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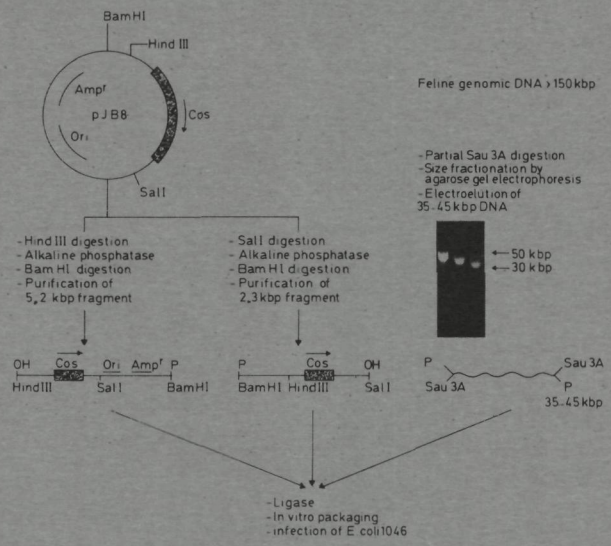
Molecular cloning and characterization of the loci



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JOSEPH STEPHAN VERBEEK

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Molecular cloning and characterization of the loci

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Chapter 1

GENERAL INTRODUCTION

THE MOLECULAR BASIS OF CANCER

I. Introduction

Experimental carcinogenesis, histological and cytogenetic studies and epidemiological observations indicate that the development of tumors is a multistep process The number and the nature of the steps is unknown But the general idea is that each step creates a subclone of cells with a new phenotype that replaces its predecessors as the result of a selection process (1-5) In separate steps, cells might acquire the power for uninhibited proliferation, invasiveness, and ultimately full metastatic potential But how many alterations are necessary on a molecular level to create such spectrum of newly acquired properties and what is their nature? That changes on the DNA level should be responsible for tumorigenesis is based on the following observations

a) In many cancer cells chromosomes are damaged and often the damage increases during tumor development (6, 7),
b) The carcinogenicity of substances is closely related to their mutagenic potential (8),
c) An inherited predisposition for some cancers has been observed (9, 10)

Moreover, xeroderma pigmentosa patients show a clear connection between susceptibility to cancer and impaired ability to repair damaged DNA (11)

Each step in the tumor progression is thought to be the result of the mutation, activation or deletion of particular genes Since about 10 years, molecular biological cancer research is focused on a restricted number of genes, the so called (proto-) oncogenes In general, understanding oncogenesis on a molecular level is understanding the consequences of genetic alterations for the expression and the biochemical functions of these genes Definition of the oncogenes originated from tumor virus research

II. Molecular genetic analysis: Activation of (proto-)oncogenes

A Animal cancer RNA tumor viruses

Mammalian tumor viruses belong taxonomically to diverse families They are divided in two groups the DNA - and RNA tumor viruses Among them the DNA viruses (herpes-, adeno-, hepatitis and papovaviruses) form a very heterogeneous group (12) The RNA tumor viruses on the otherhand belong to a single taxonomic group of RNA viruses, the retroviruses They provide the most coherent view of oncogenesis now available and showed us possible ways to cancer inducing mechanisms without virus intervention (13, 14)

Retroviruses are RNA viruses with a typical lifecycle After infection of the target cell, the single stranded RNA virus genome is copied by the viral enzyme reverse transcriptase in a double stranded circular DNA molecule that subsequently integrates

into the host genome The potential oncogenicity of this type of viruses is based on the close interaction between virus genome and host genome (15) The resulting provirus behaves as a cellular gene that can be transcribed into single stranded RNA This RNA is either packaged in virus particles or used as a polycistronic mRNA for the production of the viral proteins the *gag* coded core proteins, the *pol* coded reverse transcriptase and the *env* coded envelope proteins The retroviruses can be divided in two groups by their capability to induce transformation the acutely transforming and the slowly or non-transforming viruses The former group is able to induce transformation in tissue culture cells and tumorigenesis in animals with a short latency period between infection and the appearance of the tumor In general, this group is replication defective and hence dependent for propagation on a helper virus belonging to the replication competent latter group That latter group lacks the transforming activity in tissue culture and does not induce tumors in animals or only after a long latency period

1 Gene transduction

Many different acutely transforming retroviruses (exhaustively described in ref 13) causing carcinomas, sarcomas, leukemias and lymphomas in animals like chicken, mouse, rat, cat and monkey were found Structurally their genomes differ from the genomes of their non-acutely transforming counterparts on the one hand by missing usually some virus-specific sequences, on the other hand by possessing unique sequences directly related to their acutely transforming properties These sequences are highly similar to normal genomic sequences of non-infected tissue of the host Moreover these sequences are very well conserved between the different species of eukaryotes suggesting an important role in fundamental processes of the eukaryotic cell

The insight that the acutely transforming viruses are the result of recombination between the helper virus genome and the host genome by which viral sequences are replaced by particular cellular sequences marked the beginning of a new and important era in fundamental cancer research Retroviruses are able to act as vectors for cellular genes, a phenomenon known from bacteriophages as gene transduction Moreover they contain strong transcriptional promoters and have a high mutation rate As part of a retrovirus, a cellular gene can escape the controlling mechanisms of the cell and can give rise to mutated products that act in an abnormal way Looking for acutely transforming retroviruses in natural occurring tumors is selecting for retroviruses that have recombined with normal cellular genes with a potential oncogenicity (proto-oncogenes) In other words, independent isolates of acutely transforming retroviruses did for us what we never could have done with our limited techniques Out of a haystack of ten thousends of genes of the eukaryotic genome they picked up about twenty genes with a potential tumorigenicity

A widely accepted model for the formation of recombinant retroviruses consists of the following steps (15)

a) Accidental integration of a provirus upstream of a cellular gene in the same transcriptional orientation, thus establishing within a tissue a somatic clonal line with a new genotype,
b) Initiation of the transcription of a viral/cellular hybrid transcript that after processing can be packaged in a virion by the presence of virus specific packaging signals

Because retroviruses are diploid, a heterozygotic genome consisting of a heterodimer of a hybrid transcript and a wild-type RNA can be formed,

c) Template switching of the reverse transcriptase during reinfection and perhaps some other recombination-events yielding a recombinant virus genome consisting of a cellular sequence in between viral sequences

The chance to occur for the rare events described in b) and c) may be increased by enhanced proliferation of cells of the new genotype by promoter- or enhancer insertion (see below) On the other hand, recombination between the provirus and trivial genes would remain unnoticed

2 Insertional mutagenesis

The second group, the slowly transforming viruses, lack these transduced cellular genes The mechanisms underlying their oncogenicity is based on the features of the inverted repeats at both ends of the proviral genome This DNA structure plays not only an important role during the integration of the provirus in the host-genome, but it also contains regulatory sequences for transcription a strong promoter, an enhancer and a termination signal (16) The integration of such a structure in the surrounding of a cellular gene may strongly influence the expression of the gene

In retrovirus-induced lymphomas and leukemias in mouse and chicken, proto-oncogenes that are homologous to viral oncogenes of acutely transforming retroviruses, are frequently activated by proviral integrations (17, 18) By its mechanism of proviral integration at numerous, if not random sites in the genomic DNA (19) the retrovirus is also a powerful tool to find new cellular DNA sequences with a potential oncogenicity, by applying the 'transposon tagging' technique The virus-induced tumors tend to be clonal for the proviral integration sites The infected but normal tissue will never be clonal for these integrations Using the proviral DNA as a probe, the proviral integration sites in the cellular DNA of a tumor can be analyzed by cloning the viral-host DNA junctions When the cellular DNA of multiple independent tumors shares a common proviral integration domain it may be assumed that these specific integrations are involved in the tumorigenesis of this particular type of tumors The final step is to identify the genomically linked gene (20)

The pattern of proviral integration-sites within the common genomic domain is characteristic for the hidden gene and reflects the underlying mechanisms by which the integrated provirus influences the expression of the gene In general, the promoter insertion model (21) allows the virus genome to integrate in a 5' (left) 3' (right) orientation upstream of and in rather close proximity of coding sequences of the gene Normally transcription of the viral RNA initiates within the 5' LTR and terminates in the 3' LTR At low frequency a hybrid mRNA is generated by transcription initiated in the 5' LTR promoter, which proceeds past the 3 poly(A) addition signal into the adjacent cellular gene Abnormal splicing events from a donor sequence within the provirus (e g within the *gag* domain) to an acceptor sequence in the downstream cellular gene result in a closer juxtaposition of viral and cellular gene sequences in the chimeric mRNA Sometimes (e g ALV induced B-cell lymphomas) insertional activation results from transcription initiating within the 3' LTR and continuing in the adjacent cellular gene Efficient utilization of the 3' LTR promoter may require deletion of critical regions within the provirus that abrogates the experimentally established suppression of initiation of

transcription at the 3' LTR (17, 22) Proviral integration within the transcribed region of a gene results in a 5' truncated mRNA When only non-coding sequences are missing it may influence RNA stability and translation efficiency as is proposed for the *myc* gene (23) When the proviral integration maps more downstream and 5 coding sequences are missing it can even give rise to truncated proteins, as is described for the *myb*- (24) and *erb*-genes (25) The 5 truncated c-onc and the viral *gag* sequences remaining in these hybrid transcripts are about the same as those that are found in rapidly transforming recombinant retroviruses It shows that not only an alteration in expression pattern but also a specific truncation can play an important role in the conversion of a normal cellular protein in a product involved in tumorigenesis It may cause an alteration of the substrate specificity and cellular location, directed by the newly acquired leader protein, viral gag

The relevance of viral integrations downstream of a gene in both transcriptional orientations or upstream in the opposite transcriptional orientation compared to the gene and dispersed over rather large regions can be explained by the transcriptional enhancer function of the LTR that acts orientation independent in cis, over a rather long distance (26, 27) Enhancer insertion within the 3' end of a gene results in truncation of the gene transcripts by use of the polyA addition signal of the LTR Truncation of the carboxy terminal sequences of the *myb*-protein by proviral integration in a 3' coding exon probably contributes to the transformation in addition to the effect of transcriptional enhancement (24) All carefully mapped proviral integrations in the 3' noncoding region of the *pim*-1 gene in MLV induced T cell lymphomas (28) are found upstream of recently described mRNA destabilising ATTTA motifs (29) strongly suggesting that changes in mRNA stability may also play an important role in transformation (30)

B Human cancer

The success of the animal retrovirus system as a model system for transformation induced an intensive search for human retroviruses But until now only one human retrovirus has been found to be associated with cancer HTLV (human T-cell leukemia virus), that can be isolated from patients with an endemic form of T-cell leukemia (31) The viral genome does not show homology with any of the known (proto-)oncogenes

An explanation for this phenomenon could be that many animal systems are manipulated Laboratory animals were exposed to extremely high amounts of viruses and inbred strains were made with a high amount of integrated proviruses in the germline (endogenous viruses) and specific susceptibilities to virus infections (32) Recombinant viruses were propagated many life cycles under special laboratory circumstances And also poultry breeding, which forms a rich source of recombinant tumor viruses, is quite artificial Large populations of inbred animals live together in very close contact with each other In nature, recombinant retroviruses should hardly be able to survive

Nevertheless, the relationship between the animal system with its high-tumorigenic retroviruses and human cancer in which viruses seem to play a modest role could be demonstrated Experiments of gene transfer in tissue-culture (33, 34), and studies of highly regular chromosomal anomalies, characteristic for some malignancies (35, 36)

showed that the same cellular genes can be involved in both virus induced animal cancer and non-viral human cancer

1 Gene transfer

When normal tissues are cultured *in vitro* almost all cells will divide a few times and die subsequently Sometimes a few cells will overcome what is called a crisis in which they undergo changes that are not fully understood and start growing As a result different stabilized cell lines can be obtained Besides a variable amount of properties still remaining from the original primary cell new characteristics appear of which immortality is one of the most striking From many tissues, the only way to generate a stable cell line is to transform the primary cells by infection with a DNA tumor virus The changes these viruses induce are partially comparable with those found in cell lines obtained from primary cells that have survived a crisis Compared to their non-transformed counterparts the transformed cells show, depending on their state of tumorigenicity, the following growth characteristics that can be used as markers for tumorigenicity immortalisation, substrate free growth e g in suspension, loss of contact inhibition and lowering of their requirement of exogenous growth factors (37, 38)

From rat and mouse, fibroblasts cell lines were obtained that are immortalized but have maintained more or less their normal morphology and their normal growth characteristics When these so called NIH-3T3 - (39), Rat-1- (40), and Rat-2- (41) cells are infected with the highly transforming recombinant retroviruses their morphology will usually change while the growth characteristics will become more tumorigenic dependent on the virus used The real power of this transformation assay became clear when it was shown that transfection of the cells with total genomic DNA (42) from spontaneous human tumors can mimic transformation by retroviruses (43-45) The oncogenic circle was closed by the discovery that the gene that is responsible for the transforming capacity of genomic DNA of a human bladder carcinoma cell line was homologous to the already known viral H-*ras* oncogene (33, 34) The transforming capacity of this bladder carcinoma H-*ras* gene is based on a point mutation within the p21 *ras* protein (46, 47) together with a mutation in an intron resulting in a 10 fold increase in expression (48) These observations strongly support the model that transformation of a normal cell into a tumor cell is caused by dominant mutations in specific genes

Sometimes there is no accordance between the mutations in the original tumor DNA, as scored by southern blot analysis, and those identified after the transfection of the same DNA (49) On the one hand it cannot be excluded that the detected mutations are induced during the experimental manipulations On the other hand primary tumors generally consist of heterogeneous populations of cells When only a small minority of cells contains a particular mutation this will not be detected by a current physical method but can easily be scored in a sensitive bio-assay like the DNA transfection assay The recently developed PCR (polymarase chain reaction) method seems to be also very useful for this purpose (50)

In the DNA transfection assay new genes were discovered to be associated with different specific forms of human and animal cancer They include new genes like *neu* (44), *met* (51), *trk* (52), *dbl* (53) and *ret* (54) besides oncogenes already known from viral gene transduction But the general assumption that the transforming capability of

an oncogene is tissue specific and associated with only one step in transformation limits the system All rodent fibroblast cell lines used are the representatives of one particular tissue and are already partially transformed

The restrictions mentioned could be partially abandoned by the use of primary rat embryo fibroblasts (REF's) (55) These cells can only be transformed by combinations of activated oncogenes, like *ras* and *myc* (56) From these experiments and from the observation that activated c-*myc* is not able to transform NIH-3T3 cells it seems clear that *myc* is involved in the early step of immortalisation and *ras* in the later step of morphological transformation of the cell The inability of the co-transfected cells to seed tumors with unlimited growth in nude mice suggests that the cooperative action of *myc* and *ras* is not sufficient for a fully transformed phenotype (57) Based on the results with different combinations of oncogenes some of these genes can be classified in a group of *myc*- and others in a group of *ras*- like genes This functional assay showed also a surprising relation between some oncogenes and the transforming genes of DNA tumorviruses *Adenovirus E1a* and *Polyoma large-T* can replace *myc* by helping *ras* to transform the REF cells (55, 58) It should be noted that all three interchangeable proteins are associated with the cell nucleus (59, 60, 61)

The so-called tumorigenicity assay (62, 63) is a useful alternative for the detection of transforming genes NIH-3T3 cells are co-transfected with cellular DNA and a plasmid containing a selectable marker and subsequently cultured under selective pressure The arising resistent cell-colonies are pooled and injected into nude mice The DNA of the tumors that appear is analyzed for the transfected sequences The involvement of the exogenous sequences in tumor development is tested by a second round in the tumorigenicity assay The *mcf-2* (63) and *mas* gene (64) were identified by this assay Their ability to induce tumors in the assay is the consequence of amplification and rearrangement which occur during co-transfection (65)

2 Chromosomal aberrations

In cancer cells chromosome aberrations of all kinds can be detected and mapped with high resolution using different methods of chromosome banding or staining (66) In addition techniques are developed to map genes on chromosomes by the use of somatic cell hybrids (67) or even within a chromosomal band by *in situ* hybridization (68)

Many solid tumors show an irregular and complex pattern of chromosome translocations, breakages, deletions and duplications that becomes even more complex during tumor progression On the contrary in many lymphoid malignancies chromosome anomalies are less complex and so characteristic that they are used as cytogenetic markers for a given class of malignancy (8, 69)

a Translocations

About 75% of the cases of Burkitt's lymphoma, a B-cell malignancy, show a reciprocal translocation between the chromosomes 8 and 14 t (8,14) (q24,q32) (70, 71) Distal parts of the q arms of each chromosome are exchanged resulting in a cell with a 8q-and a 14q+ chromosome besides two normal homologs In the remaining 25% two other translocations of about the same fragment of chromosome 8 are seen Here either chromosome 2 or chromosome 22 is the recipient of the chromosome 8 fragment

t(2,8) (p11,q24) is about twice as frequent as (8,22) (q24,q11) (72) Because the human immunoglobulin genes are mapped on the chromosomes 2, band p11 (Ig Kappa) (73), 14, band q32 (Ig heavy chain) (74), and 22, band q11 (Ig lambda) (75) it can be concluded that these translocations are non-random Moreover the c-*myc* proto-oncogene is located in the site of chromosome 8, band q24 (35), involved in each of these translocations

By the use of fragments of the immunoglobulin genes as probes several translocation breakpoints characteristic for different lymphoid malignancies could be cloned and studied in detail From these studies some conclusions can be made with respect to the genes that are involved, the mechanisms of translocation and the deregulation of oncogenes juxtaposed with the immunoglobulin genes on the rearranged chromosome

Not only proto-oncogenes homologous to v-onc genes like c-*myc* and v-*abl* are involved in translocations The *bcl-1* (76) and *bcl-2* (77) genes were discovered by their involvement in translocations in respectivily chronic lymphocytic leukemia (CLL) and follicular lymphoma

Antibody diversity is created by series of recombinations between the variable (V), diversity (D), joining (J) and constant (C) regions of the immunoglobulin gene during the course of B lymphocyte differentiation The hot spots of recombination within these genes coincide with the chromosomal breakpoints Mistakes of V-D-J recombinases early in B cell development and isotype switching enzymes at a later stage probably cause the characteristic translocations This is strongly supported by the observation that in T-cells in which the same recombinase enzymes are active sequenses 3' of c-*myc* can be joined to a J segment of the TCRα-locus resulting in the T-cell acute lymphoblastic leukaemia (ALL) specific translocation t (8,14) (q24,q11) (78) The juxtapositions of c-*myc*, *bcl*-1 and *bcl* 2 to an immunoglobulin gene are the clearest examples of tissue-specific oncogene activation by a purely cellular mechanism

In Burkitt's lymphoma the breakpoints are dispersed over rather big areas within the immunoglobulin genes and the *myc* gene The feature all translocations have in common is that the *myc* gene is always located 5' of an immunoglobulin constant gene The expression of the *myc* gene is driven by genetic elements, like Ig enhancers and other elements not yet identified, within the Ig loci fragments which act in cis over a long distance in a lymphoid specific and differentiation dependent way (79, 80) Loss of trans-acting transcriptional control (81, 82) and translational control (83) or an increase of mRNA stability (84) might contribute to the deregulation of the oncogene In at least one case of Burkitt's lymphoma, mutations are found in a recently discovered orientation-dependent negative regulatory element near the end of the first exon of the *myc* gene that apparently mediates premature termination of transcription (85, 86)

In human chronic myeloid leukemia (CML) a reciprocal exchange between chromosome 9, which bears the proto-oncogene c *abl*, and chromosome 22 yields an extended chromosome 9, 9q+, and a shortened chromosome 22, the Philadelphia chromosome (Ph1) (87) All chromosomal breakpoints on chromosome 22 associated with the (9,22) translocation fell within a 5 8 kb segment, that for this reason is called *bcr* (breakpoint cluster region) (88) The breakpoints in chromosome 9 however are scattered over a region of at least 50 kb within the 5' region of the *abl* gene The *bcr* spans two small exons of an unknown gene oriented in the same direction as the *abl* gene (89) As a result of the translocation a hybrid mRNA is made starting on the *bcr* gene and spliced onto a downstream coding exon of the *abl* gene In the fusion protein

a small N-terminal part of the *abl* protein is substituted by a much larger N terminal part of the *bcr* gene (90) It is intriguing that in v-*abl* the N-terminus of c-*abl* is replaced by a part of the viral *gag* protein It seems likely that the N terminal substitutions alter the substrate specificity of the gene product Perhaps the differences in alteration also explain why the v-*abl* gene preferentially transforms early B lymphoid cells whereas the *bcr-abl* gene is associated with transformed myeloid cells (91)

In Ph+ ALL, chromosomal breaks occur in the putative first exon of the large *bcr*-gene located far upstream of the two exons involved in CML (92)

b Amplification

Many tumors, particularly solid tumors, show gene amplification The prototype model system for the study of gene amplification in mammalian cells is the dihydrofolate reductase (DHFR) gene amplification that occurs *in vitro* when cells are grown under selective pressure of methotrexate (93) These gene amplifications can be detected as two different karyotypic abnormalities homogeneously staining regions and double-minute chromosomes Double-minute chromosomes are highly amplified small pieces of a chromosome that can be present in large numbers in each cell (94, 95)

Tumors sometimes show amplifications of proto-oncogenes, mostly as an occasional feature However certain tumor types show a distinct pattern of amplifications of specific oncogenes (2) In the more progressive stages III and IV of neuroblastoma tumors, which frequently carry homogeneously staining regions and double-minute chromosomes, an amplification of a member of the *myc* family, N-*myc* is seen Because the N-*myc* gene maps on human chromosome 2, while the amplified gene copies are found in homogeneously staining regions of other chromosomes or in the double-minute chromosomes, this type of amplification seems to be associated with translocation There appears to be a significant correlation between genomic amplification of N-*myc*, anatomical progression, and disease aggressiveness of primary neuroblastomas (96, 97) In small cell carcinoma of the lung the correlation between the amplification of the different members of the *myc* family, c , N , and L *myc,* and the expression of a more malignant phenotype seems to be less clear (98 99) The c-*myc* and *neu* gene are frequently amplified in primary breast cancer (100, 101) The prognostic significance of the *neu* oncogene amplification is still controversial (101 102) But in all cases the level of mRNA expression is concordant with the amplification

In vitro DHFR gene amplification by selective pressure of methotrexate occurs much more frequently in tumorigenic than in non-tumorigenic cells suggesting that tumor cells with their inherent genomic instability are predisposed to the development of gene amplification (103) This observation might explain why multi-drug resistance also based on gene amplification is a serious problem in chemotherapy (104)

The DHFR gene amplification in methotrexate resistant mouse cells is rapidly lost when selective pressure is removed from the culture medium But the oncogene amplification in cultured human cells is quite stable strongly suggesting that such amplification is providing the tumor cells with a selective growth advantage These data suggest that oncogene amplification may play a role in tumor progression

In a murine T cell leukemia, in many hemopoietic disorders in man and in Rous sarcoma virus induced tumors of the rat the commonest trisomy is trisomy of the *myc* carrying chromosome (105-108) This suggests that mitotic non-disjunction is another amplification mechanism of the *myc* gene Duplication of the rearranged *myc* allele or

even loss of the germline fragment was also observed in some retrovirus induced T cell lymphomas in the mouse (109, 110) So the germline rearranged ratio seems to be essential in these tumors This is not the case for mouse plasmacytomas and human Burkitt lymphomas where the normal *myc* allele is switched off (111) suggesting that the LTR-*myc* complex may be subject to some trans-acting regulation by the normal allele which can be overridden by the gene amplification (112)

c Deletions

Progression of chemically induced mouse skin papillomas to the carcinoma stage can be accompanied by amplification of the mutated *ras* or loss of the normal allele or both (113) Loss of the normal *ras* allele is also found in a variety of human tumors that carry mutated *ras* (114, 115) The normal allele may antagonize the tumorigenic effect of the mutated allele (112) Loss of the normal c-*myc* allele in some mouse T cell lymphomas is already discussed in section II B 2 b

Chromosome deletions and their involvement in tumorigenicity lead us to a whole new chapter of cancer research All tumor induction models described so far are based on dominant genetic alterations But evidence has been obtained for the presence of recessive genetic lesions in about 18 different human cancers with significant frequencies As yet no mutations in the conventional proto-oncogenes are found to be involved in the inheritance of cancer On the contrary, recessive mutations in about 10 different loci are associated with an inherited predisposition for different tumor types (summarized in ref 116) For instance children who inherit a deletion in the *Rb-1* allele of chromosome 13 from one of their parents will develop a retinoblastoma if the second allele is lost by a somatic mutation (117) More recently loss of the *Rb-1* gene is found to be associated with malignancies in several other tissues including osteosarcomas, soft tissue sarcomas, and small cell lung and breast carcinomas (118-121)

In *Drosophila* about 20 recessive mutations exhibit a neoplastic phenotype at late larval stages In these cases the affected tissue fails to differentiate resulting in a lethal hyperproliferation (122-124) It is unclear whether the normal function of the genes involved is to suppress cell proliferation or to induce differentiation (125)

The recessive oncogenes might be a special category of tumor suppressor genes that can antagonize the regular oncogenes (112) This category of genes has also been detected in different *in vitro* model systems Many stable somatic cell hybrids of transformed and normal cells (or even in one case of two different transformed cells) are low or non-tumorigenic (126-132) Reappearance of tumorigenicity is accompanied by the loss of certain chromosomes derived from the normal parent Moreover morphological and non-tumorigenic revertants, positively and negatively selected with different agents, have been isolated from different types of tumor cells (130, 133-138) The reversion is not necessarily caused by the loss or down-regulation of the original transforming gene Growth of some transformed cells can be suppressed by surrounding normal cells (139, 140) probably through excreted small growth regulatory polypeptides (141, 142) Finally, tumor cells blocked in their differentiation can be induced to mature by strong artificial but also natural inducers Their responsiveness may decrease during tumor progression (112)

III. (Proto-)oncogene activation and regulation of eukaryotic gene expression

The search for cancer related genetic events as described above has resulted so-far in the discovery of at least 50 genes which are somehow associated with the induction, maintenance and progression of many different types of tumors But the frequent involvement of the same proto-oncogene in the development of a wide variety of tumors suggests that the number of genes most frequently involved may be quite limited From most genes the transduced or mutated form (=oncogene) and the normal cellular counterparts (=proto-oncogene) from different species have been molecularly cloned and their structure analyzed in detail Moreover their chromosomal locations have been determined (for review see ref 13, 143, 144)

Research on the activation mechanisms of oncogenes contributes strongly to an understanding of the complex regulation of eucaryotic gene expression By structural analysis of oncogenes and functional studies on structural elements of these genes many different mechanisms of regulation of gene expression were discovered I will illustrate this taking the *myc* and *fos* genes as examples

The *myc* gene can be activated in various classes of tumors by a number of different mechanisms, including viral transduction, proviral integration (promoter and enhancer insertion), translocation and amplification (fig 1) Mobilization of the transforming potential of the c-*myc* proto-oncogene seems to be associated only with changes in the mode of its expression In normal cells the expression of the gene is subject to both transcriptional as well as posttranscriptional regulation (145)

Walking from the 5' end to the 3' end of the gene the following regulatory regions can be found (see fig 1)

