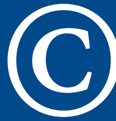

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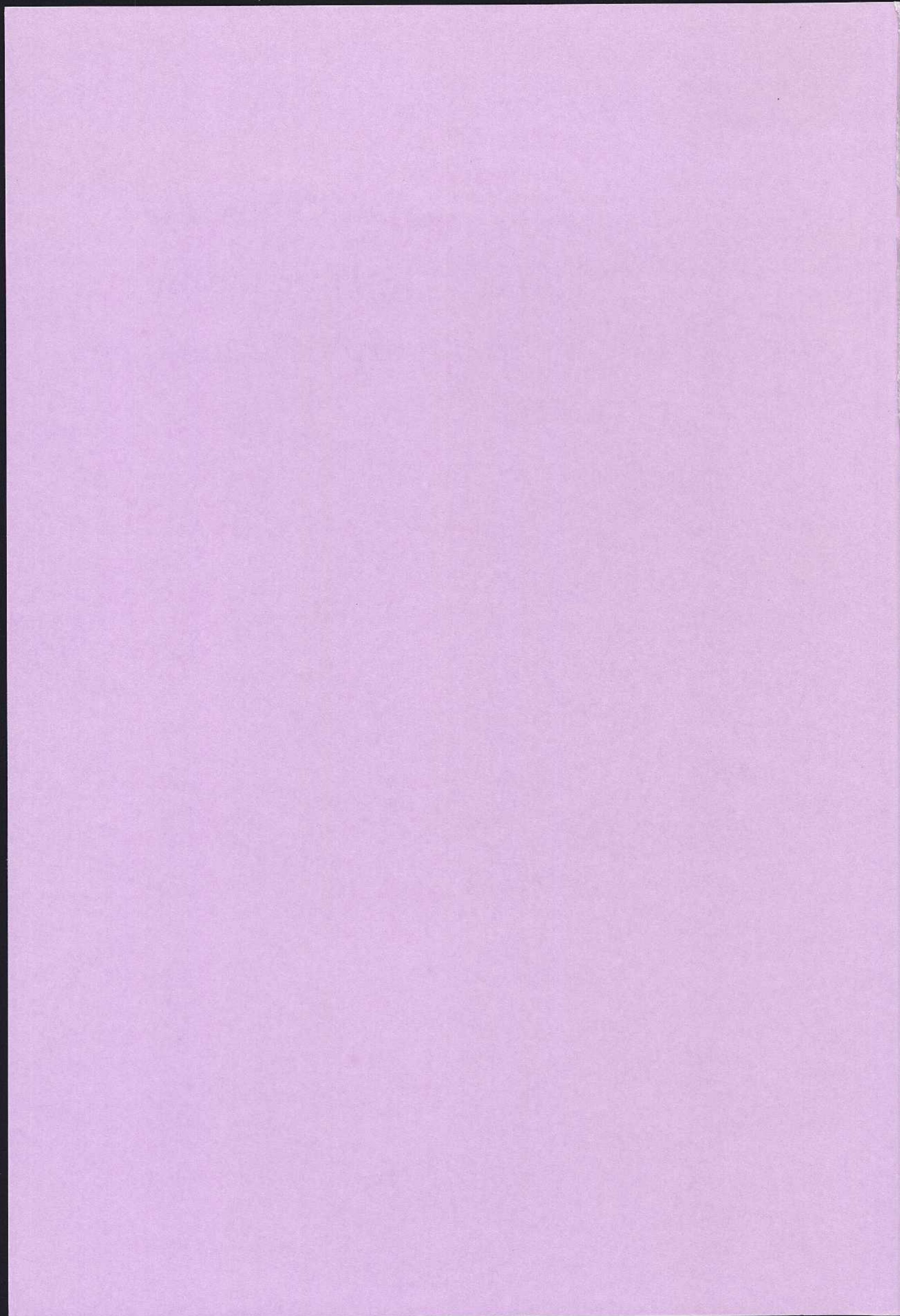
Physical Mapping and Cloning of the t(3;8)(p21;q12) Translocation Breakpoints in Pleomorphic Adenomas

Eva Röijer

Göteborg 1997



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AKADEMISK AVHANDLING
som för avläggnade av odontologie doktorsexamen med vederbörligt tillstånd
av odontologiska fakulteten vid Göteborg universitet,
kommer att offentligen försvaras i föreläsningssal 3,
Odontologiska kliniken, Göteborg
Torsdagen den 29 maj 1997, kl 13^{00}

av

Eva Röijer
Fil. kand.

Avhandlingen är av sammanläggningstyp och baseras på följande delarbeten:

I. Röijer E, Kas K, Klawitz I, Bullerdiek J, Van de Ven W and Stenman G: Identification of a yeast artificial chromosome spanning the 8q12 translocation breakpoint in pleomorphic adenomas with t(3;8)(p21;q12). Genes, Chromsomes & Cancer 17:166-171, 1996.

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Handledare:
Professor Göran Stenman

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Lunds Universitet

ABSTRACT

PHYSICAL MAPPING AND CLONING OF THE t(3;8)(p21;q12) TRANSLOCATION BREAKPOINTS IN PLEOMORPHIC ADENOMAS

Eva Röijer

Laboratory of Cancer Genetics, Department of Pathology, Göteborg University, Sahlgrenska University Hospital, SE-413 45 Göteborg, Sweden.

The pleomorphic adenoma is a benign epithelial tumor originating from the major and minor salivary glands. It is the most common type of salivary gland tumor, accounting for almost half of all neoplasms in these organs. Cytogenetically, pleomorphic adenomas are characterized by recurrent rearrangments involving the chromosome regions 3p21, 8q12 and 12q13-15. The most frequent abnormality is a reciprocal t(3;8)(p21;q12) translocation, or variants thereof in which the 8q segment is translocated to a variety of chromosome regions. The purpose of the present series of investigations was to identify the genes involved in the t(3;8)(p21;q12) using a positional cloning approach.

YAC clones corresponding to known genetic markers flanking band 8q12 were used to initiate a walk towards the 8q12 breakpoints. This resulted in the establishment of two non-overlapping YAC contigs covering approximately 75% of band 8q12. The centromeric contig covers approximately 2 Mb of genomic DNA and consists of 34 overlapping YAC clones containing at least seven putative CpG islands, and three ESTs. The telomeric contig consists of 23 YACs and covers about 5 Mb of genomic DNA. FISH mapping of YACs and cosmids from these contigs revealed that the majority of breakpoints clustered within a 300 kb subregion in the centromeric contig. Subsequent studies of new STSs and ESTs from this region led to the discovery of the *PLAG1* gene, a novel, developmentally regulated gene coding for a zinc finger protein.

The gene, which consists of 5 exons of which the first three are non-coding, spans about 35 kb, with a large intron (approximately 25 kb) between exon 1 and exon 2. The deduced amino acid sequence of the PLAG1 protein reveals seven canonical C_2H_2 zinc finger domains in the N-terminal region and a serine-rich C-terminus. There are two potential nuclear localization signals in the N-terminal region. 5' RACE analysis of tumors with t(3;8) enabled us to identify *CTNNB1* as the chromosome 3 gene fused to *PLAG1*. *CTNNB1* codes for ß-catenin, a protein interface functioning in the WG/WNT signalling pathway and in the specification of cell fate during embryogenesis.

The t(3;8) results in promoter swapping between *PLAG1* and *CTNNB1*. Fusions invariably occur in the 5' non-coding regions of both genes, exchanging regulatory control elements while preserving the coding sequences. Due to the t(3;8) *PLAG1* is activated whereas the expression of *CTNNB1* is reduced. Activation of *PLAG1* was also observed in an adenoma with a t(8;15)(q12;q14) translocation, demonstrating that *PLAG1* activation is not restricted to tumors with t(3;8). To our knowledge this is the first example of promoter swapping in solid tumors.

The identification and cloning of a novel "benign oncogene" activated by chromosome translocation in pleomorphic adenomas constitute an important step towards an increased understanding of the molecular pathogenesis of benign neoplastic growth. The results potentially allow for the design of novel diagnostic tools and targeted therapy of pleomorphic adenomas.

Key Words: Pleomorphic adenoma, chromosome abnormalities, positional cloning, fluorescence in situ hybridization (FISH), yeast artificial chromosome (YAC), PLAG1, CTNNB1.

ISBN 91-628-2514-3

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PREFACE

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CANCER GENETICS

Introduction

Cancer is nowadays recognized as being a genetic disease. At the beginning of this century, Boveri wrote ..... *Another possibility [to explain cancer] is the presence of definite chromosomes which inhibit division .... Cells of tumors with unlimited growth would arise if those "inhibiting chromosomes" were eliminated ..... and [since] each kind of chromosome is represented twice in the normal cell, the depression of only one of these two might pass unnoticed* ..... (Boveri, 1914; translation published in 1929) Today it is well established that tumors are caused by mutations and rearrangements of specific classes of genes within the chromosomes (Bishop, 1987; Weinberg 1989a, b). Three classes of tumor-associated genes have been identified in particlar, namely oncogenes, tumor suppressor genes, and DNA repair genes (Bishop, 1991; Fishel and Kolodner, 1995). It is well established that the cells in a tumor descend from a common ancestral cell which at one point, usually decades before a tumor becomes palpable, acquired a mutation that initiated a program of inappropriate growth control. The progression of such a cell into a cancer cell comes about through the accumulation of a number of additional mutations. The initial mutation can either occur in a somatic cell, which may subsequently lead to a spontaneously induced tumor, or it can occur in a germ line cell, which results in an inherited predisposition to tumor development. Many solid tumors occur in both sporadic and hereditary forms, although the latter are, as a rule, much less common.

The number of genetic events required for the conversion of a normal cell into a tumor cell is likely to vary for different cell types. In colorectal tumorigenesis for example, at least five to six events have been identified (Fig. 1) (Vogelstein and Kinzler, 1993), whereas in certain pediatric tumors, such as retinoblastoma and Wilms´ tumor, only two mutations seem to be required (Knudson, 1985, 1987). The fact that most cancers occur in the fifth to seventh decades of life is a reflection of the time it takes for an individual to accumulate enough mutations to cause malignancy. Although there seems to be some variability in the order of the mutations, there is also a definite preference, e.g. certain mutations are required for the initiation of tumorigenesis in certain cell types (Vogelstein and Kinzler, 1993).

