RETROVIRAL INDUCED LYMPHOMA: PROMOTOR-INSERTIONAL ACTIVATION OF THE CELLULAR MYC GENE BY RETICULOENDOTHELIOSIS VIRUS

By

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Department of Biochemistry

1985

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ABSTRACT

RETROVIRAL INDUCED LYMPHOMA: PROMOTOR-INSERTIONAL ACTIVATION OF THE CELLULAR MYC GENE BY RETICULOENDOTHELIOSIS VIRUS

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The avian non-acute Reticuloendotheliosis viruses(REVs) induce B-cell lymphoma in the bursa of birds 4-10 months post-infection. Recent evidence suggests that neoplasia could be caused by the activation of a cellular gene by the infecting virus. We show, by restriction enzyme analysis of tumor DNA, that REV can activate the cellular oncogene, c-myc, by insertion of the provirus proximal to the oncogene and oriented in the same transcriptional direction. We have supported our conclusion by cloning a c-myc gene activated by proviral insertion and analyzing its structure by restriction enzyme fine mapping. The structural analysis confirms our conclusion and reveals that the provirus carries a major deletion of the coding region, but has maintained both LTRs, which are identical based on DNA sequence analysis. RNA blots of poly(A) selected tumor RNA reveal novel c-myc transcripts which are consistent with initiation

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from within the LTR proximate to c-myc. However, no transcripts are seen that initiate from the other LTR indicating transcriptional surppresion by an unknown mechanism. Comparison of the DNA sequence of the LTR to a related REV strain shows major sequence divergence in the region where retroviral enhancers are commonly located. This sequence variation may account for the differences in oncogenic potential observed between strains of REVs.

TO MY MOTHER AND FATHER WHO ALWAYS BELIEVED
IN ME, EVEN WHEN THEY DIDN'T

ACKNOWLEDGEMENTS

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ABBREVIATIONS

ALV	avian leukosis virus
CSV	chicken syncytial virus
DIAV	duck infectious anemia virus
DNA	deoxyribonucleic acid
FSV	feline sarcoma virus
GTP	guanosine triphosphate
kb	kilobase
kd	kilodalton
LTR	long terminal repeat
MuLV	murine leukemia virus
MSV	murine sarcoma virus
PDGF	platlet-derived growth factor
REV	reticuloendotheliosis virus
RNA	ribonucleic acid
mRNA	messenger RNA
RSV	rous sarcoma virus
SNV	spleen necrosis virus
TS	tumor specific

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LITERATURE REVIEW

The RNA tumor virus and oncogene story dates back to 1910 (Rous 1910, 1911) when Francis Peyton Rous discovered a filterable agent(virus) that could cause sarcomas, a tumor of the connective tissue, in domestic chickens. There were other filterable agents isolated that had the capacity to induce a variety of neoplasia; leukosis in chickens (Ellerman and Bang 1908), mammary carcinomas in laboratory mice (Bitter 1936), and leukemia in newborn mice (Gross 1951, Gross 1970). In the decades that followed Rous's discovery the physical and biochemical nature of the sarcoma inducing agent was elucidated. With the invention of electron microscopy (Knoll and Ruska 1932) and sophisticated biochemical techniques the structure (Gaylord 1955) and chemical content (Crawford and Crawford 1961) of the sarcoma virus was shown to be single-stranded RNA surrounded by a protein coat.

Taxonomy

The cancer inducing viruses discovered by Rous and others are now taxonomically grouped as part of the family Retroviridae (Fenner 1975). The primary criterion for

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admission to the retroviral family is the replication of an RNA genome that procedes through a double-stranded DNA intermediate. Some other taxonomic characteristics associated with this family are: a diploid genome of positive-sense single-stranded RNA(the subunits are believed to be joined together by hydrogen bonds involving sequences located close to the 5' end of each subunit, Kung et al. 1975), and a DNA polymerase enzyme activity capable of using RNA or DNA templates(reverse transcriptase) (Fenner 1975). Retroviruses have been discovered in a wide variety of vertebrates, including snakes, fish, birds and many mammalian orders(Figure 1). However, the most experimentally useful and well studied retroviruses have been isolated from chickens, mice, cats and lower primates.

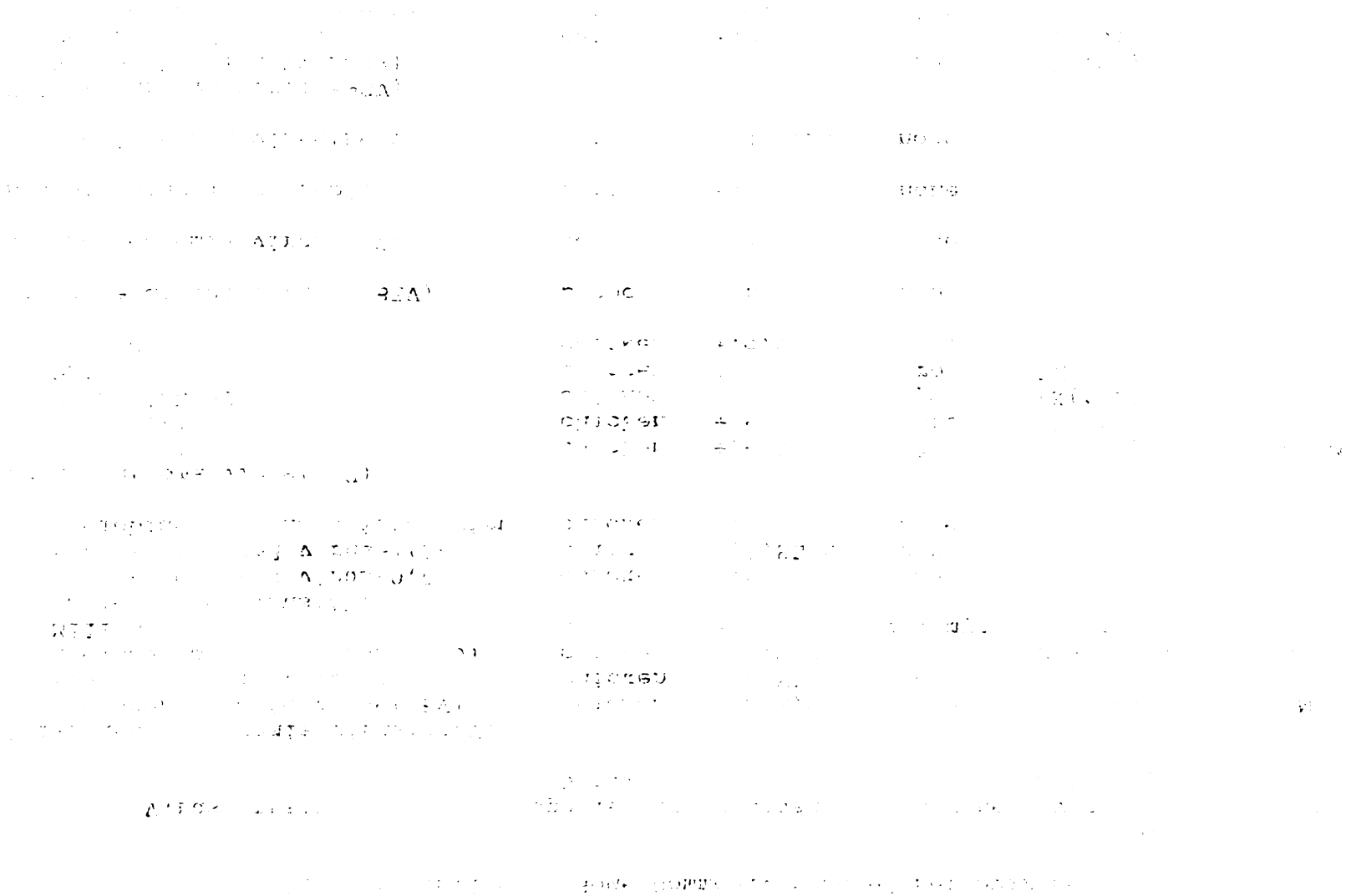
The Viral Genome

The viral RNA genome exhibits the structural features common to eucaryotic mRNA; a 5'-m^7GpppGm cap structure, a poly adenylic acid(poly A) tail and a low level of internal methylation (Teich 1982). The general structure of a replication competent retrovirus is divided into the three coding regions shown in Figure 2. The gag region encodes a polyprotein which is cleaved to form the four or five proteins which make up the major structural components of the virus particle(for review Eisenman and Vogt 1978; Dickson et al., 1982). The pol region encodes a reverse transcriptase, a RNA:DNA specific RNase H exonuclease activity (for review see, Verma 1977) and an enzyme activity necessary for integration of the viral DNA intermediate into

Characteristics of Some Commonly Studied Retroviruses

Virus Strain	Species of Origin	(a) Oncogenic	Oncogene	(b) v-onc Activity	(c) v-onc Location
Avian acute leukemia virus(AcLV)					
Erythroblastosis virus(AEV)	chicken	+,s,E,S	erbB	PK(tyr)	PM
Myeloblastosis virus(AMV)	chicken	+.s,ML	myb	?	NM
Myelocytomatosis virus(MC29)	chicken	+,s,C,M,S	myc	DNA binding	NM
Mill hill(MH2)	chicken	+,s,C,S	mil,myc	PK(ser,thr)	?
Avian leukosis virus(ALV)					
Rous associated virus-0(RAV-0)	chicken	-,n	none		
Rous associated virus-1(RAV-1)	chicken	-,l,BL,E	none		
Myeloblastosis associated virus	chicken	-,l	none		
Avian sarcoma virus(ASV)					
Fujinima(FSV)	chicken	+,s,S	fps	PK(tyr)	C,PM
Rous(RSV)	chicken	+,s,S	src	PK(tyr)	PM
Yamaguichi-73	chicken	+,s,S	yes	PK(tyr)	?
UR1	chicken	+,s,S	ros	PK(tyr)	CM
SKV770	chicken	+,s,S	ski		
Baboon endogenous virus(BEV)	baboon	-,n	none		
Bovine leukemia virus(BLV)	cow	-,l,LS	none		
Equine infectious anemia virus	horse	-,n	none		
Feline leukemia virus(FeLV)	cat	+,l,E,LS	none		
Feline sarcoma virus(FeSV)					
Snyder-Theilen(ST-FeSV)	cat	+,s,S	fes	PK(tyr)	C,PM
McDonough(SM-FeSV)	cat	+,s,S	fms	PK(tyr)	C,PM
Gardner-Rasheed(GR-FeSV)	cat	+,s,S	fgr	PK(tyr)	?

Figure 1



Characteristics of Some Commonly Studied Retroviruses

Characteristics of Some Commonly Studied Retroviruses

Virus Strain	Species of Origin	(a) Oncogenic	Oncogene	(b) v-onc Activity	(c) v-onc Location
Gibbon ape leukemia virus(GALV)	gibbon	-,l,L,LS	none		
Human T-cell leukemia virus(HTLV-I)	human	?,l,TL	none		
Murine leukemia virus(MLV)					
Abelson(Ab-MLV)	mouse	+,s,BL	abl	PK(tyr)	PM
Friend(Fr-MLV)	mouse	-,s,E			
Moloney(Mo-MLV)	mouse	-,l,TL			
Rauscher(Ra-MLV)	mouse	-,l			
Mink focus forming virus(MCF)	mouse	-,l,TL			
Spleen focus forming virus	mouse	-,s,E			
Murine mammary tumor virus(MMTV)	mouse	-,l,A			
Murine sarcoma virus(MSV)					
Harvey(Ha-MSV)	rat	+,s,E,S	ras	GTP(B),GTPase	PM
Kirsten(Ki-MSV)	rat	+,s,E,S	ras	GTP(B),GTPase	PM
Moloney(Mo-MSV)	mouse	+,s,S	mos	PK(ser,thr)	C
BALB-MSV	mouse	+,s,HS	bas		
FBJ-MSV	mouse	+,s,OS	fos	Phosphoprotein	N
3611-MSV	mouse	+,s,C,FS	raf	PK(ser,thr)	?
Reticuloendotheliosis virus(REV)					
chicken syncytial virus(CSV)	chicken	-,l,BL,LS			
duck infectious anemia virus	duck	-,l,BL,LS			
reticuloendotheliosis-A(REV-A)	turkey	-,l,BL			
reticuloendotheliosis-T(REV-T)	turkey	+,s,TL	rel		
spleen necrosis virus(SNV)	duck	-,l,BL,LS			

Figure 1 (cont'd)

Characteristics of Some Commonly Studied Retroviruses

Virus Strain	Species of Origin	(a) Oncogenic	Oncogene	(b) v-onc Activity	(c) v-onc Location
Simian sarcoma virus(SSV)	wooley monkey	+,s,FS	sis	PDGF agonist	C
Viper retrovirus(VRV)	viper	?			
Visna Virus	sheep	-,n			

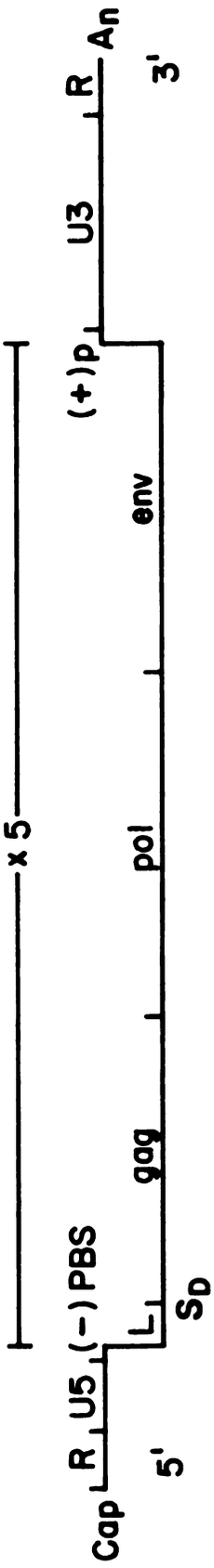
(a) "+" transforms cells <u>in vitro</u>. Malignant tumors appear after short(s) or long(l) latency, n=means no tumors. Type of neoplasia: A=adenocarcinoma, BL=B-cell lymphoma, C=carcinoma, E=erythroleukemia, FS=fibrosarcoma, HS=hemangiosarcoma, LS=Lymphosarcoma, M=myelocytoma,ML=myeloblastic leukemia, OS=osteosarcoma, S=sarcoma, TL=T-cell lymphoma.

(b) PK=protein kinase, the amino acid subject to phosphorylation is given in parentheses. GTP(B)=binds to guanosine triphosphate, PDGF=platelet-derived growth factor

(c) C=cytoplasm, CM=cytoplasmic membranes, PM=plasma membrane, N=nucleus, NM=nuclear matrix

Figure 1 (cont'd)

Figure 2. Structure of a viral RNA genome from a replication competent retrovirus. Some important structural features of a replication-competent retrovirus are illustrated. cap: 5' cap nucleotide; R: short terminal repeat; U5: sequence unique to 5' terminus; (-)PBS: binding site of tRNA that serves has (-) DNA strand primer; L: leader region; S_D: splice donor site; gag: coding region for viral structural proteins; pol: coding region for reverse transcriptase; env: coding region for envelope glycoproteins; (+) P: polypurine tract for priming of (+) DNA strand; U3: sequence unique to 3' terminus, contains viral transcriptional regulatory sequences; A_n: poly(A) tract at 3' terminus. The coding region is expanded 5x inorder to show the non-coding regions at the 5' and 3` termini. The lengths of all regions are generalized. The lengths of these regions vary amoung retroviruses.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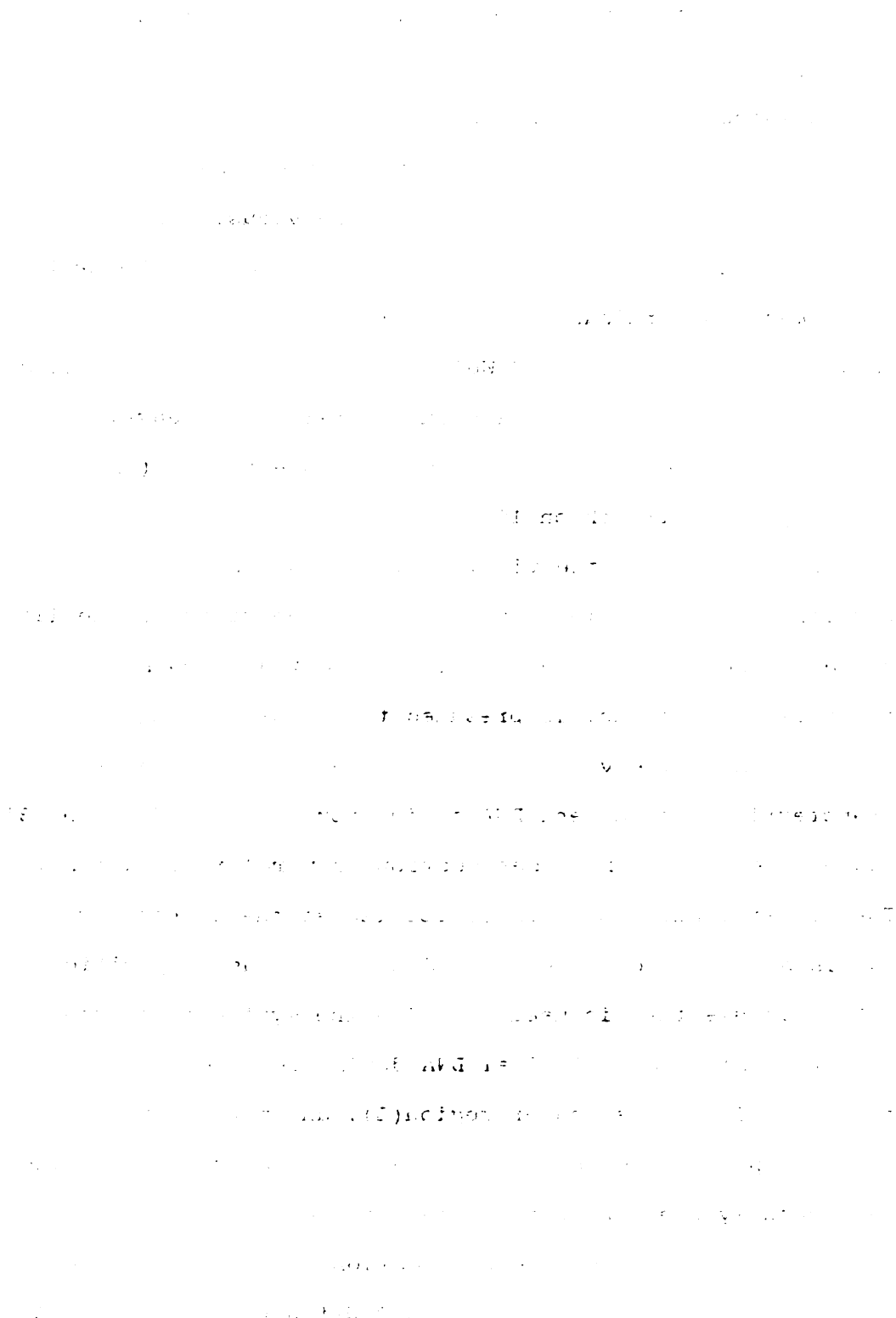
the host cellular DNA (Schwartzberg et al., 1984;Donehower et al., 1984). The env region encodes a polyprotein that is glycosylated, cleaved and transported to the plasma membrane (Hayman et al. 1978) to form a component of the viral membrane that surrounds the core of the virus. The envelope glycoproteins are targets for neutralizing antibodies and are a major determinant of the host range of the virus (Payne and Biggs 1964,1966;Weiss 1982). Treatment of intact virions with protease results in the removal of envelope proteins and the concommitant loss of infectivity (Rifkin and Compans 1971;Dickson 1982).

In addition to the viral replication genes other portions of the genome serve important functions in the life cycle of the virus. The R region, repeated at both ends of the viral RNA genome, is presumed to be used during replication of the viral RNA genome to provide a means for transferring the nascent DNA chain from the 5' end to the 3' end of the viral genome(see section on viral replication). The primer binding site(PBS), located at the 3' boundary of U5, is a stretch of 18 nucleotides that binds a specific tRNA molecule that is used to prime the synthesis of the first(minus) strand of viral DNA during reverse transcription. The leader region(L), an untranslated sequence preceeding the coding region of the viral proteins, has recently been shown to contain recognition sequences necessary for the proper encapsidation of the RNA genome into virons (Shanks and Linial 1980;Watanabe and Temin 1982) and a splice donor site used for the generation of



subgenomic viral mRNAs. The U3 region contains eucaryotic regulatory sequences important for transcriptional initiation, capping and polyadenylation of the viral RNA transcript (Varmus 1983). In addition, the U3 region has been shown to contain "enhancer" sequences that can elevate gene transcription irrespective of the position or the orientation of this sequence to the affected gene.

Enhancer Sequences

Enhancers are short cis-acting regulatory sequences that appear to increase transcription from eukaryotic promotors irrespective of their orientation and relatively independent of the location of the affected promotor. Enhancer sequences were first discovered and have been most throughly characterized in Simian Virus 40(SV 40), a small DNA tumor virus, (Benoist and Chambon 1981;Gruss et al., 1981). The transcriptional regulatory sequences of the early region of SV 40 contains several important structural features, including two 72 base-pair repeats. The deletion of both 72 bp repeats results in a drastic reduction in the transcriptional activity of the SV 40 early region genes. Interestingly, the transcriptional activity can be restored by reinserting the 72 bp repeats at a variety of positions and in either transcriptional orientation relative to the SV 40 early region RNA cap site (Moreau et al., 1981;Fromm and Berg 1982). These experiments with SV 40 laid the foundation for the operational definition of enhancers as a sequence that can increase the transcription of a gene independent of orientation and that their influence is not

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The molecular mechanism of enhancer activity is not known, although several models have been proposed. Perhaps the most popular model, suggested by Moreau et al.(1981), is that enhancer sequences provide a bi-directional entry site for either RNA polymerase II or one of its subunits. This hypothesis is consistent with the position and orientation independence of enhancer activity and is supported by the observation that enhancement decreases when functional promotors are inserted between the enhancer and the initation site of the gene being assayed (Moreau et al., 1981;Wasylyk et al., 1983). Another hypothesis suggests that enhancers may alter chromatin structure and/or superhelicity to create an 'open window' within the nucleosome structure, thereby increasing the accessiblity of the DNA within the enhancer for binding of macromolecules involved in transcription. This might be accomplished indirectly if the enhancer sequence contains a topoisomerase binding site or directly if the enhancer sequence contains, for example, alternating purine and pyrimidine tracts which favor a left-handed form of the double-stranded helix(Z-DNA). It has been proposed that Z-DNA assumes a more "open" conformation then the normal right-handed B-form of the helix and that it can not form around nucleosomes (Groudine 1983). Perhaps in support of all these hypotheses is the observation that the enhancer sequences are generally hypersensitive to DNase digestion, a result that is

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interpreted to mean that the DNA sequences of enhancers are in regions of the chromatin that are "open". For example, hypersensitive regions reside close to the SV 40, polyma, MMTV and immunoglobulin enhancers within cell types in which promotors under their control are active, but not when promotors are inactive. Consistent with the Z-DNA hypothesis is the recent finding that anti-Z-DNA antibodies (Lafer et al., 1981) bind to some viral enhancer regions (Nordheim et al., 1982;Nordheim and Rich 1983). These hypotheses are not necessarily mutually exclusive. The generation of an open chromatin structure and sequence-specific interaction between enhancers and the transcriptional machinery could act in concert to facilitate the activation of promoter elements.

