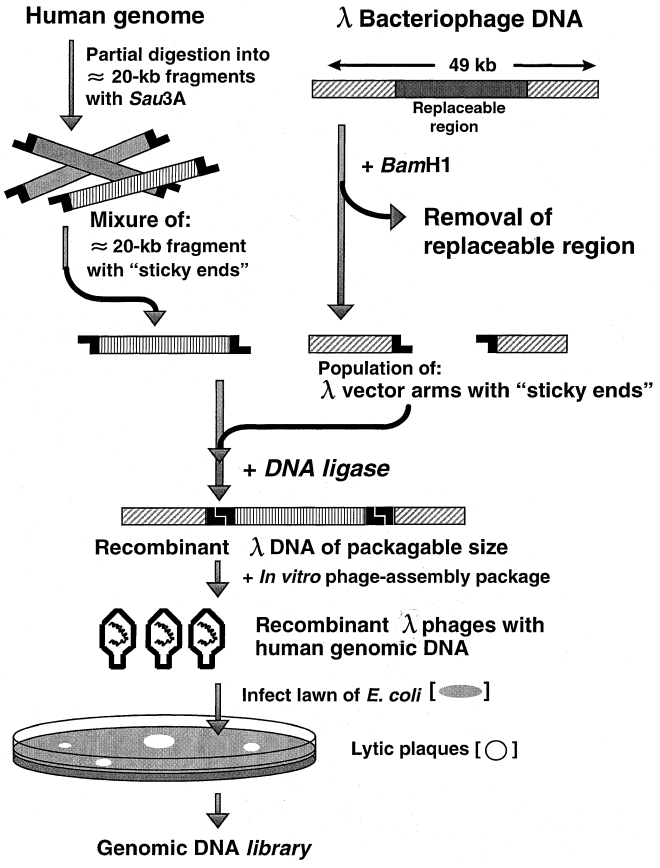

Fig. 8 Construction of a phage λ recombinants of the human genome. Partial digestion of the human genome with *Sau*3 A, a member of the *Bam*H I family of restriction endonucleases, provides DNA fragments of ≈20–25 kb. These human DNA fragments are ligated with phage λ arms, constructed by *Bam*H I endonuclease. Subsequently, the recombinants are assembled in vitro into infectious phages with a phage-assembly package set. The advantage of in vitro packaging is that only DNA molecules of the correct length (≈45 kb) produce mature, infectious phage λ particles. This characteristic assures that only human DNA fragments with a length of approximately 20–25 kb are transformed into the host. Recombinant λ phages are detected as plaques or "holes" in a lawn of host (*E. coli*) bacteria

