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The Role Of Mouse Mammary Tumor Virus In The Induction And Progression Of Mouse Mammary Tumors

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THE ROLE OF MOUSE MAMMARY TUMOR VIRUS IN THE INDUCTION AND PROGRESSION OF MOUSE MAMMARY TUMORS

by

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ABSTRACT

Mouse mammary tumor virus (MMTV) is a retrovirus which causes mammary tumors in many strains of mice. An essential part of the viral life cycle involves the integration of a double-stranded DNA provirus into the host cell genome. Tumorigenesis is thought to be mediated by such integrated proviruses.

Restriction endonuclease analysis of the DNA from a series of cell sublines from a single MMTV-induced mammary adenocarcinoma revealed that the original tumor was composed of tumorigenic and nontumorigenic cells whose proviral content could not be correlated with tumorigenicity. The tumorigenic sublines however contained a single provirus which was correlated with tumorigenicity. The tumorigenic potential of MMTV proviruses therefore depends on the site of integration.

In a second study it was shown that the mode of action of a germinally-transmitted locus of GR mice, Mtv-2, was through the generation of proviruses which activated the putative mammary proto-oncogenes int-1 and int-2.

It was also shown that a series of chemically or hormonally induced hyperplastic alveolar nodule outgrowth lines each had an integrated MMTV provirus at the same site in the genome. This locus was shown to be different from the putative mammary proto-oncogenes int-1 and int-2. Since this locus is associated with hyperplasia (a preneoplastic

condition), it has been designated int-H.

A portion of the int-H locus was molecularly cloned in a lambda phage vector-host system designed to prevent the deletion of those sequences capable of forming secondary structures. DNA from the int-H region has homology to a family of sequences in rat and human as well as mouse DNA. Transcripts from this region were detected in normal midpregnant mammary tissue as well as in preneoplastic tissue. This locus may code for a regulatory molecule. Proviral disruption of int-H regulation may predispose cells to becoming tumorigenic.

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I would like to express gratitude to those who made possible and pleasurable the completion of these studies. I would first of all like to thank my supervisor Dr. Vincent Morris who sparked my interest in the molecular biology of cancer and took personal pains to develop it. I am deeply indebted to Drs. Wayne Flintoff and Judith Ball for insightful and inspirational discussions. Helpful advice was also gratefully received from Drs. Emanuel Faust and Robert Anderson. Discussions with my graduate student colleagues Dennis Jackson and Sven Beushausen, while not strictly scientific, were treasured nonetheless.

The work described herein was greatly expedited by the technical assistance of Edwin Lee Chan whose generosity is exceeded only by his limitless patience.

Finally, I would like to express my eternal gratitude to my family. My father showed me by example what can be acheived through perserverance and dedication and was always totally supportive of my efforts. My mother and sister saw me through the rough times and are what gives every success its meaning.

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LIST OF ABBREVIATIONS

ALV - Avian leukosis virus.

AMV - Avian myeloblastosis virus.

bp - Base pair.

cpm - Counts per minute.

dATP - Deoxyadenosine triphosphate.

dCTP - Deoxycytidine triphosphate.

dGTP - Deoxyguanosine triphosphate.

dTTP - Deoxythymidine triphosphate.

DEP - Diethylpyrocarbonate.

DGE - Dye/glycerol/EDTA.

DNA - Deoxyribonucleic acid.

DTT - Dithiothreitol.

EDTA - Ethylenediaminetetraacetic acid.

env - Envelope glycoprotein.

gag - Group specific antigen.

HAN - Hyperplastic alveolar nodule.

kb - Kilobase pair.

Md - Megadalton.

MMTV - Mouse mammary tumor virus.

mRNA - Messenger RNA.

MW - Molecular weight.

nm - Nanometer.

O.D. - Optical density.

PEG - Polyethylene glycol.

[illegible] - [illegible]

pol I - DNA-dependent DNA polymerase I.

PSB - Plaque storage buffer

RNA - Ribonucleic acid.

rpm - Revolutions per minute

SDS - Sodium dodecyl sulphate

SSC - Standard sodium citrate

TE - Tris/EDTA.

Tris - Tris (hydroxymethyl)aminomethane.

tRNA - Transfer RNA.

UV - Ultraviolet.

v/v - Volume per volume.

w/v - Weight per volume.

CHAPTER 1

Introduction

Fifty years have passed since Bittner (1936) demonstrated that a milk-borne agent was responsible for the high incidence of mammary tumors in certain strains of mice; an agent he was shortly to prove to be a virus. It must have seemed to Bittner at the time that with the discovery of such a virus, the problem of mammary cancer was close to being soluble. Even in the 1940's it would not have been an extreme flight of fancy to imagine that if the details of the life cycle of this infectious agent could be worked out, the events critical for tumorigenesis would be understood. In the years following Bittner's discovery, a great deal has been learned about the replication of Mouse Mammary Tumor Virus, even down to molecular detail of which he could not have conceived. The solution to the problem of mammary tumor induction has, however, remained elusive. One impediment to the solution of this problem has been the unexpected complexity of mammary tumors at the cellular level, particularly when compared to certain other solid tumors, or to leukemias. Mammary tumors are clearly comprised of different populations of cells (Soule et al, 1981; MacInnes et al, 1981) whose origins and contribution to the tumorigenic process must be considered. This necessitates discarding the naive but more intellectually manageable

concept of the tumor as a mass of errant but uniform cells which are all experiencing the same calamity. A second confounding factor to the dissection of tumorigenesis and even to the clinical management of tumors is that tumors are dynamic entities. The cells which populate a palpable tumor are the descendants of, and may be quite different from, the cell or cells in which the initial events occurred (Hill et al, 1984). Indeed with the application of selective pressure over the course of an experiment or clinical therapy the genetic constitution of the tumor cells can be shown to change again (Dexter and Calabresi, 1982). This rapid evolution of the tumor at the cellular level has made the study of tumorigenic events from mature tumors somewhat like studying Greek antiquity using the current inhabitants of Athens. Finally, the long-pursued mechanism whereby the cell regulates its division has remained largely untractable. The disruption of this process is integral to tumorigenesis, and it is unlikely that complete understanding of one can be achieved without the other.

In spite of the daunting complexity of the problem, recent advances in recombinant technology have allowed at least a partial solution to the problem of mammary tumorigenesis in the mouse model. Molecular probes have become available which allow the investigation of the mechanism whereby the virus, in the course of its replicative cycle, disrupts the regulation of cellular genes necessary for normal mammary differentiation. Much remains to be learned about the number

and function of these genes. Since cellular deregulation is a consequence of the replication of MMTV, to understand this process it is first necessary to understand how the virus manages its own affairs at the host's expense.

CHAPTER 2

Historical Review

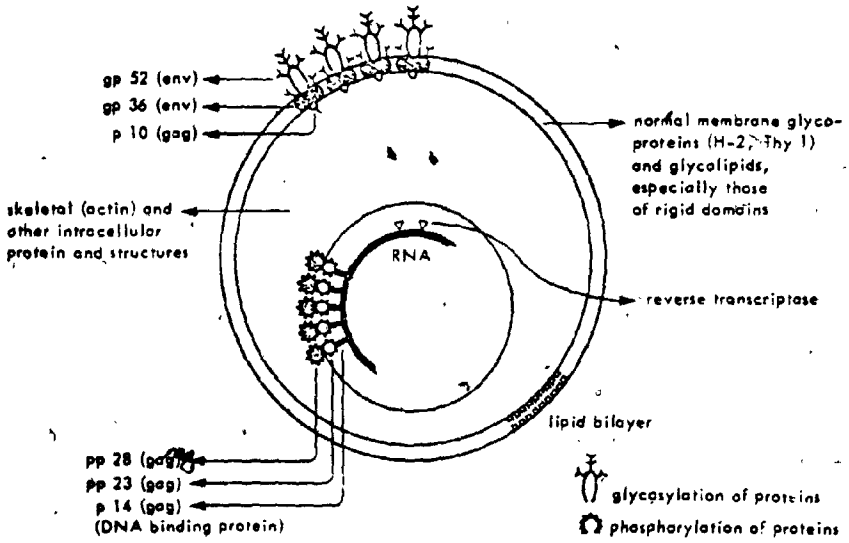
2.1 Discovery of the Mammary Tumor Agent: Systematic study of murine mammary carcinogenesis began with the inbreeding experiments of C. C. Little in the early 1900's (reviewed by Little, 1947). Strains were generated by brother-sister matings which had a high incidence of mammary tumors. Although the high tumor incidence of these mice was known to be transmitted via the female parent of cross-bred progeny, it was not until Bittner (1936) performed foster-nursing experiments that transmission was demonstrated to be via the milk. Bittner later found (1942) that the tumor-inducing agent could be passed through a filter capable of trapping bacteria and concluded that the agent was a virus. Bittner called the virus mammary tumor agent; it is currently more commonly referred to as Mouse Mammary Tumor Virus (MMTV). Dmochowski presented the first electron micrographs of the virus (1954) showing it to be an irregularly shaped body with an eccentrically placed nucleoid (the so-called B-type particle). Intracytoplasmic and budding precursors to the virus were also seen.

2.2 Structure of the MMTV Virion: Thin-section electron microscopy showed the structure of MMTV to be that of an electron dense nucleoid surrounded by a fine inner membrane

placed eccentrically within a lipid bilayer having prominent spikes (Dmochowski, 1960). These so called B type particles had a diameter of 110-130 nm and a bouyant density of 1.16-1.18 g/cm^3. They were found to be composed of 70% protein and 1.9% RNA (Sarkar et al, 1972). The lipid envelope was found to contain an imbedded 36,000 d glycoprotein (p36) anchoring a 52,000 d glycoprotein (gp52) by hydrophobic interactions (Westerbrink et al, 1979). A 10,000 d protein was also associated with the lipid bilayer. The inner core shell was mainly composed of a 27,000 d phosphoprotein. The viral RNA genome was associated with a 23,000 d phosphoprotein and an 18,000 d basic protein, while the p14 protein (14,000 d) was assumed to link the core to the viral envelope (Cardiff et al, 1978). A 10,000 d reverse transcriptase molecule was also detected in the inner core. The structure of the MMTV virion is illustrated in Figure 1. There is some controversy over the size of individual core proteins, reported as 27,000 d, 21,000 d, and 14,000 d (Dickson and Peters, 1983) or 28,000 d, 23,000 d, and 14,000 d (Bentvelzen, 1982).

2.3 Structure and Function of the MMTV Genome: The MMTV genome consists of a 70S dimer of two identical 35S subunits of positive sense RNA (reviewed by Coffin, 1982). The genome consists of three structural genes coding for three sets of proteins: the gag, or core proteins; pol or RNA-dependent DNA polymerase protein; and env or envelope proteins. A

Figure 1: Structure of the MMTV Virion. Proteins are named for their molecular weight in kilodaltons. From Bentvelzen, (1980).


gp 52 (env)
gp 36 (env)
p 10 (gag)
normal membrane glycoproteins (H-2, Thy 1) and glycolipids, especially those of rigid domains
skeletal (actin) and other intracellular protein and structures
RNA
reverse transcriptase
lipid bilayer
pp 28 (gag)
pp 23 (gag)
p 14 (gag)
(DNA binding protein)
glycosylation of proteins
phosphorylation of proteins

$tRNA^{lys}$ primer near the 5' end of the RNA genome has been found to initiate the synthesis of a DNA strand, which is made double stranded by RNAse H-mediated hydrolysis of the genomic RNA to allow strand "jumping" such that terminal homologies can prime second strand synthesis (Peters and Glover, 1982; Varmus and Swanstrom, 1982). During this synthesis, sequences at the ends of the genome become duplicated to form long terminal repeats (LTRs) containing the regulatory sequences for transcription (see Figure 2). The double stranded DNA form of the virus becomes integrated into cellular DNA in the form of a provirus (see Figure 3).

Two major classes of RNA have been detected in MMTV producing cells: a full length 7.8 kb (35S) species and a 3.8 kb (24S) species (Dudley and Varmus, 1981). The larger transcript was found to encode a gag-pol polyprotein which is proteolytically cleaved to form the components of the core. The smaller transcript was found to be a spliced derivative of the larger transcript, and encodes a polyprotein which is cleaved to generate the gp52 and gp36 envelope proteins. A third 1.7 kb transcript detected at much lower levels is described in Section 2.7.

Transcription of viral sequences has been shown to be regulated by elements in the proviral LTR (Ringold, 1983). In addition to the consensus promoter sequences TATAAA and CAAAT located 25 and 75 nucleotides upstream of the start of transcription respectively (thought to be involved in initiation by RNA polymerase II) and the polyadenylation

Figure 2: Structure of an Integrated MMTV Provirus. Numbers indicate the distance in base pairs from the left boundary of the right LTR. DR indicates a direct repeat. IR indicates an inverted repeat. PB is the primer binding site. L is a leader region. U3 and U5 are regions derived from the 3' and 5' ends of the viral RNA. R is a repeat separating U3 and U5. Integration is thought to be mediated by an endonuclease activity of the pol gene (Panganiban, 1985); this "integrase" activity may direct recombination between cellular sequences and the juxtaposed inverted repeats of the circularized provirus (the "att" site). From Michalides et al. (1983).

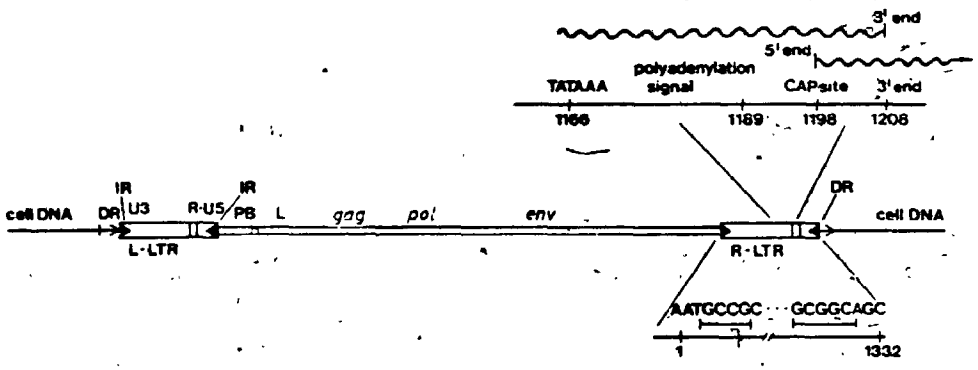
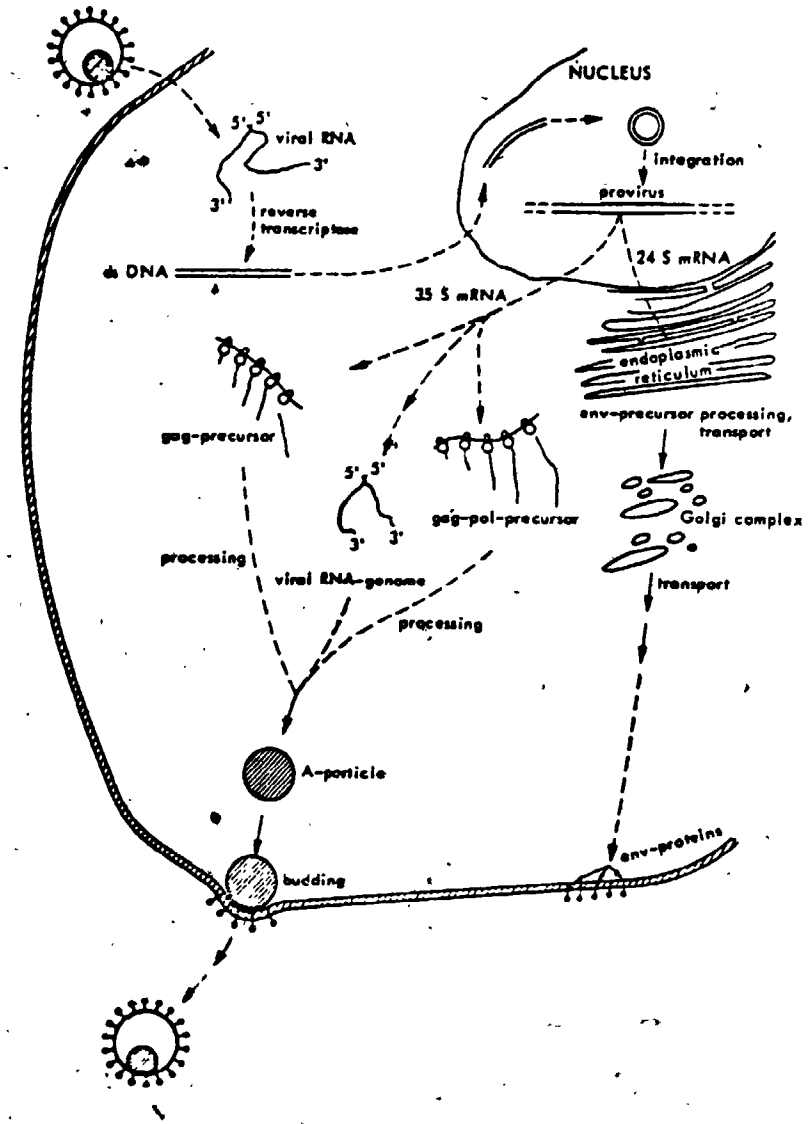

3' end
5' end
polyadenylation signal
TATAAA
CAP site
3' end
1166
1189
1198
1208
IR
IR
DR
cell DNA
U3
R-U5
PB
L
gag
pol
env
DR
cell DNA
L-LTR
R-LTR
AATGCCGC
GCGGCAGC
1
1332

Figure 3: The Life Cycle of MMTV. MMTV adsorbs to the cell via its glycoproteins and is internalized. ds DNA is synthesized during or following uncoating in the cytoplasm. Genomic and messenger RNA's are transcribed from the integrated provirus and translated in the cytoplasm into polyproteins. Glycosylation of env proteins occurs in the Golgi apparatus. The processing of gag proteins occurs in the cytoplasm and in intracytoplasmic A particles. The virions bud off at regions of env glycoprotein accumulation and complete the maturation into B particles extracellularly. From Bentvelzen (1980).


NUCLEUS
5' 5'
viral RNA
3'
3'
reverse transcriptase
ds DNA
integration
provirus
24 S mRNA
35 S mRNA
endoplasmic reticulum
env-precursor processing, transport
gag-precursor
5' 5'
3' 3'
gag-pol-precursor
Golgi complex
processing
viral RNA-genome
processing
transport
A-particle
budding
env-proteins

signal AGTAAA present in the LTR, a glucocorticoid receptor region was discovered between 140 and 190 bases upstream of the initiation point.

This region (unique to MMTV) was found to be necessary for the observed stimulation of chimaeric genes under the control of the MMTV LTR (Groner et al, 1982), and is presumably responsible for the induction of MMTV production in tumor tissue in the presence of hormone (Smoller et al, 1961). The role of hormones in tumorigenesis may thus be as an initiator of synthesis from germinal proviruses (McGrath and Jones, 1978).

2.4 Genetic Transmission of MMTV: In addition to the milk-borne route of infection, MMTV can be transmitted through the germline in the form of endogenous proviruses. Varmus et al (1972) used liquid hybridization techniques to establish the presence of genetically transmitted viral sequences in the genomes of mice. Subsequent hybridization analyses (Parks and Scolnick, 1973; Gillespie et al, 1973; Michalides and Schlom, 1975; McGrath et al, 1978) have established that all inbred strains of mice have endogenous proviruses. Morris et al (1977) quantitated the copy number of MMTV proviruses in inbred and feral strains, finding up to 10 copies per cell in inbred strains and over 30 copies per cell in the feral strain Mus cervicolor. With the exception of proviruses present in GR or in C3H and DBA mice, the endogenous proviruses do not play a role in

tumorigenesis, and are generally transcriptionally silent as evidenced by methylation patterns and chromatin structure (Traina-Dorge and Cohen, 1983; Kozak, 1985).

When C3H mice are foster-nursed on virus-free females of another strain, the incidence of mammary tumors falls from about 90% to less than 50% (Heston and Deringer, 1952; Boot and Muhlbock, 1956; Heston, 1958). It does not, however fall to the level of the virus-free strain (around 1%). The production of MMTV in the milk of C3H and DBA and the induction of tumors in foster-nursed C3H mice has been attributed to a single dominant gene Mtv-1, mapped to chromosome 7 (van Nie and Verstraeten, 1975; Verstraeten and van Nie, 1978).

The GR strain also has a high incidence of mammary tumors (90%) when foster-nursed to prevent milk-borne transmission of MMTV. High levels of MMTV virions in the milk and the development of early, hormone-dependent tumors can be transmitted by males or females to foster-nursed offspring (Muhlbock, 1965; Bentvelzen et al, 1970). A single dominant locus (Mtv-2) has been shown to be responsible for the transmission of these traits. This locus maps to chromosome 18 (van Nie et al, 1977). GR mice also harbour a locus associated with the expression of the p28 gag protein in the milk, but unrelated to tumorigenesis (Nusse et al, 1980). This locus, assumed to contain a defective provirus, has been mapped to chromosome 11.