More recently, enhancers have been found within the U3 region of a number of retroviral LTRs. The biological implications of enhancer function in this family of virus is consistent with the observation that LTRs have been shown to activate adjacent cellular oncogenes following the integration of viral DNA upstream or downstream of the affected gene (Hayward et al.;1981;Fung et al., 1981;Payne et al., 1982;Nusse et al., 1984;Swift et al., 1985). This activation, referred to as enhancer insertion, has been implicated in the formation of tumors in hosts infected with certain retroviruses.

Viral enhancers may determine the host range and/or the oncogenic potential of the virus. Studies show that the

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SV 40 enhancer is 5 times more effective in elevating gene transcription in monkey cells(CV-1) then in mouse L cells. Conversely, the enhancer of Mo-MSV is more effective in mouse cells then in monkey cells (Laimins et al., 1982). The RAV-0 and RAV-1 strains of avian leukosis virus are very similar in DNA sequence, except for the U3 region of the LTR which is highly divergent (Robinson et al., 1982). However, RAV-0 is non-oncogenic in an avian host. In contrast, RAV-1 can induce B-cell lymphoma and erythroblastosis (Fung et al., 1982,1984). Experiments with a recombinant of two strains of murine leukemia virus(MuLV), Fr-Mulv and Mo-Mulv, that induce erythroleukemia and T-cell lymphoma, respectively, were examined for their oncogenic properties (Chatis et al., 1983). It was found that a recombinant whose genome is derived primarily from Fr-MuLV, but has the enhancer region of Mo-MuLV induced T-cell lymphomas rather then erythroleukemias.

Another example of the enhancer region affecting the oncogenicity of the retrovirus was shown by Lenz et al.(1984), who observed that a recombinant of the non-oncogenic endogenous retrovirus Akv and the highly leukemogenic murine virus SL3-3 that was primarly derived from the Akv genome, was leukemogenic. Nucleotide sequencing showed that the recombinant differed from Akv only in the region thought to contain the enhancer sequence of SL3-3. It appears that the observed difference in oncogenicity is due to sequences present in the LTR of SL3-3

that exhibit significantly enhanced transcriptional activity in T-cells compared with the corresponding region of the Akv LTR (Celander and Haseltine 1984).

Experiments with recombinants of two strains of MuLV, a strain that replicates efficiently in the thymus of mouse(T^+) and a strain unable to replicate in this organ(T^-), show that the region associated with the enhancer of the T^+ strain is sufficient to allow the replication of the T^- strain in the thymus (DesGroseillers et al., 1983).

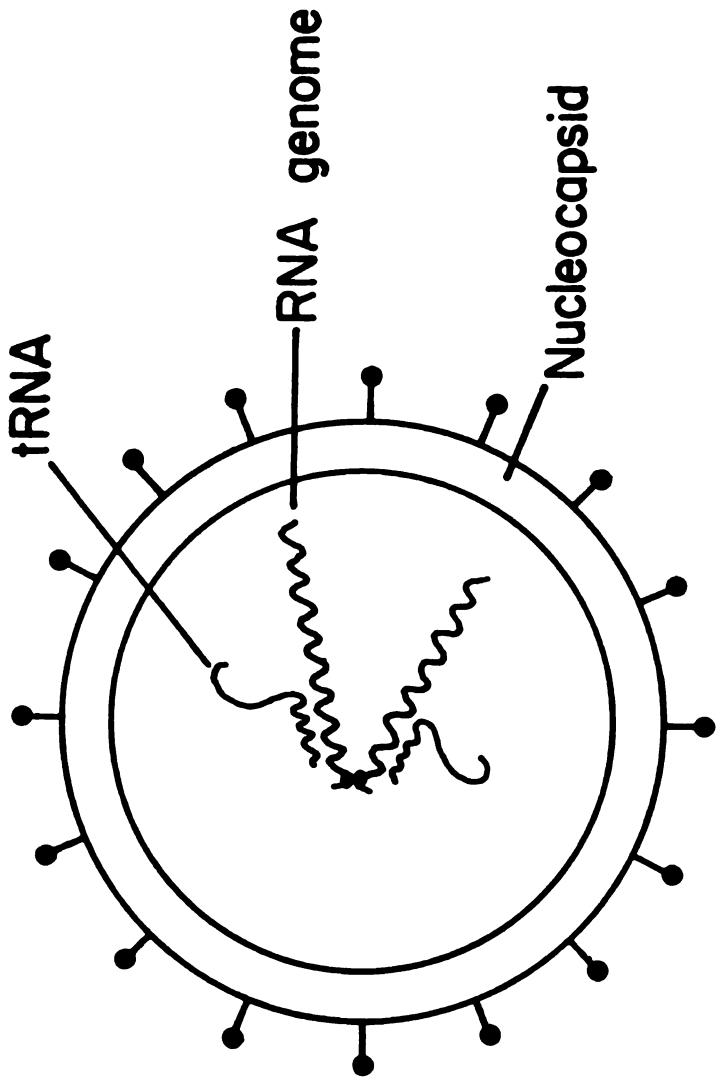
Several groups have recently reported experiments which demonstrate that sequences which lie adjacent to the immunoglobulin heavy chain locus are functionally analogous and structurally homologous to viral enhancers. As well as conforming to the operational definition of an enhancer Gilles et al.(1983) and Banerji et al.(1983) have shown that the immunoglobulin heavy chain enhancer has a somewhat more restricted tissue specificity and functions most efficiently in cells of the B lineage. There is evidence based on in vivo dimethyl sulfate protection experiments and genomic sequencing (Ephrussz et al., 1984) that alterations in the octamer CAGGTGGC found in the mouse immunoglobulin heavy chain enhancer correlates with the B verus non-B lineage specificity observed by Gilles et al.(1983) and Banerji et al.(1983). These results are consistent with the binding of a tissue-specific molecule to the enhancer region.

Virus replication

The first step in the life cycle of the virus is the adsorption and penetration of the host cell by the virus particle. The architecture of a type C retrovirus is shown in Figure 3 with protruding spikes covering the surface of the viron that represent the envelope glycoproteins. The adsorption of the virus to the cell surface involves some form of env glycoprotein-cellular receptor interaction. This interaction, between the viron envelope glycoproteins and specific receptor sites on the host cell surface is the major determinant of host susceptibility to infection (Weiss 1982). Susceptible cells can be rendered resistant to a specific retrovirus infection by pre-infection with a virus bearing the same viral glycoprotein specificity, apparently by blocking the cell receptor (Rubin 1960). This interference based on subgroup specificity for cell receptors can be used to divide a group of related viruses into different classes.

It has been suggested that virons enter the cell by first becoming attached to cell surface receptors located primarily in regions of coated pits (Marsh and Helenius 1980). In some cases, the internalized virons appear first in intracellular coated vesicles, then in large uncoated collection vacuoles and finally in secondary lysosomes. In the lysosomes(pH<5), the glycoproteins of the viral envelope can become strongly fusogenic. They may fuse rapidly with the lysosomal membrane(<1 min) thereby permitting transfer of the viral genome to the cytoplasm in such a way that the

Figure 3. The general structure of a C-type retrovirus.
Two viral RNA subunits with bound tRNA molecule are surrounded by a nucleocapsid core, host membrane and viral envelope proteins. The spikes and knobs represent the two envelope glycoproteins found on the surface of viral membrane. For the Reticuloendotheliosis group the knobs represent a glycoprotein of ca. 73 kilodaltons. This glycoprotein is anchored in place by a smaller glycoprotein, represented by the spikes, of ca. 22 kilodaltons.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viral genome is constantly protected from the hydrolases present in the lysosomal environment (Lenard 1982). After the viron is uncoated, the single stranded RNA genome is available in the cytoplasmic compartment in a form suitable for conversion to double-stranded DNA.

The synthesized linear double-stranded viral DNA intermediate is co-linear with the viral RNA with respect to the coding region(Figure 4). However, the ends of the viral RNA are different from the ends of the viral DNA. The viral RNA has a short terminal repeat(R), while the viral DNA has a long terminal repeat(LTR). The general structure of a LTR with some important regulatory features is depicted in Figure 5.

The current model for the conversion of the single-stranded viral RNA to a linear DNA duplex is complex. A model for the synthesis of the minus strand of viral DNA is presented in Figure 6. The final product of this complex process is a blunt-end linear duplex with two copies of the U3-R-U5 region which constitutes the long terminal repeat(LTR), as was seen previously(Figure 4). This molecule is transported to the nucleus and converted to one of two monomeric closed circular forms; containing either one copy of the LTR or two copies of the LTR linked in tandem.

Viral Integration

The process of retroviral integration into the host cellular DNA has two direct consequences for the structure

COMPLIANCE CERTIFICATE FOR EU DECLARATION OF CONFORMITY

We hereby declare that the machinery described in this manual complies with the essential health and safety requirements of the EU Machinery Directive 2006/42/EC.

Name and address of the person authorized to compile the technical file:

Place and date of issue:

Signature:

Name and function of signatory:

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A copy of this certificate must be kept with the machinery at all times.

Any modifications to the machinery must be approved by the manufacturer and recorded in the technical file.

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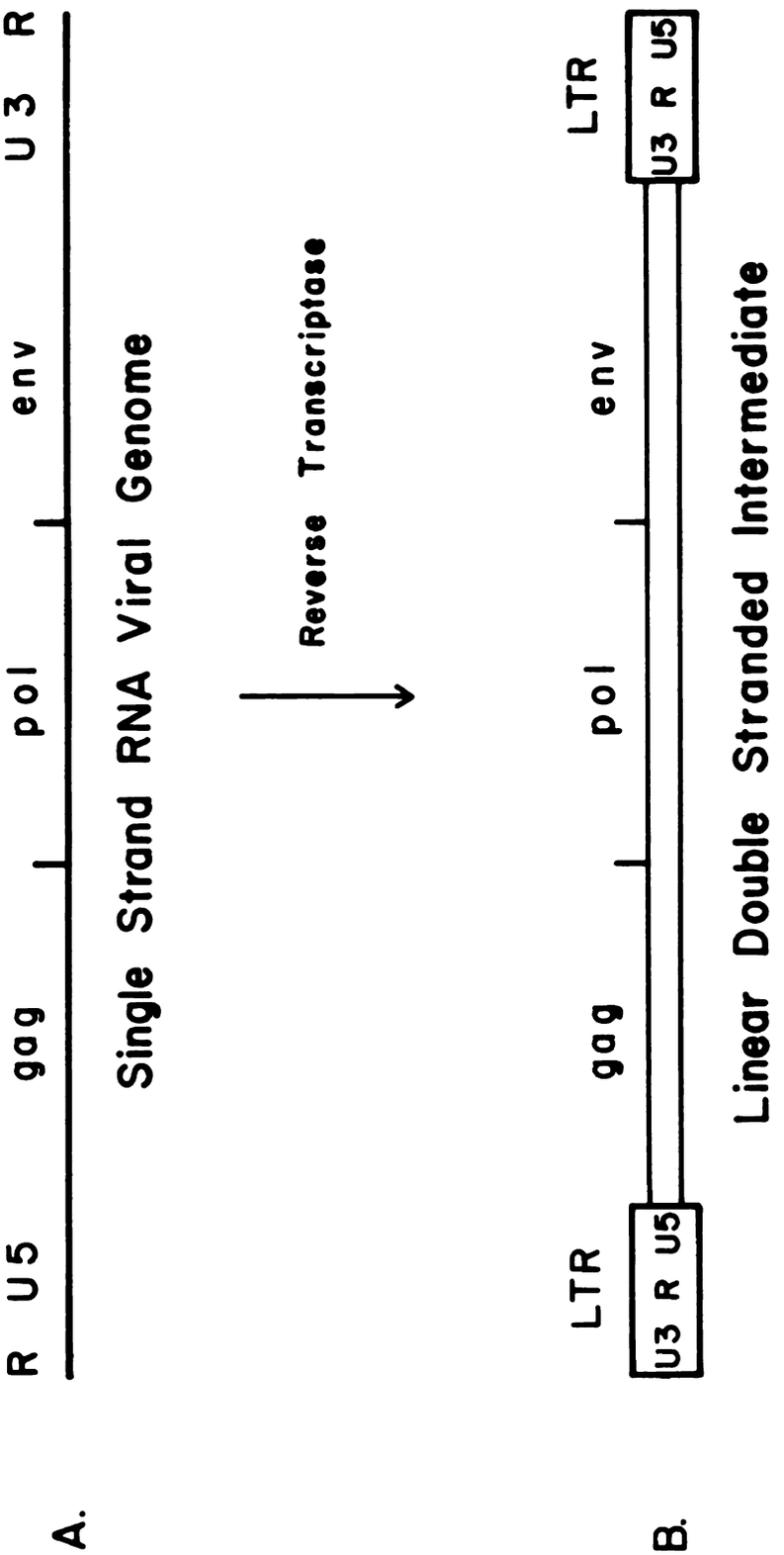
Figure 4. Comparison between the viral RNA genome and the viral DNA intermediate. The short repeat(R) in the vRNA is important for the synthesis of the vDNA. The long terminal repeat(LTR) is required for the integration of the vDNA into host cellular DNA and control of vRNA synthesis. The vRNA has only one copy of each of the U5 and U3 regions. While the vDNA carries a copy of each region at both ends of the DNA separated by the R region. This duplication of the U3 and U5 in the vDNA is a direct consequence of the unique mechanism used to convert the single-stranded RNA genome to double-stranded DNA(see section on viral replication).


A.
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Single Strand RNA Viral Genome
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B.
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U3 R U5
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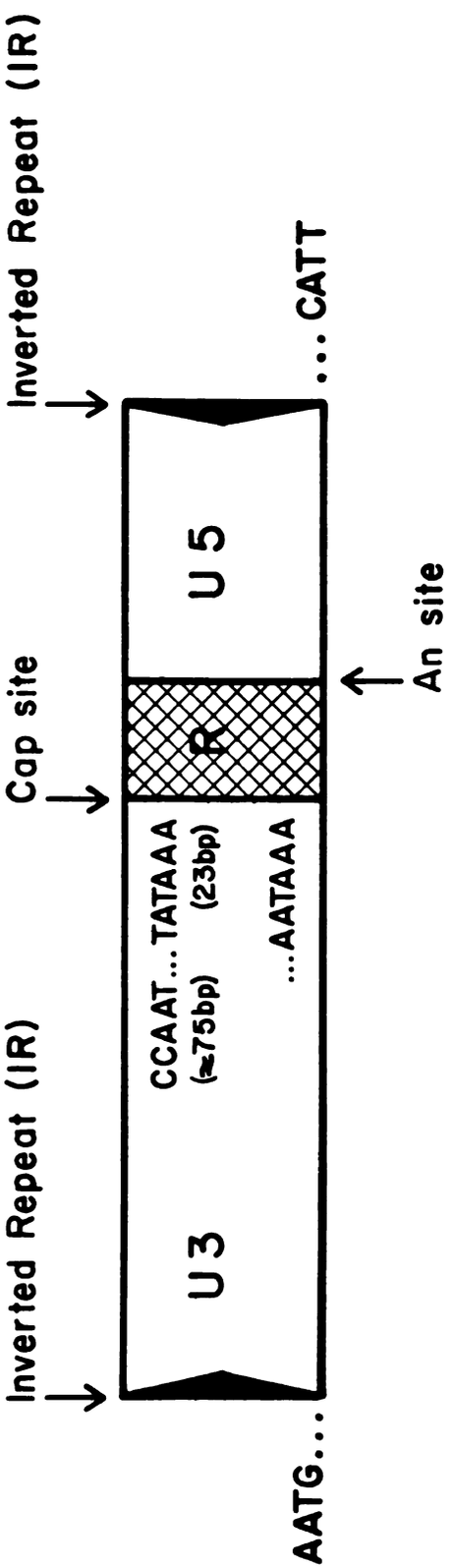
U3 R U5
Linear Double Stranded Intermediate

Figure 5. General structure of a retroviral LTR. The important features of the U3, R, and U5 are shown schematically. The LTRs terminate with short inverted repeats that almost always end with the nucleotides shown. The sequences: CCAAT and TATAAA are implicated in the initiation of eucaryotic RNA synthesis. The AATAAA sequence has been implicated as a eucaryotic polyadenylation signal. The length of U3, R and U5 vary between retroviruses. However, the length of U5 is relatively constant between related retroviruses. The polyadenylation signal is sometimes located in the R region in cases were R is long.


Inverted Repeat (IR)
Inverted Repeat (IR)
Cap site
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U5
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(≈75bp) (23bp)
...AATAAA
AATG...
... CATT

Figure 6. Synthesis of the minus-strand of viral DNA.
RNA is shown as wavy lines and DNA as straight lines with arrows denoting the direction of synthesis. (-)PBS is the tRNA primer binding site. (+) is the putative (+) strand primer site. Short vertical lines designate the boundaries of U3, R, and U5.

A) The figure at the top represents a viral RNA genome before replication begins. The first step in the synthesis of the viral DNA starts with the addition of deoxynucleotides to the 3' end of a tRNA molecule that is bound to a region called the primer binding ite(PBS) located immediately 3' to the U5 region.

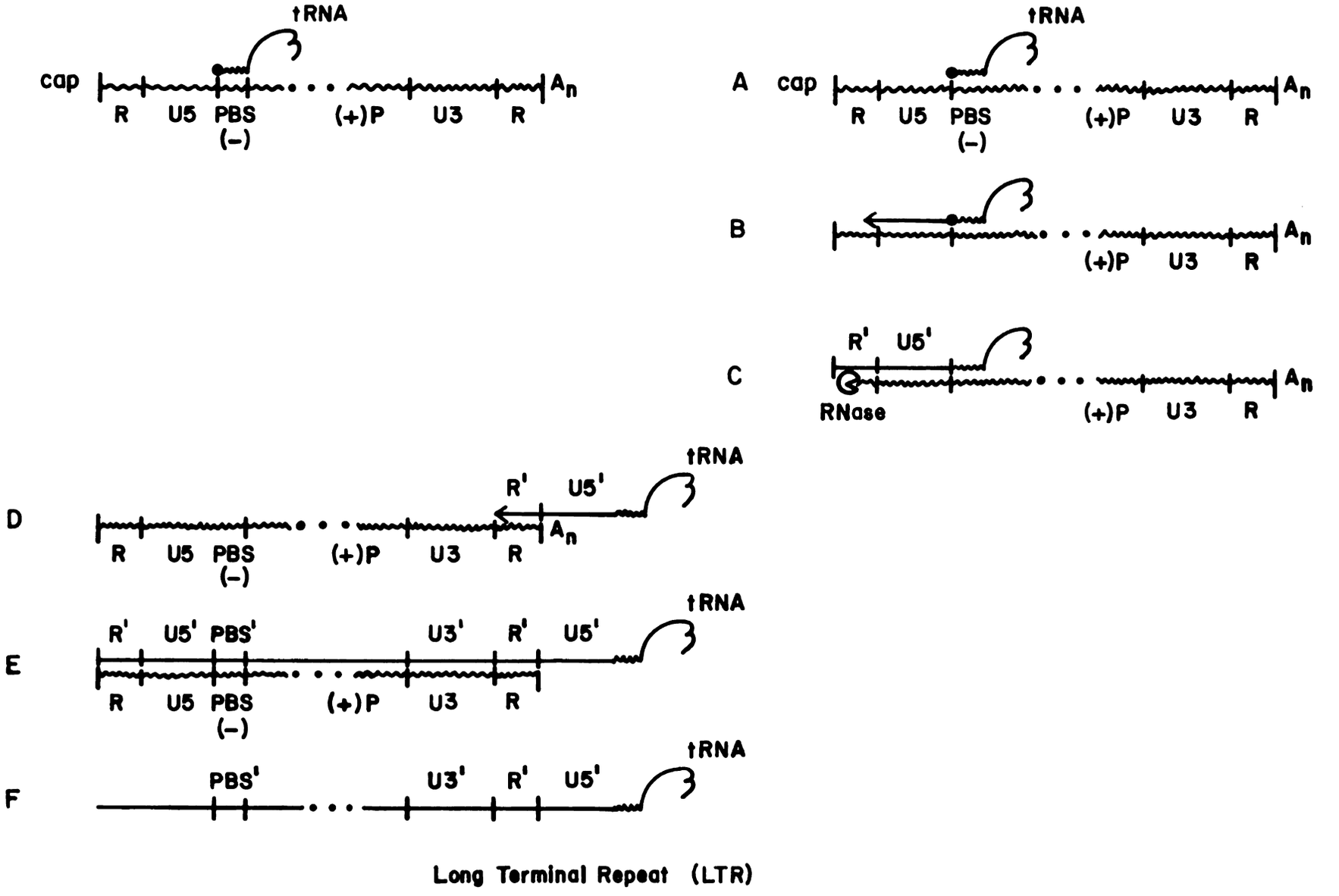
B) Synthesis terminates at the 5' end of the molecule.

C) The RNase activity of reverse transcriptase digests the R and U5 regions.

D) The newly synthesized DNA(-strand) along with its attached tRNA primer "jumps" to the 3' end of the viral RNA so that the complementary copy(the DNA copy) of the R region base pairs with the F region at the 3' end of one of the viral RNA subunits.

E) The 3' hydroxyl of the R region of the nascent minus strand of DNA is used as a primer for the synthesis of an almost complete strand of viral DNA.

F) In order to complete the synthesis of the viral strand it is necessary to initiate synthesis of DNA at the U3 boundary using the viral DNA strand as a template.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of the retroviral DNA and the host cellular DNA. By comparing unintegrated viral DNA to the integrated viral DNA it has been shown that the terminal 2 bp from each inverted repeat are lost from the viral DNA. In addition, a short direct duplication of the cellular DNA results. Every proviral-host junction examined has lost exactly 2 bp, however the size of the direct duplication varies between different retroviruses (Varmus 1983). It was shown elegantly by Panganiban et al. (1984) that a closed circular molecule with the two LTRs in tandem is the precursor to the integrated retroviral DNA. The fact that the precursor to the retroviral DNA is a closed circular molecule and the integrated viral DNA is always colinear with the linear duplex synthesized in the cytoplasm(Figure 4) implies that viral integration involves some enzymatic activity that recognizes specific sequences, presumable at the LTR circle junction. These observations are consistent with an earlier proposition that the 3' portion of the retroviral pol gene encodes an integrative function, since the protein product of this region from the RSV and the MuLV genome display endonuclease and DNA binding activities (Grandgenett et al., 1978;Misra et al., 1982;Nissen-Meyer et al., 1980;Kopchick et al., 1981). More recently, Schwartzberg et al.(1984) and Donehower et al.(1984) have examined deletion mutants of MuLV that are defective for integration. Both have shown that the deletions map in the 3' pol region of the retroviral genome. Neither the late stages of the viral

life cycle(transcription, translation and virion assembly) nor early events such as adsorption and reverse trancription of the RNA viral genome are affected by the deletions. Therefore, it is believed that one or more of the steps involved in proviral integration has been pertrubed.