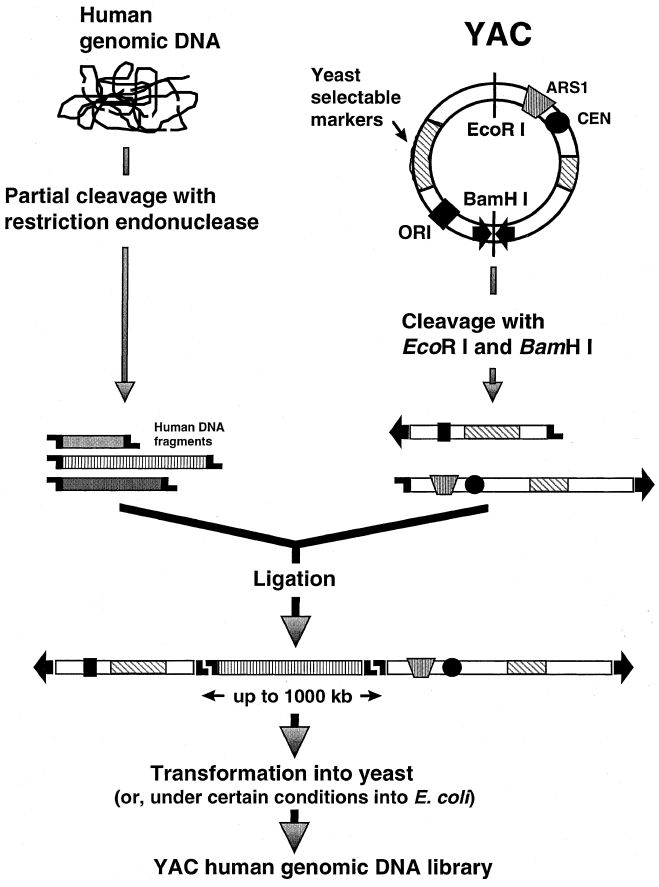

Fig. 9 Simplified scheme of cloning into a yeast artificial chromosome (YAC). The depicted YAC is a general outline showing specific YAC-associated elements. Cloning into YACs allows for the construction of a genomic human DNA library containing very large DNA restriction fragments. A YAC is a hybrid of bacterial plasmid DNA and yeast chromosomal DNA. A "model" YAC consists of: (a) selectable markers, like TRP1 and URA3 (genes involved in the biosynthetic pathways of tryptophan and uracil, respectively), (b) a centomere (CEN), (c) a (yeast) autonomously replicating sequence (ARS), (d) telomeres (*arrows*), (e) multiple restriction sites, like *EcoR* I and *Bam*H I, and (f) bacterial plasmid sequences, such as the bacterial plasmid origin of replication (ORI). The DNA sequences of the bacterial plasmid regions of the YAC are not explicitly shown

plasmid recombinant DNA technology and cloning" (Fig. 9). As a result a genomic DNA library, containing large foreign, human DNA of up to 1000 kb (when propogated in yeast), is constructed. An additional, but significant advantage of recombinant DNA YACs, transformed into yeast, is the possibility to study the genetic control of (foreign) complete genes (with promoters, introns, etc.) in an intracellular eukaryotic environment and the functional activity of proteins that require specific eukaryotic post-translational modifications (e.g. glycosylation).

The choice of the vector–host system to be used in basic and applied, medical research depends on its inherent properties and the particular goal to be achieved. The goals range from the construction of genomic and cDNA libraries, DNA sequencing, study of regulation of gene expression, detection and mapping of genes related to inherited and acquired diseases, (bulk) production of genes, proteins and recombinant virus vaccines, development of genetic probes, etc.

The polymerase chain reaction (PCR)

The polymerase chain reaction has had an enormous impact on gene cloning because it allows the isolation of a gene from as little as one cell [44, 55, 56]. The technique furnishes a relatively simple, efficient *amplification method* of specific DNA sequences [for review see 4]. Briefly, the reaction is initiated by binding of a set of specific single-stranded oligonucleotides (the primers) to the opposite strands of denaturated DNA, that is to the upper and lower strands. This primer set determines which particular DNA segment will be duplicated.

Subsequently, the actual transcription of the specific DNA sequence occurs by a thermostable enzyme, Taq polymerase. Repeating this procedure 30 to 35 times results in an exponential increase of a specific DNA fragment. The method can also be applied for mRNA amplification after reverse transcription of mRNA into double-stranded cDNA: the reverse transcriptase polymerase chain reaction (RT-PCR) [4, 71]. Although PCR and RT-PCR have simplified recombinant DNA technology considerably, it should be appreciated that these methods require pre-existing knowledge about (at least part of) the nucleotide sequences of the DNA/RNA to be amplified.

Step 4: identification of (recombinant DNA) genes of interest (Fig. 3)

Once the vector, plasmid, phage, YAC, etc. has transformed into host cells and the recombinant DNA-containing cells (colonies) have been recognized (i.e. a genomic or cDNA library has been established) (Figs. 7–9), the human genes or DNA fragments of interest have to be identified within a population of thousands of host organisms. Methods to identify a specific gene or nucleotide sequence (DNA fragment) use radiolabelled or "fluorescence"-labelled probes. The choice of a particular method of identification depends, however, on the availability of a suitable (constructed) probe. If such a probe is available, the basic technique is relatively simple and easy to perform (Fig. 10). Initially, the recombinant plasmid-containing bacterial colonies or recombinant DNA-containing phages ("plaques") are replicated to filters. After lysis, specific DNA or protein is detected by labelled nucleic acid or antibody probes, respectively. Possible (complementary acting) nucleic acids probes are: (1) specific cDNAs, (2) purified mRNA, (3) nucleic acid sequences from other organisms (especially from genes encoding evolutionary conserved proteins), and (4) chemically synthesized oligonucleotides (15–20 nucleotides long), which are deduced from a known amino acid sequence of a particular protein. Alternatively, specific proteins can be detected on the filters provided that the protein (encoded by the inserted, foreign gene) is expressed in the vector host and a (monoclonal) antibody is available. Once, a target DNA sequence of interest has been identified, it is available in large quantities for detailed, specific studies or clinical applications.