The BALB/c strain typically has a very low tumor incidence

(Medina and DeOme, 1968; Morris et al, 1980) with the exception of the BALB/cV subline (Slagle and Butel, 1985) which has a 45% incidence. Aging BALB/c mice also have been reported to have a higher incidence (7-30%), although the lack of acquired germline integrations in these mice has led to speculation that they have become infected with exogenous MMTV (Morris et al, 1980). The BALB/c genome has been found to contain two complete MMTV proviruses plus a defective provirus comprised mainly of LTR sequences (Cohen et al, 1979a). Units I and III (also known as Mtv-6 and Mtv-9) have been mapped to chromosomes 16 and 12 respectively (Callahan et al,1984). Unit II has yet to be mapped, and has no Mtv designation. A summary of the endogenous loci relevant to this thesis is presented in Table 1.

2.5 Structure and Composition of Tumors Induced by MMTV: Mammary tumors induced by MMTV are primarily of the adenocarcinoma type (Nandi and McGrath, 1973) which derive from the secretory epithelial cells of the mammary gland. Such virus-induced tumors tend to be of types A and B as classified by Dunn (1959). Type A tumors are well differentiated, display small acini and tubules composed of small cuboidal cells and occasionally display secretory activity. Type B tumors are less differentiated but usually retain tubule structures and are often cystic. The relatively undifferentiated C type tumors are seldom induced by MMTV. MMTV also induces tumors which superficially

Table 1

Characteristics of Some Endogenous Loci[1]

Proviral locus	Phenotype Expression	Eco RI fragments	Strain
Mtv-1	late tumors	6.5 - 4.5	C3H,DBA
Mtv-2	early tumors	11.4 - 7.0	GR
Mtv-3	gag proteins	21.0 - 7.0	GR
Mtv-7	-	16.7, - 11.7	GR
Unit I[2] (Mtv-6)	-	16.7	BALB/c
Unit II	-	7.8 - 6.7	BALB/c
Unit III (Mtv-9)	-	9.6 - 7.6	BALB/c

[1] Data from Kozak (1984) and Gray et al (1986).

[2] Nomenclature of Cohen and Varmus (1979).

resemble adenocarcinomas, but are at least partially adenoacanthomas, having stratified squamous epithelium. It is clear from the complex structure of mouse mammary tumors that different populations of cells are present in the tumors as has been verified experimentally.

Heppner (1982) has described a series of subpopulations of cells derived from a single mammary adenocarcinoma induced by MMTV, and propagated separately in culture. These lines differ from each other ultrastructurally, antigenically, karyotypically, and in a number of _in vitro_ growth characteristics. They also differ in tumorigenic and metastatic ability. Interactions among subpopulations have been demonstrated experimentally both _in vivo_ and _in vitro_ in this system. The presence of one subpopulation in the flank of a mouse increased the sensitivity of a second subpopulation in the other flank to the antimetabolic agent cyclophosphamide. Similarly, sensitivity of a subpopulation _in vitro_ to methotrexate could be influenced by the presence of a second subpopulation despite the physical separation of the cells in these subpopulations. MacInnes _et al_ (1981) have reported the interaction of hormone dependent and independent subpopulations of cells in GR mammary tumors such that both cell types persist despite selective pressure applied against one cell type during _in vivo_ passaging. This observation implies an interdependance of cell types in the tumor, although the basis of this interaction is unknown. Experiments conducted by Slemmer

(1981) demonstrated that in mammary adenocarcinomas in the C3H mouse strain neoplastic cells retained dependence on association with normal cells throughout tumor progression and metastasis. Again, the contribution of the normal cells to the maintainance of the tumor is unknown.

The heterogeneity of cells in mouse mammary tumors can be explained by a polyclonal origin and/or by genetic divergence during progression. With respect to the former possibility, while a polyclonal origin has been demonstrated for some hereditary (Fialkow et al, 1971) and experimental (Reddy and Fialkow, 1979) tumors, most benign and malignant tumors studied with cell markers have a clonal origin (Fialkow, 1979; Nowell, 1976). The origin of MMTV-induced tumors from one or a few cells was suggested by Cohen et al (1979) on the basis of restriction endonuclease analysis of such tumors. This view is supported by the data of Hynes et al (1979), Groner and Hynes (1980), Cohen and Varmus (1980), and Fanning et al (1980a,b). Although Cohen et al (1979) gave evidence that in the normal lactating mammary gland from 5-20% of cells are infected with MMTV, only a few of these infected cells undergo malignant transformation. The heterogeneity of mammary tumors must therefore arise through genetic events occurring in the course of progression. Nowell (1976) has suggested that the cells within a tumor are genetically unstable, a hypothesis supported by the data of Cifone and Fidler (1981). These authors showed that clones of high metastatic potential had a higher spontaneous

mutation rate than clones of low metastatic potential indicating that genetic instability was correlated with progression. Hill et al (1984) have proposed a "dynamic heterogeneity" model to explain the appearance of metastatic variants in a tumor on the basis of rapid turnover of genetic variants in tumor progression. It has not yet been determined if mammary tumor heterogeneity involves genetic instability.

2.6 Steps in Mammary Tumor Progression: The multistep nature of cancer was recognized as early as the turn of the century by Haaland (1911) who recognized that mammary tumors are preceded by distinct hyperplastic changes in epithelial cells. Subsequent to this observation two step models were proposed for experimental skin cancer in rabbits (Rous and Kidd, 1941; MacKenzie and Rous, 1941; Friedwald and Rous, 1950), mice (Berenblum and Shubik, 1947) and rats (Isaacs, 1985). Experimental liver cancer in mice has also been shown to develop in discrete stages (Epstein et al, 1967; Merkow et al, 1967). More recently, transfection analyses have suggested that a two step transformation can be effected with oncogenes from two groups (Land et al, 1983). Acute transforming retroviruses which appear to achieve conversion to the tumorigenic phenotype in one step may represent the end-product of a series of genetic events which are collectively but not separately transforming (Temin, 1983).

Human cancer is also thought to involve progressive steps

as evidenced by conditions recognized as precancerous. These include actinic keratoses which may progress to squamous cell carcinoma, uterine dysplasia which may progress to epidermoid carcinoma, and villous adenoma which may become adenocarcinoma of the colon (Willis, 1967). Human precancerous mammary lesions are thought to include the fibrocystic disease complex and a number of so-called carcinomas in situ (Wellings, 1980; Lagios, 1983). The most thoroughly studied model of tumor progression, however, remains the mouse mammary system, largely due to the distinctive focal epithelial hyperplasia known as the hyperplastic alveolar nodule (HAN). The cellular and molecular biology of this system has been extensively studied by DeOme and coworkers (1959), Medina (1973,1978) and Cardiff (1984). Hyperplastic alveolar nodules were found to appear spontaneously at a high frequency in high mammary tumor-incidence mice which carry milk-borne MMTV, and at a much lower frequency in low tumor-incidence strains (Pitelka et al, 1960). Nandi (1961) showed that MMTV was responsible for the induction of HANs by increasing the frequency at which HANs appeared in low tumor-incidence mice foster-nursed on a MMTV transmitting strain. Faulkin (1966) induced HANs by nonviral means through the oral administration of a chemical carcinogen, 3 methylcholanthrene. HANs have also been induced by 7,12 dimethylbenzanthracene, urethane, 1,2 benz[a]pyrene, and 2,7 flourendiamine (Medina, 1973). Prolonged hormone stimulation

was found to potentiate either viral or chemical induction of HANs. This effect was acheived by pituitary isograft under the kidney, freeing the pituitary from hypothalamic control.

The preneoplastic nature of HAN tissue was convincingly shown by DeOme et al (1959) in experiments where nodules were transplanted to isogenic mice whose mammary fat pads had been cleared of glandular tissue. These mice developed mammary tumors sooner and at a higher frequency in fat pads receiving HANs than in contralateral fat pads receiving normal mammary gland tissue. This transplantation technique was serially repeated by Medina to establish HAN outgrowth lines. Samples of HAN tissue induced by chemical carcinogens or pituitary isograft (see Table 2) were transplanted from one fat pad to another, always giving rise to a HAN which was allowed to fill the fat pad before further transplantation (Medina, 1973). With extended passaging, outgrowth lines were established which are the in vivo equivalent of established cell lines. Whereas normal mammary cells could be serially transplanted in vivo 5 or 6 times (Daniel et al, 1968), HAN outgrowths behave like neoplastic tissue in having indefinite division potential. In contrast to neoplastic cells, however, HANs will not grow subcutaneously, intraperitoneally, or in nonmammary fat and are restricted to growth in the mammary gland as are normal alveolar cells (Medina, 1978). Faulkin and DeOme (1960) found that the spatial organization of normal mammary tissue

Table 2

Origin and Tumorigenicity of BALB/c Hyperplastic Alveolar Nodules (HANs)

HAN line	Type	Origin	Tumorigenicity[1]
D1	Alveolar	Hormonally induced	12
D2	Alveolar	Hormonally induced	51
C4	Alveolar	DMBA[2] induced	84
C5	Alveolar	DMBA induced	85
Normal midpregnant BALB/c mammary tissue			1

[1] Percentage of HAN outgrowths in cleared mammary fat pads of syngeneic BALB/c females in which palpable tumors were detected in 12 months. Data from Pauley and Socher (1980).

[2] 7,12 - Dimethylbenzanthracene.

and HAN outgrowths was regulated by local factors produced by ducts whereas neoplastic cells grew unrestrained by these factors. The properties described above as well as properties of hormonal responsiveness and tumorigenic potential were found to be stable characteristics for each HAN outgrowth over many serial transplantations (Medina, 1978).

The administration of carcinogens not only increases the frequency at which normal cells undergo the transition to preneoplasia, but also the frequency of transition from preneoplasia to the fully neoplastic phenotype if a second dose is given. Medina (1978) showed that if DMBA or MMTV was administered to mice carrying transplanted HAN outgrowths, there was a significant increase in the frequency at which these mice developed tumors as compared to mice not receiving carcinogens (Table 3). Cardiff (1984) has used data from HAN experiments to diagram schematically the progression of mouse mammary tissues from normality through hyperplasia to tumors (Figure 4). Since HANs do not invariably form tumors, but rather do so at some characteristic rate, Cardiff has suggested that the term "preneoplastic" should be replaced with "protoneoplastic" which he believes to imply tissue not obliged to progress. Likewise since the role of MMTV in the natural course of progression is to induce noduligenesis, he believes that MuMHV (Murine Mammary Hyperplasia Virus) should replace MMTV as the name of the virus. It remains to be seen whether

Table 3

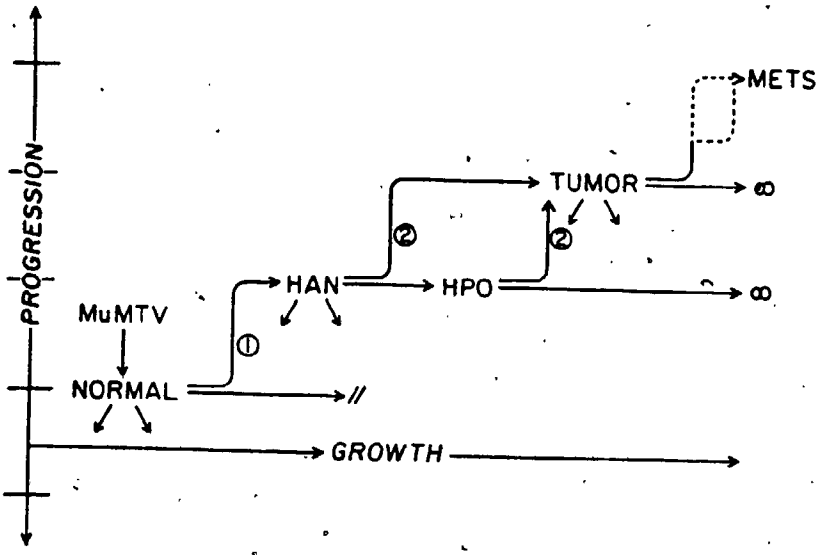
Enhancement of Mammary Tumor Progression by Viral and Chemical Carcinogens

HAN line	Carcinogen	Tumorigenicity[1]
D1	-	4
D1	MMTV	75
D1	DMBA[2]	71
D2	-	45
D2	MMTV	81
D2	DMBA	80
C4	-	84
C4	DMBA	97

[1] Percentage of HAN outgrowths in cleared mammary fat pads of syngeneic BALB/c females in which palpable tumors were detected in 12 months. Data from Medina (1978).

[2] 7,12 - Dimethylbenzanthracene

Figure 4: Progression of Mammary Cells Towards Neoplasia. Transitions theoretically require mutations, or "hits". Possible divergent pathways are indicated with arrows. HAN indicates hyperplastic alveolar nodule. HPO indicates hyperplastic outgrowth. METS indicates metastatic cells. From Cardiff (1984).


PROGRESSION
METS
TUMOR
∞
②
②
HAN
HPO
∞
MuMTV
①
NORMAL
//
GROWTH

these suggestions will receive any support.

2.7 Models of Tumor Induction by MMTV: MMTV is a replication competent retrovirus which lacks a classical oncogene, yet is responsible for a high incidence of mammary tumors in strains of mice which transmit it. The mechanism by which this is accomplished has been the source of much speculation, which developed along two lines. One body of thought is centred on an open reading frame in the proviral LTR for which no function has been assigned; the other on a form of insertional mutagenesis.

The investigation of the open reading frame (orf) began with the discovery that four methionine-rich proteins unrelated to known viral proteins could be translated *in vitro* from viral RNA partially degraded to reveal internal methionine codons (Dickson and Peters, 1981; Sen *et al*, 1981). These proteins were related, and thought to be the products of internal initiation within a reading frame of about 1000 nucleotides, sufficient to code for the largest (36 kd) species. The orf proteins were found to map to the unusually long LTR of MMTV when these products were generated by coupled *in vitro* transcription and translation of cloned LTR sequences (Dickson *et al*, 1981). Sequencing of cloned DNA revealed that a continous open reading frame did indeed exist, beginning in the env sequences adjacent to the 3' LTR of the provirus and extending for 960 nucleotides. An

orf transcript of 1.6 kb was detected in various normal, preneoplastic and neoplastic mouse mammary tissues (Wheeler et al, 1983; van Ooyen et al, 1983). This transcript contained sequences homologous to the 5' end of the viral genome spliced to the orf sequences at a site 80 bases upstream of the LTR. The protein product of this transcript has not been detected in vivo. Dickson and Peters (1983) have speculated on possible roles for the orf proteins, if they do exist in the mammary gland. They suggested a mitogenic role for the protein, which might profit the virus in dissemination and might predispose infected cells to uncontrolled proliferation. Alternatively, the protein may play some role in the process of integration, although other retroviruses accomplish integration without such a protein. Finally, the orf protein may be part of the system whereby viral gene expression is regulated by glucocorticoids. The detection of orf transcripts in normal tissue and the unique features of MMTV regulation make this seem the most plausible hypothesis (a further complexity is the regulation of orf expession but not structural gene expression by the host Lps locus as reported by Carr et al (1985)).

The investigation of insertional effects was inspired by analogy to other transforming retroviruses lacking onoogenes, particularly avian leukosis virus (ALV). ALV-induced tumors, like mammary tumors, are clonal in nature and appear after a long latency period. Tumors induced by ALV were found to contain ALV proviruses situated

immediately adjacent to c-myc, a known cellular proto-oncogene (Haywared et al, 1981; Payne et al, 1982), such that the level of c-myc transcription was elevated by the '3' proviral promoter.

An analogous model for the induction of tumors was suggested by results reported by Morris et al (1982; See also Section 4.1). The position of integrated MMTV proviruses was found to be more important than the number of proviruses in contributing to tumorigenesis. In other studies, comparison of MMTV-host cell junction fragments in a number of MMTV-induced tumors did not reveal a pattern of similar sized fragments indicative of integration at the same site in the genome (Cohen and Varmus, 1979; Fanning et al, 1980; Michalides et al, 1981; Morris et al, 1980; Altrock et al, 1982). Nusse and Varmus (1982) approached the question more directly by cloning cellular sequences from a junction fragment and using them to screen tumors for integrations in the vicinity of those sequences in the genome. A majority of tumors were found to have integrations within 20 kb of this locus, which they called int-1. A second locus, int-2, was reported by Peters et al (1983) to have similar properties. At least 75% of MMTV induced tumors examined to date have proviral integrations at one or both of the int loci. These loci are separate, mapping to chromosomes 15 (int-1) and 7 (int-2) (Peters et al, 1984). Both contain open reading frames which are transcribed in tumors, but not in normal tissues (Nusse et al, 1984;

Dickson et al, 1984). Both genes are highly conserved in humans, as is true for the known oncogenes (Bishop, 1983). The int genes are not related to the transforming gene isolated from mammary tumors by Lane et al (1981), nor to any other known oncogene. No int protein product has been isolated from normal or neoplastic tissue, and the function of the int genes in normal tissue is completely unknown.

The orientation of proviruses in the vicinity of int genes is almost invariably in the direction opposite to the direction in which these genes are transcribed. Proviruses were detected both upstream and downstream of the coding region, and at distances of up to 10 kb. The data indicate that the increase in int transcription in tumors is not the result of downstream promotion as seen in ALV induced tumors. Rather it is thought that transcriptional enhancer elements in the MMTV LTR function at a distance to increase transcription of the int genes (Nusse et al, 1984; Dickson et al, 1984). The MMTV LTR is known to function as a glucocorticoid dependent enhancer (Kennedy et al, 1982; Lee et al, 1984) with the ability to influence both chromatin structure (Zaret and Yamamoto, 1984) and the rate of initiation by RNA polymerase II (Firzlaff and Diggelmann, 1984). The frequency at which proviruses are found in the vicinity of the int loci in tumors and their arrangement thus argue strongly for the enhancement model of tumorigenesis, but important questions remain. First of all, the function of the genes is unknown. Secondly, there remain

a number of tumors for which no integration can be detected at an int locus. It is possible that proviruses reside further out in the flanking cellular sequences, or at some other important locus. Finally, since mammary tumors are the culmination of progression, the stage at which int activation is critical must be determined.

2.8 Objectives of the Investigation: In the context of the preceeding discussion, an investigative course of action was suggested which seeks to address each of the major points therein. With respect to the problem of tumor heterogeneity, it was decided to determine what relationship exists between the tumorigenic potential of tumor cell populations and their complement of MMTV proviruses. To determine the mode of action of a tumorigenic endogenous provirus, tumors induced by the Mtv-2 locus of GR mice were examined at the DNA and RNA levels with probes from mammary proto-oncogenes. Finally, the role of MMTV in the induction of hyperplastic alveolar nodules was investigated at the molecular level as an approach to describing the earliest events in progression from the normal to the transformed phenotype.

CHAPTER 3

Materials and Methods

3.1 Reagents: Restriction endonucleases were purchased from Boehringer Mannheim, except for Pst 1 and Bam H1 which were obtained from Bethesda Research Laboratories. Calf intestinal alkaline phosphatase was also purchased from Boehringer Mannheim. T4 DNA ligase was purchased from Collaborative Research. Agarose was obtained from the Marine Colloids Division of FMC Corporation. Isotopes were purchased from Amersham. NZ amine was purchased from Humko Sheffield Chemical Division of Kraft Incorporated. All chemicals were purchased from Fisher Scientific unless otherwise indicated, and were of the highest grade possible. The preparation of solutions is described in Section 3.24.

3.2 Preparation of High Molecular Weight DNA: High molecular weight DNA was prepared from mouse tissue following a modified procedure of Marmur (1961). Approximately 1 g of tissue was minced with scalpel blades in a petri dish, then washed into a glass dounce homogenizer with 5 ml of sterile 75 mM NaCl, 24 mM EDTA (ethylene diamine tetraacetic acid) pH 7.4. The tissue fragments were disrupted by six strokes of a motorized teflon pestle. Nuclei were then pelleted from the suspension in 30 ml Corex tubes by centrifugation at 5900 g for 10 minutes at room

temperature. The supernatant was removed by suction, and the nuclei were resuspended in 10 ml of 0.1 M NaCl, 20 mM Tris-HCl pH 7.4, and 10 mM EDTA. DNA was released by pronase (1 mg/ml) and SDS (sodium dodecyl sulphate 1% w/v) treatment in an overnight incubation at 37 C. Proteins and lipids were removed by three extractions with equal volumes of phenol:chloroform (1:1 v/v - phenol was saturated with 10 mM Tris-HCl pH 7.4, 1 mM EDTA). Phases were separated by centrifugation at 300 g in a clinical centrifuge. Wide bore pipettes were used to minimize shearing. DNAs were then dialysed against 5 mM Tris-HCl pH 7.4, and 0.1 mM EDTA, with dialysis solution replaced at least three times in three days. All solutions were sterilized by autoclaving; glassware was sterilized by dry heat (275 C for 4 hours).