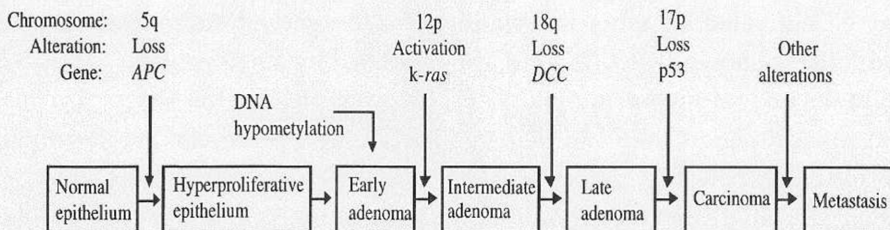

Fig. 1. Proposed model for the development of colorectal carcinoma (from Vogelstein and Kinzler, 1993).

The cell cycle

All living organisms are dependent on the ability of cells to produce daughter cells. In the development of a zygote into an adult organism, the cell divisions in the different tissues and organs are strictly regulated so that cells only reproduce when instructed to do so by other cells in their vicinity. In cancer cells this carefully controlled program is violated, cancer cells no longer respond to the usual proliferation controls and instead they follow their own internal reproduction program. The cell cycle machinery, which seems to be very similar in most eukaryotic organisms, was first studied in detail in yeast cells. The cell cycle is subdivided into four stages: G_1, S, G_2 and M (Fig. 2). Most differentiated, adult cells are in the G_1-phase (gap 1). In the absence of stimulatory signals the cell will arrest early in G_1 in a phase called G_0. During G_1 the cell checks the integrity of the DNA, synthesizes and accumulates the material necessary for cell division, and prepares for DNA-synthesis. When stimulated by mitogens, and under the right conditions, the cell will pass through the restriction point, START, or "point of no return" and proceed to the S-phase (for synthesis) where the chromosomal DNA is replicated. The cell thereafter enters a second, shorter gap phase (G_2) after which it moves on to mitosis (M). During the M-phase the cell devide into two identical daughter cells containing a complete set of the genetic material (Watson *et al.*, 1992; MacLachlan *et al.*, 1995).

Cell cycle control

The regulation of the mammalian cell cycle is very complex (Fig. 2). At first, one molecule, cdc2, was thought to be the only key player. Today, seven related so-called cyclin-dependent-kinases (CDKs) are known to play a central role in regulating the entrance into the various stages of the cell cycle. The CDKs are activated by larger proteins, termed cyclins. CDKs must

also be activated by phosphorylation of a conserved threonine. Recent studies have shown that CDKs are also regulated by small proteins acting as cyclin-dependent-kinase inhibitors. In, for example, G_1 the D-type cyclins bind to CDK4 and CDK6 and thereby induce a new cell cycle. The resulting complex acts on the growth-inhibitory molecule pRB. When pRB is phosphorylated by the complex it cannot bind and inactivate the transcription factor E2F, a factor necessary for progression into the S-phase. This action thus releases the braking effect of pRB and enables the cell to progress to the next phase. As shown in Fig. 2 the expression of cyclin D is followed by the induction of the E-, A-, and B-type cyclins. Abnormal expression of the various proteins involved in the cell cycle machinery is often seen in human cancer (MacLachlan *et al.*, 1995).

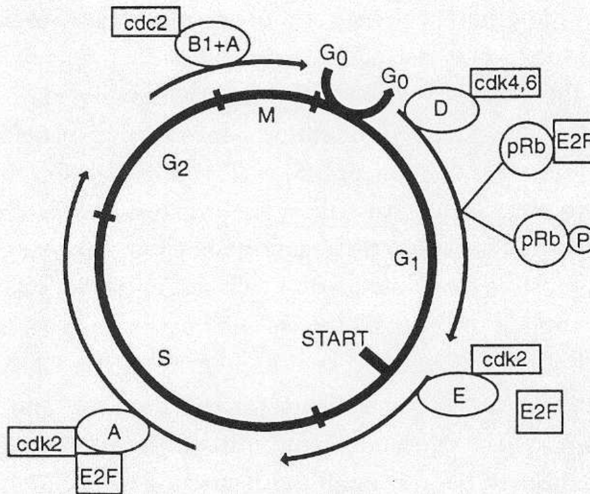

Fig. 2. Schematic view of the mammalian cell cycle (modified after MacLachan *et al.*, 1995).

Oncogenes

Oncogenes are normal cellular genes (also called proto-oncogenes) which are highly conserved through evolution. The human genome contains about 100 known proto-oncogenes that may be converted into oncogenes by specific genetic alterations. Oncogenes are very heterogeneous with regard to their functions. Their protein products may act at different levels in the cellular machinery. They are key factors in signal transduction pathways, starting with growth factors and their receptors via complex systems of cytoplasmic transducers to transcription factors in the nucleus (Weinberg,

1991; Morris, 1992a, b; Sleeman, 1992; Levine and Broach, 1995). These very complex cellular systems are strictly regulated, and it is the abnormal expression or the expression of altered protein products of genes within these systems that conduce to neoplasia (Bishop, 1991). Proto-oncogenes can be activated by several different mechanisms, including point mutations, gene truncation, gene amplification, and gross chromosomal rearrangements such as translocations, insertions, and inversions. Activated oncogenes may thus encode normal gene products, mutated gene products as well as novel fusion proteins. An oncogene can be activated by more than one mechanism. The *MYC* gene, for example, is amplified in certain tumors and activated by chromosomal translocations in others. *MYC* may also act as a retroviral oncogene (Heim and Mitelman, 1995; Cooper, 1990). On the basis of sequence and functional similarities, oncogenes can be grouped into gene families. They can also be grouped into classes depending on the site of action of their protein products(Weinberg, 1989a; Cooper, 1990).

The first oncogene was discovered in 1976 when Bishop, Varmus and co-workers found that the transforming gene of Rous sarcoma virus, a retrovirus that causes cancer in chicken, was a cellular gene named *SRC* (Stehlin *et al.*, 1976). Since then more than 20 retroviral oncogenes have been identified, including *MYC, ERBB, MOS* and *ABL*. They are all integrated into the genome of acutely transforming viruses, and are expressed as independent translation products or as fusion proteins containing viral sequences (Cooper, 1990). These viruses are capable of inducing tumors in animals with short latency periods and to efficiently transform cells in vitro. The discovery of these genes provides important evidence that cancer is a genetic disease caused by specific genes.

Growth factors. The first known physiological function of an oncogene product was described for the *SIS* gene. *SIS* was shown to be homologous to the platelet-derived growth factor (PDGF) B-chain (Doolittle *et al.*, 1983; Waterfield *et al.*, 1983). The biologically active PDGF molecule is a dimer of A- and/or B-chains. The A- and B-chains are encoded by different genes located on different chromosomes (*PDGFA* maps to 7p22 and *PDGFB* to 22q13; Stenman *et al.*; 1992a; Swan *et al.*,1982). Only cells expressing a PDGF receptor on its surface, such as fibroblasts and smooth muscle cells, are susceptible to transformation by SIS (Cooper, 1990). Other known examples of oncogenes encoding growth factors are *INT2, HST* and *FGF5*, which are all members of the fibroblast growth factor family (Nguyen *et al.*, 1988; Marics *et al.*, 1989), as well as the epidermal growth factor (*EGF*) and the

related transforming growth factor-α (*TGF*-α) (Derynck, 1988; Di Marco *et al.*, 1989).

Growth factor receptors. There are a number of growth factor receptors with protein-tyrosine kinase activity that can act as oncogenes. Binding of a ligand to such a receptor stimulates the tyrosine kinase activity. Mutations in these genes can result in constitutive activation of the receptor tyrosine kinases. Examples of such genes are *ERBB, FMS, ERBB2, TRK, RET, MET,* and *KIT*. The *ERBB* gene, which codes for the epidermal growth factor receptor (EGFR), is activated by a deletion of the ligand-binding domain of the receptor (Wells and Bishop, 1988). The *EGFR* gene is often amplified in, for example, malignant gliomas (Collins, 1995). Similarly, the *FMS* oncogene, which is homologous to the macrophage colony-stimulating factor (CSF-1) receptor (Sherr *et al.*, 1985), is activated by a point mutation in the extracellular domain of the receptor. A third example of an oncogene coding for a growth factor receptor is *ERBB2* (also referred to as *HER2/NEU*). *ERBB2* is a member of the ERBB-like gene family but is distinct from *ERBB*. *ERBB2* is often amplified and/or overexpressed in breast and ovarian cancer (Slamon *et al.*, 1989) as well as in malignant salivary gland tumors (Stenman *et al.*, 1991b).

Cytoplasmic oncoproteins. Oncogenes acting in the cytoplasm are often protein-tyrosine kinases that function as signal transducers and transmittors activating protein cascades. The *SRC* oncogene is the protype of this group. The members of the SRC subfamily - *SRC, YES, FGR, LCK, FYN, LYN* and *HCK* - are all associated with the plasma membrane and are activated by phosphorylation at important tyrosine residues. They also contain amino-terminal regulatory domains designated SH-2 and SH-3. These domains are also conserved in the ABL oncogene product, while only the SH-3 domain is conserved in the FES oncogene product (Cooper, 1990). Other kinases, such as MOS and RAF, are soluble cytoplasmic proteins with serine/threonine kinase activity (Cooper, 1990; Bishop, 1991). The MOS protein is a cytostatic factor (CSF) which arrests vertebrate eggs in the meiotic cycle (Sagata *et al.*, 1989). The RAF protein is involved in a cytoplasmic kinase cascade and is activated by interaction with active RAS protein (Marshall, 1996).

Another group of cytoplasmic oncogenes are the *RAS* genes (*HRAS, KRAS* and *NRAS*) which encode proteins of 21 kD located on the inner surface of the plasma membrane (Barbacid, 1987; McCormick, 1989). RAS proteins bind guanine nucleotides and have intrinsic GTPase avtivity. They also show structural and functional similarities to the G-proteins. The *RAS*

genes are activated by point mutations, preferentially occurring at codons 12, 13 and 61 (Bos, 1988). Most mutations seem to result in a decrease in the GTPase activity and/or increase the rate of bound GDP for free GTP.