There are structural similarities between the termini of the integrated retroviral DNA and transposable elements found in bacteria, yeast and insects (Ju and Skalka 1980; Varmus 1983). Both have a short inverted repeat at each terminus and both integration and transposition result in direct duplications of the adjacent cellular DNA. In addition, with the exception of the mouse mammary tumor virus(MMTV) LTR, all proviral inverted repeats examined are bounded by TG....CA (Temin 1980;Majors and Varmus 1981;Hughes 1981;Scott 1981;Shimothono and Temin 1980) the same 2 bp found at the end of all eukaryotic transposable elements. This has lead to the proposal that the provirus could "jump" in and out of the cellular genome in a fashion similar to transposable elements. However, it is known that the terminal 10-12 nucleotides of the LTR, located in the inverted repeat region(Figure 5), are required for integration of the viral DNA (Panganiban et al., 1984) and integration of the provirus results in the loss of the two terminal nucleotides from the inverted repeats in the LTR. Taken together, these results would generally preclude the direct DNA-DNA "transposition" of the provirus by the same mechanism used for the original integration event.

Acute-Transforming Viruses

The family of RNA tumor viruses can be divided into two classes based on their biological properties; the rare and highly oncogenic class of acute-transforming viruses that harbor oncogenes and the almost ubiquitous class of non-acute transforming virus that do not harbor oncogenes. Acute transforming viruses, with exception of some Rous strains, are replication defective, induce neoplastic disease in host animals rapidly(2-4 weeks) and transform the appropriate target cells in tissue culture (Hanafusa et al., 1977;Graf and Bueg 1978;Shih and Scolnick 1980). In contrast, the group of non-acute transforming virus are replication competent, induce neoplastic disease in the host after a prolonged latent period(4-10 months) and they do not transform cells in tissue culture.

All of these differences in biological activity between the two classes of retrovirus can be accounted for by the presence of specific oncogene(s) within the genome of the acute transforming virus (Hanafusa et al., 1977;Vogt 1977;Bishop and Varmus 1982). It was demonstrated by Stehelin et al.(1976) that the viral oncogene of RSV(v-src) has a homologous counter-part(c-src) in normal uninfected avian cells. Hanafusa et al.(1977) showed that transformation defective mutants of the acute transforming virus RSV that retained a portion of v-src could recombine with c-src to regenerate the acute transforming virus. These were the first clues that the origin of the viral

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oncogenes found in the acute transforming viruses were, in fact, derived from cellular genes already present in an infected host. Since that time other viral oncogenes have been shown to have homologous cellular counterparts in a large range of vertebrates (Spector et al., 1978, Rosseul et al., 1979;Bishop and Varmus 1982;Figure 1).

It was suggested by Graf and Bueg (1978,1980) that the susceptibility of target cells to transformation by a particular retroviral oncogene might correlated with the expression of the homologous cellular oncogene in the uninfected target cell. This was predicated on the assumption that the cellular oncogenes were involved with and expressed at specific stages of cellular differentiation. Therefore, the expression of the homologous viral oncogene interfered with further differentiation, resulting in continued cellular proliferation. However, this appears not to be the case for a number of acute transforming viruses. For example, avian myeloblastosis virus(AMV) transforms cells in the myelomonocytic lineage, but the homologous cellular oncogene(c-myb) is expressed at high levels in cells from the erythroid lineage (Gonda and Bishop 1983).

Acute-Transforming Virus Formation: Transduction

Since all oncogenes harbored by acute-transforming viruses have homologous cellular counter-parts, it is believed that acute-transforming viruses arose by capturing or transducing the cellular gene. It is apparent from the

Figure 7. Transduction of a cellular oncogene.
The process may begin with the continued passage in-vivo of a non-transforming virus. During the viral replication and infection cycle the virus has the opportunity to integrate many times in the host cellular DNA. The open box represents the U3 region and the closed box represents the R-U5 region of the viral LTR. A cellular oncogene is shown with 2 exons separated by an intron. The A_n represents the polyadenylated tail of eucaryotic mRNA.

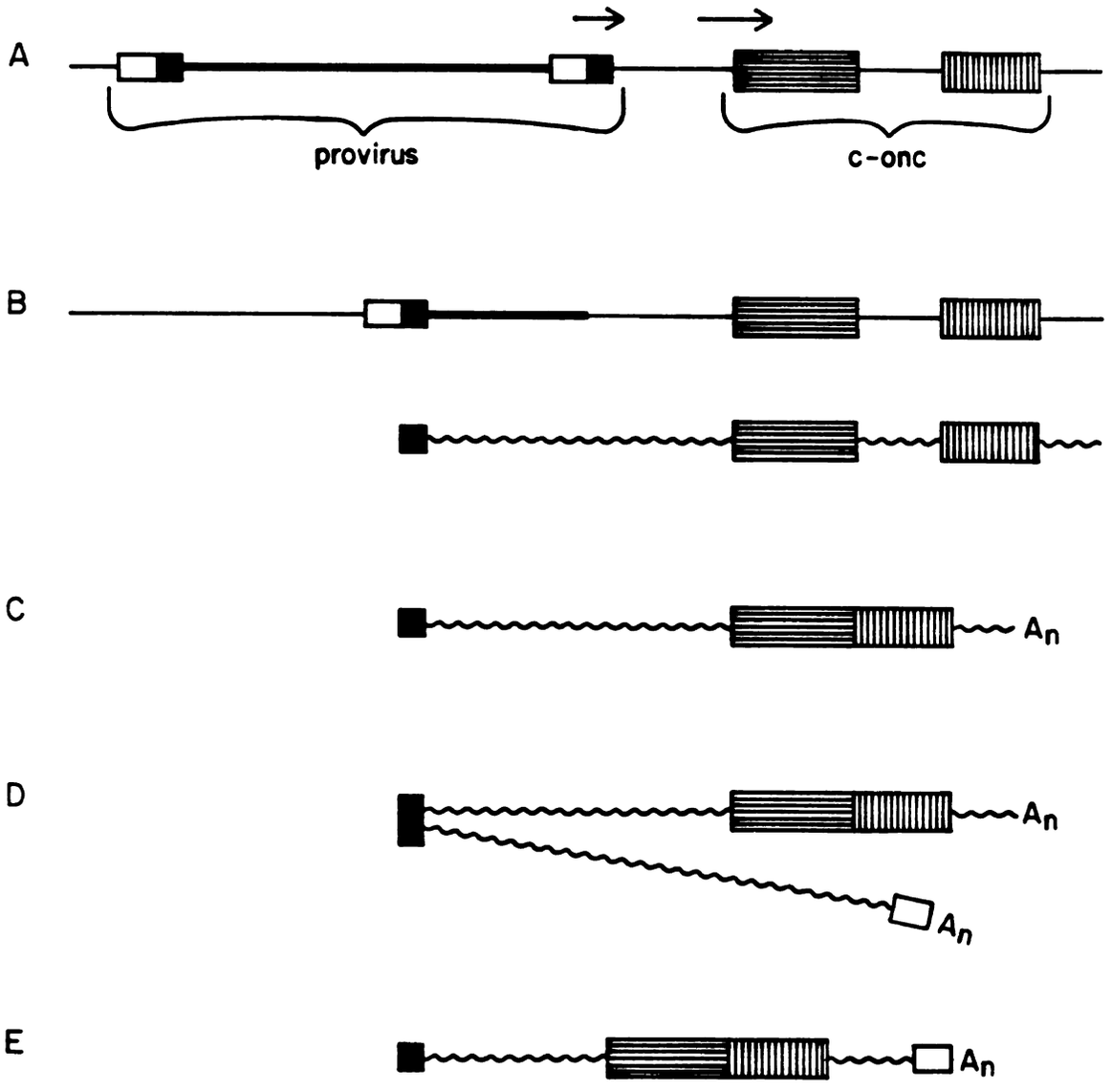
A) On occasion the virus may integrate upstream and in the same transcriptional orientation of a cellular oncogene.

B) A deletion that includes the 3' LTR could fuse the proviral and cellular oncogene DNA, such that a hybrid RNA transcript is formed.

C) The hybrid viral-oncogene transcript could be post-transcriptionally modified, i.e., removal of introns, capping, and poly adenylation, and transported to the cytoplasm as mRNA.

D) The 5' viral portion of the hybrid RNA permits the formation of heterodimers with the genome of a superinfecting or co-resident retrovirus and packaging of the hybrid RNA into virions.

E) The high frequency of retroviral recombination during replication may allow the joining of the sequences in the hybrid RNA on the 3' side of the oncogene to a U3-R sequence from a normal viral subunit, thereby creating an acute-transforming virus structure.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structure of the acute and the non-acute transforming virus that at least two recombination events must occur in order to capture an oncogene and form an acute-transforming virus genome structure. Retroviruses are adept at recombination: their genomes frequently exchange domains of unpredictable size during mixed infections (Coffin 1979). Their proviral forms can enter the host genome with great facility (Bishop 1978;Varmus and Swanstrom 1982) and furthermore, they are diploid, the linkage between viral genomes could assist cross-over between heterozygous combinations of different RNAs within the virions (Weiss et al., 1973;Hunter 1978).

Support for this model comes from two fronts. First, the comparison of viral and the homologous cellular oncogene (Wyke 1983;Wang et al.,1984) have shown that the viral gene probably derives from the cellular gene via normal RNA processing events. Second, structures similar to those shown in Figure 7 have been observed in some tumors (Payne et al., 1982;Pachl et al., 1983).

Reticuloendotheliosis Viruses.

Reticuloendotheliosis viruses(REVs) are a group of avian retroviruses related by their similar morphology (Kang et al., 1975), common group-reactive antigen (Maldonado and Bose 1976) and extensive RNA sequence homology (Kang and Temin 1974). The histopathological lesions consist of marked proliferation of reticuloendothelial cells(RE) around blood vessels and lymph follicles, especially in the liver and spleen (Sevoian 1964). The proliferating cells are

usually fusiform (Theilen 1966), with basophilic cytoplasm (Robinson and Twiehaus 1974). The nuclei are large and have prominent nucleoli (Olson 1967). In more extensive lesions, large areas of central necrosis are observed (Theilen 1966), usually in the gastrointestinal tract, bursa of Fabricius, thymus, heart and bone marrow (Olson 1967).

REVs can be propagated in many different avian hosts and cell types and although REVs natural hosts are birds, there is much data to indicate that they evolved from a mammalian C-type retrovirus. The major core protein(p29) of REV is antigenicllly distinct from the Avian Leukosis Virus core protein p27 (Maldonado and Bose 1971,1976;Moelling et al., 1975), but exhibits 40% sequence homology with MLV p30 core protein (Hunter 1978;Charman et al., 1979) and over 50% sequence homology with the major core protein of the endogenous retroviruses of macaques (Oroszlan and Gilden 1980).

The REV reverse transcriptase is antigenicllly unrelated to other avian retroviral reverse transcriptase enzymes (Misutani and Temin 1973,1974,1975;Kieras and Faras 1975;Moelling 1975;Waite and Allen 1975), but it is antigenicllly cross-reactive with reverse transcriptase from mammalian retroviruses (Bauer and Temin 1980). In addition, REV reverse transcriptase has a divalent-cation requirement for manganese, as do most of the reverse transcriptases associated with C-type mammalian retroviruses (Kang et al., 1975;Moelling et al., 1975;Waite and Allen 1975).

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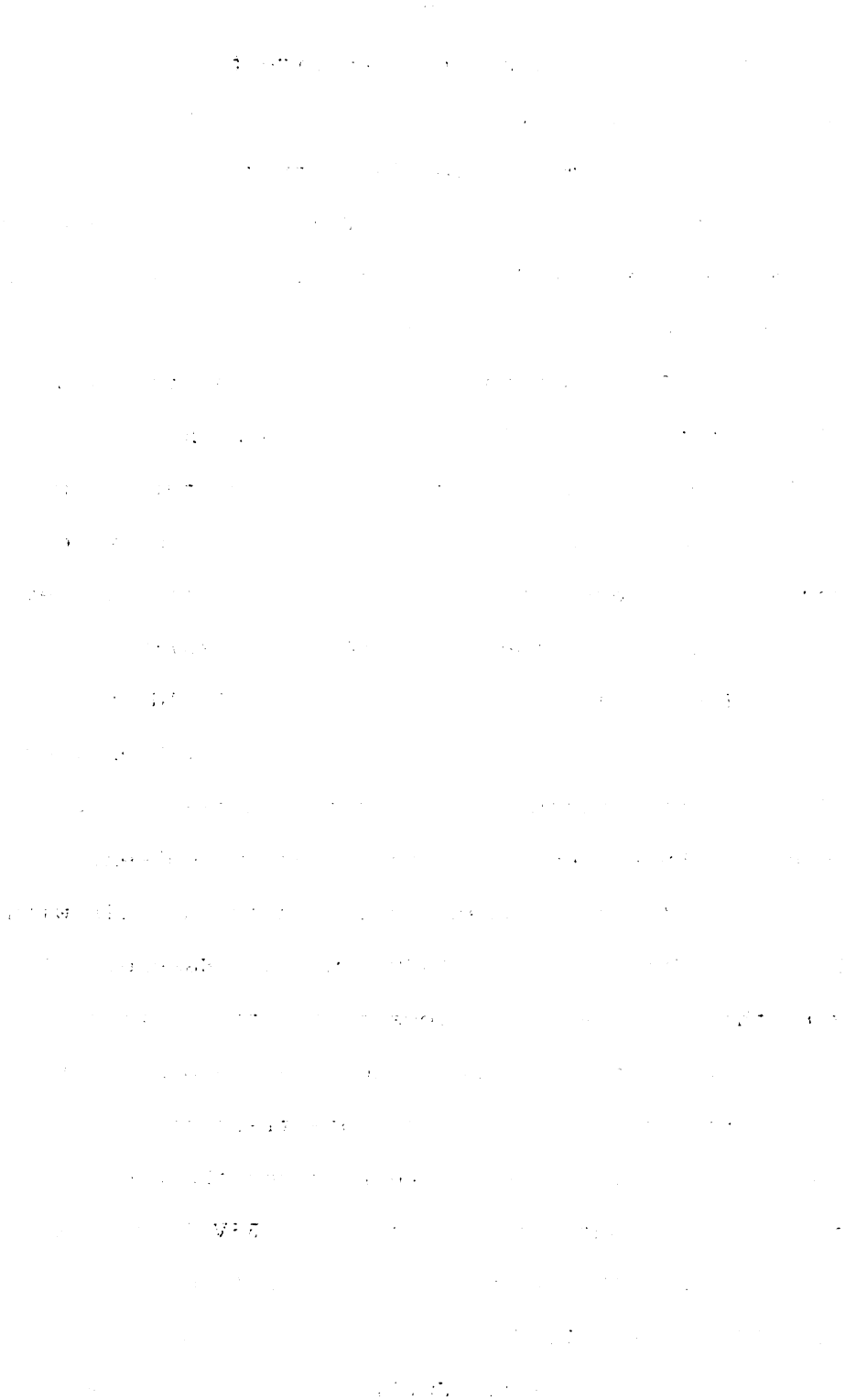
Furthermore, the tRNA primer binding site for the reverse transcriptase of REV is $tRNA^{pro}$, the same as the primer binding site used by many mammalian retroviruses (Waters 1975). Recent studies show that REV-A, the helper virus of REV-T, can replicate in dog and rat cell lines and can act as a helper virus for the replication-defective Moloney-Murine Sarcoma Virus (Varmus and Swanstrom 1982).

The prototype virus is considered to be REV strain T, originally isolated by Twiehaus in 1958 from turkeys with lymphoproliferative lesions(1958). REV-T is highly lethal for young chicks, ducklings, goslings, pheasants, turkeys, quail and guinea keets (Sevoian 1964;Olson 1967;Taylor and Olson 1972;Theilen 1966,1977;Rodriguez and Field 1969; Rodriguez 1971;Mcdougall 1980,1981;Bagust 1981;Dren 1983) inducing an acute systemic proliferative disease which is characterized by RE lesions in the liver and spleen.

Initial attempts at transforming cultured cells with REV-T failed (Witter et al., 1970;Temin and Kassner 1974). However, this was due to the loss of the transforming virus during serial passage. It has since been recognized that REV-T is a replication defective acute transforming virus (Chen et al., 1981) and therefore requires the presence of a helper virus for continued replication. REV-T can transform avian fibroblasts (Franklin 1977) and hematopoietic cells *in vitro* (Franklin 1974;Hoelzer et al., 1980;Lewis et al., 1981;Shibuya et al., 1982). Until recently, REV-T was the only member of the REV group that has been characterized as



an acute transforming virus. However, an Australian strain of REV can also induce neoplasia in inoculated chickens within 8-20 days post-infection (Ratnamohan et al., 1982).

The REV-T genome appears to be derived from REV-A (Rice et al., 1982), the helper virus found associated with REV-T, but with two major differences: an insertion in the env region of a 1.4 kb DNA segment derived from normal turkey DNA (designated the c-rel locus) and a deletion of about 3.0 kb of helper virus related sequence. By making deletions and substitutions in an infectious clone of REV-T, recovering the mutated virus by transfection and assaying the transforming capacity in chicken spleen cells, Chen et al.(1982) showed that only the v-rel gene and a small amount of helper virus is necessary to transform cells in-vitro. An interesting result from these studies has been the observation that sequences present in the helper virus of REV-A can suppress the transforming capacity of v-rel and therefore the deletion of REV-A sequence is necessary for the transforming capacity of REV-T.

Recently, attention has been focused on the replication-competent non-acute transforming members of the REV group. The four best characterized strains are: spleen necrosis virus(SNV) isolated by Trager in 1959, chicken syncytial virus (CSV) isolated by Cook in 1969, duck infectious anemia virus (DIAV) isolated by Ludford in 1969 and reticuloendotheliosis associated virus (REV-A) isolated by Hoelzer in 1979. CSV, isolated from a domestic chicken

with nerve lesions, can induce syncytia in cultured chicken embyro fibroblasts(CEF). Both SNV and DIAV were isolated from a duck infected with Plasmodium lophurae. SNV induces a rapid enlargment and necrosis of the spleen resulting in death, and DIAV induces an acute anemia. It is interesting that a new REV isolate (Li et al., 1983), RU-1, was also isolated from a duck infected with Plasmodium lophurae. The significance of this observation is unclear. All of these REV strains have the capacity to suppress the immune system response, but the suppression appears to be non-specific. In contrast, REV-A induces an immunodepressive runting disease (Hoeltzer et al., 1979), by activation of a suppressor cell population in the spleen, within 3 days post-infection (Carpenter et al., 1981;Rubin et al., 1978;Carpenter;Kempf et al., 1982). The nucleotide sequence of these four REVs show greater than 90% homology (Kang and Temin 1973). Natural occurrence of REVs have been reported in chickens (Cook 1969;Ratnamohan et al., 1980;Witter et al., 1981,1982), ducks (Grimes and Purchase 1973;Paul 1978), turkeys (Robinson and Twiehaus 1974;Paul et al., 1976;Sarma et al., 1975;McDougall et al., 1978) and Japanese quail (Carlson et al., 1974;Shat et al., 1976). In addition, new natural isolates of REV have been described (Ratnamohn et al., 1980;Mcdougall et al., 1978;Li et al., 1984;Grimes et al., 1979;Witter and Crittenden 1979). The non-acute REVs can induce B-cell lymphomas or lymphosarcomas, depending on the genetic background of the chicken, characterized by a

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long latent period of 4 to 12 months post-infection (Fadly et al., 1982, Nazerian et al., 1982). The B-cell lymphomas resemble those induced by avian leukosis virus(ALV) on the basis of organ distribution, pathology, latency, surface immunoglobin(IgM) production and activation of the same cellular oncogene, c-myc (Grimes et al., 1979;Witter and Crittenden 1979;Fadley et al., 1982;Hayward et al., 1981;Norri et al., 1981;Fung et al., 1981;Payne et al., 1982). However, the lymphosarcomas have a different organ distribution and pathology. Consistent with these biological observations is the recent finding that the activated oncogene is not c-myc (Swift unpublished data).

Cellular Oncogenes

Oncogenes were first identified as the genetic component of acute transforming viruses responsible for the capacity of these viruses to induce neoplasia rapidly in the infected host and to transform cells in vitro. Additional oncogenes have been identified by experimental designs involving the transfection of tumor DNA into NIH 3T3 cells or by the examination of common proviral integration sites in neoplasms induced by non-acute transforming viruses (Figure 8). The prototype viral oncogene is v-src, the viral oncogene of an avian acute transforming virus(RSV). Stehelin et al.(1976) and Spector et al.(1978) showed that both avian and other vertebrate DNAs contain nucleotide sequences homologous to the v-src gene. This has become a common theme as additional retroviral oncogenes have been

Characteristics of Some Oncogenes not Associated with Acute Transforming Viruses

Oncogene	Origin	Activity
Transfection of DNA		
B-lym	ALV-induced B-cell lymphomas	Related to transferrin proteins
T-lym	Mouse T-cell lymphomas	Related to Qa/Tla genes
mam	MMTV-induced mammary carcinomas	
neu	Ethylnitrosourea-induced neuroblastomas	Related to EGF receptor
N-ras	Human neuroblastomas,leukemias,sarcomas	Part of ras gene family
N-myc	Human neuroblastomas	Related to c-myc
Insertional activation (inducing provirus)		
Pim 1(MCF)	Mouse T-cell lymphomas	
pMo-1C(MoMuLV)	Rat T-cell lymphomas	
Int-1(MMTV)	Mouse adenocarcinomas	
Int-2(MMTV)	Mouse adenocarcinomas	
Mlvi-1(MoMuLV)	Rat T-cell lymphomas	
Mlvi-2(MoMuLV)	Rat T-cell lymphomas	

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identified. In fact, the homologous sequence to a number of oncogenes have been observed in many vertebrate DNAs (Bishop and Varmus 1982), Drosophila DNA (Shilo and Weinberg 1981;Shilo et al. 1984), Dictyostelium DNA (Reymond et al., 1984) and yeast DNA (Papageorge et al., 1984;Defeo-Jones et al., 1983).

The conservation of the cellular oncogenes in such evolutionary diverse species coupled with the observation that viral oncogenes and several of their "activated" cellular counterparts can induce uncontrolled cellular proliferation and interfere with or arrest cell differentiation processes (Graf et al., 1978) has led to the proposal that these cellular homologs of viral oncogenes function in normal pathways of cellular proliferation and differentiation. This proposal is very attractive in light of recent findings that link the oncogene products of c-fos, c-myc, c-ras, and c-sis with cellular growth control and differentiation (Waterfield et al., 1983;Doolittle et al., 1983;Downward et al., 1983;Kelly et al. 1983;Campsi et al. 1983;Weston et al. 1984;Muller and Muller 1984).

An early suggestion for the mechanism of oncogenesis by viral oncogene products proposed that oncogenes might be analogs of the small polypeptide growth factors that stimulate cell division after binding to receptors on the the cell surface (Todaro et al. 1976;for review see Heldin and Westermark 1984). Specificially, it was proposed that v-onc products from MuSV and FeSV, acting as agonists,

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blocked the EGF receptors resulting in a relentless stimulation of cell division. Though this does not appear to be the mechanism in the case of the MuSV and FeSV gene products, it may very well explain the mechanism of transformation by the simian sarcoma virus gene product, v-sis.