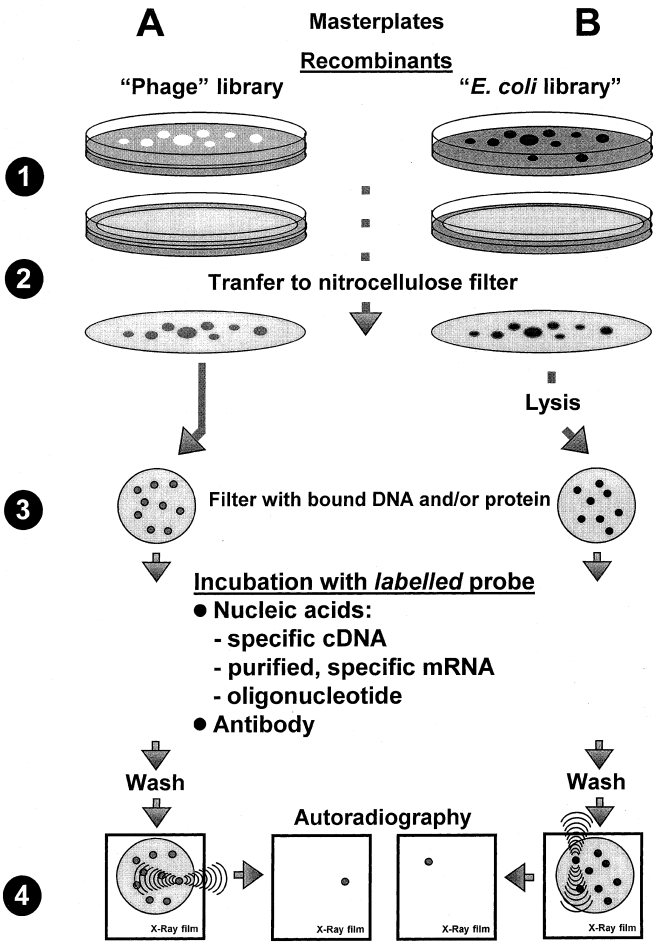

Fig. 10 Identification of bacteria and phages harbouring a specific foreign DNA. Phage "colonies" (plaques) (*A*) and bacterial colonies (*B*) are replicated onto nitrocellulose filters (*1*, *2*). Subsequent lysis (in the case of bacterial colonies; not necessary for phage plaques) results in filters bound with DNA or protein (depending on the method of lysis) (*3*). Spots on the filter that contain either specific target DNA sequences homologous to a specific nucleic acid probe or an antigen (protein) recognized by a specific antibody are detected by autoradiography (*4*)

Illustrations of the application of recombinant DNA technology and cloning in uro-oncology

Identification of prostate cancer-associated genes

Accumulation of genetic alterations (mutations, gene duplication, chromosomal aberrations, etc.) is a well-recognized feature of neoplastic tissue, including bladder [11, 37], prostate [15, 28, 46] and renal [23, 35, 80] neoplasia. In addition, gene expression and tumour progression can be affected by (altered) extra- and intercellular conditions (epigenetic events) [61, 62]. Differential gene expression between tumour cells and their normal counterparts or between tumours cells with a different degree of differentiation can be approached by comparing the steady-state level of mRNA transcripts between the various tissues by techniques known as the "differential hybridization analysis", subtraction hybridization analysis and more recently the "subtraction enhanced-display technique" [26]. These methods, comparing the different tissues, allow the investigator to determine whether an initially unknown gene is expressed more or less abundantly in one tissue compared with another, that is whether the expression of a gene is either up- or downregulated.

Differential hybridization analysis has been recently successfully applied by Bussemakers et al. [8], comparing the gene expression (at mRNA level) of a metastatic rat prostatic tumour (MatLyLu) with a non-metastatic rat prostatic tumour (Dunning R-3327-H) (Fig. 11). "Overexpression" was found in several clones, related to the metastatic tumour. Detailed analysis of one "clone", including DNA sequencing [57] and comparison with available nucleotide sequences stored in international databases (DNA banks) revealed a similar or related gene encoding for a protein known as High Mobility Group protein I(Y), a non-histone chromosomal protein [30]. Interpretation of these and additional data [70] suggested that overexpression of this gene may be functionally related to the metastatic ability of prostate tumours and may serve as a "marker" for advanced prostate cancer.