Nuclear regulatory proteins. Oncogenes also encode proteins localized in the nucleus (Eisenman, 1989; Cooper, 1990). These proteins contain structural motifs such as helix-loop-helix, leucine zipper, zinc finger, and homeobox domains, indicating that they are involved in DNA-binding and transcriptional regulation (Bishop, 1991). Examples of such oncogenes are *JUN*, *FOS*, *MYC*, *MYB*, *ETS*, *SKI*, and *REL*. The products of the JUN and FOS gene family are components of the AP-1 transcription factor. They can be activated as oncogenes simply by overexpression of the normal gene products. The *MYC* genes, in particular *MYC* and *NMYC*, are activated in a variety of human tumors as a consequence of chromosomal translocation (Burkitt´s lymphoma) and gene amplification (neuroblastoma, lung cancer, breast cancer) (Cooper, 1990; Schwab and Amler, 1990; Rudduck *et al.*, 1992). Recent studies have also shown that members of the ETS-family of transcription factors are frequently fused to the *EWS* gene in Ewing sarcomas (Kaneko *et al.*, 1996).

Tumor suppressor genes

The tumor suppressor genes, sometimes also referred to as anti-oncogenes, encode for growth-inhibitory proteins whose loss or inactivation is associated with tumor development (Hinds and Weinberg, 1994). Inactivation of these genes can occur through deletions or point mutations. Important evidence in support of the existence of genes with tumor-suppressing activity comes from several independent lines of research (Knudson, 1985, 1987; Sager, 1985, 1989; Sandberg, 1990; Seizinger *et al.*, 1991; Stanbridge, 1992): 1) somatic cell fusions between tumorigenic and non-tumorigenic cells often result in a non-tumorigenic hybrid. When such hybrids give rise to tumors in animals this usually involves the loss of a specific chromosome from the non-tumorigenic cell, suggesting that this chromosome contains a suppressor gene; 2) there are a number of human tumors that characteristically show both cytogenetically and molecularly detectable deletions, e.g. deletions of 3p sequences in lung cancer and complete or partial loss of chromosome 22 in meningioma. These losses indicate the positions of putative tumor suppressor genes; 3) the findings of constitutional and/or tumor-associated deletions in patients with hereditary cancers, e.g. retinoblastoma and Wilms´ tumor.

One of the best studied examples of hereditary cancers is the retinoblastoma. About 60% of retinoblastomas are sporadic cases with no family history of the disease, and with a single tumor in one eye. The remaining 40% of the cases are familial with bilateral tumors and often more than one independently derived tumor in one eye. In 1971, Knudson proposed a "two-hit" model for the development of retinoblastoma. He postulated that in the hereditary cases the first hit was a mutation of one allele in the germ line and that the second hit was a somatic mutation of the other allele. In the sporadic cases he proposed that both mutations were somatic. This model was later confirmed by the cloning of the *RB1* gene (Friend *et al.*, 1986; Lee *et al.*, 1987a, b) and the subsequent genetic studies of retinoblastoma families (cf. below).

Compared to oncogenes, relatively few tumor suppressor genes have so far been cloned. Examples of such genes are the retinoblastoma gene (*RB1*), the Wilms' tumor gene (*WT1*), the *TP53* gene, the familial adenomatosis polyposis coli gene (*APC*), the deleted in colorectal cancer gene (*DCC*) (Levine, 1993; Cho and Fearon, 1995), the breast cancer susceptibility type 1 (*BRCA1*) and type 2 (*BRCA2*) genes (Hall *et al.*, 1990; Wooster *et al.*, 1994), and the neurofibromatosis type 1 (*NF1*) and type 2 (*NF2*) genes (Cawthon *et al.*, 1990; Trofatter *et al.*, 1993; Rouleau *et al.*, 1993). In the following I will give a short description of some of these genes.

The Retinoblastoma gene (RB1). The retinoblastoma locus was orignally mapped on the basis of cytogenetically visible deletions involving chromosome band 13q14. Cloning of the *RB1* gene (Friend *et al.*, 1986; Lee *et al.*, 1987a, b) revealed that it encodes a 105 kDa nuclear phosphoprotein that is regulated by cell cycle dependent phosphorylation (Fig. 2). The protein is active in growth suppression by preventing transcriptional activation of several cell cycle regulatory genes. *RB1* is ubiquitously expressed. The gene is defective in all retinoblastomas as well as in a number of other cancers, including small cell lung carcinoma, bladder cancer, breast cancer, and osteosarcoma (secondary to retinoblastoma) (Marshall, 1991).

The Wilms' tumor gene (WT1). Wilms' tumor (WT) is a rare childhood tumor of the kidney that is often seen together with different congenital malformations and mental retardation (WAGR syndrome). Similar to retinoblastomas, WT occur both in familial and sporadic forms. There are at least two loci on chromosome 11 associated with the hereditary forms of WT, one at 11p13 (*WT1*) and one at 11p15 (*WT2*). The *WT1* gene is a member of the early growth response gene family (*EGR* genes) (Call *et al.*, 1990). It encodes zinc finger proteins expressed in the developing kidney,

spleen, gonads, and the uterus (Aaronson, 1991; Levine, 1993). The WT1 proteins act as transcription factors. Recently, the *WT1* gene was shown to be fused to the *EWS* gene as a result of a t(11;22) in desmoplastic small round cell tumors (Ladanyi and Gerald, 1994).

The TP53 gene. TP53 is the most commonly mutated gene in human cancer. The gene, which maps to 17p13.1, consists of 11 exons. It encodes a nuclear phosphoprotein that can act as a sequence-specific transcription factor. The protein is ubiquitously expressed, and has a key function in the cellular response to DNA damage (Levine, 1993; Haffner and Oren, 1995). Depending on the degree of damage p53 may induce G_1 arrest or apoptosis. Because of its important cellular functions TP53 has been named "The Guardian of the Genome". Most inactivating mutations in *TP53* are found in the highly conserved exons 4 to 9 (Hollstein *et al.*, 1991). Germ line mutations in *TP53* are found in patients with the Li-Fraumeni syndrome. Patients with this syndrome run an increased risk of developing a variety of mesenchymal and epithelial neoplasms at multiple sites (Malkin *et al.*, 1990).

The APC gene. Familial adenomatosis polyposis (FAP) is a cancer syndrome characterized by the presence of hundreds to thousands of colonic polyps which, in untreated individuals, leads to colorectal cancer (Fig. 1). The disease is caused by inactivating mutations in the *APC* gene located at chromosome 5q21. Almost all of the mutations, frameshift or base substitutions, are found in the 5' half of the coding sequence, leading to expression of truncated proteins. The wild type protein controls cell growth by regulation of cell adhesion, cytoskeletal anchoring and signalling. APC binds to and down-regulates ß-catenin, a protein functioning in the WG/WNT signalling pathway. Truncated APC proteins are still able to bind catenins, but have lost their ability to alter intracellular catenin distribution and to bind microtubules (Kinzler *et al.*, 1991; Levine, 1993; Polakis, 1995).

DNA repair genes

DNA is the only macromolecule capable of being restored after damage. Complementary base pairing is the key feature in directing the repair of the original structure. DNA damage may be caused by a variety of factors, including environmental chemicals, UV-light, ionizing radiation, cosmic rays, X-rays as well as by depurination and deamination which frequently occurs in human cells (Singer and Berg, 1991; Fishel and Kolodner, 1995). In eukaryotic cells there are a number of mechanisms by which different DNA

lesions may be repaired. Mismatch repair, for example, recognizes normal nucleotides which are unpaired or paired with a non-complementary nucleotide. Several human mismatch repair genes, which are homologous to components in *Saccharomyces cerevisiae* have been identified. Germ line mutations in at least four such genes, *MSH2, MLH1, PMS1*, and *PMS2*, have recently been identified in the hereditary nonpolyposis colon cancer syndrome (HNPCC) (Fishel and Kolodner, 1995). Nucleotide and base excision repair recognizes chemically modified nucleotides and bases, or adjacent nucleotides fused to each other (Fishel and Kolodner, 1995). Patients with the autosomal recessive disease Xeroderma Pigmentosum (XP) are extremely sensitive to UV-light and develop a variety of skin cancers (Sancar, 1994). XP-patients can be divided into seven different complementation groups, XP-A to XP-G, each carrying a mutation in a different gene (Hanawalt, 1994).

TUMOR CYTOGENETICS

Extensive cytogenetic studies of human cancers, particularly myelo- and lymphoproliferative disorders, have unequivocally confirmed Boveri´s original prediction, and it is now well established that the occurrence of clonal, acquired chromosome changes is a characteristic feature of neoplastic cells (Sandberg, 1990; Heim and Mitelman, 1995). An impressive number of nonrandom chromosome abnormalities have been identified among the more than 22,000 cytogenetically analyzed neoplasms included in the latest edition of the "Catalog of Chromosome Aberrations in Cancer" by Mitelman and co-workers (Mitelman, 1994). However, only 27 % of these are solid tumors, the remaining 73% being hematologic disorders and lymphomas. The reason for this discrepancy can no doubt be attributed to the well-known technical difficulties in analyzing solid tumors, and in particular carcinomas. In spite of the extensive literature on chromosome changes in human neoplasia, there is thus still a need for continued cytogenetic analysis of solid tumors.

Chromosome abnormalities in tumors can be divided into two main categories, i.e. primary and secondary abnormalities. Primary abnormalities are tumor specific and are often found as the sole anomalies, suggesting that they are of pathogenetic importance. Secondary abnormalities are, in contrast, not related to the establishment of the tumor, but rather to the progression of the tumor. Secondary abnormalities might be non-random and have been shown to be of prognostic significance in certain neoplasms. The fact that chromosome changes are tumor specific makes them useful as diagnostic markers.