1) P_0, promotor region found in humans but not in mice Two minor transcripts of 2 5 and 3 2 kbp starting in this not well characterized CG-rich promotor region show some 5' heterogeneity (146) and are generated by differential splicing The function of the one, respectivily, the two additional open reading frames present in these transcripts is unknown (146, 147),
2) Negative regulatory elements (148),
3) P_1, a promotor just at the beginning of the first (non-coding) exon, 0 5 kbp downstream of P_0,
4) P_2, a second promotor within the non-coding exon, 150 nucleotides downstream of P_1 (149, 150) Transcription normally starts at both of the two well characterized 'TATAA-box' promoters with a 4 1 preference for transcripts initiated from P_2 (151),
5) A modulator region with positive effects on transcription (152),
6) A structural element, just at the end of exon-1 involved in a partial block of elongation It is not known whether this is only a pause in elongation or a premature termination The resulting discontinuous transcription appears to be regulated by a secondary structure of the RNA which prevents elongation and also by short-lived proteins, since inhibitors of protein synthesis can partially release this block (153, 154),
7) A region within the first intron with many possible 'cryptic' promoters activated only after loss of P_1 and P_2 (155, 156, 157),

8) A region at the end of the non-coding 3' part of exon-3 with mRNA destabilizing motifs (29) The *myc* mRNA turnover in normal cells is extremely high (15-20 minutes) (158, 159) Inhibition of protein synthesis in most cell types results in a dramatic stabilization of c-*myc* mRNA and increase of protein level (159, 160) suggesting that the controling element is itself a protein involved in RNA turnover (145) The role of non-coding part of exon-1 in RNA stability is quite unclear When the 5' exon-1 was attached to an otherwise stable transcript the instability was not increased but when the 3' non-coding end was appended instability was increased (161) The 10-fold higher translation efficiency of transcripts starting at P_2 compared to P_1 transcripts (162) might be explained by a difference in stability (145),

9) Several DNAse-hypersensitive sites have been identified in c-*myc* chromatin of mouse (163) and man (164) which are thought to indicate places where an open chromatin structure would make the DNA accessible to controlling protein molecules The sites close to P_1 and P_2 are thought to be promoter sites where RNA polymerase binds and initiates mRNA synthesis The role of the sites upstream and downstream are controversial They should bind positive and negative regulatory proteins (165) By employing the gel retardation technique at least 7 different nuclear factors were identified that bind to DNA sequences within 1 1 kbp 5' of P_1 Two of them bind to negative transcription elements with the opposite properties of a transcriptional enhancer One element is very similar to a DNA sequence with dyad symmetry located within the polyomavirus enhancer At least two transcription factors interact with the c-*myc* first exon One of these may also bind to the SV40 enhancer, whereas the other may be a more specific factor (166)

Transcription of the complementary strand, so-called anti-sense transcription has been detected, in man only upstream of P_1, but in mouse at several regions (153, 167) The role of antisense transcription of c-*myc* is so far totally unknown

But even such a complex system of many different close linked elements does not guarantee the living cell the right control of gene expression in all circumstances as might be deduced from the fact that at least three loci, *Mlvi-1, Mlvi-4,* and *pvt-1/mis-1* are identified which might act in cis on the c-*myc* gene expression (20, 168-172)

There is also a fast growing amount of data on *myc* gene regulation on the transcriptional level The three exons that comprise the c-*myc* gene in many different species contain only one ORF (open reading frame) beginning with an ATG This highly conserved ORF begins near the 5' end of exon-2 and extends through approximately two thirds of exon-3 Recently the use of a non-AUG initiation codon (CTG) near the 3' end of exon-1 is observed resulting in the generation of a c *myc* isoform in which the amino acids coded by about 15 codons upstream of an ORF within exon 1 are added (173) This can also partially explain the conserved complex protein pattern after SDS-polyacrylamide electrophoresis of immunuprecipitated c-*myc* proteins from cells of different species In MEL (Friend murine erythroleukemia) cells a temporal shift in the relative abundance of the two isoforms of the c-*myc* protein during the induction of differentiation was observed The decrease in the lower molecular weight species during differentiation may represent a shift to the preferential utilization of the CTG start site within exon-1 (174)

A controversial aspect of the *myc* translation is the role of the about 70 nucleotides long complementary regions found in opposite orientations in exon-1 and exon-2 of the human c-*myc* sequence (83) It was proposed that normal c *myc* mRNA might be poorly translatable by forming a stem-loop structure that would render the initiator codon in exon-2 inaccessible Transcripts of recombined *myc* genes without exon-1 sequences are not able to form a hairpin and therefore more efficiently translated However different studies came to opposite conclusions (162, 175, 176)

The most common site for breakpoints, proviral integrations and recombinations in viral gene-transduction is a quite broad area including the first non coding exon and the 5' half of intron-1 (165) In most if not all cases of Burkitt's lymphoma mutations are found within exon-1 (111) All together this strongly suggests that normal c-*myc* transcription is mainly regulated in or around exon-1 Recently it was shown that the removal or specific mutation of exon-1 found in Burkitt's lymphoma correlates also with suppression of synthesis of the less abundant larger isoform of the *myc* protein Thus alterations on the protein level may contribute to the oncogenic activation of c-*myc* (173) In Burkitt's lymphoma expression of the recombined locus results mostly in complete down-regulation of the normal allele (146, 163) There is no agreement about the mechanisms that cause this allelic exclusion (111)

Despite the substantial amount of data on *myc* gene expression it might be concluded that, "c-*myc* gene regulation still holds its secret" (cited from 177)

Regulation of the expression of the *fos* gene is also extensively studied Expression of c-*fos* is one component of a larger program of gene activation induced by a variety of agents ranging from mitogens, hormones, ionophores, differentiation-specific agents, stress, drugs, etc In all cases induction is very rapid and transient (145, 178, 179)

Studies of the DNA sequences required for the responses to membrane-transduced signals (180-183) indicate that more then one growth-factor responsive regulatory element contributes directly to the regulation of the *fos* gene Its SRE (serum responsive element) a region some 300 bp 5 to the cap site, contains a DSE (dyad symmetry element) DSE is a palindromic sequence that is crucial for the response to serum and that binds SRF (serum regulatory factor) SRF is a protein always present in HeLa cells and inducible by EGF (epidermal growth factor) in some other cells (184, 185) A DSE is also located in the 5' flanking region of mammalian and Xenopus actin genes that are also responsive to serum (186, 187) A binding site 346 bp upstream of the cap site binds SIF (serum inducible factor) which is a factor present in NIH-3T3 cells transformed by the *sis* oncogene (188) In extracts from the mouse B lymphoma cell line WEH1231, three protein complexes are identified binding to the c *fos* promotor/enhancer region One is probably SRF while the others appear to provide basal levels of c-*fos* transcriptional activity by binding to two regions some 100 and 150 bp upstream of the cap site and to a region 60 nucleotides upstream of the cap site (not conserved in humans) harboring a potential binding site for transcription factor Sp1 (178, 181, 183) Negatively acting factors binding to the 5' region between 100 and 711 nucleotides upstream of the cap site seem to be also involved in the regulation of the c-*fos* gene expression (189, 190) Although some observations are suggestive of an autoregulatory function of the c-*fos* protein this is not required for the induction of c-*fos* by growth factors, which is even increased in the presence of protein synthesis inhibitors (145)

The mRNA destabilizing UAAAU motif mentioned earlier (29) is also present in the *fos* messenger Activation of the transforming potential of c-*fos* requires not only linkage of a transcriptional enhancer but also elimination of the multiple ATTTA motifs within a 67 bp element of the non-coding region 123-189 bp upstream from the polyA addition site (145)

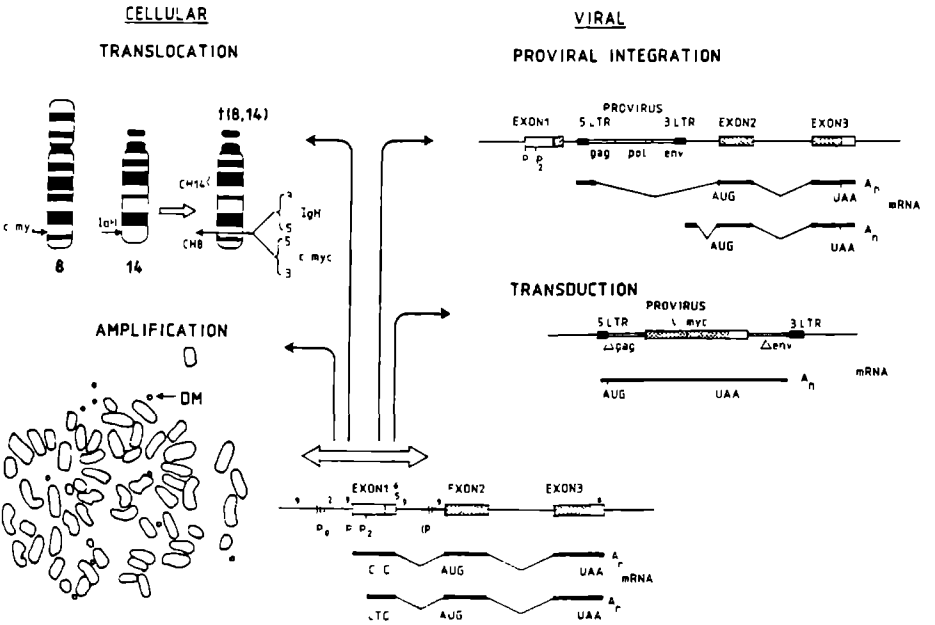

Fig. 1. The activation of the c-*myc* gene For explanation, see text
Counterclockwise from top left

- t(8,14) translocation present in about 75% of the Burkitt's lymphomas The c-*myc* locus is fused to the immunoglobulin heavy chain locus (IgH)
- Metaphase spread of small cell lung carcinoma cells that harbor DM s (Double Minutes) with amplified c-*myc*
- Structure of the c-*myc* gene Exons$_{1\,3}$ are indicated by boxes and introns by horizontal lines 1 Region involved in most recombination events, 2 dehancer sequence, 5 modulator region with positive effects on transcription, 6 block or pausing in elongation of RNA transcription, 8 mRNA destabilizing motifs, 9 nuclease hypersensitive sites
- Structure of the acutely transforming avian virus MC29 that harbors the exons 2 and 3 of the c-*myc* gene
- Integration of an ALV proviral genome within the first intron of the c-*myc* gene Mostly transcription is initiated within the 3' LTR 5' LTR initiated transcription resulting in the synthesis of a fusion protein is rare in this system (17)

IV. The (proto-)oncogene products: The normal cellular function of proto-oncogenes

Starting with the molecular clones and data of the primary structure of the oncogenes there are many ways to disclose the real identity of the oncogene products Computer comparison of the predicted amino acid sequences of oncogene products with accumulated amino acid sequence data revealed a striking similarity of some oncogene products with known proteins Proto-oncogenes have been found to be evolutionarily conserved in not only the genome of vertebrates but also in lower eukaryotes The genetic tools available in *Drosophila melanogaster* and *Saccharomyces cerevisiae* provide a powerful approach to study proto-oncogene function

A Growth factors

The simian sarcoma virus v-*sis* oncogene is a transduced form of the PDGF-2 (platelet-derived growth factor-2) gene (191)

At least three oncogenes encode proteins that are structurally similar to bFGF (basic fibroblast growth factor) One is found to be activated in the human cancer Kaposi s sarcoma (192) and in a form of human stomach cancer, hence the name *hst* (193) Another one is the mouse mammary tumor gene *int 2* (194) The third one was isolated from human bladder cancer cells (195) bFGF is the prototype for a family of heparin-binding growth factors that not only stimulate the division of certain cell types but are also potent angiogenic agents that foster blood vessel formation essential for solid tumor growth to a significant size (195) bFGF may act also as an autocrine growth factor for human melanomas (196)

In *Drosophila* at least 12 genes have been identified homologous to well known proto oncogenes (125) But only the homolog of the extremely well conserved mouse mammary oncogene *Int* 1 maps to an already known *Drosophila* locus with well defined mutations the segment polarity gene *Wingless* involved in pattern formation in segments during embryogenesis (197)

B Growth-factor receptors

The *mas* gene product (64) shares a conserved structural motif of seven hydrophobic membrane-spanning α-helices with a class of receptors coupled to GTP-binding proteins (198) like mammalian opsins (199), adrenergic receptors (200), the muscarinic receptor family (201, 202) and the substance K receptor (203) The *mas* gene shows the greatest homology to the substance-K receptor (a tachikinin neuropeptide receptor) By the use of transiently expressed *mas* genes in *Xenopus* oocytes and a stably expressed *mas* gene in transfected mammalian cell lines it could be demonstrated that the gene

product is a functional angiotensin receptor with mitogenic activity (204) This class of receptors operates by activating a membrane bound transducing protein termed a GTP binding or G protein (205)

The v-*erb*B protein is a truncated version of the EGF (epidermal growth factor) receptor lacking the ligand (EGF) binding domain (206, 207)

The similarity in tissue distribution and biochemical properties of the c-*fms* proto-oncogene product and the CSF-1 (Mononuclear phagocyte colony stimulating factor) receptor together with the fact that antibodies prepared against a recombinant v-*fms* encoded polypeptide specifically react with the CSF-1 receptor indicate that the product of the c-*fms* proto-oncogene and the CSF-1 receptor are possibly identical molecules (208, 209)

Both the EGF- and the CSF-1-receptor are protein tyrosine kinases which general and specific properties are described in section IV E

C. Nuclear proteins

The v-*erb*A gene product is a mutated ligand-independent form of a high-affinity nuclear receptor for thyroid hormone as could be demonstrated by structural and functional studies (210-213) In humans, the gene most closely homologous to v-*erb*A, TRα, is located on chromosome 17 whereas a slightly more distantly related gene, TRβ, resides on chromosome 3 (213-216) Even more sequences that cross-hybridize have been found on both chromosomes 3 and 17 The different members of the gene family seem to be expressed in a tissue specific manner (145)

In a wide variety of cell types the gene c-*fos* is usually rapidly and transiently induced by a variety of external signals The induced *fos* protein is subject to extensive post-translational modifications, mainly phosphorylations of serine residues Because phosphorylation is less extended in transforming *fos* products than in their normal cellular counterparts and has an inhibitory effect on the binding of the *fos* proteins to DNA cellulose it might be assumed that this modulates some biological properties of the *fos* protein by interfering with the direct or indirect *fos*-DNA interaction (217-219)

The *fos* protein forms an extremely stable complex with the *jun* protein (p39) that is associated with chromatin (220) The *jun* oncogene product is related to the yeast transcription factor GCN4, while the central best conserved part of the *fos* protein also shows limited homology with GCN4 (221) The consensus DNA-binding site of the GCN4 protein is closely related to that of the mammalian transcription factor AP-1 (222-224) AP-1 preparations contain several proteins (225) In fact the name AP-1 stands for a whole system of regulation of transcription whose complexity is only partially understood The transcription factors seem to interact as dimers with half-sites of palindromic recognition sequences (226) As a general model to explain the dimer formation the 'leucine-zipper' is proposed (227), a periodic repetition of leucine residues, present at every seventh position over a distance of eight helical turns of an α-helix, aligns along one face The leucine containing region of GCN4 is required for dimerisation (228) The putative zipper region, plus an adjacent highly charged domain containing stretches of acidic and basic amino acids, is similar in GCN4, *jun*, *fos* and

myc Replacement of the individual leucines within the leucine repeat structure of the *fos* protein destroys the ability to form a complex with *jun*, strongly suggesting that the repeat of leucine residues also stabilises the interaction between the *fos* and *jun* protein Nuclear protein complexes containing both *fos* and *jun* bind to the TRE (TPA responsive element) with the consensus DNA sequense TGACTCA The *fos-jun* complex can bind to DNA more tightly than either protein alone The TRE specific stimulation of transcription induced by the *jun* protein is enhanced by the presence of the *fos* protein (229, 230) The highly conserved basic motif adjacent to the leucine repeat seems to be a potential DNA binding site Mutations in this motif abolish DNA binding potential without affecting complex formation (230) *fos* and *jun* together with their closely related *jun*-B and *Fras* (*fos* related antigens) are elements of the 'AP-1 system' in which protein complexes with multiple and variable subunits bind to variants of the AP-1 binding site, thereby modulating gene transcription (226) In yeast it was shown that *fos* and *jun* can function as transcriptional activators (231)

The *myc, myb* and *ski* gene products show also nucleic acid binding properties Despite considerable knowledge of the basic molecular biology of these genes their normal cellular function is unknown They all seem to play a role in the control of the normal cell-cycle and might act as nuclear switches in signal-transduction systems by which short-term events that occur on receptor occupation are coupled to long-term alterations in gene expression

myb is also a member of the class of sequence specific DNA binding factors presumably involved in gene regulation The DNA binding domain of v-*myb*, localized in the highly conserved amino-terminal region recognizes the DNA sequence pyAAC$^{G}/_{T}$G (232) The v-*myb* protein contains also a potential α-helical 'leucine zipper' sequence (227) but the amino-terminal DNA binding domain shows no significant homology with consensus sequences of either helix-turn-helix' or 'zinc-finger-type' (233) DNA binding proteins like SP-1

The c-*myc* RNA and protein levels in human and avian cells have been shown to be invariant throughout the cell cycle, except for a transient increase observed upon mitogenic stimulation (160, 234-237) Not only the *myc* mRNA but also the protein shows a very brief half-life (20-30 minutes) (238, 239) The so called PEST region, rich in proline, glutamic acid, serine, and threonine, and flanked by clusters of positively charged amino acids might be responsible for rapid protein degradation because it has been identified in 10 unstable proteins, including c-*myc*, c-*fos* and E1a (240) At a position equivalent to the basic motif of *jun* and *fos* the *myc* protein shows substantial homology with the 'helix-turn-helix' DNA binding region of the cII protein of phage λ suggesting that the *myc* protein possesses a 'helix-turn helix' related stucture (230) The observation that c-*myc* is expressed at low levels in quiescent cells and rapidly increases upon exit from the G_0 state and its decline in terminal differentiation suggest that activation of c-*myc* expression may be related to the competence of cells to enter and progress through the cell cycle rather than with proliferation per se (234, 237, 241, 242, 243)

p53 is a cellular nuclear protein without DNA binding properties that forms stable complexes with the DNA tumor virus proteins *SV40 large-T* antigen and *adenovirus E1b*

proteins (244-247) Formation of these complexes stabilizes p53, increasing its half-life and its steady-state levels in the cell (100-fold higher) In some non-virally transformed cell lines also an increased half-life and stability of *p53* was detected These p53 proteins appeared to be mutated All mutations cluster between amino acids 120 and 230 (out of 390 amino acids) Binding studies showed that the mutant proteins bind poorly or not at all to the *SV40 large-T* antigen In these cases the functional analog of the DNA tumor virus antigens appears to be hsp70 (a heat-shock protein) (248-250) In cooperation with activated *ras*, mutant p53 clones that code for a p53 with an increased hsp70 affinity transform efficiently primary cells in vitro (251) A higher expression of the mutant p53 contributes to an incease of the transformation frequencies (252) The normal mouse or human p53 genes are not able to cooperate with an activated *ras* oncogene to transform primary cells However overproduction of the wild-type human p53 protein is sufficient to confer a tumorigenic phenotype on established rodent fibroblasts as was shown by a tumorigenicity assay (253) Moreover the normal mouse p53 is capable of rescuing primary rat cells from senescence (254) Additional experiments are necessary to test the hypothesis that the p53 gene belongs to the recessive oncogenes

The *Rb-1* gene product is also like the *myc, myb* and *fos* proteins a nuclear phosphoprotein associated with DNA binding activity The protein forms stable complexes with several DNA virus products like *adenovirus E1a* proteins (255) and *SV40 large-T* antigen (256) *E1a* proteins and *large-T* antigen are nuclear proteins that stimulate cell growth and activate and repress transcription (257, 258) Both contain a 10 amino acid region in which mutations reduce or abolish both *Rb-1* protein binding and transforming activity (256, 259) Moreover, a mutant *Rb-1* gene was discovered in a bladder carcinoma that encodes a protein that cannot bind *E1a* proteins (260) The *polyomavirus large-T* antigen/*Rb-1* protein complex may also involve other cellular components Immunoprecipitations of the complex contain also a fraction of the cellular nuclear *p53* presumingly because it was bound to *large-T* (256) Both *E1a* proteins and *SV40 large-T* antigen immortalizes primary cells, but *large-T* antigen also transforms cells by itself, while *E1a* proteins require a second oncogene such as *ras* (55, 58) to generate a transformed phenotype The data mentioned may be explained by the following model *Large-T* antigen and *E1a* protein may inactivate the *Rb-1* protein by occupying an essential site on the protein, while also carrying out other activities affecting cell growth (260)

D The *ras* gene family

From studies with yeast mutants it could be deduced that the members of the complex *ras*-family belong structurally and in some functional aspects to the signal-transducing guanine-nucleotide-binding (G) proteins (261, 262) From earlier work however it seems that *ras* actions are not initiated by stimulation of a known second messenger pathway, such as control of adenylate cyclase or inositol phosphate production (263) Recent work has demonstrated that changes in inositol-lipid metabolism may result from indirect consequences of *ras* activation (264) *ras* proteins

do appear to cause rapid changes in phospholipid metabolism, leading to phospholipase A_2 stimulation (265) and increased production of diaglycerol from an unknown source (266, 267) Activation of the transforming potential of H-*ras*, K-*ras* and N-*ras* on the protein level is based on very specific point mutations that cause a dramatically decreased ability to hydrolyse GDP or/and changes in the ability to interchange prebound GDP or GTP resulting in the stabilization of *ras* proteins in their GTP-bound active state (268)

E The protein kinases

By protein purification and enzyme assay for catalytic activity and, especially, by comparison of the deduced protein sequences of the oncogene products among themselves and with other sequences about 18 different oncogenes could be placed within a large gene family of protein kinases with a conserved catalytic domain The majority belongs to the class of tyrosine specific kinases (prototype *src* (269)) while only a few belong to the class of cytoplasmic serine/threonine specific protein kinases like *mos* and *raf*. Within the primary structure of the 250-300 amino-acid residues long catalytic domain, mostly located near the carboxyl terminus, certain short amino acid stretches are characteristic for each class (270)

Novel protein kinases are isolated by the use of two different experimental approaches Screening cDNA libraries by using the conserved catalytic domain of protein kinase genes as hybridization probes under low stringency conditions or, more recently, by using degenerate oligonucleotides as probes (270-276) and complementation or suppression of genetic defects in invertebrate regulatory mutants (277-279) The number of proven or putative protein kinases has risen to nearly a hundred (280) Primary structure alignments of many protein kinases were used to construct a phylogenetic tree of the protein kinase catalytic domains (281) In *Drosophila* over 6 genes show homology with protein kinase genes like *src, abl, erbB, raf*, the insulin receptor gene, and protein kinase C genes Each of these genes is more similar to its vertebrate counterpart than to the other members of the *Drosophila* protein kinase family This indicates that the major gene duplication events which gave rise to the family took place before the divergence of *chordates* and *arthropods*, over 800 million years ago In the meantime it suggests that the *Drosophila* genes are functional homologs of the vertebrate genes On the contrary in yeast these tyrosine specific protein kinases are not found at all suggesting that tyrosine kinases represent a class of enzymes specific for process control in multicellular organisms This is supported by the fact that many of the tyrosine kinases are growth-factor receptors (125, 280)

The detailed alignments of many catalytic domains showed that eleven major conserved subdomains can be distinguished separated by regions of lower conservation wherein fall larger gaps or insertions An arrangement of alternating regions of high and low conservation is a common feature of homologous globular proteins (282) and gives some clues to higher order structure Within the subdomains highly conserved individual amino acids are found which probably play important roles in catalysis Many of these most highly conserved residues directly participate in ATP (adenosine triphosphate) binding and phosphotransfer Others may play a role in recognition of the correct

hydroxyamino acid because they are specifically conserved in either the protein-serine/threonine or the protein-tyrosine kinase (281) Very near the amino terminus of the catalytic domain the consensus Gly X-Gly-X-X-Gly, known from many nucleotide binding proteins, is found (283) Fifteen to thirty positions on the carboxyl-terminal site of the consensus an invariant lysine is located that appears to be directly involved in the phosphotransfer reaction (284) For different kinases it was shown that the ATP analog p-fluorosulfonyl-5 -benzoyl adenosine that reacts with this lysine inhibits enzyme activity (285-287) Amino acid substitutions, including arginine, result in loss of protein kinase activity (284, 288-293) In many protein kinases an autophosphorylation site is found in the second half of the catalytic domain near what is thought to be the catalytic site (294) For several protein tyrosine kinases like *src* phosphorylation of the tyrosine residue at this position leads to increased catalytic activity (295-300) On the other hand in the gene products of *fms*, *kit* and *ret* as well as the receptors EGFR, *neu*, and PDGFR, the site is not present Usually spacing between the different subdomains is conserved but there is no experimental evidence whether this is critical The PDGF receptor, *fms*, *kit* and *ret* stand out from the other protein tyrosine kinases by a much longer spacer region located approximately in the middle of the catalytic domain (269)

Outside the catalytic domain sequences are located probably involved in the cellular localization and substrate specificity of the enzyme The *src,yes,fes,fgr* and *abl* gene products shares several conserved domains with phophatidyl-inositol-specific phospholipase C, which suggests some shared forms of regulation (301) In their carboxy-terminal end downstream of the catalytic domain some protein tyrosine kinases contain one or more tyrosine residues whose phosphorylation regulates kinase activity Depending on the kinase, the effect of phosphorylation can be positive or negative Phosphorylation of Tyr-527 of the c-*src* protein inhibits its kinase activity (302, 303), whereas autophosphorylation of Tyr-1150 and Tyr-1173 of respectively the insulin and EGF receptor enhances their kinase activities (304) Viral transforming gene products generally lack this regulatory domain Loss of the domain may lead to uncontrolled activity In general covalent modification of plasma membrane receptors by phosphorylation plays a key role in the regulation of receptor function Negative modulations are associated with heterologous phosphorylation of serine/threonine residues of the receptor proteins (305)

It is extremely hard to find a specific substrate for a particular protein kinase In the living cell phosphorylation of proteins is a usual manner to modulate protein activity A single protein kinase can phosphorylate different proteins on different sites within the protein Almost every protein is phosphorylated to some extent Some 90% of the protein-linked phosphate is coupled to serine, 10% to threonine and only 0 05% to tyrosine Arginine linked phosphate is found also Many physiological processes are regulated by protein phosphorylation metabolic pathways such as glycogen metabolism, protein synthesis, alterations in cytoskeletal organization and signal transduction (306) An attractive hypothesis to explain the rapid, protein synthesis independent, induction of genes by growth factors is phosphorylation of preexisting transcription factors in the nucleus Although many nuclear proteins are phosphorylated (e g the proto-oncogene products of *fos* and *myc*) in mammals so far little is known about the consequences this has on protein activity, with the noticeable exception of CRE (cyclic AMP responsive element) binding protein (307) SNF1 is a yeast protein kinase that seems to play a role in transcriptional regulation (308)

In yeast, one critical point for controlling cell division is in the early G1 It is controlled by a cascade of at least three protein kinases (309-311) The last protein of this cascade, called cdc2 in fission yeast, occupies a central role in controlling the cell cycle because it is not only required for G1-S but also G2 M transitions A mutation in cdc2 can be complemented by a human gene (278, 279) Moreover recently was shown that a homolog of the yeast kinase cdc2 is a subunit of the cell-cycle regulated and M-phase-specific H1K (histone H1 kinase) described in a wide variety of eukaryotic cell types and MPF (maturation promotion factor), a major control element of the G2-M transition in activated or fertilized *Xenopus* eggs (312) All these 32-36kDa proteins have a perfectly conserved 16 amino acids long peptide in common outside the consensus sequences of the kinase domain The striking homology between cell cycle regulating factors of yeast and higher eukaryotes implies that at least some aspects of cell cycle control are the same in yeast and vertebrates

RNA expression of the evolutionarily less well conserved c-*mos* gene can hardly be detected in adult tissues The highest levels of expression are found in gonadal tissues restricted to male and female germ cells The *mos* protein can be detected during progesterone-induced maturation of *Xenopus laevis* oocytes Microinjection of *mos*-specific antisense oligonucleotides in oocytes inhibits meiotic maturation by blocking germinal vesicle breakdown It has been suggested that pp39 *mos* acts directly or indirectly upstream of MPF to activate pre-MPF (313)

Protein kinases are intrinsic parts of many signal transduction pathways of the cell. Extracellular signal molecules (ligands) activate target cells by binding to cell surface receptors which transduce a signal into the cell by phosphorylation of target proteins (internal effectors) In most cases this is mediated by transducers like G-proteins (e g proteins of the *ras* family) and one or more second messengers such as cyclic AMP, diacylglycerol and Ca^{2+} Cyclic nucleotides generated by hormone dependent membrane associated cyclases activate the cyclic nucleotide regulated protein kinases A and G Diacylglycerol, produced by hormone- or growth factor-induced turnover of phosphoinositides, activates protein kinase C (PKC) In the presence of Ca^{2+}, the ubiquitous Ca^{2+} binding protein calmodulin activates at least five protein kinases phosphorylase kinase, myosin light-chain kinase and calmodulin-dependent protein kinases I, II and III (314, 315)

In vitro at least overexpression of PKC can result in a transformed phenotype (316, 317) Moreover tumor promoters that activate PKC have the same effects on proliferation as activated *ras* But, surprisingly, PKC does not correspond to a previously identified oncogene (318), nor do the other second messenger dependent protein kinases

The protein kinases that are recognised as proto-oncogenes products are involved in the transduction of external stimuli, using, at least partially, other pathways as mentioned above The tyrosine-specific kinases among them are divided into two groups the integral membrane tyrosine-kinases (receptors) encoded by the *erb*-B-, *fms*-*kit*-, *ros*-, *neu* and *met* genes, and the cellular tyrosine kinases encoded by the *src*, *yes*, *abl* and *fes/fps* genes (269) From the latter group at least two members *src* and *abl* are bound to the inner surface of the cell membrane by their myristilated N-terminal ends (covalently bound fatty acid) Characteristic domains can be distinguished in the receptor molecules a) an intracellular tyrosine kinase domain, involved in the phosphorylation of one or more specific substrates, b) a transmembrane domain, c) an extracellular domain involved in ligand binding that may contain one or more cysteine-

rich regions. The *fms*- and *kit*-oncogene products and the PDGF receptor lack cysteine rich domains (269).