3.3 Preparation of RNA: RNA was isolated following a modified procedure of Strohman et al (1977). Tissues were rapidly frozen in liquid nitrogen, then transferred to a cold solution of 7.6 M guanidine hydrochloride : 2 M potassium acetate (19:1 v/v). The tissues were immediately disrupted with a Virtis homogenizer. The solution was transferred to a 30 ml Corex tube, a 60% volume of 95% ethanol was added, and the tube was stored at -20 C overnight. The RNA was pelleted by centrifugation at 3000 g for 20 minutes (at -20 C), and the supernatant was removed. The pellet was resuspended by vortexing in 4.75 ml 8 M guanidine hydrochloride 0.25 M EDTA pH 7.0; 2 M potassium

acetate (pH 5.0) was added, and the solution was stored overnight at -20 C. RNA was pelleted as before, and resuspended in 20 mM EDTA, pH 7.0. The solution was then chloroform:butanol (4:1 v/v) extracted with centrifugation at 4000 g for 10 minutes to separate phases. Residual RNA was recovered from the nonaqueous phase by re-extraction with 3 ml of 20 mM EDTA pH 7.0 which was pooled with the previous aqueous phase. Freshly prepared 4.5 M sodium acetate pH 6.0 was added to a final concentration of 3.0 M, and the solution was stored at -20 C overnight. RNA was pelleted by centrifugation at 16,000 g for one hour at -20 C. The pellet was washed with 95% ethanol, dried, and resuspended in 0.9 ml distilled water. Two molar potassium acetate pH 5.0 was added to a final concentration of 0.2 M. Following the addition of 2 volumes of 95% ethanol, the solution was placed at -20 C overnight. RNA was pelleted at 16,000 g as before. The pellet was dried and resuspended in 0.1 ml 3 mM EDTA pH 7.0. All glassware used for RNA extraction was dry-heat sterilized (275 C for 4 hours). All solutions were treated with 0.001 volumes of diethyl pyrocarbonate (DEP, Sigma) to inactivate ribonucleases.

3.4 Determination of Nucleic Acid Concentration: The approximate concentration of nucleic acid in a solution was determined by measuring optical density at 260 nm. For the measurement of DNA concentration, the following formula was used:

[DNA] = O.D. at 260 nm x dilution factor x 50 μg / O.D.

For the measurement of RNA concentration, the formula was:

[DNA] = O.D. at 260 nm x dilution factor x 40 μg / O.D.

3.5 Restriction Endonuclease Digestion: For the purpose of restriction endonuclease analysis by blotting, 5 to 10 μg of each DNA sample was used. A tenfold concentrated solution of the incubation buffer (as specified by the manufacturer) was added to the sample prior to the addition of the restriction endonuclease (the activity of each enzyme was established by digesting 1 μg of λ DNA with 1 unit of enzyme for one hour, and visualizing the digestion products on an agarose gel stained with ethidium bromide). Murine DNA samples were typically digested with a ten-fold excess of enzyme for three hours at 37 C. DNA was precipitated by the addition of one-tenth volume of 3 M NaCl and two volumes of 95% ethanol (-20 C overnight). Precipitates were pelleted by centrifugation for 15 minutes at 15,000 g. DNAs were resuspended in 30 ul TE (10 mM Tris-HCl pH 7.4, 1 mM EDTA). Five μl of DGE (dye glcerol EDTA) was added to each sample prior to heating to 68 C for 10 minutes. Samples were then loaded onto 0.7% agarose gels (in 40 mM Tris-acetate pH 8.0, 2 mM EDTA) and electrophoresed for approximately 18 hours at 40 V (these conditions varied depending on the resolution required).

3.6 Transfer of DNA to Nitrocellulose: DNAs were transferred

to nitrocellulose using the procedure of Southern (1975) as modified by Cohen (1979). DNA was denatured by submerging the gel in a solution of 1.5 M NaCl, 0.5 M NaOH for one hour. The gel was neutralized by soaking it for a further three hours in 0.5 M Tris-HCl pH 7.2, 3 M NaCl. The gel was placed on a wick soaked in 6X SSC (standard sodium citrate; 1X is 0.15 M NaCl and 0.015 sodium citrate) and overlayed with a presoaked nitrocellulose sheet (Schleicher and Scheull BA-85, 0.45 μm pore size) followed by a 4 inch stack of paper towels. Transfer took place overnight, whereupon nitrocellulose sheets were baked in vacuo at 80 C and stored in a dessicator.

3.7 Transfer of RNA to Nitrocellulose: RNA samples were diluted with DEP-treated water to a volume of 25 μl and heated to 68 C for ten minutes. An equal volume of 20X SSC was added, and the RNA was applied to presoaked nitrocellulose sheets in a Schleicher and Schuell slot-blot apparatus. The sheets were then baked in vacuo for 2 hours at 80 C.

3.8 Preparation of [^{32}P] MMTV cDNA: cDNA was synthesized from MMTV (C3H) RNA provided by the Office of Program Logistics, National Institutes of Health, Bethesda, Maryland. Calf thymus DNA fragments were prepared by the method of Taylor et al (1976) and were used to randomly prime synthesis by avian myeloblastosis virus reverse

transcriptase (Life Sciences, St. Petersburg, Florida) [^{32}P] dCTP (410 Ci/mM) was incorporated by mixing 100 µCi with 0.32 µg MMTV RNA (boiled to remove secondary structure) in a solution of 40 mM Tris-HCl pH 8.1, 7 mM $MgCl_2$, 1 mM dithiothreitol, 4 mM KCl, 80 µg/ml of each of the deoxynucleotide-triphosphates dATP, dGTP and dTTP, 0.7 mg/ml calf thymus primers and 0.13 mg/ml actinomycin D. The reaction took place at 37 C for 45 minutes. RNA was hydrolysed by the addition of 3 M NaOH to a final concentration of 0.6 M in a 2 hour incubation at 37 C. The solution was then neutralized by the addition of 25 µl 0.2 M Tris-HCl pH 7.4 and 3 M HCl until neutral. The reaction mix was applied to a Sephadex G75 column (Pharmacia) and eluted with a column buffer containing 0.3 M NaCl, 0.02 M Tris-HCl pH 7.4 and 3 mM EDTA. A 36 ml void volume was discarded, and 20 1 ml fractions were collected in dry-heat sterilized scintillation vials. Fractions containing peak radioactivity were pooled, and cDNA was precipitated by the addition of 200 µg yeast RNA and 2 volumes of 95% ethanol (overnight at -20 C). cDNA was pelleted by a 30 minute centrifugation at 16,000 g (4 C). The pellet was dried <u>in vacuo</u> and resuspended in 300 µl 3 mM EDTA. A 1 µl aliquot was spotted on a glass-fibre filter in a scintillation vial, and following the addition of scintillant the radioactivity of the cDNA was quantitated in the scintillation counter. Specific activity of a cDNA probe was typically 10^8 cpm/µg RNA template.

3.9 Preparation of [^{32}P]-labelled DNA by Nick Translation: Plasmid DNA was labelled by nicking with DNAse I and filling in gaps with DNA polymerase I (Klenow fragment). Twenty-four units of DNA polymerase I and 1 µl of 10 µg/ml stock of DNAse I were added to a solution of 50 mM Tris-HCl pH 7.4, 5 mM $MgCl_2$, 50 µg/ml bovine serum albumin (BSA, fraction 4), 3 µg/ml dTTP, 3 µg/ml dGTP, 13 mM beta mercaptoethanol, 250 µCi [^{32}P]dCTP, 250 µCi [^{32}P]dATP, and 0.1 µg template DNA. This mixture was incubated at 15 C for 90 minutes, then at 68 C for 10 minutes. Unincorporated nucleotides were removed by centrifugation through a G75 spin-column as described by Maniatis et al (1982). A 1 µl aliquot of solution was spotted on a glass-fibre disc. Scintillation fluid was added, and radioactivity measured in a scintillation counter. The specific activity was typically 10^8 cpm/µg template DNA. Nick translated probes were boiled for 5 minutes prior to use.

3.10 Hybridization of [^{32}P] Probes to Nucleic Acids: Nitrocellulose sheets were preannealed overnight at 42 C with a solution of 6X SSC, 50% (v/v) formamide, 1X Denhardt's buffer (0.2% w/v of each of bovine serum albumin, ficoll, and polyvinylpyrollidone), 20 µg/ml yeast tRNA, and 2 µg/ml sheared salmon sperm DNA. Sufficient solution was added to wet the sheets which were placed in sealable plastic bags. The following day a corner of the bag was

opened, and MMTV cDNA or a nick translated probe (boiled 5 minutes) was introduced through the opening, which was then resealed. Approximately 10^6 cpm of probe was added per blot in most cases. Annealing occured for 2 days at 42 C whereupon excess probe was removed by washing in a solution of 2X SSC for one hour at room temperature followed by a wash in 0.1 X SSC and 0.1% SDS at 50 C for one hour and a rinse in 0.1X SSC. Fragments homologous to the probe were then visualized by autoradiography.

3.11 Growth of λ Phage: Stocks of phage obtained from various sources were initially plaque purified to ensure uniformity. Serial ten-fold dilutions of phage in 1 ml aliquots of plaque storage buffer (PSB) were performed. One hundred ul of each dilution was mixed with 100 ul of overnight bacterial culture in a 5 ml tube, and phage were allowed to adsorb for 15 minutes at 37 C. 3 ml of molten NZ overlay (0.7% agarose) was added, and the contents of the tube poured out on prewarmed NZ plates (1.5% agar). Following overnight incubation, isolated plaques were picked with sterile Pasteur pipettes and placed in 1.5 ml Eppendorf tubes containing 1 ml PSB and 1 drop chloroform. Phage were allowed to elute from the agar plug overnight at room temperature. A single plaque typically contained 10^6 pfu as evidenced by titration of the eluent.

A primary infection was performed by adsorbing 0.1 ml of the plaque eluent (approximately 10^5 pfu) with 0.1 ml overnight culture of E. coli (approximately 10^8 viable

cells) and inoculating into 50 ml NZ broth in a 250 ml flask. Cultures were rotated at 150 rpm in a 37 C waterbath overnight. The lysate was cleared of bacterial debris by centrifugation in a fixed-angle rotor at 3000 g for 10 minutes. Lysates were then titred as before, and stored over chloroform at 4 C. Titres were typically in the order of 10^9 to 10^{10} pfu/ml.

A large scale infection was performed by adsorbing 10^9 pfu of phage with 1 ml overnight culture of E. coli. This inoculum was then added to 1 l of NZ broth in a 6 l flask. The culture was incubated overnight in a reciprocating 37 C floor shaker at 100 rpm. The lysate was clarified and titred as before. A titre of 10^9 pfu/ml was required before proceeding to attempt purification.

3.12 Isolation of λ DNA: Ribonuclease A and DNAse I were added to the lysate at a concentration of 10 mg/l, and the lysate was incubated with slow rotation at 37 C for one hour. Twenty-four g/l NaCl was added, followed with 100 g/l polyethylene glycol (PEG; Fisher Carbowax 8000). After the PEG had dissolved, the lysate was placed at 4 C overnight. Phage were removed from the two-phase system by centrifugation in 250 ml bottles at 16,000 g for 20 minutes. The bottles were drained by inversion for 5 minutes, and the remaining liquid was removed. The pellets were resuspended by repeated pipetting in 20 ml total volume ice-cold PSB. The phage suspension was then loaded on a preformed CsCl

block gradient consisting of layers of 3 ml 1.7 g/ml CsCl (in PSB), 3 ml 1.5 g/ml CsCl, and 4 ml 1.45 g/ml CsCl in a 38 ml ultracentrifuge tube. The tube was filled to the top with PSB, and centrifuged at 70,000 g for 2.5 hours at 4 C. The tube was illuminated from below. The bluish-white phage band was collected from the interface between the 1.5 and 1.45 g/ml layers. Approximately 1 ml of phage from this gradient was the placed in a 5 ml ultracentrifuge tube, which was topped up with 1.5 g/ml CsCl and centrifuged at 125,000 g for 24 hours (to equilibrium). Phage were collected as before and dialysed against 10 mM $MgCl_2$, 50 mM Tris-HCl pH 8.0, and 10 mM NaCl to remove CsCl (two 1 l changes at room temperature).

DNA was extracted from phage as described by Maniatis et al (1982). The phage were transferred to a 10 ml centrifuge tube. 0.5 M EDTA pH 8.0 was added to give a final concentration of 20 mM. Proteinase K (Boehringer Mannheim) was added to a final concentration of 50 µg/ml. SDS (10% stock solution) was added to a final concentration of 0.5%. The solution was mixed by inversion and placed at 65 C for one hour. The solution was then extracted once with an equal volume of phenol (equilibrated with TE), once with phenol:chloroform (1:1 v/v), and once with chloroform. Phases were separated by centrifugation at 300 g in a clinical centrifuge. The aqueous phase was transferred with a wide-bore pipette to minimize shearing of DNA. Residual phenol was removed by sequential dialysis against three 1 l

volumes of TE overnight at 4 C. DNA concentration was determined as in Section 3.4.

3.13 Preparation of λ Arms: The nonessential "stuffer" region of the λ vector genome (see Appendix 2) was removed as follows. One hundred ug vector DNA was digested with a ten-fold excess of restriction endonuclease as in Section 3.5, ethanol precipitated, and resuspended in 100 µl TE. A 1 µl aliquot of the DNA was electrophoresed through a 0.5% minigel which was then stained with ethidium bromide and visualized on a UV transilluminator to ensure complete digestion. The DNA was heated to 68 C for 10 minutes to denature cohesive ends. DNA was loaded on a continuous 5-20% potassium acetate (in TE) gradient in a 5 ml ultracentrifuge tube which was centrifuged at 400,000 g for 2.5 hours at room temperature. Under these conditions (modified from Maniatis et al (1982)) it was possible to pellet the left and right arms. The central stuffer fragment was removed with the supernatant, which was poured off and discarded. The pellet was resuspended in 50 µl TE, and 1 µl was analysed on a minigel as before to demonstrate the loss of the central fragment. The yield from this procedure was typically 50%.

3.14 Preparation of Insert DNA by Total Digestion: Exogenous proviral junction fragments identified by Southern blotting analysis in totally digested DNA were isolated from low

melting agarose gels (Seaplaque, Marine Colloids Division, FMC Corporation). λ DNA cut with Hind III served as a marker to localize the region of the gel in which the desired fragment was present. A 5 mm slice of the gel was removed and placed in a 10 ml tube. The tube was placed at 68 C for 10 minutes, or until the agarose was competely melted. 3 ml of low salt buffer (0.2 M NaCl, 20 mM Tris pH 7.4, 1 mM EDTA) was added, and the DNA was bound to an Elutip'd column (Scleicher and Schuell) attached to a 10 ml syringe. Agarose was washed through with an additional 3 ml of low salt buffer, and the DNA was eluted with 0.4 ml of high salt buffer (1 M NaCl, 20 mM Tris-HCl pH 7.4, 1 mM EDTA) into a 1.5 ml Eppendorf tube. DNA was then precipitated by the addition of 2 volumes of ethanol with storage overnight at -20 C. DNA was resuspended in a small volume of TE at approximately 500 μg/ml.

3.15 Preparation of Partially Digested Insert DNA: Twenty kb fragments of partially digested murine DNA were prepared following Maniatis et al (1982). Conditions were established to generate a maximum proportion of partially digested Sau 3A1 fragments of 20 kb by serial two-fold dilutions of enzyme in a 1 μg reaction for one hour at 37 C. The products of digestion were analysed on a 0.4% agarose gel with markers to indicate the 20 kb region. Similar conditions were then used to digest 500 μg of DNA, and the extent of digestion was monitored as before. The DNA was extracted

twice with equal volumes of phenol:chloroform (1:1 v/v) as in Section 3.12. The DNA was ethanol precipitated and spun as in Section 3.5, and washed once with 70% ethanol. The DNA was then redissolved in 500 µl TE, heated to 68 C for 10 minutes, and cooled to room temperature. The DNA was loaded on a 38 ml 10-40% sucrose (in TE) density gradient and centrifuged at 100,000 g for 24 hours at room temperature. The bottom of the tube was then punctured with a needle, and 1 ml fractions were collected. Ten µl of each fraction was mixed with 10 µl water and 5 µl DGE and analysed on a 0.4% agarose gel as before to determine which fractions contained 20 kb DNA. These fractions were pooled, dialysed against 4 l TE at 4 C to remove sucrose, and ethanol precipitated as before. DNA was then redissolved in TE at a concentration of approximately 500 µg/ml. The yield from 500 µg starting material was typically around 10 µg.

3.16 Ligation of Arms and Inserts: Concentrations of arms and inserts were calculated following the method of Maniatis et al (1982). For equations used and a sample calculation, see appendix 3. For totally digested DNA, it was determined that for the production of concatamers the concentration of a 10 kb insert should be 24 µg/ml and the concentration of arms should be 550 µg/ml. Therefore a 10 µl reaction was set up containing 0.24 µg (0.5 µl) insert and 5 ug (5 µl) vector arms with distilled water to 16 µl. The DNAs were heated to 68 C for 10 minutes and cooled to room

temperature. Two λl of 10X ligation buffer (0.2 M Tris pH 7.5, 0.1 M $MgCl_2$, 50 mM DTT, 10 mM ATP) was added, followed by 2 λl (4.8 Weiss units) T4 DNA ligase. Ligation occured at 15 C overnight (except in the case of Eco RI cohesive ends which were ligated at 12 C). Ligation was confirmed by the presence of very high molecular weight concatamers when an aliquot of the sample was analysed on a 0.4% minigel.

3.17 In Vitro Packaging: Packaging reagents were prepared according to Protocol II of Maniatis et al (1982). Sonicated extracts from BHB 2690 (the prehead donor) were prepared by inducing a 1 l culture of the lysogen by elevating the temperature from 32 C to 45 C for 15 minutes when the culture was in midlog phase (reading 60 in the Klett colorimeter). The culture was then incubated at 37 C for 3 hours with vigourous aeration. Cells were recovered by centrifugation at 4000 g for 10 minutes in 250 ml bottles. The supernatants were drained, and residual liquid was removed. The pellets were resuspended in 3.6 ml total volume sonication buffer (20 mM Tris-HCl pH 8.0, 1mM EDTA, 5 mM beta mercaptoethanol). The suspension was transferred to a 10 ml centrifuge tube (on ice) and disrupted with a needle sonicator at maximum power with six bursts of ten seconds separated by cooling periods of 30 seconds. The suspension was then clarified by centrifugation at 30,000 g for 10 minutes at 4 C. To the supernatant was added an equal volume of sonication buffer and one-sixth volume of freshly

prepared packaging buffer (6 mM Tris-HCl pH 8.0, 50 mM spermidine, 50 mM putrescine, 20 mM $MgCl_2$, 30 mM ATP, 30 mM beta mercaptoethanol). Fifteen µl aliquots of the sonicated extract were frozen in 1.5 ml Eppendorf tubes by immersion in liquid nitrogen and stored at -70 C. The preparation of freeze-thaw exracts from BHB 2688 (the packaging protein donor) was similar except that induced cells were resuspended in 3 ml ice-cold sucrose solution (10% sucrose w/v in 50 mM Tris-HCl pH 8.0) and distributed to six 1.5 ml tubes. 25 µl lysozyme solution (2 mg/ml in 0.25 M Tris pH 8.0) was added to each tube and these tubes were immersed in liquid nitrogen. The solution was allowed to thaw, whereupon 25 µl packaging buffer was added to each tube. The extracts were pooled and centrifuged at 275,000 g for one hour at 4 C in the ultracentrifuge. Ten µl aliquots of the supernatant were frozen in 0.5 ml Eppendorf tubes by immersion in liquid nitrogen and stored at -70 C.

Recombinant phage were assembled by mixing 1 µl ligated DNA with the contents of 1 tube each of freshly thawed sonicated and freeze-thawed extracts, and incubating at room temperature for one hour. One ml of PSB and a drop of chloroform were then added, and the titre was determined as in Section 3.11.

3.18 Screening Plaques by Hybridization: Volumes of in vitro packaged phage containing approximately 10^5 pfu were pipetted into 10 ml tubes. Three hundred µl of overnight

culture was added to each tube; the phage were allowed to adsorb for 15 minutes at 37 C and 6.5 ml NZ overlay was added to each tube, which was inverted to mix the contents. The contents were then spread on 150 mm plates containing NZ agar. Incubation was at 37 C overnight.

Plaque lifts were performed as described in Maniatis et al (1982). Nitrocellulose filters (Millipore HATF) were trimmed to fit inside the 150 mm plates and marked assymetrically with fluorescent ink. Filters were then placed in contact with the surface of the agar such that the marks were aligned with similar marks on the bottom of the plate. When the filters had wet evenly, they were inverted and floated in a Pyrex dish containing 1.5 M NaCl, 0.5 M NaOH for 30 seconds. Filters were transferred to a neutralizing solution of 1.5 M NaCl, 0.5 M Tris-HCl pH 8.0 for 5 minutes, and finally to a solution of 2X SSPE (0.36 M NaCl, 20 mM NaH_2PO_4 pH 7.4, 20 mM EDTA). Filters were air dried, baked in vacuo at 80 C for 2 hours, and hybridized as in Section 3.10.