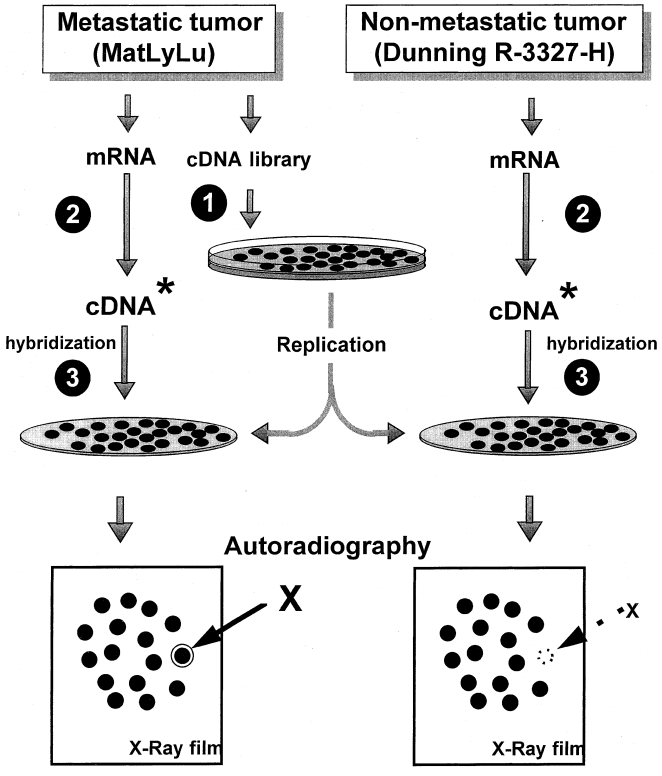

Fig. 11 The technique of differential hybridization analysis. Shown is a simplified outline of the cloning of a gene, identical or related to the gene encoding the High Mobility Group protein I(Y) [HMG-I(Y)], possibly correlated with the metastatic capacity of prostate cancer, as reported by Bussemakers et al. [8]. A cDNA library was constructed from a metastatic rat prostatic tumour (MatLyLu) and plated on the master plate (*1*). Replicates of the master plate (≈10 000 recombinant clones) were prepared on nitrocellulose filters. Radioactive-labelled cDNA (*cDNA**) was prepared from both the metastatic tumour and a non-metastatic rat prostatic tumour (Dunning R-3327-H), comparable to that illustrated in Fig. 5 (*2*). One set of filters was hybridized to each cDNA probe (*3*), followed by autoradiogaphy. Comparison of the X-ray films revealed several colonies whose RNAs were more extensively expressed in the metastatic MatLyLu tumour compared with the non-metastatic R-3327-H tumour (differential expression) (X vs x) (*4*). DNA sequencing and comparison with databases revealed the gene encoding for HMG-I(Y), possibly correlating with metastatic ability (see text) [8, 30]

Diagnostic and/or prognostic tools: in situ hybridization

A technique especially well suited for analysis of eukaryotic (human) genomes is fluorescence in situ hybridization (FISH). FISH is a technique which provides visualization of chromosomal aberrations in metaphase chromosomes and in interphase nuclei, in routine paraffin sections (Fig. 12) [20, 29, 51]. Biotinylated or fluorescein-labelled probes of specific nucleic acid sequences and/or chromosome-specific probes allow for diagnosis of numerical chromosomal aberrations in interphase cells [12], for combining with G banding, "painting of entire chromosome [13], etc. Currently, techniques are under development which increase the resolution to the theoretical limit of 1 bp in interphase nuclei [21]. An extension of the FISH technique, known as comparative genomic hybridization (CGH) is based on quantitative two-colour in situ hybridization and is especially suited for the detection of genetic imbalances in tumours [16, 31]. As an example of the power of the FISH technique, several studies, using formalin-fixed tissue specimens, indicated the loss of chromosome 9 as an early event in bladder cancer development [58], which appears to confirm the results obtained with the far more tedious conventional chromosomal staining. Recent observations suggest that the FISH technique is a useful tool for diagnosis, early detection, and management of bladder cancer using urinary cell samples [40].