Chromosome changes in malignant neoplasms

One of the most well-known examples of a primary chromosome abnormality in man is the Philadelphia (Ph) chromosome in chronic myeloid leukemia (CML) resulting from a t(9;22)(q34;q11) (Nowell and Hungerford, 1960; Rowley, 1973). Molecular cloning of the translocation breakpoints revealed that the *ABL* oncogene at 9q34 was translocated to chromosome 22q11, where it was fused to a novel gene named *BCR* (Shtivelman et al., 1985). The *BCR/ABL* fusion gene has also been detected in Ph-negative CML as well as in cases with variant translocations where there is no cytogenetic evidence of involvement of chromosome 9 (van der Plas *et al.*, 1991). Typical nonrandom, secondary chromosome changes in CML are an extra Ph chromosome, +8 and i(17q) (Sandberg, 1990).

The most well-known tumor-specific translocation in lymphomas is probably the t(8;14)(q24;q32) in Burkitt´s lymphoma (Zech *et al.*, 1976). This translocation leads to the juxtaposition of the *MYC* gene at 8q24 to the immunoglobulin heavy chain locus at 14q32 (Dalla-Favera *et al.*, 1987). The translocation leads to a head-to-head fusion of the two genes on chromosome 14. In the less frequent variant translocations t(2;8)(p12;q24) and t(8;22)(q24;q11) the breakpoint is proximal to *MYC*. In these cases the lambda and kappa immunoglobulin light chain genes at 2p12 and 22q11 respectively are fused in a head-to-tail fashion to *MYC* on chromosome 8. The functional consequences of the three translocations seem to be very similar, i.e. overexpression of *MYC* (Croce, 1993; Heim and Mitelman, 1995).

Bone and soft tissue sarcomas is a third group of neoplasms for which a reasonable number of tumors have been analyzed. The chromosomal pattern in many sarcomas is in several respects comparable to that in the leukemias. It is therefore not surprising that many of the tumor specific abnormalities seen in sarcomas are now routinely used as diagnostic markers in many laboratories. One such example is the t(11;22)(q24;q22) which is typically seen in Ewing sarcoma, neuroepithelioma and Askin´s tumor (Aurias *et al.*, 1984; Turc-Carel *et al.*, 1984; Meis-Kindblom *et al.*, 1996). Other examples include the t(X;18)(p11;q11) found in both mono- and biphasic synovial sarcomas (Turc-Carel *et al.*, 1986a, 1987), the t(2;13)(q37;q14) in alveolar rhabdomyosarcoma (Seidal *et al.*, 1982; Turc-Carel *et al.*, 1986b), the t(12;16)(q13;p11) in myxoid liposarcoma (Turc-Carel *et al.*, 1986c), the t(12;22)(q13;q22) in clear-cell sarcoma of tendons and aponeuroses (Bridge *et al.*, 1990; Stenman *et al.*, 1992b), the t(9;22)(q22;q12) in extraskeletal myxoid chondrosarcomas (Hinrichs *et al.*, 1985; Stenman *et al.*, 1995), and the t(11;22)(p13;q12) in desmoplastic small round cell tumors (Sawyer *et al.*, 1992; Shen *et al.*, 1992). However, it should also be mentioned that there are several types of sarcomas, including osteosarcomas and chondrosarcomas, for which no specific abnormalities have so far been identified.

Carcinomas are cytogenetically both more complex and heterogeneous than many other neoplasms. So far, very few recurrent abnormalities have been identified. In small cell lung cancer for instance, an interstitial deletion del(3)(p14p23) is frequently found (Whang-Peng *et al.*, 1982), and in renal cell carcinomas terminal deletions with breakpoints at 3p13 or distal to this band or a der(3)t(3;5)(p13;q22) are recurrent (Kovacs *et al.*, 1993). Subsets of breast carcinomas show trisomy 8 or del(3)(p12p14), sometimes as the sole anomalies (Pandis *et al.*, 1995; Bullerdiek *et al.*, 1993a), and among malignant salivary gland tumors a recurrent t(6;9)(q22-24;p13-23) has been

found in adenoid cystic carcinomas (Nordkvist *et al.*, 1994), and a t(11;19)(q14-21;p11) in mucoepidermoid carcinomas (Nordkvist *et al.*, 1995).

Chromosome changes in benign tumors

Studies during the last two decades have demonstrated unequivocally that chromosome abnormalities are a characteristic feature not only of malignant tumors but also of benign tumors. For several benign tumor types, such as meningiomas, pleomorphic adenomas, lipomas, and uterine leiomyomas, extensive cytogenetic data are now available (Mitelman, 1994). The meningioma, a tumor originating from the meningeal coverings of the brain, was the first benign tumor reported to have a characteristic chromosome abnormality, i.e. complete or partial monosomy 22 (Mark *et al.*, 1972; Zankl and Zang, 1972). Eight years later, Mark and co-workers (Mark *et al.*, 1980) reported on a second type of benign tumor characterized by specific chromosome abnormalities, namely pleomorphic adenomas of the salivary glands. In the original report they described three cases with a t(3;8)(p21;q12) or a variant thereof. Later studies have shown that this is the most frequent abnormality found in these tumors (Sandros *et al.*, 1990; Bullerdiek *et al.*, 1993b), and this translocation is also the subject of the present thesis. Further details of the chromosomal pattern in pleomorphic adenomas will be presented below.

Subsequent studies from several independent groups have revealed that lipomas and uterine leiomyomas also display specific chromosome abnormalities. About 50-80% of the lipomas are cytogenetically abnormal (reviewed by Sreekantaiah *et al.*, 1991; Mandahl *et al.*, 1994). At least five cytogenetic subgroups have been distinguished: (1) lipomas with translocations of 12q13-14, most common in the form of a t(3;12)(q27-28;q13-15); (2) lipomas with rearrangements of 6p; (3) lipomas with rearrangements of 13q; (4) lipomas with ring chromosomes (commonly found in atypical lipomas); and (5) lipomas with sporadic rearrangements. It should be pointed out that there is also an overlap between several subgroups (Mandahl *et al.*, 1994). In uterine leiomyomas abnormal karyotypes have been reported in 20-50% of the cases (Mark *et al.*, 1990; Nilbert and Heim, 1990; Sreekantaiah and Sandberg, 1991). About 20% of these have a t(12;14)(q14-15;q23). Other non-random changes are deletions of 7q, mostly a del(7)(q21.2;q31.2), rearrangements of 6p12-21, trisomy 12, and various structural rearrangements of 1p, 2p, and 13q.

Clinical, histopathological and cytogenetic aspects of pleomorphic adenomas

Pleomorphic adenomas (previously also designated mixed tumors) are painless, slow-growing benign tumors originating from the major and minor salivary glands. It is the most common type of salivary gland neoplasm and accounts for about half of all cases. Over 60% of the tumors are found in the parotid gland. Pleomorphic adenomas are usually diagnosed in patients in the age range 30 to 50 years, but may develop at almost any age. They are more common in females, with male-to-female ratios varying from 1:3 to 1:4 (Waldron, 1991). The tumors, which are usually encapsulated, are mainly composed of epithelial and myoepithelial cells forming a variety of patterns in a matrix of mucoid, myxoid, chondroid, and sometimes even osteoid tissue. Typically, they show a wide morphologic spectrum, some tumors being principally myxoid, while others are highly cellular. The epithelial cells may form ducts, nests or solid sheets of cells. Immunocytochemically, the myoepithelial cells stain positive for cytokeratin, S-100 protein, glial fibrillary acidic protein, actin, and vimetin. Ductal epithelial cells and solid cellular nests are strongly immunoreactive for cytokeratin. Pleomorphic adenomas are treated by surgical excision. If radically removed the prognosis is excellent. Benign pleomorphic adenomas rarely undergo malignant transformation. In the AFIP series of 326 carcinoma ex pleomorphic adenoma cases (Gnepp and Wenig, 1991), these represented 4.5 % of all pleomorphic adenomas. Carcinoma ex pleomorphic adenoma is a very aggressive tumor, and in cases in which the capsule has been penetrated the prognosis is poor .

Chromosomal aberrations in pleomorphic adenomas were first reported by Scappaticci et al. in 1973, but in the absence of chromosome banding, they could not identify any specific abnormalities. Today, about 500 pleomorphic adenomas have been cytogenetically analyzed, mainly by two independent laboratories in Sweden and in Germany (Sandros *et al.*, 1990; Bullerdiek *et al.*, 1993b; Mark *et al.*, 1997). These studies have revealed that about 20 to 50 % of the adenomas have apparently normal karyotypes. The remaining cases, which are all cytogenetically abnormal, can be divided into three major subgroups.

The first and largest subgroup comprise cases with rearrangements involving band 8q12. The most common abnormality in this subgroup is a t(3;8)(p21;q12). This was in fact the first tumor specific translocation identified in a benign human tumor (Mark *et al.*, 1980). In addition to the t(3;8) there are also a large number of variant translocations in which the 8q12->qter segment has been translocated onto a wide range of other

chromosomal bands. It should also be pointed out that there are two minor clusters of breakpoints at 8p12-23 and 8q21-22. Finally, there are a few cases on record with trisomy 8.

The second cytogenetic subgroup of adenomas comprises cases with rearrangements involving 12q13-15. The preferential abnormality in this subgroup is a t(9;12)(p12-22;q13-15) or an ins(9;12) with similar breakpoints (Stenman *et al.*, 1994; Geurts *et al.*, 1997b). Similar to the first subgroup there are also variant translocations involving different translocation partners. Two additional, minor clusters of breakpoints located at 12p13 and 12q24 are, together with 12q13-15, sometimes involved in inversions. In addition, there are a few known adenomas with interstitial deletions of 12q13-15. One of these cases contained double-minute chromosomes (dmin), and it was suggested that the dmin originated from the deleted 12q segment (Mark *et al.*, 1982). This adenoma was the first benign tumor reported to contain dmin.

The third and smallest subgroup of adenomas comprises cases without the involvement of 3p21, 8q12 or 12q13-15. These cases have instead sporadic numerical and/or structural rearrangements affecting other chromosomes or chromosome segments.