3.19 Preparation of Plasmids: Plasmid stocks were prepared from single colonies streaked on antibiotic plates and were used to inoculate 20 ml overnight cultures (antibiotics were present in all media used subsequently unless otherwise stated). The overnight culture was then used to inoculate a 1 l culture in a 6 l flask incubated at 37 C on a reciprocating floor shaker until the culture was in log phase (Klett = 80). At this point the plasmid was amplified

by the addition of spectinomycin at 170 mg/l. Cells were harvested by centrifugation in 250 ml bottles at 16,000 g for 10 minutes at 4 C. The pelleted cells were then resuspended in 5 ml per bottle of ice cold sucrose (25% w/v in 50 mM Tris-HCl pH 7.5), and harvested again by centrifugation as before. The cells were resuspended in 10 ml total volume sucrose solution. Five ml of lysozyme solution (20 mg/ml in 0.25 M Tris-HCl pH 7.5) was added, and the solution was mixed slowly on ice for 5 minutes. 10 ml 0.25 M EDTA was added, followed by 15 ml Brij-sodium deoxycholate (1% w/v Brij-58, 10 mM sodium deoxycholate, 10 mM Tris-HCl pH 7.5, 1 mM EDTA). The solution was mixed by pipetting up and down once, then kept on ice for 10 minutes. Cell walls and chromosomal DNA were removed by centrifugation at 41,000 g at 4 C. To the supernatant was added NaCl at the rate of 3% w/v, and PEG at the rate of 10% w/v. The solution was kept on ice for 2 hours with occasional mixing, then centrifuged at 1000 g in a clinical centrifuge for 10 minutes. The pellet was thoroughly resuspended in 13 ml TE. CsCl was added at the rate of 1 g/ml to give a final density of 1.55 g/ml. Ethidium bromide was added to 600 ug/ml. Plasmid DNA was separated from chromosomal DNA by centrifuging to equilibrium (2 days) at 100,000 g at 4 C. The plasmid band was visualized with UV light and collected with a hypodermic needle. Ethidium bromide was removed by 3 extractions with 1-butanol followed by 3 extractions with phenol. DNA was dialysed against

several 1 l changes of TE at 4 C. The concentration of plasmid DNA was determined as in Section 3.4.

3.20 Restriction Endonuclease Digestion and Phosphatase Treatment of Plasmids: Plasmids were digested with a ten fold excess of restriction endonucleases as in Section 3.5. Zinc sulphate was added after one hour of incubation to a final concentration of 5 mM, and the solution was made alkaline by the addition of a one-tenth volume of 1 M Tris-HCl pH 9.0. One unit of calf intestinal alkaline phosphatase was added per microgram of plasmid DNA. The solution was then incubated for a further hour at 37 C. Enzymes were removed by extractions with equal volumes of phenol, phenol:chloroform (1:1 v/v) and chloroform. Phases were separated with 3 minute centrifugations at 15,600 g. The DNA was then precipitated from the aqueous phase by the addition of NaCl to 0.3 M and 2 volumes of 95% ethanol with storage overnight at -20 C. The DNA was pelleted by centrifugation for 15 minutes in the microfuge, dried, and resuspended at approximately 100 ug/ml in TE.

3.21 Ligation of Insert DNA into Plasmid Vectors: Insert DNA (typically isolated as restriction fragments from low melting agarose as described in Section 3.14) was ligated to a ten fold molar excess of plasmid vector DNA to minimize circularization of the inserts. A 20 ul reaction contained 1 ug (10 ul) of vector DNA, 0.1 ug (1 ul) insert DNA with

sterile distilled water to 16 µl. The DNAs were heated to 68 C for 10 minutes to denature cohesive ends, then cooled to room temperature. Two ul 10X ligation buffer (described in Section 3.16) and 2 µl T4 DNA ligase (4.8 Weiss units) were added, and the reaction was incubated at 15 C overnight, except in the case of Eco RI cohesive ends, in which case incubation was at 12 C overnight.

3.22 Transformation of Plasmids into E. coli: Plasmids were introduced into E. coli following a modified procedure of Mandel and Higa (1970). An overnight culture of HB101 was inoculated into a 250 ml sidearm flask containing 40 ml of LB broth. The culture was incubated at 37 C with shaking at 50 rpm until the culture was in midlog phase (Klett = 70). The culture was chilled on ice for 20 minutes, and harvested by centrifugation at 5000 g for 5 minutes at 4 C in a fixed angle rotor. The cells were resuspended in 20 ml ice-cold 0.1 M $MgCl_2$, and centrifuged as before. The cells were resuspended in 2 ml ice-cold 0.1 M $CaCl_2$, and kept on ice for at least 30 minutes. Two hundred µl of cell suspension was mixed with 0.1 µg plasmid DNA in 100 µl TE. The suspension was incubated on ice for 30 minutes, at 42 C for 2 minutes, then on ice for an additional 30 minutes. 1 ml of LB broth (without antibiotics) was added, and the cells were incubated at 37 C for one hour. Cells were serially diluted in broth, and ten-fold dilutions were plated by adding 6.5 ml molten top agar (0.7%) and spreading on antibiotic

plates.

3.23 Antibiotic Screening and Colony Hybridization:
Transformants were screened for the presence of inserts by picking colonies with sterile toothpicks and replica plating on two types of plates: the first of which contained the antibiotic for which the plasmid-borne resistance gene had presumably been disrupted by the insert, and the second of which contained a second antibiotic for which the resistance gene was intact. Colonies displaying the predicted sensitivity were picked from the second plate and replated on another containing the same antibiotic. The presence of the insert was confirmed by hybridizing insert probe to a nitrocellulose filter applied to the plate and processed as in Section 3.18 (with an additional 3 minute bath in 10% SDS prior to base treatment).

3.24 Solutions and Media:
TE: 10 mM Tris-HCl pH 7.4 and 1 mM EDTA.
SDS: 10% w/v sodium dodecyl sulphate in water.
Phenol: Distilled at 180 C; stored at 4 C in the dark. Prior to use phenol was saturated with an equal volume of TE.
Pronase: 10 mg/ml in 20 mM Tris-HCl pH 7.4. Pronase was self-digested at 37 C for 2 hours, then stored at -20 C.
Ethidium bromide: 10 mg/ml stock solution of ethidium bromide in water diluted in TAE to 10 ug/ml.

DGE: 5 parts 0.125% bromophenol blue (in 25% glycerol) and 2 parts 0.2 M EDTA.

SSC: 0.15 M NaCl and 0.015 M sodium citrate.

DEP: All solutions not suitable for autoclaving were sterilized by the addition of diethyl pyrocarbonate to 0.1% v/v. Solutions were vigourously shaken, then allowed to stand overnight before use.

NZ: 1% w/v NZ amine, 0.5% yeast extract, 10 mM $MgCl_2$, and 10 mM NaCl. The pH of NZ broth was adjusted to 7.5 with NaOH prior to autoclaving.

LB: 1% w/v Bacto-tryptone (Difco), 0.5% yeast extract, and 10 mM NaCl. The pH was adjusted to 7.5 with NaOH prior to autoclaving.

Antibiotics: 25 mg/ml stock solutions. Ampicillin and streptomycin were dissolved in water and sterilized by filtration. Chloramphenicol was dissolved in 100 % ethanol, and tetracycline in 50% ethanol. All were stored at -20 C. Ampicillin was used at 50 µg/ml, chloramphenicol at 10 µg/ml, tetracycline at 15 µg/ml, and streptomycin at 25 µg/ml. Antibiotics were added to agar immediately prior to the pouring of plates, when the temperature of the agar was no higher than 50 C.

PSB: 100 mM NaCl, 10 mM $MgCl_2$, 20 mM Tris-HCl pH 7.5, and 0.01% (w/v) gelatin.

CHAPTER 4

Results

4.1 Analysis of MMTV Sequences in Tumorigenic and Nontumorigenic Cells from a Mammary Adenocarcinoma: The role of integrated MMTV proviruses in subpopulations of an MMTV induced mammary tumor was investigated to determine if a correlation could be made between proviral content and tumorigenicity. The cells chosen for this study were primary cultures derived by Soule et al (1981) from a single mammary adenocarcinoma (number 262) dissociated in methylcellulose. This tumor arose in a BALB/c mouse foster-nursed on a C3H mouse (BALB/cfC3H) such that it received infectious MMTV via the C3H milk. Single colonies from the trypsin-dissociated tumor were picked from methylcellulose cultures and established as monolayer cultures. The tumorigenicity of these cultures was determined in three hosts: 6 week-old BALB/c males; 6 week-old Swiss athymic females; and 3 to 4 week-old BALB/c females. Table 4, which reviews the data of Soule et al (1981), shows that dissociated cells from the original mammary tumor (262) formed secondary tumors in 4 of 4 BALB/c male and 3 of 3 athymic Swiss female mice. The MC3 subline formed secondary tumors in 5 of 5 athymic Swiss female mice and 4 of 6 BALB/c females and males. The MC4 subline formed tumors in 4 of 6 athymic female mice, 1 of 4 BALB/c females, and 1 of 9 BALB/c males. All of the tumors

Table 4

Tumorigenicity of Mammary Tumor Subcultures in Three Hosts[a]

Cells[b]	BALB/c male	BALB/c female	Athymic Swiss female
Dissociated Tumor 262	100%	not done	100%
MC-3 cell line	67%	not done	100%
MC-4 cell line	11%	25%	67%
MC-5 cell line	0%	0%	0%

[a] Percentage of animals having palpable tumors after 31 weeks.

[b] 2×10^6 cells injected either subcutaneously (BALB/c males, Swiss females) or into mammary fat pads (BALB/c females). Data from Soule et al (1981).

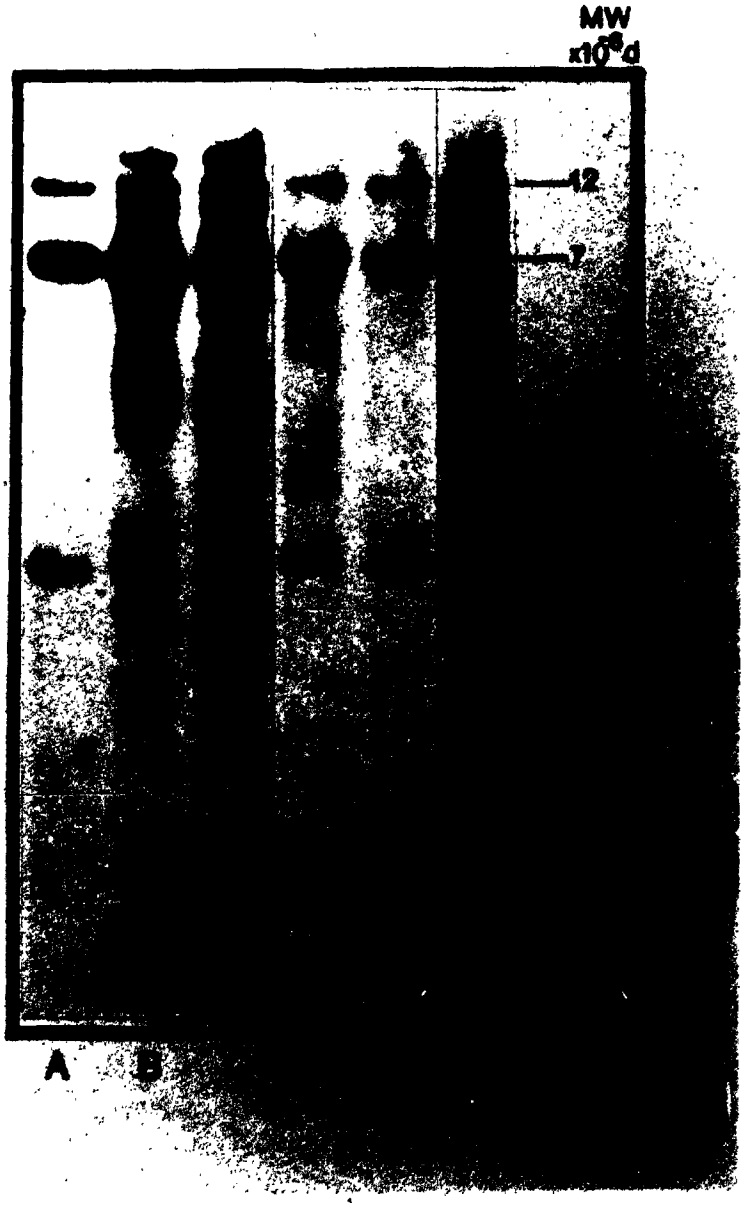
which resulted from the injection of MC3 or MC4 cells were carcinomas. MC5 cells were not tumorigenic in any of these systems.

To determine whether there was a correlation between proviral copy number and tumorigenicity, the proviral content of tumor 262 and its sublines was analysed by restriction endonuclease digestion with the enzyme Eco RI which generates 2 proviral junction fragments per integrated provirus (Cohen et al, 1979). Digestion with this enzyme (Figure 5) revealed that tumor 262 contained 5 to 6 acquired MMTV proviruses in addition to the endogenous complement of 2 complete copies and a subgenomic fragment in BALB/c (Cohen et al, 1979). The original mammary tumor and its dissociated cells were found to have identical Eco RI restriction patterns indicating that no major rearrangements occured in the proviruses during dissociation. The nontumorigenic subline MC5 was found to contain in excess of 7 acquired copies of the MMTV provirus as evidenced by greater than 14 novel MMTV Eco RI fragments in addition to the 5 fragments of endogenous origin. Surprisingly, in the DNA of the highly tumorigenic sublines MC3 and MC4 no newly acquired MMTV fragments could be detected. A similar pattern emerged from digestion with Bam H1 (Figure 6) which also generates two junction fragments per integrated provirus. No novel MMTV junction fragments could be detected in MC3 or MC4 DNA, but a large number were detected in the DNA of the MC5 subline. Both the tumor and its dissociated cells were again found to

Figure 5: Comparison of Eco RI digestions of BALB/c mammary tumor and methylcellulose colony subline DNAs. DNA was extracted from each sample, electrophoresed in a 0.7% agarose gel, transferred to nitrocellulose, and annealed with MMTV [^{32}P] cDNA (specific activity approximately 10^8 cpm/μg template RNA). All lanes contained 5 μg DNA. (A) BALB/c (low mammary tumor incidence) spontaneous mammary tumor DNA; this DNA has the same MMTV-specific Eco RI pattern as BALB/c normal organ DNA (Morris et al; 1980). (B) DNA from the dissociated cells of tumor 262. (C) DNA from tumor 262. (D) MC5 subline DNA. (E) MC4 subline DNA. (F) MC3 subline DNA. The endogenous MMTV proviral junction fragments of 10, 6, 5, 4.7 and 4 Md are 16.7, 9.6, 7.8, 7.6, and 6.7 kb in size, respectively.


MW
x10⁻³d
10
A
B

Figure 6: Bam HI digestions of BALB/c normal organ, mammary tumor, and tumor subline DNAs. The analysis was performed as in Figure 5. (A) BALB/c normal organ DNA. (B) DNA from cells dissociated from tumor 262. (C) DNA from tumor 262. (D) MC4 subline DNA. (E) MC3 subline DNA. (F) MC5 subline DNA. The endogenous MMTV junction fragments of 12, 7, 2.5 and 0.7 Md are 18.2, 14.4, 4.3 and 1.1 kb in size; respectively.


MW
x10⁻³d
12
7
A
B

contain about a dozen additional MMTV fragments indicative of 6 acquired proviruses.

An exogenous source of the acquired proviruses (from infectious MMTV rather than from amplification of endogenous proviral sequences) was confirmed by the presence of a 1.1 kb (0.7 Md) Bam HI fragment (Figure 6) and a 4.1 kb (2.5 Md) Pst I fragment (Figure 7) in DNAs containing acquired copies. Each of the fragments is generated by cleavage at an additional site in proviruses of exogenous origin (Cohen et al, 1979) and can therefore be used to identify exogenous proviruses. These fragments were detected in DNA from the original tumor and its dissociated cells and in MC5 DNA, but not in DNA from the MC3 or MC4 sublines.

The results thus far present a paradoxical situation. Whereas the nontumorigenic subline contained numerous acquired copies of exogenous MMTV proviruses, no such proviruses could be detected in the highly tumorigenic sublines; a finding opposite to the intuitive notion that a direct correlation should exist between proviral content and tumorigenicity. The apparent absence of exogenous proviruses in the tumorigenic sublines would seem to question whether such proviruses were responsible for this phenotype.

This paradox was resolved when tumors resulting from the injection of MC3 or MC4 cells by Soule et al (1981) were examined. Eco RI digestion of such tumors (Figure 8) revealed that DNA from such tumors contained one acquired MMTV provirus. The proviral junction fragments were of

Figure 7: Examination of DNA samples for acquired MMTV DNA sequences. The analysis was performed as in Figure 5, except Pst 1 was used to digest DNA samples. (A) DNA from dissociated cells from tumor 262. (B) DNA from tumor 262. (C) BALB/c normal organ DNA. (D) MC4 subline DNA. (E) MC3 subline DNA. (F) MC5 subline DNA. (G) DNA from MC3 tumor 2; this mammary tumor resulted from injection of 10^6 MC3 cells into a BALB/c female mouse. (H) DNA from MC3 tumor 1; this mammary tumor resulted from injection of 10^6 MC3 cells into a Swiss athymic nude mouse. Pst fragments of 3.3, 3.1, 2.5, 1.1, 0.9, 0.6 and 0.5 Md are 5.4, 5.1, 4.1, 1.8, 1.7, 1.5 and 0.8 kb in size, respectively.

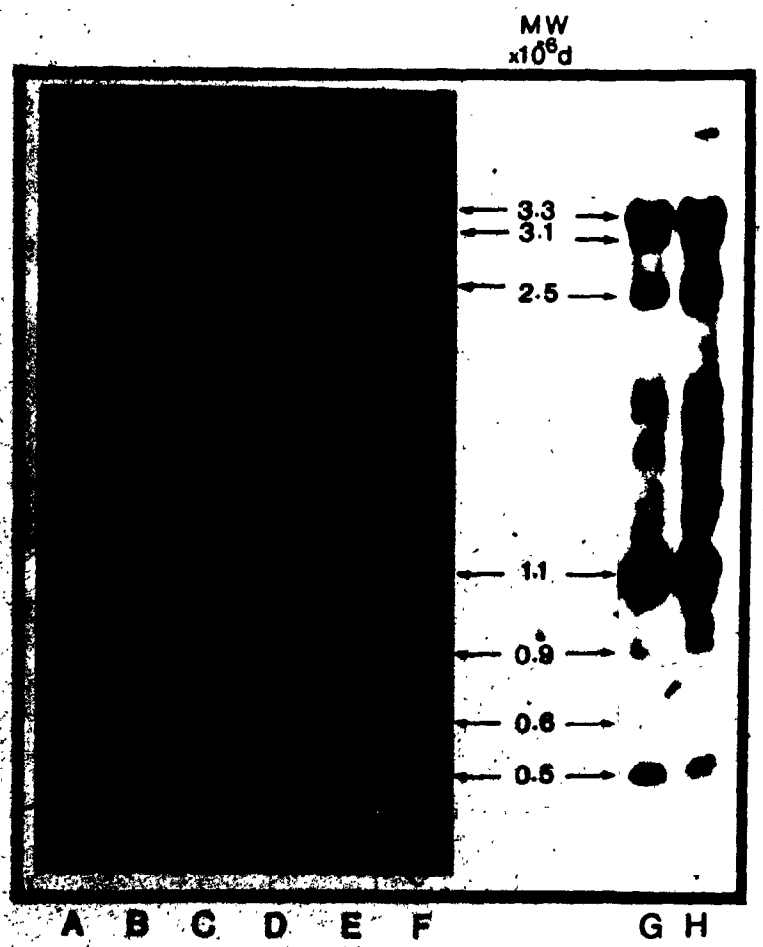
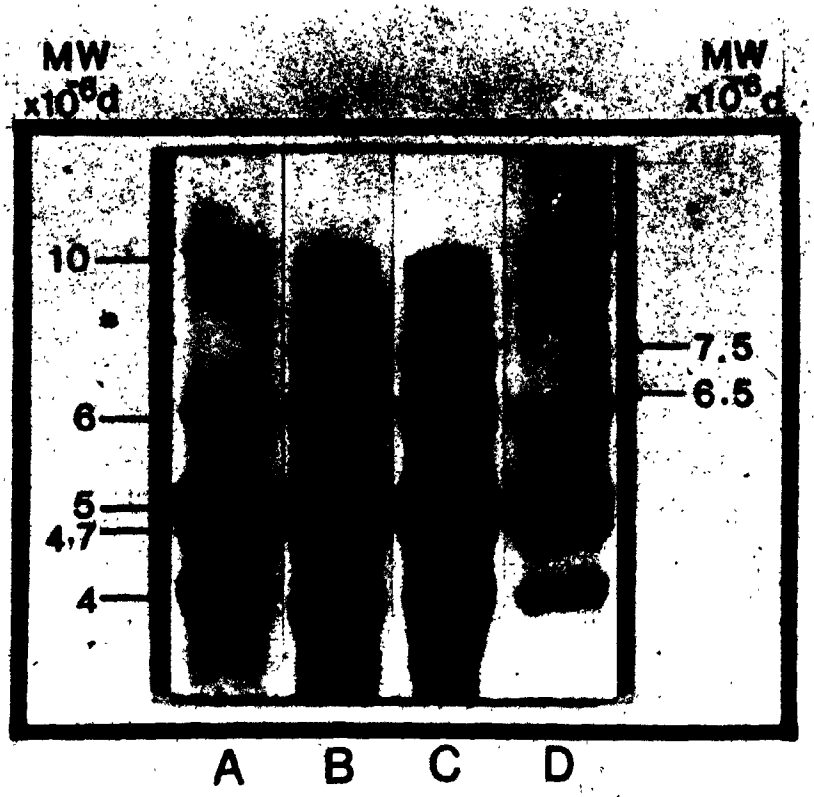

MW
x10^6d
3.3
3.1
2.5
1.1
0.9
0.6
0.5
A B C D E F G H

Figure 8: Eco RI digestion of MC3 cell and MC3 tumor DNAs. The analysis was performed as in Figure 5. (A) MC3 subline DNA. (B) MC3 tumor 2 DNA. (C) MC3 tumor 1 DNA. (D) BALB/c normal organ DNA. The endogenous BALB/c junction fragments of 10, 6, 5, 4.7 and 4 Md (16.7, 9.6, 7.8, 7.6 and 6.7 kb) are indicated with lines. Arrows designate acquired proviral junction fragments of 7.5 and 6.5 Md (11.5 and 10 kb). The 6.5 Md junction fragment is partially obscurred by the 6 Md endogenous fragment in this exposure. The large number of proviruses in MC5 cell DNA (see Figure 5) make it impossible to determine whether the junction fragments detected in MC3 tumors are also present in MC5 DNA.