Recent experiments by Waterfield et al.(1983) and Doolittle et al.(1983) show that there is a striking near identity of the amino acid sequence of v-sis(the oncogene of SSV) and the B polypeptide chain of platelet-derived growth factor(PDGF). In addition, the v-sis gene product may be functionally equivalent to PDGF in many respects since conditioned media from SSV-transformed cells contain a PDGF like growth factor that will interact with specific PDGF receptors on responsive cells (Huang et al. 1984) and whose activity is neutralized by PDGF specific antibodies (Deuel et al., 1983).

The structural homology between v-sis and PDGF, taken together with the functional similarity, lends support to the proposal that v-sis protein functions as an agonist of the PDGF receptor resulting in the continued stimulation of cell division. The observation that addition of antibodies to the culture media of SSV-transformed cells does not slow down their proliferation may imply that v-sis activates the PDGF receptor from the cytoplasmic side of the plasma membrane or that continued expression of v-sis is not necessary for maintenance of a transformed phenotype.

The ras oncogenes are a multigene family which encode highly related proteins of 21-24 kd that have the capacity to transform cells in vitro and in vivo. They have been localized at the cytoplasmic side of the plasma membrane (Willingham et al. 1980) and ras-related proteins can be detected in normal mammalian cells (Furth et al. 1982), Drosophila and Yeast (DeFeo-Jones et al. 1983;Papageorge et al. 1984). Furthermore, the level of conservation of the gene between species is such that the human ras gene is capable of substituting for some functions of the inactivated endogenous yeast ras genes (Kataoka et al., 1985). Studies of temperature sensitive mutants of the viral ras protein from the murine acute-transforming virus Ha-MSV have shown that ras proteins have the biochemical property of binding GTP and autophosphorylation of threonine (Scolnick et al. 1979). Epidermal growth factor or insulin stimulates the GTP-dependent phosphorylation of the viral ras protein and the guanine nucleotide binding activity in ras-transformed cells (Kamata and Feramisco 1984). While the function of the ras proteins is not yet clear, several observations concerning their role in cell proliferation have been described.

The transformation of some mammalian cells can occur if the ras gene is expressed at an abnormally high level (DeFeo et al, 1981;Chang et al., 1982;Spandidos et al., 1984) or if mutations alter the protein primary structure (Furth et al., 1982;Tabin et al., 1982;Yuasa et al., 1982;Santos et al.,

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1983). A particularly potent mutation is one that changes a glycine at residue 12 to a valine. This mutated or activated ras gene was first isolated from an urinary bladder carcinoma cell line, T24 (Tabin et al., 1982).

Consistent with these observations from human tumors and cell lines are the results of Feramisco et al.(1984) which show that microinjection of purified T24 mutant ras protein into a variety of quiescent somatic cells induces dramatic morphological changes followed by transient proliferation of the cells. In contrast, the microinjection of the normal ras protein has little effect on the cells. In BPA31 cells, a chemically transformed Balb/c 3T3 cell line, the c-ras^{Ki} is expressed in a cell cycle-dependent manner. The relative abundance of c-ras^{Ki} mRNA begins to increase in mid- to late G_0/G_1.

Recently, a number of laboratories have reported that the purified ras protein possess GTPase activity that is decreased by the same mutations that activate the ras protein, a valine at residue 12 or threonine at residue 59 (Gibbs et al., 1984;Sweet et al., 1984;McGrath et al., 1984). This is intriguing since there is some structural homology between ras proteins and the G protein, which is known to regulate the hormone sensitive adenylate cyclase activity (Gilman et al. 1984).

Activation of the G protein requires GTP binding and appears to utilize the hydrolysis of GTP to end a cycle of activation of the adenylate cyclase activity. If the ras

protein has an analogous regulatory function in some proliferation control pathway, then the deficiency in GTPase activity of the T24 mutant could result in prolonged stimulation of a normally regulatable activity.

These observations suggest that the ras proteins may be involved in growth factor receptor activity or in the signal transduction that may follow receptor activation with a mechanism similar to that employed by the G protein (Kamata and Feramisco 1984).

FBJ murine osteosarcoma virus(FBJ-MuSV) is an acute retrovirus that transforms fibroblasts cells in vitro and causes osteosarcomas in new born mice (Finkel and Biskis 1966). FBJ-MuSV harbors the oncogene v-fos, that is derived from the cellular gene c-fos by an out of frame deletion of 104 bp (Curran and Miller 1984). The c-fos gene product is a nuclear phosphoprotein that may be involved in cell differentiation and cell proliferation. The level of the c-fos transcripts is 100-fold greater in human term fetal membranes than in other normal human tissues and cells. These levels of c-fos expression in human amniotic and chorionic cells are close to the level of v-fos expression that results in the induction of osteosarcomas in mice and transformation of fibroblasts in-vitro. Transcription of c-fos is detected at very high levels in murine extra-embryonic tissues, especially amnion, viseral yolk sac and mid-gestation fetal liver. The level of c-fos expression in fetal membranes increases markedly at late

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stages of gestation (Muller et al., 1983) and in cells of monomyelocytic lineage, high levels of c-fos are detected only at late stages of differentiation (Muller et al., 1984b). Also, a rapid induction of the expression of the c-fos gene is observed when the promyelocytic cell line HL-60 (Collins et al., 1977,1978) or monocytic cell line U-937 (Sundstrom and Nilsson, 1976) is induced with phorbol esters to differentiate into nondividing, adherent macrophages (Mitchell et al., 1985).

Recent experiments show that stimulation of a murine cell line with PDGF or whole serum results in a transient 15 fold increase of c-fos mRNA within minutes of stimulation (Greenberg and Ziff 1984). Perhaps most intriguing are the recent experiments of Muller and Wagner(1984) that strongly suggest that c-fos plays a pivotal role in cell differentiation. Using F9 cells, a teratocarcinoma cell line, they showed that transfection of the c-fos gene into F9 cells results in two phenotypically distinct cell types. One consists of proliferating cells with normal F9 morphology and the other comprises enlarged, flat cells that assume an epithelial morphology and cease to proliferate. The morphologically altered F9 cells develop an ordered array of intermediate filaments that carry several antigens characteristic of endoderm tissue. Therefore, high levels of c-fos expression in F9 cells can result in phenotypic properties characteristic of differentiated cells.

The c-myc Gene

The structure and function of the c-myc gene has been the subject of intense investigation and is implicated in a number of vertebrate neoplasms through a plethora of mechanisms. C-myc is a normal cellular gene that has been conserved during evolution and is expressed in all tissue types examined (Bishop and Varmus 1982;Persson et al., 1984). Alterations at the c-myc locus have been observed in many B-cell malignancies of chicken, human and mouse origin. These alterations include the proximate integration of a retrovirus (Hayward et al., 1981;Fung et al., 1982,1984;Noori-Daloii et al., 1981;Payne et al., 1982;Corcoran et al., 1984;Li et al., 1984;Steffen 1984), point mutations (Rabbits et al., 1983;Westway et al., 1984), chromosomal translocations (Shen-Ong et al., 1982,Adams et al., 1982;Taub et al., 1982, for review see Klein 1983;Perry 1983;Leder et al., 1983) and amplification (Noori-Daloii et al., 1981;Collins and Groudine 1982;Little et al., 1983;for review see Klein 1981;Schimke 1984).

The c-myc gene is the cellular homolog of the oncogene v-myc, carried by the acute transforming virus, MC-29. This virus has a very wide oncogenic spectrum, inducing solid tumors composed of malignant myelocytes (Beard 1980), hepatomas, endotheliomas, carcinomas (Mladenov et al, 1967), and sarcomas (Graf and Bueg 1978). This is in contrast to the observation that the activated c-myc gene is almost always associated with the induction of tumors of B-lymphoid cells. The cause of this variance in target cell

The c myc Gene

susceptability is unknown, however, a MC29 mutant that induces mainly B and T cell lymphoid tumors and some myelocytomas has recently been isolated (Enrietto et al., 1983) that may address this question.

The c-myc protein has been localized to the nuclear matrix of normal and transformed cells (Hann et al., 1983; Donner et al., 1982;Alitio et al., 1982;Persson et al., 1984;Eisenman et al., 1985) and appears to display DNA binding properties (Donner et al., 1982;Persson et al., 1984). This is consistent with earlier reports that the v-myc gene product is predominatly located in the nucleus (Donner et al., 1982;Abrams et al., 1982). The data to suggest that the c-myc gene product is a DNA binding protein is supported by affinity chromatography assays that show c-myc protein binds to single stranded and double stranded DNA (Persson et al., 1984) and the structural similarity of the carboxy terminal region of the c-myc protein to other DNA binding proteins (Pabo and Sauer 1984).

A comparison of the DNA sequence of v-myc to the chicken c-myc gene reveal seven amino acid changes and 10 nucleotides at the 5' end of v-myc not shared with c-myc (Watson et al., 1983). The 10 extra nucleotides are apparently derived from the first intron by differential splicing during transduction of the c-myc gene (Shih et al., 1984). In addition, the v-myc gene lacks the first exon of the c-myc gene (Shih et al., 1984). Though the first exon is non-coding, it could serve an important function in c-myc

regulation at the transcriptional, post-transcriptional or translational level. It has been suggested that a stem-loop structure of the c-myc mRNA, between the first and second exon, hinders translation of the gene, thereby causing translational suppression that could be important in the regulation of the levels of c-myc protein (Saito et al., 1983). However, Nilsen and Maroney (1984) showed that the presence or absence of the first exon did not affect initation or elongation of the c-myc gene product. Also, Hann and Eisenman (1984) could detect equal levels of c-myc protein in cells having an intact and unmutated first exon compared with cells lacking exon 1, suggesting that the predicted stem-loop structure does not affect translation.

The expression of c-myc mRNA has been examined in many different cell types. The level of c-myc mRNA in non-neoplastically transformed cells is low during quienscence and increases 10-30 fold prior to cell division (Kelly et al., 1983;Campisi et al., 1984). An interesting experiment by Kelly et al. (1983) indicated that the c-myc gene may be involved in the normal response of quiescent lymphocytes and fibroblasts to a growth signal appropriate to those cells. They have been able to show that platelet-derived growth factor(PDGF), a fibroblast mitogen, and concanavalin A and lipopolysaccharide, two lymphocyte mitogens, stimulate the expression of c-myc in those cells that normally respond to them. All three mitogens are believed to act early in the cell cycle to induce

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competence; and all stimulate transient expression of c-myc within 1-2 hours, with a return to baseline well before the onset of DNA synthesis. The activation of c-myc by PDGF is particularly interesting because the sequence of the B-chain of PDGF and the gene product of the c-sis oncogene show a high degree of homology (Doolittle et al., 1983;Waterfield et al., 1983).

In a related experiment, Makino et al.(1984) examined the expression of c-myc in liver tissue induced to regenerate by partial hepatectomy. This system allows the observation of how cells in vivo, that are reasonably synchronized, respond when they move from a differentiated resting state(G_0) to a proliferative state. The results show that the expression of c-myc is increased 10-15 fold within 1-3 hours after partial hepatectomy. This expression begins to decrease rapidly after 4 hours, to a level of less than two fold the normal level after 8 hours, at which time DNA replication has still not begun. A still larger increase in c-myc transcription(600 fold) is observed when protein synthesis is inhibited by an injection of cycloheximide. These results are consistent with previous findings in vitro with fibroblast or lymphocyte cells pre-treated with cyclohexamde and stimulated by PDGF. The large increase in c-myc expression after pre-treatment with cycloheximide is believed to imply that c-myc transcription is under negative regulatory control by a protein with a high turnover rate.

During differentiation of F9 teratocarcinoma cells to nonproliferating endoderm cells, expression of c-myc mRNA decreases upon terminal differentiation (Muller et al., 1983). The c-myc gene product has also been implicated in the terminal differentiation of mouse erythroleukemia(MEL) cells (Lachman and Skoultchi 1984). It has been observed that c-myc mRNA expression shows a rapid bi-phasic change in MEL cells induced to differentiate by dimethyl sulphoxide or hypoxanthine that may be important in the irreversible commitment of MEL cells to terminally differentiate. In addition, Reitsma et al.(1983) have shown that treatment of HL-60 cells with a vitamin D metabolite results in the rapid differentiation to monocyte-like cells that is preceded by a decrease in the expression of c-myc. These observations suggest that c-myc may be involved in some aspect of cell differentiation.

Proviral Insertional Activation.

It has been demonstrated that avian leukosis viruses(ALVs), a group of non-acute transforming viruses, frequently induce B-cell lymphomas after a latency of 4-10 months post-infection (for review see Purchase and Burmeister 1978;Crittenden 1980). Since ALVs do not harbor transforming genes, the mechanism by which this retrovirus induced neoplasia was unclear.

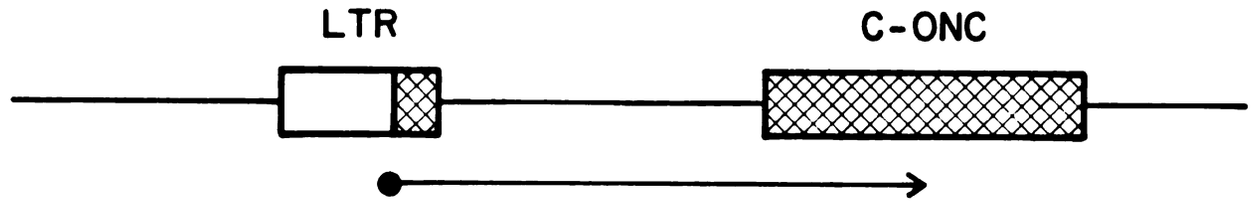
It was shown by Hayward et al.(1981) that ALV, RAV-1 strain, induce B-cell lymphomas by activation of the c-myc gene. Their results showed that the majority of bursal

Frontal Insertional Activation.

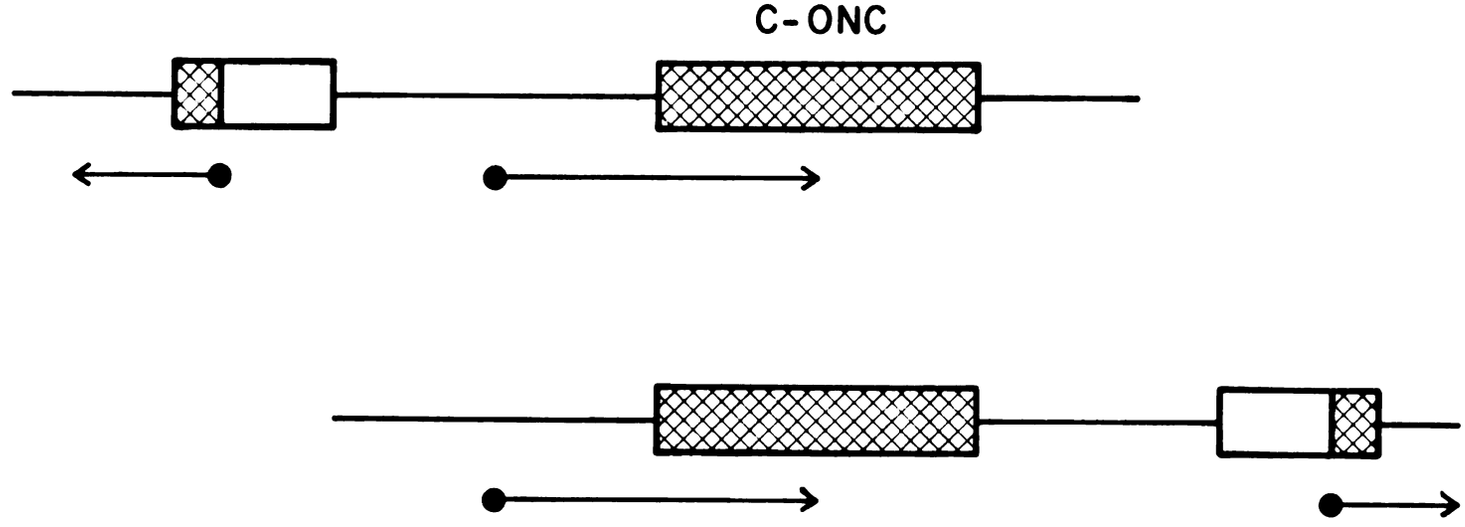
Figure 9. Two configurations of proviral DNA that result in enhanced expression of a cellular oncogene.
The promotor-insertion model(top) predicts that the proviral LTR will be found upstream from the cellular oncogene(c-onc) with the transcriptional orientation identical to the c-onc (indicated by the arrow).

The enhancer-insertion model(bottom) predicts that the proviral LTR could be oriented in the opposite transcriptional direction (as indicated by the direction of the opposing arrows) or downstream of the c-onc gene.

Promotor - Insertion



Enhancer - Insertion



tumors contained proviral sequences inserted near the c-myc gene. Subsequently, they showed that most proviral insertions occur upstream of c-myc and oriented in the same transcriptional direction. Consistent with this configuration, in most tumors, an elevated expression of a novel c-myc mRNA is detected that contains the R and U5 regions of the viral LTR. Therefore, the insertion of proviral regulatory sequences upstream of c-myc results in initiation of transcription from within the viral LTR and continues into the adjacent c-myc gene. This model of cellular oncogene activation is referred to as promotor-insertion activation(Figure 9A). Similar results have been reported by Fung et al.(1982). In addition, Swift et al.(1985) have reported that promotor-insertion is the predominant mode of c-myc activation by chicken syncytial virus, an avian non-acute transforming virus unrelated to ALVs(see section on Reticuloendotheliosis virus).

In contrast to these results, Payne et al.(1982) have reported, using a RAV-2 strain of ALV, two configurations of proviral insertion adjacent to c-myc that are inconsistent with the promotor-insertion model. In one configuration the provirus is inserted upstream of c-myc, but the viral LTR is in the opposite transcriptional orientation relative to the c-myc gene. In this orientation, transcription of c-myc can not initiate from the viral promotor. In the second configuration, the provirus is found downstream of the c-myc gene and in the same transcriptional orientation(Figure 9B). In both cases, the elevated expression of c-myc mRNA

is observed. These exceptions prompted the hypothesis, later found to be correct, that the LTR contains 'enhancer' sequences that can elevate the level of gene expression independent of the position or orientation of the 'enhancer' relative to the affected gene(see section on Enhancers). This mechanism of gene activation is referred to as enhancer-insertion.

Though the promotor-insertion configuration predominates in B-cell lymphomas induced by avian retroviruses, it appears that this system my not represent the most common configuration of proviral oncogene activation. In the small percentage of T-cell lymphomas, induced by murine non-acute transforming viruses, that show c-myc alterations, enhancer-insertion is the predominant mode of activation. The murine provirus is located upstream of the c-myc gene and in the opposite transcriptional orientation (Corcoran et al., 1984;Li et al., 1984;Steffen 1984). In addition the majority of reported activations of other putative oncogenes by proviral insertion(Figure 8) show a preference for the enhancer-insertion mode of transcriptional elevation.

Translocation.

A substantial body of evidence indicates that chromosomal translocations involving the c-myc locus and one of the immunoglobulin loci are common in certain types of B-cell lymphomas found in mice and men (Klein 1983;Perry 1983). Most of the translocations that are observed in the human disease, Burkitt's lymphoma, involve the movement of

Translocation.

part of chromosome 8, containing the c-myc gene, to the heavy-chain locus of chromosome 14 and in rare cases to the light-chain locus of chromosome 22 and 2 (Hollis et al., 1984). As a result of the translocation, alterations in c-myc expression have been observed in either the amount of c-myc mRNA or the size of the c-myc transcript. An early hypothesis for the elevated levels of c-myc mRNA was the influence of the cellular enhancer located in the heavy-chainswitching region. However, subsequent reports (Battey et al., 1983;Rabbitts et al., 1983;Gelmann et al., 1983) indicate that the translocation of c-myc often results in the movement of the cellular enhancer to chromosome 8 where it can no longer influence the transcription of c-myc on chromosome 14. Another hypothesis is that the c-myc regulatory sequences are altered due to the translocation. There is strong, but circumstantial, evidence for the suggestion that the first exon serves a regulatory function, since the c-myc gene has 3 exons, but only the second and third exons code for the c-myc protein.

The first exon of c-myc is highly conserved between mice and man, but is absent from v-myc (Shih et al., 1984). Therefore, exon 1 may serve a vital function in the normal cellular context that is either unnecessary or even deleterious to the transforming activity of v-myc. The translocations that are typical of mouse plasmacytomas result in the separation of some or all of exon 1 from exon 2 and exon 3. In contrast to the mouse plasmacytomas, in some

Burkitt's lymphoma, all three exons are translocated intact. However, DNA sequencing and S_1 mapping reveal mutations in the intact exon 1 in five Burkitt's lymphoma cell lines.

A Burkitt's lymphoma cell line carring an 8:14 translocation has been identified that has all three c-myc exons and the nucleotide sequence of the first exon is identical to the normal c-myc sequence with the exception of two single-base substitutions (Wiman et al., 1984). As suggested by the authors, it is possible that one or both of these subsitutions are in the region involved with c-myc regulation. It is also possible that sequences proximal to the first exon are also important in regulating c-myc expression. Interestingly, a similar situation has been reported in a mouse plasmacytoma, but in this case only one base substitution is observed (Stanton et al., 1984). Perhaps, only by coincidence, the next four nucleotides at this site are identical to those found in the Burkitt's lymphoma just mentioned.

Amplification.

The concept of gene amplification as a model for the generation and/or progression of cancer has been considered (Pall 1981;Klein 1981) and is supported by a number of results. Perhaps the first report of amplification of the c-myc oncogene was observed in CSV-induced bursal lymphomas (Noori-Dalloii et al., 1981). There are now a number of examples of oncogene amplification in both fresh tumors and established cell lines (Noori-Daloii et al., 1981;Collins and Groudine 1982;Little et al., 1983;Lee et al., 1984;Kohl et al., 1983;Schwab et al., 1983a;Schwab et al.,

1983b;Alitalo et al., 1983). In the vast majority of the amplifications the oncogene c-myc or a closely related gene, N-myc, is involved.

The c-myc gene has been shown to be amplified in the human promyelocytic leukemia cell line, HL-60 (Collins and Groudine 1982). This is interesting in view of the complementation experiments of Land et al.(1983) and Ruley(1983), since coexisiting with the amplified c-myc gene is an activated N-ras gene (Murray et al., 1984). Other examples of c-myc amplification have been reported in two neuroendocrine tumor cell lines derived from a colon carcinoma (Alitalo et al., 1983) and in 8 out of 18 human lung cancer cell lines (Little et al., 1983). However, most intriguing is the consistent amplification of the N-myc gene in tumors of neural origin. The N-myc gene is amplified in 14 out of 18 neuroblastoma cell lines and 5 out of 8 fresh neuroblastomas (Yunis et al., 1984;Schwab et al., 1985). In contrast, only 4 out of 36 nonneuroblastoma human tumors and tumor cell lines tested showed N-myc amplification, of which three were of neuroectodermal origin (Kohl et al., 1984). In addition, Brodeur et al.(1984) find N-myc amplified in 24 of 48 tumors from late stage retinoblastoma tumors, but find no amplification in 15 tumors from early stages of retinoblastoma tumors. The authors conclude that N-myc amplification may be involved in the progression of these tumors to later, and more deadly, stages of retinoblastoma.