The normal cellular function of most of the proto-oncogenes coding for protein kinases is unknown. As mentioned before, *fms* and *erb*B code for receptors with known ligands and recently evidence is provided that in mouse the c-*kit* protein is encoded by the Dominant-White Spotting (W) Locus (319, 320). Mutations at this locus have pleiotropic effects on both embryonic development and the regulation of hematopoiesis in adult life. Animals homozygous for mutations in the locus are sterile, have extensive white-spotting and a severe anemia that results in prenatal death (321, 322, 323). The phenotype can be attributed to the failure of stem cell populations to migrate and proliferate effectively during development (323, 324, 325).

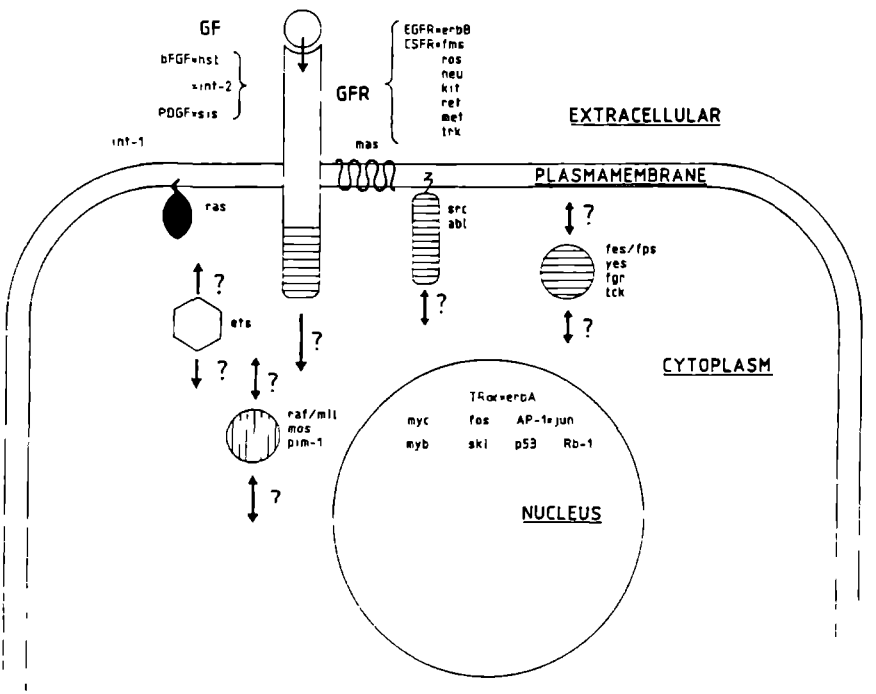

Fig. 2. Schematic drawing of the predicted cellular localization of the proto-oncogene products GF = Growth Factor, GFR = Growth Factor Receptor

V. Conclusions

In summary, oncogene products are components of very complex signal transduction circuitries which not only regulate cellular proliferation but also the development from oocyte to adult multicellular organism including differentiation, regeneration, recruitment and ageing

We distinguish the following classes of proto-oncogene products

a) polypeptide hormones acting on the cell-surface (*sis, hst, int-2*),
b) receptors for this type of hormones (*ros, met, trk, erB, neu, fms, kit, sea, ret, mas*),
c) links of the signal transduction pathways between these receptors and the cell-nucleus (members of the *ras* family via second messengers and tyrosine kinases bound to the inner surface of the cell membrane (*src, abl*) together with cytoplasmic tyrosine - (*fes,yes,fgr*) and serine/threonine kinases (*mos, raf, pim*-1)),
d) nuclear thyroid hormone receptors (*erb*A) and nuclear factors which mediate the long term effects in the nucleus of growth factors and other transmembrane signaling agents (*myc, myb, fos, ski, jun, Rb-1* and *p53*) (Fig 2)

The argument to collect this wide variety of genes under the common name (proto) oncogenes is based only on the common feature that these genes could be involved in tumor development when they are hit by particular genetic events

There are different explanations as to how genetic alterations might cause malfunction of these genes Disconnection of gene expression modulating elements and the structural parts of oncogenes can result in constitutive expression (some c-*myc* translocations) or abundant expression (retroviral transduction, gene amplification and translocation into the vicinity of a strong transcriptional enhancer) of a normal gene product Mutations can also induce constitutive activity of the gene product itself by inactivating or even removal of the elements that normally are involved in the limitation of the duration of their own action (point mutations in *ras* genes, removal of phosphorylation-sites in tyrosine kinases, removal of ligand-binding regions in receptors like *erb-B* and *erb-A* together with conformational changes characteristic of the active state of the receptor as is found also in the *neu* protein)

Probably DNA tumor viruses cause the same effects by the physical interaction of particular viral gene products (Polyoma virus middle-T, SV40 large-T, Adenovirus E1a and E1b) with cellular proteins involved in cell-growth and differentiation (*src, Rb-1* and *p53*)

It seems reasonable to assume that mutations that change the substrate specificity of proteins like the tyrosine kinases or the specificity of a transcriptional factor may play an important role but so far no evidence has been found for that

The genetic alterations mentioned result in an inaccurate behavior of the oncogenes Some can block the differentiation programm of the cell on a specific stage A constant stimulation of proliferation by constantly produced growth factors or growth factor receptors or signal transducers in an 'on-state' may achieve a similar effect The mechanism by which activated nuclear oncogenes that can mediate the response to growth factors favors proliferation over maturation is less well understood

The effect of many different mechanisms is the expansion of a specific cell compartment The enlarged but still restricted cell population will persist until another (onco)gene becomes activated During a microevolution, the cells will develop step by step towards a fully transformed phenotype What oncogenes contribute to the development of a more malignant phenotype depends on the differentiation stage of the cell Activated oncogenes can only contribute to transformation if they are permitted to act on their target within a specific so called 'differentiation window' (326), at a point where they can prevent further maturation Temperature-sensitive mutants of v-*src* and v-*erb*B transform cells and block their maturation in tissue culture at the permissive temperature But terminal differentiation is induced when the cells had been incubated for even a short time at the non-permissive temperature After the maturation has started subsequent reexpression of the transforming protein could not longer stop this process *In vivo* tumor induction experiments in B-cells suggest also strongly that the transformation of cells in different stages of differentiation in the same lineage is brought about preferentially by different oncogenes (e g *abl, myb, myc*) (5)

VI. Perspectives

In a short time fundamental cancer research became part of developmental biology Studying oncogene activity is studying the effect of mutations in signal transduction genes There are several other approaches to study signal transduction Carcinogenes like tumor promotors activate specific components of the signal transduction pathways (protein kinase C for instance (327)) and peptide analogs of their own putative pseudosubstrate regions will inhibit specifically protein kinases (315)) Together with these experimental approaches oncogene research will help to understand the mechanisms by which cell division and differentiation are regulated and will add a new dimension to the understanding of cell to-cell communication

Lower eukaryotes like yeast, *Nematodes* and *Drosophila* are very useful model systems to study cell and developmental biology The striking homology between cell cycle controlling proteins of yeast and vertebrates suggest that vertebrate cells will have a commitment point in G1 analogous to the yeast 'start' and a second control acting in G2 at the initiation of mitosis A model is proposed that places the cdc2 homolog at a point in G1 just downstream of a convergence point of various parallel signal transduction pathways originating from different growth factors and from other processes important in cell growth control, while the G2-M transition is also controlled by a cdc2 homolog (MPF) (311) The cdc2 homolog is a protein kinase controlled by phosphorylation So in this model a signal transduction pathway originating from a receptor on the cell surface leads to the regulation of nuclear proteins by phosphorylation

Many components of the signal transduction circuitries are already identified but little is known about their connection to one another Observations on revertants of tumor

cells may give some understanding From cultured v-K-*ras* transformed fibroblasts non-transformed revertants were obtained that still express the v-*ras* transforming protein The dominantly acting cellular gene or genes that cause the reversion could also cancel the transforming action of other members of the *ras* family and *src* and *fes* but not *sis, fms, raf, polyoma virus* and *SV40* (130) Revertants of v-*fos* transformed fibroblasts were resistant to transformation by v-*fos*, v-H *ras*, v-*abl* and v-*mos* but could be transformed by *trk* and *polyoma middle-T* (138) Oncogenes that are not able to transform revertants would presumably interact at a point upstream of the block within the pathway On the contrary, oncogenes that still transform the revertants may use a pathway totally independent of the blocked path Alternatively, the re-transforming oncogenes may use the same pathway but interact with this path downstream of the block Blocking of *ras* activity *in vitro* by microinjection of *ras* specific monoclonal antibodies showed that transformation of NIH-3T3 cells by v-*src*, v-*fms* and v-*fes* is dependent on an active cellular *ras* protein (328) Only v-*raf* and v-*mos*, both serine/threonine protein kinases located predominantly in the cytoplasm are dominant over the *ras* antibody block These observations can be explained by a pathway in which all the blocked oncogenes are positioned upstream of the *ras* protein and the dominant oncogenes are located downstream from *ras* (329)

A second handle on gaining information on the relationship between different oncogene products is synergism Synergism between oncogenes was first shown by transfection of REF cells with activated *ras* and *myc* genes (see section II B 1) Some acutely transforming viruses transduce more than one gene AEV (avian erythroblastosis virus) contains two genes, v-*erb*A and v-*erb*B The v *erb*B gene is sufficient for transformation In contrast, v-*erb*A alone has no transforming capacity but enhances the erythroid transformation by v-*erb*B but also other non-related growth promoting oncogenes by completely arresting the transformed erythroblasts at an immature stage in differentiation (330) Comparison of the transforming activities *in vivo* and *in vitro* of naturally occurring v-*mil*/v-*myc* (MH2) transducing viruses and v-*mil* (v-*raf*) or v-*myc* transducing viruses showed clearly the synergism between the two oncogenes Their combined action is associated with the induction of factor-independent growth in culture A model is proposed in which are provided in the G1 phase of the cell cycle both a 'competence' or early G1 signal (= v-*myc*) and a 'progression or late G1 signal (= v-*raf*) (329) 'Competence' growth factors like PDGF might induce a family of genes including *fos* and *myc* which make the cell responsive to a second group of factors, the 'progression' factors such as EGF Sequential action of competence and progression factors is required for induction of cellular DNA synthesis EGF causes the previously PDGF-treated cells to proceed into the S-phase (331-334)

Tumor models based on insertional mutagenesis by proviruses have also clearly shown the cooperative action of activated (putative) oncogenes MoMuLV induced rat thymic lymphomas containing provirus insertions in *Mlvi*-1 always contain proviral insertions in a second locus, *Mlvi*-2 in the same population of tumor cells (171) An other example is *pim*-1/c-*myc* (or N-*myc*) synergism in MoMuLV induced T-cell lymphomas in mice (26, 335) A high percentage of primary tumors contains cells with proviral integrations near both *pim*-1 and c-*myc* or N-*myc* Transplantation of these, primary, tumors resulted in selective outgrowth of minor subclones of tumor cells carrying insertions in an unknown locus suggesting that this so called *pim*-2 locus is associated with later stages of disease (336) This also supports a multistep transformation model The chance that two oncogenes become activated by proviral

integrations within one cell is extremely small A single insertion in the first common integration site in the genome of the target cell near a gene that stimulates proliferation results in expansion of a cell clone that will serve as a target for second proviral integrations A second hit may cause an additional growth advantage by which a new subclone of cells with a new phenotype is created The new subclone may replace its predecessors as the result of a selection process or becomes part of a balanced polyclonal cell population If the growing cell population allows that the process is repeated a polyclonal or monoclonal tumor with many proviral integrations within the genome will arise (337)

The cooperativity of oncogenes is also studied in transgenic mice Transgenic mice with an activated oncogene have proven to be good model systems to study tumor-development *in vivo* (for review ref 338-340) Transgenic mice with a rearranged *myc* (Eμmyc) gene activated by the immunoglobulin enhancer as a transgene develop B-cell-lymphomas with a very high incidence after a rather short latency period The preB-cell compartment of polyclonal origin is greatly enlarged in the prelymphomatous state (341, 342) It could be deduced from the following observations that for the development of tumors *Eμmyc* expression is not sufficient

a) Tumor induction is a stochastic process tumors appear after a variable latency period,
b) A distinct pre-neoplastic state can be recognized,
c) The tumors are monoclonal

The preB cells of the prelymphomatous state are used for different *in vitro* studies The cells appeared not to be autonomous, since they died rapidly when cultured without feeder cells With feeder cells initially the preB cells grew like normal cells but after 14-20 weeks they started to grow to 10 fold higher densities, implying a reduced requirement for growth factors And after 25 weeks the one remaining culture had become feeder-independent and tumorigenic Deregulated c *myc* expression in B-cell precursors seems to increase the rate of cell turnover, thereby increasing the frequency of genetic changes resulting in autonomous growth (343) But it is not excluded that *myc* also can influence directly or indirectly the stability of the genome of the prelymphomatous cell

In imitation of *in vitro* transfection experiments, transgenic mice are made with activated *ras* and *myc* genes by interbreeding established strains of mice carrying either a *myc*- or a *ras*-gene-construct with a MMTV promotor The tumor formation appeared to be much more rapid in the F_1 generation than in the parent strain carrying a single oncogene Moreover other tumor types developed in the animals suggesting that cooperation of both proteins resulted in a greater penetrance in several tissues (344) As could be expected the cooperative action of *myc* and *ras* seems not to be sufficient for tumor formation The tumors appeared to be clonal

In another approach, the potential of bone marrow cells from the Eμ-*myc* transgenic mice mentioned before in the pre-neoplastic state were compared with those of their normal littermates for transformation by Harvey murine sarcoma virus and Abelson murine leukemia virus by assessing colony formation in semi-solid medium The Eμ-*myc* bone marrow appeared to yield more lymphoid colonies than normal marrow after infection with Harvey virus, and confirmed the results with the *ras/myc* transgenic mice However, the number of lymphoid colonies induced by Abelson virus was not enhanced

Probably the primary target of Abelson virus is more primitive than the expanded pre-B cells of the Eμ *myc* mice and is therefore not present at increased frequency in the Eμ-*myc* marrow (345) The last mentioned *in vitro* result could be confirmed *in vivo* by Abelson virus infection of newborn mice from matings between normal and Eμ *myc* transgenic mice The Eμ *myc* transgenic mice did not show a higher susceptibility to lymphoma induction by Abelson virus compared to the non transgenic littermates (J S Verbeek, unpublished)

The bone marrow cells of pre-neoplastic Eμ-*myc* mice and normal mice were also infected with constructed retroviral vectors containing the *bcl*-2 coding sequence Only Eμ-*myc* marrow cultured after infection in soft agar in the absence of mitogenic stimuli yielded compact colonies containing non-adherent cells with a lymphoid morphology The colonies could survive for several weeks in liquid culture but did not proliferate, even in the presence of stroma cells or many different hemopoietic growth factors In only three independent experiments infected bone marrow cells from Eμ-*myc* mice directly seeded into liquid medium continued to proliferate, infected normal cells never did The infected Eμ-*myc* populations appeared to represent oligoclonal immortal cell lines with a pre-B lymphocyte phenotype that yielded tumors in nude and syngeneic mice Together, these data strongly suggest that *bcl* 2 cooperates with c-*myc* to immortalize pre-B cells but again the enforced expression of two cooperative oncogenes is not sufficient for autonomous growth and tumorigenicity Infection of different growth factor dependent hemopoietic cell lines with the *bcl*-2 virus showed that *bcl*-2 expression allows prolonged survival of factor starvation with nearly all the cells in G_0 As a model to explain the contribution to transformation of constitutive *bcl*-2 expression induced by the 14,18 translocation it is proposed that *bcl*-2 maintains the affected cell clone until other genetic alterations generate the more aggressive later stage (346)

To find new combinations of cooperative oncogenes in T-cell lymphomas newborn mice from matings between normal and *pim* 1 transgenic mice were infected with MoMuLV Using MuLV as an insertional mutagen the system was asked what gene would be preferentially activated in addition to the transgen The transgenic mice appeared to be highly susceptible for MoMuLV induced T cell lymphomas as could be deduced from a strongly reduced latency between virus infection and tumor induction as compared to non-transgenic littermates A further analysis showed that in 85% of the *pim*-1 lymphomas c-*myc* is activated by proviral insertion The remaining 15% contained proviral insertions within N-*myc* The lymphomas in the non transgenic littermates carried less frequent proviral *myc* integrations So in *pim*-1 transgenic mice *myc* activation seems to induce the highest selective growth advantage compared to other gene activations suggesting a strong synergistic action between *pim* 1 and *myc* (347)

Transgenic mice carrying a human c-*myc* gene activated with a murine immunoglobulin heavy chain enhancer were crossed with transgenic mice carrying a functional rearranged human gene that encodes the membrane-bound form of the immunoglobulin heavy chain (mIgμ) In the *myc*/mIgμ double transgenic mice mIgμ delayed the onset of malignancy and reduced its incidence, moreover influences also the phenotype of the tumors that did occur The specific reduction of pre-B lymphoblastic malignancies might be explained by assuming that mIgμ expressing pre-B cells should bypass entirely the stage in which heavy chain rearrangements occur by which the size of the population of cells that is most susceptible to transformation by the *myc* transgene is reduced (348) This is again an indication that differentiation may suppress tumor inducing mechanisms

The possibilities to develop transgenic mouse models will enormously increase when the techniques for homologous recombination in embryonic stem cells become operational, because this will create the possibility to switch off specific genes

The examples collected here are very recent and partially preliminary data, real experiments with a topping of inspiring imagination that at least show the way to fruitful strategies to uncover the signal transduction pathways used in the induction, outgrowth and metastasis of different types of tumors

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Chapter 2

fes/fps, A PROTO-ONCOGENE THAT ENCODES A CYTOPLASMIC TYROSINE-SPECIFIC PROTEIN KINASE

I. Introduction

A *fes/fps* transducing viruses

The *fps* oncogene was discovered as part of five isolates of avian sarcoma virus (ASV) and the *fes* oncogene as part of three isolates of feline sarcoma virus (FeSV) Later it appeared that both oncogenes are homologous to the same evolutionary well conserved cellular locus It is unclear whether the structurally closely related avian sarcoma viruses PRCII and PRCIV are really independent isolates (1) All viruses are found in tumors of field animals except for 16L that was isolated from a chicken tumor induced in the laboratory after an avian sarcoma virus infection (2) The *fps* containing avian sarcoma viruses induce myxosarcomas (2, 3, 4) and the *fes* containing feline sarcoma viruses induce fibrosarcomas (5, 6, 7). All viruses induce efficiently morphological transformation in cultured fibroblast cells They are replication defective and dependent for reproduction on a helper virus, ALV (avian leukemia virus) and FeLV (feline leukemia virus) respectively From PRCII, FSV (Fujinami sarcoma virus), ST-FeSV (Snyder-Theilen feline sarcoma virus) and GA-FeSV (Gardner-Arnstein feline sarcoma virus) sequence data are available A detailed comparison revealed that all four sequences have exactly the same *fes/fps* 3' termini The recombination took place within the same viral *gag* domain, p27 for ASV and p30 for FeLV but not in the same position of the *gag* sequence The left hand junction of *fes* and *gag* sequences might have been formed by homologous recombination at the DNA level (8) The right-hand junction appears to be generated by recombination at the level of RNA (8, 9) A poly(A) addition signal of cellular origin is present in PRCII and SFV The 5' termini of *fps* sequences in PRCII and FSV are the same but in the other viruses they differ

B The cellular homolog c-*fes/fps*

The cellular homologs of *fes/fps* from chicken, man and cat have been molecularly cloned and characterized in detail The gene is mapped to human chromosome 15 (10, 11) and to mouse chromosome 7 (12, 13) All cellular genes show the same overall structure (1) The size of each of the 18 coding exons is identical in chicken, man and cat The 5' border of the 5' non-coding exon remains to be established (1, 8, 14, 15) Exon-2 contains a potential ATG initiation codon at the start of an open reading frame of about 2 5 kb that ends in an TGA termination codon in exon-19 FSV contains all 19 chicken c-*fps* exons In PRCII the 3' end of exon-2, the complete exons 3, 4, 5, 6, 7, 8 and the 5' end of exon-9 are missing (16, 17) GA-FeSV contains 16 nucleotides of the 3' end of exon-1, the complete exons 2, 3 and 4, the 5' end of exon-5, the 3'end of exon-9, and the complete exons 10 to 19 The c-*fes* homologous sequences of ST-FeSV start at the 3' end of exon-8 and end at the stopcodon in the 19th exon (8) From these data it can be concluded that the 5' end (exon-1 to 8) of *fes/fps* is not necessary for transformation Except for the internal deletion the sequences of PCRII v-*fps* and

chicken c-*fps* differ only in 4 out of 1700 shared nucleotides resulting in no difference in their putative proteins (9, 18) FSV shows 26 amino-acid substitutions compared to chicken c-*fps* protein (18) GA-FeSV and ST-FeSV have only a few amino-acid substitutions compared to the feline c *fes* protein but both contain deletions (8) These viruses have in common that the *fes/fps* sequences are linked to amino-terminal *gag* sequences

C The *fes/fps* related gene products

All protein products of the different v-*fes/fps* genes are associated with a tyrosine-specific kinase activity and are capable of phosphorylating themselves and exogenously added substrates (19, 20, 21, 22, 23, 24) Beside the highly conserved catalytic domain at the carboxyl-terminal end, a stretch of about 100 amino acids located directly upstream of the catalytic domain, called SH2, was found to be conserved among all cytoplasmic tyrosine-specific protein kinases such as the *src ,yes,abl* and *fgr* products (25) and also in the *fes/fps* protein (26) The function of this region, that is also present in PLC (phospholipase C-148) is unknown (27)

Both the FSV and FeSV *fes/fps* proteins seem to be associated with the plasma membrane (28, 29) The amino terminus of the more tightly associated FeSV *gag* protein is myristylated (30) while the amino terminus of the avian *gag* proteins is acetylated at the first methionine residue (1)

A 98 kDa (NCP98) and a 92 kDa (NCP92) c-*fes/fps* protein can be detected in respectively normal chicken and normal murine and human cells (31, 32) They also have tyrosine-specific protein kinase activities that phosphorylate themselves and exogenous substrates in an immune-complex assay (24, 33, 34) The specific activity may be lower then that of v-*fes/fps* proteins (35) Moreover NCP98 lacks detectable tyrosine phosphorylation in vivo (34) The proteins are predominantly present in granulocytes and macrophages (31, 32, 36)

D Activation of the oncogenetic potential of *fes/fps*

Various DNA constructs were made to test what genetic changes are responsible for the transforming activity of the v-*fes/fps* genes By replacing the entire *src* sequences in RS-RSV (Rous sarcoma virus) with the v-*fps* sequences of FSV a virus was constructed ,named F36, that codes for a 91 kDa v-*fps* protein without *gag* (37) The virus is able to induce tumors in chickens and to transform cells *in vitro* But when the chicken c-*fps* sequences were substituted for the v-*src* sequences of RS-RSV the resulting virus, named Fc51, is not able to induce tumors *in vivo* or to transform cells *in vitro* (35) When the same c-*fps* sequences are linked at the 5' end to *gag* sequences, the resulting FSV-like viral construct is again able to transform cells (35) Comparable results are found with the human c *fes/fps* gene in retroviral vectors (38, 39)

In all cases the transforming potential of the *fes/fps* containing viruses is strongly associated with their tyrosine-specific kinase activity (22, 40, 41,42, 43, 44, 45, 46) F36 is highly active in a kinase assay but Fc51 has a significantly reduced kinase activity

From these data it can be concluded that the transforming potential of *fes/fps* could be activated by scattered mutations in the 5' non-catalytic domain of the gene (F36) or fusion to *gag* sequences In the natural occurring virus isolates fusion to *gag* sequences seems to be the first step in the activation of the transforming potential of the *fes/fps* gene followed by selection of mutations (FSV) or deletions (PRCII, GA-FeSV and ST-FeSV) which contribute to an increase of the transforming activity of the gene

E Proposed function of the *fes/fps* protein

Chicken myeloid cells infected with v-*fes/fps* expressing retroviruses do not require exogenous growth factors to differentiate *in vitro* (47) Thus the *fes/fps* protein may be related to cellular responses to growth factors specific to these cell types

In the proximity, within less than 1 1 kb , of *fes/fps* a gene is located, named *fur*, that codes for a protein with a transmembrane domain (48, 49) There is no evidence that the *fes/fps* protein and this *fur* protein form a membrane bound receptor complex Moreover, both genes differ in their tissue-specific expression (48) So far p56 *lck* is the only non-receptor protein kinase that is found to be associated with a membrane protein (the T cell surface antigens CD4 and CD8) (50)

To study the activated *fes/fps* gene *in vivo* transgenic mice have been made with an oncogenic *gag-fes/fps* gene construct under the transcriptional control of the human ß-globin promoter The many different phenotypes observed were probably caused by the surprisingly widespread expression of the transgene In 8 to 50 weeks, 10 to 50% of the animals developed a variety of tumors of lymphoid or mesenchymal origin (i e thymoma, lymphoma, fibrosarcoma, angiosarcoma, neurofibroma) Others show strong cardiovascular abnormalities or neurological defects (51)

Like that of all other tyrosine-specific protein kinases that are not integral membrane proteins the normal physiological role of c-*fes/fps* is still unknown The difference in biological activity between the normal cellular gene product and the oncogenically activated tyrosine kinase is not understood Identification of the physiologically significant substrates of these tyrosine kinases in their 'normal' as well in their 'activated' status may resolve these questions Identification of the highly concerved cellular phosphoprotein, designated P150, that appears to be a substrate for the v-*fes* associated tyrosine specific protein kinase activity in tissue culture cells, might be a good starting-point (52, 53)

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II. Structural analysis of a variant clone of Snyder-Theilen feline sarcoma virus

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Structural analysis of a variant clone of Snyder–Theilen feline sarcoma virus

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Summary

A variant clone of Snyder–Theilen feline sarcoma virus (ST-FeSV) encoding a polyprotein with a molecular weight of approximately 104 kDa (P104) was compared to the P85 encoding prototype clone of ST-FeSV. Analysis of chimeric genes constructed with the viral oncogenes of the two clones indicated that the variant clone coded for a larger polyprotein than the prototype clone because of genetic differences in its 3′ portion. Comparative DNA sequence analysis revealed that one nucleotide just upstream of the termination codon TGA in the prototype proviral DNA was deleted from the variant clone resulting in a 468-bp larger open reading frame. Furthermore, it appeared that the U3 regions of the long terminal repeats (LTRs) of the variant clone contained an insertion of 71 bp as compared to the LTRs of the prototype clone. In addition, both clones differed also from each other with respect to genetic sequences deleted from their *env* gene regions.