MW
x10⁶d
MW
x10⁶d
10
6
5
4,7
4
7.5
6.5
A
B
C
D

identical size in all 4 tumors tested (two are shown in Figure 8). Similar results were obtained when Bam HI was used to digest the tumor DNAs (Figure 9). These results suggest that the cells in the MC3 and MC4 sublines which are tumorigenic and ultimately predominate in the tumor have an acquired provirus associated with tumorigenicity. These cells are apparently a minor population of the sublines grown in culture (discussed in Section 5.1).

The transcriptional activity of acquired MMTV proviruses in MC3 and MC5 sublines was quantified by hybridization kinetics (Table 5). Whereas normal BALB/c mammary cells generally produce very low levels of MMTV RNA corresponding to approximately 1 genome equivalent per cell (Varmus et al, 1973), the nontumorigenic MC5 subline produced greater than 500 genome equivalents per cell and could be stimulated by dexamethasone treatment to produce greater than 2000 genome equivalents per cell. The highly tumorigenic MC3 subline produced very low levels of MMTV RNA (less than 1 genome equivalent per cell). There is therefore no correlation between levels of MMTV RNA transcription and tumorigenicity in the tumor cell sublines.

4.2 Mechanism of Tumor Induction by Mtv-2: The Mtv-2 locus of GR mice has been shown to be responsible for germline transmission of the high tumor incidence of GR mice, as described in Section 2.4. It has not been previously determined whether this locus has direct tumorigenic

Figure 9. Bam HI digestion of BALB/c and MC3 tumor DNAs. The analysis was performed as in Figure 5. (A) BALB/c normal organ DNA. (B) MC3 tumor 1 DNA. (C) MC3 tumor 2 DNA. The endogenous BALB/c junction fragments of 12, 7 and 2.5 Md (18.2, 14.4 and 4.3 kb) are indicated with lines. Arrows designate acquired proviral junction fragments of 9.2 and 5.4 Md (16 and 8.3 kb).

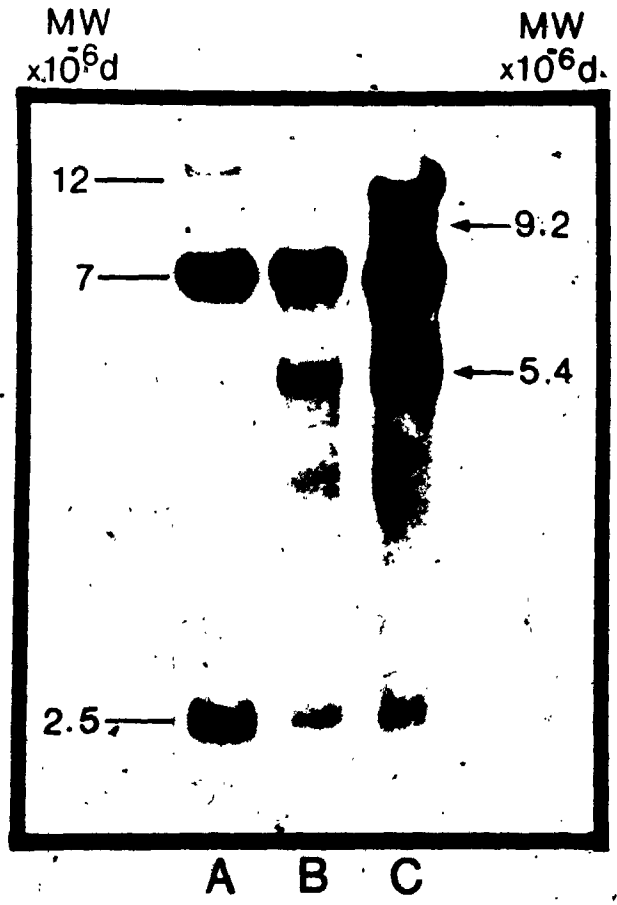

MW
x10⁻⁶d
MW
x10⁻⁶d.
12
7
2.5
9.2
5.4
A
B
C

Table 5

MMTV RNA in Mammary Tumor Subcultures

Source of RNA	Percentage Hybridization[a]	Crt	70S Genome Equivalents/Cell[b]
MC5 - dex[c]	50	1.7×10^{1}	575
	90	5.6×10^{2}	
MC5 + dex[d]	50	4.9	2050
	90	1.7×10^{1}	
MC3 ± dex	10	10^{4}	1
MC3 tumor[e]	11	10^{4}	1
	50	5.2×10^{4}	
BALB/cfC3H	50	10^{1}	1000
mammary tumor	90	10^{2}	
MMTV virion	50	2×10^{-2}	-
RNA	90	2×10^{-1}	

[a] Values derived from percentage annealing vs Crt curves. Data from Morris et al (1982).

[b] Calculated by the procedure of Varmus et al (1973).

[c] MC5 grown without dexamethasone

[d] MC5 grown with 10^{-5} M dexamethasone

[e] Tumor resulting from injection of 10^{6} MC3 cells into athymic Swiss mouse.

potential as a result of its expression or its effects on neighbouring genes or whether its tumorigenicity depends on the production of MMTV genomic RNA which is reverse transcribed and integrated as a provirus at some other proto-oncogenic locus. To address this problem, a series of tumors from GR mice foster-nursed on C57Bl mice (GRf mice) were examined at the DNA and RNA levels. Since C57Bl mice do not transmit milk-borne MMTV (Nandi and McGrath, 1973), all acquired proviruses in tumor DNA can be assumed to be derived from the Mtv-2 locus of the GR mice in which they arise.

The proviral content of GRf tumors was determined by restriction endonuclease analysis using the enzyme Eco RI which cleaves the MMTV provirus at a single site generating 2 junction fragments per provirus (Cohen et al, 1979). Southern transfers of Eco RI digested tumor DNAs were annealed with a cloned DNA fragment derived from the MMTV LTR labelled with ^{32}P by nick translation (Figure 10). Four GRf tumors were found to contain a single acquired MMTV provirus (tumors t1, t8, t14, and t15); five contained 2 acquired proviruses (tumors t2, t11, t12, t13, and t17) as did a benign cyst adenoma (t16); two tumors (t6 and t9) and one mammary hyperplasia (t7) contained 3 acquired proviral copies. As reported by MacInnes et al (1981), the autoradiographic signal from acquired proviruses relative to endogenous proviruses varies significantly indicating that the tumors were heterogeneous with respect to the number of

Figure 10: Presence of newly acquired MMTV DNA sequences in abnormal growths from GRf mammary glands. DNA was extracted, digested with Eco RI, electrophoresed in 0.7% agarose gels and transferred to nitrocellulose prior to annealing with a [^{32}P] MMTV LTR probe (approximately 10^8 cpm/ug template specific activity). All lanes contained 5 ug of DNA. (A) GR liver DNA. (B) tumor t17. (C) benign cyst adenoma t16. (D) tumor t15. (E) tumor t14. (F) tumor t13. (G) tumor t12. (H) tumor t11. (I) tumor t9. (J) tumor t8. (K) hyperplasia t7. (L) tumor t6. (M) tumor t2. (N) tumor t1.

A B C D E F G H I J K L M N

cells containing acquired proviruses. It is conceivable that proviruses resident in cells forming a minor population of the tumor could escape detection in this analysis. The copy numbers above therefore represent minimum values.

Cloned fragments from the int-1 and int-2 loci were labelled with ^{32}P by nick translation and annealed to Southern transfers of Eco RI or Bgl II (data not shown) digested tumor DNAs to determine if there were proviral integrations at these loci in GRf tumors (Figure 11). Integrations in the vicinity of these loci can be detected by this method (Nusse and Varmus, 1982; Peters et al, 1983) since the introduction of proviral sequences alters the restriction pattern of the region. This results in the appearance of an additional band on the autoradiogram corresponding to the altered int locus. Integrations could be detected at one or both int loci in 10 of 11 B-type mammary tumors tested, the exception being t15. Two tumors (t8 and t17) and a mammary hyperplasia (t7) were found to contain proviral integrations at both int loci. It is possible that tumor t15 contained proviral integrations outside the detection limits of the probes used (from 5 to 10 kb upstream and downstream of these fragments).

RNA expression from the int genes in GRf tumors was examined by slot blot hybridization (Figure 12). Unselected tumor RNA was applied to nitrocellulose filters and hybridized with probes from the coding regions of the int loci (Nusse and Varmus, 1982; Dickson et al, 1984). All six

Figure 11: Presence of newly acquired MMTV DNA sequences in the vicinity of the int-1 or int-2 loci. The analysis was performed as in Figure 10, except that 10 μg of each sample was digested with Eco RI. DNA fragments were annealed with either [^{32}P] labelled int-1 or int-2 probes (approximately 10^8 cpm/μg template specific activity) (A) GR liver DNA. (B) tumor t17. (C) benign cyst adenoma t16. (D) tumor t15. (E) tumor t14. (F) tumor t13. (G) tumor t12. (H) tumor t11. (I) tumor t9. (J) tumor t8. (K) hyperplasia t7. (L) tumor t6. (M) tumor t2. (N) tumor t1. All samples except t15 and t16 contain MMTV proviral integrations in int-1 or int-2. The integration of a provirus was detected at int-1 in sample t17; however the altered int-1 specific fragment is obscurred by the homologous allelic fragment in this exposure. An altered int-2 specific fragment in t7 (lane k) is too faint to be seen at this exposure.

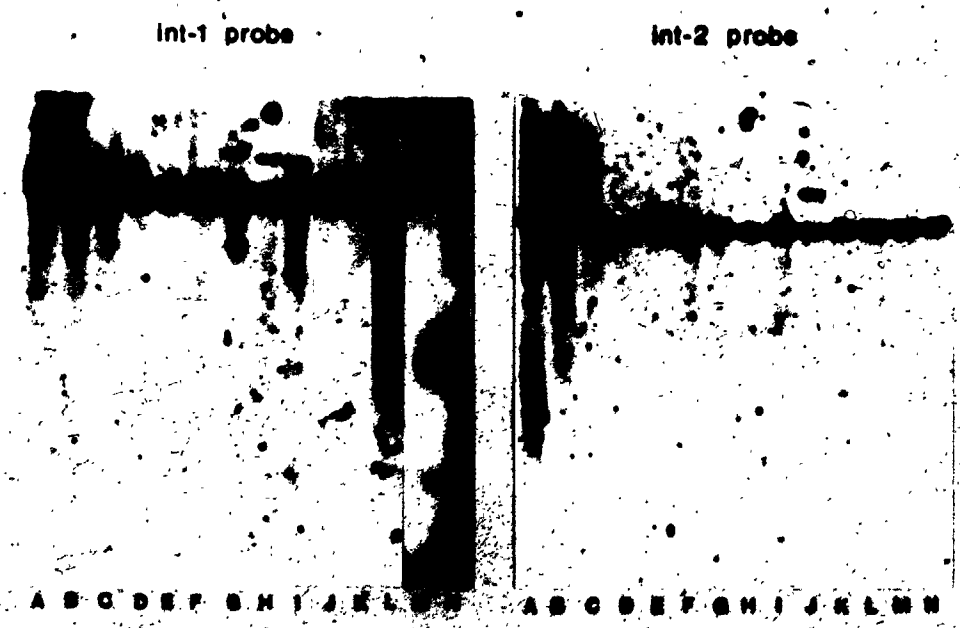
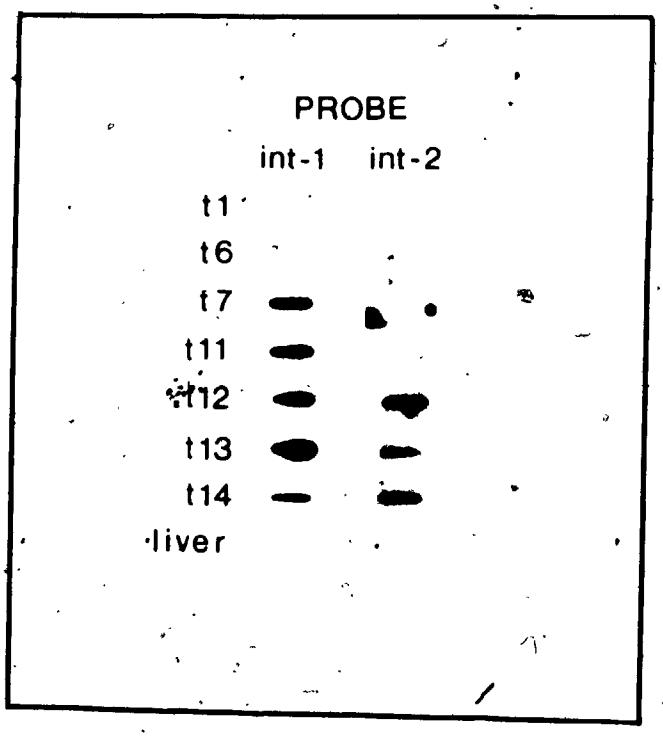

Int-1 probe
Int-2 probe
A B C D E F G H I J K L
A B C D E F G H I J K L M N

Figure 12: Slot blot analysis of GRf liver and abnormal growths from GRf mammary glands. 5 μg of RNA from each tissue was applied to nitrocellulose strips and annealed with [^{32}P] labelled int-1 or int-2 probes (approximately 10^8 cpm/μg template specific activity). The presence of RNA in all samples was confirmed by hybridization to dihydrofolate reductase cDNA.

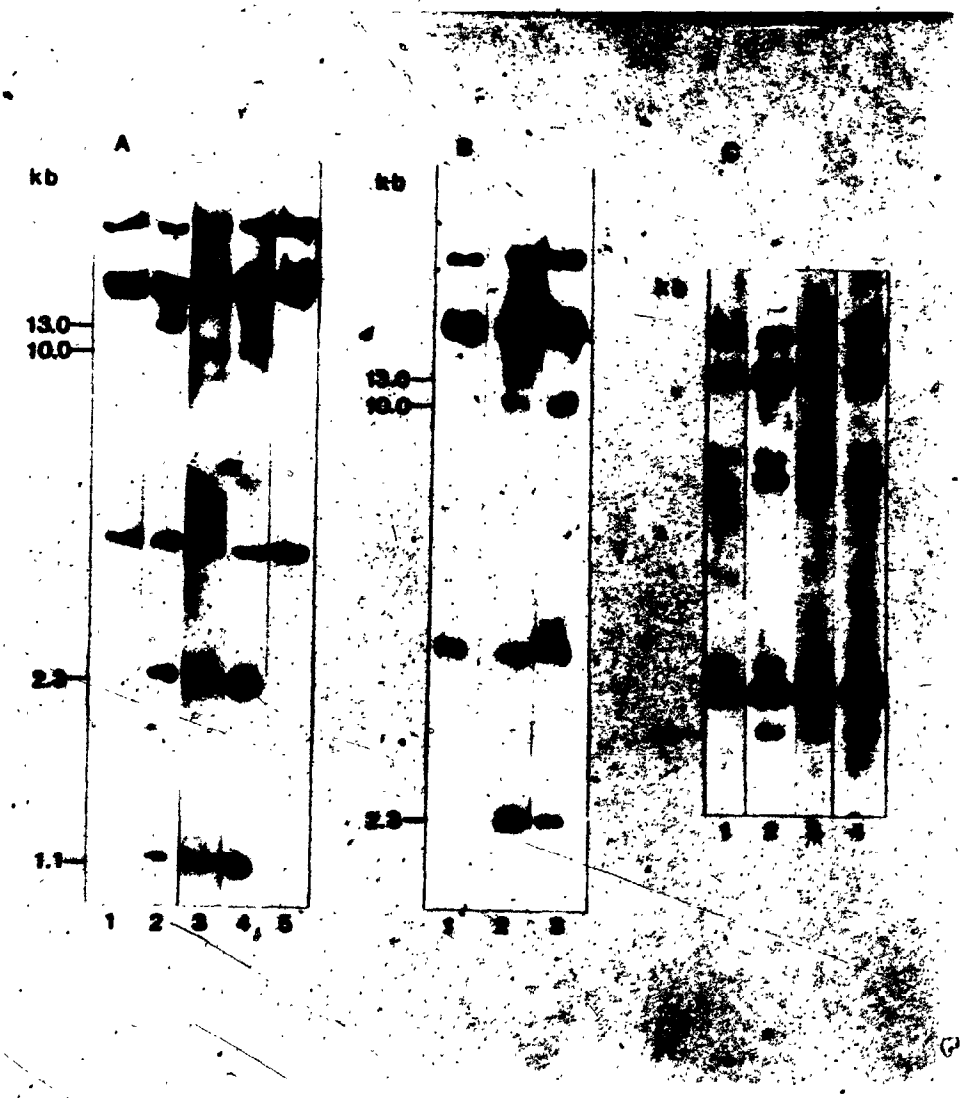

PROBE
int-1
int-2
t1
t6
t7
t11
t12
t13
t14
liver

tumors and the mammary hyperplasia for which RNA was available contained int-1 transcripts. Five of the six tumors which contained int-1 RNA also contained int-2 RNA (in no case was int-2 RNA alone detected). All tumors in which int RNA could be detected contained at least one provirus in the vicinity of an int locus, however in some cases int RNA could be detected in a tumor lacking a provirus at the corresponding locus (for example tumor t6 contained both int-1 and int-2 RNA, but integration could only be detected at the int-1 locus). The presence of RNA in all slots was confirmed by hybridization to a [^{32}P] cDNA probe prepared against dihydrofolate reductase RNA (the gift of Dr. W. Flintoff, University of Western Ontario) which detected transcripts in all samples.

4.3 Analysis of MMTV Proviral Integration Sites in Hyperplastic Alveolar Nodule (HAN) Outgrowths: A series of preneoplastic HAN outgrowth lines generated by Medina (1973) was examined to determine if integration at a specific locus was responsible for the distinctive phenotype of these tissues. These HAN outgrowth lines (described in Table 2) were induced by two distinct carcinogenic regimens: oral administration of 7,12-dimethylbenzanthracene or prolonged hormonal stimulation. As such, genomic rearrangements at a common locus in separate HAN lines would strongly implicate that locus as being involved in the transition from normality to preneoplasia.