There are thus three preferential chromosome regions, 3p21, 8q12, and 12q13-15, that are repeatedly rearranged in pleomorphic adenomas. The fact that these are often found as the sole karyotypic anomalies indicates that they are primary cytogenetic events of pathogenetic importance. Secondary chromosome changes, including dicentrics, rings and dmin, have been found in about one-third of the cases with clonal changes. So far, no non-random secondary abnormalities have been recognized. Interestingly, Bullerdiek and co-workers (Bullerdiek *et al.*, 1993b) have been able to correlate the karyotypes of a large number of adenomas with different clinicopathological parameters. They found that tumors with 8q12 abnormalities generally occurred in younger patients compared with those with normal karotypes. In addition, they found that tumors with normal karotypes were often more stroma rich than were the tumors with 8q12 abnormalities.

MOLECULAR BASIS OF CHROMOSOME ABNORMALITIES IN SOLID TUMORS

Compared to leukemias and lymphomas little is known about the molecular basis of chromosome abnormalities in solid tumors. Most of our knowledge in this field derives from recent studies of certain types of bone and soft tissue sarcomas. The perhaps most well-known translocations for which the affected genes have been identified are summarized in Table 1.

Table 1. Summary of recurrent translocations and the genes involved in solid tumors.

Tumor type	Chromosome rearrangement	Genes involved	Reference
Ewing sarcoma	t(11;22)(q24;q12)	*EWS/FLI1*	Zucman *et al.*, 1992
	t(21;22)(q22;q12)	*EWS/ERG*	Sorensen *et al.*, 1994
	t(7;22)(p22;q12)	*EWS/ETV1*	Jeon *et al.*, 1995
	t(17;22)(q12;q12)	*EWS/EIAF*	Kaneco *et al.*, 1996
Clear cell sarcoma	t(12;22)(q13;q12)	*EWS/ATF1*	Zucman *et al.*, 1993
Extra skeletal myxoid chondrosarcoma	t(9;22)(q22;q12)	*EWS/TEC*	Labelle *et al.*, 1995
Desmoplastic small round cell tumor	t(11;22)(p13;q12)	*EWS/WT1*	Ladanyi and Gerald, 1994
Myxoid liposarcoma	t(12;16)(q13;p11)	*FUS/CHOP*	Rabbitts *et al.*, 1993
			Crozat *et al.*, 1993
	t(12;22)(q13;q12)	*EWS/CHOP*	Panagopoulos *et al.*, 1996
Dermatofibrosarcoma protuberans	t(17;22)(q22;q13)	*COL1A17PDGFB*	Simon M-P *et al.*, 1997
Alveolar rhabdomyo sarcoma	t(2;13)(q35;q14)	*PAX3/FKHR*	Galili *et al.*, 1993
			Shapiro *et al.*, 1993
	t(1;13)(p36;q14)	*PAX7/FKHR*	Davis *et al.*, 1994
Synovial sarcoma	t(X;18)(p11.2;q11.2)	*SYT/SSX1*	Clark *et al.*, 1994
	t(X;18)(p11.2;q11.2)	*SYT/SSX2*	Crew *et al.*, 1995
Lipoma	t(3;12)(q27-28;q13-15)	*HMGIC/LPP*	Petit *et al.*, 1996
Uterine leiomyoma	inv(12)(q15;q24.1)	*HMGIC/ALDH2*	Kazmierczak *et al.*, 1995
Pleomorphic adenoma	t(3p;10p;12q)	*HMGIC/FHIT*	Geurts *et al.*, 1997a
	ins(9;12)(p23;q12q15)	*HMGIC/NF1B*	Geurts *et al.*, 1997b

Of these, the t(11;22)(q24;q12) found in Ewing sarcoma and peripheral primitive neuroectodermal tumor (PNET) is the prototype. Extensive studies mainly from Gilles Thomas and his co-workers, have shown that the gene affected on chromosome 22 in a number of sarcomas with translocation breakpoints at 22q12 is the *EWS* gene, which encodes an RNA-binding protein. In the t(11;22) translocation *EWS* is fused to the *FLI1* gene at 11q24. *FLI1* encodes a DNA-binding protein belonging to the ETS-family of transcription factors. In the hybrid transcripts produced by the der(22) the RNA-binding domain of *EWS* is replaced by the DNA-binding domain of *FLI1*. Similarly, in three variant translocations other members of the ETS-family, *ERG*, *ETV1*, and *EIAF*, are fused to *EWS*. These translocations lead to constitutive expression of chimeric EWS proteins with presumed transforming properties. Indeed, it was recently shown that the *EWS/FLI1* fusion gene can transform NIH3T3 cells (May *et al.*, 1993). Collectively, these findings show that fusion of *EWS* to different members of the ETS-gene family results in very similar disease phenotypes, i.e. Ewing sarcoma or PNET (Sorensen *et al.*, 1994; Jeon *et al.*, 1995).

Recent studies have demonstrated that *EWS* is also involved in several other sarcomas with translocations affecting chromosome band 22q12, such as clear cell sarcoma, desmoplastic small round cell tumor, and extra skeletal myxoid chondrosarcoma (Table 1). However, in these tumors *EWS* is fused to other transcription factors not belonging to the ETS-family. The molecular consequences of these translocations are very similar, i.e. they lead to fusion genes where the N-terminal RNA-binding domain of EWS is substituted by the DNA-binding domains of different transcription factors.

Another tumor type with a comparable translocation is myxoid liposarcoma (MLS). In the MLS-specific t(12;16)(q13;p11) the *CHOP* gene (also called GADD153) on chromosome 12 is fused to FUS (also called TLS) on chromosome 16 (Crozat *et al.*, 1993; Rabbitts *et al.*, 1993). *CHOP* codes for a dominant negative transcription factor, and *FUS* for an RNA-binding protein with homology to EWS. It has been shown that the TLS-CHOP fusion protein can function as a transcriptional activator in vitro (Zinszner *et al.*, 1994). Recent studies have also shown that the N-terminal domain of EWS, both in vitro and in vivo, can replace the TLS part of TLS-CHOP (Zinszner *et al.*, 1994; Panagopoulos *et al.*, 1996).

A common theme for several sarcoma associated translocations thus appears to be the fusion of genes containing RNA-binding domains (e.g. *EWS* or *TLS*) with transcription factor genes containing DNA-binding domains (e.g. *FLI1*, *ERG*, *ATF1*, *WT1*, or *CHOP*). The target specificity of the

transcriptional activation seems to be determined by the gene supplying the DNA-binding domain, whereas the transactivation potential and expression level seem to be determined by the gene supplying the N-terminal domain and the promoter region (Ladanyi, 1995).

As mentioned previously, there are several benign tumor types with consistent rearrangements of 12q13-15, including pleomorphic adenomas, lipomas, uterine leiomyomas, pulmonary chondroid hamartomas, endometrial polyps, aggressive angiomyxomas, and hamartomas and fibroadenomas of the breast (Mitelman, 1994). Recently, two groups identified the *HMGIC* gene at 12q15 as the target gene in these tumor types (Ashar *et al.*, 1995; Schoenmakers *et al.*, 1995). Subsequent studies have demonstrated that *HMGIC* is in fact involved in all of the above-mentioned tumor types (Bol *et al.*, 1996; Kazmierczak *et al.*, 1996; Staats *et al.*, 1996). *HMGIC* belongs to the high mobility group (HMG) protein gene family, which comprises proteins that are nonhistone components of chromatin (Grosschedl *et al.*, 1994). The HMGI-C protein binds in the minor groove of DNA and is thought to function as an architectural factor in the nuclear scaffold (Saitoh and Laemmeli, 1994). It is also suggested to be of importance in the assembly of stereospecific transcriptional complexes (Tjian and Maniatis, 1994; Giancotti *et al.*, 1991). Interestingly, mice in which both *Hmgic* alleles have been knocked out show the pygmy phenotype (Zhou *et al.*, 1995).

Most breakpoints in *HMGIC* are located within the third large intron, resulting in the separation of the three predicted DNA-binding domains (exons 1 to 3) in the amino-terminal region from the acidic, carboxy-terminal domain. So far, four fusion partner genes of *HMGIC* have been identified, i.e. *LPP*, *ALDH2*, *FHIT*, and *NF1B* (Table 1). Interestingly, the latter two have been identified in pleomorphic adenomas; *FHIT* in an adenoma with a complex t(3p;10p;12q) and *NFIB* in two adenomas with ins(9;12). In the first case, the first three exons of *HMGIC*, encoding the three AT-hook domains, were fused to exons 9 and 10 of the *FHIT* gene, which encodes the last 31 carboxy-terminal amino acids. In the latter case, the first three or four exons of *HMGIC* were fused to exon 12 of *NF1B*. Since no common functional domain has been identified so far among the known translocation partners, the critical event seems to be the separation of the DNA-binding domains from the acidic carboxy-terminal tail of HMGI-C (Geurts 1997a).

THE HUMAN GENOME PROJECT

The human genome project (HUGO) is an international research project the purpose of which is to map and sequence the entire human genome. It involves a number of large research institutions, mainly in the United States, Europe and Japan. The project, which is expected to cost $3 billion, was initiated in the United States in 1988 with funding from the National Institute of Health (NIH) and the Department of Energy (DOE). In 1989 the National Center for Human Genome Research division of the NIH was established. The goals of the project are to construct genetic and physical maps, to determine the complete sequence of the human DNA and to study the genomes of nonhuman model organisms. To accomplish these goals the development of new technology will be necessary, in particular improvements in the efficiency of DNA sequencing. The extensive knowledge of the human genome and its expected 100,000 genes that will come from the genome project will have an enormous impact on our understanding of human biology and diseases. It will profoundly alter our approach to medical care by shifting our focus from treatment of established diseases to disease prevention (Guyer and Collins, 1993; Olson, 1993; Hoffman, 1994). However, these new developments also raise a number of important ethical, legal, and social questions that need to be resolved.

Genetic maps

Genetic linkage maps are based on the frequency at which polymorphic markers are coinherited. During meiosis, genetic recombination or crossing over lead to separation of markers that were on the same chromosome in the parents. Closely linked markers are coinherited more often than distant ones. A major breakthrough that made it possible to construct human linkage maps was the discovery of minor DNA sequence variations (polymorphisms), which normally occur, and constitute the basis for human variability. Polymorphisms, such as microsatellite repeats and CA repeats, can be easily detected using the polymerase chain reaction (PCR). Much of the initial work on the construction of genetic maps of the entire human genome was done at the French institute Généthon, established and funded by the French muscular dystrophy association (Dib *et al.*, 1996). The first linkage map of the entire human genome was published in 1987 (Donis-Keller *et al.*, 1987). The latest map, published last year, consists of 5,264 microsatellites with an average spacing of 1.6 cM (Dib *et al.*, 1996).