CHAPTER II

LINKAGE OF CHICKEN C-MYC GENE TO CSV

The family of RNA tumor viruses is generally separated into two classes that appear to cause neoplasia by different molecular mechanisms. The class of acute transforming virus, with the exception of some Rous strains, are replication defective, harbor oncogene(s) for cell transformation and induce neoplasia in the host with a very short latency (2 weeks) between infection and neoplasia. In contrast, the class of non-acute transforming virus are replication competent, do not harbor an oncogene and can induce neoplasia, albeit, with a long latency of 4-10 months post-infection.

Chicken syncytial virus(CSV) is an avian non-acute transforming virus genetically unrelated to avian leukosis virus(ALV). However, similar to ALV, if affects the primary lymphoid organ for B-cell maturation, the bursa of Fabricus, inducing a malignant B-cell lymphoma in chickens 4-6 months post-infection.

It has been demonstrated by Hayward et al.(1981) that in the majority of tumors the ALV(RAV-1 strain) provirus integrates near c-myc, the cellular homolog of the oncogene of the acute-transforming virus MC-29. Analysis of the RNA from these tumors shows that c-myc transcription was elevated (Neel et al., 1981). These observations have led Hayward and co-workers to propose that increased production of c-myc protein triggers the transformation process.

In the present study we have characterized the structure of the CSV provirus in the induced tumors and report here that the proviral DNA is integrated adjacent to c-myc in over 90% of the tumors examined. In addition, similar to the results seen in the ALV system deletions of proviral DNA occurs frequently in these tumors.

MATERIALS AND METHODS

Induction of Lymphoid Leukosis.

Day-old chicks from line $15I_5x7_1$(Regional Poultry Research Laboratory) were inoculated intra-abdominally with 10^5 infectious units of end-point purified CSV. The chickens were free of common avian pathogens and reared in plastic canopy isolators. Both the virus stock and the tumor samples were shown serologically and biochemically to be free of common avian pathogens and reared in plastic canopy isolators. Both the virus stock and the tumor samples were shown serologically and by hybridization analysis to be free of avian leukosis virus. Approximately 20 weeks post-infection birds that developed lymphoma were sacrificed and both tumor and non-tumor tissue were collected for analysis. All tissue samples were immediately transferred to liquid nitrogen and then stored at - 70° C until use.

DNA Extraction and Enzyme Digestion.

Approximately 0.5g of frozen tissue was pulverized in a stainless steel tissue smasher in the presence of liquid

Induction of Lymphoid Leukosis.

DNA Extraction and Enzyme Digestion.

nitrogen. The powdered tissue was transferred to a sterile tube containing 20ml SET buffer/0.5% SDS,10mM EDTA,20mM Tris pH 7.5 and 200ug per ml proteinase K/ preheated to 60°C. After incubation for 1hr at 60°C, 500ug/ml of pronase was added and the mixture incubated overnight at 37°C with constant shaking. The mixture was extracted with phenol/chloroform and the DNA concentrated by precipitation with 2.5 volumes of ethanol. DNA was digested with a two fold excess of restriction endonuclease for 3 hours. The digested DNAs were fractionated on 0.7% agarose gels, immobilized on nitrocellulose and hybridized with the appropriate nick-translated probe.

RNA Isolation.

Tissue was pulverized similar to DNA, but the powdered tissue was transferred to 10 mls of a 4 M guanidinium thiocyanate mixture (Chirgwin et al., 1979) pre-heated to 60°C. The mixture was disrupted by homogenization in a polytron mixer. An equal volume of phenol preheated to 60°C was added and the mixture passed through a syringe fitted with a 25-gauge needle until the viscosity of the suspension was reduced. The mixture was extracted twice with chloroform and the aqueous phase transferred to a sterile tube. One gram of CsCl/2.5 mls of mixture was added and then the contents was transferred to a sterile polyallomer centrifuge tube with 1.5 mls of 5.7 M CsCl in 0.1 M EDTA(pH 7.5). The tube was spun at 35k rpm in a SW50.1 rotor for 12 hrs. The supernatant was removed and the pelleted RNA was suspended in a small volume of 4 M

2NA Isolation.

guanidinum thiocynate. A 1/40th volume of glacial acetic was added and the contents transferred to a 1500ul eppendorf. The mixture was precipitated in 0.5 volumes of ethanol over night at -20°C. The tube was spun 5 minutes in a microfuge. The precipitation step was repeated and RNA was suspended in, DEPC treated, sterile water.

Hybridization and Nick-Translation.

Filter hybridizations were carried out in 50% formamide, 5xSSPE/20x=3.6M NaCl, 0.2M NaH_2PO_4 pH 7.4, 0.02M EDTA/, 1xDenhardts/100x=10g polyvinyl pyrolidone, 10g Ficoll and 10g BSA in 500ml DDH_2O/ and 100ug per ml single-strand salmon sperm DNA at 42°C. The filters were washed at room temperature for 10 minutes in 2xSSPE and then washed twice in 0.1xSSPE, 0.1% SDS for 45' at 50°C with shaking. The SNV_t probe, a gift from Dr. H. Temin, consisted of permuted copy of the SNV genome cloned into the Sal I site of pBR322. The c-myc probe, gift from Dr. T. Robbins, consisted of a subclone of the second exon of c-myc. The probe was cloned into the Sac I and Sal I sites of pDH24. All probes contained in recombinant plasmids were digested with the appropriate restriction endonuclease(s) and gel purified. The gel purified fragments were generally nick-translated to a specific activity $>10^8$ cpm/ug of DNA (Rigby et al., 1977).

Re-hybridization of Filters.

To remove the hybridized radioactive probe from nitrocellulose filters for subsequent re-hybridizations, the filters were placed in a baking dish with 20 mM Tris and 20

Hybridization and Nick-Translation.

Re-hybridization of Filters.

mM EDTA, pH 7.5 and heated in a Tappan(Model 120) microwave oven, set on high, for 25 minutes. After cooling the filters were placed on 3MM paper to air dry.

RNA Dot Blot.

The RNA samples were denatured for 25' at 60^{o}C in 15xSSPE and 2.2M formaldehyde. Dilutions of RNA were made in wells of a microtiter plate. Nitrocellulose was equilibrated with 15xSSPE and placed on a Minifold apparatus(S&S). A 100 ul of each dilution was placed in the Minifold wells. After suction was removed from the Minifold, the nitrocellulose was air dried and baked for 2 hours in a vacuum oven at 80^{o}C. Hybridization and washing conditions were similar to DNA, except the hybridization temperature was 45^{o}C.

RESULTS

Linkage of c-myc to CSV.

A map of the CSV genome is presented in Figure 10 and a map of the chicken c-myc locus is presented in Figure 11. As indicated by the restriction enzyme maps of CSV and c-myc, neither have Eco RI sites in their coding regions. To examine the linkage of CSV proviral sequence to nearby cellular sequence the restriction endonuclease Eco RI was used. Since this endonuclease does not cleave within the CSV genome the whole proviral sequence and adjacent cellular sequences are obtained on a single Eco RI fragment.

The tumor DNA was digested with Eco RI, fractionated on a 0.7% agarose gels and transferred to nitrocellulose. Hybridization of these filters to the SNV_t probe is shown in Figure 11A. With most tumor samples, a single band of variable size is observed. Each band represents a tumor-specific(TS) fragment that corresponds to CSV proviral sequence. Only one proviral band is seen in most tumors, indicating that the CSV induced bursal tumors are derived from the outgrowth of a single transformed cell.

Mississippi Code 1972 Annotated Title 97. Criminal Offenses Chapter 29. Burglary and Unlawful Entry Generally § 97-29-1

§ 97-29-1. Burglary of dwelling house; penalty.

Every person who shall break and enter the dwelling house of another, in which there shall be at the time some human being, with intent to commit any crime, or who shall enter such dwelling house by any false or fraudulent pretense, or who shall enter such dwelling house and commit any crime therein, shall be guilty of burglary and, upon conviction thereof, shall be punished by imprisonment in the penitentiary for not more than twenty (20) years.

Source: Codes, 1942, § 2087; Laws, 1912, ch. 130; Laws, 1926, ch. 152; Laws, 1930, ch. 157; Laws, 1942, ch. 249; Laws, 1966, ch. 509, § 1; Laws, 1977, ch. 464, § 1; Laws, 1985, ch. 492, § 1; Laws, 1994, ch. 587, § 1; Laws, 1995, ch. 3, § 1; Laws, 1995, ch. 565, § 1; Laws, 1995, ch. 566, § 1; Laws, 1995, ch. 567, § 1; Laws, 1995, ch. 568, § 1; Laws, 1995, ch. 569, § 1; Laws, 1995, ch. 570, § 1; Laws, 1995, ch. 571, § 1; Laws, 1995, ch. 572, § 1; Laws, 1995, ch. 573, § 1; Laws, 1995, ch. 574, § 1; Laws, 1995, ch. 575, § 1; Laws, 1995, ch. 576, § 1; Laws, 1995, ch. 577, § 1; Laws, 1995, ch. 578, § 1; Laws, 1995, ch. 579, § 1; Laws, 1995, ch. 580, § 1; Laws, 1995, ch. 581, § 1; Laws, 1995, ch. 582, § 1; Laws, 1995, ch. 583, § 1; Laws, 1995, ch. 584, § 1; Laws, 1995, ch. 585, § 1; Laws, 1995, ch. 586, § 1; Laws, 1995, ch. 587, § 1; Laws, 1995, ch. 588, § 1; Laws, 1995, ch. 589, § 1; Laws, 1995, ch. 590, § 1; Laws, 1995, ch. 591, § 1; Laws, 1995, ch. 592, § 1; Laws, 1995, ch. 593, § 1; Laws, 1995, ch. 594, § 1; Laws, 1995, ch. 595, § 1; Laws, 1995, ch. 596, § 1; Laws, 1995, ch. 597, § 1; Laws, 1995, ch. 598, § 1; Laws, 1995, ch. 599, § 1; Laws, 1995, ch. 600, § 1; Laws, 1995, ch. 601, § 1; Laws, 1995, ch. 602, § 1; Laws, 1995, ch. 603, § 1; Laws, 1995, ch. 604, § 1; Laws, 1995, ch. 605, § 1; Laws, 1995, ch. 606, § 1; Laws, 1995, ch. 607, § 1; Laws, 1995, ch. 608, § 1; Laws, 1995, ch. 609, § 1; Laws, 1995, ch. 610, § 1; Laws, 1995, ch. 611, § 1; Laws, 1995, ch. 612, § 1; Laws, 1995, ch. 613, § 1; Laws, 1995, ch. 614, § 1; Laws, 1995, ch. 615, § 1; Laws, 1995, ch. 616, § 1; Laws, 1995, ch. 617, § 1; Laws, 1995, ch. 618, § 1; Laws, 1995, ch. 619, § 1; Laws, 1995, ch. 620, § 1; Laws, 1995, ch. 621, § 1; Laws, 1995, ch. 622, § 1; Laws, 1995, ch. 623, § 1; Laws, 1995, ch. 624, § 1; Laws, 1995, ch. 625, § 1; Laws, 1995, ch. 626, § 1; Laws, 1995, ch. 627, § 1; Laws, 1995, ch. 628, § 1; Laws, 1995, ch. 629, § 1; Laws, 1995, ch. 630, § 1; Laws, 1995, ch. 631, § 1; Laws, 1995, ch. 632, § 1; Laws, 1995, ch. 633, § 1; 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Laws, 1995, ch. 712, § 1; Laws, 1995, ch. 713, § 1; Laws, 1995, ch. 714, § 1; Laws, 1995, ch. 715, § 1; Laws, 1995, ch. 716, § 1; Laws, 1995, ch. 717, § 1; Laws, 1995, ch. 718, § 1; Laws, 1995, ch. 719, § 1; Laws, 1995, ch. 720, § 1; Laws, 1995, ch. 721, § 1; Laws, 1995, ch. 722, § 1; Laws, 1995, ch. 723, § 1; Laws, 1995, ch. 724, § 1; Laws, 1995, ch. 725, § 1; Laws, 1995, ch. 726, § 1; Laws, 1995, ch. 727, § 1; Laws, 1995, ch. 728, § 1; Laws, 1995, ch. 729, § 1; Laws, 1995, ch. 730, § 1; Laws, 1995, ch. 731, § 1; Laws, 1995, ch. 732, § 1; Laws, 1995, ch. 733, § 1; Laws, 1995, ch. 734, § 1; Laws, 1995, ch. 735, § 1; Laws, 1995, ch. 736, § 1; Laws, 1995, ch. 737, § 1; Laws, 1995, ch. 738, § 1; Laws, 1995, ch. 739, § 1; Laws, 1995, ch. 740, § 1; Laws, 1995, ch. 741, § 1; Laws, 1995, ch. 742, § 1; Laws, 1995, ch. 743, § 1; Laws, 1995, ch. 744, § 1; Laws, 1995, ch. 745, § 1; Laws, 1995, ch. 746, § 1; Laws, 1995, ch. 747, § 1; Laws, 1995, ch. 748, § 1; Laws, 1995, ch. 749, § 1; Laws, 1995, ch. 750, § 1; 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Laws, 1995, ch. 790, § 1; Laws, 1995, ch. 791, § 1; Laws, 1995, ch. 792, § 1; Laws, 1995, ch. 793, § 1; Laws, 1995, ch. 794, § 1; Laws, 1995, ch. 795, § 1; Laws, 1995, ch. 796, § 1; Laws, 1995, ch. 797, § 1; Laws, 1995, ch. 798, § 1; Laws, 1995, ch. 799, § 1; Laws, 1995, ch. 800, § 1; Laws, 1995, ch. 801, § 1; Laws, 1995, ch. 802, § 1; Laws, 1995, ch. 803, § 1; Laws, 1995, ch. 804, § 1; Laws, 1995, ch. 805, § 1; Laws, 1995, ch. 806, § 1; Laws, 1995, ch. 807, § 1; Laws, 1995, ch. 808, § 1; Laws, 1995, ch. 809, § 1; Laws, 1995, ch. 810, § 1; Laws, 1995, ch. 811, § 1; Laws, 1995, ch. 812, § 1; Laws, 1995, ch. 813, § 1; Laws, 1995, ch. 814, § 1; Laws, 1995, ch. 815, § 1; Laws, 1995, ch. 816, § 1; Laws, 1995, ch. 817, § 1; Laws, 1995, ch. 818, § 1; Laws, 1995, ch. 819, § 1; Laws, 1995, ch. 820, § 1; Laws, 1995, ch. 821, § 1; Laws, 1995, ch. 822, § 1; Laws, 1995, ch. 823, § 1; Laws, 1995, ch. 824, § 1; Laws, 1995, ch. 825, § 1; Laws, 1995, ch. 826, § 1; Laws, 1995, ch. 827, § 1; Laws, 1995, ch. 828, § 1; 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Laws, 1995, ch. 907, § 1; Laws, 1995, ch. 908, § 1; Laws, 1995, ch. 909, § 1; Laws, 1995, ch. 910, § 1; Laws, 1995, ch. 911, § 1; Laws, 1995, ch. 912, § 1; Laws, 1995, ch. 913, § 1; Laws, 1995, ch. 914, § 1; Laws, 1995, ch. 915, § 1; Laws, 1995, ch. 916, § 1; Laws, 1995, ch. 917, § 1; Laws, 1995, ch. 918, § 1; Laws, 1995, ch. 919, § 1; Laws, 1995, ch. 920, § 1; Laws, 1995, ch. 921, § 1; Laws, 1995, ch. 922, § 1; Laws, 1995, ch. 923, § 1; Laws, 1995, ch. 924, § 1; Laws, 1995, ch. 925, § 1; Laws, 1995, ch. 926, § 1; Laws, 1995, ch. 927, § 1; Laws, 1995, ch. 928, § 1; Laws, 1995, ch. 929, § 1; Laws, 1995, ch. 930, § 1; Laws, 1995, ch. 931, § 1; Laws, 1995, ch. 932, § 1; Laws, 1995, ch. 933, § 1; Laws, 1995, ch. 934, § 1; Laws, 1995, ch. 935, § 1; Laws, 1995, ch. 936, § 1; Laws, 1995, ch. 937, § 1; Laws, 1995, ch. 938, § 1; Laws, 1995, ch. 939, § 1; Laws, 1995, ch. 940, § 1; Laws, 1995, ch. 941, § 1; Laws, 1995, ch. 942, § 1; Laws, 1995, ch. 943, § 1; Laws, 1995, ch. 944, § 1; Laws, 1995, ch. 945, § 1; Laws, 1995, ch. 946, § 1; Laws, 1995, ch. 947, § 1; Laws, 1995, ch. 948, § 1; Laws, 1995, ch. 949, § 1; Laws, 1995, ch. 950, § 1; Laws, 1995, ch. 951, § 1; Laws, 1995, ch. 952, § 1; Laws, 1995, ch. 953, § 1; Laws, 1995, ch. 954, § 1; Laws, 1995, ch. 955, § 1; Laws, 1995, ch. 956, § 1; Laws, 1995, ch. 957, § 1; Laws, 1995, ch. 958, § 1; Laws, 1995, ch. 959, § 1; Laws, 1995, ch. 960, § 1; Laws, 1995, ch. 961, § 1; Laws, 1995, ch. 962, § 1; Laws, 1995, ch. 963, § 1; Laws, 1995, ch. 964, § 1; Laws, 1995, ch. 965, § 1; Laws, 1995, ch. 966, § 1; Laws, 1995, ch. 967, § 1; Laws, 1995, ch. 968, § 1; Laws, 1995, ch. 969, § 1; Laws, 1995, ch. 970, § 1; Laws, 1995, ch. 971, § 1; Laws, 1995, ch. 972, § 1; Laws, 1995, ch. 973, § 1; Laws, 1995, ch. 974, § 1; Laws, 1995, ch. 975, § 1; Laws, 1995, ch. 976, § 1; Laws, 1995, ch. 977, § 1; Laws, 1995, ch. 978, § 1; Laws, 1995, ch. 979, § 1; Laws, 1995, ch. 980, § 1; Laws, 1995, ch. 981, § 1; Laws, 1995, ch. 982, § 1; Laws, 1995, ch. 983, § 1; Laws, 1995, ch. 984, § 1; Laws, 1995, ch. 985, § 1; Laws, 1995, ch. 986, § 1; Laws, 1995, ch. 987, § 1; Laws, 1995, ch. 988, § 1; Laws, 1995, ch. 989, § 1; Laws, 1995, ch. 990, § 1; Laws, 1995, ch. 991, § 1; Laws, 1995, ch. 992, § 1; Laws, 1995, ch. 993, § 1; Laws, 1995, ch. 994, § 1; Laws, 1995, ch. 995, § 1; Laws, 1995, ch. 996, § 1; Laws, 1995, ch. 997, § 1; Laws, 1995, ch. 998, § 1; Laws, 1995, ch. 999, § 1; Laws, 1995, ch. 1000, § 1.

Figure 10. Restriction enzyme map of CSV. The chicken syncytial virus map was derived from single and double digests of genomic DNA from RP-13 (Nazerian et al., 1982), a CSV producing cell line, followed by Southern blot hybridization with subclones from spleen necrosis virus, a strain of REV that shares greater than 95% sequence homology with CSV. A=Ava I; B=BamHI; H3=Hind III; Sc=Sac I; Sal=Sal I.

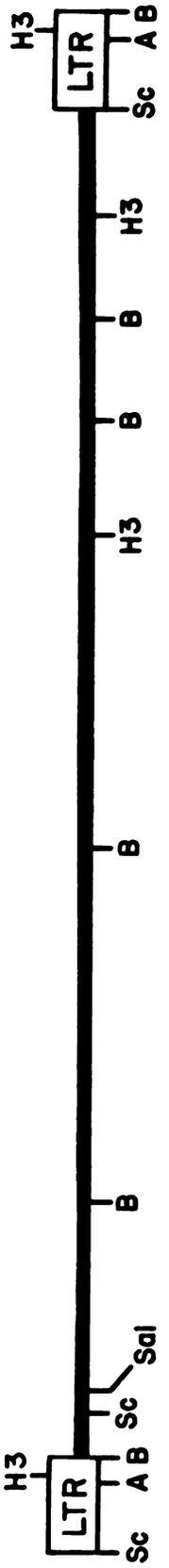
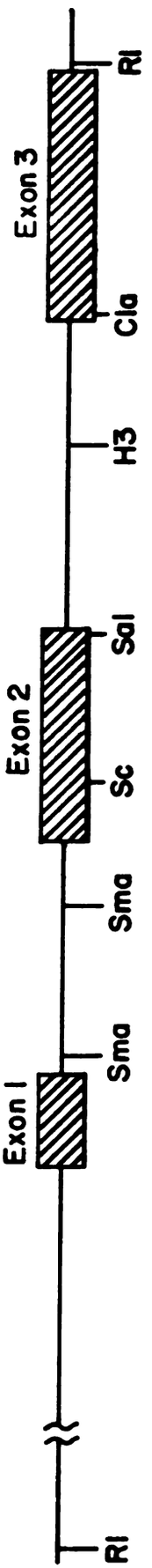

H3
LTR
A
B
Sc
H3
B
B
H3
B
B
Sal
Sc
H3
A
B
LTR
Sc

Figure 11. Restriction enzmye map of the chicken c-myc locus. The map was derived from the published DNA sequence (Watson et. al., 1983;Shih et al., 1984). Cla=Cla I; H3=Hind III; R1=Eco RI; Sc=Sac I; Sal=Sal I; Sma=Sma I.


RI
Exon 3
Cla
H3
Sal
Exon 2
Sc
Sma
Sma
Exon 1
RI

To demonstrate the linkage between the CSV provirus and the c-myc gene, the Eco RI cleaved tumor DNAs were re-hybridized with a myc specific probe. As shown in Figure 12B, each tumor contains both the unaltered c-myc allele of 13.5 kb and an altered c-myc allele that comigrates with at least one of the TS bands detected with the SNV_t probe. This data strongly implies that a CSV provirus is located adjacent to one of the c-myc alleles. These results implicate the c-myc gene in lymphomagenesis and CSV integration as the activator of this gene. The variation in relative intensity of the bands representing the unaltered and altered c-myc alleles implies that the unaltered c-myc allele is less abundant than the altered allele in some tumors. Presumably, this is due to aneuploidy for the chromosome bearing the unaltered c-myc gene. Similar results have been observed in bursal tumors induced by ALV (Payne et al., 1982).