Restriction endonuclease analysis was used to quantify the proviral content of HAN outgrowths. This revealed that the D2, C4, and C5 HANs had acquired new copies of the MMTV provirus. Bam HI junction fragments of 13.0 and 2.3 kb were detected in D2 HAN DNA (Figure 13, panels A and B) whereas C4 and C5 HANs contained junction fragments of 10.0 and 2.3 kb. The 1.1 kb internal Bam HI fragment characteristic of exogenous MMTV (Cohen et al, 1979) was also detected in D2, C4, and C5 DNA. D1 HAN DNA contained only the endogenous proviral fragments. Similar results were obtained with Bgl II digestion (Figure 13, panel C), which yielded a novel 1.6 kb junction fragment in digests of D2, C4, and C5 DNA but not in D1 DNA (the other Bgl II junction fragment may have been obscurred by an endogenous band). The total proviral content was determined by Eco RI digestion, which revealed 2 novel junction fragments of 4.7 and 10.5 kb in D2 and C5 DNA and 6 novel junction fragments in C4 DNA. Since Eco RI cleaves the MMTV provirus at a single site (Cohen et al, 1979), the number of proviruses in D2, C4 and C5 HAN DNAs must therefore be 1, 3 and 1 respectively. Slagle et al (1985) have also recently reported the presence of one acquired provirus in D2 HAN DNA. The lack of acquired proviruses in the low tumor incidence D1 HAN was reported by Ashley et al (1980) and Slagle et al (1985). Fewer junction fragments were detected in Bam HI and Bgl II digestions of C4 HAN DNA than in Eco RI digestions of the same DNA which may be the result of comigration of novel junction fragments

Figure 13: Common sizes of proviral junction fragments in HAN DNAs. DNA was extracted, digested to completion with a restriction endonuclease (Bam HI in panels A and B; Bgl II in panel C), electrophoresed through a 0.7% agarose gel, and transferred to nitrocellulose. Homologous fragments were identified by hybridization to [^{32}P] MMTV cDNA (specific activity approximately 10^8 cpm/ug RNA template). Five ug of DNA was present in each lane. (A) Lane 1, D1 HAN DNA; lane 2, D2 HAN DNA; lane 3, C4 HAN DNA; lane 4, C5 HAN DNA; lane 5 BALB/c liver DNA. (B) Lane 1, BALB/c liver DNA; lane 2, D2 HAN DNA and C4 HAN DNA mixed prior to digestion; lane 3, C4 HAN DNA and C5 HAN DNA mixed prior to digestion. The internal 1.1 kb Bam HI fragment has been run off the end of the gel in panel B. (C) Lane 1, BALB/c liver DNA; lane 2, D2 HAN DNA; lane 3, C4 HAN DNA; lane 4, C5 HAN DNA. Sizes of newly acquired proviral junction fragments are indicated (endogenous BALB/c fragments served as markers).


A
kb
13.0
10.0
2.3
1.1
1 2 3 4 5
B
kb
13.0
10.0
2.3
1 2 3
C
1 2 3 4

with endogenous junction fragments or the presence of very large junction fragments which do not transfer efficiently to nitrocellulose (personal observation).

To confirm that the acquired proviruses in D2, C4, and C5 HANs were of exogenous origin rather than the result of amplification of endogenous sequences, DNA from HANs was digested with the restriction endonuclease Pst-1 (Figure 14). The 4.1 kb internal Pst 1 fragment characteristic of exogenous MMTV proviruses (Cohen et al, 1979) was detected in D2, C4, and C5 DNA, but not in D1 HAN DNA.

The striking similarity of Bam HI, Bgl II, and Eco RI restriction patterns of D2, C4, and C5 HAN DNAs make it highly probable that acquired proviruses in these tissues reside at the same genetic locus, since random integration would be unlikely to generate so many comigrating fragments (the statistical probability of such an occurance is calculated in Section 5.3). To determine if this locus was one of the putative proto-oncogenes int-1 or int-2 (Nusse et al, 1984; Dickson et al, 1984) Eco RI digests of HAN DNAs were analysed by hybridization to cloned probes from the int loci labelled with ^{32}P by nick translation (Figure 15). Proviral integrations in the vicinity of these loci can be detected in the form of additional bands on Southern blots of DNA carrying such integrations as opposed to normal mouse DNA (Nusse and Varmus, 1982; Peters et al, 1983). No such alterations were detected in HAN DNAs indicating that the common site of MMTV proviral integration in these DNAs was

Figure 14: Presence of proviral restriction fragments in HAN DNAs which are characteristic of exogenous MMTV. Five µg of each DNA was digested with Pst I prior to analysis as in Figure 12. (1) D1 HAN DNA. (2) D2 HAN DNA. (3) C4 HAN DNA. (4) C5 HAN DNA. (5) BALB/c liver DNA. The 4.1 kb fragment is associated with exogenous MMTV and is not present in BALB/c liver or D1 HAN DNA.

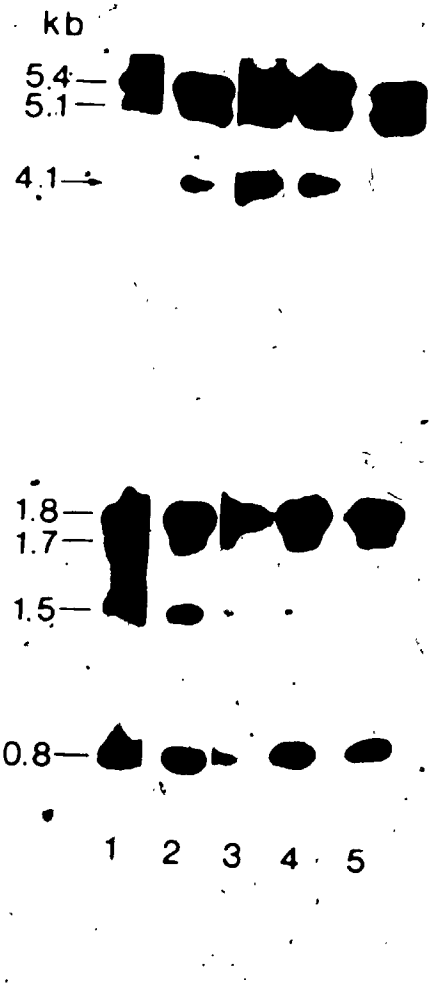
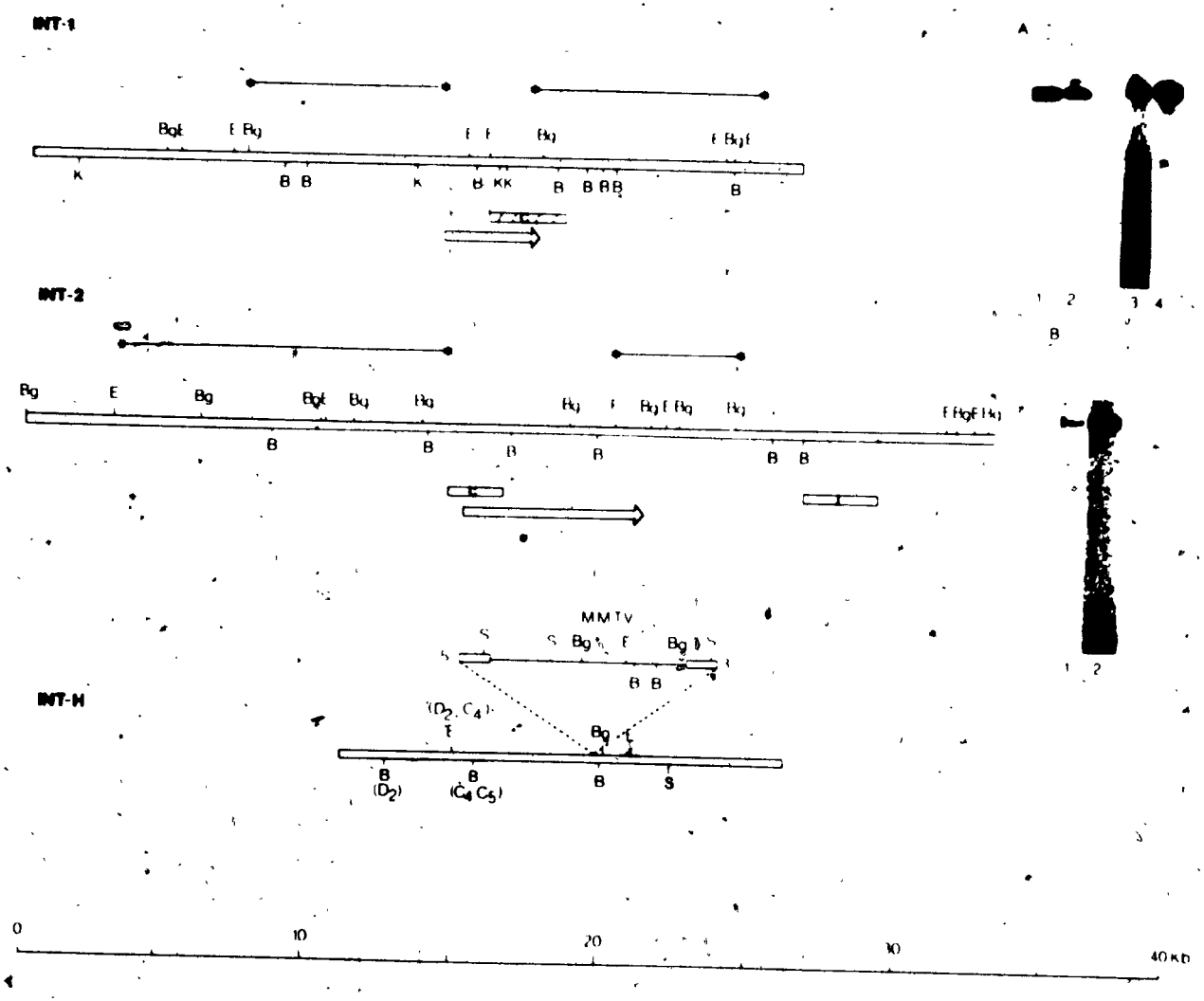

kb
5.4
5.1
4.1
1.8
1.7
1.5
0.8
1 2 3 4 5

Figure 15: Test for MMTV integrations in the vicinity of the int loci in HAN DNAs. Five ug of each DNA was digested with Eco RI or Bgl II and analysed as in Figure 10. The "c" fragments from int-1 and int-2 (boxes) were labelled with ^{32}P and used as probes. The specific activity of probes was approximately 10^8 cpm/ug template. (A) int-1 probe. Lane 1, BALB/c liver DNA digested with Eco RI; lane 2, D2 HAN DNA digested with Eco RI; lane 3, BALB/c liver DNA digested with Bgl II; lane 4, D2 HAN DNA digested with Bgl II. (B) int-2 probe. Lane 1, BALB/c liver DNA digested with Eco RI; lane 2, D2 HAN DNA digested with Eco RI. Restriction maps of int-1 and int-2 were modified from Nusse and Varmus (1982) and Peters et al (1983) respectively. Arrows refer to the transcriptional units. Regions bounded by asterisks denote areas of proviral integration in tumors. The restriction map of int-H was derived from the data given in Figures 13 and 14 and from additional experiments using pol and env specific probes (see Gray et al, 1986) Restriction sites are as follows: E is Eco RI, B is Bam HI, Bg is Bgl II, S is Sac I, K is Kpn I


INT-1
INT-2
INT-H
MMTV
A
B
1 2 3 4
1 2
0
10
20
30
40 Kb

not in the vicinity of the int loci. The common MMTV proviral integration site in preneoplastic HAN DNAs apparently represents a new locus associated with the hyperplastic phenotype; we have therefore designated this locus int-H.

4.4 Molecular Cloning of the int-H Locus: Since an analysis of the role of int-H in the induction of murine mammary hyperplasias requires cloned probes from this region, cloning strategies were devised to recover these sequences from the genomes of hyperplastic tissues. Because of the capacity of λ phage vectors to carry large DNA insert fragments and the relative ease of screening λ "libraries" with DNA probes, it was decided to attempt to clone int-H sequences in λ phage vectors.

The initial strategy was to isolate restriction fragments identified as novel proviral junction fragments by hybridization to MMTV probes as described in section 4.3. These fragments would be cloned in λ substitution vectors as described by Maniatis et al (1982). The 4.7 kb and 10.5 kb Eco RI junction fragments were chosen since they could be resolved from the endogenous BALB/c Eco RI junction fragments of 16.7, 9.8, 7.8, 7.6 and 6.7 kb. λgtWES·λB was chosen as a vector since it is capable of replicating with insert fragments of sizes from 2 to 15 kb (Leder et al, 1977). DNA was purified from agarose slices containing the appropriate fragments and ligated into λgtWES·λB arms.

Approximately 500,000 recombinant clones of this preparation were screened as plaques following *in vitro* packaging. One plaque contained sequences homologous to MMTV. This clone was found to contain a truncated junction fragment containing the 5' portion of the acquired MMTV provirus (indentified by its having the exogenous restriction pattern), but no cellular sequences from the region adjacent to it. A recombination event had apparently eliminated the sequences of interest (this phenomenon is discussed in Section 5.4), and no further attention was given to this clone.

The above findings prompted speculation that *int*-H locus contained sequences which were difficult to clone in *E. coli* hosts for unknown reasons. It was decided to attempt to clone overlapping 20 kb fragments generated by partial digestion of HAN DNA with a restriction endonuclease recognizing a tetranucleotide sequence as described by Maniatis *et al* (1982). It was hoped that an MMTV probe could be used to identify clones carrying all or some of the acquired provirus of HAN DNA and a portion of the cellular sequences flanking it without the sequences which might be interfering with the replication of clones carrying entire Eco RI junction fragments. A number of vector systems were employed without success. Finally, with the availability of vector-host systems specifically designed for the propagation of sequences which resist cloning in conventional vectors (Wyman *et al*, 1985), a successful

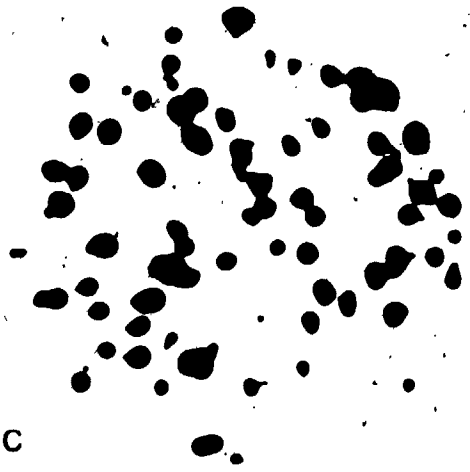
strategy was arrived at. HAN DNA partially digested with Sau 3A1 was ligated into arms prepared from the vector Charon 35. Recombinants were plated on the recA⁻recBC⁻ *E. coli* host strain DB1161. Such a strategy minimizes the deletions which occur when certain sequences are cloned in *E. coli* (Wyman *et al*, 1985).

Approximately 500,000 recombinant clones containing 20 kb Sau 3A1 fragments of D2 HAN DNA ligated into the Bam HI site of Charon 35 arms were screened on lawns of DB1161. Seven clones were found to contain sequences homologous to the probe (the identification and isolation of one such clone is illustrated in Figure 16). These clones were plaque purified and rescreened with probes derived from the cellular sequences flanking the endogenous proviruses (Units II and III) of the BALB/c mouse strain (these cloned probes were a gift from Dr. Gordon Peters, Imperial Cancer Research Fund, London, U.K.). Four clones (X1-2, X4-3, X5-1, and DY-1) contained sequences derived from the 5' end of unit III; one (X5-1) apparently contained the entire Unit III provirus with flanking sequences from both ends; one (X3-1) contained sequences from the 5' end of Unit II; one clone (X2-3) hybridized weakly with the 5' probe, but was thought to be reacting with a repeat sequence element known to be present in the Unit III 5' probe (personal communication of Dr. Gordon Peters and personal observation). DNA was isolated from a large scale culture of λX2-3 and analysed by restriction endonuclease analysis (Table 6). The results of

Figure 16: Identification and isolation of a phage clone containing MMTV sequences. (A) Approximately 10,000 recombinant phage clones were plated per 150 mm plate. (B) A plaque containing MMTV sequences is identified by the autoradiographic signal from nitrocellulose transfers. Such plaques were picked from the plate and stored in plaque storage buffer. (C) Purified MMTV-positive plaques were replated at a lower density (100 to 1000 plaques per plate) and tested to ensure purity.

A

B



C

Table 6

Restriction Endonuclease Analysis of X2-3 Insert DNA

Enzyme[a]	Eco RI	Sac I	Bam HI	Pst I
Fragments[b] (kb)	7	8*	25*	
	4.7*	5.3+	27	4.5*
	3.8	2.2		3.0*
	2.0+	2.0*		2.8
	17.5kb	17.5kb		2.4
				2.3
				2.0
				1.7*
				1.5*
				0.9*
				21.1kb

[a] Eco RI, Sac I and Pst I cleave at the boundaries of the insert; in each case phage arm fragments of 10.6 and 19.6 kb are generated. The vector's Bam HI sites are destroyed by ligation to Sau 3AI fragments; Bam HI fragments include the vector arms. Not all Pst I fragments could be mapped; one Pst I fragment may be derived from the vector arms.

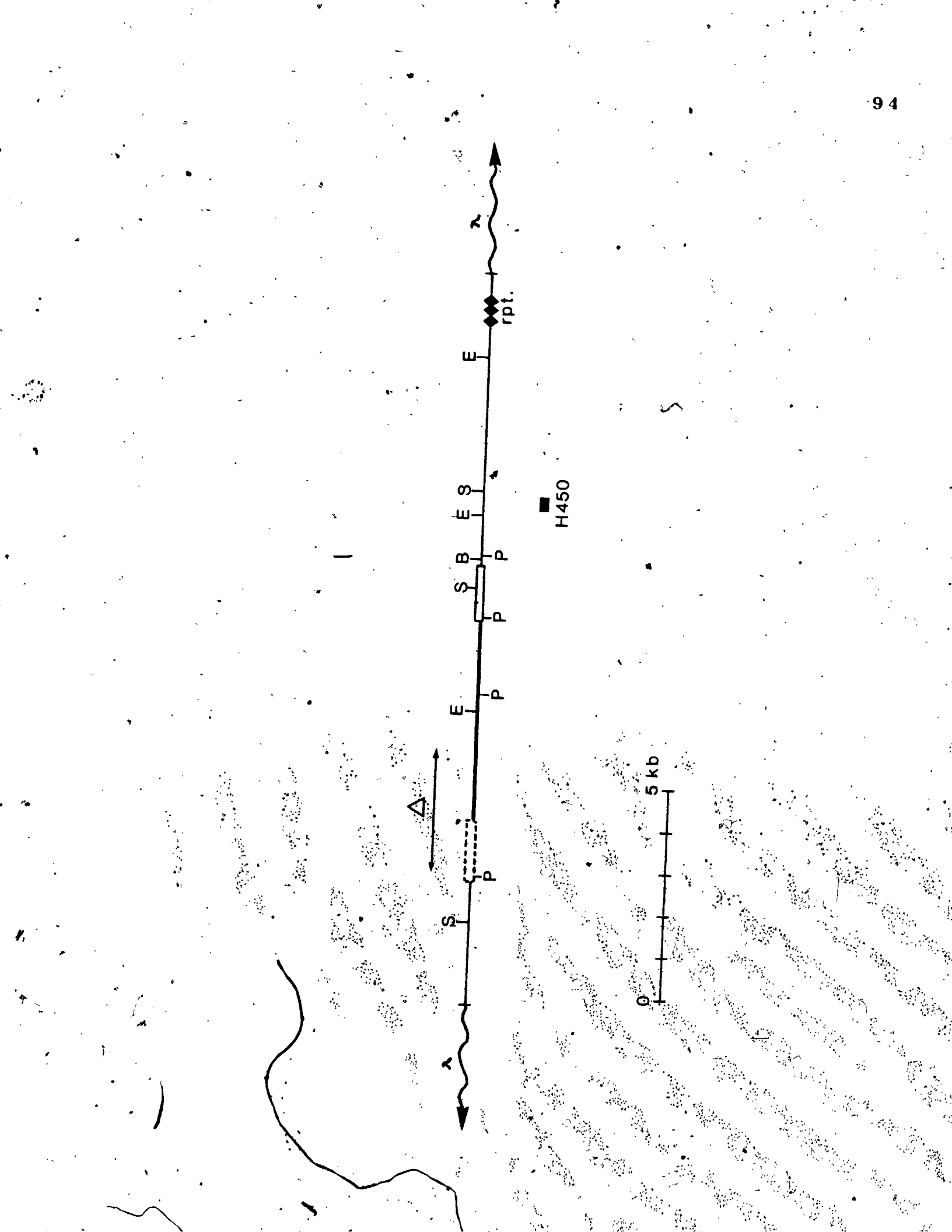
[b] Fragments hybridizing to MMTV probes are indicated with an asterisk. The + denotes fragments containing repeated sequence elements.

these and other experiments were used to generate a partial restriction map of λ X2-3 (Figure 17). A 17.5 kb DNA insert fragment was cloned containing an MMTV provirus along with flanking sequences at either end. A portion of the 5' end of the provirus had apparently suffered some deletion with the loss of two Pst 1 sites flanking the so-called "poison" sequence previously shown to be resistant to cloning in E. coli (Majors and Varmus, 1981 and 1983). In addition, the expected Bam HI sites in the proviral env gene were not detected, although hybridization to a cloned env probe indicated that no major deletion had occured in the region. Small deletions or point mutations could account for this finding.

The cellular sequences of λ X2-3 appear not to have undergone major alterations in the course of cloning as evidenced by a restriction profile of cellular sequences matching that predicted by the analysis of restriction fragments described in section 4.3. A repeated sequence element was detected at the 3' end of the insert fragment; probes containing this element hybridize to a large number of restriction framents in mouse DNA resulting in a "smear". The presence of this element may explain the weak hybridization to the Unit III 5' probe which contains repeated sequences.

A 450 bp Hind III fragment from the 3' flanking cellular sequences was used as a probe to confirm that λ X2-3 does in fact contain sequences derived from the int-H locus (Figure

Figure 17: Partial restriction map of λX2-3. Restriction sites were arranged on the basis of single and double digestions of X2-3 DNA and plasmid subclones of λX2-3 fragments. E denotes Eco RI sites; S, Sac I sites; B, Bam HI sites; P, Pst I sites. The open triangle denotes the region in which a deletion has occurred. The open boxes represent proviral LTR regions; it is not known whether the left LTR is complete. Proviral structural genes are denoted by the thick line. A repeated sequence element is denoted by closed diamonds. The phage arms are shown as wavy lines.