These maps greatly facilitate the localization of disease genes through family-based genetic linkage studies.

Physical maps

On physical maps the real distances between individual genes and markers are determined. The resolution of such maps is higher compared with linkage maps. The goal of this part of the genome project is to produce high resolution maps consisting of cloned DNA fragments that cover the entire genome and to develop unique PCR primer sets that recognize sites (sequence-tagged sites - STSs) along the entire genome (Guyer and Collins, 1993; Hoffman, 1994). The most useful types of physical maps are those that consist of cloned, overlapping DNA segments, so-called contigs. Important tools for the construction of contigs are the YAC and mega-YAC libraries developed by the Centre d´Etude du Polymorphisme Humain (CEPH) in France. YACs or yeast artificial chromosomes are vectors that allow large DNA fragments to be cloned as an additional chromosome into *Saccharomyces cerevisiae.*. The first physical maps of entire human chromosomes were published for the long arm of chromosome 21 and for the Y chromosome (Chumakov *et al.*, 1992; Foote *et al.*, 1992). Recently a YAC contig map covering about 75% of the entire human genome was described (Chumakov *et al.*, 1995). The map consists of 225 contigs with an average size of about 10 megabases. YAC contigs with varying coverage of the human genome are now available for all human chromosomes through the world wide web. Physical maps are essential tools in the identification of disease genes. Once such a gene has been localized on the genetic map the corresponding YAC clones from the physical map can be easily isolated and used in the identification of the gene in question.

Positional cloning

Isolation or cloning of a gene can be done either by positional cloning (previously also called reverse genetics) or functional cloning. In the case of functional cloning the gene product and/or the function of the gene must be known in advance. The gene is thus cloned before it is mapped. Positional cloning, on the other hand, begins with mapping the gene to a specific location on a chromosome. In this case the function of the gene is determined after it is cloned (Guyer and Collin, 1993). Positional cloning strategies rely on physical mapping information for the chromosome region of interest. During the past few years, a number of genes involved in hereditary cancer syndromes, such as the Neurofibromatosis type 1 and type

2 genes, the von Hippel-Lindau disease gene, and the Wilms' tumor gene, have been identified by positional cloning (Cawthon *et al.*, 1990; Call *et al.*, 1990; Trofatter *et al.*, 1993; Rouleau *et al.*, 1993; Latif *et al.*, 1993; Kley *et al.*, 1995). This approach has also proved to be very useful in the identification of genes involved in tumor specific chromosome rearrangements (Zucman *et al.*, 1992; Schoenmakers *et al.*, 1995). In such cases the chromosome region of interest has already been identified, and the chromosomal breakpoints can be mapped by FISH (fluorescence in situ hybridization) on tumor chromosomes using YACs derived from the region. The region encompassing the breakpoint is identified by chromosome walking . A YAC contig based on STS-content mapping is constructed, and YACs from this contig are mapped by FISH with the purpose of identifying a YAC spanning the breakpoint. This YAC is then further characterized and used to identify the relevant gene.

AIM OF THE STUDY

The primary goal of this thesis was to identify the genes involved in the recurrent t(3;8)(p21;q12) translocation in pleomorphic adenomas using a positional cloning approach.

MATERIALS AND METHODS

All studies in this thesis were performed on cultured cells, chromosome preparations, DNA and/or RNA, derived from primary tumor tissue not required for histopathological diagnosis. From each specimen analyzed, a portion was examined histopathologically to assess the tumor content. All 12 tumors used were benign pleomorphic adenomas. They all had a translocation breakpoint at 8q12, either in the form of the classic t(3;8)(p21;q12) or a variant translocation. From most tumors, metaphases stored in frozen fixative, fresh frozen tumor tissue, as well as frozen cultured cells from the same tumor ,were available. All physical mapping experiments were performed on metaphases obtained from primary cultures.

The different methods used in this thesis are described in detail in Papers I - IV or, in some cases, in references therein. Hence these will not be commented on further here.

RESULTS AND DISCUSSION

Identification of a YAC-clone spanning the 8q12 translocation breakpoint (Paper I)

As a starting point for the positional cloning effort to isolate the gene affected by the translocations involving 8q12 in pleomorphic adenomas, we isolated a YAC-clone (ICFRy900G10157) corresponding to the *MOS* proto-oncogene located at 8q11-12. This YAC also contained the proto-oncogene *LYN*. The rationale for selecting *MOS* as a starting point was an earlier report showing that *MOS* and its flanking sequences were mutated in two pleomorphic adenomas with 8q12 abnormalities (Stenman *et al.*, 1991a). FISH mapping of this YAC, together with a phage clone containing a third candidate gene *CEBPD* (CEBPD belongs to a family of leucine zipper transcription factors), on chromosomes from two adenomas with t(3;8)(p21;q12) revealed that all three genes were proximal to the breakpoints. These observations suggest that none of these genes is involved in the 8q12 translocations and that the target gene is a novel gene.

Chromosome band 8q12 is, according to radiation hybrid mapping data, flanked by the markers D8S165 and *MOS* proximally and by D8S260 distally (Sapru *et al.*, 1994). To sublocalize the breakpoints within this region we isolated a number of YACs corresponding to the genetic markers D8S260, D8S108, and D8S166 (the latter two are located about 1 Mb distal to *MOS*). FISH mapping of these revealed that they were all distal to the breakpoints in both adenomas, demonstrating that the breakpoints are located within a 1 Mb region flanked by *MOS* and D8S166.

To fine map the breakpoints we initiated a chromosome walk starting from *MOS*. Two YACs corresponding to D8S285, which maps less than 100 kb distal to *MOS*, were isolated. STS-content mapping of these revealed that YAC 166F4 also contained *MOS* and *LYN*, and that YAC 935E9 contained *LYN*, *PENK* and D8S165 but had an internal deletion of *MOS*. FISH analysis using these YACs demonstrated that they spanned the t(3;8) breakpoints. YAC 166F4, which contains *MOS*, *LYN* and D8S285, covers about 700 kb of genomic DNA. The smaller ICRF YAC, which contains *MOS* and *LYN*, but not D8S285, maps proximal to the breakpoint, indicating that the breakpoints are located between *MOS* and the distal part of YAC 166F4, a region estimated to be about 300 to 400 kb.

Mapping of the 8q12 translocation breakpoint to a 40-kb region (Paper II)

In this paper we describe the physical mapping of the breakpoints in a pleomorphic adenoma (CG682) with an insertion of a 3p segment into 8q12, i.e. a rearrangement similar to the t(3;8)(p21;q12). Based on G-banding and FISH, the karyotype of this tumor was 46,XX,der(3)del(3)(p23->24)del(3)(p21.3p14.1), der(8)dir ins(8;3)(q12;p21.3 p14.1). To further map the 8q12 breakpoint we isolated cosmids from a chromosome 8-specific library (Wood *et al.*, 1992) using STSs corresponding to markers, genes and YAC-ends mapping within YAC 166F4, which we previously showed spanned the breakpoint in adenomas with the classic t(3;8) (Paper I). FISH mapping of these cosmids revealed that the 8q12 insertion breakpoint was located within a 300-kb region flanked by *MOS* and a new STS CH129. A cosmid derived from this region, CEM23, was shown to span the breakpoint. The clustering of the 8q12 breakpoints to a limited region in tumors with the recurrent t(3;8) (Paper I), as well as in variants thereof, indicate that a common molecular mechanism underlies the different 3;8 rearrangements. Our mapping of the breakpoint in the present case to a 40-kb region will facilitate the search for the 8q12 gene.

The *FHIT* gene at 3p14.2 was recently shown to span the hereditary renal cell carcinoma associated t(3;8)(p14.2;q24) breakpoint and the *FRA3B* fragile site (Ohta *et al.*, 1996). Using a YAC containing *FHIT* we could show by FISH that *FHIT* was transposed to the der(8) but not disrupted by the 3;8-rearrangement. RT-PCR analysis showed only the normal-sized 709-bp *FHIT* fragment, indicating that *FHIT* is not affected in this case. This was also shown to be the case in adenomas with t(3;8). FISH analysis using the *FHIT* YAC and a chromosome 8 painting probe confirmed the orientation of the inserted 3p-segment into the der(8). Interestingly, we recently reported a pleomorphic adenoma with a complex rearrangement involving chromosomes 3, 10 and 12 in which the first three exons of *HMGIC* were fused to exons 9 and 10 of *FHIT* (Geurts *et al.*, 1997a).

Construction of a YAC contig and physical map covering the 8q12 translocation breakpoint cluster region (Paper III)

According to previous mapping data, band 8q12 is flanked by the markers D8S165/D8S285 proximally and by D8S260 distally (Sapru *et al.*, 1994). YACs corresponding to these markers were used for initiating the walk towards the 8q12 breakpoints. We thus started from D8S285 and constructed a 2 Mb YAC contig covering the centromeric part of band 8q12, and from D8S260 and generated a 5 Mb contig covering the telomeric part of 8q12. These two

non-overlapping contigs cover about 75% of band 8q12. Since the majority of breakpoints were found in the centromeric contig our research was focused on this.

The centromeric contig covers approximately 2 Mb of genomic DNA and consists of 34 overlapping YAC clones, with insert sizes between 169 kb and 1660 kb. It is tagged by 31 markers with an average spacing of 65 kb. Initially, we walked bidirectionally and this allowed us to link this contig to an existing chromosome 8q11 YAC contig (MIT contigs WC8.7 and WC-157). All markers and genes that were or became publicly available during our work were STS content mapped in the contig and those found positive were sublocalized by (primer) hybridization to YAC Southern blots. Three genes, *MOS*, *LYN* and *PENK*, and three expressed sequence tags (ESTs) could be positioned in the contig. One EST corresponded to *LYN*, one to *PLAG1* (Paper IV), and one did not resemble any currently known gene. A restriction map generated with rare-cutter enzymes revealed at least 7 putative CpG islands. Four of these correspond to known genes (*MOS*, *LYN*, *PENK*, and *PLAG1*), while the others might represent the 5' end of novel genes.