Feline sarcoma virus; V-*fes* oncogene; Genomic organization

Introduction

Acutely transforming RNA tumor viruses have acquired their malignant potential upon insertion of proto-oncogene sequences into their genomes (Bishop and

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Varmus, 1982, Fishinger, 1982) Three independent FeSV isolates, including the Gardner–Arnstein strain (GA-FeSV) (Gardner et al., 1970), the Snyder Theilen FeSV strain (ST-FeSV) (Snyder and Theilen, 1969) and the Hardy–Zuckerman 1 strain (HZ1-FeSV) (Snyder et al., 1984), captured sequences from the feline c-*fes* proto-oncogene (Frankel et al., 1979; Franchini et al., 1981; Hardy et al., 1981; Hampe et al., 1982; Roebrock et al., 1987). All isolates induce fibrosarcomas in vivo and transform fibroblasts in cell culture (Gardner et al , 1970; Snyder and Theilen, 1969; Snyder et al., 1984) The major translation products of GA-FeSV and HZ1-FeSV are polyproteins of 115 kDa (Stephenson et al., 1980; Van de Ven et al., 1980a) and 100 kDa (Snyder et al., 1984), respectively. In the case of ST-FeSV, two clones are described (Reynolds et al., 1981). One, which we will call the 'prototype' clone, codes for a polyprotein of 85 kDa (Barbacid et al., 1980; Reynolds et al., 1981; Sherr et al., 1978). The other, which we designated the 'variant' clone, encodes a polyprotein whose mol. wt. was reported earlier as 115 kDa (Van de Ven et al., 1980a). Now, based upon amino acid sequence data presented in this study, the mol. wt. of the polyprotein encoded by this clone appears to be 104 kDa. It was shown that the translation products of GA-FeSV and prototype ST-FeSV, which are studied in greatest detail, possess tyrosine-specific protein kinase activity in vitro and are capable of autophosphorylation as well as phosphorylation of certain other proteins (Barbacid et al., 1981; Beemon, 1981; Mathey-Prevot et al., 1982; Ruscetti et al., 1980; Van de Ven et al., 1980a,b; Snyder et al., 1984). Analysis of transformation-defective mutants of prototype ST-FeSV revealed that this enzyme activity which is located in the carboxy-terminal region of the polyproteins (Barker and Dayhoff, 1982; Levinson et al., 1981; Weinmaster et al., 1983) is required for malignant transformation (Donner et al., 1980; Reynolds et al., 1981). In the case of ST-FeSV, a single tyrosine acceptor site within the polyprotein is involved (Blomberg et al., 1981).

To elucidate genetic differences between the two clones of ST-FeSV, the genome of the variant clone was molecularly cloned and its genetic organization and structure compared with that of the prototype. Differences observed in coding and regulatory sequences are discussed.

Materials and Methods

Cell lines and media

Cells were grown in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% newborn calf serum (GIBCO). Cell lines used in this study included CCL64, a highly contact-inhibited mink lung cell line (Henderson et al., 1974), rat-2 (Topp, 1981), CCL64 nonproductively transformed by GA-FeSV (Van de Ven et al., 1980a), prototype ST-FeSV (Sherr et al., 1978) and by the McDonough strain of feline sarcoma virus (SM-FeSV) (Van de Ven et al., 1980a). Normal rat kidney (NRK) cells nonproductively transformed by the variant clone of ST-FeSV were obtained from E.M. Scolnick.

Purified DNA was transferred into cells by the calcium phosphate precipitation technique (Graham and Van der Eb, 1973) or by electroporation (Narayanan et al., 1986) For electroporation, cells were resuspended in Ca^{2+} and Mg^{2+} free phosphate-buffered saline to a final concentration of 2×10^7 cells/ml 50 μg of purified DNA was added to 0 5 ml of a cell suspension and incubation was for 10 min at 0 °C Electroporation was carried out with an electric pulse of 2 0 kV for 0 9 s

Preparation of probes and hybridization

S_1 and S_r are 5′- and 3′-specific probes of the v-*fes* oncogene of prototype ST-FeSV (Franchini et al., 1981) These probes were obtained from C J Sherr pCO14 is a recombinant of the prototype ST-FeSV provirus subcloned in pSVBR94 Isolation of DNA probes, nick translation, and hybridization analysis of Southern blots were carried out as described by Schalken et al (1985)

Construction of a genomic library and selection of recombinant clones

To isolate the complete proviral DNA of the variant clone of ST-FeSV, a gene library was constructed with the pJB8 cosmid vector system (Ish-Horowicz and Burke, 1981) using high-M_r DNA isolated from NRK cells nonproductively transformed by the variant clone of ST-FeSV For screening of the library, a combination of S_1 and S_r was used as a molecular probe Isolation of cosmid DNA from selected clones was performed according to the alkaline lysis procedure described by Davis et al (1979)

DNA sequence analysis

Upon ligation of DNA fragments into the polylinker region of M13mp8-19 (Yanisch-Perron et al, 1985), DNA sequences were determined by the dideoxy method as described by Sanger et al (1977) Gel readings were recorded, edited and compared using the Staden programs (Staden, 1982)

Immunoprecipitation analysis

Cell labeling, immunoprecipitation of polyproteins, and subsequent sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed as described by Van de Ven et al (1980b)

Results

Analysis of the translation products of the prototype and variant clones of ST-FeSV

In earlier studies, we reported on the characterization of the polyprotein encoded by the independently isolated variant clone of ST-FeSV (Reynolds et al, 1981, Van

de Ven et al., 1980a). In these studies, its mol. wt. appeared to be similar to that of the polyprotein encoded by GA-FeSV and was estimated to be approximately 115 kDa. In recent studies, however, we observed a slight difference in electrophoretic mobility between the polyproteins encoded by GA-FeSV and the variant ST-FeSV. This is illustrated in Fig. 1, which shows the results of an immunoprecipitation and SDS–PAGE analysis of the polyproteins encoded by GA-FeSV (lane 1), prototype ST-FeSV (lane 3) and variant ST-FeSV (lane 5). The greater mobility of the polyprotein encoded by the variant clone of ST-FeSV as compared to GA-FeSV P115 is clear. Based upon this observation and upon additional data presented below, we estimated the mol. wt. of the polyprotein of the variant clone as 104 kDa and will therefore designate the product as P104. To determine whether the difference in mol. wt. of the translation products of the prototype and variant clone was due to glycosylation, synthesis of the polyproteins was studied in cell lines cultured in the presence of tunicamycin. Goat antiserum directed against FeLV p15 (Reynolds et al., 1981) was used. As a control to test the inhibition of glycosylation by tunicamycin, we studied the synthesis of the glycosylated polyprotein encoded by the McDonough strain of FeSV. In the absence of tunicamycin, the previously described polyprotein P170 was found (Van de Ven et al., 1980c). In the presence of tunicamycin, no P170 could be detected. Instead, a polyprotein with a mol. wt. of 155 kDa containing p15, p12 and p30 antigenic determinants was observed (data not shown). Immunoprecipitation analysis of GA-FeSV and prototype ST-FeSV

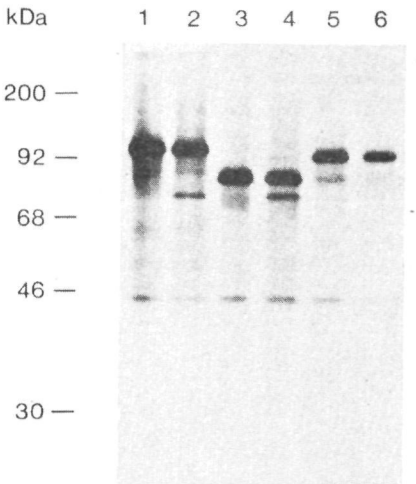


Fig. 1. Immunoprecipitation and SDS–PAGE analysis of high-molecular-weight polyproteins in mink cells nonproductively transformed by GA-FeSV (lanes 1 and 2) or prototype ST-FeSV (lanes 3 and 4) and normal rat kidney cells nonproductively transformed by the variant clone of ST-FeSV (lanes 5 and 6). Cells were grown in absence (lanes 1, 3 and 5) or presence (lanes 2, 4 and 6) of tunicamycin (5 μg/ml) for 7 h. After incubation for 4 h, [^{35}S]methionine was added (50 μCi/ml). Immunoprecipitation analysis was performed with goat antiserum directed against FeLV p15. Immunoprecipitated proteins were visualized by autoradiography using Kodak XAR-2 film.

transformed mink cells cultured in the presence of tunicamycin revealed a major band of 115 kDa (Fig 1, lane 2) and 85 kDa (Fig 1, lane 4), respectively No differences compared to the polyproteins synthesized under normal conditions could be observed Similar analysis of the variant clone of ST-FeSV in the presence and absence of tunicamycin did also not reveal any detectable change in electrophoretic mobility of the variant polyprotein (Fig 1, lanes 5 and 6) No FeLV-related proteins were precipitated with control sera or found in extracts prepared from control mink or rat cells (data not shown) These results indicated that the difference in mol wt of P85 and P104 was not due to glycosylation To explain the difference in mol wt of the two polyproteins, the possibility of a genetic modification, e g insertion or deletion of genetic sequences, or a frameshift point mutation was considered To test this possibility, molecular cloning of the variant ST-FeSV provirus was required

Molecular cloning of the proviral genome of the variant clone of ST-FeSV

To molecularly clone the proviral DNA of variant ST-FeSV, we constructed a cosmid gene library with DNA from NRK cells nonproductively transformed by variant ST-FeSV, as described in the Materials and Methods section Upon screening of the cosmid gene library (approx 750000 colonies) with a combination of the S_1 and S_r probes, three cosmid clones, designated cos 4, cos 9 and cos 11 were identified and isolated for further analysis Analysis of these cosmid clones revealed that all three cosmid clones contained the complete provirus Cosmid clone 9 was selected for further studies It appeared from restriction endonuclease analysis that the size of the proviral DNA was 4 9 kbp This is slightly smaller than the proviral DNA of prototype ST-FeSV, which is 5 3 kbp It should be noted that also a 4 3 kbp proviral DNA clone of prototype ST-FeSV has been described (Groffen et al, 1983) The difference between the 5 3 kbp and 4 3 kbp proviral DNA lies in the *env* gene region and both isolates code for the same polyprotein P85 Hybridization analysis of the proviral DNA of variant ST-FeSV with a GA-FeSV-specific probe (*Pst*I-*Sac*I fragment) (Hampe et al, 1982) revealed that such sequences were not present (data not shown) This observation indicated that sequences homologous to feline c-*fes* exon 1–6 were not represented in the variant provirus (Roebroek et al, 1987)

A restriction map of the variant proviral DNA (Fig 2) was obtained on the basis of restriction endonuclease analysis using the restriction endonucleases *Bam*HI, *Bgl*II, *Eco*RI, *Hin*dIII, *Kpn*I, *Sac*I, *Sal*I and *Xho*I or combinations of these in sequential digestions (data not shown) Comparison of the restriction maps of the prototype and variant clone of ST-FeSV provided no clues as to where to localize potential insertions or deletions

The prototype and variant strain of ST FeSV differ in the 3′ portion of the polyprotein encoding region

In an effort to determine the map position of genetic modifications that causes the enlargement of the open reading frame in the variant clone, chimeric genes were

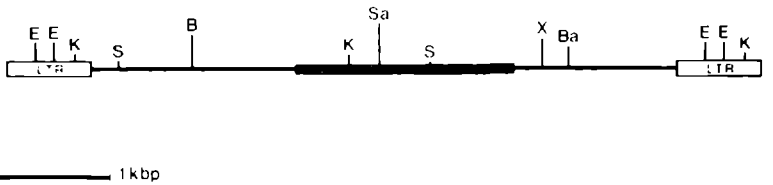


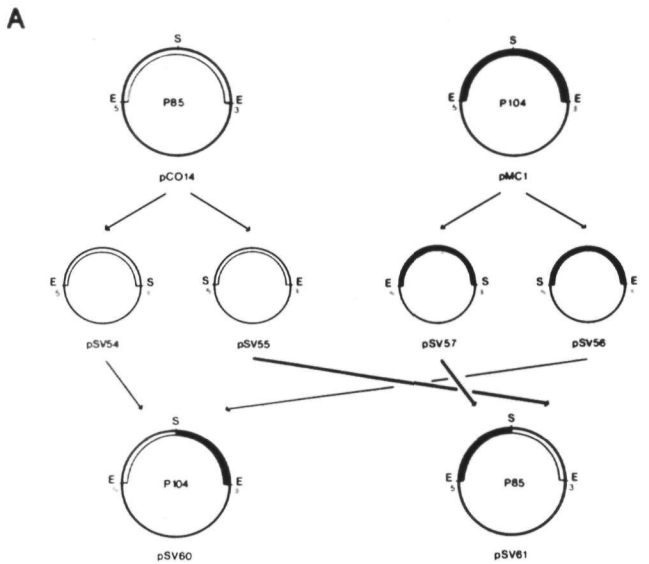
Fig. 2. Restriction endonuclease map of the proviral DNA of the variant clone of ST-FeSV. The heavy bar represents v-*fes* sequences. B, *Bgl*II; Ba, *Bam*HI; E, *Eco*RI; K, *Kpn*I; S, *Sac*I; Sa, *Sal*I; X, *Xho*I. LTR, Long Terminal Repeat.

constructed using the 5′ and 3′ halves of the proviral DNAs of the two ST-FeSV clones. A *Sal*I restriction endonuclease site present in both v-*fes* genes and an *Eco*RI restriction nuclease site located in the LTRs were used in the construction as schematically outlined in Fig. 3A. In addition to these two chimeric constructs, recombinant DNA clones that contained the parental prototype proviral DNA (clone pCO14) or the parental variant proviral DNA (clone pMC1) were also studied. Upon introduction of recombinant plasmids pSV60, pSV61, pCO14, or pMC1 into rat-2 cells, transformed clonal cell lines were obtained by selection of single cells in microtiter plates. Introduction of DNA into cells by electroporation seemed more efficient than transfection of DNA. Furthermore, linearized DNA gave better results in electroporation than closed circular DNA (data not shown). Cell lines coded rat-2–pSV60-C17, rat-2 pSV61-C18, rat-2–pCO14-C19 and rat-2- pMC1-C12, respectively, were selected for further analysis.

To characterize the translation product(s) encoded by the chimeric genes, total cell lysates were studied by immunoprecipitation and SDS–PAGE analysis. Goat antiserum prepared against GA-FeSV P115 (Fig. 3B) (Reynolds et al., 1981) or FeLV p15 (data not shown) were used. In Fig. 3B, the results of the immunoprecipitation analysis are presented. Just like cell line rat-2 pCO14-C19 (data not shown), cell line rat-2–pSV61-C18 (Fig. 3B, lane 1) appeared to express a polyprotein with a mol. wt. of 85 kDa. Cell line rat-2–pSV60-C17 (Fig. 3B, lane 2) however, expressed a polyprotein with a similar mol. wt. as P104 expressed in cell line rat-2–pMC1-C12

Fig. 3. Construction of chimeric genes and characterization of translation product(s). (A) Plasmid pCO14, containing a single copy of prototype ST-FeSV proviral DNA (open segment), was digested with restriction endonucleases *Eco*RI and *Sal*I. The v-*fes* containing DNA fragments were isolated and subcloned in pBR322, resulting in pSV54 and pSV55. Using the orientation of the prototype of ST-FeSV provirus as a reference, pSV54 and pSV55 contain the 5′ and 3′ part of the proviral DNA, respectively. Similarly, plasmid pMC1 containing a single copy of the variant ST-FeSV provirus (black segment), was digested with restriction enzyme *Eco*RI and *Sal*I. The v-*fes* containing DNA fragments were subcloned in pBR322 resulting in pSV56 and pSV57. The insert of pSV54 and the insert of pSV56 were ligated and, upon digestion with restriction endonuclease *Eco*RI, subcloned in pCO13, which resulted in pSV60. The same procedure was followed in constructing pSV61 using pSV55 and pSV57. (B) Immunoprecipitation and SDS–PAGE analysis of the polyproteins expressed in rat-2 cells transfected with pSV60 or pSV61. Cell lines rat-2–pSV61-C18 (lane 1), and rat-2–pSV60-C17 (lane 2), were metabolically labeled for 3 h with [^{35}S] methionine (50 μCi/ml). Proteins were immunoprecipitated with goat antiserum raised against FeLV p15. Autoradiography was performed as described in the legend to Fig. 1.

(data not shown). No FeLV-related proteins were precipitated with control sera or found in extracts prepared from control rat-2 cells (data not shown). These data indicated that the genetic difference between the two ST-FeSV clones that causes



the synthesis of the larger polyprotein P104 is located in the 3′ portion of their genomes

Nucleotide sequence analysis of the proviral DNA of the variant clone of ST FeSV

Although the experiments with the chimeric genes strongly suggested that genetic differences in the 3′ halves of the genomes of the two ST-FeSV clones could explain the differences in mol wt of the polyproteins, the precise nature of the genetic differences remained to be established Furthermore, the possibility of genetic modification in the 5′ portions could not be ruled out either To resolve these matters the nucleotide sequence of the proviral DNA of variant ST-FeSV was determined The results are presented in Fig 4 The sequence data of the proviral DNA of variant ST-FeSV were compared with those of the v-*fes* oncogene of the prototype strain (Hampe et al , 1982) and with FeLV helper virus sequences (Hampe et al , 1983, Nunberg et al , 1984) since the complete nucleotide sequence of the proviral DNA of prototype ST-FeSV has not been determined yet Such comparative analysis indicated that one nucleotide (G) just upstream of the termination codon, as present in the prototype strain, was deleted from the variant clone of ST-FeSV, resulting in an extension of the open reading frame with 468 bp up to the termination codon (TAA) located at nucleotide position 3776–3778 (Fig 4) Furthermore, three major other differences were observed and these are depicted in Fig 5 First of all, 71 nucleotides of the U3 region of the LTR of the variant clone were not present in the prototype ST-FeSV In addition nucleotide sequences encoding FeLV gp70 and the amino terminal portion of p15E were present in the prototype clone of ST-FeSV but not in the variant clone Finally, the 3′ portion of the *env* gene was present in the variant clone (nucleotide position 3817–4290) (Fig 4) but not in the prototype clone of ST-FeSV In addition to the four genetic differences mentioned above, the two ST-FeSV clones also differed from each other by a number of point mutations (see Discussion)

Discussion

The ST-FeSV isolate that encodes the P85 polyprotein is extensively studied and can, therefore, be considered as the prototype clone of this virus strain In this report, we describe the characterization of a variant clone of ST-FeSV It encodes a polyprotein of 104 kDa (P104) Calculation of its mol wt was mainly based upon DNA nucleotide sequence data This was a reasonable approach, since P104 is either not significantly, or not at all, glycosylated, as concluded from the studies with tunicamycin Therefore, a better estimate of the mol wt of the polyprotein of variant ST-FeSV is 104 kDa and not 115 kDa as was reported before (Van de Ven et al , 1980a)

The close relationship between the prototype and the variant clone was established in earlier studies based upon radioimmunoassay analysis of the *gag*-gene encoded domains (p15, p12) in P85 and P104 (Sherr et al , 1978, Van de Ven et al ,

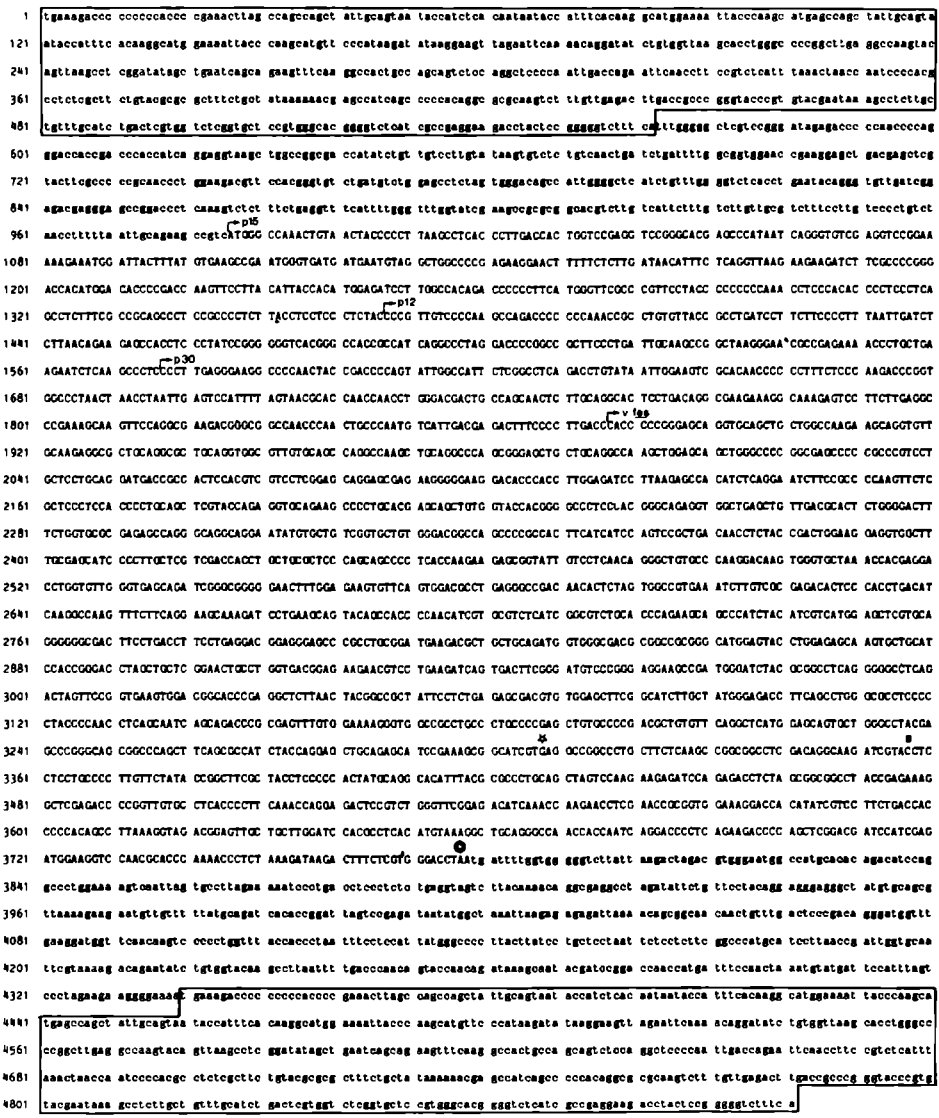


Fig 4 Nucleotide sequence of the proviral DNA of the variant clone of ST-FeSV Nucleotide sequences representing the LTRs are placed in a box Nucleotide sequences encoding the polyprotein are depicted in capitals The start of p15, p12, p30 and v-*fes* is indicated by arrows. The termination codons TAA or TGA at the ends of the open reading frames for the polyproteins of the variant and prototype clone of ST-FeSV are represented by ● and by ☆, respectively The end of v-*fes* is indicated by ■

1980a). This is now confirmed by comparative nucleotide sequence analysis of the truncated *gag*-genes in both viral genomes (Hampe et al., 1982; Fig. 4). It is furthermore evident from the observation that the same portion of the feline c-*fes*

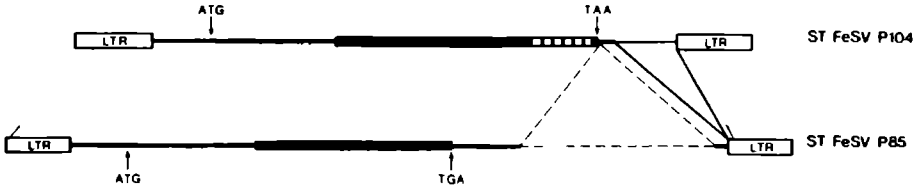


Fig 5 Schematic representation of the major genetic differences in the proviral DNAs of the variant and prototype clone of ST FeSV The heavy bars represent the v *fes* sequences LTRs are depicted as open boxes Start and stop codons of the sequences encoding the polyproteins are marked by ATG and TAA for the variant clone and ATG and TGA for the prototype clone of ST FeSV The shaded bar in the variant clone represents the extension of the polyprotein encoding sequences Lines connecting the two viral DNAs indicate deletions or insertions

proto-oncogene is present in the two clones However, there are also a number of genetic differences between the prototype and variant clone The most interesting one is the frameshift point mutation that extends the open reading frame of the variant viral oncogene with 468 bp The termination codon for the variant clone is TAA at position 3776–3778 (Fig 4) The additional 468 bp DNA region is of FeLV helper virus origin Apparently, covalent linkage of the translation product of these sequences to the protein kinase domain in the polyprotein does not influence the transforming potential of the variant clone It is possible that the extended carboxy terminal portion does not affect the tyrosine-specific protein kinase activity of the polyprotein, which activity is necessary for maintenance of the transformed state (Reynolds et al , 1981) However, it is also possible that the carboxy terminal extension is cleaved off during post-translational processing of the polyprotein resulting in the generation of P85 as in the prototype clone As can be seen in Fig 1 (lane 5), a p15-containing protein of 85 kDa can be observed in transformed cells expressing P104, suggesting that such cleavage indeed might occur Whether or not P104 is a transforming polyprotein remains to be solved

In addition to the frameshift point mutation the two clones differed from each other with respect to a number of other point mutations As far as viral oncogene sequences are concerned, these point mutations included one substitution in the p15 encoding sequence, three in that encoding p12 two in that encoding p30 and four in the *fes* sequence Seven of these mutations led to amino acid substitutions in the polyprotein Also the LTRs of the two clones and the helper virus-derived DNA regions downstream of the oncogenes appeared different The difference found in the LTRs appeared to constitute a stretch of 71 nucleotides in the U3 region, from nucleotide position 58 to 134 (Figs 4 and 5) The insert appeared to contain repeats as found in the LTRs of FeLV isolates (Nunberg et al , 1984) Apparently, this stretch does not contain control elements vital for the expression of the viral oncogene

In the helper virus sequences downstream of the oncogene, each clone had a typical deletion The variant clone missed 1420 nucleotides including sequences encoding FeLV gp70 and a small amino terminal portion of p15E The FeLV

genome sequences were flanked by the same seven nucleotides and, therefore, deletion of the FeLV sequences could have occurred during a recombination event involving these seven nucleotides. From the proviral DNA of the prototype ST-FeSV clone, 121 nucleotides encoding the amino terminal portion of p15E were deleted. All deletions appeared to be located in DNA areas not vital for the induction of malignant transformation or helper virus supported replication of the virus. Deletions in genetic regions of acutely transforming RNA tumor viruses that are not essential for replication or transformation are frequently observed.