λ
rpt.
E
S
E
B
S
P
P
P
E
P
S2
P
λ
H450
0
5 kb

18). This probe detects a 9.6 kb proviral junction fragment in Asp 718 digested D2 HAN DNA which cannot be detected in BALB/c liver DNA. The 9.6 kb Asp 718 fragment was shown to be the 3' proviral junction fragment of the int-H locus of D2. Interestingly, the normal allele of int-H could not be detected in D2 HAN DNA indicating that only the altered locus is present. This may be the result of chromosomal loss or a loss and/or duplication of the part of the chromosome on which the int-H locus resides. The 450 bp Hind III was also subcloned into the plasmid vector GB2 (Figure 19). This plasmid shares no sequence homology with the commonly used cloning vectors (Churchward et al, 1984). This subclone will be used as a probe in the future isolation of a normal int-H locus.

4.5 Preliminary Investigation into the Function of int-H:

The conservation of int-H sequences in the genomes of nonmurine species was explored by hybridizing the H450 probe to rat and human DNA restriction fragments under conditions of reduced stringency (as in Section 3.10 except that hybridization occured at 37 C and washing at 45 C instead of 50 C). A set of int-H related sequences was revealed in mouse, rat and human DNAs (Figure 20). Approximately six copies of related sequences exist in the mouse genome, with restriction polymorphisms noted between BALB/c and GR mouse strains. Approximately six related rat sequences were detected at a slightly decreased signal intensity. As

Figure 18: Demonstration that λX2-3 contains sequences derived from int-H. Lanes A, C, and E contained 5 μg of BALB/c liver DNA digested with Asp 718. Lanes B, D, and F contained 5 μg of D2 HAN DNA digested with Asp 718. The specific activity of the probes was approximately 10^8 cpm/μg template. The 3' probe contains sequences from the viral env gene. The H450 probe detects an altered int-H locus in D2 HAN DNA not detected in normal BALB/c DNA.

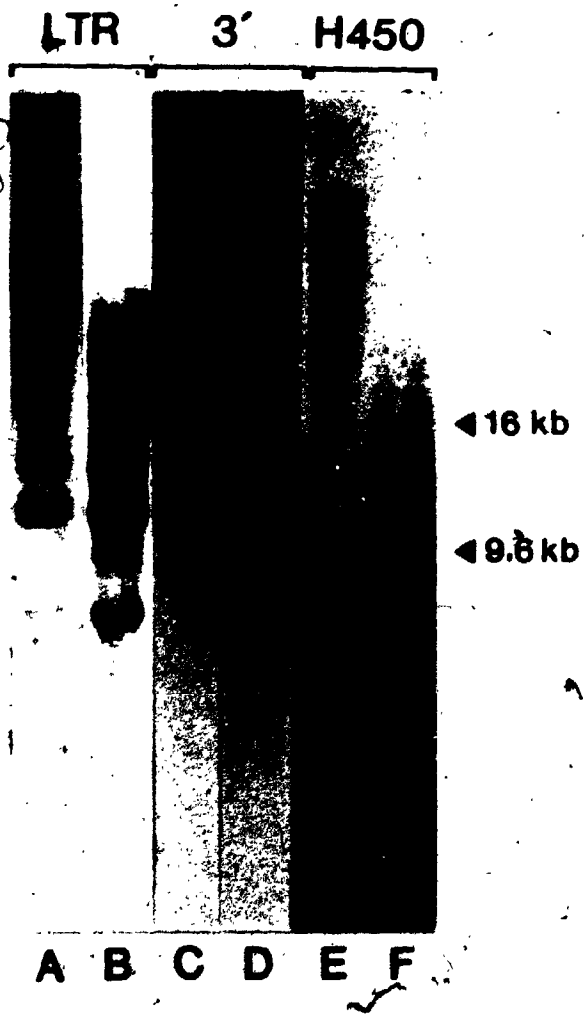
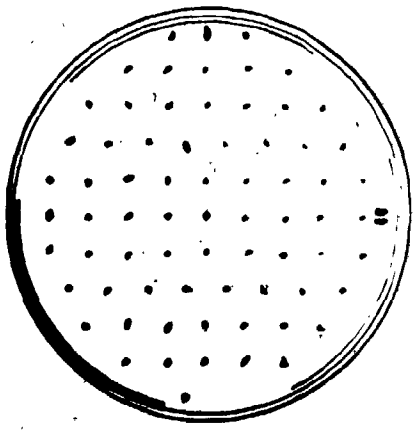
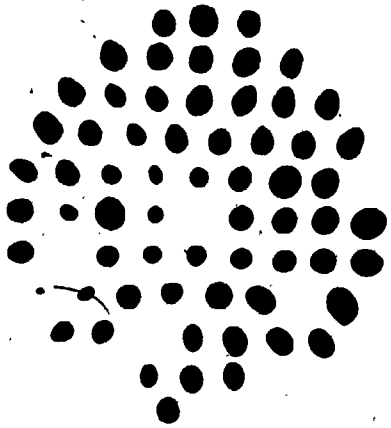

LTR
3′
H450
16 kb
9.8 kb
A B C D E F

Figure 19: Subcloning of an int-H fragment. The 450 bp Hind III fragment from λX2-3 was ligated into the Hind III site of plasmid GB2 and transformed into E. coli HB101. A series of transformants (A) were replated on streptomycin plates. (B) A nitrocellulose filter was applied to the plate, then hybridized with an H450 probe to identify transformants having H450 inserts.



A



B

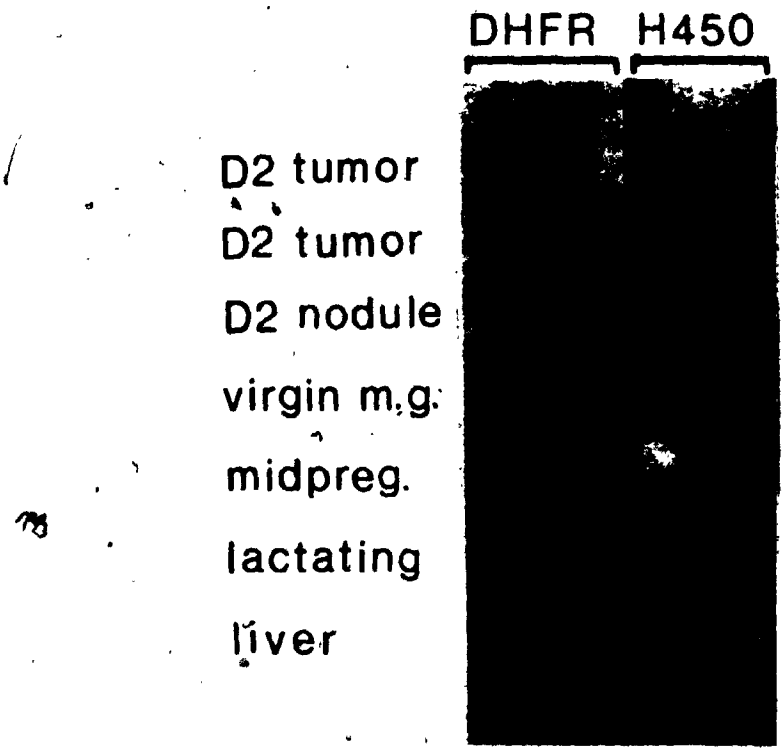
Figure 20: Detection of int-H related sequences in the genomes of rats and humans. 10 µg of each sample was digested with Eco RI or Bam HI, electrophoresed through 0.7% agarose, transferred to nitrocellulose sheets and hybridized with [^{32}P] labelled H450 DNA probe under relaxed hybridization conditions described in Section 4.5. The specific activity of the probe was approximately 10^8 cpm/µg template. Lanes A and E contained BALB/c normal mammary gland DNA. Lanes B and F contained GR normal mammary gland DNA. Lanes C and G contained normal rat mammary gland DNA. Lanes D and H contained normal human mammary gland DNA.


Eco R1
Bam H1
A B C D E F G H

predicted by increasing phylogenetic separation, human sequences hybridized to a lesser extent than rat sequences. Only two fragments related to int-H were detected in human DNA.

RNA expression from the int-H locus was examined using slot blot hybridization to the same 450 bp Hind III probe. Low levels of int-H transcripts were detected in unselected whole-cell RNA from a D2 nodule and from a midpregnant mammary gland, but not from D2 tumors, lactating mammary glands, or normal liver (Figure 21).

Figure 21: RNA slot blot analysis of int-H transcripts. Unselected whole-cell RNA was applied to nitrocellulose filters (5 μg per slot) and hybridized with 10^6 cpm of [^{32}P] labelled H450 DNA or to [^{32}P] labelled dihydrofolate reductase cDNA. The specific activity of both probes was approximately 10^8 cpm per μg of template. All tissues were from BALB/c mice.


DHFR
H450
D2 tumor
D2 tumor
D2 nodule
virgin m.g.
midpreg.
lactating
liver

CHAPTER 5

Discussion

5.1 MMTV Proviruses in Tumor Cell Subpopulations: Previous work in our laboratory has established that mouse mammary tumors contain more than one population of MMTV infected cells (MacInnes et al, 1981). These subpopulations were shown to persist despite selective pressure in the form of changes in hormonal milieu, suggesting an interdependency or interaction. Although differences were detected in the MMTV proviral content of these subpopulations, no attempt was made to correlate specific proviral patterns with tumorigenicity. This was the objective of the experiments described in Section 4.1.

Subpopulations of an MMTV-induced mammary adenocarcinoma were isolated from methylcellulose cultures of the dissociated tumor (Soule et al, 1981) and assayed for tumorigenicity by injection into syngeneic and athymic hosts. Two subpopulations (MC3 and MC4) were found to be highly tumorigenic; one (MC5) was not tumorigenic in any of the host systems. The paradoxical finding from restriction endonuclease analysis of these subpopulations was that the tumorigenic sublines MC3 and MC4 did not appear to contain any exogenous proviruses to account for their tumorigenic phenotype whereas the nontumorigenic MC5 subline had numerous exogenous proviruses. The proviral content of these

tumor sublines is reflected in MMTV RNA expression as assayed by liquid hybridization analysis. MC5 cells produce greater than 2000 70S genome equivalents per cell whereas less than 1 genome equivalent per cell was detected in MC3 RNA. The benign nature of MC5 cells despite their production of MMTV RNA may in part be explained by a lesion in processing of gp52 which precludes the production of infectious virions (Soule et al, 1981). The tumorigenic nature of MC3 and MC4 cells is more easily understood when tumors arising from the injection of these cells are analysed.

Restriction endonuclease analysis of MC3 and MC4 tumors revealed that an acquired MMTV provirus was resident in the genomes of these tissues. The proviral junction fragments generated by Eco RI or Bam H1 cleavage of 4 separate MC3 tumors were of identical size indicating that they arose from cells pre-existing in the MC3 subline since BALB/cfC3H mammary tumors contain integrated MMTV proviruses at many different sites, and there does not appear to be a preferred integration site (Cohen et al, 1979). The tumorigenic cells of the MC3 subline may contain this provirus, but may be outnumbered by normal cells which are known to populate mammary tumors (Slemmer, 1981) and persist in short-term cultures (McGrath et al, 1981). The growth of these cells may be selected for under the conditions of the tumorigenicity assay such that they predominate in the experimental tumors allowing the detection of the acquired

provirus. Similar results were obtained from the analysis of MC4 tumors.

The analysis of tumor subpopulations demonstrated that tumorigenicity does not correlate with total proviral content, but rather that a specific proviral integration site or sites may be involved in tumorigenesis. This finding preceded the discovery of the int-1 and int-2 loci (Nusse and Varmus, 1983) whose activation occurs by site specific integration of MMTV proviruses, and whose activation may be responsible for the tumorigenicity of MC3 and MC4 tumor sublines. Unfortunately, MC3 or MC4 tumors are no longer available to test this hypothesis.

The low levels of MMTV RNA expression in MC3 and MC4 tumors suggested that transcription of proviral sequences is not required for the tumorigenic process. The enhancement of expression of neighbouring genes mediated by elements in MMTV proviral LTRs has been suggested as the mechanism of int locus activation (Nusse et al, 1984; Dickson et al, 1984); no proviral transcription is required for this proposed enhancement to occur.

The striking difference in restriction patterns among tumor 262, the MC3 subline, and the MC5 subline provide very clear evidence that MMTV-induced mammary adenocarcinomas are complex structures whose components cannot be appreciated from the study of the parental tumor alone. Indeed the very cells in the tumor contributing the tumorigenic phenotype may not be detected by a holistic approach. MMTV-induced

tumors have long been described as clonal tumors (Cohen et al, 1979; Traina-Dorge and Cohen, 1983). If this is taken to mean tumors having a clonal origin it may well be true; if it is taken to mean that tumors are uniform then it is clearly false (no distinction has previously been made). The results of this study along with those of MacInnes et al (1981) and Heppner (1981) indicate that mammary tumors must be thought of as an assortment of cells rather than amassed clones of a cell. The well-differentiated glandular histology of mammary adrenocarcinomas (Dunn, 1959) suggests that this should be the case; the regular appearance of variants in cultured mammary tumor cells suggest how heterogeneity may arise (Lasfargues and Lasfargues, 1981).

5.2 Activation of the int-1 and int-2 loci in GRf Mammary Tumors: The Mtv-2 locus of GR mice has been shown to be responsible for the germline transmission of the high tumor incidence of GR mice (Michalides et al, 1981). Furthermore, the acquired proviruses detected in the tumors of GR mice have a similar restriction map to the Mtv-2 locus and may be derived from it (Fanning et al, 1980 a,b). The presence of milk-borne MMTV has previously made the role of Mtv-2 difficult to interpret, since acquired proviruses could have originated from either source. To eliminate this complication, GR pups were foster-nursed on C57/Bl females which do not transmit infectious MMTV via the milk (Nandi and McGrath, 1973). Acquired proviruses in these

foster-nursed GR mice (GRf mice) are derived solely from Mtv-2, and by studying them it has been possible to determine whether the Mtv-2 locus has direct tumorigenic potential in situ (for example by affecting neighbouring genes) or whether reintegration of Mtv-2 derived proviruses at other loci is part of Mtv-2 mediated tumorigenesis.

It has been shown in Section 4.2 that the tumors of GRf mice typically carry from 1 to 3 acquired copies of the MMTV provirus in their genomes. Since no milk-borne virus was present, these proviruses must have been derived from Mtv-2. In 10 of 11 GRf mammary tumors, MMTV proviral integration could be detected in the vicinity of one or both of the int-1 or int-2 loci. As has been reported for mammary tumors induced by milk-borne MMTV, enhanced transcription of int-1 and int-2 is generally associated with local MMTV proviral integration (Nusse et al, 1984; Dickson et al, 1984). Tumor induction by Mtv-2 therefore fits the current paradigm of int locus activation (Dickson and Peters, 1985); applying Occam's razor, there need not be any direct effect of the Mtv-2 locus other that to transcribe MMTV RNA as a source of new proviruses. It is not currently known whether the action of the Mtv-1 locus, which is associated with germline transmission of a low mammary tumor incidence in the C3H/He strain (Bentvelzen, 1974) also can be explained within the int activation paradigm.

The activation of int loci in GRf tumors can be contrasted to findings in the analysis of mammary tumors arising

spontaneously in C3Hf/Ki mice (C3H/Ki mice freed of milk-borne MMTV by foster-nursing). Popko and Pauley (1985) reported that no MMTV proviral integrations could be detected at either int locus in these tumors, nor could int-1 or int-2 RNA be detected. C3Hf/Ki mice were shown by these authors to lack both Mtv-1 and Mtv-2. Tumors arising in C3Hf/Ki mice apparently do not do so by a mechanism similar to the induction of tumors by Mtv-2; mechanisms of mouse mammary tumor induction must therefore exist outside the int activation paradigm. One GRf tumor (t15) contained no detectable int locus integration and may also have arisen by a separate mechanism. Alternatively, an int locus integration may exist in this tumor outside the limits of detection of the probes used.

The involvement of both int loci in 7 of 11 mammary tumors (considering both RNA and DNA data) parallels concerted DNA rearrangements at two distinct loci in Moloney murine leukemia virus-induced thymomas as reported by Tsichlis et al (1985). These authors have presented evidence for a synergistic effect for activated Mlvi-1 and Mlvi-2 loci ocurring in the same thymoma cells; it is possible that a synergism occurs in some GRf tumors although further experiments are required to demonstrate proviral activation of both loci in the same cells.

Finally, it is interesting that a GRf mammary hyperplasia (t7) was found to contain MMTV integrations in the vicinity of both int-1 and int-2 and contained int-1 RNA. If

progression from normality to the tumorigenic phenotype occurs in two steps in mouse mammary tissue as proposed by Cardiff (1984) (see Chapter 2), int locus activation must have occurred in the first transition in hyperplasia t7 or this tissue had already undergone the genetic event or events to affect the next transition but had yet to do so phenotypically. These themes are discussed further in section 5.5.

5.3 A Common MMTV Proviral Integration Site in Precancerous Mammary Hyperplasias: Mammary carcinogenesis is a complex process which in the mouse model may be composed of two definable stages: from normality to hyperplasia and from hyperplasia to the fully tumorigenic phenotype (Cardiff, 1984). Activation of the int-1 and int-2 loci has been proposed to be involved in the development of tumors (Nusse and Varmus, 1982; Peters et al, 1983) and can be detected at an early stage when tumors are first palpable (Peters et al, 1984 b). One hyperplasia has been described (see preceding section) in which int activation had already occurred. While considerable data has been accumulated with regard to the molecular basis of the transition to the fully transformed phenotype, much less is known about the earlier transition from normality to hyperplasia.

To address this problem, the involvement of MMTV proviruses in induction of murine mammary hyperplasia was examined by restriction endonuclease analysis of

hyperplastic alveolar nodule (HAN) outgrowth lines (created and reviewed by Medina, 1973, 1978). Exogenous MMTV proviruses were detected in the genomes of highly tumorigenic D2, C4 and C5 HANs but not in the genome of the low tumor incidence D1 HAN (see section 4.3). In spite of the diverse origins of these HANs (D2 was chemically induced; C4 and C5 were hormonally induced) comigrating proviral junction fragments could be detected in DNA from the tumorigenic HANs digested with Bam HI, Bgl II, Eco RI and Sac I. Sixteen examples of comigrating junction fragments have been discovered to date; the probability of this many comigrating junction fragments arising from digestion with hexanucleotide restriction endonuclease digestion of proviruses residing at different genomic sites (given a gel resolution of plus or minus 500 bp) is $[1000 \times (1/4)^6]^{16}$. It is therefore extremely unlikely that the acquired proviruses of D2, C4, and C5 HANs reside at different genetic loci. We have suggested that these proviruses reside at the same locus- a locus which may be involved in the induction of hyperplasias. We have therefore called this locus int-H.

The int-H locus does not appear to be part of or related to the previously described putative mammary proto-oncogenes int-1 or int-2 (Nusse et al, 1984; Dickson et al, 1984). The partial restriction map of the int-H locus cannot be superimposed on any region of either int locus. Probes derived from the int loci do not detect any rearrangement in

HAN tissue indicative of the integration of an MMTV provirus. It will be necessary to confirm by chromosome mapping that int-H is a distinct locus.

One important unresolved question regarding the involvement of MMTV in the induction of tumorigenic HANs concerns the source of this virus. The BALB/c mice in which D2, C4 and C5 HANs were generated do not produce or transmit infectious MMTV (Nandi and McGrath, 1973; Medina, 1973). No MMTV was administered intentionally at the time of induction (Medina, 1973). It has been previously noted that the acquired provirus of D2 HAN has a different restriction pattern than those of the GR, RIII and C3H variants of MMTV (Drohan et al, 1981); our data indicate that this is also true for C4 and C5 HAN. It is therefore unlikely that D2, C4 and C5 HANs became infected with a conventional strain of MMTV. It is also unlikely that the acquired provirus of these HANs is the result of amplification of an endogenous provirus since the restriction pattern of the former closely resembles exogenous MMTV (see Figure 13). The most likely source of the acquired proviruses of D2, C4 and C5 HANs is therefore an uncharacterized infectious MMTV variant. Drohan et al (1981) have reported that the BALB/cV subline of mice has become infected with such a variant; it is not known whether this variant is the same as that of tumorigenic HANs, however their restriction maps suggest that they may at least be related.

5.4 Molecular Cloning of the int-H Locus: Initial attempts to clone the int-H locus employed the phage vector gtWES B and the E coli host strain DP50SupF. (a system designed by Leder et al, 1977). Completely digested Eco RI proviral junction fragments were isolated from slices of an agarose gel, ligated into vector arms and packaged in vitro. Although one clone was identified containing a 5' junction fragment, later analysis revealed that the cellular flanking sequences from this clone had been deleted. Such a deletion was not unprecedented, having been reported in the molecular cloning of the Balbiani ring DNA from Chironomus tentans (Case, 1982) and in the cloning of an interspersed repeated DNA sequence from the Kangaroo rat Dipodomys ordii (Linn and Lark, 1982). Phage clones of human (Taub et al, 1983) and murine (Nikaido et al, 1981) immunoglobin genes have also suffered deletions in specific regions. Although efforts were made to clone int-H sequences in various vectors other than gtWES B (Charon 3A, Charon 4A, L47.1AB and EMBL3) the problem remained intractable.