Gene therapy

Most prostatic tumours are diagnosed when they are locally advanced or even metastatic. Despite the considerable improvements of the current treatment mo-

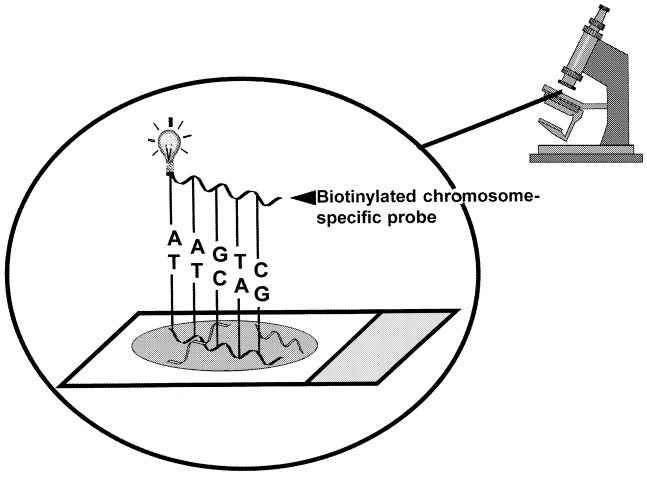

Fig. 12 Schematic representation of the fluorescence in situ hybridization (FISH) technique. A known, designed DNA or chromosomal sequence tagged with a fluorescence dye (e.g. chromosome-specific probes) hybridizes to homologous regions in a specific chromosome. Fluorescence techniques allow visualization of the target sequence

dalities, e.g. surgery, radiotherapy, chemotherapy and hormonal treatment, no effective treatment is yet available. So, it is critical to develop new therapeutic approaches, such as gene therapy, which can affect both local and metastatic prostate cancer progression. The simultaneously developed knowledge of the nature of prostate cancer-associated genes, of preparing natural and synthetic DNA sequences or complete genes (by recombinant DNA technology) and of gene delivery techniques have provided the conditions to develop gene therapeutic approaches. In cancer-directed gene therapy, DNA is transferred into cancer cells in order to kill or prevent proliferation of the malignant cells.

At present, many forms of gene therapy are under investigation [53]. An illustrative example, is an inductive, organ-specific targeting form of gene therapy and is presented here in some detail. The strategy involves the transduction of a gene encoding a prodrug-activating enzyme, a form of gene therapy known as virally directed enzyme prodrug therapy (VDEPT) or prodrug/suicide gene therapy [27]. The general strategy is based on viral-mediated transfer (as the gene vehicle) of a "suicide gene", such as the Herpes Simplex Virus-thymidine kinase gene (HSV-tk) into tumour and normal cells and subsequent administration of a non-toxic prodrug (e.g. the nucleoside analogue ganciclovir) (Fig. 13). The idea is that, after transduction, the HSV-tk gene is expressed and that the encoded thymidine kinase (TK) converts ganciclovir (GCV) to phosphorylated GCV. Phosphorylated GCV is incorporated into genomic DNA, which leads to cell death of proliferating cells (Fig. 13). Despite the far less than 100% gene transfer efficiency, it has been noted that adjacent, non-infected tumour cells are killed too, a phenomenon known as the "bystander effect" [18, 81]. Recent in vivo studies have shown that treatment of prostatic tumour-bearing mice with a replicative-defective recombinant adenovirus, carrying HSV-tk, followed by GCV administration leads to suppression of tumour growth and of spontaneous metastasis [17, 25].

A promising and elegant extension of VDEPT is an approach in which specificity for certain tumours is explored [27, 49]. Regarding prostate cancer, this goal may be obtained by constructing a recombinant DNA molecule consisting of the prostate-specific antigen (PSA) transcription unit linked to, for example, the HSV-tk gene (Fig. 13). PSA expression is under androgen control and almost exclusively expressed by the ductal epithelial cells of the normal prostate, and in prostatic tumour cells. cDNA cloning of PSA has revealed the protein-encoding region and a 5′ end with a steroid hormone response element (HRE)-like sequence [10, 38, 39, 52, 63, 82]. Recently, the transcription unit of the PSA gene has been characterized in detail [10, 50]. Moreover, Pang et al. [50] showed that this PSA promoter region was functionally active when introduced (via a plasmid) into a prostatic cancer cell line in vitro. The availability of DNA sequence of the PSA regulatory sequence offers the opportunity to recombine this region with prodrug-activating "suicide" genes and establish a prostate-specific VDEPT (Fig. 13). However, since normal prostate cells will also be sensitive to this form of VDEPT it seems likely that this treatment may be most useful for adjuvant treatment against suspected micrometastases or remaining cancer cells due to incomplete resection of primary tumours [19].