The size of the Eco RI linkage fragments vary from 8-22 kb. We assumed that the size variations are due to deletions or structural alterations of the viral or surrounding cellular sequence. In order to examine the possibility that the alteration are in the viral sequences the tumor DNA was digested with Bam HI and hybridized to the SNV_t probe(data not shown). In an unaltered provirus there are 5 internal CSV fragments produced ranging in size from 2.2 - 0.75 kb. The Bam HI digestion pattern of the tumor DNA shown in figure 12A indicated the loss of some of

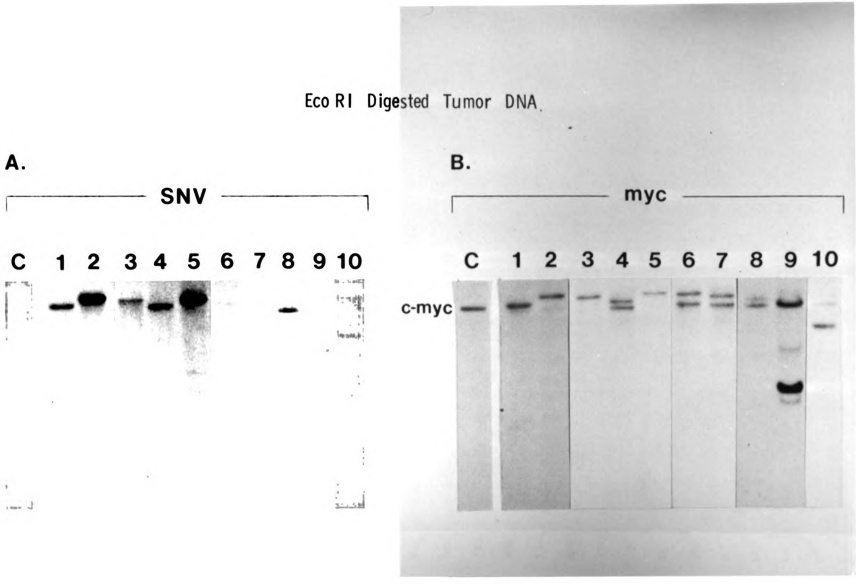
Figure 12. Hybridization of Eco RI digested tumor DNA. The structure of CSV proviruses and the chicken c-myc gene in B-cell lymphoma as analyzed using Eco RI digestion. Panel A) hybridization to the SNV_t probe. Panel B) hybridization to the chicken c-myc probe. The control lane(C) is DNA from a chicken free of REV infection. Usually, the normal c-myc bands are more intense than the tumor specific bands due to the presence of some normal tissues in the tumor nodules; in some cases (e.g. tumors 1-3 and 5), the reverse is true. Presumably, these are the result of the preferential loss of the chromosome carrying the normal c-myc allele in tumor cells, a phenomenon previously described in other chicken B-lymphomas (Payne et al., 1982).

Hybrid structured CSV parsing of bounded [illegible]

[illegible]

[illegible]

[


Eco RI Digested Tumor DNA
A.
SNV
C 1 2 3 4 5 6 7 8 9 10
B.
myc
C 1 2 3 4 5 6 7 8 9 10
c-myc

the viral specific fragments, thereby confirming that the size variations seen in the bands in Figure 12A are due to deletion or structural alteration of the integrated viral genome. The TS bands that are smaller than the unaltered c-myc allele(lane 9 and 10) are probably due to the presence of a new Eco RI site in the structurally altered viral genome.

Amplification.

An interesting result, presented in Figure 12A, is the unusual intensity of the hybridization signal of the TS band in lane 9. We believe this to be caused by the amplification of the provirus and c-myc sequences. In order to obtain a better estimate of the amount of CSV sequences present in a tumor with an intense TS band a titration experiment using SNV_t as quantization standards, was carried out(Figure 13). Thirty micrograms of DNA from tumor number 15 that showed amplification of CSV sequences (data not shown) and 30 ug of DNA from a tumor with a proviral band of similar size, but with a "normal" band intensity (tumor number 18), were digested with Eco RI and loaded on an agarose gel together with varying amounts of the purified insert of SNV_t(Figure 13, lanes c-f). The quantities of the cloned DNA in lanes c-f were such that they represent, respectively, 2, 4, 10, 20 copies per cell equivalent of CSV sequence in 30 ug of chicken DNA. When the TS bands in lanes a and b were traced densitometrically and their intensity compared with that of the standards in lanes c-f, it was found that while tumor no. 18 in lane b

Modification

Instrument

Accuracy

Precision

Sensitivity

Resolution

Range

Linearity

Reproducibility

Repeatability

Figure 13. Quantification of the CSV sequences in tumor no. 15. 30 ug each of Eco RI-digested genomic DNA from tumors no. 15 and no. 18 were analyzed in a 0.8% agarose gel, then hybridized with the SNV_t probe. The DNA concentration was determined by diphenylamine assay (Putman 1957). The cloned SNV_t DNA (lanes c-f) was used as a quantization standard. Lanes c, d, e, and f contain 0.23, 0.46, 1.15 and 2.3 ng each of purified SNV_t insert DNA. Based on a diploid genome size of 2 x 10^9 bases for chicken, these amounts of SNV_t DNA would give 2, 4, 10, and 20 copies per cell equivalent of CSV sequences in 30 ug of chicken genomic DNA. The weakly hybridizing lower band in lanes c-f represents the contaminating pBR322 vector DNA.


a b c d e f
2 4 10 20

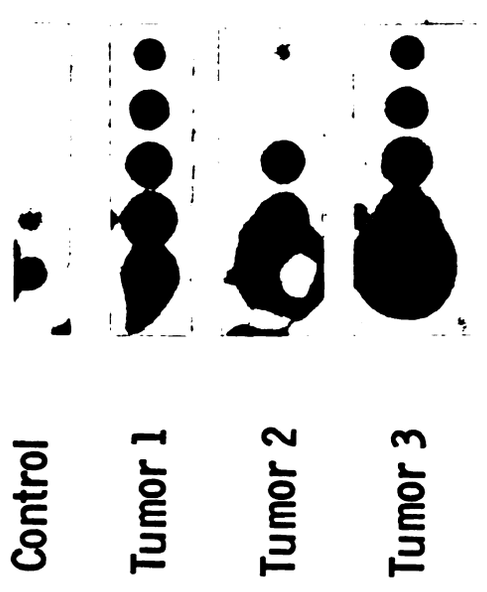
acquired only one copy of a CSV provirus, tumor number 15 appears to carry approximately eight copies of the proviral sequences.

Enhanced Expression of c-myc.

The structural analysis of the tumor DNA showing the CSV-myc linkage implies that c-myc gene expression is elevated based on the results of Hayward et al.(1981) and Payne et al.(1982). To confirm the transcriptional activation of the c-myc gene by the CSV provirus, the RNA from tumors 1, 2, and 3 were examined by RNA dot blot. The results in Figure 14 show that c-myc expression is elevated in all 3 tumors when compared with controls. Densitometric tracing indicated that the amount of elevation is 100, 80, and 50 fold, respectively.

Enhanced Examination of Cmax.

Figure 14. Quantification of c-myc RNA from tumors. Dot blot analysis of RNA from bursal tumors and non-neoplastic bursal tissue. Total poly A(+) RNA was isolated from control tissue and tumor 1, 2 and 3(see Figure 12). RNA was quantitiated by UV adsorbtion at 260 nm. An estimate of the level of c-myc RNA in the tumors was determined by spotting 2, 5, 10, 20, or 40 ug of RNA on to nitrocellulose, hybridizing with the c-myc probe, washing the filter, exposing to x-ray film and comparing the intensities of the hybridization signal to the control by densiometric scanning.


40 20 10 5 2 ug
Control
Tumor 1
Tumor 2
Tumor 3

DISCUSSION

Recent studies by Hayward et al.(1981) strongly implicate the activation of the c-myc gene in the bursal lymphomas induced by ALV infection. In this study we wished to determine whether CSV, which induces a lymphoid leukosis similar to ALV, was also integrated near the c-myc gene. Our data shows that in over 90% of the tumors examined, 28 of 30 tumors, c-myc is linked to CSV proviral sequence. These results are consistent with results from Hayward et al.(1981) and support the authors suggestion that the insertion of a provirus near c-myc is instrumental in altering the expression of the cellular gene, thereby triggering the oncogenic transformation.

The fact that CSV and RAV-1 share very little sequence homology, including their LTRs, and yet they are both found integrated near the c-myc gene in lymphoid leukosis strongly implicates this gene in the transformation of lymphocytes. In addition, our RNA dot hybridizations indicate that CSV is able to increase the transcriptional activity of c-myc as much as 100 fold, supporting the proposal that elevated

levels of c-myc gene product may trigger the progression to neoplasia.

It is very interesting that the deletions of proviral sequence observed in the CSV induced lymphomas is strikingly similar to those observed in RAV-1 induced lymphoid leukosis. These results imply that deletion is important for some aspect of the transformation process. It is possible that deletions of the viral genome that disrupt the transcriptional program of viral RNA facilitate the transcription of the downstream cellular sequences. Perhaps the transcription of viral RNA from the left LTR extending into the right LTR may affect the initiation at the right LTR. A disruption of the transcriptional program caused by a deletion in the proviral DNA may expose the right LTR and allow efficient transcription of the downstream cellular oncogene.

Alternatively, the deletion of viral sequences may play a role in the selective growth of the tumor clones. Those cells in which the expression of viral antigens is eliminated by deletion may therefore be rendered less immunogenic and able to escape the host immune response. Histopathological examination shows that at onset of the disease, there are many microscopically observed enlarged bursal follicles(considered to be transformed cells) (Cooper et al., 1968;Neiman et al., 1980). Immune selection may account for the finding that only a limited number develop into tumors.

The possiblity that gene amplification may relate to the generation and/or progression of neoplasia is supported by a variety of findings (Pall et al., 1982;Varshavsky et al., 1982). Chromosomal aberrations, including double minute chromosomes, 'homogeneously staining regions' and trisomy have been found in a number of tumors and tumor cell lines (Klein 1981). In some cases the cell lines have been shown to contain amplified oncogenes, including c-myc, c-abl and c-Ki-ras (Schwab et al., 1983). In most virally and non-virally induced murine T-cell leukemias trisomy of chromosome 15 is often the only chromosomal abnormality detected (Dofuku et al., 1975).

Our results indicating that c-myc is amplified in tumor no. 15 may point to another mechanism whereby a non-acute transforming virus can induce neoplasia. However, we have examined 30 tumors for CSV-myc linkage, in only two tumors have we observed amplification of the c-myc gene. These results indicate that if amplification is involved in a mechanism for c-myc activation for certain bursal lyphomas, it is relatively rare.

We have shown that c-myc is linked to proviral CSV sequence in over 90% of the bursal tumors examined and the majority of CSV provirus have undergone some structural alteration. In addition, the RNA dot blots indicate the enhanced expression of c-myc in these CSV induced tumors. Presently, we do not know the exact insertion site nor the orientation of the provirus. Further analysis by

restriction enzyme digestion and northern blot analysis should tell us whether the elevated expression of c-myc is consistent with promotor-insertion, enhancer insertion or perhaps some other molecular mechanism of oncogene activation. Also, it is not clear how the structural alterations in the provirus contribute to the transformation process nor what the exact nature of the alterations are, but detailed structural analysis of the molecularly cloned TS bands should provide insight into the molecular mechanism of the alteration and its possible function in the transformation process.

CHAPTER III

DETERMINATION OF TRANSCRIPTIONAL ORIENTATION AND SITE OF INTEGRATION OF CSV RELATIVE TO C-MYC

The mechanisms by which ALV activates the c-myc gene in B-lymphomas have been extensively characterized (Hayward et al., 1981;Payne et al., 1982;Fung et al., 1982;Westway et al., 1984). Although important exceptions have been noted (Payne et al., 1982;Pachl et al., 1983), ALV proviruses usually integrate upstream of the c-myc coding exons and are arranged in the same transcriptional direction, enabling the provirus to utilize its 3' LTR promoter to transcribe and deregulate the downstream c-myc gene (Hayward et al., 1981;Payne et al., 1982;Fung et al., 1982). We have previously shown that in CSV induced B-lymphomas, CSV proviruses and the c-myc gene are physically linked (Noori-Daloii et al., 1981). The arrangement and insertion sites of CSV proviruses however were not analyzed in detail.

Here we describe our analyses of the provirus-c-myc linkage structure in twenty-two CSV induced lymphomas. Our results indicate that the integration pattern of CSV proviruses show a striking similarity to those observed for ALV proviruses involved in c-myc activations.

MATERIALS AND METHODS

Induction of Lymphoid Leukosis.

Day-old chicks from line $15I_5x7_1$(Regional Poultry Research Laboratory) were inoculated and housed as described in Chapter II.

DNA Extraction and Enzyme Digestion.

The isolation of DNA from tissue and digestion with restriction endonuleases was as described in Chapter II.

Hybridization and Nick-Translation.

Conditions for hybridization and nick-translation were as described in Chapter II. The SNV-LTR probe, derived from the SNV proviral clone, was subcloned into the Sac I-Bam HI sites of pDH24. The myc5 and myc3 probes were derived from gel purified Sma I-Sac I and Cla I-Eco RI fragments from a chicken c-myc clone, gift from Dr. T. Robbins.

Re-hybridization of Filters.

The conditions for washing off hybridized probe were the same as described in Chapter II.

Induction of Lysosomal Hydrolases.

Hybridization and Nick-Translation.

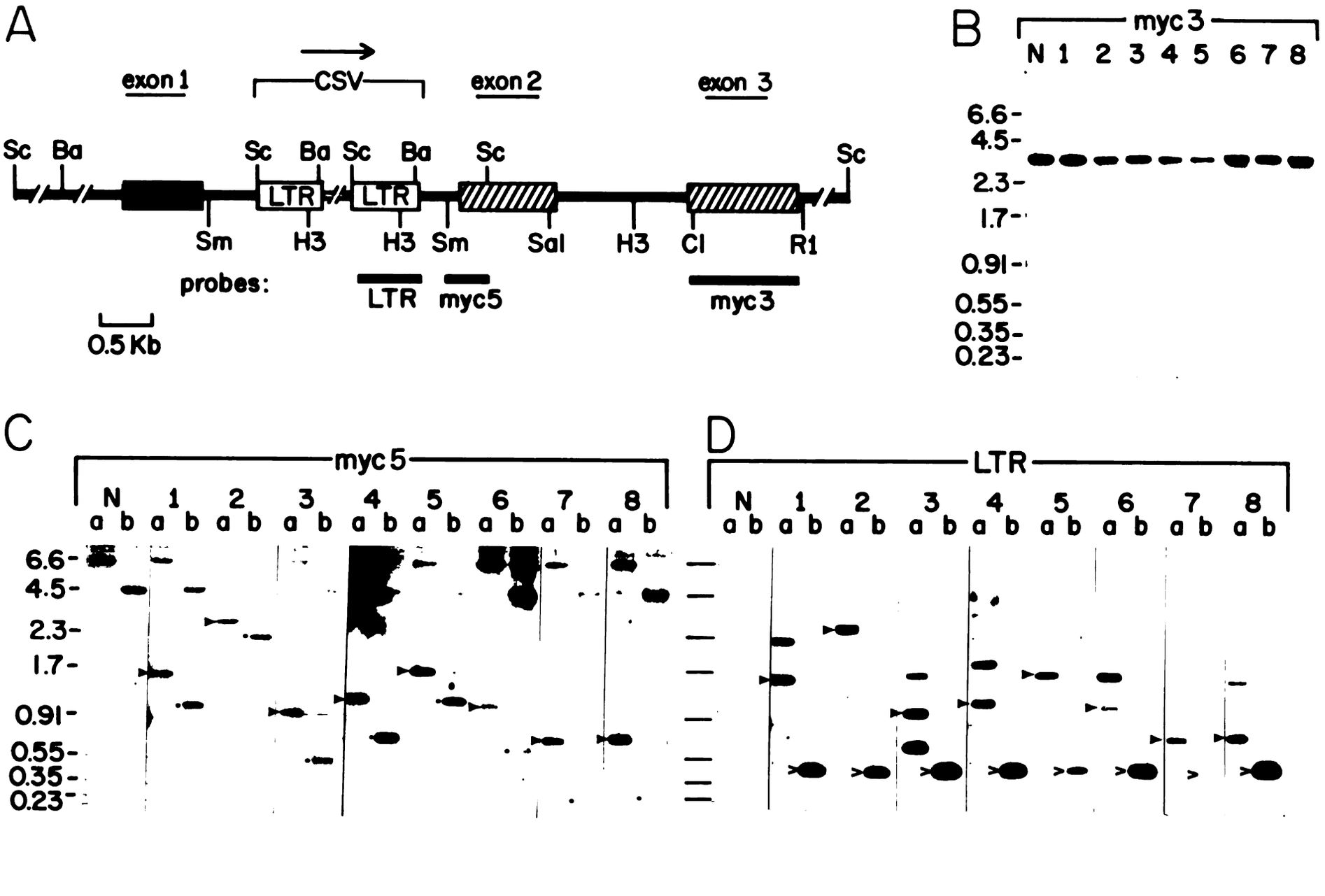
Re-hybridization of Filters.

RESULTS

The chicken c-myc gene, like its mammalian counterpart, has three exons; only the second and the third exons contain protein-coding sequences. As indicated before, the majority of the ALV proviruses in B-lymphomas are situated upstream from the second c-myc exon and are in the same transcriptional direction as the c-myc gene. Figure 15A shows what would be predicted if the CSV provirus also inserts in a similar manner. To facilitate the mapping of the proviral integration sites in a large number of samples we adopted the following strategy, relying principally on Sac I and Bam HI digestions. We took advantage of the fact that a Sac I site cleaves near the 5' end the second c-myc exon, thereby dividing the c-myc locus into a 5' and a 3' region (Watson et al., 1983), and we prepared probes (myc5 and myc3, Figure 14) specific for each region. The CSV LTR contains a Sac I and a Bam HI site near its 5' and 3' boundaries, respectively. If the diagram in Figure 15A

Figure 15. Position and orientation of the CSV proviruses in the c-myc locus. (A). A schematic diagram of a CSV provirus inserted in the first intron and oriented in the same transcriptional direction as the c-myc gene. The positions of the c-myc exons and the restriction enzyme map of the c-myc are according to Shih et al.(1984) and Watson et. al.(1983) The map of the CSV LTR was determined by single and double digestion of a cloned CSV provirus (Swift et al. in preparation) and is very similar to the published data of the closely related spleen necrosis virus LTR (Shimotohno et al., 1980). Sc=Sac I, Ba=Bam HI, H3=Hind III,Sal=Sal I. The restriction fragments from which the probes were derived are as indicated.

(B)-(D). Tumor or normal(N) control DNA were digested with restriction enzymes, and hybridized to the probes shown on the top of each panel. Samples in (B), and lanes in a of (C) and (D) were digested with Sac I. Samples in lanes b of (C) and (D) were digested with both Sac I and Bam HI. The DNA was extracted from discrete tumor nodules and analyzed by agarose gel electrophoresis and Southern-blot hybridization, as previously described (Fung et al., 1982). The normal control DNA was isolated from the bursa of an uninfected line $15I_5x7_1$ bird and treatedly exactly the same way. The molecular size markers (in kb) were based on Hind III digested lambda and Hind III/Bam HI/Bgl I digested pAT 153 DNA included in the same gel. Usually, the normal c-myc bands are more intense than the tumor specific bands (marked by or dots), due to the presence of some normal tissues in the tumor nodules; in some cases (e.g. tumors 2 and 3), the reverse is true. These presumably are the result of the preferential loss of the chromosome carrying the normal c-myc allele in tumor cells, a phenomenon previously described in other chicken B-lymphomas (Payne et al., 1982). Occasionally, extra weakly hybridizing bands can be seen in Sac digested DNA, hybridized to the myc5 probe. The exact nature of these bands are not clear, but they are likely due to the presence of repetitive sequences in the intron portion of the probe, since no such bands were detected when v-myc was used as a probe (data not shown).


A
exon 1
CSV
exon 2
exon 3
Sc Ba Sc Ba Sc Ba Sc Sc
LTR
LTR
Sm H3 H3 Sm Sal H3 Cl R1
probes:
LTR
myc5
myc3
0.5 Kb
B
myc 3
N 1 2 3 4 5 6 7 8
6.6-
4.5-
2.3-
1.7-
0.91-
0.55-
0.35-
0.23-
C
myc 5
N 1 2 3 4 5 6 7 8
a b a b a b a b a b a b a b a b a b a b
6.6-
4.5-
2.3-
1.7-
0.91-
0.55-
0.35-
0.23-
D
LTR
N 1 2 3 4 5 6 7 8
a b a b a b a b a b a b a b a b a b a b

represents the CSV/c-myc junction structure, then Sac I digestion should yield the following results. One, the 5' Sac fragment of c-myc should be shortened when compared to the normal control, due to the presence of the Sac I site in the viral LTR, but the 3' Sac fragment of c-myc should not be affected. Two, the LTR probe and the myc5 probe should cohybridize to the shortened 5' Sac I fragment. As shown in Figure 15C to 15D, the predicted results are exactly what we observed. In these experiments, Sac I digested tumor and control DNA were fractionated by electrophoresis in 0.85% agarose gels, immobilized on nitrocellulose paper and hybridized with the myc5 probe (lanes a of Figure 15C). The normal control (C) DNA displays a 6.8 kb band, corresponding to the normal c-myc locus. Each tumor sample carries an additional shortened c-myc band (indicated by). In contrast, none of the tumors showed any evidence of alteration when hybridized to the myc3 probe (Figure 15). This indicates that the proviral insertions occur 5' to the Sac I site in the second c-myc exon.

Hybridization to the LTR probe(lanes a in Figure 15D) reveals the linkage between LTR and c-myc. In each tumor sample, at least one of the bands (marked by) detectable with the LTR probe comigrates with the altered c-myc band shown in lanes a of Figure 15C. The other LTR-containing bands presumbly represent the junction fragments from the other end of the individual proviruses or from other proviruses present in the same tumor DNA. This analysis also defines the transcriptional orientation of the

DNA were fractionated by electrophoresis in 0.7%
agarose gels, transferred
hybridized with the myc1 probe (
The
corner) relative to the normal c-myc locus. Both
). In contrast, none of the tumors showed any
with the PvuII probe (bottom panel). In addition to the
myc probe (figure 1).

proviruses as the same as that of c-myc. If the provirus and c-myc were oriented in opposite transcriptional directions, then regions to which the LTR and myc5 probes hybridize would be unlinked by Sac I digestion and no cohybridization would have been detected. To further substantiate the conclusion that the LTR-c-myc linkage structure shown in Figure 15A is correct, tumor DNA was doubled-digested with Sac I and Bam HI, and hybridized to the myc5 probe (lanes b in Figure 15C), the resulting altered c-myc band(indicated by) was found to be approximately 500 bp shorter than the band in Figure 15C (lanes a) in all cases. In addition, the fragments in lanes b of Figure 15C, in general, did not comigrate with those in lanes b of Figure 15D, when the LTR probe was used as the hybridization probe. The LTR probe uniformly detects a ca. 500 bp fragment(indicated by). We anticipated this result since Sac I and Bam HI double digestion of the DNA should liberate the 500 bp fragment from which the LTR probe was originally derived. Based on all the criteria discussed above, we conclude that the proviruses have the structure depicted in Figure 15A. This type of analysis was extended to a total of 22 tumor DNA samples. With no exception, all tumor samples displayed digestion patterns similar to the eight representative samples illustrated, suggesting that the diagram in Figure 15A indeed represents the predominant configuration of the CSV proviruses involved in c-myc activation. This conclusion has received additional support

from detailed mapping and sequencing of a genomic clone (from tumor 4) carrying the entire activated c-myc gene and the inserted provirus (Swift et al., in preparation).