A potential explanation for the resistance of int-H sequences to cloning was suggested by the finding of Leach and Stable (1983) that synthetic palindromes greater than 30 bp in length were very unstable in conventional vector-host systems. These authors suggested that secondary structures such as the cruciform structure formed by palindromes are excised from recombinant phage clones through the action of host recombination nucleases. As predicted, the loss of

sequences through deletion was prevented by using a host deficient in recombination enzymes. Taking advantage of this rationale, Murray et al (1984) were able to construct wheat genomic libraries in a recombination-deficient phage vector system despite the presence in wheat DNA of a high repeat sequence content. Similarly, Wyman et al (1985) have demonstrated that a substantial proportion of the human genome (8.9%) which forms secondary structures in phage vectors can only be propagated in bacterial hosts bereft of recombination systems. It was decided to attempt to minimize the loss of cloned int-H sequences in a similar manner.

Charon 35 was chosen as a vector since it allows the cloning of 20 kb DNA fragments and can be propagated on recA- hosts (Loenen and Blattner, 1983). Charon 35 has the additional advantage of having a gam gene whose product inhibits the exonuclease V enzyme of E. coli, thus blocking the recBC recombination system (Sakaki et al, 1978). When this vector is plated on E. coli DB1161 (recA- recBc- sbc- -see Appendix 1) the ability of the host to mediate deletion is severely restricted.

One clone (λX2-3) containing int-H sequences was isolated from approximately 500,000 recombinant phage containing partially digested Sau 3A1 fragments of D2 HAN DNA ligated into the BamH1 sites of Charon 35 arms (See Section 4.4). A number of clones were isolated containing endogenous MMTV proviruses, predominantly Unit III. The uneven representation of endogenous clones (for example 5 Unit III

clones, but no Unit I clone) indicates that the presence of these clones in the D2 library was not stochastic in spite of the specialized vector-host system.

Restriction endonuclease analysis of λX2-3 indicated that some deletion or deletions had occurred at the 5' end of the provirus; this deletion removed the "poison" sequence previously shown to be difficult to clone (Majors and Varmus, 1981, 1983). Small deletions or point mutations also eliminate Bam H1 sites from the proviral env gene. It is not known how these alterations arose in the recombination-deficient host; conceivably the mutations in this host are "leaky", or other recombination pathways exist. Although a repeated sequence element was detected 3' to the provirus, it is not known whether this element of some other sequence contributed to the difficulties encountered in cloning this region.

5.5 Speculation of the Function of the int-H Locus: The molecular observations regarding the acquired MMTV provirus common to three hyperplastic alveolar nodule outgrowth lines can be related to the wealth of biological observations previously accumulated in an attempt to synthesize a molecular basis for the preneoplastic lesion. In order to do this, it is first necessary to appreciate the normal development of mammary epithelium.

Mammary tissue from virgin animals (and from males at all stages of development) is in a quiescent state characterized

by little cell division and no production of milk proteins (Vorherr, 1974). In the female, pregnancy evokes an intense stimulation of ductal and alveolar growth mediated by luteal and placental sex steroids, placental lactogen, prolactin and chorionic gonadotropin. The midpregnant mammary gland is in a state of maximal cellular proliferation; the population of mammary epithelial stem cells expands greatly, then differentiates into presecretory cells and myoepithelial cells due to the influence of prolactin, placental lactogen, insulin, cortisol and growth factors. It is not until pregnancy is at term that milk proteins (caseins, whey acidic protein, α lactalbumin and others) are produced. At the onset of lactation, the withdrawal of luteal and placental sex steroids and lactogen and increased pituitary prolactin stimulates the synthesis and release of milk fats and proteins into the alveolar lumen. Oxytocin release during suckling causes subsequent ejection. Because the secretory alveolar cells have completed their program of differentiation, cell division has largely subsided in the lactating gland.

In a number of ways, the preneoplastic HAN outgrowth lines D2, C4 and C5 resemble normal mammary epithelium at the early to midpregnant stage of development. Firstly, both types of tissues are in a state of cellular proliferation (unlike virgin tissue), but are restricted to growth in the mammary fat pad and cannot be transplanted to another site (Medina, 1978). Secondly, preneoplastic HANs and early to

midpregnant glands do not produce significant amounts of milk proteins (Hohmann et al,1972; Vorherr, 1974) which obviously distinguishes them from lactating tissue. The important difference between these types of tissues is that midpregnant mammary gland tissue can be hormonally induced to complete its developmental program and produce milk proteins whereas preneoplastic tissue cannot (see Table 7). The coordinate repression of a set of mammary-specific genes in the preneoplastic HAN outgrowths with an integrated MMTV provirus at the int-H locus (D2, C4 and C5 HANs) but not in a HAN (D1) lacking this provirus suggests that the provirus may in some way be mediating this repression. Two models for this coordinate repression are presented in Figure 22.

In the first model, the expression of a repressor molecule is responsible for restricting expression of milk protein genes in inappropriate tissues and in nonlactating mammary tissue. An activator, whose expression may be sensitive to the hormonal changes occuring late in pregnancy, is not expressed at other times. Proviral insertion in the vicinity of the repressor gene causes increased and constitutive expression of the repressor molecule such that no activation of milk protein genes can occur. This would account for the lack of inducibility of milk protein genes in HANS grown in vitro (Hohmann et al, 1972). The consequence of such an event in vivo could be developmental blockage of mammary epithelium at a proliferative stage. Cells blocked at this stage may be considered to have undergone the first

Table 7

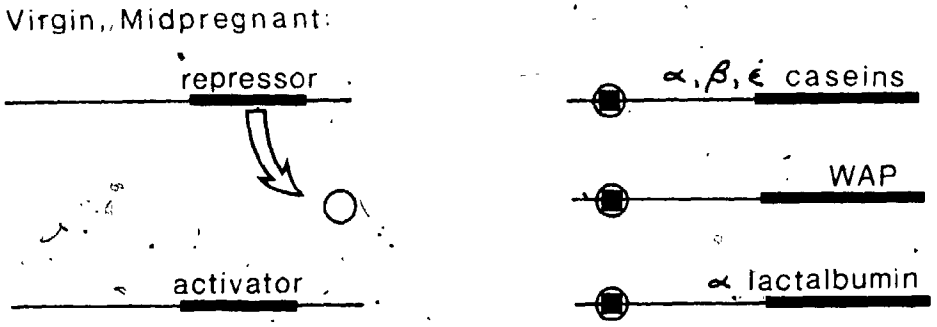
HAN	Int-H Provirus[c]	Induction of Casein[a] I	IFP	Fold induction	Casein[b] positive cells(%)
D1	-	770	5100	6.6	60-75
D2	+	560	720	1.3	1- 3
C4	+		not done		1
C5	+		not done		1
Normal BALB/c mid-pregnant mammary tissue	-	2700	7500	3	100

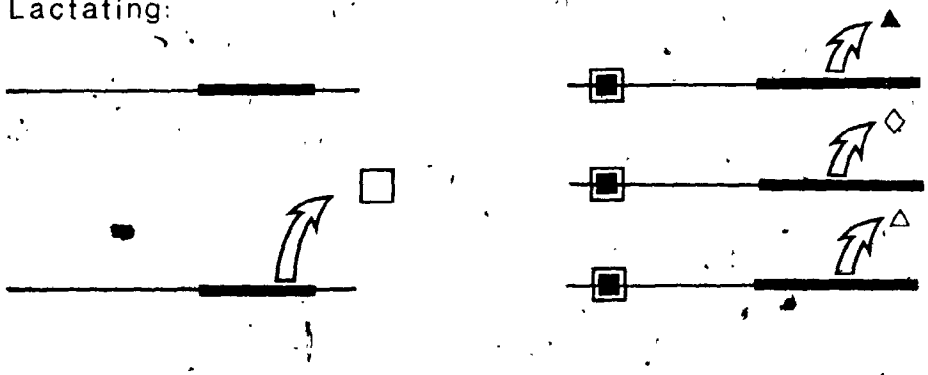
[a] cpm casein/mg wet weight of tissue explants synthesized at 48 hr *in vitro* in insulin (I) or insulin, hydrocortisone and prolactin (IFP). Data from Hohmann *et al* (1972).

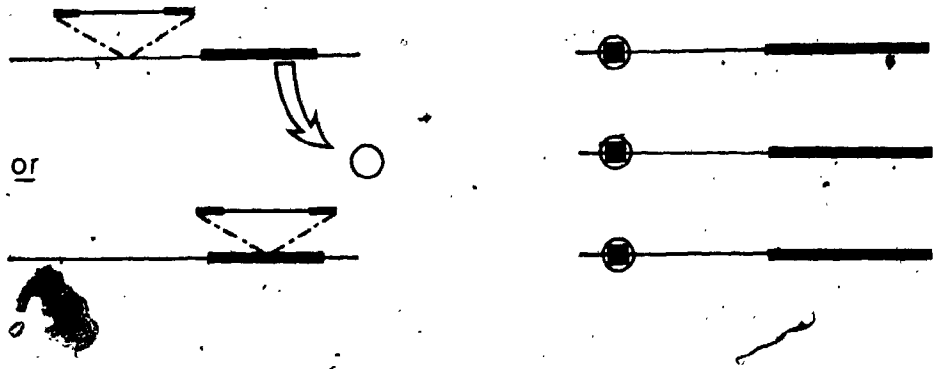
[b] Cells reactive with casein specific antibody in direct immunoperoxidase assays. Data from McGrath *et al* (1985) and Smith *et al* (1984).

[c] Presumed from analysis described in Section 4.3. Yet to be confirmed with cloned probes.

Figure 22: Models of int-H function in normal and preneoplastic tissues. Milk protein genes (α, β, and ϵ caseins, whey acidic protein (WAP) and αlactalbumin) are regulated by binding of a repressor molecule (open circle) or activator molecule (open square) to a regulatory sequence (closed box). The milk protein genes become active during the late stages of pregnancy. A proviruses is proposed to have inserted at the point indicated by the broken lines. The presence of the provirus in hyperplastic tissue results in constitutive repression of milk protein genes due to increased expression of the repressor (upper diagram) or insertional inactivation of the activator gene (lower diagram).


Virgin, Midpregnant:
repressor
activator
α, β, ε caseins
WAP
α lactalbumin


Lactating:


Hyperplastic:
or

transition from normality to preneoplasia (see Figure 4). Having already acheived a limitless potential for division (as evidenced by the serial transplantation studies of Medina (1973)), these cells may be predisposed to becoming tumorigenic upon a second "hit". The nature of this second hit is unknown, but it would presumably free the cells from the organ restricted growth characteristic of HAN tissues (Medina, 1978).

Increased expression of the putative repressor could arise as a result of proviral enhancement as has been postulated for the activation of the int loci (Dickson et al, 1984; Nusse et al, 1984) or downstream promotion is seen in the activation of the myc proto-oneogene by avian leukosis virus (Hayward et al, 1981; Payne et al, 1982).

In the second model, the putative activator gene is disrupted by proviral insertion. Ample precedent for insertional inactivation of genes by proviruses exists in the literature having been suggested as the mechanism reponsible for the dilute coat colour mutation in DBA/2J mice (Jenkins et al, 1981) and the disruption of a collagen gene in the Mov-13 strain of mice (Jaenisch et al, 1983). Integration of an MMTV provirus into the activator gene might result in a truncated, unstable or permuted gene product, if any product is made at all.

From these two models one can make predictions which can be tested experimentally. The enhancement/downstream promotion model suggests that the product of the int-H locus

(a repressor) should be present in normal nonlactating mammary tissue, preneoplastic tissue, and normal nonmammary tissue. The second model suggests that the int-H product (an activator) should be present in lactating tissue alone. Preliminary data (Figure 21) are in accordance with the first model, since RNA transcripts of int-H were detected in preneoplastic D2 HAN cells and in normal midpregnant cells, but not in cells from lactating tissue. Since the former are proliferating tissues, continuous production of the repressor may be required to maintain repression of newly synthesized copies of the mammary-specific genes. Int-H RNA levels may be lower in quiescent normal tissues.

Finally, it can be speculated that if the int-H gene product is a repressor, it may have DNA binding properties. Conservation of sequences has been observed among other DNA binding proteins, for example between the homeo box gene of Drosophila (which is a developmental regulator of segmentation) and the yeast mating-type regulatory protein both of which have DNA binding activity (Desplan et al, 1985). The family of related sequences detected by the int-H probe under relaxed hybridization conditions (Figure 20) may represent genes encoding other regulatory or DNA binding proteins. It will be very interesting to determine whether nucleic acid homology exists, and efforts are underway to sequence a portion of int-H for computerized search and comparison.

5.6 Summary and Prospectives: The analysis of mouse mammary tumor subpopulations has offered a lesson which (stated simply) is: any observation from whole tumor samples represents an average of traits from potentially dissimilar components. While seeming more like common sense than scientific revelation, this fact has not been fully appreciated in discussions of the clonality of MMTV-induced mammary tumors (Cohen et al, 1979; Traina-Dorge and Cohen, 1983) where conclusions were drawn from the analysis of whole tumor DNA.

The full understanding of the role of MMTV in the tumorigenic process was not made more distant by the finding that the tumorigenic endogenous locus, Mtv-2, operates within the int activation paradigm. It had previously been suggested that Mtv-2 mediated tumorigenesis may represent a novel mechanism of tumor induction (Michalides and Nusse, 1981).

Finally, it is hoped that the discovery of a genetic locus associated with the preneoplastic phenotype will provide a new impetus for the study of mammary tumor induction and progression. With cloned sequences in hand it should be possible in the future to learn more about the role of this locus in normal, preneoplastic and neoplastic mammary tissue.

APPENDIX 1

Escherichia coli Strains

DP50 SupF (Leder et al, 1977). Genotype: F^-, ton A53, dap D8, lac Y1, gln V44, (supE 44), Δ(gal-uvr B)47, λ^-, tyr T58, (supF 58), gyr A29, Δ(thy A57), hsd S3.

LE 392 (Murray et al, 1977). Genotype: F^-, hsdR 514, (r_K^-, m_K^-), (supE 44), (supF 58), lac Y1 or Δ(lac IZY)6, gal K2, gal T22, met B1, trp R55, λ^-.

DB 1161 (Wyman et al, 1985). Genotype: thr 1, ara 14, leu B6, Δ(gpt-proA)62, lac Y1, tsx 33, (supE 44), gal K2, λ^-, trp 27(B or C), rac^-, sbc B15, his G4, rfb D1, rec A56, srl 300:Tn 10, rec B21, rec C22, end A5, rps L31, bdg K51, xyl 5, mtl 1, arg E3, thi 1, Δ(hsd)26(r^-, m^-).

HB 101 (Boyer and Rouland-Dussoix, 1969). Genotype: F^-, hsd S20(r_B^-, m_B^-), rec A13, ara 14, pro A2, lac Y1, gal K2, rps L20(Sm^r), xyl 5, mtl 1, (supE 44), λ^-.

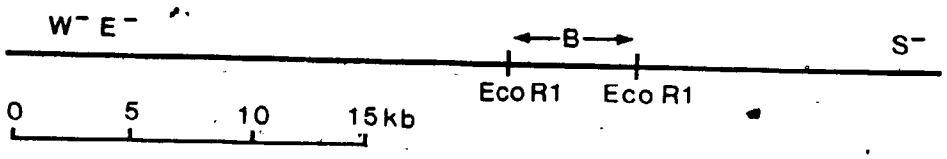
BHB 2688 (Hohn and Murray, 1977). Genotype: (N205 $recA^-$, [δimm^{434}, cIts, b2, red^-, Eam, Sam/δ]).

BHB 2690 (Hohn and Murray, 1977). Genotype; (N205 $recA^-$ [δimm^{434}, cIts, b2, red^-, Dam, Sam/δ]).

APPENDIX 2

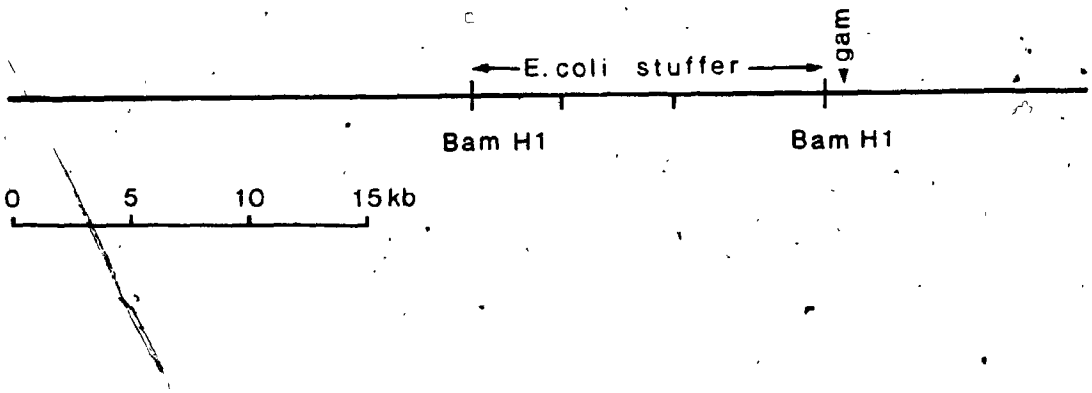
Vectors Used in the Molecular Cloning of int-H

λgtWES·λB:



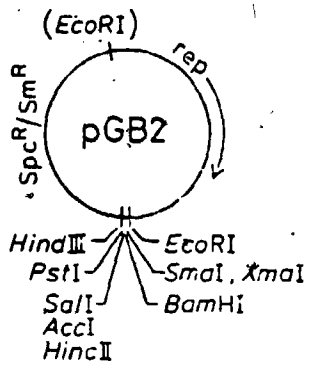
λgtWES·λB was designed by Leder et al (1977) to be incapable of propagation outside of the laboratory. Amber mutations exist in three essential genes (W, E, and S); this phage therefore requires a host strain (such as DP50 SupF) carrying an amber suppressor mutation. Recombinants are generated by replacing the nonessential "B" fragment with Eco RI fragments of 2 to 15 kb. The diagram was modified from Leder et al (1977).

λCharon 35:



λCharon 35 was designed by Loenen and Blattner (1983) to suppress rec BC mediated recombination through its gam protein function. The central "stuffer" fragment was derived from partially Bam HI digested E. coli DNA and can be replaced with fragments of 9 to 20 kb. The diagram was modified from Loenen and Blattner (1983).

pGB2:



pGB2 was constructed by Churchward et al (1984) so as not to share sequence homology with other commonly used plasmid and phage cloning vectors. pGB2 carries a resistance gene for spectinomycin and streptomycin and a polylinker into which various types of restriction fragments can be inserted. The diagram is from Churchward et al (1984).

APPENDIX 3

Determination of Ligation Conditions for the Construction of Recombinant Phage Genomes

Following the rationale of Maniatis et al (1982) pp 286-288:

if "j" is the effective concentration of one end of a DNA molecule in the neighbourhood of the other end of the same molecule,

$$j = (3/2\ \pi\ lb)^{3/2} \text{ ends/ml}$$

where l = length of DNA in centimetres, and b = length of a randomly coiled segment of DNA (which is 7.7×10^{-6} cm for λ DNA in ligation buffer). Also,

$$j_\lambda = j\lambda(MW\lambda/MW)^{2/3} \text{ ends/ml}$$

$$= 5.5 \times 10^{22}/MW^{3/2} \text{ ends/ml}$$

for a duplex linear DNA molecule with cohesive ends, if "i" is the concentration of all ends,

$$i = 2N_oM \times 10^{-3} \text{ ends/ml}$$

where N_o is Avogadro's number.

For totally digested DNA of 10 kb (6.25 Md), $j = 3.52 \times 10^{12}$ ends/ml. For λ gtWES·λB arms of 35 kb (21.9 Md), the value of $j = 5.37 \times 10^{11}$ ends/ml. To get concatamerization i should be much greater than j. Using the larger (insert) value of j, to get $i/j = 10$,

$$i = 10j = 3.52 \times 10^{8} \text{ ends/ml}$$

since i = i(inserts) + i(arms)

$$i = (2\text{NoMinserts} + 2\text{NoMarms}) \times 10^{-3} \text{ ends/ml}$$

$$= 15\text{NoMinserts} \times 10^{-3} \text{ ends/ml}$$

$$= 3.9 \times 10^{-12}\text{M}$$

Therefore the concentration of inserts should be:

$$(3.9 \times 10^{-12}\text{M}) \times (6.25 \times 10^{6}\text{ g/M})$$

$$= 2.44 \times 10^{-5}\text{ g/ml}$$

$$= 24\ \mu\text{g/ml}$$

A similar calculation for the concentration of arms DNA gives a value of 550 μg/ml.

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