A question that is not resolved by this study pertains to the way in which the prototype and variant clone of ST-FeSV were generated. As an early event in the generation of acutely transforming RNA tumor viruses, integration of the proviral DNA of a helper virus in the vicinity of a proto-oncogene is proposed. For the two cases discussed here, this would mean integration of FeLV proviruses close to c-*fes* of the cat. The possibility that the original fibrosarcoma arose as the result of the integration of two different FeLV proviruses (because of the different LTRs) close to c-*fes* is unlikely. We, therefore, hypothesize that both clones resulted from the same proviral insertion. Thereafter, two alternative routes can be considered to explain the genetic differences of the two clones. Either the two clones were generated by independent but very similar recombination events between viral and feline c-*fes* genetic sequences, maybe followed by secondary genetic modifications, or they are progenitors from the same ancestral recombination virus that arose in the primary fibrosarcoma of the cat (Snyder and Theilen, 1969). In the latter case, the differences between the two clones would be explained only by secondary genetic modifications.

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III. Molecular cloning of the feline c-*fes* proto-oncogene and construction of a chimeric transforming gene

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Molecular cloning of the feline c-*fes* proto-oncogene and construction of a chimeric transforming gene

(Recombinant DNA; cosmid gene library; malignant transformation; transfection, bacteriophage λ vectors; tyrosine-specific protein kinase)

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SUMMARY

The feline c-*fes* proto-oncogene, different parts of which were captured in feline leukemia virus (FeLV) to generate the transforming genes (v-*fes*) of the Gardner-Arnstein (GA) strain of feline sarcoma virus (FeSV) and the Snyder-Theilen strain (ST) of FeSV, was cloned and its genetic organization determined. Southern blot analysis revealed that the c-*fes* genetic sequences were distributed discontinuously and colinearly with the v-*fes* transforming gene over a DNA region of around 12.0 kb. Using cloned c-*fes* sequences, complementation of GA-FeSV transforming activity was studied. Upon replacement of the 3′ half of v-*fes*^{GA} with homologous feline c-*fes* sequences and transfection of the chimeric gene, morphological transformation was observed. Immunoprecipitation analysis of these transformed cells revealed expression of high M_r fusion proteins. Phosphorylation of these proteins was observed in an in vitro protein kinase assay, and tyrosine residues appeared to be involved as acceptor amino acid.

INTRODUCTION

Two independent isolates of FeSV, GA-FeSV (Gardner et al., 1970) and ST-FeSV (Snyder and Theilen, 1969), appear to have captured genetic sequences from the same feline proto-oncogene (Frankel et al., 1979; Hampe et al., 1982; Groffen et al., 1983b). The genetic locus involved is called c-*fes* (Frankel et al., 1979; Franchini et al., 1981). Nucleotide sequence analysis indicates that the two proviral genomes contain common as well as unique c-*fes* genetic sequences (Hampe et al., 1982). Both virus isolates encode as their major translational products high M_r polyproteins (Khan et al., 1978; Sherr et al., 1978). Serological and tryptic-peptide analyses have indicated that they are related and consist of FeLV *gag*-gene-encoded structural proteins fused to nonstructural components encoded by

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Abbreviations: bp, base pairs; FeLV, feline leukemia virus; FeSV, feline sarcoma virus; GA-FeSV, Gardner-Arnstein strain of FeSV; kb, kilobase(s) or kilobase pair(s); nt, nucleotide(s); PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulphate; S_l, left 0.5-kb *Pst*I-*Pst*I; S_r, right 0.5-kb *Pst*I-*Pst*I DNA fragment of v-*fes*ST; SSC, 0.15 M NaCl, 0.015 M Na_3 citrate, pH 7–8; ST-FeSV, Snyder-Theilen strain of FeSV

the newly acquired cellular genetic sequences (Ruscetti et al., 1980, Van de Ven et al., 1980a, Barbacid et al., 1981) Both polyproteins exhibit tyrosine-specific protein kinase activity (Van de Ven et al., 1980b, Barbacid et al., 1980a) and from analysis of transformation-defective mutants, it appeared that this enzyme activity is required for malignant transformation (Donner et al., 1980, Reynolds et al., 1981) The carboxyl portions of the viral polyproteins resemble tyrosine-specific protein kinases encoded by other retrovirus isolates as well as the catalytic chain of the mammalian cyclic 3 , 5 -adenosine monophosphate-dependent protein kinase (Groffen et al., 1983a) Tyrosine-phosphorylation of the polyproteins themselves has been reported (Van de Ven et al., 1980b, Barbacid et al., 1980a), for the ST-FeSV gene product, this seems to be limited to a single tyrosine acceptor site (Blomberg et al., 1981) An M_r 92000 cellular protein has been identified in cells of cats and related mammals as the putative c-*fes* translational product (Barbacid et al., 1980b) It is likely that this protein has some functional characteristics in common with the v-*fes* polyproteins However, its precise structure and role under normal physiological conditions is still unknown

To study directly the differences between the two transforming v-*fes* oncogenes and their immediate progenitor gene, the normal (non-transforming) feline c-*fes* proto-oncogene, we have cloned all v-*fes*-homologous sequences in the cat genome and present some of their biological and biochemical characteristics

MATERIALS AND METHODS

(a) Cells

Cells were grown in Dulbecco's modification of Eagle's medium supplemented with 10% calf serum (GIBCO) Cell lines used have been previously described and included a highly contact-inhibited mink lung cell line, CC164 (Henderson et al., 1974), a mink cell line nonproductively transformed by GA-FeSV, 64F3 (Sherr et al., 1978), rat-2 cells (Topp, 1981), and the feline fibroblast cell line AZ (kindly provided by A V Lauver)

(b) Gel electrophoresis and hybridization

Restriction endonucleases were purchased from either Boehringer or Bethesda Research Laboratories (BRL) Analysis of restriction endonuclease-digested DNA was essentially as described by Southern (1975) using molecular probes nick-translated to specific activities of about $2-5 \times 10^8$ cpm/μg, according to the method of Rigby et al (1977) Hybridization experiments were routinely performed at 42 C for appropriate periods of time in a 20 mM sodium phosphate buffer (pH 7 0) containing 5 mM EDTA, 50% formamide, 5 x SSC (0 15 M NaCl, 0 015 M Na_3 citrate, pH 7–8), 1 x Denhardt's solution (0 2 mg ml Ficoll, 0 2 mg ml polyvinylpyrrolidone, 0 2 mg ml bovine serum albumin), 100 μg ml denatured salmon testes DNA and $0\ 2-1 \times 10^6$ cpm ml nick-translated DNA Upon hybridization, filters were washed as described (Jones et al., 1980) using 0 1 x SSC at 65 C in the final step and dried, autoradiography was performed by exposing XAR-2 film (Kodak) for up to four days at −70 C with Dupont Lightning Plus intensifying screens

(c) Preparation of DNA probes

The S_1 and the S_r probes, each representing an 0 5 kb *Pst*I-*Pst*I DNA fragment of v-*fes*ST, were obtained from C J Sherr (Franchini et al., 1981) A human c-*fes* probe, PP0 1, representing genetic sequences homologous to GA-FeSV v-*fes* (nt position 1111 to 1227, Hampe et al., 1982) and subcloned in M13mp8 (Messing and Vieira, 1982), was used as an independent 5 -specific *fes* probe (A J M Roebroek, unpublished data) Other molecular probes homologous to v *fes* were prepared from pHH1, a recombinant clone of pBR322 containing the 14 4 kb *Eco*RI DNA insert of λGA-FeSV (Fedele et al., 1981) AS0 6 is an 0 6-kb *Ava*I-*Sal*I DNA fragment, BS1 8 is a 1 8-kb *Bgl*II-*Sal*I DNA fragment, PA0 3 is an 0 3-kb *Pvu*II-*Ava*I DNA fragment and RR0 5 is an 0 5-kb *Rsa*I-*Rsa*I DNA fragment isolated from GA-FeSV proviral DNA (see Fig 1) Upon digestion of DNA with appropriate restriction endonucleases, DNA probes were purified by electrophoresis through low-melting-point agarose (BRL) as described (Weislander, 1979)

(d) Construction of genomic libraries and selection of recombinant clones

To construct a feline gene library of phage recombinants, purified *Eco*RI cellular DNA fragments of about 12 kb were ligated to purified *Eco*RI vector arms of λ Charon 4A Ligation products were packaged in vitro into phage particles (Hohn, 1979), yielding 2.5×10^5 plaque forming units

Construction of a representative feline genomic library in the pJB8 cosmid vector system was performed essentially as described by Ish-Horowicz and Burke (1981) Ligated DNAs were packaged in vitro as described (Hohn, 1979) and propagated in *E coli* 1046 Approx 5×10^5 individual colonies were plated at a density of about 2.0×10^4 colonies/plate (plate diameter 15 cm)

For isolation of recombinant clones containing v-*fes*-homologous cellular sequences, replica filters of both the recombinant bacteriophage and cosmid libraries were screened as described (Benton and Davis, 1977, Hanahan and Meselson, 1980) For screening of the bacteriophage recombinants a combination of two v-*fes*-specific probes, S_1 and S_r, was used In the screening of the cosmid library an 0 7-kb *Eco*RI-*Hin*dIII DNA fragment isolated from pAO89 and representing a unique 5 DNA sequence of the insert of pSV3 (Fig 2) was included as an additional probe (probe EH0 7)

Analytical and preparative isolation of cosmid DNA from selected clones was performed according to a combination of the alkaline lysis procedure of Birnboim and Doly (1979) and the procedure described by Davis et al (1979)

RESULTS

(a) Identification of feline v-*fes*-homologous genetic sequences

To study v-*fes*-homologous genetic sequences within the cat genome, five subgenomic v-*fes* restriction fragments were used as molecular probes S_1, S_r, AS0 6, PA0 3 (GA-FeSV v-*fes*, 5 -specific) and RR0 5 (GA-FeSV v-*fes*, 3 -specific) (Fig 1) Probes were purified and tested for contaminating FeLV-derived genetic sequences, since the genome of the domestic cat (cat No 031182) used in this study contained multiple FeLV related sequences (Fig 1A, lane a) None of the probes revealed hybridization with FeLV sequences in λHF60 (Mullins et al, 1981) (not shown) In Fig 1A, the analysis of high-M_r human and cat cellular DNA digested with restriction endonuclease *Eco*RI is shown A single human *Eco*RI restriction fragment of about 12 kb (Fig 1A, lane b), which had been shown before to contain all human v-*fes*-homologous sequences (Groffen et al, 1982, Franchini et al, 1982), was clearly detected by a combination of all five probes, indicating the specificity of the molecular probes Similarly, strongly hybridizing bands of about 14 and 12 kb were detected in genomic DNA isolated from feline lung tissue (cat No 031182) (Fig 1A, lane c) Only the 12-kb *Eco*RI DNA fragment appeared to hybridize with the 3 -specific *fes* probe RR0 5 (Fig 1A, lane d) and only the 14-kb *Eco*RI DNA fragment with the 5 -specific probe PA0 3 (Fig 1A, lane e) Similar observations were made in an analysis of genomic DNA isolated from a feline fibroblast cell line (AZ) (not shown) These data show that all v-*fes*-homologous sequences within the cat genome are distributed over two *Eco*RI DNA fragments These observations imply, furthermore, the existence of only a single feline c-*fes* locus

(b) Molecular cloning of feline c-*fes* proto-oncogene sequences

For the molecular cloning of the feline c-*fes* sequences, high-M_r AZ DNA was digested with restriction endonuclease *Eco*RI and fractionated by agarose gel electrophoresis Selected DNA fractions were ligated to an equivalent amount of λ Charon 4A *Eco*RI vector arms Multiple c-*fes*-containing recombinant bacteriophages were identified upon initial screening of about 2.5×10^5 plaques with a mixture of the S_1 and S_r probes Five plaques were selected for further analysis after their inserts had been subcloned in pBR322 Restriction endonuclease analysis indicated that the feline DNA inserts in these five subclones were all different (not shown) Only one of them, designated pSV3, appeared to contain a 12-kb DNA insert as detected in genomic blot analysis (not shown)

Nucleotide sequence analysis of the 5 end of the DNA insert of pSV3 revealed the presence of a

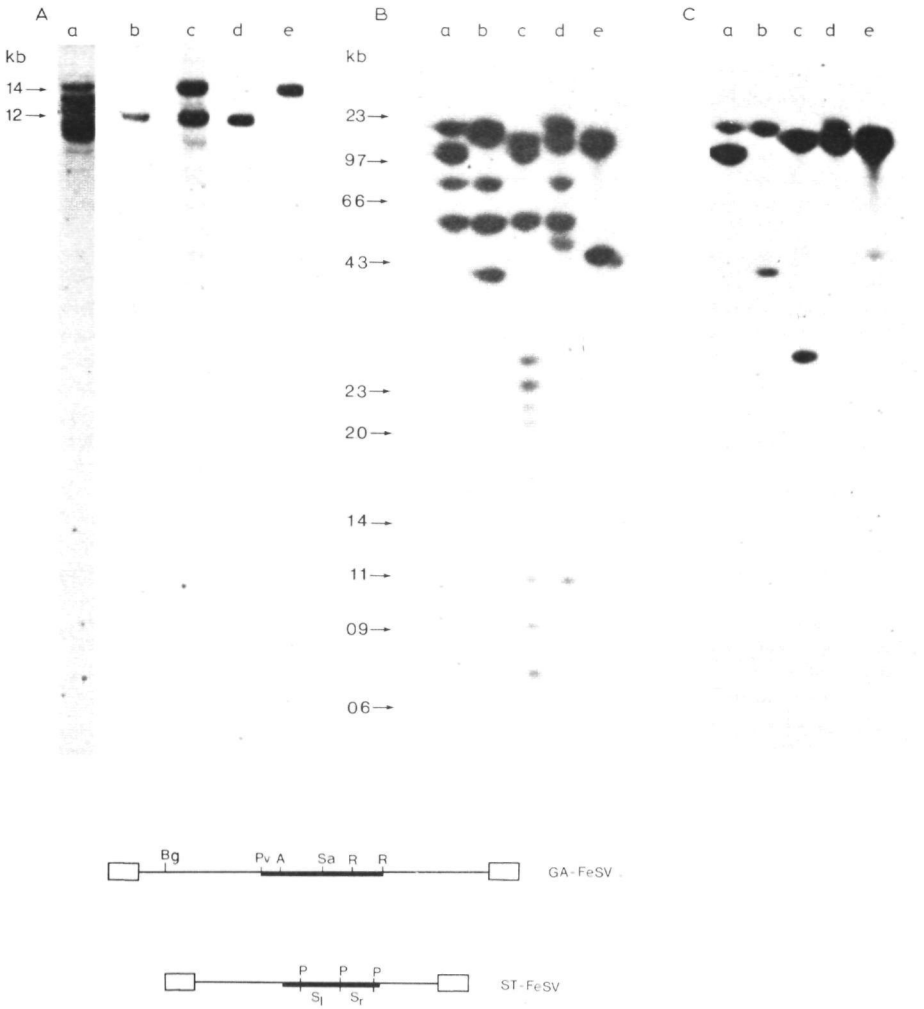

Fig. 1. Identification of v-*fes* sequences by Southern blots. (A) Identification of v-*fes*-homologous genetic sequences in feline DNA. High-M_r DNA was prepared from lung tissue of a domestic cat (No. 031182) (lanes a, c, d and e), or normal human liver (lane b), digested with *Eco*RI and electrophoresed through an 0.8% agarose gel. After transfer of DNA to nitrocellulose, filters were hybridized with nick-translated FeLV proviral DNA (lane a) or a combination of subgenomic v-*fes* probes (lanes b, c, d and e). DNA probes included AS0.6, PA0.3, RR0.5, S_l, and S_r, as schematically indicated below the autoradiogram. The upper map is the GA-FeSV proviral genome. Open boxes represent the long terminal repeats, and the solid bar represents v-fes^{GA}. The lower map is the ST-FeSV proviral genome; sizes and positions of the S_l and S_r probes are indicated. (B) *Eco*RI restriction pattern of DNA isolated from cosmid clones cos1, cos3, cos5 and cos6 and recombinant plasmid pSV3 (lanes a–e, respectively) obtained upon electrophoresis through an 0.8% agarose gel and visualization by hybridization with a mixture of the individual clones as probes. The positions of M_r markers are indicated with arrows in panel B and include *Hin*dIII-digested λ DNA and *Hae*III-digested ϕX174 DNA fragments. (C) Autoradiogram of *Eco*RI restriction pattern shown in panel B upon hybridization with the same combination of subgenomic v-*fes* probes as described under section A. A, *Ava*I; Bg, *Bgl*II; P, *Pst*I; Pv, *Pvu*II; R, *Rsa*I; Sa, *Sal*I.

v-*fes*-homologous region of about 100 nt flanked by consensus splice-site sequences (A J M R , unpublished observation) This putative feline c-*fes* exon corresponds to coding sequences in GA-FeSV proviral DNA from nucleotide position 1447 to 1543, as numbered by Hampe et al (1982) Most of the 400 5 v-*fes*^{GA}-specific nt upstream of nt position 1447 are apparently missing in the 12-kb *Eco*RI DNA fragment and are probably represented in the 14 kb fragment Because of the major rearrangements observed in most of the DNA clones, the λ Charon 4A library was not screened with a 5 -specific v-*fes* probe to isolate the 14-kb *Eco*RI DNA fragment and an alternative approach was chosen for the molecular cloning of the feline c-*fes* locus

To facilitate the isolation of all v-*fes*-homologous sequences in a single recombinant clone, the pJB8 cosmid vector system (Ish-Horowicz and Burke, 1981) was applied for the construction of a representative feline gene library propagated in *E coli* 1046 For the preparation of DNA inserts of 35–45 kb, high-M_r DNA isolated from lung tissue of a domestic cat (cat No 031182) was used Identification of cosmid clones with v-*fes*-homologous DNA inserts was performed with a combination of the S_l, S_r and EH0 7 probe Upon screening of approximately 5×10^5 colonies, four cosmid clones, designated cos 1, 3, 5 and 6, were identified and purified further for restriction analysis *Eco*RI digestion of the recombinant pJB8 cosmid clones resulted in excision of the DNA inserts and thus provided estimates of their sizes Such digestion of the four cosmid clones followed by agarose gel electrophoresis resolved multiple *Eco*RI restriction fragments in all four clones, and the sizes of the various DNA inserts were estimated to lie between 33 and 47 kb (Fig 1B) Further characterization of the DNA inserts in the cosmid clones was performed by hybridization analysis None of the cosmid clones exhibited any homology with the FeLV proviral DNA isolated from λHF60 (not shown) Using the complete GA-FeSV proviral genome as a probe, two hybridizing bands of 14 and 11 kb were observed in cosmid cos1 (Fig 1C, lane a) Cosmid cos3 contained hybridizing bands of 14 and 4 kb, cos5 of 12 and 3 kb, and cos6 of 14 and 12 kb (Fig 1C, lanes b–d) Restriction maps of the inserts were generated and the v-*fes*-homologous genetic sequences within the clones were localized using the DNA probes indicated in Figs 1 and 2 (hybridization data not shown) As summarized in Fig 2, the recombinant phage and the four cosmid clones contain overlapping cellular genetic sequences corresponding to a single contiguous region of cat cellular DNA of about 70 kb The feline c-*fes* sequences are distributed over a DNA region of about 12 0 kb and, in this respect, resemble the human c-*fes* locus Within the latter region, at least two DNA stretches of nonhomology were identified and these probably include intervening sequences It appeared that the 14-kb *Eco*RI fragment contained only GA-FeSV-specific sequences (not shown) Further analysis of the 12 0-kb DNA region with subgenomic v-*fes* probes revealed that the cloned DNA region of the cat contains the complete v-*fes* homolog in an orientation colinear with the viral oncogene (Fig 2)

(c) Construction of a chimeric gene and transfection analysis

To determine whether or not the cloned feline c-*fes* sequences can functionally complement the v-*fes* oncogene, a recombinant between the c-*fes* locus and the GA-FeSV provirus was constructed A *Sal*I cleavage site located in the middle of the v-*fes* gene of GA-FeSV was selected for use in an experiment to replace a major portion of the viral protein kinase domain, including the tyrosine phosphorylation site, with the putative cellular protein kinase domain (Hampe et al , 1982, Groffen et al , 1983a) Construction of the chimeric gene in pAO50 is outlined in Fig 3 and its genetic organization was confirmed by restriction endonuclease analysis

The chimeric gene was transfected (Graham and Van der Eb, 1973, Wigler et al , 1978) into rat-2 cells to test its transforming capacity As a positive control, the insert of pHH1 (i e , the integrated DNA provirus of GA-FeSV subcloned in pBR322) was used Transfection of recombinant plasmids pAO21 and pAO28, which contain v-*fes* or c-*fes* DNA fragments, did not result in morphological transformation of rat-2 cells Neither were foci induced when pSV3 was transfected However, recombinant plasmid pAO50, which contains the 5 half of the GA-FeSV genome fused to the 3 half of the feline c-*fes* DNA region, was able to induce foci in rat-2 cells An efficiency tenfold lower than that observed upon transfection of the GA-FeSV provirus was

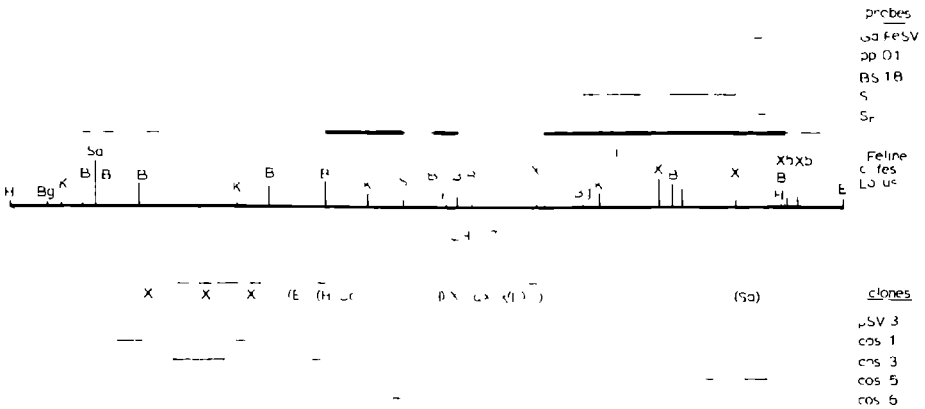

Fig 2 Restriction map of the feline c *fes* region In the center of the figure a schematic restriction map of the 20 kb *Hin*dIII *Eco*RI fragment that contains the feline c *fes* gene is depicted Above the restriction map black segments on a solid line indicated the relative positions of the v *fes* homologous regions within this 20 kb fragment At the top of the figure restriction fragments of the feline c *fes* locus that hybridize to the complete GA FeSV proviral genome pp0 1 BS1 8 S_1 and S_r are indicated The position of the 20 kb *Hin*dIII *Eco*RI fragment within a 70 kb contiguous sequence of feline DNA is indicated by the dashed lines The organization of this 70 kb sequence was deduced as a composite of individually mapped overlapping feline sequences represented within pSV3 and the four cosmid clones The 0 7 kb probe I H0 7 indicated on the map serves also as the scale for the map B *Bam*HI Bg *Bgl*II I *Eco*RI H *Hin*dIII K *Kpn*I S *Sac*I Sa *Sal*I Xb *Xba*I X *Xho*I The restriction map of the 70 kb sequence is incomplete as far as the *Eco*RI and *Hin*dIII sites are concerned as indicated by (E) and (H) symbols

repeatedly found From two independent transfection experiments, transformed clonal cell lines, designated R2-AO50-C11-1 and R2-AO50-C12-1, were obtained upon selection of single cells in microtiter plates Cells of these clones exhibited anchorage-independent growth in soft agar Southern blot analysis of DNA isolated from one such clone, R2-AO50-C12-1, revealed hybridization patterns that could be expected from integration of the intact DNA insert of pAO50 (Fig 3B) Furthermore, hybridization signals indicated the presence of multiple copies of pAO50 These transfection data indicate the possibility that the chimeric gene in pAO50 has transforming potential and that the 3 half of the normal feline c-*fes* locus encodes functional protein kinase components Similar results were reported for the human c-*fes* proto-oncogene (Sodrowski et al 1984)

(d) Immunoprecipitation analysis of translational product(s) encoded by the chimeric gene and in vitro protein kinase activity

To identify and characterize translational product(s) encoded by the chimeric gene, a total lysate from R2-AO50-C12-1 cells was analyzed by immunoprecipitation (Van de Ven et al, 1980a) (Fig 4) Anticipating that the chimeric gene would code for fusion proteins that contain both FeLV *gag*-gene related and *fes*-specific antigenic determinants, conventional antisera prepared against FeLV p15 and GA FeSV P110 and a monoclonal antiserum prepared against GA-FeSV P110 (Veronese et al, 1982) were included With all three antisera, proteins were precipitated with migration characteristics in SDS-polyacrylamide gels (Laemmli 1970) as expected for GA-FeSV-encoded P110 (Fig 4) Furthermore, an 80-kDal protein was detected (Fig 4, lane c), presumably representing a cleavage product of the 110-kDal protein No FeLV-related proteins were precipitated with control sera

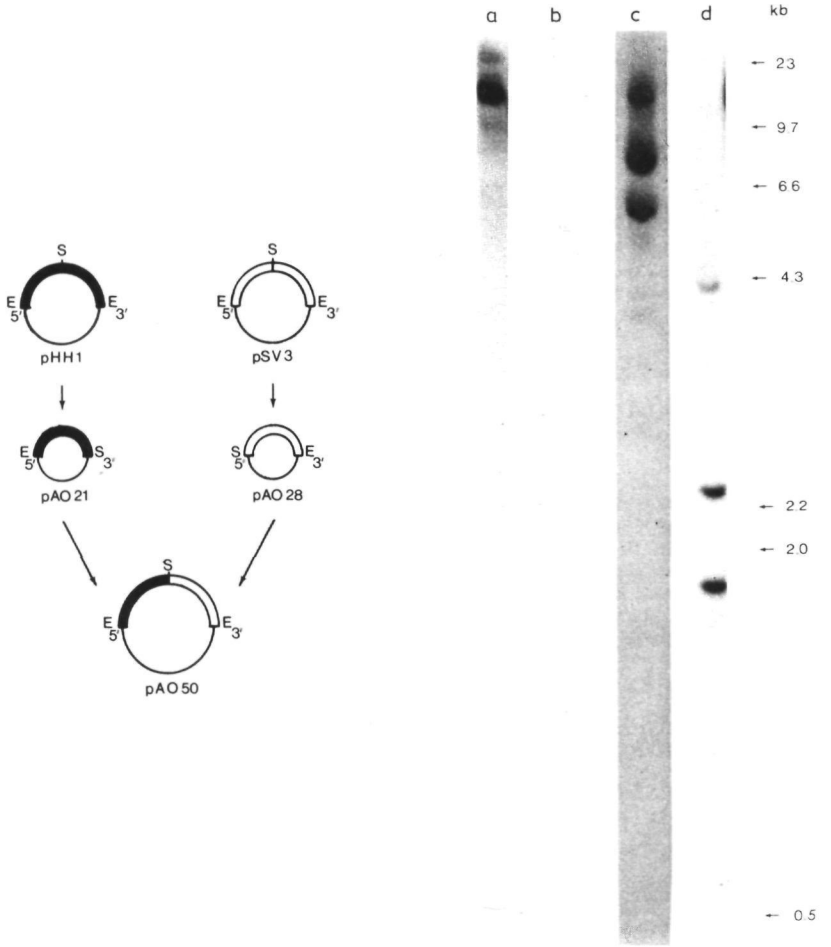

Fig. 3. Construction of a chimeric gene and identification of v-*fes*-homologous sequences. Left panel: plasmid pHH1, containing a single copy of GA-FeSV proviral DNA including mink flanking sequences within a 14.4-kb *Eco*RI DNA insert (black segment), was digested with *Eco*RI + *Sal*I and the v-*fes*-containing *Eco*RI-*Sal*I DNA fragments were isolated for subcloning. Using the orientation of the GA-FeSV genome as a reference, only the 5′ half of the pHH1 insert was subcloned. Similarly, only the 3′ half of the 12-kb feline c-*fes* insert (open segment) of pSV3 was subcloned. pAO50 was constructed as indicated. Plasmid pAT153 was used in all cloning experiments (not indicated). Right panel: identification of v-*fes*-homologous sequences in rat-2 cells transformed by pAO50-derived sequences. DNA was prepared from R2-AO50-C12-1 cells (lanes a, c, and d) and control rat-2 cells (lane b), digested with *Eco*RI (lanes a and b), *Eco*RI + *Sal*I (lane c), or *Eco*RI + *Sac*I (lane d) and examined by Southern blot analysis using the complete v-*fes* oncogene of GA-FeSV as a molecular probe. As M_r markers a *Hin*dIII digest of λ DNA was used (see right margin).