Knowing that all the proviruses are oriented in the same way as the c-myc gene, we could then use the sizes of the junction fragments to accurately locate the individual proviral insertion sites. The Sac I-Bam HI junction fragments (lanes b Figure 15C) are especially useful for this purpose, since they are usually smaller than 700 bp and readily resolved in the gel. The data summarized in Figure 16 shows that most of the insertion sites are located within 500 bp immediately 5' to the second exon of c-myc, five of the proviruses reside within the first exon and two are located further upstream. However, none are located in the 5' one third of the first intron.

is shows that most of the transition mitochondria located within

ppG DP immediately 5' to the second exon of cwnH, like a

the chromosomes reside within the first exon and two are

located further upstream. However, none are located in the

5' and third of the first intron.

The Montana Territorial Penitentiary was established by the Montana Legislature in 1867 and was located in Deer Lodge. The first prisoners were received in 1871. The prison was built of stone quarried nearby and was designed to hold 200 inmates. By the 1880s, overcrowding was a serious problem. In 1912, a new prison was built in Deer Lodge, and the old territorial prison was abandoned. The new prison was also built of stone and was designed to hold 400 inmates. It was expanded several times over the years and is still in use today.

Figure 16. **The nonrandom distribution of CSV proviral insertion sites in the c-myc locus of avian bursal tumors.**
The upper diagram shows the CSV proviral insertion sites as determined by the sizes of Bam-Sac junction fragments(see text).

0.2Kb
exon 2
exon 1
2
22
20
1
21
5
4
6
17, 18, 19
3,16
15
14
13
8,12
7
9, 10, 11

DISCUSSION

It is Interesting that the pattern of integration observed for the CSV proviruses is identical to that observed for the ALV proviruses found in the B-lymphomas induced by ALV (Shih et al., 1984). Specifically, most of the ALV proviruses are concentrated in a region 500 bp preceding the second c-myc exon and a few are scattered within the first exon. The 5' one third region of the first intron of c-myc which is devoid of CSV proviruses is also silent for ALV proviral integration. Intuitively, the insertion of a viral promoter between the natural promoter and the coding sequence of the resident gene should render the resident gene under viral control.

One would anticipate, in the c-myc case, that the viral insertion sites would be distributed along the entire region between the start of the first and second exon. This is apparently not the case in both CSV and ALV induced B-lymphomas. One possibility is that the nonrandom

Discussion

distribution of the proviruses may be a consequence of integration specificity of the viruses. However, given the dissimilarity of ALV and CSV, it is unlikely that such a specifity is based on the primary sequences of the target sites. (The terminal nucleotides, implicated in integration of CSV and ALV are very different and the viral enzymes involved in the cleavage of junction also have different recognition specificities (Shimotohno et al., 1980;Swanstrom et al., 1981)). Furthermore, the fact that CSV and ALV both have multiple insertion sites in the clustered region also argues against a strict sequence requirement for integration. Though, the local DNA structure and chromatin conformation may have a profound effect on the proviral insertion frequency. As pointed out by Shih et al.(1984), the intron region contains many complementry sequences that may form dyad symmetries. However, computer sequence analysis (Staden 1984;Swift et al., 1985) of the intron does not reveal a correlation between dyad symmetries (Staden 1984;Tinoco et al., 1973) and proviral integration sites.

There are two possiblites for the lack of integration in the 5' portion of the first exon. First, it may be that the chromatin in this region is inaccessable to the provirus during integration. Second, perhaps integration in this region may not result in cellular transformation and the formation of a tumor. The first possiblity could be mediated by a lack of specific viral integration recognition sequences in this region of the DNA. However, as mentioned above, sequence specificity during integration is not likely

to be the answer. It is also possible that the chromatin in this region adopts a structural organization, such that the proteins necessary for viral integration are unable to gain access to the host DNA.

It may be that there is some DNA sequence at the 3' end of the silent region that affects post-transcriptional processing, mRNA stablity, translation or perhaps all of the above. An example of what might cause translational inhibition is translation initiation codons in the 5' untranslated region of the mRNA. Fewer then 10% of over 200 eukaryotic mRNAs examined have ATGs in the 5' untranslated region (Kozak 1983). Recent reports demonstrate that the presence of ATGs 5'-proximal to the authentic initiation codon results in a dramatic decrease in the amount of protein synthesized (Kozak 1984;Lui et al., 1984). We find four ATGs positioned from 575 to 515 bp 5'-proximal to the second exon of c-myc, that may interfere with the translation of the c-myc gene. This location demarcates the the approximate 3' boundary of the silent region. In this regard, it is interesting that the 5' end of the silent region is bounded by a consensus splice donor site (Mount 1982), presumably used to join the first and second exon of c-myc during post-transciptional processing (Shih et al., 1984). Proviral integrations 5' to this splice donor site would allow the removal of the initiation codons during post-transcriptional processing.

The several possibilities discussed above are not mutually exclusive and more than one factor is likely to be involved in defining the activation scheme. In this regard, it is interesting to note that the situation for c-myc activation in mouse or rat lymphomas induced by murine non-acute retroviruses are rather different. In these instances, the proviruses are exclusively found to be upstream from the first c-myc exon and are arranged in the opposite orientation (Tsichlis et al., 1983;Nusse et al., 1984). Thus, enhancement, rather than promoter insertion appears to be involved in c-myc activation non-acute murine retroviruses.

In summary, the data presented in this communication show that despite its completely different origin, CSV uses a mechanism very similar to that of ALV to activate c-myc gene causing B-lymphomagenesis. The majority of CSV proviruses are arranged in a configuration that allows the use of the viral promoter for c-myc transcription, resulting in enhanced expression of a truncated c-myc message with little or no sequences derived from the first exon.

The several [illegible]
[illegible] likely to [illegible]
involved in defining the [illegible] In this regard,
it is interesting [illegible] that the activation [illegible]
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In general, the proviruses are exclusively found to be
[illegible]
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retroviruses.

In summary, [illegible] completely different origin. Cav uses a mechanism very similar to that of ALV to activate c-myc and causing B-lymphomagenesis. [illegible] majority of Cav proviruses are [illegible] in a configuration [illegible] use of [illegible] promoter for c-myc transcription, resulting in enhanced expression of a truncated c-myc [illegible] sequences derived from the first exon.

CHAPTER IV

TRANSCRIPTIONAL AND STRUCTURAL ANALYSIS OF AN ACTIVATED C-MYC GENE

We reported previously that the CSV provirus integrates near the c-myc locus in CSV-induced chicken B-cell lymphomas (Noori-Daloii et al., 1981). Based on Southern analysis, the majority of the proviruses have large deletions, are located in the first intron of the c-myc locus and oriented in the same transcriptional direction as the c-myc gene (Swift et al., 1985). In order to confirm and extend these findings, we wished to isolate from tumor DNA clones containing the CSV provirus and the perturbed c-myc allele to analyze its structural features by restriction enzyme mapping and DNA sequencing.

MATERIALS AND METHODS

Induction of Lymphoid Leukosis.

Day-old chicks from line $15I_5x7_1$(Regional Poultry Research Laboratory) were inoculated and housed as described in Chapter II.

DNA Extraction and Enzyme Digestion.

The isolation of DNA from tissue and digestion with restriction endonuleases was as described in Chapter II.

Hybridization and Nick-Translation.

Conditions for hybridization and nick-translation were as described in Chapter II. The SNV-LTR probe, derived from the SNV proviral clone, was subcloned into the Sac I-Bam HI sites of pDH24. The myc5 and myc3 probes were derived from gel purified Sma I-Sac I and Cla I-Eco RI fragments from a chicken c-myc clone, gift from Dr. T. Robbins.

Re-hybridization of Filters.

The conditions for washing off hybridized probe were the same as described in Chapter II.

Induction of Lymphoid Leukemia

DNA Extraction and Enzyme Digestion.

Hybridization and Nick-Translation.

Re-hybridization of Filters.

Construction of a Genomic Library of Tumor 713.

A library of tumor 713 genomic DNA was contructed according to methods described by Maniatis et al.(1982). High molecular weight DNA from tumor 713 was prepared (see Chapter II) and partially digested with Mbo I. The DNA was fractionated on a 10-40% sucrose gradient (Maniatis et al., 1982), fragments of 16-22 kb were pooled and ligated into the Bam HI site of phage vector BF101. Recombinant molecules were packaged in-vitro and screened for inserts homologous to both myc3 and LTR probes by in-situ hybridization (Benton and Davis 1977).

DNA Sequencing.

DNA sequencing was carried out according to the method of Maxam and Gilbert (1980). A subclone of the 5' LTR and the INT region were derived from the 2.0 kb viral Sac-Sac fragment of clone 713 after digestion with Bam HI and ligation to Bam-Sac digested pDH24. A subclone of the 3' LTR and the 3' viral junction region were derived from the 2.2 kb Sac-Sac viral-myc junction fragment of clone 713 after digestion with Bam HI and ligation to Bam-Sac digested pDH24. The subcloned DNAs were digested with either Eco RI, this site is in pDH24 adjacent to the Sac I site, or Bam HI, treated with calf intestinal phosphatase, end labeled by T4 polynucleotide kinase and digested with either Bam HI or Sac I. The fragments were gel isolated and sequenced.

Southern blot.

As described in Chapter II.

Construction of a Genomic Library of Tissue 713.

A library

[illegible] was used by Dr. [illegible].

[illegible] from tumor 713 was [illegible] (see

[illegible] was [illegible] by the DNA was

transferred [illegible] Maniatis et al.,

[illegible] 22 kb were pooled and ligated to

[illegible] Sam 81 site of Lambda Charon 21A [illegible]

were packaged and titred by [illegible] and

and [illegible] 1974).

DNA Sequencing.

[illegible] was carried out according to the method

of Maxam and Gilbert (1980). A restriction of the 81 DTE and

the INT region were removed from the [illegible] vector

fragment of clone 713 after digestion with Bam HI and

ligation to Bam-cut [illegible] pUC21. A restriction [illegible]

and the [illegible] viral insertion region were derived from the [illegible]

Sac-Sma viral [illegible] fusion fragment of clone 713 after

digestion with Sma II and [illegible] to Bam-Sac digested

pUC24. The subclones DNA were digested with either Sac II,

this site is in pUC21 adjacent to the Sac I site, or Sma II,

treated with calf intestinal phosphatase, and [illegible]

polynucleotide kinase and digested with either Sac II or

[illegible]. The fragments were gel isolated and sequenced.

Southern blot.

As described in Chapter II.

RNA Isolation.

As described in Chapter II.

Northern blot.

RNA blot analysis was performed on poly(A) selected RNA (Aviv and Leder 1972). Samples and DNA markers were denatured for 25' at 60°C in 25mM MOPS pH 7.0/1mm EDTA/2.2M formaldehyde/50% formamide. The RNA samples and markers were fractionated by electrophoresis in a 1.5% agarose gel with 2.2 M formaldehyde (Maniatis et al., 1982). Conditions for transfer and hybridization were similar to those used for DNA, except hybridization was carried out at 45°C and washing was at 55°C.

RNA Isolation.

A

Northern blot.

RNA

and Laser

EQUAL

those used for [illegible] was carried out at

RESULTS

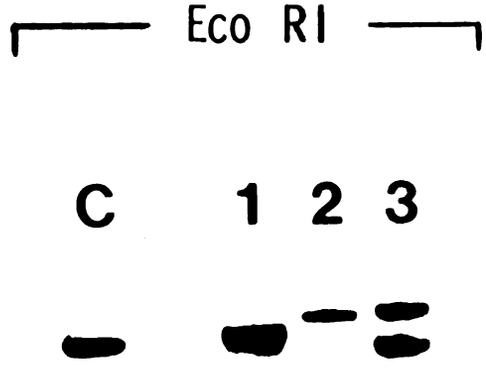
We have previously used Eco RI, a restriction enzyme which cleaves neither the CSV provirus nor the c-myc exons, to screen for CSV DNA insertion near the c-myc locus (Noori-Daloii et al., 1981). Figure 17 shows the Eco RI digestion analysis of three representive tumor DNA samples and one non-neoplastic control(C) hybridized with v-myc probe. In all lanes, including the control lane, a 13.5 kb fragment corresponding to the normal allele is seen. However, in the tumor lanes is an additional band, of varying size, but larger than the normal c-myc band. Since Eco RI does not cleave in the CSV provirus it is unlikely that the altered c-myc bands of varying mobilities can be attributed to differences in proviral insertion sites; rather, they are likely consequences of proviral or cellular DNA deletions. The c-myc band in tumor 713(Figure 17, lane 1) is only 16 kb, implying there is a deletion of ca. 6 kb of viral or cellular sequences.

Figure 17. [illegible] of the [illegible] c-myc gene in [illegible]

Eco RI digestion. Hybridization to the chicken c-myc probe. The control [illegible] is from a chicken free of ALV [illegible] normal c-myc bands are more intense than the tumor specific bands due to the presence of [illegible] tissues in the tumor nodules; in some cases (e.g. tumor 2) the reverse is true. Presumably this is the result of the pre[illegible] loss of the chromosome carrying the normal c-myc allele in tumor cells, a phenomenon previously described in other chicken lymphomas (Payne et al., [illegible]).

Figure 17. Structure of the chicken c-myc gene in B-cell lymphoma as analyzed by Eco RI digestion. Hybridization to the chicken v-myc probe. The control lane(C) is from a chicken free of REV infection. Usually, the normal c-myc bands are more intense than the tumor specific bands due to the presence of some normal tissues in the tumor nodules; in some cases (e.g. tumor 2), the reverse is true. Presumably, this is the result of the preferential loss of the chromosome carrying the normal c-myc allele in tumor cells, a phenomenon previously described in other chicken B-lymphomas (Payne et al., 1982).

Tumor DNA Used for Genomic Libraries --- Hybridized to v-myc



Cloning and mapping of the activated c-myc gene in tumor 713.

To ensure that the c-myc alteration is due to CSV proviral insertion, Eco RI digested 713 DNA was hybridized to SNV probe (data not shown). As expected, the SNV probe cohybridized to the altered 16 kb c-myc band indicating that the CSV proviral sequences are contained in the same 16 kb fragment. The fact that no other CSV-related sequences are detected indicated that the c-myc linked CSV DNA is the only provirus present in this tumor.

We have isolated the perturbed c-myc allele from a genomic library of tumor 713 DNA. The genomic library was constructed from 713 tumor DNA partially digested with Mbo I and ligated to phage vector BF101 (Mantiatis et al., 1982). The CSV linked c-myc clone was selected from the library by its ability to simultaneously hybridize to myc and REV probes. The map of this clone, lambda 713 (Figure 18), is consistent with the presence of a complete c-myc gene with all three exons intact, but between the first and second exon, a deleted CSV provirus has been inserted. Southern hybridizations with LTR probe and probes representing the first(myc1) and second(myc2) exons confirm the linkage of CSV to c-myc(data not shown). For example, Bam HI digestion yields three fragments with respective sizes of 2, 6.3, and ca. 10 kb. The 2 and 10 kb fragments are detectable by the LTR probe and the 6.3 kb fragment is detectable by myc2 probe. The 10 kb fragment is also detectable by myc1 probe,

tumor II.

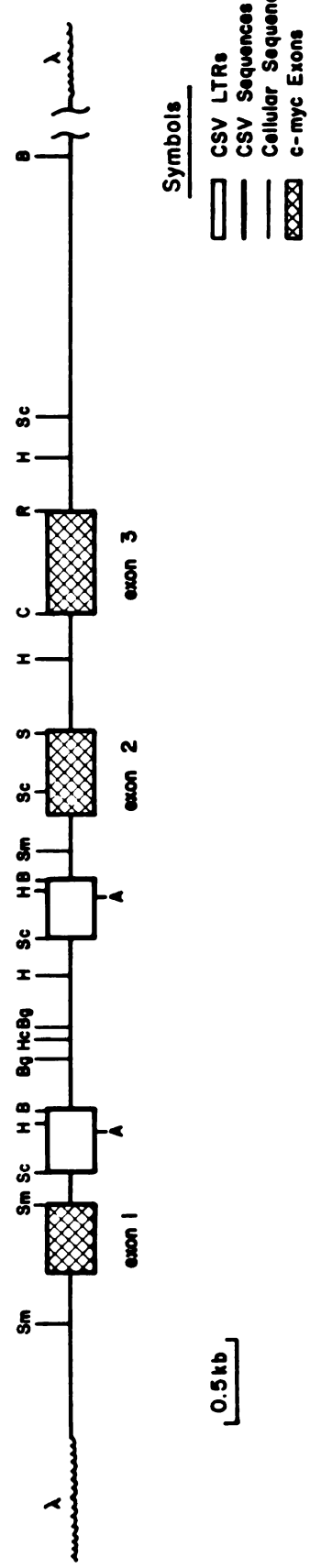
[illegible] to the [illegible] Court

[illegible]

[illegible]

[illegible]

Figure 18. Restriction endonuclease cleavage map of phage clone 713. Molecular clones that hybridized to both myc3 and LTR probes were obtained from a recombinant phage library that was constructed with tumor DNA from the bursa of chicken 10713. Fragments containing sequence homologous to c-myc or CSV were identified by Southern blot analysis with subclones of c-myc and REV probes. The cross-hatched boxes represent the three c-myc exons of chicken. The open boxes represent the LTR's of CSV. The left and right lambda arms adjacent to the insert are indicated. A=Ava I; B=Bam HI; Bg=Bgl I; C=Cla I; H=Hind III; Hc=Hinc II; R=Eco RI; S=Sal I; Sc=Sac I; Sm=Sma I.


λ
Sm
Sm Sc
H B
Bg Hc Bg
H
Sc
H B
A
Sm
H B
A
Sc
S
Sc
H
C
R
H
Sc
B
λ
exon 1
exon 2
exon 3
0.5 kb
Symbols
CSV LTRs
CSV Sequences
Cellular Sequences
c-myc Exons

thereby defining the 5' junction fragment. The 2 kb band represents the internal portion of the provirus and is only detected by LTR probe. The 6.3 kb fragment is only detected by myc2 probe, defining the 3' junction fragment. Sac I digestion yields two fragments detectable by LTR; the 2 kb internal fragment and the 1.65 kb 3' junction fragment which can be detected by both the myc2 and the LTR probe. This and other data are consistent with the physical map shown in Figure 18.

Sequencing analysis of 713 clone.

The mapping data described above suggests that the provirus has a large internal deletion, but retains two intact LTR's. The latter finding is in contrast to the structures of several ALV provirus clones isolated from chicken B-lymphomas, where the deletion usually extends to or beyond the 5' LTR (Payne et al., 1982;Pachl et al., 1983). To ascertain the intactness of both LTR's and the nature of the large internal deletion, we have determined the DNA sequence of the relevant portions of the proviral clone.

We have divided our sequencing results into four regions: 5' LTR, 5V (5' viral sequence), 3V (3' viral sequence), and 3' LTR. Using the appropriate subclones derived from the 5' and 3' LTR's, we have determined the entire sequence of the 3' LTR and all but the first seven nucleotides of the 5' LTR. The data shows that the two LTR's are identical to each other. The 3' LTR is also

identical to a CSV provirus LTR isolated from the DNA of a producer cell line(data not shown). This strongly suggests that the 713 provirus indeed contains two intact and potentially transcriptionally active LTRs.

The CSV LTR sequence (Figure 19) is 510 bp long. The U3 region is ca. 334 bp long, the terminal repeat region(R) is 76 bp and the U5 region is ca. 100 bp, based on a comparison of the U3, R and U5 regions to the SNV LTR (Shimotohno et al., 1980). The CSV LTR has the same regulatory features that are characteristic of most retrovirus LTR's (Varmus 1983). A CCAT box is present at a position 75 bp upstream of the RNA cap site and 23 bp upstream of the cap site is a TATA box. The signal sequence usually associated with eukaryotic polyadenylation, AATAAA, is located 24 bp upstream of the R-U5 junction.

A comparison of the published sequence of the SNV LTR (Shimotohno et al., 1980) to the sequence of the CSV LTR reveals several interesting features. A variability in the size of the U3 region of the viral LTRs of different recombinant clones of SNV provirus has been reported (Shimotohno and Temin 1982). In each case a deletion of one of two duplication elements consisting of a 46 bp and 26 bp unit, each is bounded by smaller penta or hexa-nucleotide repeats is observed to account for the variation. In the CSV clones analyzed here, only one copy of each repeat is present. While the 46 bp repeat in CSV is almost identical to that of SNV, the sequences of the 26 bp repeat diverge

Figure 19. Comparison of the nucleotide sequences in the LTRs of CSV and SNV. Small repeats and significant regulatory sequences are in captial letters (see text). Where the DNA sequences are indentical between CSV and SNV a period(.) is substituted for the proper letter. Deletions are indicated by hyphens(-).