Future of uro-oncology (some molecular biological aspects)

Diagnosis

The discussed methodologies have contributed immensely towards, amongst other things, a rapid, continuing increase of identification and isolation of genes associated (directly or indirectly) with the initiation and progression of genitourinary malignancies. It now seems potentially feasible to use "genetic markers" as diagnostic (and possibly a priori screening) tools. However, these markers will be tumour type specific, since surprisingly the currently available knowledge indicates a

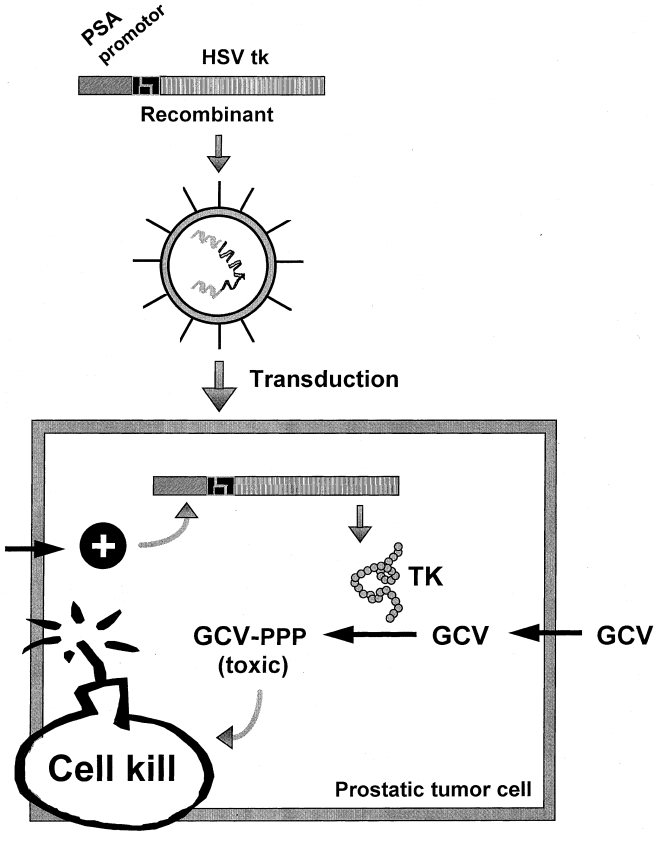

Fig. 13 Proposed scheme of prodrug/suicide gene therapy of (adjuvant) treatment of possible incompletely resected primary tumours and/or suspected micrometastases of prostate cancer. *PSA promoter* PSA transcription unit, *HSV tk* Herpes Simplex Virus-thymidine kinase gene, *TK* thymidine kinase, *GCV* ganciclovir, *GCV-PPP* phosphorylated ganciclovir. See text for detailed explanation

different pattern of genetic aberrations depending on the type (bladder, prostate, kidney, etc.) of the tumour [11, 28, 80]. Moreover, mutations within a particular gene appear to be heterogeneous, both inter- as well as intratumoral [54]. These ''obstacles'' severely limit large-scale, general genetic analysis. Recently, however, a new technology has been introduced, the so-called DNA chip technology [74]. Briefly, single-stranded nucleic acid (DNA) sequences, consisting of short oligonucleotides (10–20 bases) are attached to a silicon chip. At present, ten to hundred of thousands of different oligonucleotides can be arranged on single, commercially available DNA chips of 1 cm^2 [74]. So, in a single assay an extremely large array of well-defined oligonucleotides can be used as probes, each corresponding with specific human genes or mutated variants, such as known tumour suppressor genes (e.g. TP53, BCRA1) [24, 74]. By the mechanism of specific, complementary base pairing or hybridization between these probes and a (fluorescence-labelled) mRNA population, derived from an individual's tumour, an extensive molecular diagnosis can be accomplished. It is speculated that this new (but essentially based on the methods discussed in previous sections of this review) technology will reduce time of analysis and costs by at least a factor of ten [74]. In addition to these new prospects in clinical diagnosis, basic research will also take advantage of this technology, especially in combination with the knowledge gained of the entire human genome nucleotide sequence by the Human Genome Project, expected to be completed by the year 2005. For example, basic research in tumour biology addressing subjects like differential gene expression (see above) will be speeded up considerably.