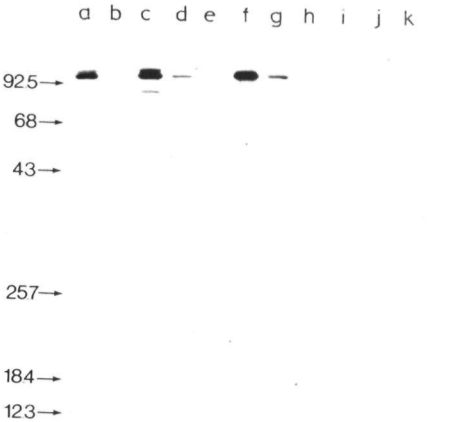

Fig. 4. Immunoprecipitation and SDS-polyacrylamide gel electrophoresis (PAGE) analysis of high-M_r fusion proteins expressed in rat-2 cells upon transfection of pAO50. Cell lines including R2-AO50-C12-1 (lanes a, c, d and e), control rat-2 cells (lanes b, j and k), mink cells nonproductively transformed by GA-FeSV (lanes f, g and h) and control CC164 mink cells (lane i) were metabolically labeled by 4 h incubation in [^{35}S]methionine-containing medium (25 μCi/ml), and proteins were immunoprecipitated with antiserum prepared against GA-FeLV P110 (monoclonal R113-263) (lanes a and b), GA-FeSV P110 (polyvalent) (lanes c, f, i and j), FeLV p15 (lanes d, g and k) or bovine serum albumin (lanes e and h). Immunoprecipitates were analyzed by SDS-PAGE as described in RESULTS, section d. M_r standards include phosphorylase B (92.5 kDal), bovine serum albumin (68 kDal), ovalbumin (43 kDal), α-chymotrypsinogen (25.7 kDal), β-lactoglobulin (18.4 kDal) and cytochrome *c* (12.3 kDal) (see left margin).

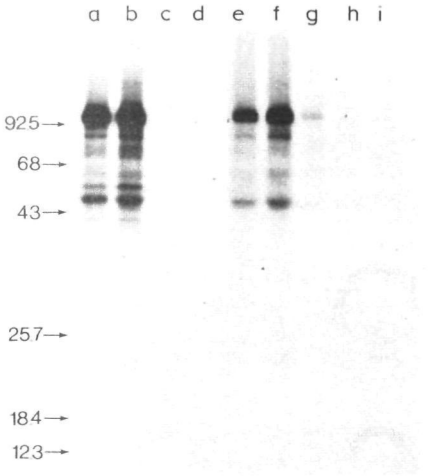

Fig. 5. Analysis of protein kinase activity associated with fusion proteins expressed in rat-2 cells morphologically transformed upon transfection of pAO50. Immunoprecipitates were prepared from extracts of GA-FeSV-transformed mink cells (lanes a–c), CC164 control mink cells (lane d), R2-AO50-C12-1 cells (lanes e–h) and control rat-2 cells (lane i) using antiserum prepared against FeLV virion proteins (lanes a, d, e and i), GA-FeSV P110 (polyvalent) (lanes b and f), FeLV p15 (lane g) or bovine serum albumin (lanes c and h). Using [γ-^{32}P]ATP as substrate, immunoprecipitates were assayed for phosphotransferase activity and analyzed by SDS-PAGE. M_r standards are as described in the legend to Fig. 4.

or found in extracts prepared from untreated rat-2 cells. These data demonstrate that the identified chimeric gene products are fusion proteins similar in structure to the GA-FeSV-encoded polyprotein P110, in that they consist of FeLV *gag*-gene-encoded structural proteins and *fes*-gene-encoded nonstructural components. In an in vitro protein kinase assay, the chimeric gene products were shown to possess such activity (Fig. 5). Phosphoamino acid analysis revealed that tyrosine residues were phosphorylated (not shown). Comparable results have been reported by Sodrowski et al. (1984) with chimeric constructs of the human c-*fes* proto-oncogene and the GA-FeSV viral oncogene.

DISCUSSION

The isolation and characterization of feline c-*fes* proto-oncogene sequences is described in this report. Genomic blot analysis indicated that the feline genome contains a single c-*fes* proto-oncogene. Its isolation was accomplished by construction of two different gene libraries. One library consisting of recombinant λ phages was instrumental in resolving the genetic organization of a major portion of the locus. The genetic organization of the complete v-*fes*-homologous region was finally resolved by analysis of recombinant clones from a cosmid gene library. A contiguous region of feline genomic sequences of 70 kb was isolated and shown to contain all v-*fes*-homologous genetic sequences detected in genomic blot analysis. The c-*fes* sequences were found to be distributed discontinuously over a 12.0-kb region, and colinearity with the v-*fes* onco-

gene was established Nucleotide sequence analysis indicates the presence of extensive noncoding or intervening sequences in the feline c-*fes* locus (A J M R , unpublished observations) With respect to these characteristics, the feline locus closely resembles the previously described human c-*fes* proto-oncogene (Groffen et al , 1982, Franchini et al , 1982) The precise size of the feline c-*fes* coding sequences remains to be determined Based upon the complexity of v-fes^{GA} and v-fes^{ST}, this size is at least 1 95 kb (Hampe et al , 1982) However, sequence analysis of the human and feline c-*fes* locus indicates that some c-*fes* coding sequences have not been captured in either of the viral oncogenes Such sequences have been found between the DNA regions that are homologous to the v-fes^{GA} and the v-fes^{ST} unique sequences and at the very 5 ends of the human and feline c-*fes* loci (A J M R , unpublished observation) Furthermore, the *fps* oncogene of Fujinami sarcoma virus, which represents the avian variant of GA- and ST-FeSV, has a complexity of approx 2 6 kb (Shibuya and Hanafusa, 1982) and the size of c-*fes*-specific mRNA detected in human acute myelogenous leukemia has also been reported to be 2 6 kb (Slamon et al , 1984)

The availability of cloned feline c-*fes* proto-oncogene sequences provided the opportunity to study characteristics of its translational product In an initial approach, the possibility was tested whether or not c-*fes* genetic sequences could functionally complement the protein kinase activity of the viral oncogene product Therefore, a chimeric gene was constructed in which v-*fes* sequences encoding a major portion of the kinase domain were replaced by feline c-*fes* genetic sequences Transfection experiments indicated that this chimeric gene in pAO50 had oncogenic potential Transfected cells exhibited a transformed phenotype, grew in soft agar and expressed a tyrosine-specific protein kinase activity associated with the high-M_r fusion protein These results demonstrate that the carboxyl half of the GA FeSV-transforming protein can be functionally complemented by feline c-*fes* protein components The observation that the fusion protein encoded by the chimeric gene is not only functionally but also structurally highly similar to GA-FeSV P110 suggests that all coding sequences present in that 3 part of feline c-*fes* have been captured in GA-FeSV These feline cellular sequences apparently encode a protein component of approx 33 kDal, and they presumably represent about one-third (carboxyl portion) of the normal cellular protein described by Barbacid et al (1980b) However, the possibility cannot be excluded that deletion of some of the c-*fes*-coding sequence is necessary to allow malignant transformation, as has been described for the *fos* oncogene (Miller et al , 1984) In that case, the same genetic sequences can be expected to be deleted from the viral as well as the newly constructed chimeric oncogene Transfection data, however, are not strongly in favor of this possibility They rather support the hypothesis that preservation of the 3 -proximal sequences that encode the protein kinase domain is a prerequisite for the malignant potential of the locus (Sodrowski et al , 1984) This is in contrast to particular genetic sequences farther upstream in the feline c-*fes* locus whose presence does not seem to be essential for induction of transformation, since both v-fes^{GA} and v-fes^{ST} contain unique oncogene-specific sequences at their 5 ends Furthermore, protein components of approx 25 kDal present in the 92-kDal normal c-*fes* cellular protein seem to be missing in the viral transforming proteins (Barbacid et al , 1980b, Hampe et al , 1982) The role of this NH_2-terminal portion of the *fes* polypeptide is not yet clear However, studies on the v-*fps* gene, which contains a major portion of the avian cellular homolog, may provide some clues Experiments by Foster and Hanafusa (1983) have indicated that without the *gag* gene sequences, the viral *fps* gene can still transform cells in culture and induce tumors in chickens Protein analysis indicates that in the generation of the *fps* viral oncogene some of the 5 c-*fps* sequences were not captured and were replaced by *gag* sequences, resulting in the removal of potential cellular regulatory elements and establishing viral control over the expression of the gene Insertion mutagenesis has identified functional regions in the 5 portion of the transforming gene (Stone et al , 1984) It is possible that the NH_2-terminal part of the protein is not only involved in regulatory functions but also in substrate recognition or in positioning of the protein in a particular cellular compartment Further studies with the molecularly cloned c-*fes* proto-oncogene should define the functional domains of the feline c-*fes* translational product both in its normal form and after the malignant conversion of this gene

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fms, A PROTO-ONCOGENE THAT ENCODES AN INTEGRAL TRANSMEMBRANE GLYCOPROTEIN WITH TYROSINE-SPECIFIC PROTEIN KINASE ACTIVITY

I. Introduction

A The *fms* transducing viruses

The *fms* oncogene has been identified in two independent FeSV isolates with very similar genomic organization SM-FeSV (Susan McDonough strain (1) and HZ5 (Hardy-Zuckermann strain 5) (2) These C type replication defective transforming retroviruses were isolated from spontaneously occurring fibrosarcomas in the domestic cat Cell-free filtrates of the original neoplasms could induce fibrosarcomas in kittens In tissue culture SM-FeSV induces morphological transformation not only in NIH-3T3 cells but also in NRK cells, mink lung epithelial cells and feline embryo fibroblasts (3, 4, 5, 6) In standard DNA transfection assays the molecularly cloned v-*fms* oncogene is biologically active as a transforming gene (3) In both SM-FeSV and HZ5 the same *gag* sequences are fused to 5' sequences of the original cellular *fms* proto-oncogene of the cat, suggesting that recombination between FeSV and c-*fms* may have been facilitated by short regions of homology between the viral *gag* and the 5 part of the cellular gene As a result the primary translation product of the viruses is a fusion polyprotein with a *gag*-coded amino terminal part (7)

B The cellular homolog c-*fms*

The feline, and human cellular homologs of v-*fms* have been molecularly cloned (8, 9, 10, 11) The human, mouse and feline cDNA sequence and the primary structure of the complete coding region of the human gene have been determined (7, 12, 13, 14) The *fms* coding sequences that map to the long arm of human chromosome 5 at band q33 2-q33 3 are arranged in 21 small exons dispersed over 34 kb of genomic DNA (7, 9, 15, 16) A non-coding exon is located 26 kb upstream of the protein coding sequences (7) The 3' end of the PDGF receptor gene is located less than 0 5 kb upstream from this exon Similarities in chromosomal localization, organization, and encoded amino acid sequences suggest that the genes encoding the CSF-1 and PDGF receptors arose through duplication (17)

The putative initiation codon falls within a Kozak consensus sequence for the initiation of translation located 102 nucleotides downstream from the corresponding FeLV recombination site of the feline locus Thus at the *gag-fms* junction the viral fusion polyprotein contains 34 feline c-*fms* derived amino acids that are not normally translated from the proto-oncogene mRNA The distal 40 amino acids of the normal human c-*fms* product are unrelated to the last eleven residues in the truncated carboxyl terminus of the SM-FeSV transforming protein (12, 18) In SM FeSV v-*fms* a 554 bp deletion of the original feline c-*fms* sequences has removed the last 50 codons of feline c-*fms* and allows the open reading frame to continue into 3' untranslated sequences until it terminates 11 codons later (7) The v-*fms* gene of HZ5 has lost only the last 24 amino acids present in c-*fms* and has replaced them with 2 novel residues (2) This extreme

C-terminus is strictly conserved between man, mouse and cat implying a conserved function for this domain A single tyrosine residue (Y969 in man), four residues away from the carboxyl terminus, may represent a negative regulatory site of phosphorylation in the normal c-*fms* gene product The proteins encoded by the SM-FeSV v-*fms* and feline c-*fms* differ by 9 dispersed amino acid substitutions (12, 14)

C The *fms* related gene products

The primary translation product of the full-sized genomic 8 2 kb SM-FeSV RNA is a 170 kDa protein in which 536 amino terminal *gag* coded residues are fused to 975 carboxyl terminal v-*fms* coded amino acids The 536 *gag* residues consist of a 77 amino acids leader sequence that includes a 20 residues long hydrophobic signal for membrane insertion and the complete sequences of the individual *gag* proteins p15, p12 and p30 and the first fourteen amino acids of *gag* protein p10 The *fms* coded part consists of a 34 residues long amino terminal segment, that is not translated from c-*fms* mRNA, followed by a hydrophobic stretch corresponding to the amino-terminal hydrophobic sequence of the c *fms* gene product (12, 19) In about the middle, the *fms* coded part contains a 26 amino acid transmembrane segment on the aminoterminal side of the kinase domain The primary translation product undergoes complex co- and post-translational processing during cellular transport (7, 20, 21) The following model is proposed (21) The nascent polypeptide, synthesized on membrane bound polyribosomes, is co-translationally translocated into the lumen of the rough endoplasmatic reticulum until the second hydrophobic segment of 26 amino acids stops the transfer and anchors the polyprotein in the membrane, thus orienting the distal 406 residues long carboxyl-terminal segment in the cytoplasm In the mean time, the 11 canonical sites for the addition of asparagine (N) linked oligosaccharides, that are clustered in the v-*fms* sequence on the amino terminal side of the transmembrane segment, are accessible to glycosyl transferases within the lumen of the endoplasmatic reticulum This results in a 180 kDa glycosylated polyprotein This gP180*gag-fms* is post-translationally cleaved at a site near the *gag fms* junction to yield a free unglycosylated 55 kDa fragment and a membrane bound 120 kDa glycoprotein The p55 *gag* peptide includes the 34 residues encoded by the 5' untranslated region of the c-*fms* mRNA and the 19 amino acids of the *fms* coded hydrophobic signal sequence Thus this model predicts that the processed v-*fms* and c-*fms* gene products have identical amino termini During the transport through the Golgi complex to the plasma membrane the N-linked carbohydrate chains of gP120v *fms* are processed to complex type oligosaccharides, which results in a mature cell-surface form of the glycoprotein of 140 kDa (20, 22, 23, 24, 25)

The v-*fms* gene products possess tyrosine kinase activity and are capable of phosphorylating themselves and exogenously added substrates The tyrosine specific phosphorylation levels are low in the v-*fms* transformed tissue culture cells (26, 27, 28, 29, 30) Both cell surface expression and kinase activity are required for transformation (27, 31, 32, 33, 34)

The human, murine and feline c-*fms* loci code for mature glycoproteins of respectively 150, 165 and 170 kDa The variation in size of the c-*fms* products in

different species may be do, at least in part, to differences in the pattern and extents of glycosylation (7) They also have an associated tyrosine-specific protein kinase activity (35)

Both the mature v-*fms* and the c-*fms* encoded proteins contain four distinct domains an extracellular (ligand binding) domain, a hydrophobic 26 residue membrane-spanning sequence, a cytoplasmic domain possessing tyrosine kinase activity, and a C-terminal tail The structure resembles that of several growth factor receptors like the EGF (epidermal growth factor) receptor In about the middle of the kinase domain a long stretch of approximately 70 residues is located that is not present in most other kinases The proteins encoded by the *kit, ret* and PDGF receptor genes contain analogous but not identical inserts (36, 37)

The v-*fms*-encoded glycoprotein differs from its cellular counterpart in the following properties

* it is partially blocked in its transport to the cell surface (23)
* it is constitutivily phosphorylated on tyrosine (28, 29)
* it induces tyrosine phosphorylation of heterologous proteins in transformed cells in the absence of the ligand CSF-1 (Colony stimulating factor 1) (27)
* it is relatively refractory to down-modulation induced by phorbol esters (38)

D The proposed functions of the c-*fms* protein

Antisera to a recombinant v-*fms*-coded polypeptide expressed in bacteria precipitate the presumptive c-*fms* product from murine macrophages The 165 kDa glycoprotein is the same size as the murine CSF-1 receptor Purified CSF-1 stimulates tyrosine-specific phosphorylation of the c-*fms* gene product of purified membrane fractions from CSF-1-dependent murine macrophages (21) The v *fms* coded glycoprotein specifically binds murine CSF-1 (39) Murine c-*fms* cDNA encodes a functional CSF-1 receptor (13) NIH-3T3 cells infected with a recombinant retroviral expression vector that expresses the human c-*fms* protein can form colonies in semisolid medium in response to human CSF-1 (40) Co-transfection of vectors expressing c-*fms* and CSF-1 genes induces transformation of NIH-3T3 cells by an autocrine mechanism Thus, it can be concluded that c-*fms* and the gene that encodes the cell surface receptor for the mononuclear phagocyte colony stimulating factor CSF-1 are the same

While the 4 kb *fms* transcript (15) is predominantly expressed in normal monocytes and macrophages(29, 35, 41, 42, 43) the *fms* RNA has also been detected in murine and human placenta and in human choriocarcinoma cell lines derived from malignant placental trophoblasts (29, 42, 44, 45) In the mouse transcription of the CSF-1 gene has been demonstrated in uterine glandular epithelium (46) The levels of uterine CSF-1 production increases during gestation and are highest at parturition (47) Thus in addition to serving as a lineage specific growth factor in hematopoiesis, CSF-1 may also play a role in normal trophoblast development during embryogenesis

The physiologically relevant substrates for the tyrosine specific kinase of the c-fms gene product are unknown Cells transformed by v-*fms* do not show markedly elevated levels of phosphotyrosine in total cellular proteins (7) Tyrosine phosphorylated proteins

have been detected in a CSF-1 stimulated macrophage cell line (26) and in v-*fms* transformed cells (27)

Binding of the ligand CSF-1 to the c *fms* encoded receptor protein stimulates rapidly transient expression of the c-*fos* proto oncogene (48, 49) and stimulates DNA synthesis (50) What signal-transduction pathway is involved is unknown Recently was shown that v-*fms* expression increases *raf*-1 phosphorylation and associated serine/threonine-specific kinase activity It is unclear whether this activation is direct or indirect (51)

E The activation of the oncogenic potential of *fms*

To determine what genetic alterations are related to the oncogenic activation of the *fms* gene, chimeric constructs between feline and human c-*fms* and SM-FeSV v-*fms* sequences were made and specific point mutations analyzed (14, 52)

The transforming ability of the various *fms* constructs was determined in three assays The efficiency of focus formation of the *fms* gene constructs was tested in standard transfection assays The cells that expressed these genes were tested for their ability to form colonies in soft agar and to induce tumors in nude mice On the basis of the results of these assays the constructs can be placed in an ascending order of oncogenicity The normal feline c *fms* gene and a feline c-*fms* gene with all point mutations found in v-*fms* do not form foci and do not form colonies in soft agar A feline c-*fms* gene with the C-terminal modification as found in v-*fms* produced transfectants with a partially transformed phenotype that form low numbers of small colonies in soft agar but cannot induce tumors in nude mice When the feline c-*fms* construct not only contains the C-terminal modification but also an amino acid substitution either at position 301 or 374 the resulting partially transformed transfectants formed tumors after a latency period of 6 to 9 weeks A double mutant that contains both aminoacid substitutions besides the C terminal modification produced fully transformed foci and the transfected cells formed tumors in nude mice with a short latency of 3 to 4 weeks

The efficiency of transformation and colony formation could be increased by adding exogenous CSF-1 or co-transfection of a CSF-1 coding gene In the presence of CSF-1 expression of a construct with a wild type c-*fms* gene results in transformation by an autocrine mechanism However, the c-*fms* construct with the truncated v-*fms* 3 end was more active than the wild-type gene was in inducing transformation by this autocrine mechanism Thus, the mutated forms of *fms* code for functional receptors that are still capable to bind their ligand

When constructs were used with human *fms* sequences the results were slightly different However the experimental procedures were also slightly different The constructs with feline c-*fms* sequences were tested in Rat 2 cells, the human c-*fms* recombinant genes in NIH-3T3 cells To test the effect of the modification of the C-terminus in v-*fms*, in the human c-*fms* constructs only tyrosine residue 969 was substituted by phenylalanine neglecting the influence of the deletion of 50 other residues The important role of this substitution was demonstrated with a chimeric construct that contains v-*fms* with a human wild-type 3' end The chimera was non-transforming But loss of Tyr969 abrogated the inhibitory effect of the human C-terminus

(40) The use of this type of chimeras is quite complicated because of the species specific amino acid substitutions that are brought together in new combinations The overall homology between human c-*fms* and v-*fms* is 84% Species specific differences in the transforming activities have also been reported for several *src* mutants (53)

A human c-*fms* gene in which leucine residue 301 was substituted by serine (as is the case in v-*fms*) induced morphological transformation, anchorage-independent growth, and tumorigenicity in nude mice Substitution of Tyr969 by phenylalanine augmented the transforming efficiency of mutant ser301 although insufficient to induce transformation by itself Analysis of the protein products of the different constructs showed that the block to glycosylational processing seen with the viral protein is due to the point mutations present in the v-*fms* gene These point mutations result also in the constitutive phosphorylation of the mature cell surface form of the receptor on tyrosine Pulse-chase experiments showed that the half-lives of the v-*fms* and c-*fms* proteins are similar but that the processing of the v-*fms* proteins is both slower and less efficient The C-terminal modification increases the relative kinase activity, the point mutations alone do not do so

From these data it can be concluded that both substitution of leucine 301 and deletion of the carboxyl terminus are important for the activation of the transforming potential of the c-*fms* protein Conversion of leucine 301 to serine in human c-*fms* is an activating mutation, whereas elimination of tyrosine 969 disrupts a negative regulatory restraint normally imposed by sequences at the receptor carboxyl terminus It was shown that the v-*fms* encoded glycoprotein also retains a high-affinity binding site for CSF-1 and is able to respond biologically to the growth factor So in its natural host both autocrine and non-autocrine mechanisms may contribute collaboratively to tumor formation

The c-*fms* gene was also found to be a target for insertional mutagenesis by F-MuLV (Friend murine leukemia virus) (54) This strongly suggests that aberrant expression of the c-*fms* gene can serve as an initiating event in leukemogenesis

A significant subset of human AMLs (acute myelogenous leukemias) that lack evidence of monocytic differentiation shows an aberrant c-*fms* expression The mechanism of activation is unknown (7) Because the genes for human CSF-1 (55), GM-CSF (56), IL-3 (57), Il-5 (58), PDGF-R (59), αFGF (acidic fibroblast growth factor) (60) and c-*fms* (CSF-1R) (15), all map to chromosome 5q (22) any of these genes might potentially be implicated in the pathogenesis of the '5q- syndrome' (61) and several forms of AML (62, 63, 64)

The transforming activity of v-*fms* has also been studied *in vivo* Mouse bone marrow cells infected with SM-FeSV were transplanted into lethally irradiated mice Several primary recipients developed splenomegaly within a month or myeloproliferative disorders that were provirus-positive without evidence of clonality Secondary recipients transplanted with spleen cells from provirus positive primary recipients developed clonal erythroleukemias or B cell lymphomas expressing the v-*fms* encoded glycoprotein Thus, v-*fms* can contribute to proliferative abnormalities of multiple hematopoietic lineages (65)

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II. Molecular cloning and characterization of feline cellular genetic sequences homologous to the oncogene of the McDough strain of feline sarcoma virus

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Molecular Cloning and Characterization of Feline Cellular Genetic Sequences Homologous to the Oncogene of the McDonough Strain of Feline Sarcoma Virus

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The organization within the cat genome of cellular genetic sequences homologous to the viral oncogene v-*fms* of the McDonough strain of feline sarcoma virus (SM-FeSV) was determined Four cosmid clones containing overlapping v-*fms* homologous cellular DNA inserts representing a contiguous region of cellular DNA of approximately 80 kbp in length have been isolated from a feline cosmid gene library Within this region of the cat genome, the c-*fms* genetic sequences are dispersed over a region of around 30 kbp and are interspersed with at least three intervening sequences

The McDonough strain of feline sarcoma virus (SM-FeSV) represents an acutely transforming RNA tumor virus that was initially isolated from a naturally occurring fibrosarcoma of a domestic cat (*10*). Its genome contains feline leukemia virus (FeLV) as well as cellular genetic sequences of cat origin (*4, 7, 8*). SM-FeSV resembles the majority of other mammalian and avian transforming retroviruses in that it is replication defective (*1, 14, 16*) The primary translational product of SM-FeSV is a polyprotein of 170,000 molecular weight, $P170^{gag\text{-}fms}$, and consists of amino terminal structural proteins encoded by the FeLV *gag* gene and a carboxyterminal component encoded by the acquired feline cellular sequences (*1, 14, 16*). With respect to its function *in vivo*, polyprotein $P170^{gag\ fms}$ lacks tyrosine-specific protein kinase activity (*2, 13, 14, 16*) although sequence homology with tyrosine-specific protein kinases encoded by other viral oncogenes such as v-*fes* and v-*src* has been observed (*7*). *In vitro*, tyrosine phosphorylation of $P170^{gag\text{-}fms}$ has been reported (*2*).