The starting point for the telomeric contig was D8S260, for which two megaYACs were isolated. An STS (CH31) corresponding to the distal end of one of these was shown to be present in two YACs containing D8S510, which links our contig to an 8q13 specific contig (Doerflinger *et al.*, 1995). The telomeric contig consists of 23 megaYACs and covers about 5 Mb of genomic DNA. One gene, *CYP7*, was shown to reside within the contig. The fact that we have only been able to identify and map five genes in the two contigs, which together cover about 75 % of band 8q12, is consistent with the view that Giemsa-positive bands are chromosome regions rather low in G+C content and poor in genes (Craig and Bickmore, 1993).

Identification of a major breakpoint cluster region in 8q12 (Paper III)

In previous studies we have shown that YAC 166F4 spans the breakpoint in two adenomas with t(3;8)(p21;q12) (Paper I) and that a cosmid clone mapping within this YAC covers the breakpoint in an adenoma with an ins(8;3) (Paper II). To extend these observations we have now analyzed five additional adenomas with different 8q12 abnormalities (for details of their karyotypes see Table 1 in Paper III). FISH analysis of these cases revealed that three of them had a breakpoint within YAC 166F4, and two cases had a breakpoint proximal to this YAC (cf. below). Fine mapping of these breakpoints using cosmids containing *MOS* (CEM1) and STS EM156 (CEM23)

revealed that the breakpoints in six of eight tumors tested were in a 300-kb region flanked by these two cosmids. The fact that both tumors with the recurrent t(3;8)(p21;q12) and those with 8q12 translocations and other translocation partners have breakpoints within the 300-kb region, indicate that this is the major breakpoint cluster region in pleomorphic adenomas with 8q12 abnormalities.

Two of the eight tumors tested had breakpoints proximal to *MOS*. In one case the breakpoint was mapped between *MOS* and YAC 898G12, and in the other case it was found to reside within YAC 943G4 (containing the *XRCC7* gene), which maps at least 2 Mb centromeric to MOS. Since these two breakpoints are clearly different from those located in the 300 kb breakpoint region, it is unlikely that they affect the same gene. Interestingly, we have recently obtained molecular data suggesting the presence of an additional breakpoint region distal to our telomeric contig. Based on FISH we have estimated that these breakpoints are 15 to 20 cM telomeric to the 8q12 breakpoint cluster region. Collectively, our findings thus suggest that there are several genes in the proximal part of 8q that are affected by chromosome rearrangements in pleomorphic adenomas.

Identification and characterization of the *PLAG1* gene (Paper IV)

In this paper we show that *PLAG1* (Pleomorphic Adenoma Gene 1) is the target gene affected by the 8q12 translocations in pleomorphic adenomas. In previous studies (Paper III) we identified a 300-kb breakpoint cluster region between *MOS* and STS EM156. To fine map this region and eventually identify the *PLAG1* gene we developed a contig consisting of 27 cosmids and 2 phage clones. The contig covers 300-kb of genomic DNA. By FISH we could show that two cosmids, 149G12 and 4H6, spanned the 8q12 breakpoints in two adenomas. BLAST searches of new STSs from the contig revealed that the right end of YAC 143D5 (STS CH283) displayed sequence identity with a publicly available EST. In Northern blot analysis using this EST as a probe, a 7.5 kb transcript was detected, representing the putative *PLAG1* gene. This transcript was detected in several fetal tissues, including lung, liver, kidney, and placenta (low levels), but not in any of the adult tissues examined, salivary gland tissue included.

Cloning and analysis of the *PLAG1* cDNA showed that it contains an open reading frame (ORF) of 1500 bp, starting with the ATG at position 481-483. An in frame stop codon (TAG) is present 9 nucleotides upstream of this ATG. The composite cDNA (7313 nucleotides) of *PLAG1* has the coding capacity for a protein with a molecular weight of 56 kD. The deduced amino

acid sequence of the PLAG1 protein reveals seven canonical C_2H_2 zinc finger domains in the N-terminal region and a serine-rich, non-finger region representing the C terminus. There are two potential nuclear localization signals in the N-terminal region. Strictly speaking, the deduced PLAG1 protein is not a Krüppel zinc finger protein, since it does not contain the characteristic H/C linker between the zinc fingers (Bellefroid *et al.*, 1989). Collectively, the structural features suggest that the PLAG1 protein is a novel member of the large zinc finger gene family. The *PLAG1* sequence is not homologous to any known zinc finger protein, although studies of anonymous ESTs have indicated that there is at least one closely related human gene, tentatively designated *PLAG2*. Cloning and characterization of this gene is now in progress.

Apart from transcriptional modulation and control of RNA metabolism, cromatin packaging might also constitute an important activity through which zinc finger proteins exert their regulatory roles (El-Baradi and Pieler, 1991). In this context it should be noted that the presumed role of HMGI-C is also to be found in chromatin modelling (Wolffe, 1994). The mammalian genome contains a large number of zinc finger genes (Bellefroid *et al.*, 1989), and the number of such genes implicated in cancer is growing steadily. For example, mutations within the zinc finger gene *WT1* predispose to Wilms´ tumor (Haber *et al.*, 1990). Furthermore, in desmoplastic small round cell tumors, *WT1* is fused to *EWS* as the result of a t(11;22)(p13;q12) (Ladanyi and Gerald, 1994), and in acute promyelocytic leukemia, a fusion occurs between the retinoic acid receptor-α locus and a Krüppel-like zinc finger gene *PML* due to a t(15;17)(q22;q11-21), or to the zinc finger gene *PLZF*, due to a variant t(11;17)(q23;q21) (de Thé *et al.*, 1991; Chen *et al.*, 1993). Similarly, in non-Hodgkin´s lymphoma, *LAZ3/BCL6*, a gene encoding a protein with seven zinc fingers, is rearranged by recurrent translocations involving 3q27 (Kerckaert *et al.*, 1993; Ye *et al.*, 1993).

Genomic organization of the *PLAG1* gene (Paper IV)

Comparison of transcribed and genomic sequences of the *PLAG1* gene revealed that it contains 5 exons. The transcriptional orientation of the gene is directed towards the centromere. The first three exons are non-coding, the fourth exon contains the translation start site (ATG), and the N terminus of the protein, including one complete zinc finger. The second finger is split by intron 4 and continues into exon 5, which contains the remaining part of the ORF and the long 3'-UTR (5533 bp). Based on our YAC and cosmid maps, the *PLAG1* locus spans about 35 kb, with a large intron (approximately

25 kb) between exon 1 and exon 2. RACE analysis has revealed two alternatively spliced mRNAs, differing from each other by the presence or absence of the non-coding exon 2.

Identification of *CTNNB1* as a fusion partner of *PLAG1* in the t(3;8) translocation (Paper IV)

Since Southern blot analysis of pleomorphic adenomas with 8q12 abnormalities had revealed rearrangements in the 5' non-coding region of *PLAG1* we performed rapid amplification of 5' cDNA ends (5'-RACE) analysis on tumors with t(3;8) to identify a possible chimeric transcript consisting of sequences from 3p21 fused to *PLAG1*. Sequence analysis of the PCR products from two tumors revealed that ectopic sequences were fused to the acceptor splice site of exon 3 of *PLAG1*. BLAST analysis of these sequences revealed that they were identical to exon 1 sequences of *CTNNB1*, a gene that has previously been mapped to chromosome 3p21 (Kraus *et al.*, 1994; Nollet *et al.*, 1996). Independent evidence of the involvement of *CTNNB1* in the 3;8-rearrangements was obtained by FISH using the two YACs 756G5 and 750D3, which contain the complete gene. Both YACs spanned the 3p21 breakpoint in an adenoma with an ins(8;3).

Northern blot analysis revealed that *CTNNB1* is ubiquitously expressed, as a 3.8 kb RNA doublet in all tissues tested, normal salivary gland tissue included. The gene codes for ß-catenin, which is a cytoplasmic protein of about 88 kD (Gumbiner and McCrea, 1993). ß-catenin has been found as a structural component of adherence junctions, binding directly to cadherins. The cadherin-ß-catenin complex mediates cell adhesion, cytoskeletal anchoring, and signalling, which are important processes in the regulation of cell growth and behaviour (Peifer, 1993; Kemler, 1993; Gumbiner, 1996).

ß-catenin has also been implicated in the specification of cell fate during embryogenesis (Miller and Moon, 1996) as well as in the WG/WNT signalling pathway by virtue of its association with the adenomatous polyposis coli protein APC (Peifer, 1996). APC may act either as a negative regulator of ß-catenin accumulation and signalling or as a negative regulator and effector, with phosphorylation of APC by protein kinase GSK3ß as a controlling element (Rubinfeld *et al.*, 1996).

Recent studies have also shown that ß-catenin directly interacts with members of the TCF/LEF family of architectural transcription factors, such as LEF-1 (Behrens *et al.*, 1996) or XTcf-3 (Molenaar *et al.*, 1996), and that following nuclear translocation, it can regulate gene expression and activate specific target genes. Interestingly, LEF-1 has also been shown to regulate

epithelial-mesenchymal tissue interactions during organogenesis (Kratochwil *et al.*, 1996). This is of particular interest since pleomorphic adenomas, which are epithelial tumors, clearly show both epithelial and mesenchymal differentiation.

Detection of CTNNB1/PLAG1 fusion transcripts by RT-PCR (Paper IV)

To extend and confirm the 5'-RACE analysis, we applied a reverse transcription-polymerase chain reaction (RT-PCR) approach using primers specific for *CTNNB1* and *PLAG1*. Analysis of 7 pleomorphic adenomas with t(3;8) (6 cases) or ins(8;3) (1 case) revealed hybrid transcripts consisting of *CTNNB1* and *PLAG1* sequences in all the tumors analyzed. Products of two different sizes were obtained, 509 bp and 614 bp. The larger product differs from the smaller by an extra 105 bp which corresponds to the alternatively spliced exon 2 of *PLAG1*. Both products were seen in five tumors, whereas in two cases only the smaller product could be detected. The identity of these PCR products was confirmed by sequence analysis. The *CTNNB1/PLAG1* fusion transcripts could not be detected in an adenoma with a variant translocation t(8;15)(q12;q14), demonstrating that these transcripts are specific for the t(3;8). The reciprocal fusion transcripts *PLAG1/CTNNB1* encoded by the der(3), could also be readily detected by RT-PCR. Collectively, these data indicate that the breakpoints in all seven tumors with 3;8-rearrangements had occurred in the 5' region of *PLAG1*, upstream of the non-coding exon 3. In *CTNNB1* the breakpoints were always in the first intron.