Therapy

The simultaneous progress in understanding of the molecular biology and immunology of cancer, and development of recombinant DNA and gene delivery technologies has accumulated in a series of safety-testing gene therapy trials. At present, many different forms of gene therapy are under investigation [53]. The central challenge is undoubtedly one of perfecting methods for delivering the therapeutic genes or the choice of a safe and effective gene delivering vehicle. A large variety of gene delivery vehicles, each with its own set of advantages and disadvantages, is currently being explored, namely retroviruses, adenoviruses, adeno-associated viruses, liposomes, polymers and ''naked'' DNA [32, 53, 83]. The choice of a particular vehicle will depend ultimately on the therapeutic goal. In general, and contrary to an inherited genetic disease, it can be argued that in gene therapy directed against tumors, where only short-term gene activity is required, non-integrating delivery vehicles, such as adenoviruses, liposomes or naked DNA may be the most likely choice [22]. At present, an ideal gene transfer system is certainly not available and vehicle systems of the future will probably be an ensemble of the most advantageous features of the various vehicles [22].

In addition to the large variety of vehicles, gene therapy for cancer encompasses many strategies, ranging from delivering genes encoding for cytokines (ex vivo and in vivo) (immunotherapeutic gene therapy) or enzymes converting non-toxic prodrugs into toxic metabolites (prodrug/suicide gene therapy) to inactivation of oncogenes (antisense gene therapy) and replacement of tumor suppressor genes [for reviews see [6, 14, 49, 53, 65, 66]. Important areas for future research, including gene therapy for genitourinary malignancies, should consist of modifying the various vehicles, increasing the efficiency of vehicle infection, and optimizing targeting and specificity. With respect to the latter, as noted, clinical studies of the prodrug/suicide gene therapy, using the PSA transcription unit in combination with the HSV-tk gene, may be a worthwhile goal for treatment of prostate cancer [50], applied as an adjuvant treatment against suspected micrometastases or tumour cells remaining after incomplete resection of primary tumours [49, 65].

Another potentially promising area may be a gene therapeutic, immunological approach which focuses on the use of autologous tumour cells, transduced ex vivo with a cytokine gene, such as T cell growth factor interleukin-2 or granulocyte-macrophage colony-stimulating factor (GM-CSF), a dendritic cell activator [53]. It is anticipated that these gene-transduced, irradiated patient's cancer cells, when returned to the patient, secrete these cytokines and stimulate immune cell activity vigorously and attack the particular patient's tumours. Recently, Simons et al. [67] provided initial data of a Phase I trial using this strategy as an approach to treat patients with metastatic renal cell carcinoma (RCC). In part of the fully evaluable patients, vaccinated with GM-CSF-transduced autologous RCC cells, an objective partial response was found. It was concluded that, in terms of feasibility, safety and bioactivity this approach is worthwhile to explore.

A new, innovative application of gene therapy has been suggested by Mickisch et al. [41, 42]. In a series of fundamental experiments, a strategy was explored with the intention of developing a potential clinical scenario which should protect autologous bone marrow (BM) cells by introducing ex vivo multidrug-resistance (MDR1) cDNA in these cells. BM cells are known to be highly sensitive to cytotoxic drugs, due to a limited expression of P-glycoprotein [42], and may complicate dose-intensification regimens for treatment of tumours which eventually develop drug-resistance. Clinical results addressing, in a Phase I context, autologous reinfusion of BM genetically altered by retroviral transfer of the MDR1 gene in patients with metastatic, refractory bladder cancer will soon become available (G.H. Mickisch, personal communication).

In summary, at present, many forms of gene therapy are under investigation, but it should be appreciated that gene therapy is not yet (technically) advanced enough for widespread application.

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