As an initial approach to determine the complexity of the feline genetic sequences homologous to the viral transforming gene v-*fms*, Southern blot analysis was performed (*15*). Analysis of genomic cat DNA (cat No 031182) digested with restriction endonuclease *Eco*RI and hybridized with a ^{32}P-labeled FeLV-specific probe (a 5.3-kbp *Xho*I/*Xho*I DNA fragment representing *gag*- and *pol*-specific genetic sequences isolated from λ-HF60 which contains the entire proviral genome of FeLV (*12*)) revealed numerous strongly hybridizing DNA fragments. This observation indicates the presence of multiple FeLV-related genetic sequences (Fig. 1, lane A). For analysis of the feline v-*fms* homologous genetic sequences, subgenomic DNA fragments as indicated in the upper part of Fig. 1 were used. These included three *Pst*I/*Pst*I restriction fragments of about 0 4, 0.7, and 1 45 kbp in size, a *Sma*I/*Pst*I DNA fragment of 0.4 kbp, and a 1.2-kbp *Hae*II/*Hae*II DNA fragment which is localized at the 5′ portion of v-*fms*. Based upon DNA sequence analysis of v-*fms*, it appeared that the 1.45-kbp DNA fragment contains about 300 bp of FeLV-specific genetic sequences at its 3′ position (*7*). It should be noted that about 40 nucleotides of the very 5′ end of v-*fms* were not

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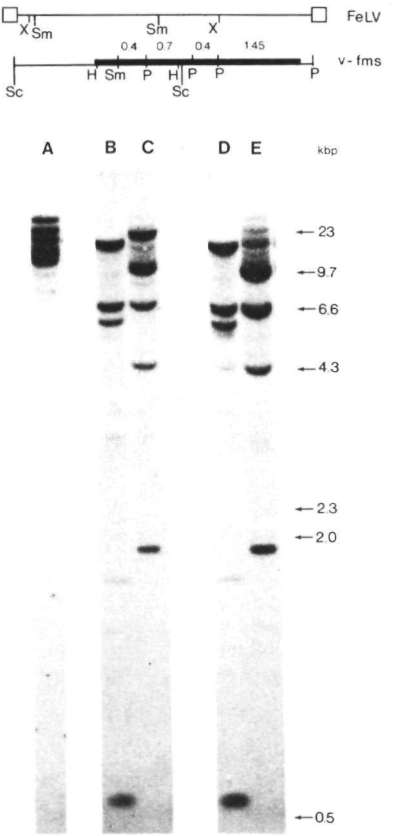

FIG. 1. Identification of v-*fms* homologous genetic sequences in cat cellular DNAs. High-molecular-weight DNAs were prepared from lung tissue of two different cats (cat No. 031182 (lanes A, D, E) and cat No. 8410141 (lanes B, C)), digested with restriction endonuclease *Eco*RI (lanes A, C, E) or *Bam*HI (lanes B, D) and electrophoresed through an 0.8% agarose gel. After transfer of DNA to nitrocellulose, filters were hybridized with a ^{32}P-nick-translated FeLV *Xho*I/*Xho*I probe (lane A) or a combination of four v-*fms* probes, including the 0.4-kbp *Sma*I/*Pst*I, the 0.7-kbp *Pst*I/*Pst*I, the 0.4-kbp *Pst*I/*Pst*I, and the 1.45-kbp *Pst*I/*Pst*I DNA fragment (lanes B–E). Molecular probes used are indicated in the upper part of the figure. Molecular-weight markers included are *Hin*dIII-digested λDNA fragments. H, *Hae*II; P, *Pst*I; Sc, *Sac*I; Sm, *Sma*I; X, *Xho*I.

included in any of the probes used in the genomic blot analysis. Total high-molecular-weight DNA of two different cats digested with restriction endonuclease *Bam*HI (Fig. 1, lanes B, D) or *Eco*RI (Fig. 1, lanes C, E) and hybridized under stringent conditions with a combination of the first four v-*fms* probes mentioned above disclosed a number of strongly and weakly hybridizing bands. As far as the strongly hybridizing DNA fragments are concerned, hybridization patterns were highly similar for both cats using restriction endonuclease *Bam*HI. They included DNA fragments of 15, 6.5, 5.5, 1.6, and 0.7 kbp. A possible restriction fragment-length polymorphism was detected using restriction endonuclease *Eco*RI. An 16-kbp DNA fragment detected in the DNA of cat No. 031182 was missing in the DNA of cat No. 8410141; however, stronger hybridization was observed in the DNA of the latter with DNA fragments of 9.7 and 6.6 kbp. In an analysis with only the 1.2-kbp *Hae*II/*Hae*II DNA fragment as a 5′-specific v-*fms* probe, hybridization was detected with a 0.6-kbp *Bam*HI and a 3.7-kbp *Eco*RI DNA fragment (data not shown). The patterns of the weakly hybridizing DNA fragments were different for the two cats. Southern blot analysis using individual probes revealed that most of these weakly hybridizing DNA fragments could only be detected with the 1.45-kbp probe (data not shown) which contains some FeLV-specific genetic sequences. Furthermore, these DNA fragments seem to comigrate with those detected with the FeLV probe and, therefore, they most likely represent FeLV-related genetic sequences.

The numerous feline DNA *Eco*RI or *Bam*HI restriction fragments hybridizing to v-*fms*, which itself has a complexity of only 3.0 kbp, could reflect the presence of extensive intervening sequences within the feline c-*fms* locus. The genomic blot analysis further indicates that the complexity of the feline c-*fms* locus can be estimated to be between 30 and 40 kbp in size, if these genetic sequences are confined to a single locus.

To facilitate a more detailed structural analysis of feline c-*fms*, a gene library of feline genomic DNA was constructed using the pJB8 cosmid vector system (*9*). In Fig.

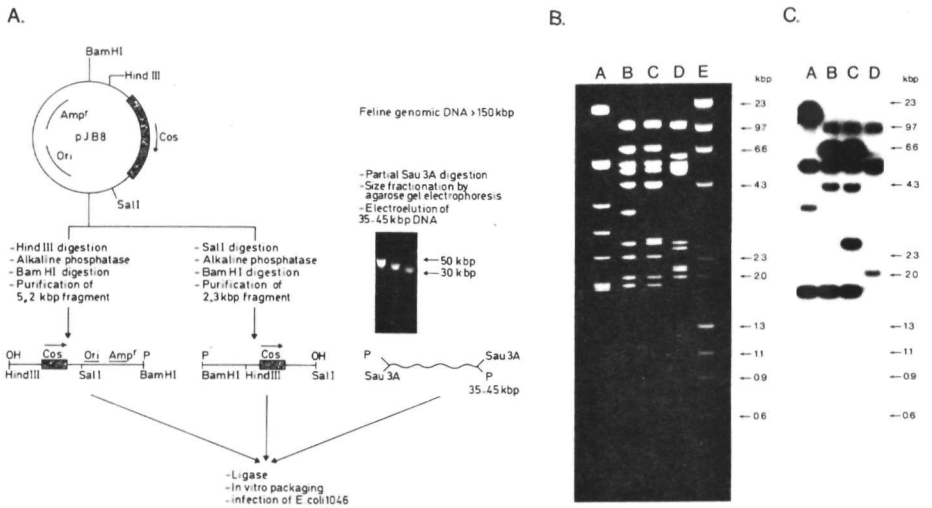

FIG. 2. Characterization of cosmid clones isolated from a feline cosmid gene library. (A) Feline *cosmid gene library construction in the* pJB8 *cosmid vector system.* DNA *isolated from lung* tissue of cat No. 031182 was used. (B) *Eco*RI restriction pattern of cosmid clones 9, 11, 14, and 18 (lanes A, B, C, and D, respectively) obtained upon electrophoresis through an 0.8% agarose gel and ethidium bromide staining. Molecular-weight markers included are *Hind*III-digested λDNA fragments (lane E). (C) Autoradiogram of *Eco*RI restriction pattern as shown in (B) upon hybridization with a combination of five ^{32}P-nick-translated v-*fms* probes, including the 3.0-kbp *Sac*I/*Sac*I, the 0.4-kbp *Sma*I/*Pst*I, the 0.7-kbp *Pst*I/*Pst*I, the 0.4-kbp *Pst*I/*Pst*I, and the 1.45-kbp *Pst*I/*Pst*I DNA fragments. Positions of the molecular probes within the SM-FeSV proviral genome are indicated in Figs. 1 and 3.

2A, a schematic representation of the procedures involved is depicted. From the cosmid library, a total of five clones, designated PFLL-clones 9, 11, 14, 16, and 18, have been identified upon screening of about 2.8×10^5 bacterial colonies with the 1.45-kbp *Pst*I/*Pst*I v-*fms* probe and these have been propagated for further analysis. Upon screening with a subclone of pBR322 containing the entire FeLV proviral genome, PFLL-clone 16 appeared to contain FeLV-related genetic sequences. None of the four other cosmid clones exhibited detectable hybridization with this FeLV probe (data not shown).

Restriction endonuclease *Eco*RI digestion of cosmid clones 9, 11, 14, and 18 followed by agarose gel electrophoresis and ethidium bromide staining of the DNA fragments gave a first characterization of the DNA inserts in the various clones (Fig. 2B), since the pJB8 cosmid vector possesses *Eco*RI cleavage sites on both sites next to its *Bam*HI cloning site. From this analysis, it appeared that the size of the cellular DNA inserts in the various cosmid clones varies between 35 and 41 kbp in length. Two of the cosmid clones, PFLL-clones 11 and 14, showed a highly similar pattern of *Eco*RI restriction endonuclease fragments, indicating the clones contain almost the same feline genetic region. For further characterization, five molecular probes with specificity for subgenomic regions of v-*fms* were used (Fig. 2C). As shown in Fig. 3, these probes (a–e) included in addition to some of those used in the genomic blot analysis a 3.0-kbp *Sac*I/*Sac*I probe containing the 5′ portion of v-*fms* (about 1.2 kbp) and some FeLV *gag*-gene-specific genetic sequences. It should be noted that the hybridizing 5.3-kbp DNA fragment present in all four clones represents cosmid vector pJB8.

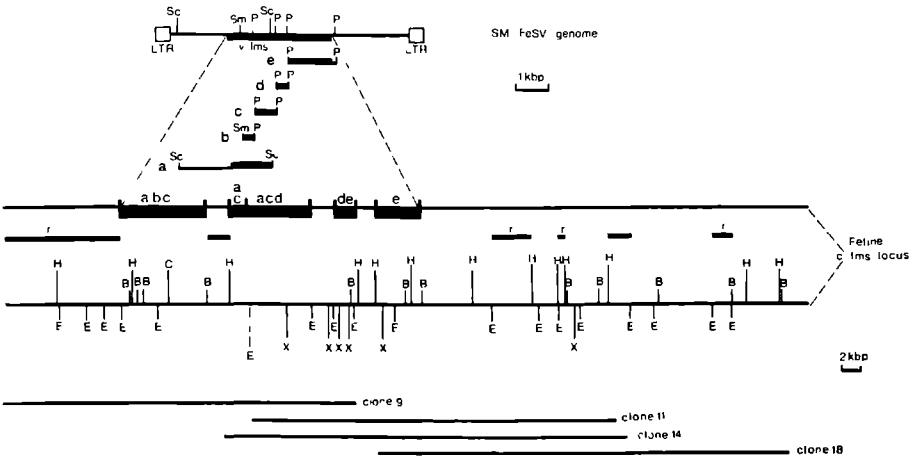

FIG 3 Restriction endonuclease map of the feline c-*fms* region In the upper part of the figure, the SM-FeSV proviral genome is shown The open boxes represent the long terminal repeats, the solid bar the acquired feline cellular sequences representing v-*fms*. Directly below the SM-FeSV genome, subgenomic restriction endonuclease fragments (a–e) used as probes in this study are indicated On a separate line, feline DNA restriction endonuclease fragments homologous to v-*fms* genetic sequences are represented as solid boxes Above each region, probes that exhibit homology are indicated Boxed areas labeled with "r" directly above the restriction endonuclease map indicate highly repetitive sequences detected in the feline c-*fms* region The *Eco*RI site involved in the restriction fragment-length polymorphism is indicated with a broken line Beneath the map, cellular inserts within cosmid clones 9, 11, 14, and 18 are shown B, *Bam*HI, C, *Cla*I, E, *Eco*RI, H, *Hin*dIII, X, *Xho*I

Clone 9 appeared to contain hybridizing *Eco*RI fragments of 16, 3.7, 1.9, and 0 65 kbp Similar analysis of clone 11 revealed hybridizing *Eco*RI fragments of 9.7, 6.6, 4.3, and 1 9 kbp Clone 14 which contains a DNA insert highly similar to the one in clone 11 appeared to contain an additional 3.0 kbp hybridizing DNA fragment. In clone 18, v-*fms* homologous *Eco*RI fragments of 9 7 and 2.1 kbp were observed. Restriction maps of the cosmid clones were generated using various combinations of the restriction endonucleases *Eco*RI, *Bam*HI, *Hin*dIII, *Xho*I, and *Cla*I. Furthermore, in Southern blotting analysis using individual probes, the v-*fms* homologous genetic sequences within the cosmid clones were localized in more detail (data not shown). As summarized in Fig. 3, the four cosmid clones contain overlapping cellular sequences corresponding to a single contiguous region of cat cellular DNA of about 80 kbp in length. Within a 30-kbp region, four distinct regions of v-*fms* homology, interspersed by at least three nonhomologous regions representing probably intervening sequences, were identified Furthermore, the feline sequences homologous to v-*fms* are colinear with the viral v-*fms* oncogene. With respect to the size of the DNA region over which the v-*fms* homologous sequences are distributed, the feline c-*fms* locus resembles the human locus (unpublished observations; *8*)

To establish whether the complete feline v-*fms* homolog is represented in a nonrearranged way in clones 9, 11, 14, and 18, the cloned sequences were compared with the DNA fragments detected in the genomic blot analysis Special attention was given to the 5′ portion of v-*fms* since a probe for the very 5′ part of the locus was excluded from the genomic blot analysis, initially. Comparison of Figs. 1 and 3C reveals that, with the exception of a

0.7-kbp *Bam*HI fragment, all hybridizing *Bam*HI and *Eco*RI restriction fragments observed in the genomic blot analysis are present in the four cosmid clones. These also include the 0.6-kbp *Bam*HI and the 3.7-kbp *Eco*RI DNA fragments detected with the 5′ v-*fms*-specific *Hae*II/*Hae*II probe (data not shown). The 0.7-kbp *Bam*HI DNA fragment appeared to be FeLV related, since it hybridized to a probe representing the complete FeLV provirus and it could also be detected in cosmid clone 16 (data not shown) which contains FeLV-specific sequences.

The presence of a restriction fragment-length polymorphism in the DNA of the two cats, as observed in the genomic blot analysis, has been confirmed by analysis of the cosmid clones. An *Eco*RI restriction site in cosmid clone 14 is missing in clone 9 and, as a result, a 16-kbp *Eco*RI DNA fragment is present in clone 9.

To determine whether genetic sequences homologous to the 5′ end of v-*fms* are also present in cosmid clone 9, the 3.7-kbp *Eco*RI DNA fragment was further analyzed (Fig. 4). Restriction endonuclease digestion of the 3.0-kbp *Sac*I/*Sac*I DNA fragment of the SM-FeSV proviral genome with *Hae*II generates a 1.8-, 1.2-, and 0.07-kbp DNA fragment and digestion with *Bal*I results in DNA fragments of 1.2, 0.9, 0.7, and 0.2 kbp. The 1.8-kbp *Hae*II DNA fragment contains the first 38 nucleotides of v-*fms* and the 0.2-kbp *Bal*I DNA fragment contains the first 130 nucleotides (*7*). Using the 3.7-kbp *Eco*RI DNA fragment isolated from cosmid clone 9 as a probe in Southern blot analysis of the DNA fragments mentioned above, the observed hybridization pattern indicated cosmid clone 9 contained most if not all of the 5′ v-*fms* homologous genetic sequences (Fig. 4B, lanes A and B). From the results obtained in this study it can be concluded that the cat genome contains a single v-*fms* homologous protooncogene which is completely represented in the four isolated cosmid clones.

In a further characterization of the feline c-*fms* region, the localization of highly repetitive feline genetic sequences was determined in a hybridization analysis using total cat DNA as a probe. Only regions with strong homology are indicated in Fig. 3.

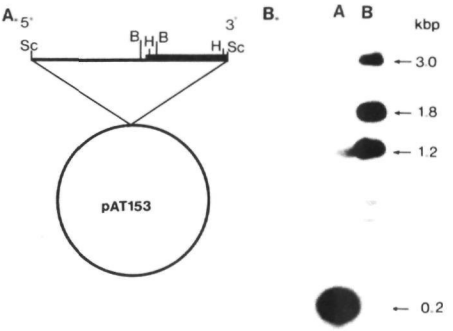

FIG. 4. Presence of genetic sequences homologous to the very 5′ end of v-*fms* in cosmid clone 9. (A) Restriction endonuclease map of the 3.0-kbp *Sac*I/*Sac*I subgenomic v-*fms* DNA fragment subcloned in pAT153. The thicker bar at the 3′ end represents 1.2 kbp of the 5′ end of v-*fms*. (B) Purified 3.0-kbp *Sac*I/*Sac*I DNA was digested with *Bal*I (lane A) or partially digested with *Hae*II (lane B) and electrophoresed through an 0.8% agarose gel. After transfer of DNA to nitrocellulose, the filter was hybridized with the ^{32}P-nick-translated 3.7-kbp *Eco*RI DNA fragment obtained from cosmid clone 9. Molecular-weight markers included are *Hae*III-digested ϕX174 DNA fragments. B, *Bal*I; H, *Hae*II; Sc, *Sac*I.

Characterization of feline c-*fms* using v-*fms* as a tool may lead to an underestimation of the complexity of c-*fms*. It is possible that during the original generation of the transforming viral oncogene or during subsequent diversion, substantial deletions have been introduced in v-*fms*, as can be seen with the oncogenes of the Gardner–Arnstein and Snyder–Theilen strain of feline sarcoma virus (*6*, *17*). Other protooncogene loci such as c-*fos*, c-*myc*, and c-*ras* have been reported to contain exon sequences not found in their related viral oncogenes (*3*, *5*, *11*). In addition, in a particular isolate of Abelson murine leukemia virus, about 800 bp of coding sequences, present in another isolate, are missing (*18*). It is possible that c-*fms* genetic sequences have been ex-

cluded from v-*fms*, in a similar way. The availability of molecular clones of feline c-*fms* will facilitate experiments to resolve this question and it is clear that precise mapping of all feline c-*fms* coding sequences within the 30-kbp region will require extensive DNA sequence analysis

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III. Molecular cloning and characterization of human cellular genetic sequences homologous to the oncogene of the McDough strain of feline sarcoma virus

A A hereditary abnormal c-*fms* proto-oncogene in a patient with acute lymphocyte leukaemia and congenital hypothyroidism

British Journal of Haematology 1985 61 135–138

A hereditary abnormal c-*fms* proto-oncogene in a patient with acute lymphocytic leukaemia and congenital hypothyroidism

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SUMMARY A patient with congenital hypothyroidism and acute lymphocytic leukaemia was found to be homozygous for a 0 4 kbp deletion in the c-*fms* proto-oncogene This was established by studying DNA from the patient's leukaemic cells, from cultured skin fibroblasts of the patient and from normal white blood cells of both parents The uncertain relevance of this finding to the condition of the patient is discussed

Proto-oncogenes are thought to fulfill important functions in growth regulation and development of multicellular organisms (Mueller *et al* 1982) They have also been implicated in tumorigenesis (Cooper 1982) In that case however, specific genetic changes such as point mutations or DNA rearrangements seem to be required (Yunis 1983)

Mutations can cause restriction fragment length polymorphisms (RFLP) that can be detected by Southern blot analysis Using the v-*fms* viral oncogene of the McDonough strain of feline sarcoma virus (SM FeSV) as a molecular probe we have analysed genomic DNA of four normal individuals and 12 cancer patients and found an abnormal form of the c-*fms* proto-oncogene in DNA of one patient

RESULTS AND DISCUSSION

The abnormal proto-oncogene was found in the DNA of a 16 year old boy who was under treatment for congenital hypothyroidism during the last 14 years and who developed an acute lymphocytic leukaemia A cell rich bone marrow aspirate showed 82% of the cells to be lymphoblasts 80% of which were positive in an immunofluorescence assay for the common ALL antigen T-cell or B-cell markers were absent Chromosome analysis of the leukaemic blood cells revealed the presence of 50 chromosomes in 13 of 14 analysed mitoses Nine metaphases showed trisomies of the chromosomes 3 8 10 and 17 All metaphases exhibited

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the presence of two chromosomes 5 on which the human c-*fms* proto-oncogene has been localized (Groffen *et al*, 1983).

Initially, restriction endonuclease analysis indicated the presence of multiple RFLPs. As can be seen in lane A of Fig 1, Southern blot analysis revealed seven bands of hybridization in

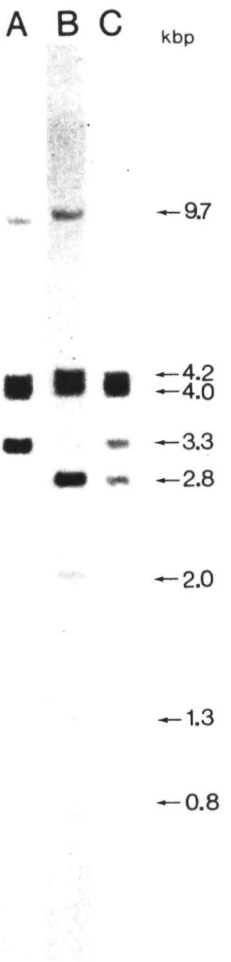

Fig 1. Analysis of human DNAs for restriction fragment length polymorphisms in genetic sequences homologous to the viral oncogene v-*fms* of the McDonough strain of feline sarcoma virus. High molecular weight DNAs were prepared from normal human liver tissue (A), from leukaemic blood cells of the patient (B), from blood cells of his mother (C). 20 μg DNA samples were digested with restriction endonuclease BamHI; DNA fragments were size selected by electrophoresis through an 0·8% agarose gel, transferred to nitrocellulose and analysed by hybridization using ^{32}P-nick-translated v-*fms* as a molecular probe. Molecular weights of human c-*fms* fragments were deduced from co-electrophoresed restriction endonuclease HindIII digested λ DNA.

human liver DNA cleaved with restriction endonuclease BamHI. Their sizes were 9·7, 4·2, 4·0, 3·3, 2·0, 1·3 and 0·8 kbp. The same hybridization pattern was observed in a restriction endonuclease BamHI digest of DNA isolated from the leukaemic blood cells of the patient, except that the 3·3 kbp band is missing and a new 2·8 kbp band was detected (lane B). Similar results were obtained using restriction endonuclease EcoRI and HindIII (data not shown). Molecular cloning of both the normal and abnormal c-*fms* loci and heteroduplex analysis (Figs 2A and 2B) indicated the observed RFLPs to be induced by a deletion. (For a schematic representation of the data see restriction map in Fig 2C.)

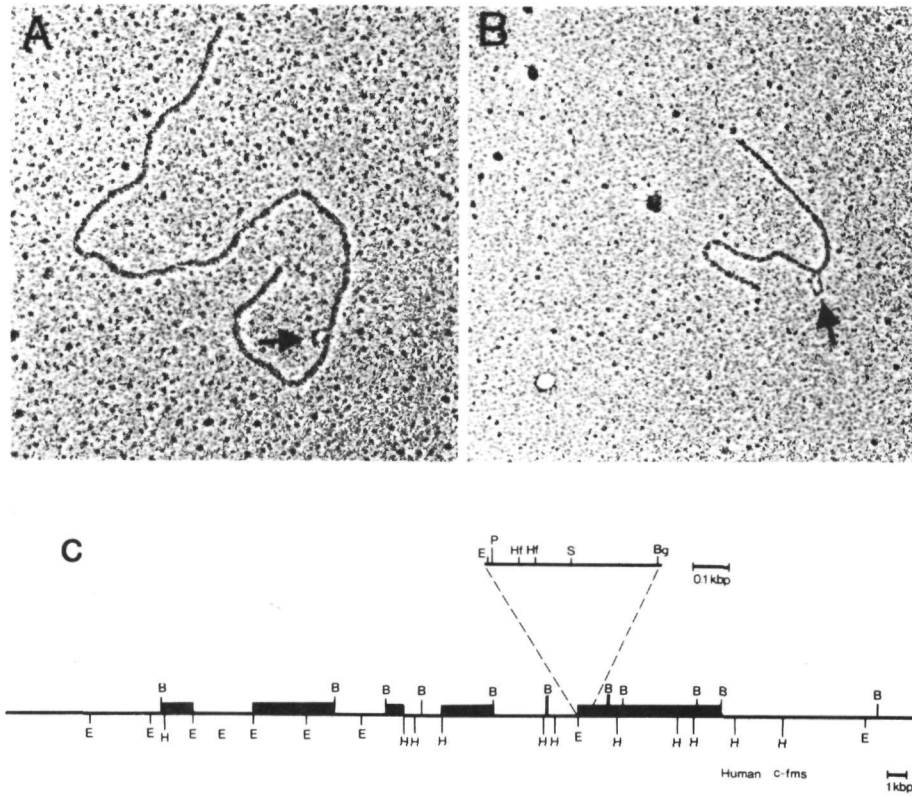

Fig 2. (A) Heteroduplex analysis of the 3·3 and 2·8 kbp BamHI DNA fragments isolated from the human c-*fms* locus and moleculary cloned in pBR322. Recombinant plasmids were linearized by digestion with restriction endonuclease SalI. (B) Heteroduplexes of the purified DNA inserts described under (A). (C) Schematic restriction endonuclease map of human c-*fms*. DNA fragments homologous to v-*fms* are represented as heavy bars. In the upper part of the figure a restriction map of the DNA sequences not found in the abnormal c-*fms* proto-oncogene is shown. The position of these sequences in the normal human c-*fms* locus is indicated by dotted lines. The two BamHI restriction sites used in the molecular cloning of the 2·8 and 3·3 kbp DNA fragment are indicated by prominent marking lines. B, BamHI; Bg, BgIII; E, EcoRI; H, HindIII; Hf, HinfI; P, PstI; S, Sau3A.