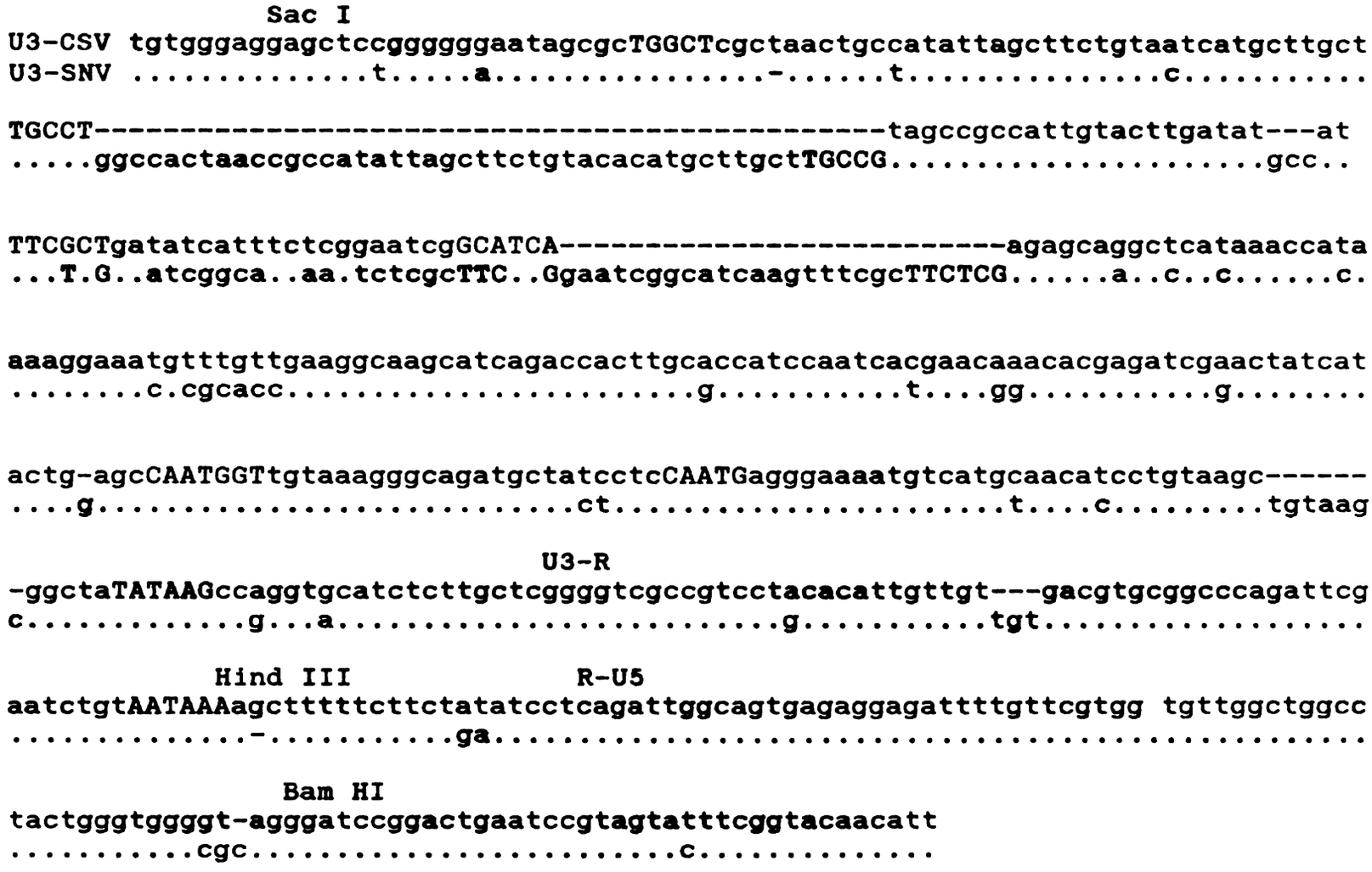

Sac I
U3-CSV tgtgggaggagctccgggggaatagcgcTGGCTcgctaactgccatattagcttctgtaatcatgcttgct
U3-SNV .....t.....a.....-.....t.....c.....
TGCCT----------tagccgccattgtacttgatat---at
.....ggccactaaccgccatattagcttctgtacacatgcttgctTGCCG.....gcc..
TTCGCTgatatcatttctcggaatcgGCATCA----------agagcaggctcataaaccata
...T.G..atcggca..aa.tctcgcTTC..GgaatcggcatcaagtttcgcTTCTCG.....a..c..c.....c.
aaaggaaatgtttgttgaaggcaagcatcagaccacttgcaccatccaatcacgaacaaacacgagatcgaactatcat
.....c.cgcacc.....g.....t....gg.....g.....
actg-agcCAATGGTtgtaaagggcagatgctatcctcCAATGagggaaaatgtcatgcaacatcctgtaagc------
....g.....ct.....t....c.....tgtaag
U3-R
-ggctaTATAAGccaggtgcatctcttgctcggggtcgccgtcctacacattgttgt---gacgtgcggcccagattcg
c.....g...a.....g.....tgt.....
Hind III R-U5
aatctgtAATAAAagctttttcttctatatcctcagattggcagtgagaggagatttgttcgtgg tgttggctggcc
.....-.....ga.....
Bam HI
tactgggtggggt-agggatccggactgaatccgtagtatttcggtacaacatt
.....cgc.....c.....

Figure 19

significantly. In fact, it accounts for 40% of the divergent sequences between the CSV and SNV LTRs, not counting deletions. Although the functions of the 46 and 26 bp repeats are not clear, they map in to the general area where retroviral enhancer sequences usually reside (Dhar et al., 1980;Schwartz et al., 1983;Hampe et al., 1983). The 72 bp repeat of Mo-MSV is in a similar location to the SNV and CSV repeats. Since the two repeats, 46 and 26 bp, add up to 72 and they are in a region where viral enhancers have been localized, a possible analogy might be made to the Moloney murine sarcoma virus (Dhar et al., 1980) which contains two 72 bp repeats that can act as a transcriptional enhancer.

The restriction map of the 713 provirus already suggests that the deletion break point probably lies very close to the 5' end, since the 5' Sac I and Sal I sites are both missing in the clone. Sequence analysis reveals that the breakpoint is only 18 nucleotides away from the 5' LTR(data not shown). The 18 nucleotides correspond to the $tRNA^{pro}$ primer binding site(PBS), but the sequences after that are completely different from the published leader and gag sequences of SNV (O'Rear and Temin 1982). We presume this stretch of sequences belongs to the pol or env genes, however, there is no available sequencing data from either CSV or related REV-A and SNV to confirm this. Nevertheless, it is clear that deletion occurs rather early, since the leader sequence, the packaging signal and the splice donor site for env gene are all lost, making this defective

geneome incapable of synthesizing properly processed and packagable transcripts. Interestingly, a deletion that begins in approximately the same place was observed in an ALV provirus integrated upstream from c-myc (Westway et al., 1984). The 3'V sequences match closely with those in the corresponding region of the SNV DNA. It represents the carboxyl terminus of the env gene and the region between env and the 3' LTR. The overall homology in this region between CSV and SNV is ca. 95%. However, only 180 bp is available for comparison from the literature.

The 5' viral sequencing data suggests that the 713 provirus carries a large deletion and is likely to contain only a portion of the env sequences. Hybridization to cloned SNV DNA with a radio-labelled DNA fragment corresponding to the internal 2 kb sequence of the 713 provirus confirms that only the env sequences are present.

Expression of proviral and c-myc sequences in tumor 713.

Having obtained the structural information about the perturbed c-myc gene, we wished to examine the mode of its activation. The RNA expression of c-myc and viral related sequence were examined by northern blot hybridization using myc3, LTR, and INT(the internal viral region of 713, Figure 20). If the CSV provirus activates c-myc in a manner similar to ALV provirus, then we would predict the presence of a 2.3 kb transcript which initiates in the 3' LTR and contains the two c-myc exons (Hayward et al., 1981). We observe two myc transcripts in lane 1 (2.3 and 2.0 kb).

The 5' viral noncoding beta augmo by that the its

However, only two to

from the literature.

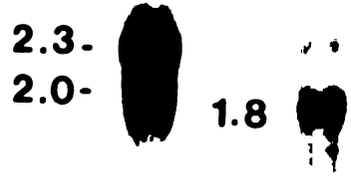
Having determined the structural information about the
, we wished to examine the mode of
. RNA
sequences were
Figure 2b). In the IRV, the internal virus region of
. If the IRV promotes activator entry in
to Any promoter, then we would
of a 3.3 kb transcript was initiated in the
minimize the two transcripts et al., . We
observe the two transcripts in time [illegible] for
. of the DNA. It is
carbonyl fluidity of the env gene and the peptide bonds on any
and . The model I hypothesis is this motion between
any and may is . However, only two to
.

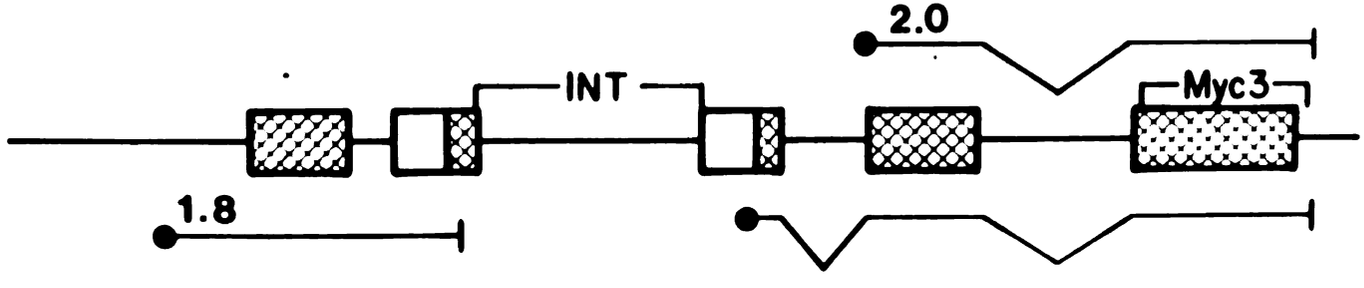
Figure 2. Northern blot of poly(A) selected RNA from tumor 113. 5 ug of poly(A) selected RNA was fractionated by horizontal electrophoresis in 1.5% agarose with 2.2 M formaldehyde. RNA was transferred to nitrocellulose filters and hybridized or re-hybridized to [illegible]

Figure 20. Northern blot of poly(A) selected RNA from tumor 713. 5 ug of poly(A) selected RNA was fractionated by horizonatal electrophoresis in 1.5% agarose with 2.2 M formaldehyde. RNA was transferred to nitrocellulose filters and hybridized or re-hybridized to probes: left) myc3; middle) LTR and right) INT.

RNA From Tumor 713

myc3 LTR INT





However, only the 2.3 kb transcript cohybridizes to the LTR probe (lane 2). These results indicate that the 2.3 kb transcript has LTR sequence covalently linked to the c-myc mRNA and therefore initiation for this transcript very likely begins at the viral promotor located in the U3 region of the LTR. This result is consistent with the promotor-insertion configuration of the 713 provirus.

Then what is the origin of the 2.0 kb myc transcript that does not contain LTR sequences? We believe that the 2.0 kb transcipt may initiate from a cryptic promotor located in the first intron of c-myc. There are many TATA sequences in this region, especially within the first 80 bp of the second exon (Shih et al., 1984). Based on the elevated level of expression, it is likely that the CSV enhancer sequences are influencing the transcription from a cryptic promotor.

We observe two LTR transcripts in lane 2, a 2.3 kb transcript and a 1.8 kb transcript. However, as mentioned above, only the 2.3 kb transcript hybridizes to the c-myc probe. Since the 1.8 kb transcript hybridizes only to the LTR probe, it is possible that this transcript initiates upstream of the first exon of c-myc and terminates in the 5' LTR of CSV. If one assumes that inititation begins 300 bp upstream of the first exon, there are TATA sequences in this location, and terminates at the U3-R junction of the 5' LTR the size of the expected transcript is ca. 1.8 kb. This utilization of the 5' LTR is analogous to the result of

Payne et al.(1982) for the case of ALV integration downstream of the c-myc gene and very common in the MMTV integration near the int-1 region (Nusse et al., 1983,1984).

Since the 5' LTR is identical to the 3' LTR and the 3' LTR is transcriptionally active, we would expect an abberrant viral transcript or transcripts initiated at the 5' LTR. However, when the northern blot was hybridized to probe INT, a probe which spans the internal region of the 713 provirus, there is no detectable viral transcript carrying the internal sequences, indicating either the transcripts are not stable, abberrant termination, possibly as a result of the deletion, or some mechanism of surppression of the transcriptional potential of the 5' LTR.

Due to the scarcity of the intact RNA samples, more detailed characterization to explore the nature of these two messages could not be accomplished. Nevertheless, it is evident that the viral genes are not present in the final transcripts and there is one c-myc message generated that is consistent with the promotor-insertion mode of oncogene activation.

DISCUSSION

It has been observed that c-myc activation, by the insertion of avian leukosis virus, is often accompanied by deletion of one of the two LTRs (Neel et al., 1982;Payne et al., 1982;Fung et al., 1982,1984). One suggestion for the observed high frequency of LTR deletion is that transcription from the 5' LTR would interfere with the amount of transcription from the 3' LTR. Cullen et al.(1984) have demonstrated that early after transfection of quail cells(QC1-3) with DNA containing a provirus-like structure and a preproinsulin II gene linked to the 3' LTR, i.e., 5' LTR-env-3' LTR-preproinsulin II, the transcriptionally active 5' LTR surppressed the transcription of the downstream preproinsulin II gene. They also observed that deletion of the 5' LTR or insertion of a transcriptional terminator from SV 40 just downstream of the 5' LTR increased transcription of the preproinsulin II gene 5-fold.

It might be expected that the presence of the 5' LTR in the 713 provirus would suppress transcription from the 3' LTR. One possiblity that could explain both the unsuppressed trancriptional activity of the 3' LTR and the unobserved viral transcript(s) is the presence of a eukaryotic transcriptional termination signal near the 5' LTR/5' viral junction. In this case, the situation would be analogous to the results observed by Cullen et al.(1984) when the SV 40 early termination region was placed near the 5' LTR junction. We have examined the 5' viral junction region for sequence homologies to termination signals found in Ad2 virus, SV 40, drosophila, yeast and phage (Hay et al, 1982;Maderious and Chen-Kiang 1984;Henikoff et al., 1983;Hatfield et al., 1983). Though we do not find any sequence similarities, there could still be sequences present in this region that attenuate or terminate transcription.

It is possible that the promotor in the 5' LTR is active in initiating transcription of the internal viral region, but during post-transcriptional processing the internal region is spliced out of the transcript using the concensus splice donor site (Mount 1982) located 195 bp downstream from the 5' LTR-PBS junction. However, this would still leave 231 bp of homologous sequence in the spliced transcript, a sufficient length to be able to detect the viral transcript with the INT probe.

An alternate possiblity is suggested by the results of Emerman and Temin(1984). They have shown that suppression of transcription of two proximal promotors does not depend on the relative position of the two promotors, but on which promotor is transcriptionally active. Using a retrovirus vector with two dominant selectable markers, i.e., 5' LTR-tk-promotor-neo-3' LTR, they have shown that suppression of expression is dependent on which gene is under selective pressure. They find suppression of the 3' promotor when selection is for the tk gene, consistent with promotor interference. However, selection for the neo gene results in suppression of tk expression by the 5' promotor. In this case, inhibition of expression cannot be attributed to promotor interference. The authors hypothesize a model in which transcription from one promotor causes a change in the chromatin structure of the surrounding DNA so that transcription from a nearby promotor will be inhibited. The activated c-myc gene could be considered a gene under selective pressure, since its consitutive expression may be required to maintain a transformed phenotype. Analogous to the results of Emerman and Temin, the transcriptional activity of the 3' LTR and the c-myc gene would result in the suppression of the viral transcript(s) from the 5' LTR. It might be possible to test this proposition by transfecting the 713 clone into an appropriate cell where the expression of c-myc is no longer required.

transcription from a nearby promoter will be inhibited. The activated chromosome could be considered a dominant trait because its constitutive expression would be required to maintain a transformed phenotype. Mutations that result in the suppression of transcription (a) from the 5' [illegible] promoter to promoter interference. The latter case, however, seems to be unlikely in this case. Lack of expression could be attributed to a mutation in the [illegible] which would result in the inability of [illegible] under restrictive pressure. They find suppression of the [illegible] mutation is dependent on which [illegible] vector with two dominant selectable [illegible] promoter is transcribed only weakly. Deletion of [illegible] on the relative position of [illegible] of transcription [illegible] promoter and initiation [illegible]

An alternate possibility is [illegible]

Several recent observations suggest that the U3 region of viral LTRs may determine the oncogenic potential of a retrovirus by conferring the capacitiy to replicate in the appropriate target cell. Murine viruses capable of inducing T-cell lymphomas replicate in the thymus, but non-leukemogenic viruses do not (Cloyd 1983;O'Donnell et al., 1983;Celander and Haseltine 1984). In addition, the capacity of Friend leukemia virus and Moloney leukemia virus to induce erythroid or lymphoid neoplasia, repectively, is probably determined by U3 sequences in the viral LTR (Chatis et al., 1984). It has also been demonstrated by Robinson et al.(1982) that the RAV-0 and RAV-1 strains of avian leukosis virus are almost indentical in DNA sequence, except for the U3 region which is highly divergent, however RAV-0 appears to be non-oncogenic in an avian host. In contrast, RAV-1 can induce B-cell lymphoma and erythroblastosis.(Fung et al., 1982,1984).

The divergence observed between the U3 region of CSV and SNV LTRs is intriguing in light of the fact that CSV and SNV have different oncogenic capacities (R.L. Witter, personal communication). SNV is approximately 3 times more potent in inducing bursal lymphoma in chicken line $15I_5x7_1$ than CSV and has greater than 3 times the capacity to induce non-bural lymphomas(thymic lymphosarcomas) in chicken line 6_3. Therefore, it is possible that the differences observed in the oncogenic capacity of SNV and CSV is related to the variation observed in the U3 region.

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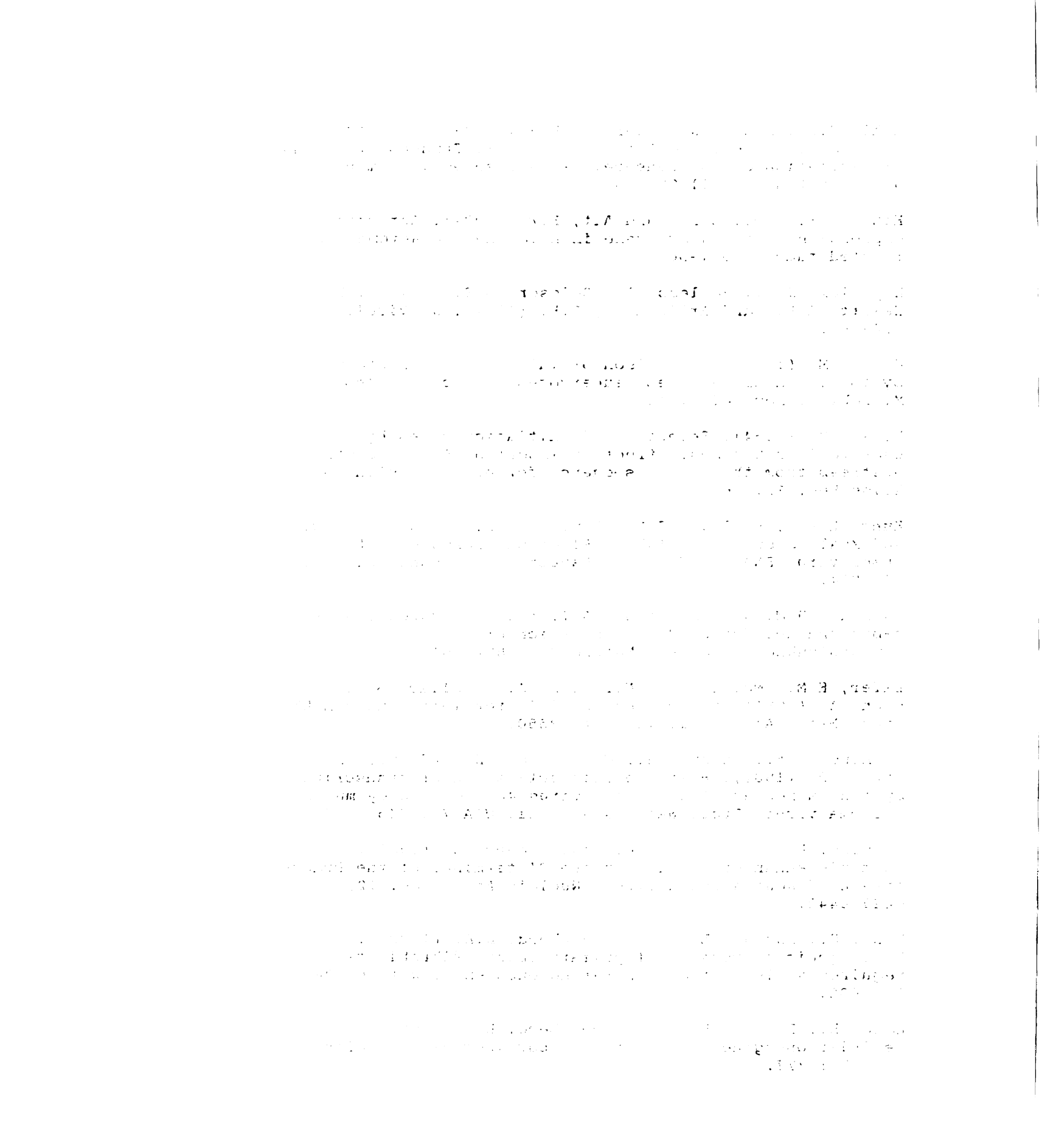

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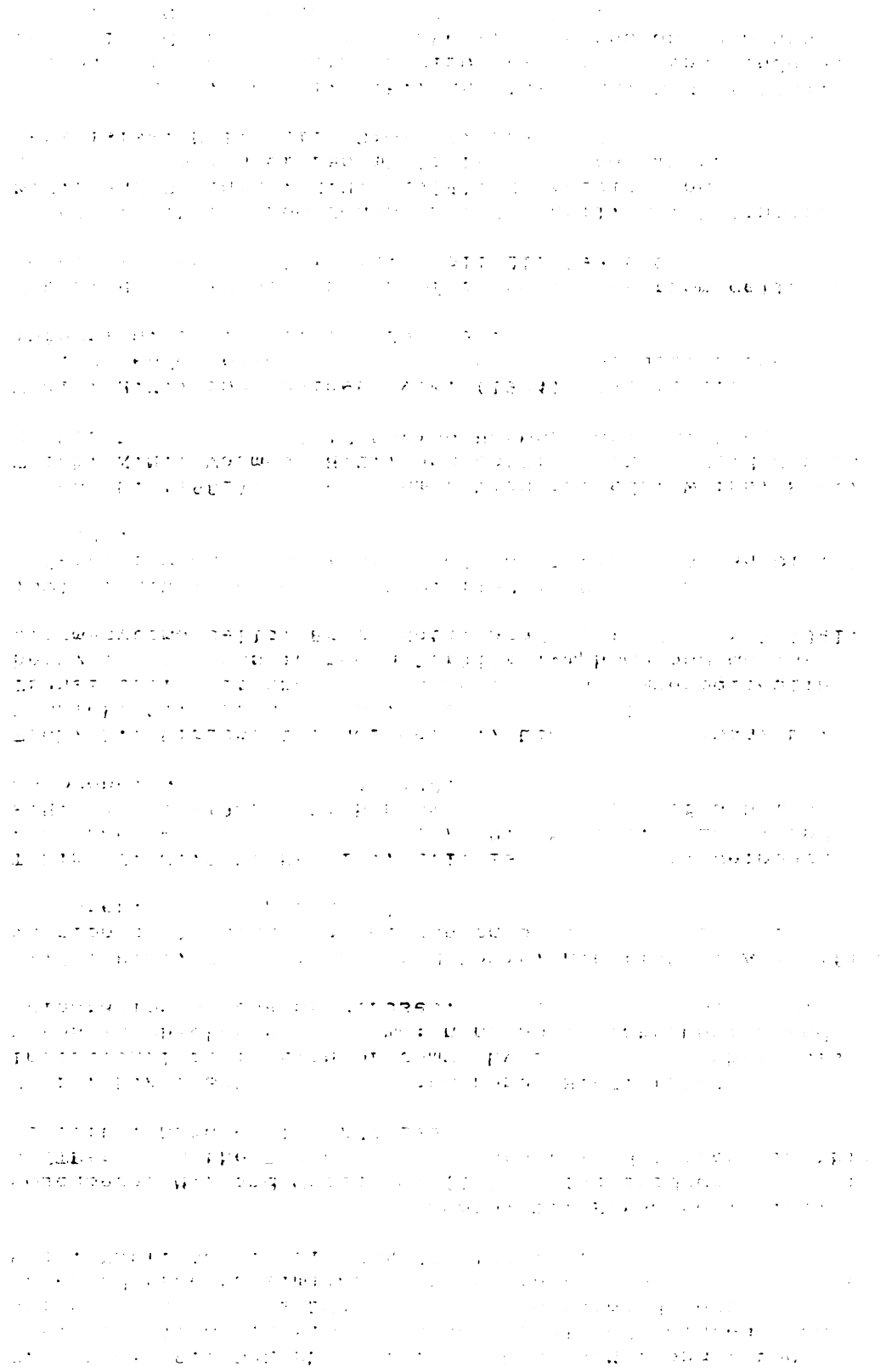

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