Activation of PLAG1 expression due to promoter swapping (Paper IV)

To evaluate the consequences of the 3;8-rearrangements on the expression of *PLAG1* and *CTNNB1* we performed Northern blot analysis. All tumors tested expressed a 7.5 kb transcript using a probe specific for the 3'UTR of *PLAG1*. This transcript was also detected with a probe specific for exon 1 of CTNNB1, demonstrating that it does not correspond to the normal *PLAG1* transcript but to a *CTNNB1/PLAG1* fusion transcript. This was also confirmed using a probe specific for exon 1 of *PLAG1*.

In contrast to tumors with 3;8-rearrangements, the adenoma with the t(8;15)(q12;q14) only expressed the normal *CTNNB1* transcripts in addition to the 7.5 kb transcript detected by the *PLAG1* 3'UTR probe. The expression levels of *CTNNB1* was about twice as high as in tumors with a *CTNNB1/PLAG1* fusion transcript. 5'-RACE analysis revealed that this tumor also has a breakpoint in the 5' region of PLAG1, and that the

translocation leads to a fusion transcript with ectopic sequences fused to exon 3 of PLAG1. The ectopic sequences consist of 38 nucleotides which do not correspond to any known gene sequences.

Collectively, our findings demonstrate that the t(3;8) translocations lead to reciprocal exchange between expression control elements of *PLAG1* and *CTNNB1*, leading to activation of *PLAG1* and reduced expression of *CTNNB1*. Since the coding sequences of both genes are invariably preserved, we have described the molecular mechanism as promoter swapping. To the best of our knowledge this is the first example of promoter swapping in solid tumors. However, in leukemias and lymphomas there are a few known examples of promoter substitutions (Aplan *et al.*, 1992; Dallery *et al.*, 1995; Galiègue-Zouitina *et al.*, 1996).

SUMMARY

The most frequent cytogenetic finding in pleomorphic adenomas is a t(3;8)(p21;q12) translocation, or variants thereof in which the 8q12->qter segment is translocated to a variety of other chromosome regions. Using a positional cloning approach we have identified the target genes affected by the t(3;8) in primary pleomorphic adenomas. Two non-overlapping YAC contigs, covering approximately 75% of band 8q12, were established. FISH mapping of YACs and cosmids from these contigs revealed that the majority of breakpoints clustered within a 300-kb subregion in the centromeric part of 8q12. Detailed mapping of this region led to the identification of the *PLAG1* gene, a novel, developmentally regulated gene coding for a zinc finger protein. 5'-RACE analysis of primary tumors with t(3;8) enabled us to identify *CTNNB1* as the chromosome 3 gene fused to *PLAG1*. *CTNNB1* codes for ß-catenin, a protein interface functioning in the WG/WNT signalling pathway and specification of cell fate during embryogenesis.

The t(3;8) results in promoter swapping between *PLAG1* and *CTNNB1*. Fusions invariably occur in the 5' non-coding regions of both genes, exchanging regulatory control elements while preserving the coding sequences. Due to the t(3;8) *PLAG1* is activated whereas the expression of *CTNNB1* is reduced. Activation of *PLAG1* was also observed in an adenoma with a t(8;15)(q12;q14) translocation, demonstrating that *PLAG1* activation is not restricted to tumors with t(3;8). To our knowledge this is the first example of promoter swapping in solid tumors.

The identification and cloning of a novel "benign oncogene" activated by chromosome translocation in pleomorphic adenomas constitute an important step towards an increased understanding of the molecular pathogenesis of benign neoplastic growth. The present findings may also have diagnostic implications. The fact that *PLAG1* is not normally expressed in adult tissues, but activated in tumors as a result of 8q12 rearrangements, makes it a potentially useful marker in the differential diagnosis of benign and malignant salivary gland tumors. The results potentially allow for targeted therapy of pleomorphic adenomas.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to all those who have participated in this work and in particular:

Professor Göran Stenman, my supervisor, for introducing me to the field of cancer genetics, for guiding me through this thesis, for his enthusiasm and encouragement, and for being a great supervisor.

Professor Wim Van de Ven, for invaluable scientific collaboration.

My co-authors, Dr. Koen Kas, Dr. Marianne Voz, Anna-Karin Åström, Eva Meyen, Professor Jörn Bullerdiek and Ingrid Klawitz for being excellent collaborative partners.

Margareta Behrendt, Marie Juberg-Ode, Anabel Peydró-Mellquist, Dr. Pelle Sahlin, Helene Sjögren and Caspar Wohlfahrt for skillful technical assistance and for being supportive lab colleagues and friends.

Professor Lennart Enerbäck, Department of Pathology, for his support and for providing a pleasant working environment.

Professor Lars-Gunnar Kindblom, Associate Professor Walter Ryd and Dr. Fabrizio Remotti for tissue collection and histopathological diagnosis.

Dr. Anders Nordkvist and Associate Professor Staffan Edström, Department of Otorhinolaryngology, for providing tumor material.

Associate Professor Joachim Mark, Barbro Wedell and Rigmor Dahlenfors for their support.

Staff and colleagues at the Department of Pathology. Special thanks to Kerstin for secretarial help and to Ali-Reza, Hermengild, Kristina, Ulla-Britt and Ingrid for all kinds of help.

My friends and colleagues at the Department of Oral Pathology.
Patrick O´Malley for excellent and much-appreciated proofreading.

All my friends outside the laboratory, in the air, on the sea, on the walls and on the ground.

My family, for their love and support.

Financial support for this work was received from the Swedish Cancer Society (Cancerfonden), the IngaBritt and Arne Lundbergs Research Foundation, the Assar Gabrielssons Research Foundation and Stiftelsen Jubileumsklinikens Forskningsfond mot Cancer.

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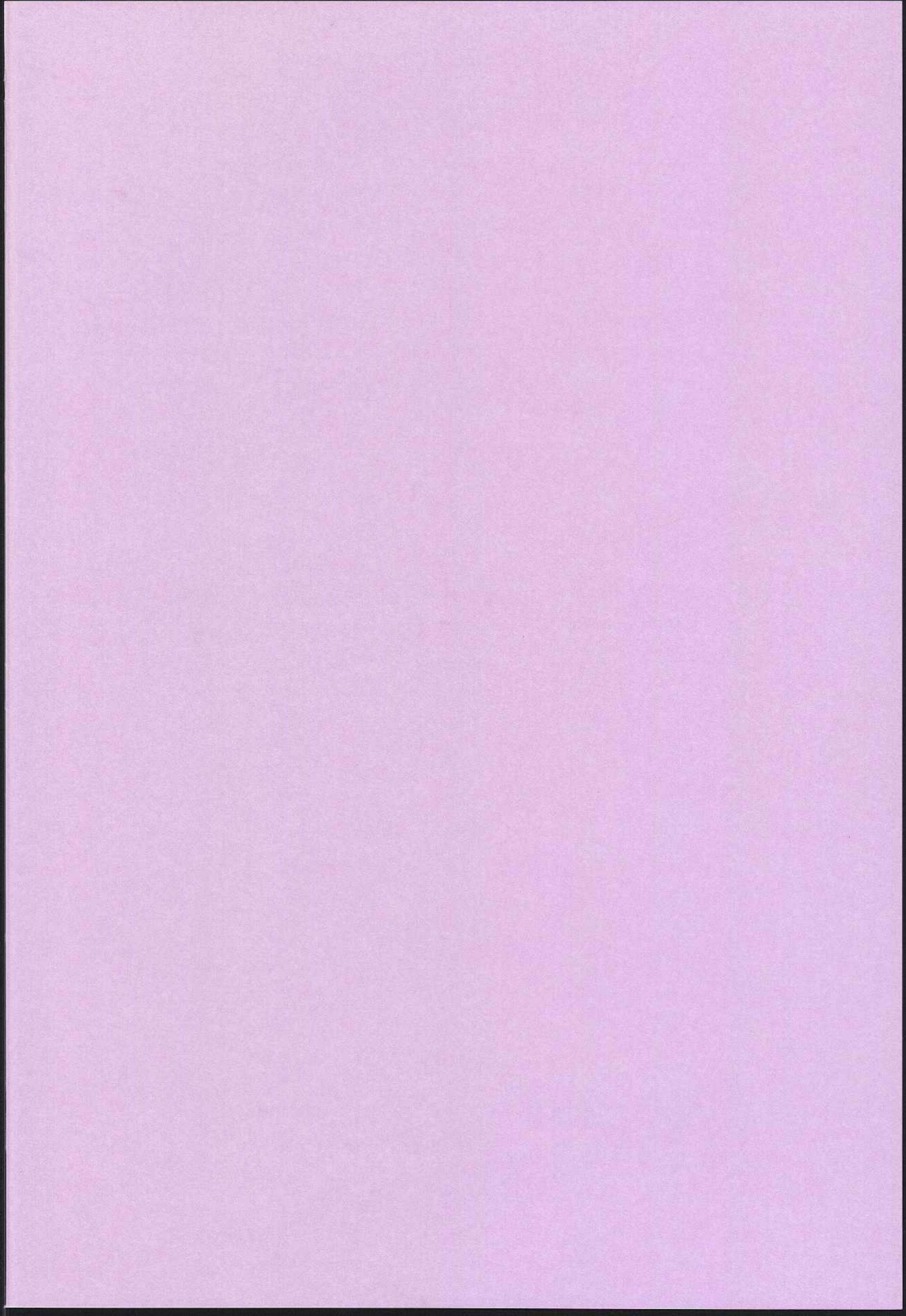

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