Based upon detailed Southern blot analysis of the DNA of the leukaemic blood cells it was concluded that these cells were homozygous for the abnormal c-*fms* gene The observation that in the DNA of the patient s leukaemic blood cells the normal c-*fms* allele was absent was of interest The possibility that highly similar or identical somatic mutations had occurred independently in both chromosomes is not very likely Furthermore a monosomy of chromosome 5 could be excluded based upon the chromosome analysis Further study revealed that the apparent homozygosity for the observed genetic change was inherited Southern blot analysis was performed with DNA isolated from skin fibroblasts of the patient and with DNA from each parent The same restriction fragment pattern as observed in the DNA of the patient s leukaemic blood cells was also observed in DNA isolated from his skin fibroblasts (data not shown) Furthermore each parent appeared to have both a normal and an abnormal c *fms* allele (Fig 1 lane C) (data DNA of father not shown)

It has been suggested that the c-*fms* encoded protein(s) may have a crucial role in mammalian development Relatively high levels of expression of c-*fms* have been reported for human placenta Expression of c-*fms* in human amnion and chorion cells appeared somewhat lower and that in a number of other organs including lymph nodes insignificant (Mueller *et al* 1983) In this respect the observation of congenital hypothyroidism in an individual homozygous for an inherited abnormal allele of the c-*fms* locus is of particular interest, although it may be accidental A possible correlation with the observed acute lymphocytic leukaemia also remains questionable Further analysis is required to establish the direct effect of the deletion on the function of the c-*fms* translational product(s) Identification of more individuals homozygous for this trait should be of value in determining the functional significance of the abnormal c *fms* proto-oncogene in the human genetic pool

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ADDENDUM

To determine the distribution of the c-*fms* RFLP, DNA from cancer patients and healthy individuals was analysed by Southern blot hybridisation with a v-*fms* DNA probe.

The genomic DNA from 31 unrelated healthy individuals and 27 cancer patients has been tested. The tumors tested were leukemias, Gravitz -, Wilms -, lung - and thyroid tumors.

The results are summarized in tabel 1.

Tabel. 1. Distribution of the *fms* alleles (n=normal, a=abnormal)

	Tumors	Healthy
heterozygotes na	5 (18,5%)	4 (13%)
homozygotes nn	22 (81,5%)	27 (87%)
homozygotes aa	0 (0%)	0 (0%)
total tested	27	31

A French research group has tested 48 unrelated healthy individuals. It appeared that 23% were heterozygotes na, 75% homozygotes nn and 2% homozygotes aa. The children from couples na x nn have also been tested Their results suggest a selective pressure in favor of heterozygotes (1).

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B Human c-*fms* proto-oncogene

comparative analysis

with an abnormal allele

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Human c-*fms* Proto-Oncogene: Comparative Analysis with an Abnormal Allele

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The organization of the human c-*fms* proto-oncogene has been determined and compared with an abnormal allele. The human v-*fms* homologous genetic sequences are dispersed discontinuously and colinearly with the viral oncogene over a DNA region of ca. 32 kilobase pairs. The abnormal c-*fms* locus contains a small deletion in its 3' portion. DNA sequencing analysis indicated that it was 426 base pairs in size and located in close proximity to a putative c-*fms* exon.

Proto-oncogenes are thought to play an important role in cell differentiation and development (5, 17–19) They can be converted to oncogenes, and as such they have been implicated in tumorigenesis (3, 12, 13, 23, 27, 28) Molecular analysis of proto-oncogenes may thus provide insight in the mechanisms involved in these processes

The proto-oncogene under investigation in the present study is c-*fms*, which is homologous to the viral oncogene v-*fms* of the McDonough strain of feline sarcoma virus (SM-FeSV) SM-FeSV, originally isolated from a naturally occurring fibrosarcoma of a domestic cat (14), is a recombinant between feline leukemia virus and cellular genetic sequences of cat origin (4, 6, 9) The primary SM-FeSV translational product was identified as a 170,000-molecular-

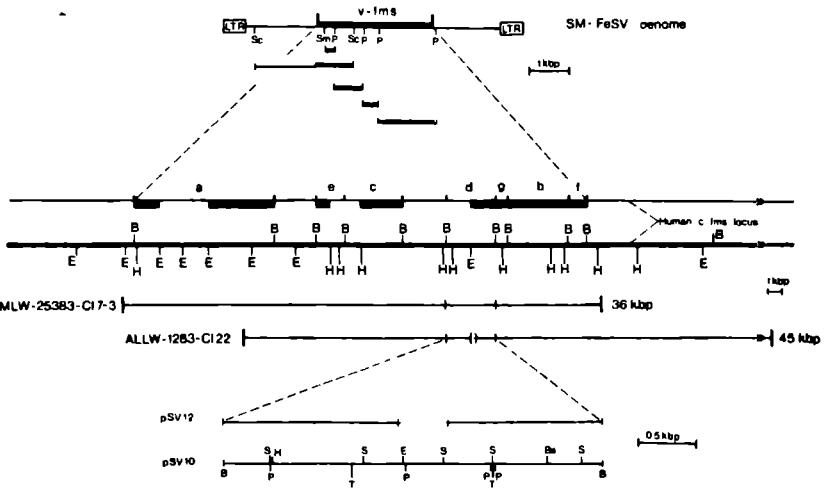

FIG 1 Restriction enzyme map of the normal and mutated human c-*fms* region In the upper portion of the figure, the SM-FeSV provirus is shown Open boxes represent the long terminal repeats (LTR) the solid bar represents the relative position of v-*fms*, and feline leukemia virus-specific sequences are represented as a solid line Directly below the SM-FeSV genome DNA fragments used as molecular probes are indicated In the center of the figure, a schematic restriction enzyme map of the human c-*fms* region and the map positions of human *Bam*HI restriction endonuclease fragments (labeled a through f, see also Fig 2) that exhibit homology with v-*fms*-specific molecular probes are shown Digestions with restriction endonucleases *Eco*RI and *Hin*dIII narrow down the ranges of homology further (solid bars) Solid lines below the restriction enzyme map of the human c-*fms* region identify the relative positions of the DNA inserts in cosmid clones CMLW-25383-C17-3 and ALLW-1283-C122 In the lower part of the figure the restriction enzyme map of the DNA insert of subclone pSV10 is indicated, and in the DNA insert of subclone pSV12 the size and the position of the deletion is given more precisely Both pSV10 and pSV12 are recombinants of pBR322 B, *Bam*HI, Bs, *Bst*EII, E, *Eco*RI, H *Hin*dIII, P, *Pst*I, S *Sau*3A, Sc *Sac*I, Sm, *Sma*I, T, *Taq*I

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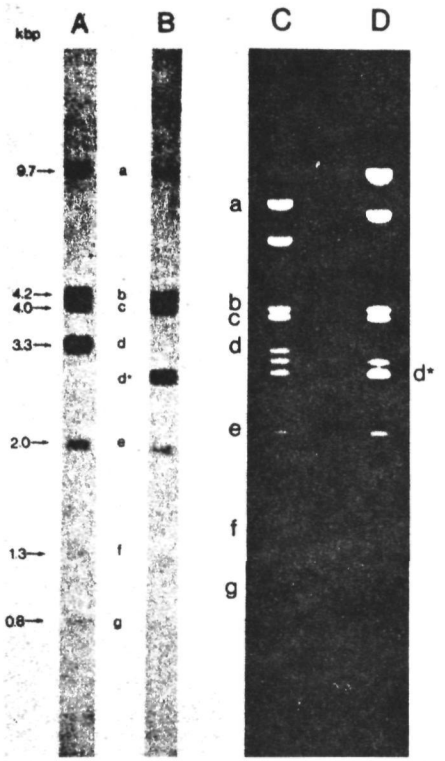

FIG. 2. Comparison of the normal and a mutated form of the human v-*fms* cellular homolog by genomic blot analysis and molecular cloning. High-molecular-weight cellular DNA purified from human liver (patient 18783) (A) or leukemic blood cells of patient 1283 (B) was digested with restriction endonuclease *Bam*HI and analyzed by Southern blot analysis. As a molecular probe, a combination of subgenomic subclones of SM-FeSV, including a 3.0-kbp *Sac*I-*Sac*I, a 0.4-kbp *Sma*I-*Pst*I, a 0.7-kbp *Pst*I-*Pst*I, a 0.4-kbp *Pst*I-*Pst*I, and a 1.45-kbp *Pst*I-*Pst*I DNA fragment representing the entire v-*fms* oncogene, as indicated in Fig. 1, was used. Hybridizing bands are labeled a through f and d*. DNA of cosmid clone CMLW-25383-C17-3 (C) and cosmid clone ALLW-1283-C122 (D) was digested with restriction endonuclease *Bam*HI, size fractionated on an 0.8% agarose gel, and stained with ethidium bromide. DNA fragments corresponding to hybridizing bands in the genomic blots (A, B) are labeled a through f and d* accordingly (hybridization data not shown). Molecular weights of DNA fragments were deduced from coelectrophoresed restriction endonuclease *Hin*dIII-digested λDNA markers.

weight polyprotein, P170$^{gag\text{-}fms}$, consisting of the amino-terminal structural proteins p15, p12, and p30 encoded by the feline leukemia virus *gag* gene covalently linked to a nonstructural component encoded by the acquired feline cellular sequence (2, 21, 25). The function of the polyprotein remains to be established, but it possesses a number of characteristics in common with growth factor receptors (1).

For detection of human v-*fms* homologous cellular genetic sequences, five probes (indicated in Fig. 1) were prepared from pCO1, which contains a 6.7 kilobase-pair (kbp) insert of SM-FeSV subcloned from a λgtWES.λB recombinant clone (4). High-molecular-weight DNAs isolated from a normal human liver (Fig. 2, lane A) was digested with restriction endonuclease *Bam*HI and analyzed by Southern blot hybridization (24). As shown, v-*fms* homologous human DNA restriction fragments of 9.7, 4.2, 4.0, 3.3, 2.0, 1.3, and 0.8 kbp (labeled a through g in Fig. 2) were resolved. DNA fragments of 9.7, 2.0, 1.3, and 0.8 kbp showed rather weak hybridization under the conditions used. These results indicate that all human v-*fms* homologous genetic sequences detectable by such genomic blot analysis are contained in seven *Bam*HI DNA fragments.

The *Bam*HI restriction fragment patterns observed in analysis of DNA samples of a number of individuals (four normal individuals and 12 cancer patients) were all identical. However, in the DNA isolated from a patient suffering from common-type acute lymphocytic leukemia and congenital hypothyroidism (patient 1283), a restriction fragment length polymorphism was observed. The 3.3-kbp *Bam*HI fragment was missing, and a new 2.8-kbp DNA fragment (labeled d* in Fig. 2) was observed (Fig. 2, lane B). In comparative analysis of the DNA of this patient and normal human liver DNA with restriction endonuclease *Eco*RI, a similar observation was made. The *Eco*RI restriction fragment pattern observed in normal human liver appeared to include DNA fragments of 16, 12, and 2.5 kbp (data not shown). The pattern obtained with DNA isolated from the leukocytes of patient 1283 lacked the 16- and 12-kbp DNA fragments and showed instead a 28-kbp DNA fragment. A similar observation was made with restriction endonuclease *Hin*dIII (data not shown). These Southern blot analyses indicate the absence of a normal c-*fms* allele in the leukemic blood cells of this particular patient. A detailed map of the human c-*fms* locus is required to define the observed restriction fragment length polymorphisms more precisely. Therefore, we undertook molecular cloning of both the normal and abnormal allele. By using the pJB8 cosmid vector system (11), genomic libraries were constructed (10, 11) with high-molecular-weight DNA isolated from purified leukocytes of patient 1283 (abnormal c-*fms* alleles) and chronic myelocytic leukemia patient 25383 (normal c-*fms* alleles). These genomic libraries have been designated ALLW-1283 and CMLW-25383, respectively. Three cosmid clones containing v-*fms* homologous genetic sequences have been isolated from the two libraries (8). Hybridization analysis with the complete provirus of feline leukemia virus as a probe revealed that none of the isolated cosmid clones exhibited any sequence homology (data not shown). Two of the three cosmid clones (CMLW-25383-C17-3 and ALLW-1283-C122) have been selected for further analysis in this study. The third cosmid clone, isolated from cosmid library CMLW-25383, contained a 39-kbp DNA insert which was partially overlapping at the 5′ end with clone CMLW-25383-C17-3. *Eco*RI restriction endonuclease digestion revealed the DNA inserts of CMLW-25383-C17-3 and ALLW-1283-C122 to be 36 and 45 kbp in size, respectively.

In Fig. 2, DNA fragments detected in genomic blot analysis are compared with those present in the two cosmid clones. Analysis of cosmid clone CMLW-25383-C17-3 (Fig. 2, lane C) upon digestion with restriction endonuclease *Bam*HI and ethidium bromide staining revealed the presence

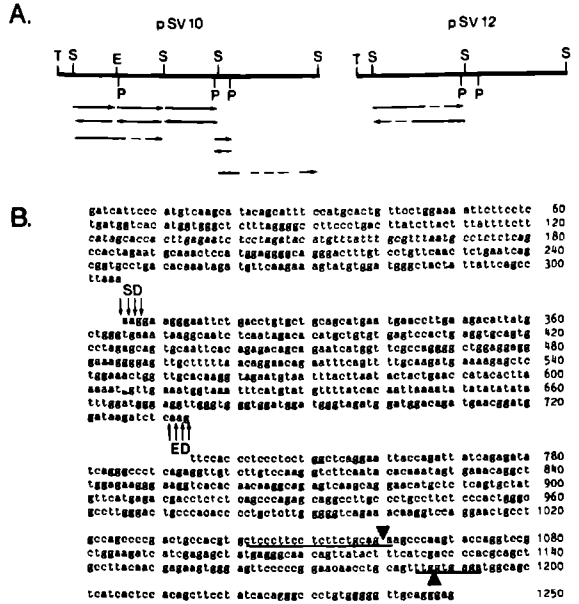

FIG 3 Nucleotide sequences of the human c-fms gene In (A) portions of the DNA inserts of subclones pSV12 and pSV10 are indicated Individual DNA fragments subcloned in bacteriophage M13mp8 and mp9 (15) are indicated by arrows Solid arrows represent stretches that are actually sequenced E, *Eco*RI, P, *Pst*I, S *Sau*3A, T, *Taq*I In (B) the nucleotide sequence of 1250 bp of c *fms* DNA isolated from patient 25383 (normal c-*fms* gene) is presented in the same orientation as the v-*fms* oncogene The nucleotide sequence was determined by the dideoxy chain termination method (22) The length of the deletion is estimated to be 426 bp and the size of the putative c-*fms* exon is 127 bp Deletion start points and end points are indicated by arrows (SD start of deletion, ED, end of deletion) Consensus splicing sequences adjacent to the exon are underlined In (C) the coding sequences of the putative c-*fms* exon are compared with the amino acid sequences encoded by the corresponding v-*fms* homologous region, as published by Hampe et al (6) Positions of nucleotide substitutions are underlined

of DNA fragments of 9 7, 7 5, 4 2, 4 0, 3 3, 3 0, 2 8, 2 0, 1 3, 1 1, and 0 8 kbp in length Hybridization with DNA probes, representing the entire v-*fms* oncogene, showed that all *Bam*HI DNA fragments detected in the genomic blot analysis (Fig 2, lane A, bands a through g) were present in this cosmid clone (data not shown) These results indicate that the complete human v-*fms* cellular homolog as detected by genomic blot analysis is represented in this cosmid clone The DNA fragments that do not exhibit v-*fms* homology are probably intervening and flanking sequences Similar comparative analysis of cosmid clone ALLW-1283-C122 (Fig. 2, lane D) with genomic DNA of patient 1283 (Fig 2, lane B) revealed that this cosmid clone contained DNA fragment d* By using restriction endonuclease *Bst*EII, *Bam*HI, *Cla*I, *Eco*RI, *Hin*dIII, *Sal*I, *Xba*I, *Xho*I, or combinations of these enzymes in a hybridization analysis with five subgenomic v-*fms* probes, the localization of *Bam*HI, *Eco*RI, and *Hin*dIII within cosmid clone CMLW-25383-C17-3 was ob-

tained (Fig. 1). As can be seen in the figure, the human v-*fms* cellular homolog within this cosmid clone is dispersed over a DNA region of ca. 32 kbp and is interspersed by at least four regions of nonhomology. These data confirm results described by Heisterkamp et al. (9). Furthermore, the human v-*fms* homologous genetic sequences appeared to be distributed colinearly with the viral oncogene (data not shown). By a similar approach, a restriction enzyme map was generated of the abnormal allele (Fig. 1), and it was established that the genetic change was a small deletion. More detailed information about the genetic organization of the region missing in the abnormal allele was obtained by DNA sequencing analysis (Fig. 3). The size of the deletion is 426 base pairs (bp) and is located in close proximity to a 127-bp v-*fms* homologous region flanked by consensus splicing acceptor and donor sequences (16) (Fig. 3B). Comparison of the sequence data of the human and viral homologous DNA regions revealed nine base substitutions (Fig. 3C). They were all found in the third position of a codon, leaving the amino acid sequence intact (Fig. 3C).

The v-*fms* oncogene, which has been derived from the feline c-*fms* locus (4, 9), was reported to be ca. 3.0 kbp in size (6). That of the human c-*fms* coding sequences remains to be determined. However, most of the 32-kbp c-*fms* region probably represents noncoding or intervening sequences, as can be concluded from the DNA sequence analysis indicating a human c-*fms* region of 1,250 bp to contain only 127 nucleotides of v-*fms* homologous coding sequences. It should be emphasized that the complexity of the human c-*fms* coding sequences may not be accurately determined with only v-*fms* as a tool. Not all coding proto-oncogene sequences are necessarily present in a viral oncogene, as was documented for the Gardner-Arnstein and the Snyder-Theilen strains of feline sarcoma virus (7), different isolates of Abelson murine leukemia virus (29), and the Finkel-Biskis-Jinkins murine osteosarcoma virus (26). In a similar way, v-*fms* may lack some feline c-*fms*-specific genetic sequences. Furthermore, v-*fms* may have been subjected to diversion during or subsequently to its generation. Species differences between humans and cats are less likely to be a major factor. DNA sequence analysis of one c-*fms* exon revealed sequence homology with the feline v-*fms* sequence of 93% with no change in the amino acid sequence. These results are typical for the high degree of conservation of proto-oncogenes during the evolution. Precise mapping of coding sequences in the human c-*fms* gene requires further mRNA and DNA sequence analysis.

It is not known whether the deletion affects the expression of the c-*fms* allele. Neither could we establish whether the physical condition of the patient was a consequence of the presence of the abnormal c-*fms* allele.

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The notion that the acutely transforming retroviruses owe their tumorigenic properties to the transduction of particular cellular genes of their host (oncogenes) marks a new stage in the research on the molecular basis of tumorigenesis Cloning of the proviruses gave us the tools in the form of DNA probes to search for the original homologous cellular genes (proto-oncogenes) in the complex eucaryotic genome Subsequently, these genes can be molecularly cloned as a first step in the process of investigating their normal cellular function and their involvement in non-viral tumorigenesis The research presented in this thesis is focused on two oncogenes, *fes* and *fms*

Three well characterized, independent FeSV (feline sarcoma virus) isolates have captured genomic sequences of the same feline locus, c-*fes* These sequences are only partially overlapping Moreover, comparison of the different v-*fes* sequences with the homologous viral oncogene v-*fps* of FSV (Fujinami sarcoma virus) suggested that in all three FeSV isolates 5 *fes* sequences are missing Therefore, another not well studied, proviral genome of a FeSV , also containing v-*fes* sequences, was molecularly cloned and structurally analyzed The virus proved to be closely related to ST-FeSV (Snyder-Theilen feline sarcoma virus) The variant clone shows a frameshift pointmutation that extends the open reading frame with 468 bp This explains why the variant virus encodes a 104 kDa fusion polyprotein and the prototype ST-FeSV a 85 kd fusion polyprotein

The proto-oncogenes c-*fes* and c-*fms* of the cat have been molecularly cloned and the human c-*fms* as well Clones from cosmid libraries that contain parts of the *fes* and *fms* loci could be identified by using FeSV oncogene sequences as molecular probes Restriction enzyme mapping and Southern blot analysis revealed that the proto-oncogene sequences are distributed discontinuously and colinearly with the viral transforming genes over long DNA regions Comparison of the DNA sequence of a putative (small) exon of human c-*fms* with the DNA sequence of v-*fms* (of feline origin) confirmed the striking conservation of the (proto-)oncogene at both the nucleic acid and aminoacid level

To determine the location of mutations that are involved in the activation of the transforming potential of the *fes* gene a chimeric v-*fes*/c-*fes* gene was constructed It was demonstrated that the carboxyl half of the GA-FeSV (Gardner-Arnstein feline sarcoma virus) transforming protein can be functionally replaced by the corresponding carboxyl portion of the feline c-*fes* protein Fusion to viral *gag* sequences therefore seems to be the most important step in the activation of the *fes* gene

Certain types of human cancer are strongly correlated with sub-chromosomal abnormalities Using Southern blot hybridization with FeSV *fes* and *fms* probes, human DNA isolated from cancer patients and from healthy individuals was screened for RFLPs (restriction fragment length polymorphism) associated with the genes mentioned A patient with congenital hypothyroidism and acute lymphocytic leukemia was found to be homozygous for a RFLP in the c-*fms* proto-oncogene With DNA from purified leukocytes of this patient a genomic cosmid library was constructed Analysis of a clone

that contained v-*fms* homologous sequences revealed that the RFLP was caused by a 426 bp deletion located in the 3' portion of the gene, in close proximity to a putative exon Genomic DNA of 27 cancer patients and 31 healthy individuals was analysed 84% did not show the polymorphism 16% appeared to be heterozygous This distribution was the same for cancer patients and healthy individuals

Met het besef dat de acuut-transformerende retrovirussen hun tumorgene eigenschappen te danken hebben aan de transductie van bepaalde genen van hun gastheer (oncogenen) ging het onderzoek naar de moleculaire basis van tumorgenese een nieuwe fase in Klonering van de provirale genomen van deze virussen gaf ons de gereedschappen, in de vorm van DNA probes, om de originele homologe cellulaire genen (proto-oncogenen) te identificeren in het complexe eukaryotische genoom Op hun beurt kunnen deze genen gekloneerd worden als een eerste stap in het onderzoek naar hun normale cellulaire functie en hun rol in niet-virale tumorgenese Het onderzoek gepresenteerd in dit proefschrift beperkt zich tot de oncogenen *fes* en *fms*

Drie goed gekarakteriseerde onafhankelijke katte-sarkoom virus-isolaten hebben in hun genoom sequenties opgenomen van hetzelfde katte locus, c-*fes* Deze sequenties zijn slechts gedeeltelijk overlappend Bovendien bleek uit een vergelijking van de verschillende v-*fes* sequenties met de sequentie van het homologe virale oncogen v-*fps,* afkomstig van het FSV (Fujinami sarkoom virus) uit de kip, dat waarschijnlijk in alle drie de katte-sarkoom virussen sequenties van het 5' deel van c-*fes* ontbreken Daarom werd een nog niet nader geïdentificeerd katte-sarcoom virus-isolaat, dat ook v-*fes* sequenties bevat, gekloneerd en de structuur geanalyseerd Hieruit bleek dat het virus nauwe verwantschap vertoont met het Snyder-Theilen katte-sarkoom virus isolaat De variant kloon bevat een puntmutatie, die leidt tot een verschuiving in het leesraam wat resulteert in een verlenging van dit leesraam met 468 baseparen Dit verklaart waarom deze Snyder-Theilen virus variant voor een 104 kDa fusie-poly-eiwit codeert en het prototype Snyder-Theilen virus voor een 85 kDa fusie-poly-eiwit

De c-*fes* en c-*fms* proto-oncogenen van de kat zijn gekloneerd alsmede het c-*fms* gen van de mens Klonen, afkomstig van cosmide-banken, die sequenties homoloog aan v-*fes* en v-*fms* bevatten, konden worden geïdentificeerd door virale oncogen sequenties, afkomstig van katte-sarcoom virussen, te gebruiken als DNA probes Een analyse met behulp van restrictie enzymen en Southern blots toonde aan dat de proto-oncogen-sequenties discontinu en colineair met de virale sequenties zijn verdeeld over grote DNA fragmenten Een vergelijking van de DNA sequentie van een verondersteld (klein) exon van het menselijke *fms*-locus met de DNA sequentie van v-*fms*, dat van de kat afkomstig is, bevestigde de opvallende conservering van deze (proto-)oncogen sequenties op het niveau van zowel het DNA als het eiwit

Om de plaats te bepalen van mutaties, die zijn betrokken bij de activatie van het transformerende vermogen van het *fes* locus, werd een chimeer v-*fes*/c-*fes* gen geconstrueerd Aangetoond werd dat de carboxy-helft van het transformerende eiwit van het Gardner katte-sarkoom virus kan worden vervangen door een overeenkomstig carboxy-deel van het katte-c-*fes* zonder de transformerende activiteit aan te tasten Fusie met virale *gag* sequenties lijkt daarom de belangrijkste stap in de activatie van het *fes* gen

Er bestaat bij de mens een duidelijk verband tussen bepaalde vormen van kanker en sub-chromosomale afwijkingen Met behulp van de hybridisatie van Southern blots met v-*fes* en v-*fms* probes, afkomstig van katte-sarkoom virussen, werd menselijk DNA, geïsoleerd uit tumorweefsel van kanker-patienten en normaal weefsel van een willekeurige groep gezonde individuen, getest op de aanwezigheid van polymorfismen

in de lengte van DNA fragmenten (RFLP), die homoloog zijn met de genoemde oncogenen Een patient met congenitale hypothyroidie en acute lymphatische leukemie bleek homozygoot te zijn voor een RFLP in het c-*fms* proto-oncogen Met het DNA, geisoleerd uit gezuiverde leukocyten van deze patient, werd een cosmide-bank gemaakt Analyse van een kloon, die sequenties homoloog aan v-*fms* bevatte, toonde aan dat dit RFLP werd veroorzaakt door een deletie van 426 baseparen gelegen in het 3' deel van het gen, vlak voor een verondersteld exon Het genomisch DNA van 27 kanker patienten en 31 gezonde personen werd geanalyseerd Hiervan bleek 84% het polymorphisme niet te vertonen terwijl 16% heterozygoot was In tumoren en gezond weefsel was de frequentie, waarmee het polymorphisme aanwezig was, dezelfde

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Na zijn middelbare school opleiding aan de Rijks Hogere Burger School te Helmond liet Sjef Verbeek zich inschrijven aan de Rijksuniversiteit te Utrecht In 1979 werd het kandidaats examen biologie, variant B4 (hoofdvak scheikunde), afgelegd Het doctoraal examen, dat in 1981 volgde, bestond uit de onderdelen Moleculaire Genetica (hoofdvak o l v Prof Dr G A van Arkel), Moleculaire Celbiologie (tweede hoofdvak o l v Prof Dr H O Voorma) en Electronen-microscopische structuur Analyse (nevenrichting o l v Prof Dr Ir P F Elbers)

Gedurende de periode september 1981 tot september 1985 werd het in dit proefschrift beschreven onderzoek verricht, onder leiding van Dr W J M van de Ven en Prof Dr H P J Bloemers in het Laboratorium voor Biochemie van de Faculteit der Wiskunde en Natuurwetenschappen van de Katholieke Universiteit van Nijmegen Bovendien werd in het kader van dit door Het Koningin Wilhelmina Fonds gesubsidieerd onderzoeks-project een stage vervuld in het laboratorium van Dr J Stephenson verbonden aan de Frederick Cancer Facility van het National Cancer Institute te Frederick, Maryland, USA

Momenteel maakt hij deel uit van de sectie Moleculaire Genetica van Het Nederlands Kanker Instituut te Amsterdam (wetenschappelijk directeur Prof Dr P Borst) In de werkgroep van Dr A Berns verricht hij onderzoek aan het modelsysteem van de transgene muis met geactiveerde oncogenen